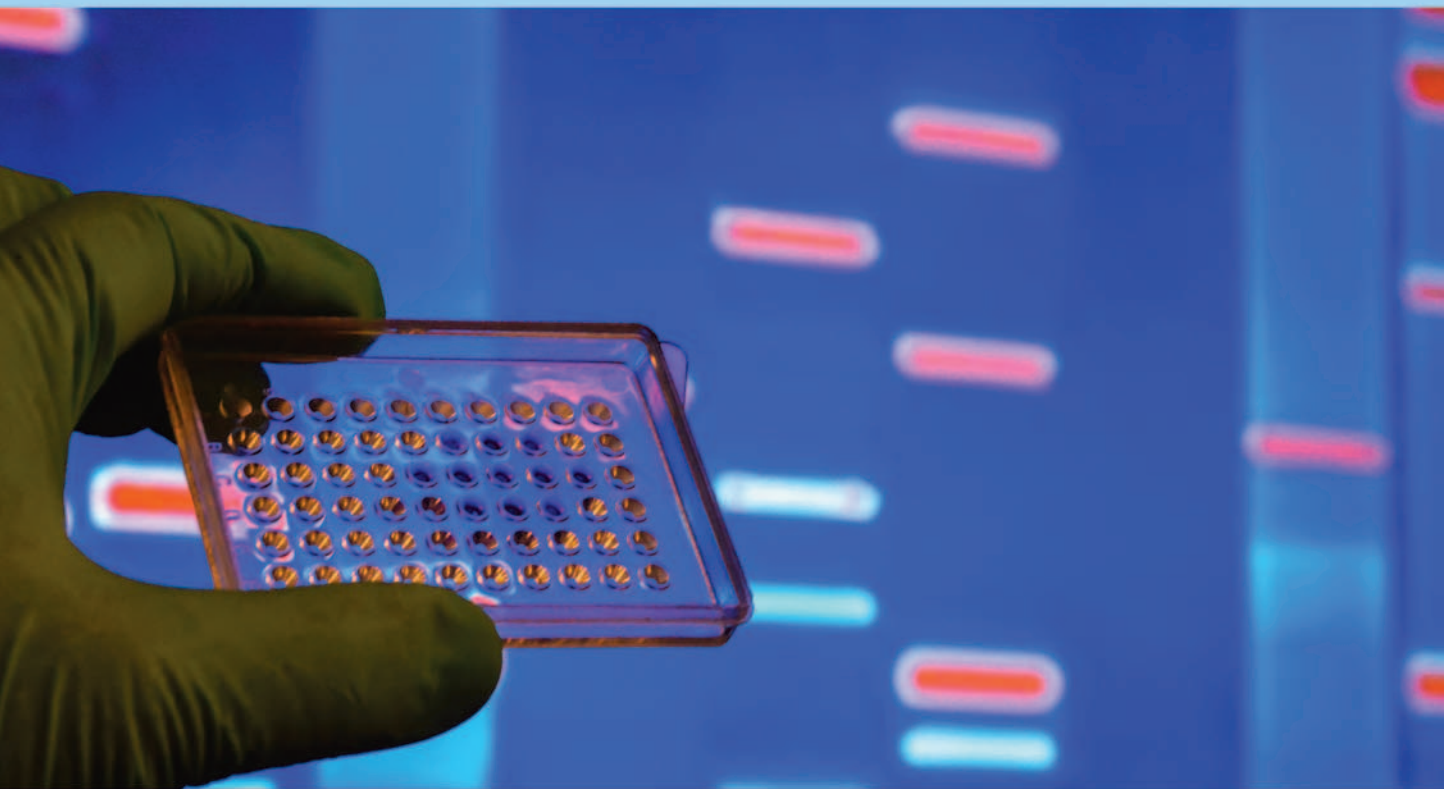


Developments in Applied Microbiology and Biotechnology



Microbial Genomics: Clinical, Pharmaceutical, and Industrial Applications

Edited by
Huseyin Tombuloglu and Amal Mahmoud



MICROBIAL GENOMICS:
CLINICAL, PHARMACEUTICAL,
AND INDUSTRIAL
APPLICATIONS

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DEVELOPMENTS IN APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

MICROBIAL GENOMICS: CLINICAL, PHARMACEUTICAL, AND INDUSTRIAL APPLICATIONS

Edited by

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An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States

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ISBN: 978-0-443-18866-4

For information on all Academic Press publications visit our website at <https://www.elsevier.com/books-and-journals>

Publisher: Stacy Masucci
Acquisitions Editor: Kattie Washington
Editorial Project Manager: Deepak Vohra
Production Project Manager: Fahmida Sultana
Cover Designer: Greg Harris

Typeset by TNQ Technologies



Dedication

“To my beloved wife, Guzin, and my daughters Hale and Busra.”

*Huseyin Tombuloglu,
Dammam, Saudi Arabia*

“To ‘حبات اللؤلؤ’ (my pearls), who have inspired me with their passion for learning and creativity—to my students at the Biology Department, College of Science, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia.”

*Amal Mahmoud,
Cairo, Egypt*

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Contents

Contributors xi

1. Introduction to microbial genomics

Ahmed Mohamed Hashem, Mohamed Awad, and
Hesham M. Abdullah

1. Introduction 1
2. Architecture of microbial genomes 3
3. Genome sequencing technologies and advances 7
4. Microbial genome annotation 12
5. Applications of microbial genomics 14
6. Current limitations and challenges of microbial genomics 17
7. Future perspectives in microbial genomics 17
- References 18

2. Viral genomics

Sara H. Mahmoud and Ahmed A. Khalil

1. Introduction 31
2. Decoding the genome: A comprehensive look at viral genomics 32
3. Exploring viral evolution through genomic analysis 35
4. From genome to pandemic: Understanding viral genomics in the era of COVID-19 43
5. Using genomics to predict the next viral outbreak 46
6. The promise of viral genomics: Developing vaccines, therapies for emerging diseases and other applications of viral genomics 48
7. Tracking the spread of viruses: How genomic sequencing is revolutionizing public health 56
8. Future directions in viral genomics 56
- References 58

3. Fungal genomics

Tarek A.A. Moussa, Mohamed S. Zaky, and Safaa A.S. Al-Qaysi

1. Introduction 71
2. Fungal genome history 72
3. Fungal genome sequencing 79
4. Importance of fungal genome databases 83
5. Genome sequencing and human health 84
6. Genomic sequences and plant health 87
7. Genomic sequence and bioactive compounds 89
8. Genomic sequence and enzyme biotechnology 89
- References 92

4. Introduction to genomic-based clinical applications

Amr Elguoshy and Ahmed Mohamed Hashem

1. Introduction 97
2. Genomics in the clinical context 98
3. Genomic basis of pathogens 99
4. Methods and strategies used for pathogen diagnosis and predicting the emergence of pathogens 100
5. Impact of genomics on pathogen diagnosis and prediction of novel pathogen emergence 103
6. Bioinformatics based clinical applications 106
7. Conclusion 110
- References 110

5. Molecular techniques for the diagnosis of viral infections

Samira Smajlovic, Galyah Alhamid, Guzin Tombuloglu,
Ahmed A. Khalil, and Huseyin Tombuloglu

1. Viral diagnosis in clinics 117
2. Nucleic acid amplification methods 118

3. CRISPR-Cas-based viral diseases diagnosis	124
4. Nanotechnology for viral disease diagnosis	127
5. Weaknesses and technical challenges of different viral diagnosis methods	130
6. Future perspectives	131
7. Conclusion	132
References	133
Further reading	140

6. Bacterial identification and diagnosis of bacterial infections through genomics, proteomics, nanotechnology, machine learning, and microelectromechanical systems

Sumayah I. Alsanie, Latifah A. Aljabari, Najla A. Aljabari, Samira Smajlovic, and Huseyin Tombuloglu

1. Introduction	143
2. Bacterial identification and characterization	144
3. Genome-based approaches	147
4. Proteomics methods	152
5. Nanotechnology in bacterial pathogen detection	159
6. Machine learning in bacterial identification and diagnosis	161
7. Microelectromechanical systems (MEMS) in bacterial identification and diagnosis	162
8. The role of microfluidics in bacterial identification and diagnosis	163
9. Raman spectroscopy in bacterial identification and diagnosis	164
10. Conclusions	164
References	164
Further reading	172

7. Fungal identification and diagnosis

Masoomeh Ghobad-Nejhad, R. Henrik Nilsson, and Alexander Ordynets

1. Fungi: An overview	173
2. Phenotypic identification of fungi in a modern view	177
3. Incorporating molecular data in fungal identification	184
4. Rapid fungal identification methods	190

5. Integrative fungal identification	194
6. Key points	196
World wide web links	197
Acknowledgments	198
References	198
Further reading	203

8. Introduction to genomics-based pharmaceutical applications

Hannah Hisham Rashwan, Alyaa Dawoud, and Mohamed El-Hadidi

1. Introduction	205
2. Genomics revolutionizing the field of pharmaceuticals	206
3. Genomics applications in the pharmaceutical fields	211
References	214

9. Antiviral history and viral targets for antiviral therapy

Akram Hegazy, Sara H. Mahmoud, Ahmed Magdy Khalil, Luis Martinez-Sobrido, and Ahmed Mostafa

1. History of viruses and antiviral therapy	219
2. Viruses' composition and specific viral targets	222
3. Natural products as antiviral agents	236
4. Perspectives and conclusions	247
Conflict of interest	248
Author contributions	248
Funding	248
References	249
Further readings	275

10. Host- and genomics-based antiviral approaches

Ahmed Mostafa, Ahmed Magdy Khalil, Akram Hegazy, Sara H. Mahmoud, and Luis Martinez-Sobrido

1. Cell signaling and virus-dependent cell-based targets	277
2. Genomics-based antiviral pharmaceutical applications	287
3. Drug repurposing for antiviral use	292
4. Perspectives and conclusions	299
References	299

11. Antibiotic resistance and target-based technologies for drug development

Maleeha Azhar, Adeeb Shehzad, Raheem Shahzad, and
Ebtesam A. Al-Suhaimi

1. Introduction 311
2. Development of antimicrobial agents for drug-resistant strains 312
3. Sources of antibacterial agents 316
4. Obstacles in the development of new antibacterial drugs and their proposed solutions 318
5. Innovative approaches for antimicrobial drug discovery 320
6. Quorum sensing and drug discovery 324
7. Nanotechnology and antibacterial drug development 326
8. Conclusion 327
- References 328

12. Discovery and development of new antibacterial drugs

Esmail Sabry Elassal, Ahmed Osama Fahmy,
Amir Nagy Mohamed Saad, Ahmed Hesham Ali,
Ahmed Hamada Elshenety, Osama Ahmed Badr,
Ahmed Noby Amer, Wafaa Mohammed Fouda, and
Yaseen Ali Mosa Mohamed Elshaier

1. Introduction 333
2. Antibacterial treatment 334
3. Bacterial resistance 336
4. Antibiotic discovery in the pre-genome era 337
5. Bacterial genomes and genome sequencing 337
6. RNA as a target for drug design 339
7. Genetic approaches to facilitate antibacterial drug development 343
8. Facilitating target-based drug discovery 351
9. The power and limits of genetic target validation 351
10. Concluding remarks 353
- References 354

13. Potential targets for the development of antifungal drugs

Aroosa Younis Nadeem, Adeeb Shehzad, Raheem Shahzad,
Meneerah A. AlJafari, and Ebtesam A. Al-Suhaimi

1. Introduction 361
2. Classification and mechanism of antifungal drugs 362
3. Antifungal targets and strategies 364
4. Therapeutic interventions against fungal drug resistance 365
5. A multifaceted approach to tackle fungal virulence 369
6. Antifungal vaccines to combat fungal infections 375
7. Novel antifungal therapeutics 376
8. Conclusions and future perspectives 376
- References 377

14. Introduction to genomics-based industrial applications

Imane Boudellioua

1. History of genomics in biotechnology 385
2. The colors of biotechnology 387
3. Classification of genomics-based applications 388
4. Regulation of genomics-based applications 390
5. The future of genomics-based applications 391
- References 392

15. Tailed phage machinery: toward advanced biocontrol

Nassereldin Ibrahim, Joel T. Weadge, and Hany Anany

1. Introduction 395
2. Bacteriophage assembly 397
3. Conformational changes during infection 409
4. Advanced applications 410
5. Conclusions 416
- References 416

16. Advances in fungal biotechnology in agriculture

Tarek A.A. Moussa, Rehab M. Hafez, Aya A. Mostafa, and
Mona M. Ali

1. Introduction 427
2. Fungi and pest control 427

3. Fungal bioremediation of organic pollutants:
Mechanisms, advantages, constraints and future
perspectives 432
4. Fungal genetic engineering 439
5. Plants genetic engineering for fungal disease
resistance 445
- References 452

Index 465

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Introduction to microbial genomics

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1. Introduction

The term “genomics” was proposed by Dr. Thomas H. Roderick in 1986 to describe the study of an organism’s complete set of genes, known as the genome, along with examining genomes from various species, including their evolution and relationships (Kuska, 1998). The development of DNA sequencing technologies since the 1970s has only made genomics possible, resulting in an exponential increase in sequencing projects. Sequencing became a standard part of biological study, resulting in the establishment of a genomic age.

Microbial genomics is a rapidly growing research field that studies the structure and function of microorganism genomes, such as bacteria, viruses, and fungi. Its history dates back to 1977, when Sanger discovered the 5368 bp genome of bacteriophage φX174, making it the first microbial genome to be sequenced (Sanger, Air, et al., 1977).

In the decades since, the number of sequenced microbial genomes has increased dramatically since the completion of the 1.78 Mbp genome of *Haemophilus influenzae* in 1995 (Fleischmann et al., 1995) and the 0.57 Mbp genome of *Mycoplasma genitalium* (Fraser et al., 1995). Microbial genomics, a prosperous scientific field, was founded at that time. Today, more than 200,000 complete or near-complete bacterial genomes have been sequenced, with more being added every day (Fig. 1.1), along with other microorganisms, including approximately 2400 for archaea and 13,000 for viruses (see Joint Genome Institute - Genomes Online Database, JGI GOLD v.7; <https://gold.jgi.doe.gov/statistics> (Mukherjee et al., 2019). Sequencing technology advances have made this exponential growth possible, making sequencing faster, cheaper, and more accurate. Additionally, the development of bioinformatics tools has made it easier to analyze and interpret the vast amounts of genomic data

^a Designing the chapter construction, writing the original draft managing the contributions of all co-authors, and taking the lead in editing and revising the chapter draft.

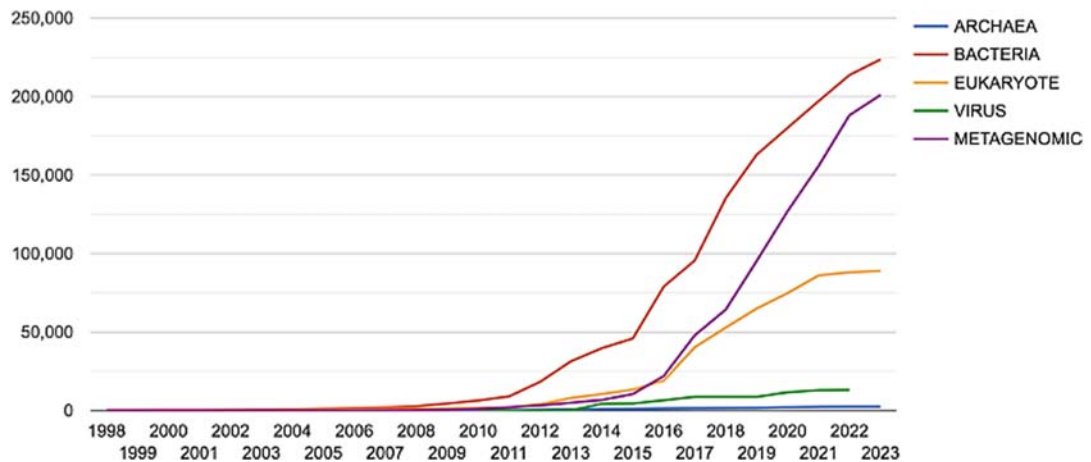


FIGURE 1.1 The completed sequencing of genomes has experienced a notable increase in growth in the past few years, exhibiting an exponential trend. Source: <https://gold.jgi.doe.gov/statistics>

generated by sequencing. This explosion in microbial genomic data has led to new insights into the biology of microorganisms and new approaches to understanding and treating infectious diseases and supplied enough information for functional and evolutionary assumptions that could be tested experimentally.

In order to get insights into the structure and function of a targeted genome, a combination of in vitro experiments and computational analysis will be utilized (Fig. 1.2). The initial step begins with DNA extraction followed by the utilization of either short- or long-read sequencing technology. The second step involves the application of bioinformatics analysis, which encompasses the process of assembling a vast number of produced reads to construct the whole chromosome or genome. In addition, the process of genome annotation involves the identification of genes and various genomic characteristics. For a comprehensive

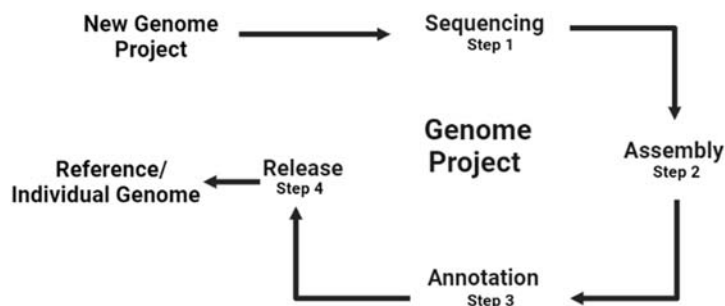


FIGURE 1.2 Diagram depicting the fundamental procedures encompassed in a standard genome study.

understanding of the latest advancements in software and databases pertaining to microbial genomics, please refer to the work of (Gao, 2019). Ultimately, the genomic data will be archived in various databases, which can be broadly categorized as either general repositories, such as NCBI (GenBank) <http://www.ncbi.nlm.nih.gov/> and EMBL-EBI <http://www.ebi.ac.uk/> or organism-specific databases, for example, FlyBase for *Drosophila* and other species <http://flybase.org/> and SGD for *Saccharomyces* Genome Database <http://www.yeastgenome.org/>.

Comparative genomics, metagenomics, functional genomics, and evolutionary genomics are all subfields of microbial genomics. Comparative genomics compares the genomes of different microbes to uncover their genome similarities and differences (Setubal et al., 2018; Tettelin et al., 2005). Metagenomics studies the collective genomes of whole microbial communities found in a particular environment without needing cultivation (Handelsman, 2004; Wajid et al., 2022). Functional genomics focuses on understanding how genes are expressed and regulated within microorganisms to determine their specific functions (Przybyla & Gilbert, 2022). Evolutionary genomics investigates the evolutionary processes that shape microbial genomes across time (Koonin et al., 2017).

The insights gained from microbial genomics have far-reaching implications in various domains. In medicine, it has led to advancements in understanding pathogenic microbes' virulence mechanisms and antibiotic-resistance genes (Alkatheri et al., 2023; Sintchenko et al., 2021). In agriculture, it has encouraged the development of novel strategies for crop improvement by harnessing the potential of helpful microbes (Ke et al., 2021; Maina & Rodoni, 2022). Additionally, microbial genomics has shed light on ecological processes such as nutrient cycling, bioremediation of pollutants, and symbiotic relationships between microbes and higher species (Freedman et al., 2017; Long et al., 2016).

2. Architecture of microbial genomes

2.1 Bacterial genome

The genome of all organisms is organized to fit into the cell space or its compartments.; This is important since the length of an organism's chromosome exceeds the size of the cell (Boccard et al., 2005; Travers & Muskhelishvili, 2005). The exact processes of DNA folding were first discovered in eukaryotes (Kornberg, 1974; Olins & Olins, 1972). As a result, nucleosomes were established as the fundamental structural components of eukaryotic folded DNA. Bacteria are unicellular organisms that lack a nucleus and other membrane-bound organelles. The bacteria genome is organized and compacted into a membrane-free area known as the nucleoid that changes shape and composition depending on the bacterial state (Dillon & Dorman, 2010). Unlike eukaryotic genomes, bacteria do not have histones or nucleosomes to package their DNA. However, bacterial genomes are still organized to allow for efficient gene expression and replication.

Most bacterial genomes comprise circular DNA molecules (Fig. 1.3) ranging in size from 0.1 to 15 Mbp (Ochman & Davalos, 2006). GC composition of genomic DNA has been widely investigated and shown to vary significantly, ranging from less than 20% to almost 75% (Almpanis et al., 2018; McCutcheon & Moran, 2010). One of the primary reasons for the short

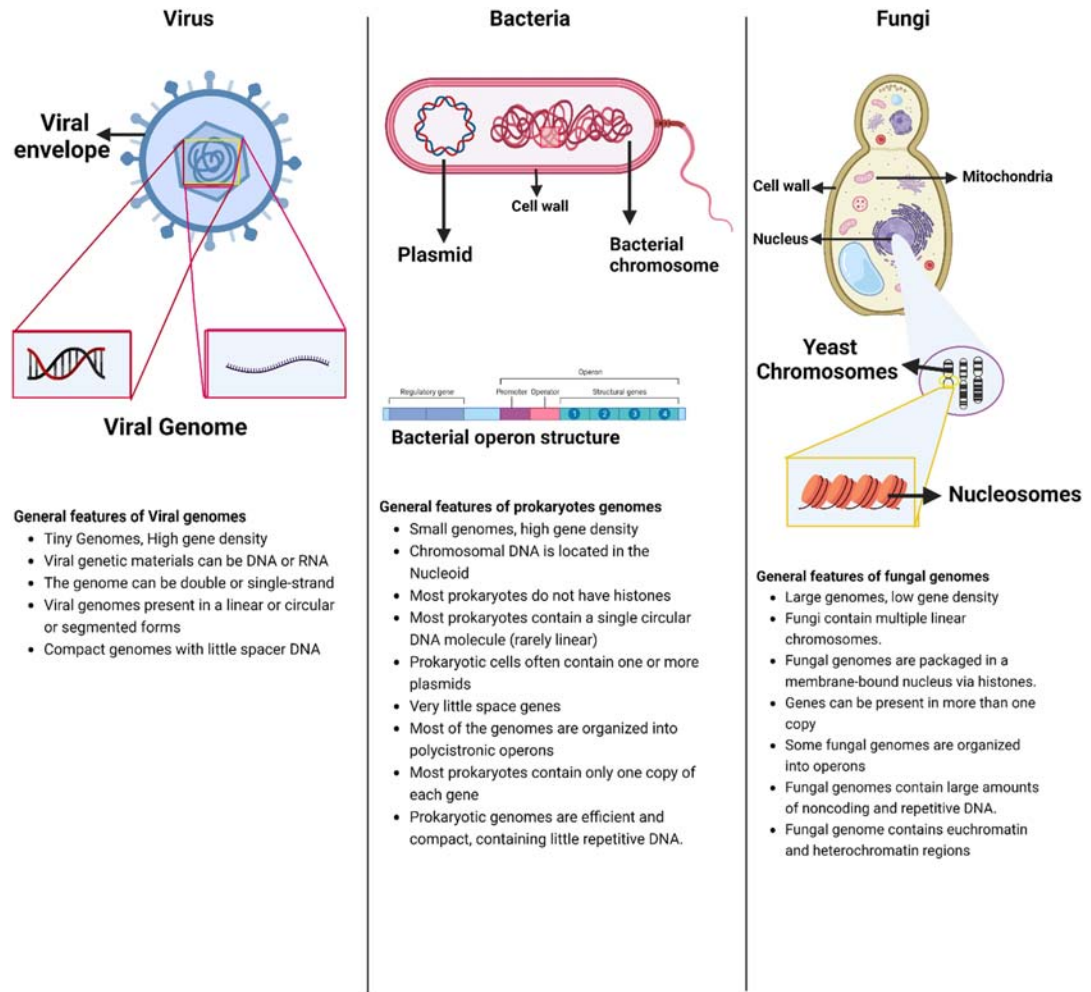


FIGURE 1.3 The illustration summarizes the common characteristics and organizational framework of viral, bacterial, and fungal genomes. This figure was created with <https://biorender.com/>.

genome size of bacteria is that most bacterial species display a mutational bias in which superfluous sequences are deleted throughout evolution, resulting in a comparatively low number of repetitive DNA (Andersson & Andersson, 1999; Martinez-Gutierrez & Aylward, 2022; Moran & Mira, 2001; Nilsson et al., 2005; Rocha, 2003). Furthermore, one-third of identified bacterial genes overlap with others (Fukuda et al., 1999; Johnson & Chisholm, 2004), significantly expanding the functional potential of a small set of genetic information. A tiny genome allows DNA transactions to occur at the right moment (Brocken et al., 2018), making bacteria adaptable to varied environments (Colizzi et al., 2023).

Comparative genomic studies revealed that bacterial genomes are highly conserved across species, with only a small fraction of genes unique to a particular species (Jordan et al., 2002).

In terms of gene content, most bacterial genomes are highly compact, with genes constituting around 90% of the genome with an average of 1 gene density per 1kbp (Ochman & Davalos, 2006); the majority of the sequence consists of functional protein-coding regions, with few noncoding or intervening sequences. These genes are organized into operons (i.e., lac operon) or clusters often associated with specific metabolic pathways or functions (Chemla et al., 2020). Bacterial genomes also contain various mobile genetic elements, such as plasmids and transposons, which can be transferred between species (Blattner et al., 1997; Smillie et al., 2010).

The organization of bacterial genome architecture is primarily achieved by binding proteins to specific DNA sequences (Dame et al., 2020). These proteins, known as nucleoid-associated proteins (NAPs), help to compact the DNA and regulate gene expression. One of the most well-known NAPs in bacteria is HU, which binds to DNA and promotes compaction by bending it into a U-shape. Another important NAP in bacteria is H-NS, which regulates gene expression by binding to specific regions of the chromosome and repressing transcription (Dillon & Dorman, 2010). H-NS also helps to organize the chromosome by forming higher-order structures such as loops and domains (Luijsterburg et al., 2006). In addition to NAPs, bacterial chromosomes are organized through supercoiling. Supercoiling occurs when the DNA helix twists upon itself, resulting in a more compact structure. This twisting can be facilitated by enzymes called topoisomerases, which break and rejoin the DNA strands to relieve tension (Shen & Landick, 2019). While bacterial chromatin may be less complex than eukaryotic cells, it still plays a vital role in regulating gene expression and ensuring efficient replication.

2.2 Viral genome

Viruses are tiny infectious agents that can replicate only inside the living cells of an organism. More in-depth ultrastructural investigations revealed that they have DNA or RNA as their genomic material packaged into a capsid protein coat (Fig. 1.3). Some viruses also have an outer envelope comprising lipids and proteins surrounding the capsid. The genome organization of viruses varies depending on their type and size. The DNA genomes can be single-stranded or double-stranded and can be circular or linear in shape (Szpara & Van Doorslaer, 2021). The RNA genomes (Fig. 1.3) can also be single-stranded or double-stranded and positive-sense or negative-sense (O'Carroll & Rein, 2016). For example, small DNA viruses such as papillomaviruses have a circular genome (McBride, 2008) that is tightly packed into the capsid. In contrast, more giant DNA viruses, such as herpesviruses, have a linear genome packaged into a more complex structure called a nucleocapsid (Fischer, 2016; Schulz et al., 2022; O'Carroll & Rein, 2016). The nucleocapsid comprises the genomic DNA wrapped around histone-like proteins, similar to chromatin in eukaryotic cells (Reuter & Krüger, 2018).

RNA viruses also have different genome organizations depending on their type. Some RNA viruses, such as retroviruses, have a single-stranded RNA genome reverse-transcribed into DNA before integrating into the host cell's genome (D'Souza & Summers, 2005). Other RNA viruses, such as the influenza virus, have segmented genomes of multiple RNA pieces (Dadonaite et al., 2019) packaged separately into individual virions.

The gene density of viruses varies widely depending on the type of virus. Some viruses have tiny genomes with only a few genes, while others have larger genomes with hundreds or even thousands of genes. For example, the smallest known virus, the circovirus, has a genome size of only 1.7 kbp (Breitbart et al., 2017; Fisher et al., 2020), while the largest known virus, the pandoravirus, has a genome size of 2.5Mbp (Fischer, 2016; Schulz et al., 2022). Generally, viruses' gene density and genome composition are highly variable and reflect their evolutionary history and adaptation to different host organisms and environments. Understanding these mechanisms is essential for developing antiviral therapies and vaccines to combat viral infections.

2.3 Fungal genome

Fungi are a diverse group of organisms that play essential roles in various ecosystems, including decomposition, nutrient cycling, and symbiosis with other organisms (Treseder & Lennon, 2015; Yu et al., 2023). Understanding the genomes of fungi is crucial for understanding their biology and interactions with other organisms (Lindahl et al., 2013; Porras-Alfaro et al., 2014; Schoch et al., 2012). Fungal genome architecture and gene density are essential aspects of fungal biology that have been extensively studied recently (Cuomo & Birren, 2010).

Fungal genomes are complex and dynamic structures crucial in regulating gene expression and other cellular processes (Wang et al., 2020a). The organization of chromatin within the fungal is the complex (Fig. 1.3) consisting of DNA, histones, and other proteins that comprise the structure of chromosomes (Allshire & Ekwall, 2015). In fungi, chromatin is organized into distinct domains associated with different gene expression levels. These domains include euchromatin, which is more open and accessible to transcription factors, and heterochromatin, which is more tightly packed and less accessible.

Fungal genomes vary significantly in size, ranging from around 10 Mb to over 100 Mb. The genome size is primarily determined by the number of genes present, ranging from a few thousand to tens of thousands (Dujon, 1996; Spanu et al., 2010). The gene density in fungal genomes is generally high, with an average of around one gene per kilobase (kb) of DNA. This increased gene density is due to the compact nature of fungal genomes, which contain relatively few noncoding regions (Stajich et al., 2007). Fungal genomes are composed of a variety of different types of DNA sequences. The most common types are protein-coding genes, which comprise most of the genome. Fungal genes are typically organized into clusters or operons, which contain multiple genes transcribed together as a single unit (Hurst et al., 2004). In addition to protein-coding genes, fungal genomes contain noncoding RNA genes, such as ribosomal RNA (rRNA), transfer RNA (tRNA) genes, and repetitive DNA sequences, which can make up a significant proportion of the genome (Donaldson & Saville, 2012; Li, O'Neill, et al., 2021, Li, Liu, et al., 2021). These repetitive sequences include transposable elements (TEs), which can move around within the genome and cause mutations or changes in gene expression.

Various factors, including histone modifications, DNA methylation, and chromatin remodeling complexes, regulate chromatin organization within the fungal genome. These factors work together to control the accessibility of genes to transcription factors and other

regulatory proteins (Allshire & Ekwall, 2015; Chang et al., 2019). One crucial aspect of fungal chromatin organization is the role played by epigenetic modifications. These modifications can be passed down from generation to generation without changes to the underlying DNA sequence (Gibney & Nolan, 2010). They can also be influenced by environmental factors such as temperature or nutrient availability (Turner, 2009).

Studies have shown that chromatin organization changes can profoundly affect fungal development and pathogenesis (Lai et al., 2022). For example, mutations in genes involved in chromatin remodeling have been linked to defects in spore formation and virulence in several fungal species (John et al., 2021; Van Der Does & Rep, 2007).

In general, comprehending the structural arrangement of the fungal genome is crucial in order to gain insights into the mechanisms by which these organisms interact with their surroundings and regulate gene expression. The continuous development of genomic technologies has yielded valuable insights into the intricate nature of this complex process, consequently opening the way for novel approaches to managing fungal infections.

3. Genome sequencing technologies and advances

3.1 First-generation sequencing

The initial experimental approach employed for ascertaining the sequence of nucleotide bases in DNA molecules was Sanger sequencing technology, which relied on the termination of DNA synthesis during chain elongation (Sanger & Coulson, 1975). The sequencing of the Φ X174 bacteriophage genome was conducted by Sanger sequencing (Sanger, Nicklen, & Coulson, 1977). The Maxam–Gilbert method was developed as an alternative approach to DNA sequencing, utilizing a chemical modification reaction to induce the cleavage of DNA molecules (Maxam & Gilbert, 1977). In 1987, Applied Biosystems, Inc. introduced a semiautomated sequencer that utilized the Sanger sequencing process. This innovation involved replacing radiolabeling with fluorescence labeling. The implementation of the Applied Biosystems sequencer resulted in a reduction in the occurrence of manual errors and had a pivotal role in advancing the field of DNA sequencing technology (Hood et al., 1987; Smith et al., 1986).

Several projects were initiated to sequence the genomes of various species using first-generation technology. For instance, the genomes of *Haemophilus influenza* (Fleischmann et al., 1995), *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium., 1998), *Drosophila melanogaster* (Myers et al., 2000), *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), and human (International Human Genome Sequencing Consortium, 2004) were targeted for investigation.

3.2 Next-generation sequencing

Despite its high accuracy, Sanger sequencing is characterized by its high cost, time-consuming nature, and labor-intensive requirements. Various next-generation sequencing (NGS) technologies have been developed to address the limitations of Sanger sequencing and provide efficient sequencing data at a large scale. The initial commercial sequencer of

the present era was the Roche 454, which was introduced in 2005. This sequencer utilized pyrosequencing technology and was founded on a sequencing-by-synthesis approach. Subsequently, in 2007, the Solexa or Genome Analyzer (GA) sequencing method emerged, encompassing the Hiseq series and Miseq. Sequencing by ligation technology was first developed in 2007 by utilizing the SOLiD sequencing platform (Shendure et al., 2005; Valouev et al., 2008). The Ion Torrent sequencer, introduced in 2010 (Rothberg et al., 2011), employed the semiconductor sequencing technology pioneered by Life Technologies.

NGS has introduced a new phase characterized by the availability of fully automated sequencers that can generate large amounts of data at a lower cost and with a level of accuracy similar to earlier sequencing techniques (Rothberg et al., 2011). The availability of NGS technology offers unprecedented possibilities in a wide range of everyday tasks and fields related to biology. The aforementioned endeavors encompassed a range of activities, including promptly detecting pathogens and microbes in various tissue and environmental samples and applying personalized medicine for illness treatment and diagnosis (Heikamp & Pui, 2018; Wilson et al., 2019). A considerable number of live organism genomes have been sequenced and subsequently employed in various omics research endeavors (Hodzic et al., 2017).

3.3 Third-generation sequencing

While NGS is a highly effective method for conducting sequencing studies, it is important to note that the read lengths of most NGS systems are often shorter compared with Sanger sequencing. The advent of the single-molecule sequencing technique has catalyzed the revolution in third-generation sequencing technology (Derrington et al., 2010; Eid et al., 2009). The Pacbio RS sequencing platform was created by Pacific Bioscience in 2011, utilizing the Single Molecule Real-Time (SMRT) technology. The Pacbio technology has the capability to produce readings that have an average length of 2 Kbp and a maximum length of 23 Kbp (Rasko et al., 2011). The portable USB Nanopore MinIon sequencer was introduced by Oxford Nanopore in 2014, utilizing the Nanopore sequencing approach (Mikheyev & Tin, 2014). The Nanopore sequencer does not possess a theoretical constraint on read length. However, in practical applications, the read length has been observed to reach up to 200 Kbp, with an N50 value of approximately 30 Kbp (Michael et al., 2018). Several recent studies have reported the presence of 1 Mbp reads in Nanopore datasets (Jain et al., 2018; Rao et al., 2021).

The MinIon USB sequencer provides the capability to do sample sequencing in real time, thereby significantly enhancing the efficiency of genomic research conducted in remote locations. Regrettably, the long-read approach exhibits a comparatively elevated error rate compared with Sanger sequencing and NGS. According to previous studies (Ferrarini et al., 2013; Laver et al., 2015), the error rate in Pacbio 8 systems is estimated to be approximately 15%, whereas in Nanopore sequencers, it surpasses 35%. Hybrid error-correction algorithms are being developed by researchers, drawing on NGS data and self-correction algorithms, in response to the significant error rate (Das et al., 2019; Morisse et al., 2021). The Sequel II system, newly introduced by Pacbio, is a methodology wherein high-quality long reads are produced by invoking consensus from subreads generated by utilizing several

enzymes that traverse a circularized template. High-fidelity (Hifi) reads often exhibit an average length ranging from 10 to 25 kilobase pairs (Kbp) and possess an error rate that is comparable with that of NGS techniques (Hon et al., 2020).

3.4 Genome assembly

In recent decades, there have been significant advancements in sequencing technologies. With the exception of small molecules such as plasmids and viral genomes, none of those aforementioned sequencing technologies possesses the capability to sequence the complete DNA molecule as a fragment with a straightforward sequence. In contrast, the utilization of sequencing technologies yields substantial quantities of data in the form of tiny reads or fragments, with lengths varying from 70 base pairs in NGS to 200 kilobase pairs in Nanopore sequencing. The effective management and comprehensive processing of sequencing data necessitate implementing robust computing processes. The process of genome assembly, as depicted in Fig. 1.4, is a computer technique employed in the field of biology. It involves utilizing fragmented reads to construct a representation of the genome that closely approximates its true structure. The process of genome assembly can be separated into two

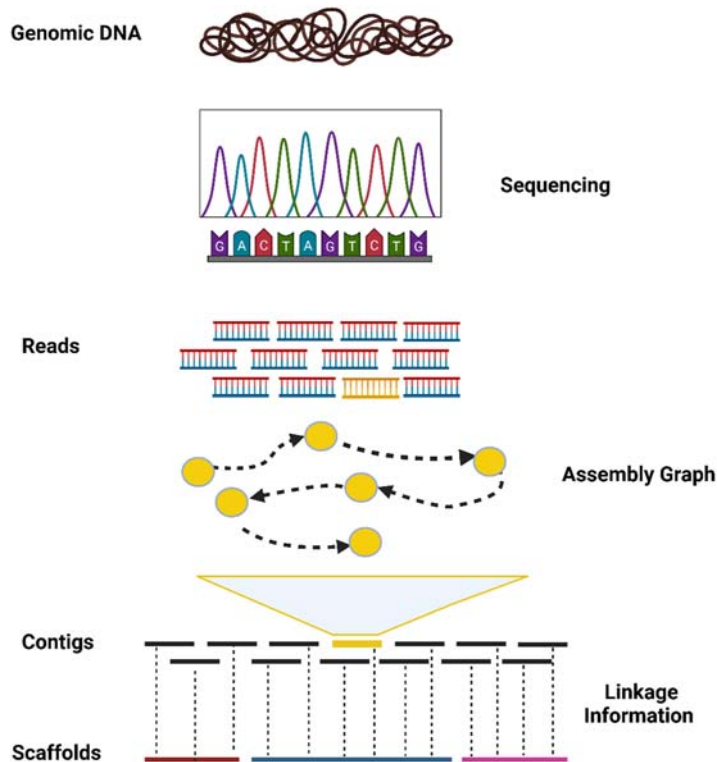


FIGURE 1.4 Schematic representation of the genome assembly pipeline. This figure was created with <https://biorender.com/>.

distinct steps, namely assembly and scaffolding. Either a de novo assembly or reference-guided assembly strategy can be employed to facilitate the two distinct phases of assembly projects.

3.4.1 *De novo assembly*

The de novo assembly process of a genome presents a substantial computational obstacle as it aims to reconstruct the DNA sequence of a specific genome by leveraging the overlaps among fragments acquired by sequencing technologies (Shafin et al., 2020). The aforementioned methodology facilitates the generation of contigs, which are extended and contiguous segments of the desired genomes. In the first-generation sequencing data analysis, researchers developed straightforward greedy assembly strategies. These techniques involved merging reads based on their most significant overlap (Pop, 2009). The utilization of high-throughput sequencing technologies has led to remarkable progress in developing genome assembly algorithms. The authors of the study (Li et al., 2012) employed graph algorithms in the construction of several assemblers, as depicted in Fig. 1.4. These assemblers may be categorized into two primary groups, namely Overlap-Layout-Consensus (OLC) and de Bruijn graph (DBG) methods. With a few exceptions, the SSAKE (Warren et al., 2007) and JR-Assembler (Chu et al., 2013) were devised utilizing the greedy algorithm.

The OLC algorithm has three distinct processes, the initial of which involves generating a pairwise alignment for all reads within a given collection of sequencing datasets (R). In (R), all entities are depicted as nodes in an overlap graph, and the alignment metadata produces directed edges that are encoded by the overlaps of the graph vertices. In the subsequent stage, the graph undergoes a cleaning process wherein branching nodes and transitively infeasible edges are resolved, resulting in the establishment of a contigs layout that consists of clear and continuous paths (see Fig. 1.4). In the final phase, the approach conducts a multiple sequence alignment to unveil the consensus sequence of each contig (Li et al., 2012). Several NGS assemblers have been developed utilizing the Overlap-Layout-Consensus (OLC) graph approach. Examples of these assemblers include Celera (Myers et al., 2000), Arachne (Batzoglou et al., 2002), Phrap (de la Bastide & McCombie, 2007), and Newbler (Margulies et al., 2005). In addition, several third-generation sequencing technologies are classified as OLC graph assemblers, such as Canu (Koren et al., 2017), Mecat (Xiao et al., 2017), Miniasm (Li, 2016), and Falcon (Chin et al., 2016).

Although the utilization of overlapping information from the full read can be advantageous, the process of Overlap-Layout-Consensus (OLC) requires substantial computing and storage resources, particularly when handling large and intricate genomes. Consequently, the de Bruijn graph (DBG) was devised with the aim of reducing computational expenses while significantly enhancing assembly outcomes. The DBG (de Bruijn graph) approach initiates by fragmenting each read within a set of reads (R) into segments of a specific length k , referred to as k -mers. The process involves creating a directed graph by using a collection of distinct $k-1$ -mers as nodes. The network's edges are formed by connecting k -mers that share a common suffix-prefix overlap of length $(k-1)$. The constructed contigs are detected by the algorithm through the analysis of well-defined graph paths (Li et al., 2012).

The de Bruijn graph has proven to be highly compatible with NGS data, leading to the development of several tools, including Velvet (Zerbino & Birney, 2008), SOAPdenovo2 (Luo et al., 2012), ABySS (Simpson et al., 2009), and AllPath-LG (Gnerre et al., 2011). Regrettably, DBG exhibits a significant vulnerability to error rates. The utilization of de Bruijn graph (DBG) for third-generation sequencing is hindered by the error rates associated with long reads technologies. The ABrujin assembler represents the initial long reads assembler developed to assess the impact of the De Bruijn Graph (DBG) on the assembly process of third-generation sequencing reads. Efficient utilization of this technique has been observed in the reconstruction of tiny genomes, such as those of *E. coli* and *C. elegans* (Lin et al., 2016). Subsequent to that, assemblers based on de Bruijn graphs (DBG), namely Flye (repeated graph) (Kolmogorov et al., 2019) and Wtdbg2 (fuzzy Bruijn graph) (Ruan & Li, 2020), have been devised and effectively employed for the construction of intricate genomes.

3.4.2 Genome scaffolding

The initial phase of de novo assembly yields a complex representation of the genome of interest in the form of several contigs. Regrettably, a significant number of investigations in the fields of comparative genomics and population genetics necessitate highly contiguous or chromosome-level assemblies. Consequently, many approaches and procedures have been developed to address the scaffolding stage (Rice & Green, 2019).

The computational biology technique known as genome scaffolding employs linkage data to infer the sequential arrangement and relative orientation of contigs within the genome being investigated (see Fig. 1.4). One commonly used approach in the development of various scaffolding software, such as Ragoo (Alonge et al., 2019), MeDuSa (Bosi et al., 2015; Rice & Green, 2019), and CSAR (Chen et al., 2018), is the utilization of information extracted from reference genomes. However, it is essential to note that discrepancies in structure and chromosomal alterations between the target and reference genomes might potentially lead to errors in the scaffolding process (Bosi et al., 2015; Ghurye & Pop, 2019; Kolmogorov et al., 2014). Consequently, de novo scaffolding techniques are favored in generating chromosome-level assemblies (Awad & Gan, 2023). The methodologies for de novo scaffolding can be classified into two categories: mapping-based approaches and sequencing-based approaches, based on the origin of the linkage data.

The scaffolding procedures that rely on mapping-based techniques utilize the linkage information derived from genomic markers acquired through mapping approaches (see Fig. 1.4). Various methods have been employed in the identification of mapping markers, including the Genetic Linkage map (Nossa et al., 2014), Radiation Hybrid map (Raudsepp et al., 2008), Fluorescence In Situ Hybridization map (FISH) (Raudsepp et al., 2008), and the more recent optical mapping methodology. The optical map is a technique that generates an organized restriction map of the target genome, which is then marked using fluorescent dyes. Several genomes, such as *Sorghum bicolor* (Deschamps et al., 2018), *Arabidopsis thaliana* (Jiao et al., 2017), and the human genome (Seo et al., 2016), have utilized optical maps to facilitate chromosome-level structuring.

The widespread utilization of sequencing-based scaffolding in genome assembly initiatives can be attributed to the cost-effectiveness of sequencing technology. The scaffolding strategy utilized in this study leverages the linkage information obtained from both ends

of a tiny genomic fragment (known as paired-end) or a long genomic fragment inserted in a Bacterial Artificial Chromosome (referred to as mate-end) (Adams et al., 2000; International Human Genome Sequencing Consortium, 2004). Furthermore, several methodologies have been developed to utilize low-coverage long reads for the purpose of scaffolding short-read assemblies. Examples of such tools include LRscf (Qin et al., 2019), SLR (Luo et al., 2019), and LINKS (Warren et al., 2015). The high-throughput chromosomal conformation capture (Hi-C) sequencing technique was introduced in 2009 as a means to investigate the three-dimensional (3D) architecture of chromosomes (Lieberman-Aiden et al., 2009). The Hi-C technique is widely employed for genomic scaffolding, being a commonly utilized approach in this field. Several tools have been developed to use HiC in genome scaffolding. Examples of these tools include LACHESIS (Burton et al., 2013), Juicer (Durand et al., 2016), and SALSA2 (Ghurye & Pop, 2019). Furthermore, several genomes have successfully attained chromosome-level assembly by utilizing Hi-C scaffolding. For instance, *Cerasus humilis* (Wang et al., 2020c), *Prunus avium* (Wang et al., 2020b), *Miscanthus lutarioriparius* (Miao et al., 2021), *Solanum melongena* (Wei et al., 2020), goose (Li et al., 2020), and human (Garg et al., 2021) are notable examples. 10XGenomics initially developed the linked-reads sequencing method as a promising means of obtaining linkage information. However, in the year 2020, the firm decided to discontinue its provision of genomic sequencing services.

The utilization of sequencing technologies for scaffolding purposes has become prevalent in genome studies due to their increased accessibility and reduced costs. The scaffolding approach utilizes the information obtained from the paired-end or mate-ends of Bacterial Artificial Chromosome (BAC), as described by the International Human Genome Sequencing Consortium in 2004. In addition, many software have been developed to utilize long reads to enhance short read assemblies. These applications include LRscf (Qin et al., 2019), SLR (Luo et al., 2019), and LINKS (Warren et al., 2015). The utilization of Hi-C sequencing, also known as high-throughput chromosomal conformation capture, has gained significant popularity as a technology for scaffolding at the chromosome 7 level.

4. Microbial genome annotation

High-throughput sequencing technologies have boosted the quantity of available microbial genomes recovered from cultured microbes, single cells, and metagenomes. As a result, quick and extensive functional gene annotation systems are required to analyze and compare these genomes. The process of identifying and annotating genes and other functional elements in a genome is known as genome annotation. Structural and functional annotation objectives can be addressed by analyzing sequence data using either a statistical model approach called *the ab initio* method (Brent, 2005; Guigó et al., 1992) or a sequence similarity strategy known as the homology-based method (Altschul et al., 1990; Söding, 2005). Both approaches involve identifying protein-coding genes, noncoding RNA genes, regulatory elements, and other genomic features (Lobb & Doxey, 2016; Lobb et al., 2020; Saikat, 2023). This information is crucial for understanding the biology and function of microorganisms and comparative genomics studies.

The process of microbial genome annotation typically involves several steps. First, the raw sequence data generated from DNA sequencing are assembled into contigs or scaffolds using bioinformatics tools such as de novo assemblers (Fig. 1.4) (Rice & Green, 2019). Once the genome assembly is complete, the next step is to predict protein-coding genes within the genome (Huynen et al., 2000). Several bioinformatics tools are commonly used for gene prediction in microbial genomes. Prodigal (Hyatt et al., 2010) <http://compbio.ornl.gov/prodigal/> is a widely used tool, which combines hidden Markov models (HMMs) and statistical models to identify protein-coding genes based on their coding potential. Other popular gene prediction tools include MicrobeAnnotator (Ruiz-Perez et al., 2021) <https://github.com/cruizperez/MicrobeAnnotator>, PGAP (Li, O'Neill, et al., 2021, Li, Liu, et al., 2021) <https://github.com/ncbi/pgap>, GeneMarkS-2 (Lomsadze et al., 2018) <http://exon.gatech.edu/genemark/genemarks2.cgi>, Glimmer (Delcher et al., 2007) <http://ccb.jhu.edu/software/glimmer/index.shtml>, and AUGUSTUS (Stanke et al., 2004) can also be accessed via the web interface at <http://bioinf.uni-greifswald.de/augustus/> and a web service at <http://bioinf.uni-greifswald.de/webaugustus/>.

In addition to protein-coding genes, noncoding RNA genes are critical functional elements within microbial genomes (Mattick et al., 2023). Tools such as tRNAscan-SE (Chan & Lowe, 2019) (<http://trna.ucsc.edu/tRNAscan-SE/>) and INFERNAL (Nawrocki & Eddy, 2013) <https://github.com/EddyRivasLab/inferral> are commonly used for predicting transfer RNA (tRNA) genes and other noncoding RNA genes such as ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs).

Once the protein-coding and noncoding RNA genes have been predicted, functional annotation can be performed to assign putative functions to these genes (Gaudermann et al., 2006); this can be done by comparing the predicted gene sequences against databases of known proteins using tools such as BLAST or HMMER (Altschul et al., 1990; Eddy, 2011). These comparisons help in identifying homologous proteins with known functions.

Functional annotation can also involve predicting protein domains using tools such as InterProScan (Jones et al., 2014) <https://www.ebi.ac.uk/interpro/search/sequence/> or PfamScan (Mistry et al., 2007) <https://www.ebi.ac.uk/Tools/pfa/pfamscan/>. These tools search for conserved domains within protein sequences that provide clues about their function.

In addition to gene prediction and functional annotation, microbial genome annotation involves identifying regulatory elements such as promoters, transcription factor binding sites, and operon structures (Fig. 1.3). Tools such as PromScan (Mistry et al., 2007) and BPROM (Solovyev & Salamov, 2011) are commonly used to predict promoter regions, while tools such as MEME (Bailey et al., 2015) <https://meme-suite.org/meme/> and RSAT (Turatsinze et al., 2008) <http://rsat.ulb.ac.be/rsat/> are used to identify transcription factor binding sites.

In general, the process of annotating microbial genomes is a complex operation that necessitates the integration of several bioinformatics tools and databases. The annotation of microbial genomes has been significantly enhanced by the accessibility of high-throughput sequencing technologies and the ongoing advancements in bioinformatics tools. Those efforts have resulted in a deeper understanding of their biology and the identification of potential applications in diverse domains, including biotechnology, medicine, and environmental science.

5. Applications of microbial genomics

Applications of Microbial Genomics: Harnessing Genetic Frontiers for Diverse Fields.

Microbial genomics has transformed many fields by offering vital insights into microbial genetic makeup, evolution, and functions. Here are some examples of microbial genomics applications in several domains (Fig. 1.5), which provide an overview of the applications of microbial genomics. The figure illustrates the diverse applications of microbial genomics, showcasing its impact across various fields. Each application presents unique opportunities for research and practical implementation, contributing to advancements in human health, industry, and environmental sustainability.

5.1 Comparative genomics

Comparative genomics plays a crucial role in understanding microbial diversity, adaptation and identifying conserved genes, variations, and unique features. It provides insights

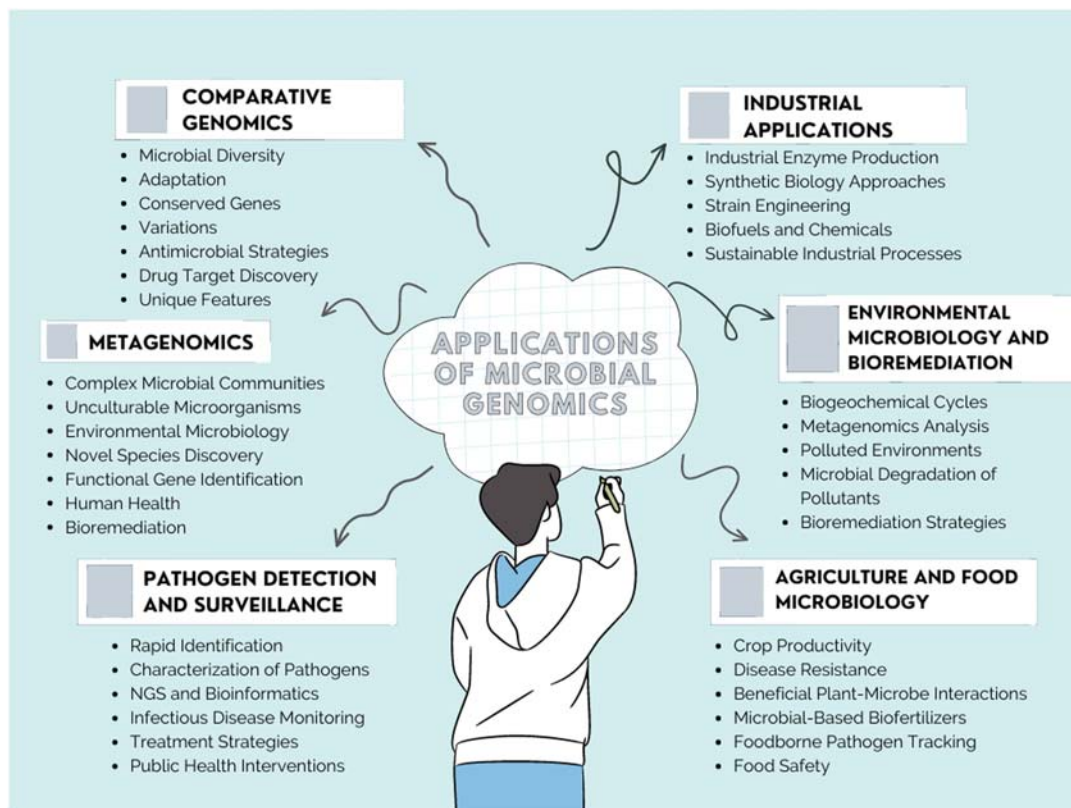


FIGURE 1.5 Exploring the frontiers: applications of microbial genomics. It is evident that microbial genomics has revolutionized various fields by harnessing the genetic frontiers.

into the genetic basis of microbial phenotypes, facilitating the development of antimicrobial strategies and the discovery of drug targets (Kobras et al., 2021; Padmanabhan et al., 2013). Comparative genomics has been used to explore the adaptation of microbes to different environments and to uncover their unique genomic signatures (Zhou & Miller, 2002).

5.2 Metagenomics

Metagenomics enables studying complex microbial communities and unculturable microorganisms by directly analyzing their genetic material. It reveals microbial communities' genetic potential and functional diversity in various environments. Metagenomic analysis has wide-ranging applications in environmental microbiology, bioremediation, human health, and the discovery of novel species and functional genes (Quince et al., 2017). It has revolutionized our understanding of microbial ecology and has the potential to uncover new insights into the interactions between microorganisms and their environments (Vecherskii et al., 2021).

5.3 Pathogen detection and surveillance

Genomic approaches have accelerated pathogen detection and surveillance by enabling rapid identification and characterization of pathogens. Next-generation sequencing technologies combined with bioinformatics analysis allow for the accurate and timely identification of infectious agents, helping to guide appropriate treatment strategies and public health interventions (Ko et al., 2022; Mohsin et al., 2021). Metagenomic surveillance and early warning systems have been utilized to monitor emerging infectious diseases and track their spread (Grubaugh et al., 2019). Additionally, next-generation sequencing has been applied to track antimicrobial resistance in pathogens, providing valuable insights for effective management and control (Zankari et al., 2012).

5.4 Industrial applications of microbial genomics

Microbial genomics has revolutionized industrial applications by providing insights into the genetic basis of microbial traits and enabling the exploitation of microbial genomes for various purposes. Industrial enzyme production has been enhanced by identifying and optimizing enzymes using genomic approaches (Douillard & de Vos, 2014; Fridman & Pichersky, 2005; Kuznetsova et al., 2005; Liu et al., 2008). Synthetic biology approaches leverage microbial genomics to engineer microbial strains to produce valuable compounds, biofuels, and chemicals. Genomics-guided strain improvement has facilitated enhanced production of biofuels and chemicals by targeting specific metabolic pathways (Aulakh et al., 2023; McCarty & Ledesma-Amaro, 2019; Nielsen et al., 2013, 2016).

5.5 Environmental microbiology and bioremediation

Microbial genomics has transformed our understanding of microorganisms' roles in biogeochemical cycles and their applications in environmental remediation. By studying

the genomes of environmental microbes, we can decipher their functional capabilities and ecological roles in biogeochemical processes (Falkowski et al., 2008; Kato et al., 2018). Metagenomic analysis has been instrumental in uncovering the microbial diversity and potential for bioremediation in polluted environments (Jung et al., 2016; Kato et al., 2018). Furthermore, genomic insights into microbial degradation pathways have paved the way for developing bioremediation strategies targeting pollutants (Deutschbauer et al., 2006; Lovley, 2003).

5.6 Agriculture and food microbiology

Microbial genomics has emerged as a powerful tool in addressing critical challenges in agriculture and food microbiology. The application of genomics in this field encompasses various aspects, including crop productivity, disease resistance, beneficial plant–microbe interactions, microbial-based biofertilizers, foodborne pathogen tracking, and food safety. By studying the genomes of agricultural microorganisms, researchers have gained insights into the genetic basis of traits related to crop productivity, such as nutrient utilization, stress tolerance, and growth promotion (Doni et al., 2022; Han & Yoshikuni, 2022; Ke et al., 2021). Genomic approaches have also facilitated the identification and characterization of genes involved in disease resistance, enabling the development of resistant crop varieties through molecular breeding and genetic engineering techniques (Iquebal et al., 2022; Keller et al., 2018). Furthermore, microbial genomics has shed light on the complex interactions between plants and beneficial microbes, revealing mechanisms contributing to enhanced nutrient uptake, disease suppression, and overall plant health (Wu et al., 2009). In addition, microbial-based biofertilizers, which harness the metabolic capabilities of specific microorganisms, have been optimized using genomics approaches, offering sustainable alternatives to chemical fertilizers (Mitter et al., 2021). In the context of food safety, microbial genomics plays a vital role in tracking and identifying foodborne pathogens, providing insights into their genetic characteristics, transmission routes, and virulence factors, thus aiding in the development of effective control measures (Guinane et al., 2019; Zhou et al., 2022). Moreover, genomics-based techniques are utilized to ensure food safety by enabling rapid and accurate detection of contaminants and pathogens in food products (Allard et al., 2018; Ronholm et al., 2016). The integration of microbial genomics into agriculture and food microbiology holds great promise for improving crop yields, enhancing disease management, promoting sustainable farming practices, and safeguarding the quality and safety of our food supply.

In conclusion, integrating genomics and microbiology has transformed our understanding of microorganisms and their applications in diverse fields. The advent of advanced genome biology and technology has empowered researchers to explore the vast genetic diversity and functional attributes of microorganisms, leading to significant advancements in pathogen detection, industrial processes, environmental remediation, and agricultural practices. Continued research in this field will undoubtedly unlock further opportunities for harnessing the potential of microbial genomics to address global challenges and improve human well-being.

6. Current limitations and challenges of microbial genomics

The advances in genomics technologies and computational biology have accelerated and expanded our understanding of the microbial world and its associations with various aspects of life. However, several significant issues and gaps still need to be addressed.

Advances in genomics technologies and computational biology have greatly expanded our understanding of the microbial world and its diverse associations with various aspects of life. However, several significant challenges and gaps in microbial genomics still need to be addressed:

One major challenge is sample bias, wherein microbial studies often rely on samples collected from easily accessible environments, such as soil or the human gut. Unfortunately, these samples may not fully represent the true diversity of microorganisms present in other habitats, limiting our understanding of microbial communities and their functions across different ecosystems.

Another hurdle is functional annotation. While sequencing technologies have made obtaining genomic data from microorganisms easier, the process of assigning functions to genes and predicting their roles in cellular processes remains complex due to a large number of unknown genes and limited experimental validation.

Analyzing metagenomics data poses another significant challenge. Microbial communities are incredibly complex and diverse, and the presence of nonmicrobial DNA contaminants further complicates the analysis. Developing robust computational tools and algorithms is crucial for extracting meaningful information from metagenomics datasets.

Horizontal gene transfer (HGT) is a well-known phenomenon among microbes, facilitating the exchange of genetic material and playing a pivotal role in microbial evolution and adaptation to changing environments. However, accurately identifying HGT events and understanding their impact on microbial genomes remain problematic.

Microbial communities are highly interactive, relying on functional interactions for various metabolic processes. Understanding these intricate interactions within complex ecosystems is essential for deciphering community dynamics and predicting how ecosystems respond to environmental changes.

Data integration becomes critical for a comprehensive understanding of microbial systems with the increasing availability of diverse omics datasets (e.g., genomics, transcriptomics, proteomics). Developing effective data integration and analysis methods is essential to uncover complex relationships between genes, proteins, and metabolites and to gain deeper insights into microbial functions and interactions.

Addressing these limitations and challenges in microbial genomics will pave the way for further advancements and a more comprehensive understanding of the microbial world and its vital contributions to our environment and health.

7. Future perspectives in microbial genomics

Looking toward the future, the opportunity of microbial genomics to enhance our comprehension of the microbial world is highly promising.

Single-cell genomics has experienced notable breakthroughs, making it an intriguing avenue to explore. This innovative methodology enables researchers to examine individual microbes, providing insights into their genetic variability, metabolic capabilities, and functional interconnections among communities. By analyzing single cells, we can acquire an unparalleled understanding of the complex mechanisms underlying microbial life.

Long-read sequencing technologies present a possible approach for addressing the limitations of current short-read sequencing technologies in resolving intricate genomic architecture, including repeated sections or extensive structural variants. Through the application of long-read sequencing techniques, it is possible to attain enhanced precision in genome assemblies and obtain a more comprehensive understanding of microbial genomes.

Functional metagenomics is an additional crucial approach involving integrating metagenomic data with available tests. This integration establishes a connection between the existence of genes and their actual functions, addressing the gap between gene presence and function. Integrating microbial community dynamics and ecosystem functioning is of utmost importance to enhance comprehension of these processes. This integration yields valuable insights into the operational mechanisms of microbial communities and their impact on surrounding environments.

The incorporation of systems biology approaches in the field of microbial genomics presents a promising avenue for further progress. The complex interaction among genes, proteins, and metabolites within microbial systems can be comprehensively elucidated using systems biology approaches. This integrated approach facilitates a more thorough comprehension of microbial functionalities and their contributions to diverse biological phenomena.

As we look forward, these forthcoming prospects in microbial genomics possess the capacity to unveil new findings and fundamentally transform our understanding of the microbial realm. The utilization of these advanced methodologies and strategies will unquestionably enhance our comprehension of microbial communities and their significant influence on multiple aspects of life.

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Viral genomics

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1. Introduction

Viral genomics is the study of the genetic material of viruses, including their DNA or RNA. It involves the analysis of the complete set of genetic instructions that a virus carries, known as its genome. The study of viral genomics is crucial for comprehending the biology of viruses, creating innovative diagnostic techniques and treatments, and managing the proliferation of viral illnesses. The field of viral genomics has been rapidly expanding due to advancements in sequencing technologies, which have made it possible to sequence viral genomes quickly and at a relatively low cost. DNA sequencing technology is utilized to characterize the viral genome, which is the genetic material of viruses, and has significantly improved our knowledge of these minute disease-causing agents. Viral genomics is important for understanding viral diseases for several reasons. By studying the genetic material of viruses, valuable insights can be obtained regarding different aspects of viral biology, such as their replication mechanisms, interactions with host cells, and pathogenicity. These insights contribute to a better understanding of viruses and their impact on various organisms. One important aspect of viral genomics is the identification of genetic variations within viral populations. This is particularly relevant for viruses that can cause severe illness, such as the flu or the coronaviruses. This can be used to develop new treatments and vaccines, track the spread of viral outbreaks, and study the evolution of viruses over time. By monitoring the genetic changes that take place within a viral population, a better understanding can be gained regarding how a virus spreads and evolves, without specifying any particular group involved in the analysis. This can be used to develop new diagnostic tests that can detect even the most recent genetic variations of a virus, as well as to design new vaccines that can protect

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against multiple strains of a virus. This can be used to develop targeted interventions and prevent further spread of the virus. Another important aspect of viral genomics is understanding how viral genes are regulated. By studying the genetic switches that control the expression of viral genes, valuable insights can be gained to develop new therapeutics capable of inhibiting virus replication. For instance, it has been discovered that certain viruses utilize specific host proteins for replication, and efforts are underway to develop drugs that can target these host proteins and impede viral replication, without specifying any particular group involved in the research. Understanding the genetic makeup of viruses can also aid in the design of vaccines that target specific viral proteins or genetic sequences, increasing their effectiveness and reducing the risk of viral escape mutants.

Overall, viral genomics is a crucial field that is advancing our understanding of viruses and their biology. This knowledge will play an important role in our understanding of viral diseases and the development of effective strategies for their prevention and treatment, new diagnostic tests, therapies, and vaccines, which can help control the spread of viral infections and improve human health.

2. Decoding the genome: A comprehensive look at viral genomics

Decoding the Genome is an important topic in the field of microbiology and infectious disease research. Viruses are obligate intracellular parasites that rely on host cells to replicate, and their genomes can be diverse and complex. Advances in genomics have revolutionized our understanding of viral biology, evolution, and pathogenesis (Chaitanya, 2019; Weitzman et al., 2010).

Viruses are small infectious agents that replicate only inside the living cells of other organisms. They can cause a wide range of illnesses in humans, from the common cold to more serious diseases such as influenza and Ebola (Lwoff, 1957). Although they are often associated with negative health effects, viruses can also have positive impacts on human health, such as by helping to fight off other infections. Viruses can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea. The study of viruses is known as virology, a subspecialty of microbiology (Notkins et al., 2012).

Virus structure is important because it determines the way a virus attacks its host cell and how to counter it. Viruses are composed of three main parts: the nucleic acid (DNA or RNA), a protein coat, and an outer cell membrane (Harrison, 2001). The composition of viruses can vary depending on the type of virus, but in general, viruses are composed of a few basic components.

1. **Nucleic Acid:** Viruses contain either DNA or RNA as their genetic material, but not both. The nucleic acid contains the genetic instructions for the virus to replicate and produce new virus particles. The nucleic acids contain a genetic blueprint for the protein coat, which is made up of 180 different proteins called capsids (Lam et al., 2018).
2. **Capsid:** The capsid is a protein shell that surrounds the nucleic acid of the virus. It provides protection for the genetic material and helps the virus to attach to and enter host cells. A virus's outer structure, "capsid," is made up of a protein shell that gives the viruses shape and protects the virus's DNA or contents from damage. Multiple copies of structural components called capsomeres compose the capsid. The capsid protects the genetic material, which may be either DNA or RNA. A virus's capsid is a protein and

lipid structure. However, some viruses have an envelope around the capsid ([Chen et al., 2011](#); [Kuznetsov et al., 2011](#); [Louten, 2016](#)).

3. **Envelope:** Some viruses have an envelope made of lipids and proteins that surrounds the capsid. This envelope helps the virus to evade the host's immune system and facilitates entry into host cells. Viruses come in a wide variety of shapes and sizes, but they all share a similar basic structure ([Castón and Carrascosa, 2013](#)). They usually have a triangular head and a long rod-like tail. The viral genome is packaged into one of these capsids and released into the host when it infects a cell ([Payne, 2022](#)). Some viruses have additional protein shells that cover their nucleic acid core and give it extra protection. This is known as a "double membrane" structure. It also increases the size of the virus and protects it from the action of the host's immune system ([Harak et al., 2015](#)). One example of a double membrane structure is Tobacco Mosaic Virus (TMV). TMV is a plant virus that affects tobacco plants. The genome of a virus is the complete set of genetic material that it carries, which can be either DNA or RNA. The genome can vary in size from a few thousand base pairs to hundreds of thousands of base pairs, depending on the virus ([Brown, 2018](#)).
4. **Surface or spike proteins:** Many viruses, particularly those with an envelope, have surface/spike proteins on their surface that help them to attach to and enter host cells. These surface proteins are often the targets of vaccines and antiviral drugs ([Vahey et al., 2019](#)).
5. **Other proteins:** Some viruses have additional proteins that are involved in viral replication and assembly ([Alberts et al., 2014](#)). M protein (matrix protein) is a structural protein found in many types of viruses, including coronaviruses, influenza viruses, and retroviruses. It plays a crucial role in virus assembly and membrane budding, which are important steps in the virus life cycle ([V'kovski et al., 2021](#)). M protein is involved in the formation of the viral envelope, which surrounds the viral nucleocapsid (the genetic material of the virus). The protein interacts with both the viral nucleocapsid and the host cell membrane to facilitate the assembly of new viral particles ([Guo et al., 2022](#)). In coronaviruses, the M protein forms a scaffold that interacts with the viral nucleocapsid and the envelope proteins to form the virus particle. It also plays a role in viral pathogenesis by interacting with host cell proteins and modulating the host immune response ([Gorkhali et al., 2021](#); [Hossain et al., 2022](#); [Yadav et al., 2021](#)). In influenza viruses, the M1 protein is responsible for maintaining the shape of the virus particle and ensuring that the viral genome is packaged correctly. It also interacts with the viral envelope proteins to facilitate virus assembly and budding ([Batishchev, 2022](#)). Generally, M protein is essential for the formation of virus particles and is considered a major driver of virus assembly and membrane budding. Understanding the role of M protein in the virus life cycle is important for the development of antiviral drugs and vaccines ([Bhatti et al., 2020](#); [Li et al., 2022](#)).

The composition of viruses is simple compared with that of cells, but it is precisely adapted to allow the virus to enter and hijack host cells for replication ([Chih et al., 2022](#)). The genome of a virus contains all the information necessary for the virus to replicate and produce new viral particles. This includes genes encoding for structural proteins that make up the virus particle, as well as enzymes and other proteins that are required for replication and other functions ([Yang et al., 2023](#)). The genomic structure of a virus can be either linear or circular and can be single-stranded or double-stranded. In some cases, the genome of a virus may be

segmented, meaning that it is divided into several distinct pieces of DNA or RNA (Roossinck, 2023). For example, the SARS-CoV-2 virus has a single-stranded RNA genome that is approximately 30,000 base pairs in length (Singh et al., 2020, pp. 5–29). The genome is composed of several genes, including those that encode for the viral spike protein, which is used by the virus to enter host cells, and the RNA-dependent RNA polymerase, which is required for viral replication (Astuti, 2020). In contrast, the human papillomavirus (HPV) has a double-stranded DNA genome that is approximately 8000 base pairs in length. The genome of HPV is circular and contains several open reading frames (ORFs) that encode for viral proteins involved in replication and pathogenesis (Marković et al., 2021) (Fig. 2.1).

RNA viruses are viruses that have RNA as their genetic material, rather than DNA (Knipe et al., 2013). RNA viruses can be further divided into several groups based on the type of RNA that they carry and their genome structure. Some of the main groups of RNA viruses include:

Positive-sense RNA viruses: These viruses have a single-stranded RNA genome that can be directly translated by host cells into viral proteins (Berman, 2012, p. 237). Examples of positive-sense RNA viruses include the coronaviruses (such as SARS-CoV-2), the picornaviruses (such as poliovirus and rhinovirus), and the flaviviruses (such as Zika virus and West Nile virus).

Negative-sense RNA viruses: These viruses have a single-stranded RNA genome that must be converted into a positive-sense RNA intermediate before viral proteins can be produced (Te Velthuis et al., 2021). Examples of negative-sense RNA viruses include the paramyxoviruses (such as measles virus and mumps virus) and the filoviruses (such as Ebola virus).

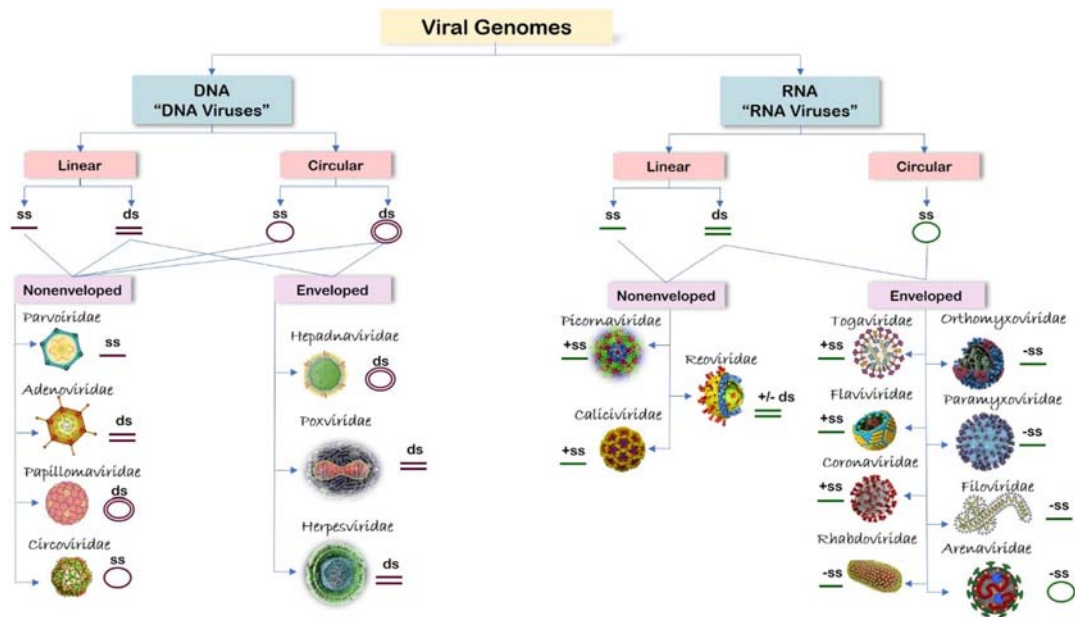


FIGURE 2.1 Decoding viral genomes: Schematic diagram unraveling the diversity of DNA and RNA viruses—enveloped and nonenveloped, double and single strands, negative and positive sense.

Double-stranded RNA viruses: These viruses have a double-stranded RNA genome that can be directly translated by host cells into viral proteins. Examples of double-stranded RNA viruses include:

1. **Reoviruses:** These are nonenveloped viruses that can cause respiratory, gastrointestinal, and neurological diseases in humans and animals (Bennett et al., 2019).
2. **Rotaviruses:** These are also nonenveloped viruses that are a leading cause of severe diarrhea in young children worldwide (Tate et al., 2016).
3. **Birnaviruses:** These are enveloped viruses that can infect fish and birds and can cause diseases such as infectious pancreatic necrosis in salmon and bursal disease in poultry (Vishwanatha et al., 2022).
4. **Orthoreoviruses:** These are nonenveloped viruses that can infect a wide range of animals, including humans, and can cause respiratory, gastrointestinal, and neurological diseases (Dermody et al., 2013).

Retroviruses: These viruses have a single-stranded RNA genome that is converted into DNA by the viral enzyme reverse transcriptase. The viral DNA is then integrated into the host cell's genome, where it can be transcribed and translated into viral proteins. Examples of retroviruses include human immunodeficiency virus (HIV) and human T-cell lymphotropic virus (HTLV) (Julia et al., 2023; Richman et al., 2020). RNA viruses are known for their high mutation rates, which can result in the emergence of new viral strains with different properties, such as increased transmissibility or virulence. This can make it challenging to develop effective treatments and vaccines against RNA viruses. However, understanding the genomic structure and replication cycle of RNA viruses can help in the development of antiviral drugs and vaccines that target specific aspects of the viral life cycle.

3. Exploring viral evolution through genomic analysis

The study of viral evolution has undergone significant advancements in recent decades, focusing on the utilization of genomic analysis to unravel the mysteries surrounding the evolutionary processes of viruses. Through the analysis of viral genomes, valuable insights are gained into their evolutionary trajectory, encompassing aspects such as their origins, genetic modifications that have transpired over time, and the mechanisms through which viruses adapt to new hosts or environments (Bedford et al., 2015; Duffy et al., 2008; Lu Ma et al., 2020, pp. 565–574). Next-generation sequencing (NGS) stands as a highly potent tool in investigating viral evolution, enabling the swift sequencing of substantial amounts of genetic material derived from viral samples. Through the comparison of sequences among diverse viral strains, valuable information is gleaned regarding the mutations that have arisen over time, facilitating a comprehensive understanding of the virus's evolutionary journey and adaptive mechanisms (Duffy et al., 2008; Fauver et al., 2020; Srinivas & Prasad, 2023). Genomic analysis plays a crucial role in tracing the dissemination of viral outbreaks. By examining the genomes of viral samples obtained from various geographical locations, valuable information is obtained regarding the specific viral strains accountable for the outbreak and their subsequent spread. This analysis aids public health officials in devising effective

strategies to contain the outbreak and mitigate its consequences (Dinh et al., 2017; Hadfield et al., 2018; Lemey et al., 2018). Genomic analysis unveils valuable insights into the mechanisms employed by viruses to adapt to novel hosts or environments. By comparing the genomes of distinct viral strains, specific mutations linked to alterations in virulence, transmission, or host range can be identified. This knowledge deepens our understanding of how viruses evolve to surmount host defenses, acclimate to new surroundings, and propagate more effectively (Baric et al., 1999; Parrish et al., 2008).

NGS has been extensively employed in investigating the evolution of diverse viruses such as influenza, HIV, Ebola, and Zika virus. For instance, NGS has proven instrumental in monitoring the global dissemination of distinct influenza virus strains, empowering the anticipation of prevalent strains in forthcoming flu seasons (Faria et al., 2016a; Goldberg et al., 2015; Handel et al., 2021; Heena et al., 2023; Quick et al., 2016). Apart from NGS, various tools are utilized, including phylogenetic analysis and molecular clock analysis, to delve into viral evolution. These approaches facilitate the reconstruction of the evolutionary lineage of viruses, estimation of mutation rates, and identification of key factors that have influenced viral evolution over time (Hadfield et al., 2018; Viral genome sequence datasets display, 2023; Holmes, 2008; Wertheim et al., 2011; Bouckaert et al., 2014).

3.1 Genomic analysis techniques

Genomic analysis techniques are essential tools used to study the genetic makeup of organisms, including viruses. Genomic analysis assumes a crucial role in investigating virus evolution, facilitating the examination of mutations and alterations that occur within the viral genome over time. Here are some commonly used genomic analysis techniques in virus evolution research.

3.2 Viral genomes' sequencing

Traditionally, Sanger sequencing has been the primary method for sequencing the genetic material of viruses. This method involves the use of dideoxynucleotides to stop the extension of a DNA chain, resulting in a set of fragments of varying lengths that represent the sequence of the DNA being sequenced. These fragments are separated by size using gel electrophoresis and then read using a fluorescence-based method. While still used today for certain applications, Sanger sequencing is limited in terms of throughput and is relatively expensive (Jakleyezqv, 2023; Luring et al., 2010).

NGS technologies have revolutionized the field of viral genomics by providing a much higher throughput and lower cost. NGS methods vary, but they generally involve breaking the viral genome into small pieces, amplifying these pieces, and then sequencing them in parallel using different approaches such as Illumina, PacBio, and Oxford Nanopore Technologies (Goodwin et al., 2016; Mardis, 2013; Shendure et al., 2008; Thomson et al., 2016).

Illumina sequencing, which is the most widely used NGS technology, works by fragmenting the viral genome, amplifying these fragments using PCR, and then sequencing them on a flow cell where they are read by fluorescently labeled nucleotides (Bentley et al., 2008; Khalifa et al., 2016; Metzker, 2010; Shendure et al., 2008). PacBio sequencing involves real-time

sequencing of individual DNA molecules, which allows for longer reads and improved accuracy (Chin et al., 2013; Eid et al., 2009; Hufnagel et al., 2020; Rhoads et al., 2015). Oxford Nanopore Technologies use nanopores to directly sequence DNA or RNA molecules as they pass through a nanopore, allowing for long reads and rapid sequencing times (Goodwin et al., 2016; Jain et al., 2015; Lu et al., 2016; Simpson et al., 2017). Here are some references for software commonly used for sequencing viral genomics:

1. CLC Genomics Workbench: <https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>
2. Geneious: <https://www.geneious.com/>
3. IDseq: <https://idseq.net/>
4. ARTIC Network: <https://artic.network/>
5. Nextclade: <https://clades.nextstrain.org/>
6. VarScan: <http://varscan.sourceforge.net/>
7. GATK: <https://gatk.broadinstitute.org/hc/en-us>

After sequencing, the data must be processed and analyzed to assemble the viral genome and identify variations. This is typically done using specialized software and bioinformatics tools that align the reads to a reference genome, detect and remove errors, and call variants. The resulting genome sequence can then be used for a range of applications, such as identifying the virus, tracking its spread, and studying its molecular biology. Overall, NGS technologies have revolutionized the field of viral genomics by providing higher throughput and lower costs compared with traditional Sanger sequencing.

3.3 Phylogenetic analysis

Phylogenetic analysis involves the reconstruction of evolutionary relationships between organisms or sequences. In the context of virus evolution, phylogenetic analysis can be used to reconstruct the evolutionary history of viruses, identify the origins of viral outbreaks, and track the spread of viral strains (Bouckaert et al., 2014; Ghafari et al., 2022; Srinivas & Prasad, 2023).

3.4 Molecular clock analysis

Molecular clock analysis is a method that uses the rate of evolution of DNA or protein sequences to estimate the time of divergence between different lineages. This technique can be used to estimate the time of origin of viral outbreaks and to track the rate of viral evolution over time (Drummond et al., 2015; Sanjuin et al., 2010; Worobey et al., 2014).

3.5 Genome assembly

Genome assembly involves the reconstruction of the complete genome sequence of a virus from short DNA or RNA reads generated by NGS. This technique is critical for studying the genetic changes that occur during viral evolution and for identifying the mutations that confer new phenotypes or virulence (Berlin et al., 2015; Li, 2011; Nagarajan et al., 2013).

3.6 Functional genomics

Functional genomics involves the study of the functional elements within a genome, such as genes, regulatory sequences, and noncoding RNAs. This technique can be used to identify the genetic changes that contribute to the evolution of viral phenotypes, such as changes in virulence or host range (Corman et al., 2019; Ford et al., 2019; Teng et al., 2021).

Various constantly evolving tools and software programs are available for genomic analysis in virus evolution research. Through the integration of various techniques, a comprehensive understanding of viral emergence, spread, and genetic alterations can be attained. The selection of suitable tools and software relies on the research objectives, data type being analyzed, and the computational resources available. Commonly used tools include those for genetic and epigenetic analysis, and there are many others with unique strengths and limitations. There are many tools and software programs available for genomic analysis, each with its own strengths and limitations.

There are a variety of bioinformatics tools and techniques used to analyze the large amounts of data generated by sequencing viral genomes. Here are some examples shown in Table 2.1.

3.7 Genomic analysis in viral evolution

Viruses are known to rapidly mutate and recombine, leading to the emergence of new strains and the evolution of existing ones. Genomic analysis can help to identify these changes and track the spread of new virus strains. For example, genomic analysis has been used to track the spread of the COVID-19 virus and its various variants, including the Alpha, Beta, Gamma, and Delta variants, by identifying the mutations in the spike protein that affect its binding affinity to the human ACE2 receptor and the antibody escape (Bedford et al., 2020; Volz et al., 2021; Tegally et al., 2020; for Immunization, 2021). Genomic analysis can help to identify mutations in the viral genome that confer drug resistance and develop new drugs that target these mutations as in the Hepatitis C virus genome (Fridell et al., 2010).

In addition, genomic analysis was used to track the origins and spread of the Ebola virus outbreak in West Africa in 2014–16 (Gire et al., 2014; Park et al., 2015; Quick et al., 2016). This was critical in developing effective control measures to contain the outbreak. The analysis can also help to identify the reservoirs and vectors of viruses, such as bats for Ebola, mosquitoes for Zika, and camels for MERS (Carroll et al., 2015; Dudas et al., 2017; Gire et al., 2014; Kuhn et al., 2013; Marí Saéz et al., 2015; Team, 2014; Tong et al., 2015). Another example of how genomic analysis has been used to understand viral evolution is in the case of the Zika virus outbreak in the Americas in 2015–16. Genomic analysis was employed to sequence the genomes of Zika virus samples obtained from diverse geographic regions, enabling a comparative study with other closely related viruses such as dengue virus and West Nile virus (Faria et al., 2016b; Metsky et al., 2017). Moreover, the origins of the Zika virus outbreak were successfully traced by examining evidence indicating the initial introduction of the virus into Brazil during 2013 or 2014, presumably originating from a Pacific region outbreak. Additionally, distinct genetic alterations within the virus were identified, linked to heightened virulence and transmission, potentially exacerbating the severity of the outbreak (Faria et al., 2016c, 2017; Lanciotti et al., 2016).

TABLE 2.1 Common genomic analysis tools used in virus evolution research.

Techniques	Function/role	Tools	References
1. Genome assemblers	One of the first steps in analyzing viral genomes is to assemble the sequencing reads into a contiguous sequence. Genome assemblers are software programs that take short DNA or RNA reads generated by NGS and assemble them into a complete genome sequence.	SPAdes, ABySS, and Velvet	Bankevich et al. (2012) , Simpson et al. (2009) , Zerbino (2008) , and Zerbino et al. (2008)
2. Genome annotation	Once the genome has been assembled, the next step is to annotate the genes and other functional elements. This can be done using different tools, which uses a variety of gene prediction algorithms to identify open reading frames and other features.	Prokka	Seemann (2014)
3. Comparative genomics	Comparing viral genomes can provide insights into their evolution, pathogenicity, and host range. This can be done using tools, which align multiple genomes to identify conserved regions and differences between them.	Mauve or MAFFT	Katoh et al. (2009)
4. Alignment software	Alignment software is used to compare DNA or protein sequences to identify similarities and differences.	ClustalW, MAFFT, and MUSCLE	Edgar (2004) , Edgar et al. (2006) , Katoh et al. (2019) , and Larkin et al. (2007)
5. Phylogenetic analysis software	Phylogenetic analysis software is used to reconstruct evolutionary relationships between organisms or sequences.	BEAST, PhyML, MrBayes, and RAxML	Drummond et al. (2012) , Ronquist et al. (2012) , and Stamatakis (2014)
6. Molecular clock analysis software	Molecular clock analysis software is used to estimate the rate of evolution of DNA or protein sequences and to calculate the time of divergence between different lineages.	BEAST and MEGA	Drummond et al. (2007) and Kumar et al. (2018)
7. Variant callers	Variant callers are software programs that identify single nucleotide polymorphisms (SNPs) and other genetic variations in DNA or RNA sequences.	GATK, FreeBayes, and VarScan	Garrison et al. (2012) , Koboldt et al. (2012) , and McKenna et al. (2010)
8. Gene ontology analysis software	Gene ontology analysis software is used to annotate genes and other genomic features with functional annotations.	DAVID, PANTHER, and Gene Ontology	Consortium (2019) , Huang et al. (2009) , and Mi et al. (2013)
9. Genome browser	Genome browsers are web-based tools that allow visualization and exploring genomic data.	UCSC genome browser, Ensembl genome browser, and NCBI genome data viewer	Kent et al. (2002) , Rangwala et al. (2021) , and Zerbino et al. (2018)

(Continued)

TABLE 2.1 Common genomic analysis tools used in virus evolution research.—cont'd

Techniques	Function/role	Tools	References
10. Structural variant callers	Structural variant callers are software programs that identify larger genomic variations, such as insertions, deletions, inversions, and duplications, that are not easily detectable by variant callers.	Lumpy, Delly, and Manta	Chen et al. (2016) , Layer et al. (2014) , and Rausch et al. (2012)
11. Transcriptome assemblers	Transcriptome assemblers are software programs that take short RNA reads generated by NGS and assemble them into a complete transcriptome, which is the set of all RNA transcripts produced by a cell or tissue.	Trinity, Oases, and Trans-ABYSS	Grabherr et al. (2011) , Robertson et al. (2010) , and Schulz et al. (2012)
12. Gene expression analysis software	Gene expression analysis software is used to quantify and compare the expression levels of genes or transcripts across different conditions or tissues.	DESeq2, edgeR, and Limma	Chen et al. (2014) , Love et al. (2014) , Ritchie et al. (2015) , and Robinson et al. (2010)
13. Epigenetic analysis software	Epigenetic analysis software is used to analyze epigenetic modifications, such as DNA methylation and histone modifications, that can influence gene expression and genomic stability.	Bismark, HOMER, and ChIP-seq Peak Caller	Heinz et al. (2010) , Krueger et al. (2011) , and Zhang et al. (2008)
14. Pathway analysis	Pathway analysis can be used to identify the biological pathways that are affected by viral infection. This can be done using tools, which provide curated databases of biological pathways and allow for the identification of pathway enrichment or depletion.	KEGG or Reactome	Kanehisa (2002)
15. Functional enrichment analysis	Functional enrichment analysis can be used to identify the biological processes, molecular functions, or cellular components that are overrepresented among a set of genes or proteins.	GO enrichment analysis or KEGG pathway enrichment analysis	Kanehisa (2002)
16. Genome annotation software	Genome annotation software is used to annotate genomic features, such as genes, promoters, enhancers, and regulatory elements, with functional annotations.	MAKER, AUGUSTUS, and Prokka	Cantarel et al. (2008) , Seemann (2014) , and Stanke et al. (2008)
17. Data visualization	Data visualization can be used to explore and communicate the results of bioinformatics analysis. This can be done using tools, which create visualizations of genomic data and network graphs, respectively.	Circos or Gephi	Cherven (2013) and Krzywinski (2011)
18. Network analysis software	Network analysis software is used to analyze and visualize complex biological networks, such as protein-protein interaction networks and metabolic pathways.	Cytoscape, Pathway Tools, and STRING	Karp et al. (2002) , Shannon et al. (2003) , and Szkarczyk et al. (2019)

TABLE 2.1 Common genomic analysis tools used in virus evolution research.—cont'd

Techniques	Function/role	Tools	References
19. Genome-wide association study (GWAS) software	GWAS software is used to identify genetic variants associated with complex traits or diseases in large populations.	PLINK, GCTA, and SNPTEST	Marchini (2010), Purcell et al. (2007), and Yang et al. (2013)
20. Metagenomic analysis software	Metagenomic analysis software is used to analyze microbial communities in various environments, such as soil, water, and human gut.	QIIME, MG-RAST, and MetaPhlAn.	Buvé et al. (2014), Meyer et al. (2008), and Truong et al. (2015)
21. CRISPR/Cas analysis software	CRISPR/Cas analysis software is used to analyze and annotate CRISPR arrays and cas proteins in bacterial and archeal genomes.	CRISPRCasFinder, CRISPRDetect, and CRISPRone	Biswas et al. (2016), Couvin et al. (2018), and Gupta et al. (2021)
22. Viral quasispecies analysis software	Viral quasispecies analysis software is used to analyze the diversity and evolution of viral populations within an infected host.	QuRe, VIRUSorter, and ViQuaS	Londhe et al. (2021)
23. Antibiotic resistance analysis software	Antibiotic resistance analysis software is used to analyze genomic data to identify antibiotic resistance genes and mutations in bacterial genomes.	ResFinder, ARG-ANNOT, and AMRFinder	Alcock et al. (2020), Feldgarden et al. (2021), and Zankari et al. (2012)
24. Epistasis analysis software	Epistasis analysis software is used to analyze genetic interactions between different genetic variants that affect complex traits or diseases.	PLINK, MDR, and BOOST	Purcell et al. (2007), Ritchie et al. (2001), and Wan et al. (2010)
25. Protein structure prediction	Protein structure prediction can be used to predict the 3D structure of viral proteins, which can help to understand their function and identify potential drug targets. This can be done using tools, which use algorithms to predict protein structures based on sequence data.	Phyre2 or I-TASSER	Manjusha et al. (2019) and Zheng et al. (2019)
26. Epitope prediction	Epitope prediction can be used to identify potential antigenic sites on viral proteins, which can inform the development of vaccines or diagnostic assays. This can be done using tools, which predict the binding of peptides to major histocompatibility complex (MHC) molecules.	NetMHC or ABCpred	Mohammed et al. (2021) and Soria-Guerra et al. (2015)
27. Genome editing software	Genome editing software is used to design and optimize guide RNAs for CRISPR/Cas or other genome editing tools, and to predict off-target effects.	CRISPOR, CHOPCHOP, and sgRNACas9	Concordet et al. (2018), Labun et al. (2019), and Xie et al. (2014)

Influenza virus: Genomic analysis has been used extensively to study the evolution of influenza viruses, which undergo frequent genetic changes due to their high mutation rates. Through the analysis of genomes from diverse influenza virus strains, specific mutations linked to alterations in virulence, host range, and transmission have been identified (Bedford et al., 2015; Koelle et al., 2015; Smith et al., 2009a; Taubenberger et al., 2010). Influenza virus has a segmented genome consisting of eight RNA segments, which allows for genetic reassortment or “antigenic shift” to occur when two different influenza viruses infect the same host cell. This can lead to the emergence of new strains that can cause pandemics (Bouvier et al., 2008; Medina et al., 2011).

The evolution of influenza virus over time and the emergence of new strains can be tracked through genomic analysis. For instance, in 2009, a novel H1N1 influenza virus emerged, resulting in a global pandemic that rapidly spread across the world. Genomic analysis revealed that this virus was a reassortant, containing genetic material from human, swine, and avian influenza viruses. Through tracing the origins and transmission patterns of the virus, targeted interventions were devised to control the pandemic and preempt potential future outbreaks (Garten et al., 2009; Team, 2009; Weaver et al., 2016).

Genomic analysis has also been used to identify changes in the surface proteins of influenza virus that can lead to the emergence of new strains. The surface proteins hemagglutinin (HA) and neuraminidase (NA) are the primary targets of the immune response to influenza virus, and mutations in these proteins can allow the virus to escape immunity. Sequencing the genome of influenza virus strains enables the identification of mutations in the HA and NA genes, which could potentially enable the virus to evade the immune response (Garten et al., 2009; Nelson et al., 2008; Russell et al., 2008; Smith et al., 2009b; Xu et al., 2008). Another important application of genomic analysis of influenza virus has been in the development of vaccines. The World Health Organization (WHO) monitors the evolution of influenza virus and recommends changes to the vaccine formulation each year to ensure that the vaccine is effective against circulating strains. Genomic analysis is used to identify the strains that are most likely to be circulating in the next flu season, and this is used to develop the vaccine (Abou El Naja et al., 2022; Control and C, 2021; Treanor, 2004).

So, we can summarize some examples of how genomic analysis has been used to understand influenza virus evolution in these points:

1. Next-generation sequencing has been used to sequence and compare the genomes of different influenza virus strains. This has enabled the identification of genetic changes that have transpired over time, encompassing point mutations, deletions, insertions, and reassortments, without specifying any particular group involved in the analysis (Taubenberger et al., 2010; Team, 2009; Yoon et al., 2014).
2. Phylogenetic analysis has been used to reconstruct the evolutionary history of influenza viruses. Comparing the genetic sequences of distinct influenza virus strains enables the construction of evolutionary trees that illustrate the relationships between different strains (Bhatt et al., 2011; Hou et al., 2016; Rambaut et al., 2008; Tong et al., 2012).
3. Genomic analysis has also been used to study the evolution of specific influenza virus genes, such as the hemagglutinin (HA) and neuraminidase (NA) genes. These genes

encode proteins that are important for viral entry and release and are often the targets of host immune responses (Bhatt et al., 2011; Neumann et al., 2004; Yao et al., 2022; Zhu et al., 2014).

4. Genomic analysis has also been used to study the evolution of influenza viruses in different host species, including birds, humans, and pigs. Comparing the genetic sequences of influenza viruses from diverse host species provides valuable insights into the adaptation of influenza viruses to novel hosts (Medina et al., 2011; Neumann and Kawaoka, 2004; Shih et al., 2007).

Genomic analysis assumes a critical role in comprehending the evolution of influenza and SARS-CoV-2 and formulating strategies to curb their transmission. By shedding light on the genetic alterations transpiring within viral populations over time, genomic analysis aids in the development of enhanced tools for the prevention and treatment of viral infections.

4. From genome to pandemic: Understanding viral genomics in the era of COVID-19

The story of SARS-CoV-2, the virus responsible for the COVID-19 pandemic, is one of rapid evolution and global spread. Here is a brief summary of how the virus went from being a novel coronavirus to a pandemic that has affected millions of people worldwide.

4.1 Genomic origin

SARS-CoV-2 is a betacoronavirus that is closely related to the SARS-CoV virus that caused the SARS outbreak in 2003. The virus likely originated in bats and was transmitted to humans through an intermediate host, possibly a pangolin, at a wet market in Wuhan, China, in late 2019 (Lam et al., 2020; Wu et al., 2020; Zhang, Wu, & Zhang, 2020; Zhou et al., 2020).

Viral genomics has helped identify the origin and spread of SARS-CoV-2. The genome of the virus was sequenced early in the pandemic, aiding in the identification of the virus as a novel coronavirus and its potential origins in bats, with transmission to humans potentially occurring through an intermediate host, possibly a pangolin. This determination is supported by the discovery of a bat coronavirus that closely resembles SARS-CoV-2, as well as the knowledge that pangolins are traded in the Wuhan wet market where the initial cases of COVID-19 were reported. Furthermore, the spread of the pandemic has been effectively tracked, new variants of the virus have been detected, and a comprehensive understanding of the transmission dynamics of the virus has been achieved, without specifying any particular group involved in the research (S. Liu et al., 2020; R. Lu et al., 2020; Wang et al., 2020; Zhu et al., 2014).

4.2 Outbreak in China

The first cases of COVID-19 were reported in Wuhan in December 2019, with the initial cases linked to the Huanan Seafood Wholesale Market. The Chinese government quickly

implemented strict measures to contain the outbreak, including lockdowns and travel restrictions, but the virus continued to spread (Lu, Ma et al., 2020, pp. 565–574; Yang et al., 2020).

4.3 Global pandemic

By March 2020, the virus had spread to every continent, leading the World Health Organization to declare a global pandemic. Countries around the world implemented measures to slow the spread of the virus, including social distancing, mask mandates, and widespread testing. Genomic analysis has played a critical role in understanding the origins and evolution of SARS-CoV-2, the virus responsible for the COVID-19 pandemic. By sequencing the genomes of viral samples obtained from diverse geographic regions, multiple genetic lineages of the virus have been identified, and the global spread of the virus has been traced (Andersen et al., 2020; Fauver et al., 2020; Forster et al., 2020; Grubaugh et al., 2020; Phillips, 2021; Pinoli et al., 2021; Van Dorp et al., 2020). Genomic analysis has also provided insights into the mechanisms by which the virus has adapted to infect humans and spread more efficiently (Hu et al., 2020; Li et al., 2020; Z. Liu et al., 2021; Pachetti et al., 2020; Plante et al., 2021).

Additionally, genomic analysis has been instrumental in the development of diagnostic tests, therapies, and vaccines for SARS-CoV-2. By identifying the crucial genes and proteins involved in viral replication and pathogenesis, targeted therapies and vaccines have been developed to cater to the specific genetic characteristics of the virus (Petherick, 2020; Russell et al., 2020; Wiersinga et al., 2020).

Genomic analysis of SARS-CoV-2, the virus responsible for the COVID-19 pandemic, has provided important insights into its evolution and transmission patterns. The SARS-CoV-2 genome spans approximately 30,000 nucleotides and exhibits numerous mutations. These mutations have enabled the tracking of its global spread and provided insights into its evolutionary trajectory over time (Bedford et al., 2020; S. Liu et al., 2020; R. Lu et al., 2020; Wang et al., 2020).

4.4 Identification of new variants

One of the most important applications of genomic analysis of SARS-CoV-2 has been in the identification of new variants of the virus. These variants can arise when the virus replicates and mutates, and some variants may be more transmissible or more virulent than others. By sequencing the genomes of SARS-CoV-2 from diverse samples, new variants can be identified, and their spread can be tracked without the direct involvement of individuals conducting the research (Korber et al., 2020; Webb et al., 2021).

For example, the Alpha variant (B.1.1.7) was first identified in the United Kingdom in December 2020 and has since spread to many other countries. Genomic analysis has shown that the Alpha variant has multiple mutations in the spike protein of the virus, which may make it more transmissible than earlier versions of the virus. Similarly, the Delta variant (B.1.617.2) was first identified in India in December 2020 and has since become the dominant variant in many countries. The Delta variant has mutations in the spike protein that may make it more transmissible and potentially more resistant to some COVID-19 vaccines (England, 2021a,b; Khandia et al., 2022; Y. Liu et al., 2021).

4.5 Vaccine development

The rapid development of vaccines has been a significant scientific achievement in the fight against COVID-19. Multiple vaccines have been authorized for emergency use by various regulatory agencies, with the Pfizer-BioNTech, Moderna, and Johnson & Johnson vaccines being widely used in many countries. Vaccination campaigns are ongoing, but challenges remain in ensuring equitable distribution and overcoming vaccine hesitancy (Polack et al., 2020; Baden et al., 2021; Sadoff et al.).

The utilization of viral genomics has played a crucial role in the advancement of effective treatments and vaccines for COVID-19. Through the examination of the genomic composition of the virus, potential targets for drugs have been identified, and vaccines have been developed to specifically target distinct components of the virus. For example, the Pfizer and Moderna vaccines use mRNA technology to target the spike protein of SARS-CoV-2. This technology was made possible by the identification of the genomic sequence of the virus (Anderson et al., 2020; Cao et al., 2020; Jackson et al., 2020; Zhu et al., 2020). In addition, the antiviral drug remdesivir was developed based on its ability to target the viral RNA polymerase, which is critical for the replication of the virus. This and other drugs have been used to treat COVID-19 patients and reduce the severity of the disease (Beigel et al., 2020; Pruijsers et al., 2019).

In addition to the development of vaccines, genomic sequencing has facilitated the discovery of novel treatments for COVID-19. Potent neutralizing antibodies against SARS-CoV-2, the causative virus of COVID-19, have been identified through high-throughput single-cell sequencing of B cells from convalescent patients. These antibodies could potentially be used to treat severe cases of COVID-19 or to prevent infections in high-risk individuals (Cao et al., 2020; Chen et al., 2021; L. Liu et al., 2020; Suryadevara et al., 2021; Zost et al., 2020).

While much of the focus on COVID-19 has been on the acute phase of the illness, there is growing recognition of the long-term consequences of the disease, known as postacute COVID-19 syndrome (PACS). Patients with PACS may experience a range of symptoms, including fatigue, cognitive impairment, and respiratory problems, for several months after their initial infection. Genomic sequencing could potentially be used to understand the underlying mechanisms of PACS and develop more targeted treatments for affected individuals (Carfi et al., 2020; de Araújo et al., 2021; Huang et al., 2021; Nalbandian et al., 2021).

Finally, genomic sequencing has been employed to investigate the immune response to COVID-19, shedding light on the topic without the direct involvement of individuals conducting the research. Robust T-cell immunity has been identified in convalescent individuals who experienced asymptomatic or mild cases of COVID-19. These findings indicate that even mild infections may provide a certain level of immunity against the virus. They have also identified targets of T-cell responses to SARS-CoV-2 in humans with COVID-19 disease and unexposed individuals, which could inform vaccine development and public health strategies (Grifoni et al., 2020; Lipsitch et al., 2020; Neidleman et al., 2021; Sekine et al., 2020).

In conclusion, genomic epidemiology has been a critical tool in the fight against COVID-19, providing valuable insights into the origin, transmission, and evolution of the virus. It has also played a significant role in vaccine development, treatment development, and

understanding the long-term consequences of the disease. As the pandemic continues to evolve, genomic sequencing will likely continue to play a key role in our efforts to control the spread of the virus and develop more effective treatments and vaccines.

5. Using genomics to predict the next viral outbreak

Genomics has become an increasingly important tool for identifying emerging viral threats. One approach is to conduct active surveillance for new viruses in animal populations that may pose a risk to human health. Another approach is to identify viruses that have the potential to mutate and spread more easily in humans. In 2012, a novel strain of avian influenza, H7N9, was detected in human populations in China, without the active involvement of anyone in this identification process. Through genome sequencing, it was determined that the virus had undergone genetic alterations, enabling it to enhance its infectivity in human hosts. This was critical in developing effective strategies for controlling the spread of the virus (Gao et al., 2013). Once a new viral threat has been identified, genomic surveillance can be used to track its spread and evolution. This involves sequencing the genomes of viruses collected from infected individuals and comparing them with other sequences in a database to identify patterns of transmission and evolution (Dudas et al., 2017; Lemey et al., 2018; Quick et al., 2017). For example, genomic surveillance played a critical role in tracking the spread of the Ebola virus during the 2014 outbreak in West Africa. Sequencing was utilized to identify multiple independent introductions of the virus into the region and to trace its transmission between countries. This guided the response to the outbreak, including the allocation of resources to the most impacted areas and the formulation of effective treatment strategies, without direct involvement of individuals conducting the research (Gire et al., 2014).

Genomic surveillance is also being used to track the spread of COVID-19. Thousands of viral genomes from around the world have been sequenced to identify various lineages and variants of the virus. This played a pivotal role in comprehending the global spread of the virus and formulating effective public health responses, without direct involvement of individuals conducting the research (Faria et al., 2021; Hadfield et al., 2018; Rambaut et al., 2020).

Some of the key ways that genomics can aid in predicting the next viral outbreak include:

1. Identifying new viruses: By sequencing the genetic material of viruses found in animals or humans, the genetic material of viruses can be analyzed to identify new viral strains and evaluate their potential to cause disease, without specifying any particular group involved in the analysis.
2. Tracking the spread of outbreaks: Genomics can be used to track the spread of viral outbreaks by comparing the genetic sequences of viruses from different individuals or locations. This can help to identify the source of an outbreak and determine how it is spreading.
3. Developing diagnostic tests: Once a new viral strain has been identified, genomics can be used to develop diagnostic tests that can quickly identify the virus in infected individuals.

4. Developing treatments and vaccines: Genomics can also aid in the development of treatments and vaccines by providing insights into the genetic makeup of the virus and how it interacts with the host immune system.

There are several genomics prediction tools that can be used to predict the next viral outbreak. These tools use various data sources and analytical methods to identify and analyze emerging viral strains and predict their potential to cause disease. Here are some examples in [Table 2.2](#).

In conclusion, genomics has the potential to revolutionize the way we predict and respond to viral outbreaks. By identifying emerging viral threats and tracking their spread, genomics can enable more targeted and effective public health interventions. However, there are also challenges to using genomics for outbreak response, including the cost and complexity of sequencing viral genomes and the need for more research to understand how to interpret genomic data. Nevertheless, the potential benefits of genomics for outbreak response make it a critical area of research for the future.

TABLE 2.2 Some common tools that are used in prediction of the next viral outbreak.

Prediction tool	Role	References
1. Global Initiative on Sharing All Influenza Data (GISAID)	This is a global database of influenza virus genomes that is freely available around the world. GISAID enables tracking the evolution and spread of influenza viruses and identifying emerging strains that have the potential to cause a pandemic, without specifying any particular group involved in the tracking and identification process.	Elbe et al. (2017) , Khare et al. (2021) , and Shu et al. (2017)
2. Nextstrain	This is an open-source platform that facilitates tracking the spread of viral outbreaks in real-time. Nextstrain utilizes genomic data to reconstruct the evolutionary history of viral strains and monitor their geographical spread, without specifying any particular group involved in the tracking and analysis. This can help to identify the source of an outbreak and track how it is spreading.	Hadfield et al. (2018) and Nextstrain (2021)
3. Metagenomics	This is a technique that involves sequencing all the genetic material in a given sample, such as a swab from an infected person or an environmental sample. Metagenomics can be used to identify new viral strains and assess their potential to cause disease.	Bragg et al. (2014) , Mokili et al. (2012) , and Steele et al. (2005)
4. Machine learning algorithms	These are computational models that can be trained on genomic data to predict the emergence of new viral strains or the likelihood of an outbreak. Machine learning algorithms can be used to analyze large datasets of genomic data and identify patterns that are indicative of an emerging viral threat.	Bonaccorso (2017) , Mahesh (2020) , and Mohammed et al. (2016)

(Continued)

TABLE 2.2 Some common tools that are used in prediction of the next viral outbreak.—cont'd

Prediction tool	Role	References
5. Viral Epidemiology Signature Analysis (VESPA)	This is a computational tool that can be used to identify key genetic signatures associated with viral outbreaks. VESPA uses machine learning algorithms to analyze genomic data and identify patterns that are indicative of an emerging viral threat.	Barik et al. (2020, 2022) and Gaballah et al. (2018)
6. Phylogeotool, or Phylogeographic Tool	Refers to a software or tool used in the field of phylogeography. Phylogeography is the study of the historical processes that have shaped the geographic distribution of genetic lineages, typically within a species. It combines principles from phylogenetics (the study of evolutionary relationships) and biogeography (the study of species distribution patterns). Some examples include BEAST (Bayesian evolutionary analysis by sampling trees), BEAST2, Phylogeographer, and GeoPhyloMaker. The most appropriate tool based on their specific research questions, data types, and analytical requirements can be selected.	Baele et al. (2018) , Libin et al. (2017) , and Theys et al. (2019)
7. Influenza Risk Assessment Tool (IRAT)	This is a tool developed by the US Centers for disease control and prevention (CDC) that can be used to assess the pandemic risk posed by new influenza strains. IRAT uses a combination of genomic, epidemiological, and clinical data to evaluate the likelihood that a new influenza strain will cause a pandemic.	Burke et al. (2018) and Trock et al. (2012)
8. Genome Detective	This is a web-based platform that allows users to analyze genomic data and identify potential pathogenicity and drug resistance of a viral strain. Genome Detective uses a combination of genomic and bioinformatics tools to analyze genomic data and identify key genetic features that are associated with pathogenicity and drug resistance.	Cleemput et al. (2020) and Vilsker et al. (2019)

6. The promise of viral genomics: Developing vaccines, therapies for emerging diseases and other applications of viral genomics

Viral genomics plays a crucial role in advancing our understanding of viruses and their interactions with hosts. Sequencing and analyzing viral genomes enable the identification of new viral species, tracking viral evolution, and gaining insights into the interactions between viruses and host cells, without the direct involvement of individuals conducting the analysis. This knowledge can be used to develop targeted vaccines and therapies and inform outbreak response strategies. The scope of viral genomics extends beyond developing vaccines and therapies for emerging diseases, with the potential to improve our understanding of virology and enhance prevention, diagnosis, and treatment of viral infections ([Vilsker et al., 2019](#); [Weiss et al., 2020](#)).

Viral genomics has numerous applications in a variety of fields, including:

1. **Diagnosis of viral infections:** Viral genomics can facilitate the accurate diagnosis of viral infections by identifying specific viral genetic sequences present in patient samples (N. Zhang et al., 2020). This can help healthcare professionals to distinguish between different viruses that may cause similar symptoms, such as respiratory infections, and enable prompt treatment and appropriate public health measures to be implemented. In addition, viral genomics can provide insights into the spread and epidemiology of viral infections, which can inform surveillance and control strategies (Attwood et al., 2022; Telenti et al., 2021).
 - (a) Influenza: Viral genomics can be used to identify specific strains of influenza virus responsible for seasonal outbreaks. By sequencing the viral genome from patient samples, one can determine whether the circulating strains are a good match for the strains included in the current influenza vaccine (Klingen et al., 2018; Vijaykrishna et al., 2015).
 - (b) COVID-19: Viral genomics has been critical for identifying and tracking the spread of SARS-CoV-2, the virus responsible for COVID-19. By sequencing the viral genome from patient samples, one can identify new variants of the virus and track their spread across different geographic regions (Lythgoe et al., 2021; Martin et al., 2020; Robishaw et al., 2021).
 - (c) Hepatitis C: Viral genomics can be used to identify specific genotypes of the hepatitis C virus, which can inform treatment decisions. Some genotypes of the virus are more responsive to certain antiviral therapies than others, so accurate genotyping is important for selecting the most effective treatment regimen (Chevaliez, 2011; Chevaliez et al., 2009; Messina et al., 2015; Thomson et al., 2016).
 - (d) Human papillomavirus (HPV): Viral genomics can be used to identify specific strains of HPV responsible for cervical cancer and other HPV-related cancers (Hu et al., 2018; Zhou et al., 2022).

Viral genomics can provide important insights into the identity, spread, and epidemiology of viral infections, which can inform clinical decision-making and public health interventions.

2. **Epidemiology and surveillance:** Viral genomics can be used to track the spread of viral infections, study transmission patterns, and identify the source of outbreaks (Agoti et al., 2017; Hill et al., 2021).

Viral genomics can be used for epidemiology and surveillance purposes, which include.

- a. **Tracking the spread of viral infections:** Through sequencing the viral genome from patient samples across different geographic regions, one can reconstruct the transmission pathways of viruses and track their spread. This can provide important insights into the dynamics of viral outbreaks and inform public health measures aimed at controlling the spread of the virus (Wohl et al., 2020).

For example, during the COVID-19 pandemic, viral genomics has been used extensively to track the spread of SARS-CoV-2 across different regions and countries (Lo et al., 2020).

Similarly, viral genomics has been used to track the spread of other viral infections, such as influenza and hepatitis C ([Hadfield et al., 2019](#)).

- b. Studying transmission patterns: Viral genomics plays a crucial role in understanding the transmission of viruses within populations. By analyzing the genetic sequences of viruses from different patients, clusters of infections can be identified, and the mode of transmission, whether it is through direct contact or a common source, can be determined ([Hill et al., 2021](#); [Ke et al., 2023](#)).
 - c. Identifying the source of outbreaks: Viral genomics can be used to identify the origin of viral outbreaks, such as the source of a foodborne illness or a zoonotic infection ([Aggarwal et al., 2021](#); [Freimanis et al., 2016](#)).
 - d. Monitoring the emergence of new strains: Viral genomics can be used to monitor the emergence of new strains of viruses, such as influenza viruses or coronaviruses ([John et al., 2021](#); [Moratorio et al., 2018](#)).
3. Vaccine development: Viral genomics can be used to develop vaccines by identifying the specific genetic sequences that encode viral antigens and using them to create vaccine candidates. Also, it can be developed through identification of specific viral proteins or mechanisms that can be targeted by the immune system. This approach involves sequencing the viral genome and identifying proteins that are unique to the virus or are essential for its replication ([Gebre et al., 2021](#); [Sette et al., 2010](#); [Travieso et al., 2022](#); [Uddin et al., 2020](#)).

Once these proteins are identified, vaccines can be developed that target these proteins, either by using the proteins themselves as the vaccine or by using genetic engineering to produce proteins that are similar to the viral proteins. These vaccines can then be used to stimulate an immune response and protect against infection ([Seib et al., 2012](#)).

One of the major advantages of genomics in vaccine development is its ability to identify potential targets for vaccines. When a new virus emerges, genomic sequencing can be used to identify the essential elements of the virus that are susceptible to targeting by the immune system. These elements usually consist of proteins located on the virus's surface, which can be recognized by antibodies or other immune cells ([Rinaudo et al., 2009](#)).

To illustrate, the development of vaccines against the human papillomavirus (HPV) and hepatitis B virus (HBV) was informed by viral genomics. By sequencing the viral genomes of these viruses, specific viral proteins that can be targeted by the immune system can be identified. Vaccines against these viruses were then developed using either the proteins themselves or genetically engineered versions of the proteins and have been shown to be highly effective in preventing infection ([Tsai, 2015](#)).

Similarly, viral genomics is being used to develop mRNA COVID-19 vaccines. These vaccines were developed based on the genetic sequence of the SARS-CoV-2 virus, which was rapidly sequenced and shared globally in the early stages of the pandemic. By targeting the spike protein on the surface of the virus, the mRNA vaccines are able to train the immune system to recognize and respond to the virus, providing protection against COVID-19 ([Gao, 2021](#); [Hussain et al., 2022](#)).

The SARS-CoV-2 virus has a single-stranded RNA genome of approximately 30 kilobases, encoding for 29 proteins, including the spike (S) protein, envelope (E) protein,

membrane (M) protein, and nucleocapsid (N) protein. The S protein, which is responsible for binding to the human receptor and mediating viral entry, is the main target of vaccines (Shamabadi et al., 2023).

The first generation of COVID-19 vaccines was based on the traditional approach of using inactivated or attenuated viruses, viral proteins, or virus-like particles. However, with the viral genome sequence at hand, genomic information was utilized to advance vaccine platforms, including mRNA and viral vector vaccines (Nooraei et al., 2021; Peng et al., 2021; van Riel et al., 2020).

mRNA vaccines, such as the Pfizer-BioNTech and Moderna vaccines, utilize the genomic information of the S protein to create a small piece of messenger RNA (mRNA) that encodes the protein. The mRNA is then encapsulated in lipid nanoparticles and injected into the body. Once inside the cells, the mRNA is translated into the S protein, triggering an immune response (Fang et al., 2022; Jain et al., 2021).

Similarly, viral vector vaccines, such as the Oxford-AstraZeneca and Johnson & Johnson vaccines, use a modified version of a harmless virus to deliver the genetic code for the S protein. The virus's genome is modified to include the genetic sequence for the S protein, and when injected into the body, the virus delivers the genetic material into cells, leading to the expression of the S protein and an immune response (Chavda et al., 2022).

In addition to the development of vaccines, genomics has also been critical in understanding the genetic variability of the SARS-CoV-2 virus and its potential impact on vaccine efficacy. Mutations in the S protein have been identified, including the B.1.1.7, B.1.351, and P.1 variants, which have raised concerns about vaccine efficacy. Genomic surveillance has been used to monitor the spread of these variants and to identify any potential changes in vaccine efficacy (Akkiz, 2021; Mengist et al., 2021).

Also, Ebola virus is a highly deadly virus that causes severe hemorrhagic fever in humans and other primates. The virus is endemic to several countries in Africa and has caused several outbreaks over the years, with the most recent outbreak occurring in West Africa in 2013–16 (Cenciarelli et al., 2015; Ohimain et al., 2021). During this outbreak, several experimental vaccines were developed, including the rVSV-ZEBOV vaccine, which was eventually used in the outbreak response. This vaccine was developed using a viral vector approach, in which a harmless virus is used to deliver genes from the Ebola virus that stimulate an immune response. The genes utilized in the rVSV-ZEBOV vaccine were identified through genomic sequencing of the Ebola virus, enabling the identification of key components of the virus that are likely to be targeted by the immune system (Gélinas et al., 2020; Mbala-Kingebeni et al., 2021).

Genomics is also playing an important role in the development of vaccines for other emerging viruses, such as Zika virus and Chikungunya virus. Both of these viruses are transmitted by mosquitoes and have emerged as significant public health threats in recent years. Genomics has been employed to identify key targets for vaccines, such as the envelope protein of the Zika virus and the E1 and E2 proteins of the Chikungunya virus. Several vaccine candidates are currently in development for both of these viruses, and early results have been promising (Garg et al., 2020).

Influenza vaccines are typically developed based on the antigenic properties of the virus, which are determined by two surface glycoproteins: hemagglutinin (HA) and

neuraminidase (NA). The HA protein is the primary target of the immune system and is responsible for binding the virus to host cells, while the NA protein helps the virus to exit infected cells and spread to new hosts. The HA protein is the main target of the immune response, and it is the primary component of most influenza vaccines. However, the virus can undergo genetic changes in the HA protein that can alter its antigenicity and escape from the immune system. Genomic surveillance can help to identify these changes and track the evolution of the virus (Wei et al., 2020; Wohlbold et al., 2014).

The use of genomics in influenza vaccine development has led to the development of new approaches to vaccine design, such as the use of recombinant HA proteins, virus-like particles (VLPs), and DNA vaccines. Recombinant HA proteins are generated by expressing the HA gene in a recombinant system and purifying the protein for use as a vaccine. VLPs are self-assembling structures that mimic the structure of the virus but do not contain the viral genome, making them safer for use as vaccines. DNA vaccines are based on the injection of DNA encoding the HA protein, which is then expressed by host cells and elicits an immune response. Genomics has enabled the identification of new targets for vaccine development, such as conserved regions of the virus that are less likely to mutate and escape from the immune system (Keshavarz et al., 2019; Scorza et al., 2016).

One of the most significant contributions of genomics to influenza vaccine development has been the development of universal influenza vaccines that provide broad protection against multiple strains of the virus. Universal influenza vaccines aim to target conserved regions of the virus that are shared among different strains and are less likely to mutate. By targeting these conserved regions, universal influenza vaccines could provide long-lasting protection against multiple strains of the virus, including those that may emerge in the future. Genomics has played a critical role in identifying these conserved regions and developing new vaccine candidates that target them (Scorza et al., 2016).

4. Drug discovery: Viral genomics can be used to identify potential targets for antiviral drugs by studying viral genetic sequences and the molecular mechanisms by which they replicate and infect cells (Watanabe et al., 2015).

Viral genomics has also been instrumental in the development of antiviral therapies for emerging diseases. In the early stages of the Ebola outbreak in West Africa, sequencing was employed to identify potential drug targets within the genome of the virus. This led to the development of the antiviral drug remdesivir, which has shown promise in treating Ebola and other viral diseases.

There are a few ways in which viral genomics has facilitated drug discovery efforts.

- a. Identifying potential drug targets: By sequencing the genome of a virus, specific genes or proteins that are essential for the virus to survive and replicate can be identified. These essential components can then be targeted with drugs to prevent the virus from spreading (Beltran et al., 2013).
- b. Predicting drug resistance: Viruses can quickly evolve and develop resistance to drugs. By analyzing the genetic makeup of a virus, drugs are likely to be effective and which may be prone to resistance can be predicted. This can inform drug development efforts and help to design drugs that are less likely to be rendered

ineffective by viral mutations (Kryazhimskiy et al., 2011; Robson, 2020; Romano et al., 2012).

- c. Developing personalized medicine: analysis of the genetic makeup of a virus and the host, personalized medicine can be developed to target the specific strain of the virus and the unique genetic makeup of the individual. This approach may be particularly useful in the development of antiviral therapies for chronic viral infections, such as HIV or hepatitis C (Rajpoot et al., 2018).
- d. Rapid identification of new viruses: Advances in sequencing technology have made it possible to rapidly sequence the genomes of newly emerging viruses (Radford et al., 2012; N. Zhang et al., 2020).

This part of the antiviral will be discussed in more details in Chapter 10.

5. Evolution and phylogenetics: Viral genomics plays a crucial role in studying the evolution and phylogenetics of viruses, providing insights into their adaptation and evolution over time. It has played a significant role in the study of viral evolution and phylogenetics. Evolution refers to the changes that occur in the genetic makeup of a virus over time, while phylogenetics is the study of the evolutionary relationships between different strains of the virus (Geoghegan et al., 2018).

Sequencing of the genomes of different strains of a virus, the genetic mutations that have occurred over time can be analyzed, leading to the reconstruction of the virus's evolutionary history. This can be used to identify the virus's origins, track its spread between different populations, and gain a deeper understanding of how it has evolved to adapt to new environments or hosts (Engel et al., 2016).

In addition to providing insights into viral evolution, viral genomics can be used to study the genetic diversity of a virus (Volz et al., 2013).

Phylogenetic analysis can also be used to inform drug development efforts by identifying potential drug targets that are conserved across different strains of the virus. For example, if a particular gene or protein is essential for the survival of the virus and is conserved across different strains, it may be a promising target for drug development (Culligan et al., 2014; Shahbaaz et al., 2016).

6. Environmental monitoring: Viral genomics can be used to monitor viral diversity and distribution in different environments, including oceans, soil, and air (Nayfach et al., 2021).

By sequencing the genomes of viruses found in various environmental samples, one can identify the different types of viruses present in a particular environment and monitor changes in their diversity and abundance over time. This can be used to identify new or emerging viruses, monitor the spread of known viruses, and understand the factors that drive viral diversity in different environments (Fierer, 2017; Rodriguez-Brito et al., 2010).

In addition to identifying potential sources of viral outbreaks, environmental monitoring can provide insights into the ecological roles that viruses play in different ecosystems. For example, viruses are known to infect bacteria and can play an important role in controlling bacterial populations and nutrient cycling in the ocean. Also, sequencing the genomes of viruses in wastewater can help identify the presence of viruses that may be causing outbreaks of waterborne diseases. Similarly, analyzing the viral genomes present in animal samples such as bats or birds can help identify

potential sources of emerging viruses that can jump to humans (Martínez-Porchas and Vargas-Albores et al., 2017; Zhang et al., 2022, p. 100062).

Environmental monitoring can also be used to study the effects of human activities on viral diversity and distribution. For example, deforestation, urbanization, and agriculture can all alter the composition of viral communities in the soil and water, potentially leading to the emergence of new viruses or the spread of known viruses into new environments (Ortiz et al., 2021).

7. Agricultural applications: Viral genomics can be used to study viral pathogens affecting crops and livestock, leading to the development of improved diagnostic tools and strategies for disease management (Fornace et al., 2013; Lucas, 2011).

Viral genomics has numerous agricultural applications, including the study of plant and animal viruses, the development of viral-resistant crops, and the identification of viral pathogens that can affect livestock and crops (Simón-Mateo and García et al., 2011).

One of the major applications of viral genomics in agriculture is the study of plant viruses. Plant viruses can cause significant economic losses in agriculture by reducing crop yields, stunting plant growth, and affecting crop quality (Tatineni et al., 2023).

Viral genomics can also be used to develop viral-resistant crops. This can help reduce the use of pesticides and other chemicals, leading to more sustainable agricultural practices (Khalid et al., 2017).

In addition to studying plant viruses, viral genomics can also be used to identify viral pathogens that affect livestock and crops. For example, viral genomics can be used to identify the causative agents of diseases such as foot-and-mouth disease in cattle or tomato yellow leaf curl virus in tomato crops. This can be used to develop strategies for disease prevention and control, including the development of vaccines or the use of resistant crop varieties (Babu et al., 2018; Balke et al., 2019).

Generally, viral genomics has numerous agricultural applications that can help increase crop yields, reduce the use of chemicals and pesticides, and prevent the spread of viral diseases in livestock and crops.

8. Forensic virology: Viral genomics can be used to trace the origin and spread of viruses in bioterrorism or criminal cases.

Viral genomics plays an important role in forensic virology, which involves the use of viral genetic material as evidence in legal investigations. Forensic virology can be used to investigate a range of issues, including the source of viral outbreaks, the transmission of viral infections, and cases of bioterrorism (Hietpas et al., 2005).

Viral genomics can be used in forensic investigations to identify the genetic fingerprint of viruses found at crime scenes or in biological samples taken from victims or suspects. By analyzing the genomes of these viruses, forensic investigators can identify the specific strain of virus involved and track its spread over time. This can be used to identify the source of the virus and the individuals or groups responsible for its transmission (Budowle, Murch, & Chakraborty, 2005; Schmedes et al., 2016).

Viral genomics can also be used to investigate cases of bioterrorism, where viruses may be used as weapons. By analyzing the genomes of viruses used in these attacks, forensic investigators can identify the specific strain of virus and trace its origin to a

particular laboratory or individual. This can help law enforcement agencies to identify and prosecute the perpetrators of these attacks (Oliveira et al., 2020).

In addition to investigating viral outbreaks and bioterrorism cases, viral genomics can be used in forensic investigations of other types of crimes. For example, viral genetic material found at a crime scene can provide evidence linking a suspect to the crime or help to identify the source of an outbreak of viral illness in a particular community (Budowle et al., 2005).

9. Oncolytic virotherapy: Viral genomics can be used to engineer viruses that selectively target cancer cells, leading to the development of oncolytic virotherapies.

Oncolytic virotherapy is a promising approach to cancer treatment that involves using viruses to selectively target and destroy cancer cells. Viral genomics plays a critical role in the development and optimization of oncolytic viruses for use in cancer therapy (Wang et al., 2022).

By studying the genomes of different types of viruses, those that have natural oncolytic properties or engineer viruses with these properties can be identified. Oncolytic viruses can be designed to target specific cancer cells, while leaving healthy cells intact, through genetic modification of the virus (Jafari et al., 2022).

Viral genomics can also be used to optimize the safety and effectiveness of oncolytic viruses. For example, the genes responsible for virus replication and immune evasion to increase the therapeutic potential of the virus can be identified and modified.

Viral genomics can be used to monitor the response of cancer cells to oncolytic viruses and to predict the outcome of treatment. By sequencing the genomes of cancer cells before and after treatment, changes in the tumor microenvironment and the evolution of viral resistance in cancer cells can be identified. This can be used to develop more effective treatments and to monitor patient response to therapy (Lee et al., 2020).

10. Host–virus interactions: Viral genomics can be used to study the interactions between viruses and their host cells, providing insight into the mechanisms of viral pathogenesis (Feschotte et al., 2012).
11. Emerging viruses: Viral genomics can be used to identify and characterize newly emerging viruses, allowing for early detection and rapid response to potential outbreaks (Murphy et al., 1994).

One of the primary ways that viral genomics is used in studying emerging viruses is through viral surveillance. By monitoring the genetic diversity of viruses in different populations, one can identify new or changing viral strains and track their emergence and spread. This information can be utilized to develop new diagnostic tests, vaccines, and antiviral drugs to combat these emerging viruses.

Viral genomics can also be used to study the molecular mechanisms by which emerging viruses infect and replicate in host cells (Grubaugh et al., 2019).

12. Public health policy: Viral genomics can inform public health policies by providing data on viral transmission dynamics and identifying high-risk populations for targeted interventions (Cevik et al., 2021).
13. Viral metagenomics: Viral genomics can be used to study the diversity and distribution of viral communities in complex environments, such as the human microbiome or soil (Park et al., 2011).

7. Tracking the spread of viruses: How genomic sequencing is revolutionizing public health

Tracking the spread of viruses has always been a critical aspect of public health, but recent advances in genomic sequencing have revolutionized our ability to do so. By analyzing the genetic material of viruses, the spread of infectious diseases can be tracked more accurately, identify the source of outbreaks, and develop more effective strategies for controlling them.

Genomic sequencing involves analyzing the entire genetic material of a virus to identify its specific strain and track its evolution. This process is much faster and more accurate than traditional methods of pathogen identification, such as culturing and antibody testing, which can be time-consuming and may miss new or mutated strains of a virus (Maljkovic Berry et al., 2020).

One of the most significant applications of genomic sequencing in public health is pathogen surveillance. By collecting and analyzing samples from infected individuals, specific strain of a virus causing an outbreak can be identified and track its spread over time. This can be used to identify the source of an outbreak, track the movement of infected individuals, and inform public health responses, such as quarantine measures or vaccination campaigns.

Genomic sequencing can also be used to identify new or emerging viral threats. This can be used to develop new diagnostic tests, treatments, and vaccines, and to inform public health policy decisions.

Another important application of genomic sequencing is in the development of personalized medicine. By analyzing an individual's genetic makeup, one can identify genetic variations that may impact their response to certain drugs or infections. This can be used to develop personalized treatment plans that are tailored to the individual's genetic profile, improving treatment outcomes and reducing the risk of adverse reactions.

In summary, genomic sequencing is revolutionizing public health by providing faster and more accurate methods of tracking the spread of viruses, identifying new and emerging threats, and developing personalized treatments. By incorporating genomic sequencing into public health surveillance and response strategies, we can improve our ability to control infectious diseases and protect public health.

8. Future directions in viral genomics

As genomic sequencing technology continues to advance, there are many exciting future directions in viral genomics that hold promise for improving our understanding of viruses and their interactions with hosts. Here are some potential areas of focus.

1. **Single-cell sequencing:** With the advancement of single-cell sequencing technology, the genetic material of individual cells, including infected cells, can now be analyzed. This has the potential to unveil new insights into the diversity of viral populations within a host and their interactions with host cells, without specifying any particular group involved in the research.
2. **Long-read sequencing:** Existing sequencing technologies have limitations when it comes to accurately sequencing long stretches of DNA or RNA. However, long-read

sequencing technology has the potential to overcome this limitation, enabling the acquisition of more complete and accurate genomic data from viruses, without specifying any particular group involved in the research.

3. **Metagenomics:** Metagenomics involves sequencing the genetic material of entire communities of organisms in a given environment. This approach has the potential to reveal the diversity and distribution of viruses in different environments, such as soil or water, and their potential interactions with other microorganisms.
4. **Machine learning and artificial intelligence:** Machine learning and artificial intelligence techniques can be used to analyze large datasets generated by viral genomics studies, identifying patterns and relationships that may not be apparent using traditional methods.
5. **Multimomics approaches:** Multimomics approaches involve integrating data from different types of -omics studies, such as genomics, transcriptomics, and proteomics. This approach can provide a more comprehensive understanding of the molecular mechanisms underlying viral infections.
6. **Epigenomics:** Epigenomic studies entail analyzing modifications to DNA or histones that can impact gene expression. By examining the epigenomic changes that transpire during viral infections, valuable insights can be gained into the host–virus interaction and potentially uncover new targets for antiviral therapies, without specifying any particular group involved in the research.
7. **Real-time sequencing:** Real-time sequencing technology has the potential to allow for rapid sequencing and analysis of viral genomes during outbreaks, providing valuable information for public health responses.
8. **Spatial genomics:** Spatial genomics technology can be used to map the location of viral infection within tissues or organs, providing a more detailed understanding of the spatial distribution of viruses within a host.
9. **Host genomics:** Host genomics involves analyzing the genetic material of the host organism in addition to the viral genome. This approach can provide insights into host factors that may influence susceptibility to viral infections or response to treatment.
10. **Personalized medicine:** With the increasing availability of genomic sequencing data, there is potential for the development of personalized treatments for viral infections based on an individual's genomic profile.
11. **Vaccine development:** Viral genomics can be used to identify key genetic elements of a virus that are necessary for its replication or pathogenicity. This can be used to develop new vaccines that target these elements, potentially leading to more effective and targeted vaccination strategies.
12. **Evolutionary dynamics:** Viral genomics can provide insights into the evolutionary dynamics of viruses, including their origin, transmission patterns, and adaptation to new hosts or environments. Understanding these dynamics can inform public health responses to outbreaks and guide the development of new treatments and vaccines.
13. **Viral engineering:** With the ability to manipulate viral genomes, viral engineering has the potential to create new viruses or modify existing ones for various purposes, such as vaccine development or oncolytic therapy.

The future of viral genomics is bright, with many exciting directions for research and development that hold promise for improving our understanding of viruses and their interactions with hosts, and for developing new treatments and public health strategies to combat viral infections.

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Fungal genomics

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1. Introduction

Fungi are the largest group of eukaryotic organisms that range from yeast and slime molds to mushrooms. These organisms are majorly classified as monophyletic Eumycota groups, and their diversity ranges from 500,000 to 9.9 million, spanning over 1 billion years of evolutionary history. They are abundant worldwide due to their small size and cryptic lifestyle in soil and dead and decomposing matter, as symbionts with algae, fungi, bryophytes, pteridophytes, higher plants, and animals. These organisms dominate Earth from polar to temperate and tropical habitats. Due to their ecological dominance, they play a central role in human endeavors. The fungus (mushroom and truffle) is directly used as human food, and yeasts are used in the bread industry. The fungi also carry out nutrient cycling by decomposing organic matter. They also produce antibiotics, enzymes, mycotoxins, alkaloids, polyketides, and other chemical compounds.

Two decades ago, witnessed a massive evolution in screening and studying the fungal genome and ended up doing many trials of constructing a comprehensive genomic database. The total amount of genome sequences has raised since sequencing the whole genome of unicellular growth-budding yeast of small genome size, *Saccharomyces cerevisiae* (*S. cerevisiae*), as the first fungus's complete sequencing conducted in 1996, in addition to intensive genetic and molecular analyses (Goffeau et al., 1996). Although sequencing of the yeast's genome revealed only a simple overview of the fungal kingdom, this was the spark that ignited the global studies not only of fungi and yeast's genome but also of total eukaryote whole genome sequences, gene function, and expression. Thereafter, the completion of the sequencing of the whole genome of fission yeast, *Schizosaccharomyces pombe* (*S. pombe*) (Wood et al., 2002), then *Neurospora crassa* (*N. crassa*) (Galagan et al., 2003), helped in giving more clear insight into the genomics of the whole fungal kingdom. The *N. crassa* genome was

the first filamentous fungus to be sequenced and shown to have around 2 times as many genes as *S. cerevisiae* and *S. pombe*; however, it didn't have homologs to known proteins for over 40% of these genes (Galagan et al., 2005).

2. Fungal genome history

It is worth mentioning that the fungal genome would for sure give an indication and in-depth understanding of sequences of common eukaryotes' fundamental cellular processes. Not only that, but also, it would solve many of the challenges we face in health, agriculture, enzyme biotechnology, bioenergy, and ecological diversity (Sharma, 2016).

Capitalizing on the importance of fungal genomics, the very beginning of 2000 witnessed the involvement of a consortium of mycologists in collaboration with scientists from the MIT Center for Genome Research (now the Broad Institute) and Whitehead Institute in order to launch the fungal genome initiative (FGI) (<http://www.broad.mit.edu/annotation/fgi/>) for listing and grouping the sequenced genomes of fungi throughout the kingdom in a specific database, which would serve as assistance to the many fungal areas of study. Interestingly, selection of organisms to be sequenced wasn't done haphazardly, yet groups of organisms were created based on specific criteria in order to increase the potentiality of their comparative genomics' combined value, evolutionary studies, and medical applications. Out of 23 fungal genomes that were released in 2014, only two fungal genomes were available at the launch of FGI. A process of double check and matching of the sequenced genomes from different associations including the Washington University Genome Sequencing Center, TIGR, Joint Genome Institute (JGI), Génolevures, the Marine Biological Laboratories, the Stanford Genome Technology Center, Sanger Institute, Duke Center for Genome Research, and the University of British Columbia (Galagan et al., 2005) (Tables 3.1 and 3.2).

One resource has been launched to collect the whole genome representing *S. cerevisiae* strain S288C, which is named Saccharomyces Genome Database (SGD). It has been acting as a source of all data related to sequence information, functional annotations, mapping, protein expression data, mutant phenotypes, physical and genetic interactions, and the primary literature from which these data are derived (<http://www.yeastgenome.org/>) (Goffeau et al., 1996).

Another search tool was available and used by the SGD project, which collected around 50,000 literature under the name of Textpresso (<http://textpresso.yeastgenome.org>) (Müller et al., 2004). Also, each and every pathway's complete description, with molecular structures, and listing of full reference was available through the (<http://pathway.yeastgenome.org>) interface (Cherry et al., 2012). Different types of genomic information were listed by GBrowse as a genome browser, which was used by SGD (<http://browse.yeastgenome.org>) (Stein et al., 2002). All SGD curated interactions, whether genetic or physical, were listed in bulk through the BioGRID database (Biological General Repository for Interaction Datasets; <http://www.thebiogrid.org/>) (Stark et al., 2011). Also, many resources on the web were launched to group and collect genomic data on fungi, some of them listed in Table 3.3 (Sharma, 2016).

In 2012, around 2650 projects were accomplished by JGI or Department of Energy (DOE) in US, and then 2014 witnessed increasing by threefold, melted out generating genome sequence database of fungi, algae, and plants, including 456 trillion nucleotides (Nordberg et al., 2014).

TABLE 3.1 Complete fungal and Oomycete genomes in 2005.

Genus/species	Taxonomy	Sequencing center(s)
<i>Ashbya gossypii</i> (aka <i>Eremothecium</i>)	Saccharomycetes	Biozentrum an Syngenta AG
<i>Aspergillus fumigatus</i>	Eurotiomycetes	Sanger Institute & TIGR
<i>Aspergillus nidulans</i>	Eurotiomycetes	Broad Institute & Monsanto
<i>Aspergillus terreus</i>	Eurotiomycetes	Broad Institute
<i>Botrytis cinerea</i>	Leotiomycetes	Broad Institute & Syngenta AG; Genoscope
<i>Candida albicans</i>	Saccharomycetes	Stanford genome technology center, Sanger Institute & broad Institute
<i>Candida glabrata</i>	Saccharomycetes	Génolevures
<i>Candida guilliermondii</i> (anamorph of <i>Pichia</i>)	Saccharomycetes	Broad Institute
<i>Candida lusitanae</i> (aka <i>Clavispora</i>)	Saccharomycetes	Broad Institute
<i>Candida tropicalis</i>	Saccharomycetes	Broad Institute & Génolevures
<i>Chaetomium globosum</i>	Sordariomycetes	Broad Institute
<i>Coprinus cinereus</i>	Homobasidiomycetes	Broad Institute
<i>Coccidioides immitis</i>	Eurotiomycetes	Broad Institute
<i>Coccidioides posadasii</i>	Eurotiomycetes	TIGR & Broad Institute
<i>Coprinus cinereus</i>	Homobasidiomycetes	Broad Institute
<i>Cryptococcus neoformans</i> (anamorph of <i>Filobasidiella</i>)	Homobasidiomycetes	Broad Institute, Genome Sciences Center Canada, Duke Center for Genome Center, Research, Stanford Genome Technology center & TIGR
<i>Debaryomyces hansenii</i>	Saccharomycetes	Génolevures
<i>Encephalitozoon cuniculi</i>	Microsporidia	Gene Scope
<i>Fusarium graminearum</i> (aka <i>Gibberella zeae</i>)	Sordariomycetes	Broad Institute
<i>Kluyveromyces lactis</i>	Saccharomycetes	Génolevures
<i>Magnaporthe grisea</i>	Sordariomycetes	Broad Institute
<i>Neurospora crassa</i>	Sordariomycetes	Broad Institute
<i>Phanerochaete chrysosporium</i>	Homobasidiomycetes	Joint Genome Institute
<i>Phytophthora ramorum</i>	Oomycete	Joint Genome Institute
<i>Phytophthora sojae</i>	Oomycete	Joint Genome Institute
<i>Podospora anserina</i>	Sordariomycetes	CNRS & Genoscope
<i>Rhizopus oryzae</i>	Zygomycota	Broad Institute
<i>Saccharomyces bayanus</i>	Saccharomycetes	Washington University Genome Sequencing Center, Broad Institute, Génolevures

(Continued)

TABLE 3.1 Complete fungal and Oomycete genomes in 2005.—cont'd

Genus/species	Taxonomy	Sequencing center(s)
<i>Saccharomyces castellii</i>	Saccharomycetes	Washington University Genome Sequencing Center
<i>Saccharomyces cerevisiae</i>	Saccharomycetes	Stanford Genome Technology Center, Sanger Institute & Broad Institute
<i>Saccharomyces kluyveri</i>	Saccharomycetes	Washington University Genome Sequencing Center & Génolevures
<i>Saccharomyces kudriavzevii</i>	Saccharomycetes	Washington University Genome Sequencing Center
<i>Saccharomyces mikatae</i>	Saccharomycetes	Broad Institute & Washington University Genome Sequencing Center
<i>Saccharomyces paradoxus</i>	Saccharomycetes	Broad Institute
<i>Schizosaccharomyces pombe</i>	Schizosaccharomycetes	Sanger Institute
<i>Sclerotinia sclerotiorum</i>	Leotiomycetes	Broad Institute
<i>Stagonospora nodorum</i> (anamorph of <i>Phaeosphaeria</i>)	Dothideomycetes	Broad Institute & International Stagonospora nodorum Genomics Consortium
<i>Uncinocarpus reesei</i>	Eurotiomycetes	Broad Institute
<i>Ustilago maydis</i>	Ustilaginomycotina	Broad Institute, Bayer Crop Science AG, & Exelixis
<i>Yarrowia lipolytica</i>	Saccharomycetes	Génolevures
<i>Zygosaccharomyces rouxii</i>	Saccharomycetes	Génolevures

TABLE 3.2 Complete fungal and Oomycete genomes project, in 2005. pointing species of all four major fungal groups; ascomycetes, basidiomycetes, zygomycetes, and chytrids, they represented human and plant pathogens, saprophytes, and model organisms.

Genus/species	Taxonomy	Sequencing center(s)
<i>Alternaria brassicicola</i>	Dothideomycetes	Washington University Genome Sequencing Center
<i>Aspergillus clavatus</i>	Eurotiomycetes	TIGR
<i>Aspergillus fischerianus</i>	Eurotiomycetes	TIGR
<i>Aspergillus flavus</i>	Eurotiomycetes	TIGR
<i>Aspergillus niger</i>	Eurotiomycetes	Joint Genome Institute
<i>Aspergillus parasiticus</i>	Eurotiomycetes	University of Oklahoma
<i>Batrachomyces dendrobatidis</i>	Chytridiomycete	Broad Institute & Joint Genome Institute
<i>Blastomyces dermatitidis</i> (anamorph of <i>Ajellomyces</i>)	Eurotiomycetes	Washington University Genome Sequencing Center
<i>Candida dubliniensis</i>	Saccharomycetes	Sanger Institute
<i>Candida parapsilosis</i>	Saccharomycetes	Sanger Institute

TABLE 3.2 Complete fungal and Oomycete genomes project, in 2005. pointing species of all four major fungal groups; ascomycetes, basidiomycetes, zygomycetes, and chytrids, they represented human and plant pathogens, saprophytes, and model organisms.—cont'd

Genus/species	Taxonomy	Sequencing center(s)
<i>Fusarium oxysporum</i>	Sordariomycetes	Broad Institute
<i>Fusarium verticillioides</i>	Sordariomycetes	Broad Institute & Syngenta AG
<i>Glomus intraradices</i>	Glomeromycetes	Joint Genome Institute
<i>Histoplasma capsulatum</i> (anamorph of <i>Ajellomyces</i>)	Eurotiomycetes	Broad Institute & Washington University Genome Sequencing Center
<i>Kluyveromyces marxianus</i>	Saccharomycetes	Génolevures
<i>Kluyveromyces thermotolarans</i>	Saccharomycetes	Génolevures
<i>Kluyveromyces waltii</i>	Saccharomycetes	Broad Institute
<i>Laccaria bicolor</i>	Homobasidiomycetes	Joint Genome Institute
<i>Lodderomyces elongisporus</i>	Saccharomycetes	Broad Institute
<i>Melampsora larici-populina</i>	Urediniomycetes	Joint Genome Institute
<i>Mycosphaerella fijiensis</i>	Dothideomycetes	Joint Genome Institute
<i>Nectria haematococca</i>	Sordariomycetes	Joint Genome Institute
<i>Nosema locustae</i> (aka <i>Antonospora</i>)	Microsporidia	Marine Biological Laboratory
<i>Peronospora parasitica</i>	Oomycetes	Washington University Genome Sequencing Center
<i>Phakopsora meibomia</i>	Urediniomycetes	Joint Genome Institute
<i>Phakopsora pachyrhizi</i>	Urediniomycetes	Joint Genome Institute
<i>Phytophthora capsici</i>	Oomycete	Joint Genome Institute
<i>Phytophthora infestans</i>	Oomycete	Broad Institute & Sanger Institute
<i>Pichia angusta</i>	Saccharomycetes	Génolevures
<i>Pichia farinose</i>	Saccharomycetes	Génolevures
<i>Piromyces</i> sp.	Chytridiomycete	Joint Genome Institute
<i>Pneumocystis carinii</i>	Pneumocystidomycetes	Broad Institute & University of Cincinnati
<i>Puccinia graminis</i>	Urediniomycetes	Broad Institute
<i>Pyrenophora tritici-repentis</i>	Dothideomycetes	Broad Institute
<i>Saccharomyces exiguus</i>	Saccharomycetes	Génolevures
<i>Saccharomyces servazzii</i>	Saccharomycetes	Génolevures
<i>Schizosaccharomyces japonicus</i>	Schizosaccharomycetes	Broad Institute
<i>Schizosaccharomyces octosporus</i>	Schizosaccharomycetes	Broad Institute
<i>Trichoderma reesei</i> (anamorph of <i>Hypocrea jecorina</i>)	Sordariomycetes	Joint Genome Institute
<i>Trichoderma virens</i>	Sordariomycetes	Joint Genome Institute
<i>Xanthoria parietina</i>	Lecanoromycetes	Joint Genome Institute

TABLE 3.3 List of some fungal genome databases and used functions.

Genome	Database	Functions	References
<i>Candida albicans</i>	http://www.candidagenome.org/(http://genolist.pasteur.fr/CandidaDB/)	Functional information about genes and proteins; biochemical pathways; and the textpresso full text literature search tool	Skrzypek et al. (2010)
<i>Aspergillus</i> spp.	http://www.aspergillus-genomes.org.uk , http://www.cadre-genomes.org.uk	Manage genomic data and to offer web-based tools for analysis and visualization of genomic features; provides access to annotated genomes, which are of particular importance to the <i>Aspergillus</i> genetics community (<i>A. fumigatus</i> Af293 and A1163, <i>A. nidulans</i> , <i>A. oryzae</i> , and <i>A. niger</i>); of these, two are clinical isolates (<i>A. fumigatus</i> Af293 and A1163)	Gilsenan et al. (2012)
<i>Phytophthora</i> spp.	http://www.pfgd.org	Allows intuitive 25 navigation of transcript and genomic data; automated analyses are performed on all sequence data	Gajendran et al. (2006)
<i>Saccharomyces cerevisiae</i>	http://genome-www.stanford.edu/Saccharomyces/ , http://www.yeastgenome.org , http://pathway.yeastgenome.org	SGD collects and organizes biological information about genes and proteins of the yeast <i>S. cerevisiae</i> . It provides genome-wide computational analyses for <i>S. cerevisiae</i> , or for comparative studies; genetics and cellular biology of other fungal genera that are important to basic research, human health, and industry	Cherry et al. (2012), Engel et al. (2010)
Fungi	http://FungiDB.org	Functional genomic resource for pan fungal genomes; Fungi DB provides a data-mining interface to the comparative and functional genomic data of multiple species of fungi that differs from the species focused resources of SGD, CGD, and AspGD	Stajich et al. (2012)
TIGR fungal database	www.tigr.org/tbd/fungal	Collection of species-specific databases that use a highly refined protocol to analyze EST sequences	Galagan et al. (2005)
Fungi	http://www.genomesonline.org	Centralized monitoring of genome and metagenome projects worldwide	Pagani et al. (2012)
Fungi	http://www.broad.mit.edu/FGI/	Genome sequence of fungi from throughout the kingdom	Li et al. (2021), Stajich, (2017)
<i>Saccharomyces cerevisiae</i>	http://www.thebiogrid.org/	BioGRID contains high-throughput interaction data for <i>S. cerevisiae</i> and other model organisms	Breitkreutz et al. (2008), Stark et al. (2011)

TABLE 3.3 List of some fungal genome databases and used functions.—cont'd

Genome	Database	Functions	References
Fungi	http://mips.gsf.de/projects/fungi/	Munich Information Center for Protein Sequences	Mewes et al. (2011)
<i>Ustilago maydis</i>	http://mips.gsf.de/genre/proj/ustillago/	MUMDB: <i>Ustilago maydis</i> genome database	Mewes et al. (2011)
<i>Schizosaccharomyces</i>	http://www.pombase.org/pombe	PomBase is a comprehensive database for the fission yeast <i>Schizosaccharomyces pombe</i> , providing structural and functional annotation, literature curation, and access to large-scale data sets	Lock et al. (2018)
Fungi	http://jgi.doe.gov/fungi	MycoCosm is a fungal genomics portal, developed by the US Department of Energy Joint Genome Institute to support integration, analysis, and dissemination of fungal genome sequences and other “omics” data by providing interactive web-based tools. The JGI genome Portal automatically generates and monitors BioSample and BioProject submissions to NCBI	Grigoriev et al. (2011), Nordberg et al. (2014)
Fungi	http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genome-initiative	Fungal genome Initiative	Cuomo and Birren (2010)
Fungi	http://genome.jgi-psf.org/programs/fungi/index.jsf	JGI MycoCosm	Ahrendt et al. (2023)
Comparative Fungal Genomics Platform	http://cfgp.riceblast.snu.ac.kr/main.php	CFGP	Park et al. (2008)
Fungi	http://fungalgenomes.org/wiki/Fungal_Genome_Links	Fungalgenomes.org site	Stajich (2017)
Fungi	http://fungi.ensembl.org/index.html	Ensembl fungi	Ahrendt et al. (2023)
Yeast and other fungi	http://www.mips.biochem.mpg.de	MIPS contains information on an up-to-date database of the complete genome, classification of protein sequence (ProtFam), and collection of protein sequence databases. Provide information about open reading frames	Mewes et al. (2011)
<i>Fusarium graminearum</i>	http://mips.gsf.de/genre/proj/fusarium/	FGDB: <i>Fusarium graminearum</i> genome database	Güldener et al. (2006)

Sixty-three fungal whole genome sequences out of 650 genomes were added by JGI to the public databases in 2014. The JGI also initiated after that the large-scale genomic project of 1000 fungal genomes to reveal the needed compressive information for studying complex metagenomes (Sharma, 2016).

Another source of genomic databases is the *Candida* Genome Database (CGD, <http://www.candidagenome.org/>), which was launched mainly for the opportunistic pathogen, *Candida albicans* (Jones et al., 2004). New features like Textpresso and Biochemical Pathways were added to CGD to leverage the comparative genomic analysis conducted to on eight closely related *Candida* species (Butler et al., 2009; Skrzypek et al., 2010). The process of merging CADRE, the Central *Aspergillus* Data Repository (<http://www.cadre-genomes.org.uk>), and the website of *Aspergillus* (<http://www.aspergillus.org.uk>), capitalized on cross-linking with medical information and together melted out a unique resource for industrial and clinical aspects of the genera (Gilsenan et al., 2012/2012).

Thereafter, numerous databases specified in the genomic sequencing database and its analysis have been generated and updated since the bioinformatics revolution in the last decade, which offered great assistance to the comparative analysis through the fungal species. According to Liolios et al. (2007), the Genome OnLine Database is a specific site launched for projects of whole genome-sequencing (<http://www.genomeonline.org>), which also has a mirror site at the Institute of Molecular Biology and Biotechnology, Crete, Greece (<http://gold.imbb.forth.gr/>) and their associated metadata, around the world. In 2023, it encompasses more than 15,980 fungal organisms' genome submitted of more than 24,900 sequencing projects, out of 2500 studies and 4200 analysis projects conducted.

The recent revolution in genomic sequencing led to a massive increase in fungal functional genomic data like proteomic, microarray, RNA sequence, etc., so investigating and extracting the information from data sets of multiple genomes needed to be integrated to facilitate the usage. As a comprehensive resource for fungal genomic and functional genomic data, FungiDB (<http://fungidb.org>) was developed as an online resource out of integration with the NIAID-funded Eukaryotic Pathogen Bioinformatic Resource Center (<http://eupathdb.org>) (Aurrecochea et al., 2010). It is worthy to mention that the genomes in the website are mainly related to *Aspergillus*, *Candida*, and *Saccharomyces* genome resources and originally collected through (<http://broadinstitute.org>) the Broad Institute (Engel et al., 2010; Skrzypek et al., 2010). Similarly, a Web Development Kit (WDK) search engine adopted the same database (Fischer et al., 2011).

Serving as the interface for mining the data, not only species-based resources like SGD, CGD, and AspGD, the FungiDB not only acts as a source of comparative and functional genomic data for multiple species of fungi, yet also it offers an integrated query system as part of the WDK and GUS database structures. FungiDB has an edge over other online database resources that don't have the data mining engine nor the capabilities of comparisons across the species; only they provide complementary data queries, such as Ensemble Fungi (Kersey et al., 2010), the JGI's MycoCosm (<http://jgi.doe.gov/fungi>), and IMG tools (Markowitz et al., 2010), or Microbes Online (Dehal et al., 2010/2010). Then FungiDB witnessed obtaining the genomic sequence data directly from the providers at sequencing centers, GenBank, and associated functional data repositories (GEO or SRA) in addition to SGD, CGD, and AspGD key models. Out of 263 annotated fungal genomes in 2022, only 17 annotated fungal genomes were available in FungiDB and listed in Tables 3.4 and 3.5 (Stajich et al., 2012).

TABLE 3.4 Sample genomes were published in 2011.

Phylum	(sub)Class	Species	Genome size (Mbs)	No. of genes
Ascomycota	Eurotiomycetes	<i>Aspergillus clavatus</i> NRRL1	27.86	9413
		<i>Aspergillus flavus</i> NRRL3357	36.79	12,730
		<i>Aspergillus fumigatus</i> Af293	29.39	10,067
		<i>Aspergillus nidulans</i> FGSC A4	30.5	10,865
		<i>Aspergillus niger</i> ATCC 1015	37.2	6679
		<i>Aspergillus terreus</i> NIH 2624	29.33	10,564
		<i>Coccidioides immitis</i> H538.4	27.73	10,640
		<i>Coccidioides immitis</i> RS	28.95	9878
	Sordariomycetes	<i>Fusarium graminearum</i> PH-1 (NRRL 31084)	36.45	13,605
		<i>Fusarium oxysporum</i> (f.sp.4287)	61.4	17,466
		<i>Fusarium verticillioides</i> 7600 (<i>Gibberella moniliformis</i>)	41.78	14,457
		<i>Magnaporthe oryzae</i> 70–15 (<i>Magnaporthe grisea</i>)	41.7	11,385
		<i>Neurospora crassa</i> OR74A	41.04	10,154
	Saccharomycetes	<i>Candida albicans</i> SC5314	14.32	6444
		<i>Saccharomyces cerevisiae</i> S288c	12.16	6926
Basidiomycota	Pucciniomycetes	<i>Puccinia graminis</i> f.sp. <i>tritici</i> CRL 75–36-700–3	88.65	21,065
	Tremellomycetes	<i>Cryptococcus neoformans</i> var. <i>grubii</i> H99	18.88	7121
Mucoromycotina (Zygomycota)		<i>Rhizopus oryzae</i> RA 99–880	46.09	17,652

Additional information is available at <https://fungidb.org/GenomeDataTypes>

3. Fungal genome sequencing

The massive advancement that happened in bioinformatics and genome sequencing technology was the cornerstone of the evolution of comparative genomic analysis of the fungal kingdom. The shotgun metagenomic technique is one of these technologies used for whole genome sequencing, which is called whole genome shotgun (WGS), and is considered a huge advancement over conventional cloning methodology that was used for eukaryotic genomes sequencing. This low-cost sequencing technology (WGS) generates genome sequences with long-range contiguity by applying incomparable sequencing accuracy. In the clone-by-clone sequencing technique, the mapping is selective for each clone, which requires excessive work to get the target sequencing templates. Not only is preparation of different shotgun libraries needed for each clone, but it also needs to be tested, sequenced, and assembled. A recent new effective and efficient technology edged over WGS in time and cost, called

TABLE 3.5 Fungal genome assemblies in GenBank and EMBL-Bank.

Genome	Size (Mb)	Genes	Strains	Phylum	Subphylum
<i>Ashbya gossypii</i>	9.6	4726	1	Ascomycota	Saccharomycotina
<i>Candida albicans</i>	14.3–14.4	6094–6160	2	Ascomycota	Saccharomycotina
<i>Candida glabrata a</i>	12.3	5202	1	Ascomycota	Saccharomycotina
<i>Candida guilliermondii</i>	10.6	5920	1	Ascomycota	Saccharomycotina
<i>Candida lusitanae</i>	12.1	5941	1	Ascomycota	Saccharomycotina
<i>Candida parapsilosis</i>	13.1	5733	1	Ascomycota	Saccharomycotina
<i>Candida tropicalis</i>	14.6	6258	1	Ascomycota	Saccharomycotina
<i>Debaryomyces hansenii</i>	12.2	6272	1	Ascomycota	Saccharomycotina
<i>Kluyveromyces lactis</i>	10.6	5076	1	Ascomycota	Saccharomycotina
<i>Kluyveromyces waltii</i>	10.9	10,721	1	Ascomycota	Saccharomycotina
<i>Lodderomyces elongisporus</i>	15.5	5802	1	Ascomycota	Saccharomycotina
<i>Pichia stipitis</i>	15.4	5841	1	Ascomycota	Saccharomycotina
<i>Pichia pastoris</i>	9.4	5313–5450	2	Ascomycota	Saccharomycotina
<i>Saccharomyces bayanus</i>	10.2–11.9	11,992	2	Ascomycota	Saccharomycotina
<i>Saccharomyces castellii</i>	11.4	4700	1	Ascomycota	Saccharomycotina
<i>Saccharomyces cerevisiae</i>	10.7–12.3	5196–5904	7	Ascomycota	Saccharomycotina
<i>Saccharomyces kluyveri</i>	11.3	5321	1	Ascomycota	Saccharomycotina
<i>Saccharomyces kudriavzevii</i>	11.4	nd	1	Ascomycota	Saccharomycotina
<i>Saccharomyces mikatae</i>	12.6	10,311	1	Ascomycota	Saccharomycotina
<i>Saccharomyces paradoxus</i>	12.2	10,554	1	Ascomycota	Saccharomycotina
<i>Saccharomyces pastorianus</i>	22.4	14,152	1	Ascomycota	Saccharomycotina
<i>Vanderwaltozyma polyspora</i>	14.7	5652	1	Ascomycota	Saccharomycotina
<i>Yarrowia lipolytica</i>	20.5	6448	1	Ascomycota	Saccharomycotina
<i>Alternaria brassicicola</i>	30.3	10,688	1	Ascomycota	Pezizomycotina
<i>Ascosphaera apis</i>	21.5	nd	1	Ascomycota	Pezizomycotina
<i>Aspergillus clavatus</i>	27.9	9125	1	Ascomycota	Pezizomycotina
<i>Aspergillus flavus</i>	37.1	12,074	1	Ascomycota	Pezizomycotina
<i>Aspergillus fumigatus</i>	28.8–29.2	9631–9906	2	Ascomycota	Pezizomycotina
<i>Aspergillus nidulans</i>	30.1	10,560	1	Ascomycota	Pezizomycotina
<i>Aspergillus niger</i>	33.9	14,165	1	Ascomycota	Pezizomycotina

TABLE 3.5 Fungal genome assemblies in GenBank and EMBL-Bank.—cont'd

Genome	Size (Mb)	Genes	Strains	Phylum	Subphylum
<i>Aspergillus oryzae</i>	37.1	12,079	1	Ascomycota	Pezizomycotina
<i>Aspergillus terreus</i>	29.3	10,406	2	Ascomycota	Pezizomycotina
<i>Blastomyces dermatitidis</i>	66.6–75.4	9522–9555	2	Ascomycota	Pezizomycotina
<i>Blumeria graminis</i>	161.4	nd	1	Ascomycota	Pezizomycotina
<i>Botrytis cinerea</i>	42.7	16,448	1	Ascomycota	Pezizomycotina
<i>Chaetomium globosum</i>	34.9	11,124	1	Ascomycota	Pezizomycotina
<i>Coccidioides immitis</i>	27.7–29.0	10,355–10,608	4	Ascomycota	Pezizomycotina
<i>Coccidioides posadasii</i>	25.5–28.6	9897–10,060	11	Ascomycota	Pezizomycotina
<i>Colletotrichum graminicola</i>	51.6	nd	1	Ascomycota	Pezizomycotina
<i>Fusarium graminearum</i>	36.5	13,332	1	Ascomycota	Pezizomycotina
<i>Fusarium oxysporum</i>	61.4	17,735	1	Ascomycota	Pezizomycotina
<i>Fusarium verticillioides</i>	41.8	14,179	1	Ascomycota	Pezizomycotina
<i>Histoplasma capsulatum</i>	30.4–38.9	9233–9532	5	Ascomycota	Pezizomycotina
<i>Magnaporthe grisea</i>	41.7	11,074	1	Ascomycota	Pezizomycotina
<i>Microsporium canis</i>	23.2	8765	1	Ascomycota	Pezizomycotina
<i>Microsporium gypseum</i>	23.2	8876	1	Ascomycota	Pezizomycotina
<i>Nectria haematococca</i>	54.4	15,707	1	Ascomycota	Pezizomycotina
<i>Neosartorya fischeri</i>	32.6	10,407	1	Ascomycota	Pezizomycotina
<i>Neurospora crassa</i>	39.2	9826	1	Ascomycota	Pezizomycotina
<i>Paracoccidioides brasiliensis</i>	29.1–32.9	7876–9136	3	Ascomycota	Pezizomycotina
<i>Penicillium marneffei</i>	28.5	nd	1	Ascomycota	Pezizomycotina
<i>Pyrenophora tritici-repentis</i>	37.8	12,171	1	Ascomycota	Pezizomycotina
<i>Sclerotinia sclerotiorum</i>	38.3	14,522	1	Ascomycota	Pezizomycotina
<i>Stagonospora nodorum</i>	37.2	16,597	1	Ascomycota	Pezizomycotina
<i>Talaromyces stipitatus</i>	35.7	12,449	1	Ascomycota	Pezizomycotina
<i>Trichoderma atroviride</i>	36.1	11,100	1	Ascomycota	Pezizomycotina
<i>Trichoderma reesei</i>	34.1	9129	1	Ascomycota	Pezizomycotina
<i>Trichoderma virens</i>	38.8	11,643	1	Ascomycota	Pezizomycotina
<i>Trichophyton equinum</i>	24.1	8560	1	Ascomycota	Pezizomycotina
<i>Trichophyton tonsurans</i>	23	nd	1	Ascomycota	Pezizomycotina

(Continued)

TABLE 3.5 Fungal genome assemblies in GenBank and EMBL-Bank.—cont'd

Genome	Size (Mb)	Genes	Strains	Phylum	Subphylum
<i>Uncinocarpus reesii</i>	22.3	7798	1	Ascomycota	Pezizomycotina
<i>Verticillium dahliae</i>	33.8	10,535	1	Ascomycota	Pezizomycotina
<i>Verticillium albo-altrum</i>	32.8	10,221	1	Ascomycota	Pezizomycotina
<i>Schizosaccharomyces japonicus</i>	11.3	4814	1	Ascomycota	Taphrinomycotina
<i>Schizosaccharomyces octosporus</i>	11.2	4925	1	Ascomycota	Taphrinomycotina
<i>Schizosaccharomyces pombe</i>	12.6	4962	1	Ascomycota	Taphrinomycotina
<i>Coprinus cinereus</i>	36.3	13,392	1	Basidiomycota	Agaricomycotina
<i>Cryptococcus neoformans</i>	17.5–19.1	6210–6967	4	Basidiomycota	Agaricomycotina
<i>Laccaria bicolor</i>	64.9	20,614	1	Basidiomycota	Agaricomycotina
<i>Moniliophthora perniciosa</i>	26.7	16,329	1	Basidiomycota	Agaricomycotina
<i>Phanerochaete chrysosporium</i>	35.1	10,048	1	Basidiomycota	Agaricomycotina
<i>Postia placenta</i>	90.9	17,173	1	Basidiomycota	Agaricomycotina
<i>Puccinia graminis</i> f. sp. <i>tritici</i>	88.6	20,567	1	Basidiomycota	Pucciniomycotina
<i>Malassezia globosa</i>	8.9	4285	1	Basidiomycota	Ustilaginomycotina
<i>Ustilago maydis</i>	19.7	6522	1	Basidiomycota	Ustilaginomycotina
<i>Batrachochytrium dendrobatidis</i>	23.7	8794	1	Chytridiomycota	Chytridiomycota
<i>Spizellomyces punctatus</i>	24.1	8804	1	Chytridiomycota	Chytridiomycota
<i>Rhizopus oryzae</i>	46.1	17,467	1	Zygomycota	Mucoromycotina
<i>Encephalitozoon cuniculi</i>	2.9	1997	1	Microsporidia	
<i>Enterocytozoon bieneusi</i>	3.9	3632	1	Microsporidia	
<i>Nosema ceranae</i>	7.9	2614	1	Microsporidia	

Data updated from www.broadinstitute.org, www.jcvi.org, www.genolevures.org, www.genome.jgi-psf.org, www.genome.wustl.edu, www.ebi.ac.uk, www.yeastgenome.org, www.candidagenome.org, www.agd.vital-it.ch, and www.aspergillusgenome.org

high-throughput sequencing (HTS) methods, even overcomes WGS's problems like unnecessary overlapping and errors originating both with the maps and sequencing. HTS surpasses the other techniques in improving the quality and continuity of the yield sequences and leverages the development that happened in the applied assembling algorithms in addition to the inclusion of end sequences from large insert clones (Aparicio et al., 2002; Jaffe et al., 2003; Mullikin & Ning, 2003).

However, still some challenges exist in the WGS genome sequencing technique, mainly the repetitive sequences that emerged through sequencing, especially those associated with telomeres, centromeres, and rDNA arrays that yielded high-identity repeated sequences. In the case of fungal genome sequencing, the probability of this problem decreased whenever the level of repetitive sequence moderated. For bacterial sequencing, the probability of such

difficulties decreased as these regions are not cloned, or even are being cloned yet eventually not assembled (Galagan et al., 2005).

In case of diploid genomes, the repetitive sequences have a great relation with the presence of polymorphism in the chromosomal regions, where assembly problems occurred due to incorrectly merging between regions with low polymorphism; on the other hand, the regions with high polymorphism showed no problems. So, it is difficult to differentiate between alleles through prologs. To minimize such problems, only haploid forms or closely related ones were sequenced. Algorithms were developed to precisely assemble the diploid's genome sequence (Vinson et al., 2005), to overcome the obstacle of unavoidable diploid forms as for *Candida albicans* (Braun et al., 2005; Jones et al., 2004).

4. Importance of fungal genome databases

Being eukaryotic representative, the fungal comparative genomic database analysis provides an outstanding chance to study the prevalence, diversity, biology, and evolution of this medically, industrially, environmentally, and biotechnologically important kingdom. The genomic information not only acts as a tool to understand the physiological, metabolic, and genetical trends, as well as revealing the cellular mechanisms of how eukaryotic organisms operate and react, but also is considered a potential tool in the biotechnological, medical, and fermentation industries (Annaluru et al., 2014). The investigation and analysis of whole fungal genomic sequencing helped in proper fungal behavioral understanding in different cases, which led to solving issues related to agriculture, biotechnology, bioenergy, health, and industry (Fig. 3.1). The importance of fungal genome sequencing as a tool is not only stemming from revealing data but also helping in gene predictions and determining which organism may have the susceptibility to acquire specific traits based on specific factors, so fungal genomics have great impacts on human health, environment, and industry. The more fungal genomes are being sequenced, the more we know about genes responsible for antibiotics, bioactive compounds, enzymes, and their pathways (Sharma, 2016).

The genome sequencing data enriched the field of studying the genetic comparison across the kingdom of fungi to figure out the evolutionary relatedness between the species, which has a great impact on medical and industrial applications. Many potential active metabolites are being extracted from filamentous groups, including enzymes, bioactive compounds, and acids. Studying the comparative genomic data across the groups led to discovering the genetic redundancy across fungal group and gene horizontal and vertical transition, which helped in revealing many enzymatic production capabilities and their metabolic pathway, and other key factors for pathogenicity roles as the comparative genomic analyses revealed clues about the pathogenesis mechanisms, which is needed for disease management (Jones et al., 2004). Also genomic study applications helped in modeling the central carbon metabolism, which modified the industrial citrate and citric acid synthesis (Pel et al., 2007).

The whole genome sequencing of *N. crassa*, as a model fungus, was the spark that ignited the study of plant and animal pathogenesis, biotechnology, molecular genetics, molecular cell biology, and gene silencing. Analysis of genome sequencing of *N. crassa* showed a perfect model system of eukaryotes to study, owing to its encoded protein similarities to proteins

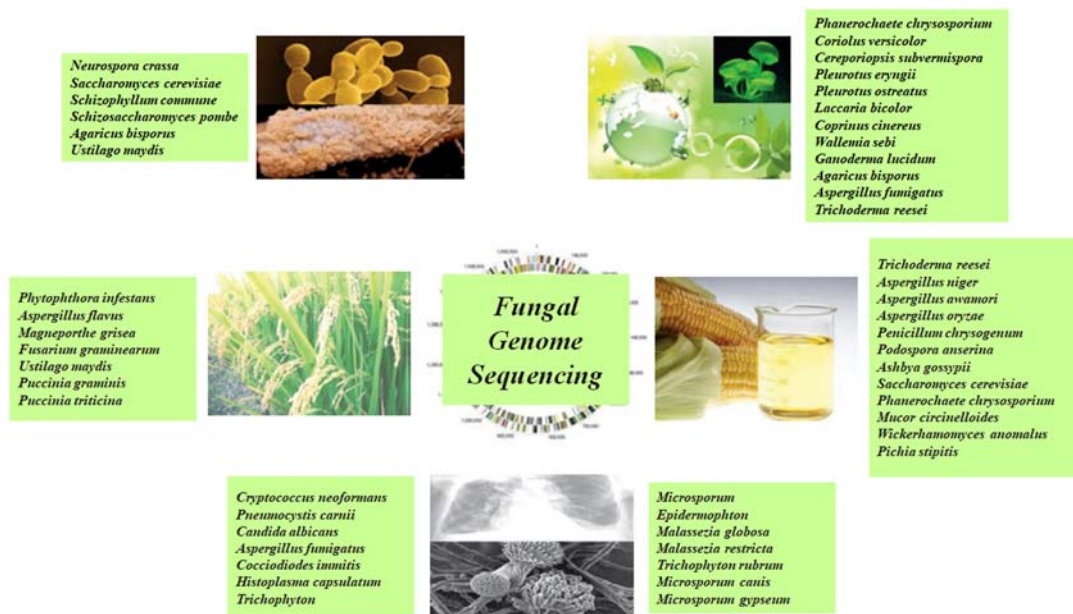


FIGURE 3.1 Some important fungal group associated with different fields.

in animals, plants, and other filamentous fungi, yet it reported no homologs to the yeasts proteins (Galagan et al., 2003).

The whole genome sequencing comparative analysis distinguished the fact that although fungi appear to be morphologically and physiologically similar, they are very divergent at the genome level. For instance, it was being thought that ascomycetes species; *Magnaporthe grisea* and *N. crassa* shared the same ancestor (Berbee & Taylor, 2000; Heckman et al., 2001), yet studying their comparative genomic sequencing showed only 47% of amino acid identity between them (Dean et al., 2005). Also, 68% of amino acid identity was reported when genomic sequencing comparison were made between *A. nidulans*, *Aspergillus fumigatus* (*A. fumigatus*), and *A. oryzae* (Galagan et al., 2005).

5. Genome sequencing and human health

A wide range of human acute and chronic diseases are mainly caused by fungi; based on this fact, scientific endeavors were focused on fungal genome sequencing for proper understanding of fungal pathogenicity patterns, and much biomedical research conducted could counter this threat (Table 3.6). Out of being one of the most common human pathogens, which is infecting the skin and mucosal layer, *Candida albicans* is considered one of the first eukaryotic pathogens to be selected for genome sequencing and its comparative analysis, sequencing the whole genome for *Candida* of 14 Mb, melted out many genes responsible for sensation, e.g., the calmodulin pathway, which contains a protein kinase, in addition to pH regulatory genes responsible for acidity adaptation (Jones et al., 2004).

TABLE 3.6 Whole genome sequencing of human fungal pathogens (Sharma et al., 2016).

Organism	Genome size (Mb)	GenBank accession/release date	Center/consortium	Significance
<i>Aspergillus fumigatus</i> Af293	30	6/26/2003	Wellcome Trust, Sanger Institute (UK) and TIGR	Cause invasive Aspergillosis in the immunocompromised patient; produce tryprostatins, with spirotryprostatin B being of special interest as an anticancer drug
<i>Blastomyces dermatitidis</i> ATCC18188	73	ADMK01000000/ March-2010	NIAID	Cause disease in immunocompetent people, causative agent of blastomycosis
<i>Candida albicans</i> SC5314	14	AACQ01000000	Stanford University	Severe systemic disease in immunocompromised postsurgery, or patient taking broad-spectrum antibiotics
<i>C. tropicalis</i> CBS 94	15	—	NHGRI	Virulent in neutropenic host
<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21	19.05	7/13/2004	Stanford University	Fatal meningitis in humans in immunocompromised individuals
<i>Coccidioides immitis</i> H538.4	28	AASO01000000	NIAID	Causes coccidioidomycosis, also known as “valley fever”
<i>Penicillium chrysogenum</i> Wisconsin 54–1255	34.1	—	DSM Anti-infectives, The Netherlands	Human allergens
<i>Geomyces destructans</i> 20,631–21	35	AEFC01000000/ August 2010	NHGRI and USGS	Fungal skin infection
<i>Histoplasma capsulatum</i> G186AR	30	ABBS02000000/ may-2008		Fungal respiratory infections (histoplasmosis)
<i>Paracoccidioides brasiliensis</i> Pb03	29	ABHV01000000	NIAID	Paracoccidioidomycosis (PCM)
<i>Trichophyton rubrum</i> CBS118892	22.5	ACPH01000000	Broad Institute, NHGRI	A dermatophyte, cause human fungal infections
<i>Wangiella</i> (<i>Exophiala</i>) <i>dermatitidis</i> NIH/ UT8656	30	AFPA01000000	Broad Institute, NHGRI	Emerging human mycosis in immunocompromised patient (phaeohyphomycosis)
<i>Trichophyton tonsurans</i>	23	ACPI01000000	Broad Institute, NHGRI	Causes inflammatory or chronic noninflammatory finely scaling lesions of skin, nails and scalp, e.g., Tinea capitis

(Continued)

TABLE 3.6 Whole genome sequencing of human fungal pathogens (Sharma et al., 2016).—cont'd

Organism	Genome size (Mb)	GenBank accession/release date	Center/consortium	Significance
<i>Microsporium gypseum</i>	23.2	ABQE01000000	Broad Institute, NHGRI	Produces a single inflammatory skin or scalp lesion
<i>Arthroderma benhamiae</i>	22.2	ABSU00000000/ 2009	Institute of Microbiology and Department of Dermatology and Allergology, Germany	Infect keratinized tissue i.e., skin, nails and, rarely hair
<i>Trichophyton verrucosum</i>	22.5	ACYE00000000/ 2009	Institute of Microbiology and Department of Dermatology and Allergology, Germany	Highly inflammatory, involving the scalp, beard or exposed areas of the body, i.e., Nails and skin

As immunocompetent mammals and human pathogens, both *Coccidioides immitis* and *Coccidioides posadasii* cause human pulmonary infection (Hector & Laniado-Laborin, 2005; Sharpston et al., 2009). Surprisingly, the genome sequencing of soil saprophytes *Coccidioides* species ended up not only revealing the pathogenicity behavior but also claiming gene transitions occurred for a few genes that have transformed them from plant-based to animal-based (Sharpston et al., 2009). Another human pathogen belongs to Ascomycetes, *A. fumigatus*, as a saprotrophic fungus that causes aspergillosis, which is devastating lung infections in terms of morbidity and mortality (Latgé and Chamilos, 2019). *A. fumigatus* is mainly infecting susceptible patients suffering from neutropenia and taking drugs like corticosteroids with T cell problems (Ronning et al., 2005). The genomic sequencing of *A. fumigatus* revealed species-specific chromosomal islands, which led to their adaptation to different habitats (Fedorova et al., 2008; Ronning et al., 2005).

Cryptococcus neoformans (*C. neoformans*), a basidiomycetous yeast that is a chest infection pathogen, may also cause central nervous system disease. Interestingly, the 20 Mb genome of *C. neoformans* was sequenced in 2005 by Loftus et al., which turned out 6500 intron-rich gene structures (Loftus et al., 2005). The genome analysis revealed the intensive presence of transposon, which is known to be causative of karyotyping instability, that eventually led to many changes like virulence (Loftus et al., 2005; Ormerod et al., 2013). In 2012, Martinez and his colleagues sequenced the whole genome of the dandruff and seborrheic dermatitis-causing pathogens *Malassezia globosa* (*M. globosa*) and *Malassezia restricta*, which belong to basidiomycete fungi.

Comparative genomic sequencing analysis of *M. globosa* (as a human pathogen) and *Ustilago maydis* (*U. maydis*) (as maize pathogen) showed high degree of rapprochement, which gives a clear clue of ancestral shift between different hosts (Xu et al., 2007). Interestingly, the phylogenetic analysis of genomic data from *M. globosa* and *U. maydis* showed both sharing the same gene cluster responsible for specific proteins that were known to be functionally specific to plant interactions. Martinez and his colleagues in 2012 conducted

comparative genome analysis to dermatophytes and related species; *Trichophyton species* and *Microsporium species* ended up the duplication of dermatophytes genome throughout the group evolution, and many genes responsible for enzymatic secretion were appeared and fostered, which all led to evolve fungal virulence and pathogenicity (Martinez et al., 2012).

6. Genomic sequences and plant health

The plant infection control endeavors stem from its economic importance in order to pursue sustainable food production and decreasing the consumption of irrigation water and fertilizers (Table 3.7). Among the top common and important fungal plant-pathogenic are: *Fusarium oxysporum* causing *Fusarium* wilt to many plants, *Magnaporthe oryzae* (*M. oryzae*) causes rice blast disease, *Botrytis cinerea* causes gray mold disease to more than 200 important crops, *Blumeria graminis* causing powdery mildew disease on several important small grain cereals and several grass, *Mycosphaerella graminicola* (*M. graminicola*) causes the *Septoria tritici* blotch, which is a devastating wheat leaves' disease that leads to dramatic decreases in wheat yield, and *U. maydis* that infect the maize and form corn smut diseases. Some of the key factors that hampered controlling the plant infection were understanding the genetic and biochemical basis of pathogenicity, besides the lack of clear knowledge of the exact infection mechanisms (Dean et al., 2012).

A genomic analysis of the plant pathogen and causative agent of rice blast disease, *M. oryzae* (38.8 Mb), was conducted and gave a clear image of the invasion and cooccurrence of such pathogens with the economically important cultivated crop (Dean et al., 2005). Contrary to *M. oryzae*, studying the whole genome sequence of *U. maydis* (19.8 Mb) showed obvious clues about its ability to survive without causing big damage to the host (Hertz-Fowler & Pain, 2007).

Comparative genomic analysis as well as transcriptomics of some rust-causing fungal species were conducted and analyzed out of their economic importance, giving dozens of rust candidate secreted effector proteins were noticed through the species (CSEPs). Capitalizing on the integration between the technology of genomics, transcriptomics, and effector-directed annotation of *Puccinia striiformis* f. sp. *tritici* isolates, all led to the capability of construction of a CSEPs mining framework through closely related isolates then linking these to their pathogenicity profiles (Cantu et al., 2013). Another deep genomic analysis of plant pathogens (36 Mb) *F. graminearum*, which acquired the capabilities to adapt a wide range of habitats and cause economic losses for many crops, revealed the mechanism of adaptation and accommodation of such pathogens and the host plants. Interestingly, specific genomic regions related to nutrition and lineage were detected when genomic comparative analysis was done for *F. graminearum*, *F. verticillioides*, and *F. oxysporum* f. sp. *lycopersici*; these regions are considered key factors in acquiring virulence traits and pathogenicity (Ma et al., 2010; Pain & Hertz-Fowler, 2008). The comparative genomic analysis of the plant pathogen and cause of *Septoria tritici* blotch in wheat, *M. graminicola*, has distinguished a possible mechanism of pathogenicity through detecting structural rearrangements of dispensable chromosomes. These chromosomes were reported to be horizontally transferred and then recombined, which highlighted new attributes of genetic structure. This mechanism was

TABLE 3.7 Whole genome sequencing of some pathogenic plant-fungi^a (Sharma, 2016).

Organism	Genome size (Mb)	GenBank accession/ release date	Center/consortium
<i>Pyrenophora semeniperda</i> (anamorph <i>Drechslera campanulata</i>)	32.5 Mb	ATLS000000000	National research Initiative, United States Department of Agriculture
<i>Phytophthora infestans</i>	240	AATU01000000/9/2009	Broad Institute
<i>Aspergillus flavus</i>	—	—	—
<i>Magnaporthe grisea</i> 70–15	40	10/31/2003	International rice blast genome consortium
<i>Fusarium graminearum</i>			Broad Institute
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> NRRL 26406	54.03	AGNE000000000.1	Broad Institute
<i>F. pseudograminearum</i> CS3220	37.16	CBMC010000000/2013	Bioplatfroms, Australia
<i>Ustilago maydis</i> 521	20.5	7/29/2003	Broad Institute
<i>Puccinia graminis</i>	—	—	—
<i>Mycosphaerella graminicola</i> (anamorph <i>Septoria tritici</i>)	39.7	2011	DOE Joint genome institute
<i>Alternaria brassicicola</i> ATCC 96836	29.6	October 03, 2009	Washington University genome center (WUGC)
<i>Alternaria arborescens</i> CBS 102605	34	AIIC01000000	Ohio Agricultural research and development center research

^a*Pyrenophora semeniperda* (anamorph *Drechslera campanulata*) is necrotrophic, fungal seed pathogen; has a wide host range within the Poaceae family; *Phytophthora infestans* causes the most destructive disease of potato; annual worldwide potato crop losses due to late blight are conservatively estimated at \$6.7 billion; *Aspergillus flavus* infection causes ear rot in corn and yellow mold in peanuts; *Magnaporthe grisea* 70-15 causes rice blast fungus, rice rotten neck, rice seedling blight, blast of rice, oval leaf spot of Gramineae, pitting disease, ryegrass blast; *Fusarium graminearum* causes head blight, crown rot, and root rot of cereals; *Fusarium oxysporum* f. sp. *melonis* NRRL 26,406 is an important group of fungal plant pathogens cause various diseases on nearly every economically important plant species and pose health hazard to humans and livestock. Producing mycotoxins, besides their economic importance, species of *Fusarium* also serve as key model organisms for biological and evolutionary research. *F. pseudograminearum* CS3220 causes crown rot disease: in Australia, crown rot is responsible for annual losses of approx. \$100 million in the wheat and barley industries; *Ustilago maydis* 521 causes smut on maize and teosinte; *Puccinia graminis* causes disease of bread wheat, durum wheat, barley, and triticale; *M. graminicola* (anamorph *Septoria tritici*) is wheat plant pathogen causing Septoria leaf blotch; *Alternaria brassicicola* ATCC 96,836 causes black spot disease on virtually every important cultivated Brassica species; *A. arborescens* CBS 102,605 is a fungal plant pathogens cause serious agricultural losses worldwide, major pathogen of tomato.

detected through a small number of genes in only eight dispensable chromosomes, but the other essential chromosomes were syntenic (Stukenbrock et al., 2010). The genomic analysis of *M. graminicola* also reported that the genes responsible for secretion of lysis enzymes that would break the host cell walls were present but in a lower number than would be expected, and that may be a kind of maneuver against plant defense mechanisms, which is considered evolution (Goodwin et al., 2011).

7. Genomic sequence and bioactive compounds

Many fungal species are distinguished by the presence of unique pathways for the production of many bioactive compounds, named secondary metabolites; as being natural products, these secondary metabolites were categorized and listed under the main groups of alkaloids, flavonoids, peptides, terpenes, and polyketides (Keller et al., 2005). Besides, it produces several pigments, phenolic compounds, and mycotoxins such as aflatoxins (Lackner et al., 2012). Genome-mining and comparative genomic sequencing analysis uncovered the fact that normal and conventional culturing condition aren't the proper way to investigate and estimate the fungal capabilities to synthesize bioactive secondary metabolites, regarding the possibility of the presence of many gene clusters that are silent under normal cultivation conditions (Brakhage, 2013), SMURF (Khaldi et al., 2010), antiSMASH (Medema et al., 2011), and Fungi Fun (Priebe et al., 2011), were kinds of bioinformatic algorithms developed as endeavors done to screen, detect, and identify these gene clusters responsible for synthesizing secondary metabolism. The genome sequencing analysis contributed to screening and investigating the fungal group's capabilities to secrete many active secondary metabolites. For instance, predict the capabilities of the basidiomycetes group to produce polyketides based on tracking the polyketide pathway; this group of natural products includes many important antibiotics like tetracycline and erythromycin, in addition to products of antitumor activity like epothilone and dyslipidemia drug like as lovastatin (Lackner et al., 2012).

A whole genome sequence of medicinal importance of the fungus *Ganoderma lucidum* strain 260,125–1 (43.3 Mb) was done, which ended up detecting and identifying a set of potential gene clusters involved in putative secondary metabolism (Chen et al., 2012; Sanodiya et al., 2009). Another screening and analysis for genome sequences of (28.15 Mb) *Omphalotus olearius* was done and revealed two clusters of genes that are responsible for the biosynthesis of the antitumor agent illudin. Integration between genomic and biochemical data melted out a framework that was developed to list all the data of terpenomes from the Basidiomycota group and helped to target specific genes for the synthesis of new novel bioactive compounds (Wawrzyn et al., 2012).

8. Genomic sequence and enzyme biotechnology

Spring out of its commercial and biotechnological importance, the application of enzymes improves and accelerates many industrial operations and make them more efficient by reducing the amount of energy needed and minimizing undesired waste and byproducts (Table 3.8). It's worth to mention that fungi have the capability to produce all kinds of carbohydrate-active enzymes (CAZymes), and whole genome sequencing facilitates searching for enzymes' synthetic abilities on gene level (Cantarel et al., 2009). *Aspergillus* genus had a head start and was among those that took intensive concentration for genomic sequencing owing to the excessive and diverse enzymatic yield they produced. Interestingly, as an important source of citric acid and many economic-important enzymes, *Aspergillus niger*, that produces glucoamylase, which lysis the starch in food and ethanol production,

TABLE 3.8 The whole genome sequencing of fungi is used in enzyme biotechnology^a (Sharma, 2016).

Organism	Genome size (Mb)	GenBank accession/ release date	Center/consortium
<i>Saccharomyces cerevisiae</i>	12	1996	International effort
<i>Trichoderma reesei</i> QM6a	33	April 08, 2005	DOE- Joint Genome Institute
<i>Aspergillus niger</i> CBS 513.88	37	3/28/2006	DSM, The Netherlands
<i>A. oryzae</i> RIB40	37	12/20/2005	NITE (National Institute of Advanced Industrial Sciences and technology)
<i>Penicillium chrysogenum</i> Wisconsin 54–1255	34.1	—	DSM Anti-infectives, The Netherlands
<i>Podospora anserina</i>	35.5	—	—
<i>Ashbya gossypii</i>	—	—	—
<i>Phanerochaete chrysosporium</i> RP-78	30	2004	DOE – Joint Genome Institute
<i>Wickerhamomyces anomalus</i> (formerly <i>Pichia anomala</i>)	25.47	2012	
<i>Agaricus bisporus</i>	36	AEOK00000000 and AEOL00000000	DOE – Joint Genome Institute
<i>Pichia angusta</i> CBS 4732	9		BRG Grant (resources génétiques des microorganismes No. 11–0926-99)
<i>P. farinose</i> CBS 7064	13.9	—	Genolevures
<i>P. guilliermondii</i> ATCC6260	12	3/17/2005	Broad Institute
<i>P. pastoris</i> DSMZ 70382	9.4	4/14/2009	Natural Resources and Applied Life Sciences Vienna, Austria
<i>P. stipitis</i> CBS 6054	15.4	2/23/2007	DOE – Joint Genome Institute
<i>Morchella conica</i> CCBAS932	—	PRJNA217205/1/14/2014	1000 Fungal genomes/JGI
<i>Penicillium chrysogenum</i> (<i>Penicillium rubens</i>) NCPC10086	32.3	APKG00000000	Natural Science Foundation of China
<i>P. roqueforti</i> FM164, <i>P. camemberti</i> FM 013	28, 34	HG792015–HG792062, HG793134–HG793313	ANR grant ‘Food Microbiomes’ (ANR-08-ALIA-007–02)
<i>Mortierella alpina</i> isolateCDC-B6842	39.53	AZCI00000000	National Institute of Allergy and Infectious Diseases, National Institutes of Health Department of Health and Human Services

^a*S. cerevisiae* is the most useful yeast, having been instrumental to wine-making, baking and brewing since ancient times; most intensively studied eukaryotic model organisms in molecular and cell biology; *Trichoderma reesei* QM6a Secretes large amounts of cellulolytic enzymes; *Aspergillus niger* CBS 513.88 is soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose; known for citric acid and gluconic acid production; causes black mold of onions; *A. oryzae* RIB40 ferments soybeans, also used to saccharify rice, other grains, and potatoes in the making of alcoholic beverages such as huangjiu, sake, makgeolli and rice vinegars; *Penicillium chrysogenum* Wisconsin 54–1255 is the source of several β -lactam antibiotics, e.g., penicillin; industrially used to produce enzymes, i.e., polyamine oxidase, phospho-gluconate dehydrogenase, and glucose oxidase; *Podospora anserina* used in biomass degradation; *Ashbya gossypii* used in Riboflavin-overproduction; *Phanerochaete chrysosporium* RP-78 degrades lignin to carbon dioxide; used in bioremediation; *Wickerhamomyces anomalus* (formerly *Pichia anomala*) is a biocontrol agent; antifungal agent; wine fermentation; phytate digestion; source of protein and vitamins; *Agaricus bisporus* is xylan, cellulose, pectin decomposer; secondary decomposer fungus, plays an ecologically significant role in the biomass degradation; also used for bioremediation of substrates contaminated with heavy metals; *Pichia angusta* CBS 4732 is a methylotrophic yeast, well studied for structure, function, and biogenesis of the peroxisomes; *P. farinose* CBS 7064 produces glycerol from glucose; *P. guilliermondii* ATCC6260 converts xylose to xylitol; riboflavin synthesis; *P. pastoris* DSMZ 70,382 Used in biofuel, biochemical, and genetic research; also used in biotech industry; *P. stipitis* CBS 6054 ferments xylose to ethanol; *Morchella conica* CCBAS932 used in cellulase and laccase production; *Penicillium chrysogenum* (*Penicillium rubens*) NCPC10086 used in penicillin biosynthesis; *P. roqueforti* FM164 and *P. camemberti*, used for the maturation of soft cheeses; *P. camemberti* and *P. roqueforti* are used as starter cultures for cheeses; *Mortierella alpina* isolateCDC-B6842 used in commercial applications, such as with biodiesel and other lipid based products.

this fungus was among seven aspergilli species whose genomes were early sequenced and annotated (Gilsenan et al., 2012).

Among important fungal enzymes, the hydrolytic enzymes that cause degradation of the cell wall, as it is considered a crucial factor in fungal pathogenicity, due to their hydrolysis attributes that enable the fungus to penetrate the plant cell wall application, have been reported as a key factor in bioethanol production from woody material. Many studies concentrated on screening the activity of hydrolytic enzymes from fungal origins and resulted in different preferences of different fungal species toward each and every type of plant biomass (Battaglia et al., 2011; Zhao et al., 2014). Many comparative analyses focused on tracking groups of enzymes across the fungal kingdom. CAZymes was one of the most studied enzymes, with more than 300 ones were detected out of proteomes predicted from 187 CAZyme families, and it was observed that the presence of some CAZyme families was specifically related to a definite phylum (Zhao et al., 2014). The Ascomycota division encompasses a group of fungi that secrete polysaccharides-hydrolytic enzymes from cellulases and hemicellulases. *Podospora anserina*, as a fungus of this division, was recorded to have a maximum number of cellulose-degrading enzymes (Espagne et al., 2008).

Fungal enzymes contribute to many important bioprocesses, like biofuel production throughout fermentation of the fungus that led to produce alcohol and ethanol, or even by degradation of lignocellulosic plant stocks. The Fungal Genomics Program was launched out of integration between the JGI and the US DOE, for the sake of utilizing the fungal genome sequencing analysis to figure out their presence, prevalence, abundance, and applications for bioenergy (Grigoriev et al., 2011). For a long time, many yeast species like *S. cerevisiae*, *S. bayanus*, and various hybrids, such as *S. carlsbergensis*, were fermented to produce ethanol from poly, di, and monosaccharides, but they didn't have the capabilities of fermenting xylose, arabinose, and cellobiose. Capitalizing on the genome sequencing and genome mining technologies, the sequencing of the complete genome of *Pichia stipitis* revealed the presence of many genes responsible for cellobiose-degrading enzymes (Kurtzman & Robnett, 2010; Smith et al., 2008). On the other hand, a comparative genomic technique was applied to the responsible genes for xylose consumption after sequencing two xylose-fermenting organisms, *Spathaspora passalidarum* and *Candida tenuis*, that was done by DOE-JGI. Besides, genomic analysis of five species of *Hemiascomycete* with different xylose-consumption phenotypes showed many genes responsible for xylose ingestion (Wohlbach et al., 2011). These species are ubiquitous filamentous ascomycetes important to the biotechnology, biomedical, and food industries. They commonly occur as food spoilage agents and opportunistic pathogens and are widely used as versatile cell factories.

Genomic sequencing (32.19 Mb) of *Penicillium chrysogenum* Wisconsin 54–1255 resulted in defining many genes contributing to the synthesis of penicillin (Van Den Berg et al., 2008). Then comparative genome analysis was done among *P. roqueforti* and *P. camemberti* (both have been used in manufacturing cheese) and *P. rubens* (as the main source of penicillin synthesis). The analysis resulted in the presence of an island that accommodates around 250 genes that exist in the genome of the species *P. roqueforti* with 575 kb of length; some of these genes in the genomic island contributed to competition with other microorganisms (Cheeseman et al., 2014).

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Introduction to genomic-based clinical applications

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1. Introduction

The beginnings of genomics-based medical applications can be attributed to the completion of the Human Genome Project in 2003 (Gonzaga-Jauregui et al., 2012; Hood & Rowen, 2013). The primary objective of this project was to undertake the sequencing and mapping of the entirety of the human genome, providing scientists with an extensive understanding of the genetic composition of our genomes (Engel, 1993). The successful completion of this study represented a turning point in the field of medicine since it facilitated the emergence of personalized medicine and targeted therapies designed based on an individual's unique genome (Gonzaga-Jauregui et al., 2012).

The discipline of medicine has been significantly transformed by implementing clinical applications based on genomics. Next-generation sequencing (NGS) technologies have made it possible to analyze large amounts of genomic data quickly and cost-effectively (Xuan et al., 2013). The integration of genomics, which focuses on the comprehensive analysis of an individual's genetic makeup, with clinical practices has given rise to a nascent domain. This has led to breakthroughs to enhance the efficacy of diagnostic procedures, therapeutic interventions, and preventive measures in identifying disease-causing genetic mutations and developing targeted therapies for various conditions such as cancer, cardiovascular diseases, rare genetic disorders, and infectious diseases (Brittain et al., 2017; Hilt & Ferrieri, 2022).

Understanding the structure and function of pathogen genomes, encompassing bacteria, viruses, fungi, and other infectious species, can yield valuable insights into their virulence, drug resistance, and transmission patterns. Several genomic technologies and strategies have been employed for pathogen diagnosis, including polymerase chain reaction (PCR)-based assays (Lei et al., 2021; Li et al., 2022; Liu et al., 2018), microarrays (McLoughlin, 2011), whole genome sequencing (WGS) (McLoughlin, 2011; Tagini & Greub, 2017), and

metagenomics (Gu et al., 2019). The advancements in these technologies, combined with the development of computational biology, have significantly improved the speed and accuracy of pathogen identification compared to conventional methods. Furthermore, genomics has drastically altered drug development through target identification (Patel, 2016), which provides valuable insights into potential drug targets based on genomic data. Target identification involves analyzing the genomes of individuals to identify specific genes or proteins that play a role in disease development or progression, thereby resulting in the design of treatments that selectively target these genes or proteins, leading to more effective and personalized treatments.

One example of genomics-based clinical treatments can be observed by applying targeted therapies for cancer treatment. Through genomic analysis of tumor samples, researchers can detect and characterize distinct mutations or variations in genes associated with cancer (Nakagawa & Fujita, 2018; Patel, 2016). This data aids in advancing pharmaceuticals targeting these altered genes or proteins, resulting in more efficient therapies and reduced toxicity. For instance, pharmaceutical drugs like imatinib, which is used in treating chronic myeloid leukemia by specifically targeting the BCR-ABL fusion gene (Stagno et al., 2016), and trastuzumab, which is utilized in managing HER2-positive breast cancer (Von Minckwitz et al., 2019). Another area where genomics has significantly impacted drug development is in adverse drug reactions (ADRs) (Cacabelos et al., 2019). Genetic differences can potentially increase a patient's susceptibility to ADRs or modify their response to a particular treatment. Genomic analysis has the ability to detect these genetic variants, resulting in well-informed decisions regarding drug selection and dosage. For example, conducting a genotypic test for HLA-B*5701 before administering abacavir has decreased the risk of severe hypersensitivity reactions in individuals diagnosed with HIV (Parczewski et al., 2010).

In conclusion, the field of genomics has had a significant advancement in clinical applications subsequent to the successful completion of the Human Genome Project. Recent advances in Genomic technologies and computational methods have experienced considerable progress involving enhanced diagnostic capabilities, tailored therapeutic interventions, and preventive measures. Genomics-based methodologies substantially influence public health; they bring a transformative shift in healthcare provision and have resulted in enhanced patient outcomes. The ongoing investigation of the complexities of the human genome will lead to significant advancements in genomics-based clinical applications that hold immense promise for the future of medicine.

2. Genomics in the clinical context

Genomics is the comprehensive study of an organism's entire genetic material, including its structures, functions, interactions, and variations. In healthcare, genomics refers to applying genomic information to improve medical diagnosis, treatment, prevention, and overall understanding of health and diseases. This involves analyzing the DNA sequences, gene expressions, and genetic variations to better understand the mechanisms involved in the onset and progression of diseases, diagnose diseases, predict disease risks, use pharmacogenomics, and develop personalized treatment strategies.

Genomics enhances disease diagnosis by identifying genetic markers associated with various conditions. In the case of hereditary diseases like cystic fibrosis, genomics enables accurate early diagnosis, facilitating prompt intervention and family planning (Castellani et al., 2008). Additionally, genomics aids in diagnosing rare diseases with genetic origins. Whole exome sequencing or WGS can identify rare genetic mutations responsible for conditions like familial hypercholesterolemia, leading to more accurate diagnosis and better management (Cuchel et al., 2014; de Paiva Silvino et al., 2020). Furthermore, genomics aids in predicting disease risks. For example, genetic variants associated with increased susceptibility to certain diseases like Alzheimer's can help individuals and healthcare providers implement preventive measures early on (Lambert et al., 2013).

Genomics guides drug selection and dosages based on a patient's genetic profile. For instance, genetic variations in drug-metabolizing enzymes can influence a person's response to medications, allowing physicians to prescribe the most effective and safe drugs for that individual (Relling & Evans, 2015). Moreover, genomics allows tailoring medical treatments based on an individual's genetic makeup. For example, certain cancer treatments, such as targeted therapies, are designed to target specific genetic mutations driving tumor growth, leading to more effective and less toxic treatments (Collins & Varmus, 2015).

The role of genomics in advancing medical practice is evident through its application in precision medicine, pharmacogenomics, diagnostics, rare disease management, and predictive medicine. These examples highlight how genomics has transformed healthcare by offering more personalized, effective, and patient-centered approaches to treatment and prevention.

3. Genomic basis of pathogens

A pathogen is a biological agent that can cause disease for its host. Pathogens such as a virus, bacteria, fungi, or parasites can be transmitted from one host to another through various means, including direct contact, airborne droplets, contaminated food, or various vectors like mosquitoes and ticks (Sherris, 1990). They can cause a wide range of infectious diseases, from common colds and flu to more severe illnesses like tuberculosis, HIV/AIDS, and COVID-19.

The genomic basis of pathogens, including bacteria, viruses, fungi, and other infectious agents, refers to the genetic information encoded in the DNA or RNA of microorganisms that enables them to cause disease, evade host defenses, and adapt to various environments. Understanding the genomic features of pathogens provides insights into their pathogenicity, virulence, and mechanisms of infection.

Bacteria are single-cell organisms with a relatively simple genomic structure, consisting of a single circular DNA molecule located in the cytoplasm, carrying essential genes that are responsible for the bacterium's survival, replication, and virulence. Additionally, bacterial genomes can also contain plasmids, which are smaller, circular DNA fragments that may carry additional genes, such as those for antibiotic resistance (Brock & Madigan, 1988).

Viruses, on the other hand, have a much simpler genomic structure consisting of either DNA or RNA, which can be single-stranded or double-stranded molecules enclosed within a protein coat (Flint, 2000). Viral genomes are relatively compact, containing genes encoding

proteins necessary for attachment, entry into host cells, replication, and assembly of new viral particles. Unlike cells, viruses lack the cellular machinery required for independent metabolism, making them obligate intracellular cells that rely heavily on host cellular machinery for replication and transcription (Flint, 2000).

Unlike bacteria and viruses, fungi, which include yeasts, molds, and other microorganisms, have more complex genomes that can be relatively large and may include multiple linear chromosomes and exhibit variations in gene content and arrangement.

Fungi genomes encode proteins that are responsible for a wide range of functions, including those related to nutrient acquisition, sexual and asexual reproduction, the production of virulence factors, and interactions with other organisms (Basenko et al., 2018; Moore, 2013, pp. 1–231). Other infectious agents, such as parasites, can have intricate genetic structures due to their multicellular nature (Truant et al., 2016).

4. Methods and strategies used for pathogen diagnosis and predicting the emergence of pathogens

Before the era of genomics, the gold standard for pathogen diagnosis was based on culturing the infectious agent from clinical specimens and characterizing its phenotypic properties, such as colony morphology, biochemical reactions, and antimicrobial susceptibility. This approach was the foundation of clinical microbiology and allowed for the isolation and identification of a wide range of bacterial, fungal, and parasitic pathogens. However, this process was time-consuming and could lead to delays in diagnosis, potentially affecting patient management. Additionally, it may not be suitable for fastidious or unculturable pathogens. Despite these limitations, culture-based methods remain important for certain pathogens and still serve as a reference for validating newer diagnostic approaches (Leber & Burnham, 2022; Tille, 2014). Fig. 4.1 presents a comprehensive schematic representation that encapsulates the evolving landscape of methods and approaches utilized for pathogen diagnosis and prediction.

4.1 Polymerase chain reaction (PCR) and nucleic acid amplification techniques

The development of PCR in the 1980s marked a significant milestone in molecular diagnostics (Mullis et al., 1986). PCR allowed for the amplification of specific DNA sequences from pathogens, enabling their rapid detection and identification. Targeting conserved regions of pathogen genomes, PCR has been widely used for diagnosing bacterial, viral, and parasitic infections. Over the years, various modifications of PCR, such as real-time PCR (Liu et al., 2018), quantitative PCR (Wu, Tian, et al., 2020), digital PCR (Lei et al., 2021; Li et al., 2022), and high-resolution melting (Tamburro & Ripabelli, 2017), have been introduced, further improving sensitivity and quantification of pathogen nucleic acids. In the case of COVID-19, real-time PCR assays targeting specific regions of the SARS-CoV-2 genome have become crucial for diagnosing and monitoring the disease (Sule & Oluwayelu, 2020). PCR-based techniques have significantly transformed the field of pathogen diagnostics by

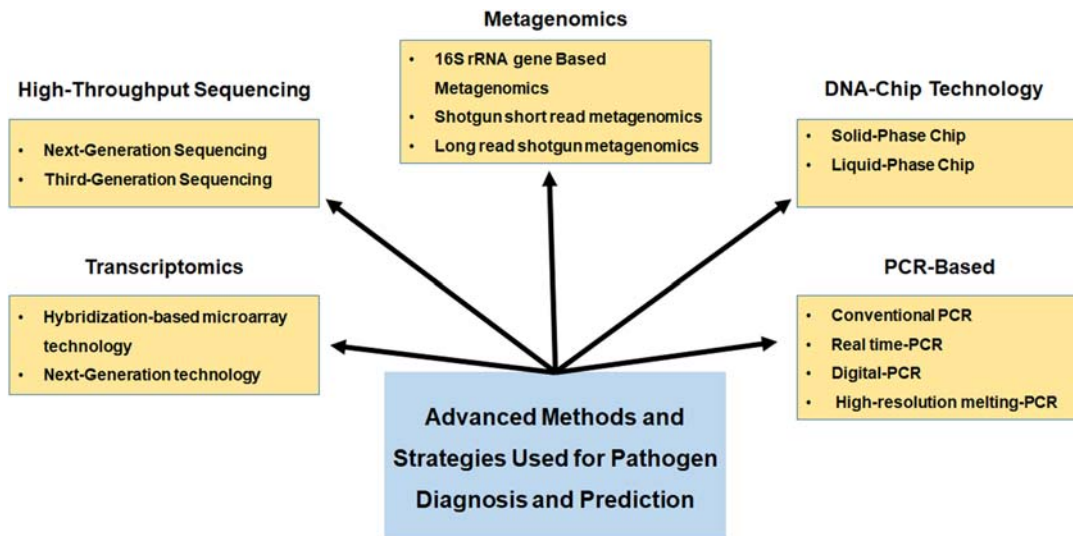


FIGURE 4.1 Schematic representation depicting the recent methods and approaches utilized for pathogen diagnosis and prediction.

offering accelerated and more precise results in contrast to conventional culture-based methodologies. Additionally, nucleic acid amplification techniques have proven to be effective in the rapid and simple identification of known infectious disease pathogens, as well as the detection of drug resistance (Bang et al., 2023; Tamburro & Ripabelli, 2017).

4.2 Microarrays and DNA chips

Microarray technology emerged in the late 1990s as a high-throughput method for analyzing gene expression profiles (Lockhart et al., 1996). In the context of pathogen diagnosis, microarrays were adapted to detect multiple pathogen targets simultaneously. DNA microarrays, also known as DNA chips, encompass both solid-phase and liquid-phase chips, which operate on the fundamental principle of nucleic acid molecular hybridization. The detection of gene sequences in samples is achieved by the process of hybridization, wherein target sequences within the samples are allowed to bind with complementary probes that are immobilized on different surfaces, allowing for the simultaneous detection of various pathogens in a single test. This approach facilitated the diagnosis of infections with multiple agents or emerging pathogens (Bednar, 2000). Nevertheless, the extensive implementation of microarrays encountered obstacles from financial constraints, restricted probe coverage, and the requirement for specialized equipment (Call, 2005).

4.3 Next and third-generation sequencing (NGS)

The introduction of NGS technology during the 2000s brought about a significant transformation in the field of genomics, enabling exploration of the WGS for pathogenic organisms

(Mardis, 2008). NGS allows for rapid and cost-effective sequencing of large volumes of genetic material, making it an ideal tool for pathogen diagnosis (Ki, 2021). WGS provides a comprehensive view of the entire pathogen genome, enabling accurate identification, subtyping, and characterization of pathogens, including drug resistance and virulence determinants. NGS methodologies have been used in the investigation of several pathogens, encompassing bacteria, viruses, and fungi. These procedures have contributed to the advancement of our comprehension regarding the genomic variety and evolutionary dynamics of these organisms (Mardis, 2008).

In 2014 (Lu et al., 2016), third-generation sequencing tools, including those from Oxford Nanopore Technologies and PacBio, were released. This novel method detects changes in electrical current when DNA passes through the nanopore, thereby identifying the DNA sequence. High-throughput methods produce long reads of DNA molecules at a lower cost because they don't use specialized procedures like labeling DNA molecules. There are benefits to TGS that NGS lacks; for example, long reads and direct RNA molecule sequencing are two features that NGS lacks.

4.4 Metagenomics and shotgun sequencing

Metagenomics is a powerful approach that involves the direct sequencing of all genetic material in a clinical sample, without the need for culturing or specific target amplification. The methods of metagenomic NGS can be broadly categorized into two distinct approaches, which are determined mainly by the specific target or type of information acquired during the sequencing process (Chiu & Miller, 2019). The utilization of the PCR in targeted metagenomic sequencing involves the amplification of specific regions, such as the 16S rRNA gene (Chiu & Miller, 2019; Shahi et al., 2019) or the internal transcribed spacer (Alanagreh et al., 2017), which enables the classification of pathogens based only on taxonomical criteria. Shotgun metagenomics involves sequencing the entire genomic content of a sample, enabling the identification and characterization of pathogens even in complex microbial communities (Joseph & Pe'er, 2021). Shotgun metagenomics offer a broad access to various therapeutically significant genomic characteristics, including resistance genes, identification of virulence factors, and strain-level typing of all microbial communities present in the sample. This approach has been particularly valuable for diagnosing infections with unculturable or difficult-to-detect pathogens as it was estimated that less than 1% of microorganisms could be cultured using these methods (Wooley et al., 2010). Therefore, selecting these two ways should be based on the specific information needed for the intended goal.

4.5 Transcriptomics

Transcriptomics sequencing, alternatively referred to as RNA sequencing (RNA-seq), has emerged as a highly powerful technique that has dramatically altered the areas of genomics and molecular biology. The process involves utilizing high-throughput sequencing techniques to analyze RNA molecules, thereby offering a comprehensive overview of gene expression within a specific cell or tissue (McDermaid et al., 2019). The utilization of transcriptomics sequencing has been extensively employed in fundamental scientific investigations to examine gene regulation and discover new biomarkers (Zhao et al., 2022).

However, there is an increasing understanding of its potential in the clinical context for identifying pathogens.

Transcriptomics sequencing has numerous advantages. Transcriptomic sequencing can offer a comprehensive perspective of the complete transcriptome within an infected sample (Ren et al., 2021). This implies that the technology has the capability to not only detect the presence of known pathogens through the identification of their distinct RNA sequences but also to discover a novel or unexpected pathogen that might be present in the specimen. This approach enables the identification of viral and bacterial infections without previous knowledge or specific primers. In addition, transcriptomics sequencing has the potential to offer valuable insights into the dynamics of host-pathogen interactions and the immune responses induced during the course of an infection (Pang et al., 2022; Westermann & Vogel, 2021). Through the examination of gene expression profiles in infected tissues or cells, scientists are able to identify the essential genes that play a role in the activation or repression of immune responses. This understanding has the potential to facilitate the development of targeted medicines aimed at modulating host immune responses in order to enhance the efficacy of combating infections.

Despite the above advantages, there remain obstacles that must be addressed before transcriptomics sequencing can be widely adopted as a standard method for pathogen identification in the clinical field. These factors encompass the standardization of protocols, the optimization of bioinformatics pipelines for data analysis, and the consideration of cost-effectiveness. Nevertheless, due to technological developments and cost reductions, transcriptomics sequencing exhibits significant potential as a beneficial instrument for precise and rapid pathogen identification in the therapeutic domain.

The historical evolution of traditional and novel genomics and proteomic-based diagnostic methods for pathogens in clinical settings highlights the continuous advancements in technology and their impact on pathogen detection and identification. From culture-based methods to the emergence of molecular techniques like PCR, microarrays, NGS, and Mass spectrometry, the field of genomics and proteomics has transformed pathogen diagnosis, allowing for faster, more accurate, and comprehensive analysis of pathogens in the clinical laboratory. These advancements continue to shape the future of diagnostics, providing valuable tools for infectious disease management and public health surveillance.

5. Impact of genomics on pathogen diagnosis and prediction of novel pathogen emergence

The field of genomics has changed the process of pathogen identification and detection within the clinical setting. By utilizing advanced techniques and acquiring valuable insights, genomics has dramatically improved our capacity to rapidly and precisely identify infectious diseases. This alteration has resulted in enhanced healthcare provision to patients, rapid implementation of interventions, and improved management of diseases. In the following points (Fig. 4.2), we will illustrate the impact of genomics on identifying and detecting pathogens in clinical settings.

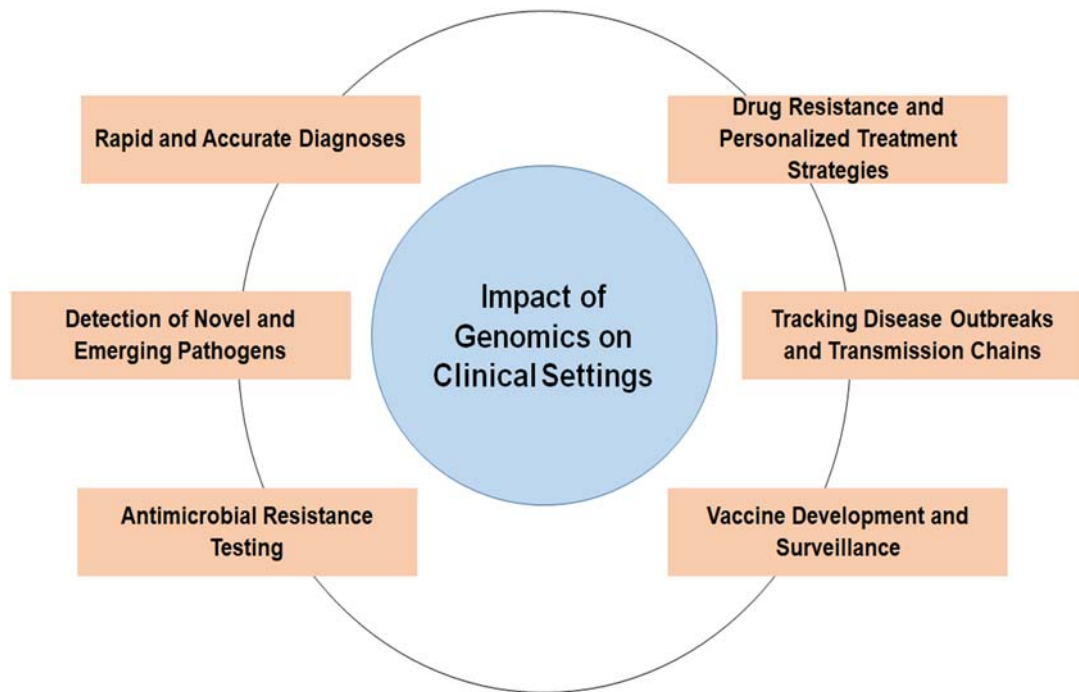


FIGURE 4.2 A diagram illustrating the impact of genomics on clinical practices.

5.1 Rapid and accurate diagnoses

Genomics enables clinicians to identify pathogens with unprecedented speed and accuracy. For example, NGS technologies can sequence the entire genome of a pathogen within hours, compared to days or weeks required by traditional methods. This speed is crucial for diseases with critical treatment timelines, such as sepsis. In a study, researchers used metagenomic sequencing to identify pathogens in critically ill patients with suspected infections, leading to quicker and more accurate diagnosis (Grumaz et al., 2016). Moreover, NGS was employed during the Zika virus outbreak to quickly identify and characterize viral strains (Faria et al., 2016).

5.2 Detection of novel and emerging pathogens

Genomics enables the identification of novel or previously unknown pathogens, which is crucial for managing outbreaks and designing effective interventions. Metagenomic sequencing can unveil the complete genetic content of a sample, revealing known and novel pathogens. During the 2018 Ebola virus outbreak in the Democratic Republic of Congo, genomic sequencing played a pivotal role in identifying the Zaire ebolavirus responsible for the epidemic (Mbala-Kingebeni, Aziza, et al., 2019; Mbala-Kingebeni, Pratt, et al., 2019).

More recently, NGS played a vital role in the identification of a new coronavirus responsible for the COVID-19 outbreak ([Wu, Zhao, et al., 2020](#)).

5.3 Tracking disease outbreaks and transmission chains

Genomics aids in tracking the spread of pathogens and understanding their transmission dynamics. By comparing genomic sequences from different cases, researchers can reconstruct transmission chains and identify sources of infection and surveillance in the epidemic. The integration of genomic epidemiology with artificial intelligence (AI) played a crucial role in unraveling the transmission routes of Ebola during the West African outbreak ([Dudas et al., 2017](#)).

5.4 Antimicrobial resistance testing

Genomics has greatly transformed antimicrobial resistance testing by providing a comprehensive view of the genetic determinants of resistance. WGS allows for the identification of specific resistance genes, mutations, and mechanisms, enabling clinicians to tailor antibiotic treatments to the individual patient's resistance profile. In tuberculosis management, genomics plays a crucial role in detecting drug-resistant strains and guiding treatment decisions ([Farhat et al., 2016](#)).

5.5 Drug resistance and personalized treatment strategies

Genomics analysis plays a crucial role in the identification of drug-resistance mutations in pathogens, as well as the detection of genetic variants that impact patient reactions to drugs. This information guides physicians in customizing treatment techniques that are most suitable and in avoiding therapies that are ineffective. In the case of HIV, genotypic resistance testing plays a crucial role in customizing antiretroviral regimens for patients based on their specific viral resistance profile ([Mullis et al., 1986](#)).

5.6 Vaccine development and surveillance

Genomics has accelerated vaccine development and surveillance efforts by providing insights into pathogen evolution and genetic diversity. Sequencing pathogen genomes helps identify potential vaccine targets and monitor changes in viral or bacterial populations. This is exemplified by the rapid development of COVID-19 vaccines, where genomic analysis informed vaccine design ([Corbett et al., 2020](#)).

Overall, by allowing culture-independent diagnostics, improving epidemic investigations, guiding antibiotic resistance testing, and easing vaccine development, genomics has altered pathogen identification and detection in the clinical setting. The above mentioned examples illustrate the significant influence that genomics is having on our ability to diagnose, monitor, and respond to infectious diseases in real-time, all of which will ultimately lead to better patient outcomes and public health responses.

6. Bioinformatics based clinical applications

Bioinformatics plays a crucial role in the analysis of genomic data derived from pathogens, facilitating the transformation of such information into discoveries that carry clinical significance, as illustrated in Fig. 4.3. Through the utilization of computational methods on extensive genomic datasets, scientists are able to determine crucial genetic characteristics, gain insights into pathogenic mechanisms, predict drug resistance, and provide guidance for treatment approaches.

6.1 Comparative genomics

Comparative genomics is a powerful tool for studying pathogen evolution and identifying genetic factors associated with pathogenicity. By comparing the genomes of different strains or species, researchers can identify key genomic variations that may contribute to pathogen emergence or virulence. Bioinformatic resources, such as Pathosystems Resource Integration Center (Wattam et al., 2017), the Integrated Microbial Genomes and Microbiomes, and MicroScope (Vallenet et al., 2009), provide tools for comparative genomics analysis, allowing researchers to explore genomic variations and identify potential markers of pathogen emergence.

6.2 Phylogenetic analysis

Phylogenetic analysis is used to reconstruct evolutionary relationships among pathogens based on their genetic sequences. By constructing phylogenetic trees, researchers can identify

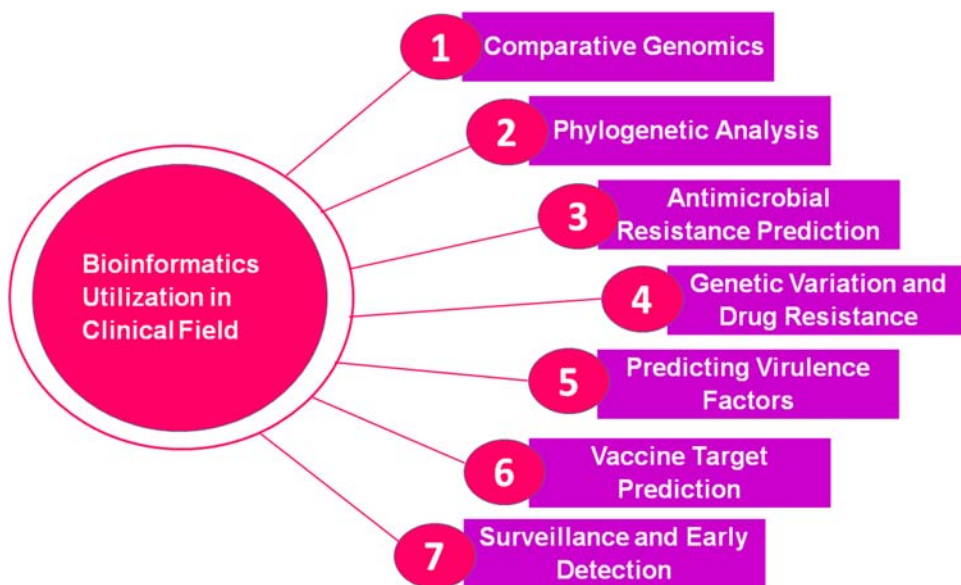


FIGURE 4.3 A diagram depicting the application of bioinformatics in medical practices.

closely related strains or species and track the spread and emergence of pathogens. Several bioinformatic tools such as Molecular Evolutionary Genetics AnalysisX (Kumar et al., 2018) and Bayesian Evolutionary Analysis Sampling Trees (Drummond et al., 2005) can construct phylogenetic trees and provide insights into pathogen emergence patterns.

6.3 Antimicrobial resistance prediction

Bioinformatic tools like the Comprehensive Antibiotic Resistance Database (Jia et al., 2017) (<https://card.mcmaster.ca/>) and ResFinder (Zankari et al., 2012) (<https://cge.food.dtu.dk/services/ResFinder/>) leverage genomic data to predict antimicrobial resistance in bacterial pathogens. By analyzing resistance genes, mutations, and genetic determinants, these resources can assist in the diagnosis of antimicrobial-resistant infections and guide treatment decisions. The prediction of emerging resistance patterns helps healthcare professionals identify potential threats and implement appropriate infection control measures.

6.4 Genetic variation and drug resistance

Bioinformatics tools are employed for the purpose of analyzing the genetic diversity exhibited by human pathogens and making predictions regarding mutations that confer resistance to drugs. The Stanford HIV Drug Resistance Database serves as a valuable tool for researchers and specialists by allowing them to input HIV genomic sequences and obtain predictions on drug resistance profiles. This resource greatly assists in the process of selecting appropriate antiretroviral medicines that are likely to be helpful in combating the virus (Mardis, 2008).

6.5 Predicting virulence factors

Bioinformatics tools can predict virulence factors in pathogens by analyzing their genetic content. For example, the Virulence Factors Database (VFDB) catalogs virulence-related genes and their functions in various pathogens (Chen et al., 2005). Analyzing pathogen genomes against VFDB helps identify potential virulence determinants and understand their roles in disease.

6.6 Vaccine target prediction

Bioinformatics assists in predicting potential vaccine targets by analyzing pathogen genomes for antigenic and conserved regions. For instance, researchers use tools like Vaxign to predict vaccine candidates based on specific properties and structural features in pathogen proteins (He et al., 2010).

6.7 Surveillance and early detection

Bioinformatic resources can aid in the surveillance and early detection of emerging pathogens. By monitoring genomic data from various sources, such as sequencing databases or

environmental samples, potential pathogen candidates can be identified and their potential to cause outbreaks can be assessed. For example, Nextstrain (Hadfield et al., 2018) (<https://nextstrain.org/>) is a platform for real-time tracking of pathogen evolution, which combines genomic data, phylogenetics, and visualization to provide insights into the spread and evolution of pathogens such as viruses. Additionally, the Global Initiative on Sharing Avian Influenza Data (Elbe & Buckland-Merrett, 2017) (<https://www.gisaid.org/>) is a platform for sharing and analyzing influenza genetic sequence data. PANGOLIN (Rambaut et al., 2020) (<https://cov-lineages.org/>) is another resource that utilizes phylogenetic analysis and classification algorithms for tracking and understanding the global spread of SARS-CoV-2 variants based on their genetic similarity.

By continuously monitoring the genetic diversity and evolutionary patterns of these viruses, these resources can predict the emergence of new viral strains or the potential for outbreaks in specific regions. This information aids in the early detection and diagnosis of viral infections, guiding public health interventions, and the development of targeted diagnostic assays.

6.8 Challenges and ethical considerations in genomics data management, privacy, and data sharing

While genomics holds great promise in clinical practice, its implementation raises significant challenges and ethical considerations related to data management, privacy, and data sharing. Balancing the potential benefits with privacy and ethical concerns is crucial to ensure responsible and effective use of genomic data.

The first noteworthy challenge pertains to data security and privacy, given that genomic data is sensitive and uniquely identifiable. Protecting patient privacy while enabling data analysis and sharing is challenging. Therefore, patient consent, data encryption, and secure storage are essential to prevent unauthorized access and data breaches (Mohammed Yakubu & Chen, 2020). Another ethical aspect revolves around informed consent and patient autonomy. Explaining the complexities of genomics to patients for informed consent can be difficult. Patients may not fully comprehend the implications of sharing their genetic information. Therefore, ensuring that patients have comprehensive information about the purpose of data sharing, potential risks, and benefits should give them the autonomy to decide whether to share their data (Compagnone et al., 2017).

The secondary use of data poses yet another ethical quandary. Genomic data collected for clinical purposes may be used for research or other commercial applications, raising concerns about patient control over their data. Therefore, clear policies and agreements should delineate the intended uses of data and allow patients to provide input on how their data is shared (Hazin et al., 2013).

The intricate matter of data sharing and ownership adds further layers of complexity. Determining who owns and controls genomic data is complex. Researchers, patients, and institutions may have differing views on data ownership, thus establishing clear data-sharing agreements, specifying ownership rights, and respecting patients' preferences regarding data sharing and control (Belani et al., 2021).

De-identification and reidentification represent an additional facet of the ethical landscape. De-identified genomic data can potentially be reidentified, compromising patient privacy.

Therefore, employing robust de-identification techniques and raising awareness among researchers about reidentification risks (Emam, 2011).

Addressing these challenges and ethical considerations requires collaboration between researchers, healthcare professionals, policymakers, and patients. Striking a balance between advancing medical knowledge through genomics and protecting patients' privacy and autonomy is crucial for the responsible integration of genomics into clinical practice.

6.9 Future directions and innovations

The integration of AI and other omics with genomics is poised to reshape the landscape of pathogen identification and diagnosis. In the future, AI-driven innovations offer new avenues for faster, more accurate, and comprehensive pathogen detection and characterization (Obermeyer & Emanuel, 2016).

Future directions and innovations in pathogen diagnosis and prediction through Genomics are poised to revolutionize infectious disease management. Genomic technologies are advancing rapidly, enabling more rapid and comprehensive pathogen identification. The integration of high-throughput sequencing, metagenomics, and advanced bioinformatics tools allows for the detection of known and novel pathogens, even in complex samples. For instance, the use of nanopore sequencing has demonstrated its potential to provide real-time pathogen identification in resource-limited settings, enhancing outbreak response capabilities (Quick et al., 2017). This trend is expected to continue with the development of portable sequencing devices that enable rapid on-site diagnosis and monitoring, thereby transforming the way we identify and respond to pathogens.

The integration of genomics with AI holds tremendous promise for predictive modeling and risk assessment. AI algorithms can analyze large-scale genomic and epidemiological data to forecast the emergence of novel pathogens and the potential for outbreaks. By learning from historical data and recognizing patterns in genomic variations, AI-driven predictive models could provide timely alerts and inform public health strategies. For example, AI-based prediction and diagnostic models have been successful in forecasting infectious diseases such as dengue fever (Gupta et al., 2023). As AI continues to evolve, its integration with genomics could enable proactive measures to prevent or mitigate the impact of infectious disease outbreaks.

In addition to diagnostics and prediction, genomics is set to play a pivotal role in tailoring treatment approaches. Precision medicine, which involves tailoring treatments based on an individual's genomic profile, is gaining momentum in infectious disease management. Genomic data can guide the selection of appropriate antimicrobial agents and predict drug resistance patterns, enabling more effective and targeted therapies. For instance, in tuberculosis treatment, genotypic drug resistance testing has shown promise in guiding the selection of appropriate drug regimens (Miotto et al., 2017). This approach can minimize treatment failure and reduce the development of drug resistance. As genomics continues to reveal intricate details of pathogen biology, personalized treatment strategies are expected to become a cornerstone of infectious disease management.

Moreover, the integration of genomics with other omics disciplines, such as proteomics, metabolomics, glycomics, and lipidomics, holds the promise to revolutionize pathogen identification and diagnosis in the future. Through the analysis of a person's genetic composition

in conjunction with their molecular reactions to pathogens, we can attain a thorough comprehension of how hosts and pathogens interact, facilitating the more precise and speedy identification of infectious agents. This multiomic approach will not only enhance our ability to identify novel pathogens and track their evolution but also enable the development of personalized treatments and diagnostics, ultimately reshaping the landscape of infectious disease management toward more precise and effective strategies.

7. Conclusion

Genomics has brought about a transformative impact on pathogen identification, diagnosis, and prediction, revolutionizing our understanding and management of infectious diseases. The integration of advanced genomic technologies, such as high-throughput sequencing and bioinformatics, has significantly accelerated the process of identifying pathogens. This has enabled the detection of both known and novel pathogens with unparalleled accuracy and speed, even in complex clinical samples. Moreover, genomics has ushered in an era of precision diagnostics by providing insights into the genetic makeup of pathogens. The ability to sequence and analyze pathogen genomes has paved the way for personalized medicine approaches in infectious disease management. Genomic data offer critical information about antimicrobial resistance, virulence factors, and potential treatment targets. This enables clinicians to tailor treatment strategies based on the specific genetic characteristics of pathogens, optimizing therapeutic outcomes and minimizing the development of drug resistance. Furthermore, genomics-driven predictions powered by AI have transformed our ability to forecast the emergence of novel pathogens and disease outbreaks.

In conclusion, genomics has brought about a paradigm shift in pathogen identification, diagnosis, and prediction. The fusion of genomic technologies, personalized medicine approaches, and AI-driven predictions has not only enhanced our understanding of infectious diseases but also provided powerful tools for effective disease management and outbreak control. These transformative advancements hold the potential to reshape the landscape of global health and infectious disease response.

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Molecular techniques for the diagnosis of viral infections

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1. Viral diagnosis in clinics

Viral infections manifest with many symptoms, often mirroring other illnesses. This diagnostic ambiguity underscores the crucial importance of an accurate viral diagnosis. Timely and precise identification of the causative virus not only facilitates targeted treatment but also aids in preventing the unnecessary use of antibiotics, which can contribute to antimicrobial resistance (Ventola, 2015; WHO, 2019). Therefore, accurate viral diagnosis within clinical settings is paramount to guiding effective patient management and safeguarding public health. Moreover, early and accurate virus identification enables healthcare providers to initiate appropriate antiviral therapies, manage symptoms effectively, and prevent complications. Beyond individual care, diagnostics play an instrumental role in public health interventions. Rapid identification of viral outbreaks empowers health authorities to implement timely measures to contain the spread, thereby mitigating the impact on populations (Lee et al., 2010; Lipsitch et al., 2003).

The diagnostic supply for viral infections is diverse, encompassing various approaches that cater to different scenarios. Classical methods like microscopy and viral culture remain foundational, allowing direct visualization and isolation of viral particles. Modern molecular techniques, such as PCR assays, enable the detection of viral genetic material with exceptional sensitivity. Immunological methods, including enzyme-ELISA and lateral flow assays, detect

viral antigens or antibodies in patient samples (Bermejo-Martin et al., 2010; Karagoz et al., 2023; Yu et al., 2020). In addition, emerging technologies have revolutionized viral diagnosis. For instance, next-generation sequencing offers unparalleled insights into viral genomes, aiding in identifying novel strains and studying viral evolution. Additionally, the integration of CRISPR-based diagnostics presents rapid and precise tools for detecting viral nucleic acids (Mardis, 2008; Zhang et al., 2020).

The importance of an accurate viral diagnosis within clinical and public health contexts cannot be overstated. As we explore the diverse landscape of diagnostic methods available, we gain a comprehensive understanding of how these techniques work in harmony to combat viral infections and safeguard public well-being. This chapter delves into the significance of precise viral diagnosis, emphasizing its pivotal role in individual patient care and broader public health strategies.

2. Nucleic acid amplification methods

Nucleic acid amplification methods are the most widely used for pathogen detection and the diagnosis of infectious diseases. These methods generate millions of copies of the targeted nucleic acids to be detected by various techniques like agarose gel electrophoresis, fluorometric, and colorimetric techniques, or are even coupled with immunoassays. The targeting of the viral DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) is commonly achieved with the aid of short single-stranded oligonucleotides (primers) that are specifically designed to anneal to the gene of interest. The exponential amplification occurs when the polymerase enzyme creates new strands each time primers bind to their target gene region. For RNA viruses, a reverse transcription step precedes the reaction to create a complementary strand of DNA, which can be done separately (two-step) or in the same reaction (single-step). Nucleic acid amplification methods are either PCR-based (PCR and ddPCR) or isothermal (LAMP, RPA, HDA, and RCA). Ideally, diagnostic tests must be highly sensitive, specific, simple, and accessible. This subchapter reviews the molecular detection methods with applications in viral diagnosis, as well as the advantages and disadvantages of each.

2.1 PCR-based nucleic acid amplification methods

2.1.1 *Polymerase chain reaction*

The polymerase chain reaction (PCR) was first developed as an alternative to the specific restriction endonucleases for the synthesis of DNA sequences by Mullis in 1983. PCR requires a thermal cycler to carry the reaction that consists of repetitive cycles of three main steps: denaturation of the double-stranded DNA (dsDNA) as the temperature rises; annealing, in which the temperature is reduced to enable primers hybridizing to their target sequence on the single-stranded DNA (ssDNA); and polymerase extension, where the DNA polymerase aids in creating new strands as the temperature increases. The number of the target dsDNA exponentially doubles with each cycle, creating millions of PCR products in a relatively short time (Mullis et al., 1986). A reverse transcriptase (RT) enzyme is included in the reaction if an RNA virus is the target of interest (RT-PCR). A reverse transcription step

precedes the reaction to create a complementary DNA strand. PCR products can be detected by agarose gel electrophoresis or fluorescence. Fluorometric detection enables real-time monitoring of the results by using target-specific probes or fluorescent dyes. Probes are labeled with a fluorescent reporter at the 5'-end and a quencher at the 3'-end, and once they bind to their target sequence, they are displaced and hydrolyzed during the polymerase extension phase, cleaving the fluorescent reporter that the PCR instrument can detect. Several target-specific probes labeled with different fluorescent dyes can be used when designing multiplex assays (Alvarez & Nourbakhsh, 2014; Bustin & Nolan, 2020).

Since its development, RT-PCR has been the most widely used molecular technique for the detection of a variety of viruses like Zika virus (ZIKV) (Alzate et al., 2022; Rademan et al., 2019; Viedma et al., 2019), HIV (Bertine et al., 2017; Graf et al., 2019), Dengue virus (DENV) (Alshabi et al., 2022; Anwar et al., 2022; Kann et al., 2021), SARS (Drosten et al., 2003; Huang et al., 2005; Yam et al., 2005), Hantavirus (Madai et al., 2021; Nunes et al., 2019; Vaheri et al., 2008), West Nile virus (Gdoura et al., 2022; Xu et al., 2022), and SARS-CoV-2 (D'Cruz et al., 2020; Kudo et al., 2020; Tombuloglu et al., 2021, 2022; Yanase et al., 2022) among many others (Artika et al., 2020). Aside from virus-specific assays, multiplex RT-PCR assays are being developed for differential diagnosis of various viral infections with similar symptoms, which is crucial for accurate diagnosis and, subsequently, correct treatment plan (Chen et al., 2021; Chung et al., 2021; Ni et al., 2021). Also, multiplex RT-PCR assays are designed to target different genes of the same virus, which is advantageous when viral mutations are recurrent to avoid false-negative results (Tombuloglu et al., 2021, 2022). Many developed multiplex RT-PCR assays for the differential diagnosis of viral pathogens (Chen et al., 2021; Chung et al., 2021). For instance, Chung et al. designed a dual multiplex RT-PCR assay that targets the N1 and N3 genes of SARS-CoV-2, the M gene of influenza A/B, and the N gene of respiratory syncytial viruses simultaneously, with a reasonable limit of detection (LoD) for each target (Chung et al., 2021). Similarly, Ni et al. developed a multiplex RT-PCR assay for the differential diagnosis of SARS-CoV-2 and influenza A/B by targeting *N* and *ORF1ab* of SARS-CoV-2 and *M* and *NA* genes of influenza A/B, respectively (Ni et al., 2021).

Since the declaration of the COVID-19 pandemic, RT-PCR has been declared the gold standard technique by the WHO (World Health Organization) and CDC (Center for Disease Control) due to its exceptionally high sensitivity and specificity (Rabaan, Smajlović, et al., 2023). However, the RT-PCR technique requires a thermal cycler and experienced personnel for operation, making it expensive, laborious, and unsuitable for point-of-care testing (POCT). Furthermore, RT-PCR tests struggle to catch up to the expanded number of tests required per day, as seen during the COVID-19 pandemic, because it can take >24 hours to get the results, which is insufficient when a large number of tests must be performed rapidly (Alhamid, Tombuloglu, Rabaan, et al., 2022; Bustin & Nolan, 2020). In addition, RT-PCR is prone to false-negative results that arise from low viral load samples, inappropriate sample handling, viral mutations, and the time of testing from the onset of infection (Rabaan, Alshahrani, et al., 2023; Tombuloglu et al., 2022). The recurrent viral mutations cause a reduction in the sensitivity of RT-PCR assays, leading to false negative results (Chen et al., 2022; Sharma et al., 2022). For instance, the del31/33 mutation of the SARS-CoV-2 Omicron variant significantly reduced the diagnostic performance of the commercial RT-PCR kits (Ippoliti et al., 2022), while other kits completely failed in detecting some variants due to the mismatching between

primers binding to the mutated regions (Chen et al., 2022). Multiplex assays have a higher advantage because they target more than one gene to avoid false-negative results. To ensure diagnostic accuracy, incorporating an internal control validates the success of the reaction (Tombuloglu et al., 2021, 2022). Also, the results must be combined with clinical observations that can be verified using other detection kits, routine quality control, and genomic surveillance to monitor the regional viral strains (Chen et al., 2022). Confirmation of the diagnosis is important as many different management approaches can be taken, and misdiagnosis could result in insufficient use of public health resources (Rojas et al., 2020).

2.1.2 Droplet digital PCR

Hindson et al. originally developed droplet digital PCR (dPCR) as an improved dPCR version. This technology generates water-oil emulsions, each carrying an amplification reaction, to determine the DNA concentration in a sample by analyzing positive-to-negative droplets using Poisson's statistics. The reaction setup consists of a droplet generator cartridge with microwells, in which samples are loaded. This system uses microfluidics to separate or partition each microwell into 20,000 droplets. Once generated, the droplets are transferred to a PCR plate to start the amplification reaction. Subsequently, the PCR products are loaded into a droplet reader, where a two-color detection system is used to read data using a fluorescent probe. If at least one copy is present in a single droplet, it will emit a fluorescent signal indicating a positive reaction; otherwise, the droplet is negative (Hindson et al., 2011).

ddPCR overcomes the false negative drawback of RT-PCR because it can detect down to a single DNA copy due to many partitions, which helps detect viral infections in patient samples with low copy numbers. Bruner et al. used ddPCR to quantify intact and defective HIV-1 proviruses to find a strategy to target the intact proviruses (Bruner et al., 2019). Others detected the ZIKV in the central nervous system of mice using ddPCR, which enabled the quantification of the viral infection (Enlow et al., 2020). ddPCR was also used to detect SARS-CoV-2 in samples with low RNA abundance. Studies reported that ddPCR detected the virus in samples that were otherwise RT-PCR-negative (Behera et al., 2021; Falzone et al., 2020; Liu et al., 2020; Suo et al., 2020; Yu, Yan, Wang, Yang, Wang, Tang, Gao, Wang, Ma, Xie, Wang, et al., 2020).

ddPCR provides precise quantitative data regarding viral copy numbers in clinical samples, providing insights into disease prognosis and appropriate treatment plans. In addition, ddPCR has lower background readouts than RT-PCR, which effectively reduces the generation of false positive results (Liu et al., 2020). However, the ddPCR instrument is expensive, laborious, and requires experienced personnel. Additionally, the reaction takes up to two times longer than RT-PCR to get the results. ddPCR can be used as a confirmatory test if RT-PCR results are contradictory (Behera et al., 2021; Falzone et al., 2020; Liu et al., 2020).

2.2 Isothermal amplification methods

2.2.1 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a molecular diagnostic technique developed by Notomi (2000) that amplifies nucleic acids under isothermal reaction conditions. The use of four to six primers that target specific gene regions increases the

amplification rate and target specificity. With a variety of detection methods like turbidity, colorimetry, fluorometry, and agarose gel electrophoresis, as well as minimum instrument requirements for the reaction, the LAMP technique is gaining popularity in viral diagnostics (Alhamid, Tombuloglu, Rabaan, et al., 2022; Notomi et al., 2015). Once LAMP primers anneal to the template, new strands are created and displaced. The released strands have complementary sequences on either or both ends, forming stem-loop and dumbbell structures, respectively, which serve as amplification starting points. The repeated annealing and displacement cycles create millions of DNA structures with alternate lengths and inverted repeats. Reverse transcription LAMP (RT-LAMP) amplifies RNA targets in a single reaction tube by adding a RT enzyme (Notomi, 2000; Notomi et al., 2015). One of the most straightforward detection techniques is the colorimetric LAMP because the naked eye can see the results as the color changes with the presence of viral nucleic acids by adding indicator dyes to the reaction (Alhamid & Tombuloglu, 2022).

RT-LAMP is a simple and cost-effective technique that gained popularity in viral detection, including SARS-CoV-2 (Alhamid & Tombuloglu, 2022; Alhamid, Tombuloglu, Motabagani, et al., 2022; Alhamid, Tombuloglu, Rabaan, et al., 2022), MERS-CoV (Lee et al., 2016; Mao et al., 2018); HIV-1 (Gomes et al., 2020; Zhang et al., 2022), among others that ultimately showed comparable diagnostic performance to RT-PCR. In cases of pandemics, using RT-LAMP resolves the overwhelming demand for rapid tests required per day because this technique is cheap, does not require experts to perform the test, and is suitable for POCT (Alsaeed et al., 2024). Home-use lyophilized LAMP assays can be developed for self-testing (Song et al., 2022), and they can withstand crude samples because they are tolerant to inhibitors (Odiwuor et al., 2022).

The most common disadvantage of the LAMP technique is the false positive results from using six primers that form unwanted secondary structures (Meagher et al., 2018). Like RT-PCR, the sensitivity of LAMP decreases when samples with low viral loads are tested. In addition, some studies reported intermediate results (between positive and negative) when incorporating pH-sensitive dyes, indicating lower sensitivity of the pH-dependent colorimetric RT-LAMP (Aoki et al., 2021; de Oliveira Coelho et al., 2021). Efforts to tackle these limitations suggested using quenched fluorescent primers, not exceeding 30–60 minutes incubation time, and reducing the number of primers to avoid mis-amplifications (Alhamid et al., 2023; Urrutia-Cabrera et al., 2021).

2.2.2 Recombinase polymerase amplification

Recombinase polymerase amplification (RPA), as the name implies, is driven by a recombinant enzyme to isothermally amplify nucleic acids at low reaction temperatures (25–43°C). This technique was initially developed to overcome the complicated DNA amplification requirements, such as sophisticated equipment and sample-handling procedures. Instead of the denaturation step, recombinase-primer complexes scan dsDNA and facilitate strand exchange at the complementary sites (Piepenburg et al., 2006). The recombinase protein (T4 UvsX)-primer complex is inserted into the template and forms a D-ring structure, where chain displacement and strand synthesis start by incorporating DNA polymerases (*Bacillus subtilis* Pol I). The D-ring structure stays put with the help of the single-stranded binding protein. The repetition of these steps creates exponential amplification of the target region of the

template. Coupled with a probe-based approach, this technique can quantitatively detect viral DNA/RNA in a short turnaround time (Piepenburg et al., 2006; Tan et al., 2022).

Combining RPA with lateral flow and CRISPR/Cas assays for result visualization is common in viral detection applications (Tan et al., 2022). Cherkaoui et al. developed a one-pot duplex RT-RPA assay for SARS-CoV-2 detection that targets two genes simultaneously (*E* and *RdRp*), coupled with fluorescent detection. They also utilized LFA for the detection of SARS-CoV-2 in resource-limited areas. Their assay rapidly detected 50 copies as early as 14 minutes (Cherkaoui et al., 2021). Another study also developed a duplex RT-RPA and colorimetric LFA to detect *ORF1ab* and *N* genes of SARS-CoV-2 and influenza A/B viruses. The assay can detect 10 RNA copies in 1 hour. Additionally, RPA can amplify nucleic acids under constant low temperatures; even using body heat seems to be sufficient (Sun et al., 2022). Crannell et al. proved the efficiency of RPA when incubating reactions using only body heat when detecting the *pol* gene of HIV-1, yielding high sensitivity (Crannell et al., 2014). Lillis et al. also developed an instrument-free incubation for detecting HIV-1 *pol* gene at ambient temperatures, showing consistent results between 31–43°C (Lillis et al., 2014).

This technique can detect the virus from crude samples cheaply because it does not require a thermal cycler and can be lyophilized and stored at room temperature, eliminating the expensive shipping costs. All these features qualify RPA for POCT for viral diagnosis. Yet, due to low-temperature reactions, primer mismatching is a common issue. Another drawback is the few available RPA kits, all developed for research purposes only, which limit their use for diagnostic applications. Also, using crude samples might reduce the sensitivity in some cases (Cassedy et al., 2021). Nonetheless, this technique proved sensitive, specific, and fast and can be used for POCT. With flexible reaction temperature ranges that do not affect its sensitivity, RPA does not require precise temperature control. The RPA technique in viral diagnosis might be used more often once enough testing and validation are performed (Cassedy et al., 2021; Tan et al., 2022).

2.2.3 Helicase-dependent amplification

DNA replication in vivo is driven by DNA polymerase and accessory proteins, one of which is DNA helicase, responsible for unwinding the double helix structure. Helicase-dependent amplification (HDA) mimics the in vivo mechanism to amplify nucleic acids under isothermal conditions (60–65°C). In this technique, dsDNA is denatured by a thermostable helicase enzyme, and ssDNA-binding proteins coat each strand. Then, two primers hybridize into their complementary sequences in the template strands. DNA polymerase extends the primers annealed to the template strand, and a dsDNA is created, which acts as a substrate by DNA helicases, creating a simultaneous chain amplification reaction. The addition of thermostable RT-HDA enables the detection of RNA viruses. The amplified products can be detected in real-time by fluorescence or lateral flow strips (Vincent et al., 2004).

HDA does not require a thermal cycler and is cheap, simple, and suitable for POCT to detect viral diseases. A study compared the performance of HDA and the gold standard RT-PCR in detecting herpes simplex viruses 1 and 2 (HSV-1, HSV-2), and varicella zoster virus from skin lesions without DNA extraction in HDA tests. Both methods showed agreement, indicating a comparable performance between HDA and RT-PCR (Jevšnik et al., 2020). Slinger et al. also used HDA assays to detect HSV-1 and HSV-2 in 177 swab specimens. Compared to the conventional viral culture technique, the HDA assay was significantly more

sensitive and faster (1–2 hours compared to 2 days) (Slinger et al., 2019). Others used the RT-HDA technique coupled with a dipstick to detect SARS-CoV-2 visually and obtained a LoD of 6 copies/ μ L (Shanmugakani & Wu, 2022). Similarly, Zasada et al. detected SARS-CoV-2 using multiplex RT-HAD, targeting *E*, *RdRp*, and *N* genes, coupled with lateral flow assay, and obtained LoD of 3 copies/reaction. RT-HDA was more sensitive than RT-LAMP when both assays were tested simultaneously (Zasada et al., 2022).

The most common disadvantage of HDA is the limited speed and processivity of the helicase enzyme, making this technique inefficient at amplifying long amplicons. The long primer bases and isothermal reaction conditions make this technique prone to nonspecific amplification and subsequent false positive results, which is why low primer concentrations are preferred. Using chemically inactive primers containing a 3'-end blocking group is a proposed method to prevent primers' self-annealing. Efforts to overcome these limitations are being imposed by modifying reaction conditions, as reviewed by Barreda-García et al. (2018). HDA reagents are expensive, and there is a lack of validated assays to diagnose viral pathogens. Nevertheless, this technique has a high sensitivity, rapid turnaround time (30–90 minutes), can tolerate crude samples, pathogens can be detected at low concentrations, and is easy to use, especially when coupled with LFA (Barreda-García et al., 2018; Shanmugakani & Wu, 2022).

2.2.4 Rolling circle amplification

Rolling circle amplification (RCA) is a unidirectional isothermal amplification of circular nucleic acids that creates long concatemers ssDNA containing hundreds of tandem repeats of sequences complementary to the circular template (Fire & Xu, 1995). Depending on the target, various DNA polymerase enzymes can be used in an RCA reaction: Φ 29, Bst, and vent exo-DNA polymerase or T7 RNA polymerase for RNA templates (Mohsen & Kool, 2016). Φ 29 is the most frequently used because it has the best processivity and strand displacement activity (Blanco et al., 1989). Reactions can occur at room temperatures up to 65°C (depending on the enzyme used). They are composed of three main steps: (1) Circular template ligation, which is conducted by enzymatic ligation either mediated by the template (T4 DNA ligase) or by using a template-free special ligase (circLigase); (2) primer-induced single-stranded elongation; and (3) displacement and amplification mediated by DNA polymerase enzyme. The resulting product is a linear DNA with tens to thousands of tandem repeats, which can be detected in real-time with fluorescence or agarose gel electrophoresis. This technique helps detect small trances of DNA/RNA, and the reaction can occur on solid surfaces, such as beads, microwell plates, microfluidic devices, and paper strips, enabling a wide range of applications (Garafutdinov et al., 2021).

Unlike PCR, RCA uses one primer once, and it is the only isothermal amplification method that uses a circular template, presenting unique applications that include repeating probes, branched RCA, Padlock probes-RCA, and hyperbranched RCA (Mohsen & Kool, 2016). RCA offers great sensitivity and specificity in the detection of biological targets. Kim et al. used the RCA method with fluorescent and microfluidic-based detection of four gene targets of SARS-CoV-2 (*E*, *N*, *RdRp*, and *ORF1ab*) from infected patients' facemasks. T4 ligase was used for ligation, while Φ 29 polymerase was used for strand displacement and amplification. The microfluidic-based RCA was conjugated with a padlock probe and Bst 3.0 DNA polymerase (Kim et al., 2022). Also, others developed a novel strategy, multiregion padlock hybridization RCA, specifically for SARS-CoV-2 detection. They used Φ 29 polymerase and

SplintR ligase to ligate the padlock probe that hybridizes with the target RNA. The multipadlock can detect multiple genes, making it helpful in detecting mutated regions efficiently (Kumari et al., 2022).

It is possible to use more than one primer to increase the efficiency of this technique further, in which case it is called hyperbranched RCA. RCA products can also be detected colorimetrically from the oxidation of chromogenic substrates or the hybridization of metal nanoparticles. Also, analytes in complex media can be detected without isolation from their pure form (Garafutdinov et al., 2021). All these features pave the way for various applications for viral diagnosis and analyzing bio-analyte varieties. Despite the advantages, RCA's efficiency depends on the circular template's size. Also, the potential of RCA in viral diagnosis is still not fully explored, and this technique suffers from complicated reaction schemes. More studies are required to optimize and improve this technique in clinical settings to broaden its diagnostic potential (Garafutdinov et al., 2021; Kumari et al., 2022).

Beyond the aforementioned techniques, nested PCR, multiplex PCR, and real-time PCR variants have found utility in viral diagnostics. In conclusion, PCR-based methods have revolutionized viral diagnostics by offering rapid and accurate detection of viral genetic material. From the gold standard, RT-PCR, to innovative approaches like RT-LAMP and ddPCR, these techniques have transformed our ability to identify and manage viral infections in clinical settings (Espy et al., 2006).

3. CRISPR-Cas-based viral diseases diagnosis

3.1 CRISPR-Cas technology in general

CRISPR (clustered regulatory interspaced short palindromic repeats) is a group of DNA sequences that originated in prokaryotes previously infected with bacteriophages and were subsequently incorporated into their genomes as a defense mechanism to recognize and eliminate foreign nucleic acid sequences by using the endonuclease activity linked with an enzyme called Cas. Cas is developed in prokaryotes to cleave invading viruses' nucleic acids like scissors (Sande et al., 2021).

CRISPR-Cas is an essential genome editing technique that permits an organism's DNA levels to be altered. Among numerous CRISPR-Cas groups, the CRISPR-Cas9 system has demonstrated a significant pace of advancement during its history. Other technologies, such as CRISPR-Cas12 and CRISPR-Cas13, have recently been presented (Hirano et al., 2022).

CRISPR-Cas technology has been used to diagnose and treat various infectious and noninfectious disorders (Hirano et al., 2022). It has been employed effectively in patient-derived hematopoietic stem and progenitor cells, liver illnesses such as viral hepatitis caused by hepatitis B and C viruses (HBV and HCV), and neurological disorders such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease (Jamehdor et al., 2020). Recently, it advanced in virology, including research into enteric viruses (Hirano et al., 2022).

Infectious agents, particularly viruses and bacteria, are responsible for many hospitalizations and deaths globally. Providing accurate and fast diagnostics for various infectious illnesses is difficult. CRISPR-Cas systems have been engaged in diagnostic applications to detect nucleic acid-based pathogenic biomarkers with single-base precision (Sande et al., 2021).

In this subchapter, we recap CRISPR-Cas technology's uses in viral disease diagnosis, its limitations, and future perspectives in the context of viral disease diagnosis.

3.2 Viral disease diagnosis

3.2.1 Virus detection using CRISPR-Cas9-based assay

The ZIKV was discovered by amplification of the viral RNA utilizing nucleic acid sequence-based amplification with the inclusion of RT, RNase H, and T7 polymerase followed by CRISPR-Cas9 cleavage of the region next to the PAM site (Pardee et al., 2016). On the other hand, unlike conventional amplification methods, EXPAR (exponential amplification reaction) is a new, highly effective, and fast isothermal amplification technology that allows for constant temperature operation without the need for exogenous primers. Furthermore, the CRISPR-Cas9 initiated isothermal EXPAR (CAS-EXPAR) uses the EXPAR amplification mechanism, allowing pathogen identification with a sensitivity of 1 amol-10 fmol. Because of its excellent specificity, this approach was demonstrated to identify methylated DNA as well (Huang et al., 2018).

At the same time, in response to the increasing number of COVID-19 cases, the Drug Controller General of India permitted FELUDA (FnCas9 editor-linked uniform detection), a CRISPR-Cas9-based diagnostic assay that permits fast detection of SARS-CoV-2 within an hour at a small expense and with no use of complicated equipment. FELUDA can identify single nucleotide variations with 100% accuracy and 97% specificity throughout viral load amounts (Azhar et al., 2021).

3.2.2 Virus detection using CRISPR-Cas12 and CRISPR-Cas13-based assays

A technology that combines RPA and CRISPR-Cas13 to identify target RNA or DNA sequences was recently developed. This platform is known as SHERLOCK (specific high-sensitivity enzymatic reporter unLOCKing). Besides being transferrable and exceptionally sensitive in detecting DNA or RNA from genuine medical samples, this technology can identify numerous targets using a multiplex fluorescence-based detection method (Gootenberg et al., 2018; Kellner et al., 2019) created a multiplexed CRISPR-Cas13 test (SHERLOCKv2-version 2) able to identify four targets in one reaction with reporters for LwaCas13a, PsmCas13b, CcaCas13b, and AsCas12a in FAM, TEX, Cy5, and HEX channels, respectively. Synthetic (ZIKV ssRNA was detected in the HEX channel, as was synthetic DENV ssRNA in the FAM channel.

A comparable method, DETECTR (DNA endonuclease targeted CRISPR transreporter), combines isothermal RPA with Cas12a enzymatic activity. DETECTR was utilized to identify human papillomavirus (HPV) 16 from HPV 18 in crude DNA extracts from cultured human cells and clinical samples (Chen et al., 2018). Unlike DETECTR, HOLMES (a one-hour low-cost, multipurpose highly efficient system) employs LbCas12a and preamplification using PCR (Li et al., 2018). HOLMESv2, a significantly modified version of HOLMES, allows for one-pot reactions and utilizes thermostable AacCas12b, with LAMP (LAMP of DNA) for preamplification (Li, Li, Wu, et al., 2019; Li, Li, Wang, et al., 2019). Fig. 5.1.

Coming back to SHERLOCK (Myhrvold et al., 2018), went one step further by combining SHERLOCK with HUDSON (heating unextracted diagnostic samples to annihilate nucleases), which eradicates the requirement for nucleic acid extraction and permits pathogen

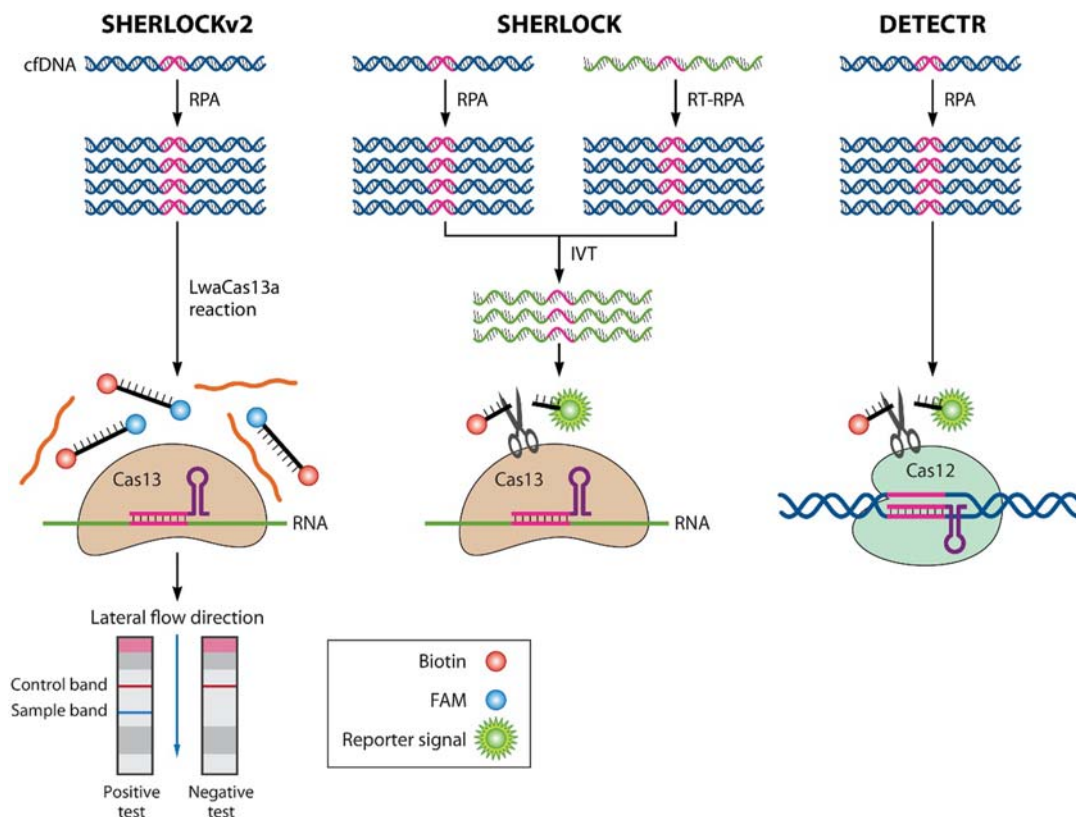


FIGURE 5.1 Comparison of CRISPR-Cas technologies for nucleic acid detection in SHERLOCKv2, SHERLOCK, and DETECTR assays (Mustafa & Makhawi, 2021).

identification straight from body fluids. HUDSON coupled with SHERLOCK enabled precise recognition of DENV in patient samples of saliva, whole blood, and serum within 2 hours. The authors additionally showed method specificity and flexibility by discriminating between four DENV serotypes and establishing a 1-week assay to detect six prevalent HIV (human immunodeficiency virus) RT mutations. SHINE (SHERLOCK and HUDSON integration to navigate epidemics) was recently used to identify SARS-CoV-2 in clinical specimens that had not been extracted (saliva and nasopharyngeal swabs) (Arizti-Sanz et al., 2020a, Arizti-Sanz, Freije, Stanton, Petros, et al., 2020).

STOPcovid (SHERLOCK testing in one pot), a diagnostic test for SARS-CoV-2, was created to decrease the number of fluid handling procedures by Joung et al. (2020). It employs thermostable AacCas12b and RT-LAMP preamplification. The findings of the lateral flow readout may be detected in 70 minutes, while the results of the fluorescence readout can be acquired in 40 minutes (Joung et al., 2020).

CARMEN (combinatorial arrayed reactions for multiplexed evaluation of nucleic acids) uses microfluidics and CRISPR-Cas13a-based SHERLOCK technology. It enables the detection of over 4500 targets per chip in a fast, scalable, sensitive, high-throughput, low-cost

manner (Ackerman et al., 2020). CARMEN enabled reaction miniaturization, lowering costs and minimizing reagent and sample usage. This approach was used to detect 169 human-associated viruses and identify drug-resistant HIV mutations and subtype coronaviruses and influenza A strains (Ackerman et al., 2020). Table 5.1.

3.2.3 CRISPR-Cas applications in COVID-19 diagnosis

CRISPR-based detection approaches, notably those based on Cas12 and Cas13, provide reliable and quick detection and encourage advanced strategies for detecting SARS-CoV-2.

Viral RNA from nasopharyngeal swabs can be amplified and transferred for fluorescence detection utilizing the CRISPR-Cas12a detection platform (DETECTR), delivering quick findings (Broughton et al., 2022, pp. 870–874). A lateral flow test can also be employed for respiratory tract extract detection. A quick, naked-eye visual detection approach (CRISPR-Cas12a-NER) has also been developed, resulting in an uncomplicated and trustworthy field identification tool (Wang et al., 2020).

Joung et al.'s SHERLOCK detection technology unites isothermal nucleic acid amplification with Cas13a protein for excellent sensitivity and specificity in identifying SARS-CoV-2 (Joung et al., 2020). Fozouni et al. used numerous crRNAs with distinct specific sequences to enhance Cas13a protease activation for precise SARS-CoV-2 identification (Fozouni et al., 2021). Additionally, using SHINE, SARS-CoV-2 RNA may be detected in unextracted materials (Arizti-Sanz et al., 2020a, Arizti-Sanz, Freije, Stanton, Petros, et al., 2020). It can identify viral RNA in patient samples fast and sensitively, with minimal tools lowering the possibility of contamination. During the pandemic, this approach was in high demand, and it has increased diagnostic testing capacities for infectious illnesses.

3.2.4 Conclusion

CRISPR-Cas-based diagnostic approaches are adaptable, low-cost, and do not require specialized equipment and knowledge. Because of the CRISPR technology's nature, it may be used in point-of-care (POC) diagnostic sensing platforms. Due to their excellent single-molecule sensitivity, CRISPR-related nucleases may 1 day be employed for direct analytical assessment of nucleic acids. However, substantial study is required for each infectious illness to select the optimum Cas enzyme combination and create the best system.

4. Nanotechnology for viral disease diagnosis

Using nanoparticles with a high surface-to-volume ratio allows for the quick, real-time, and sensitive detection of different viral diseases. Because the sample amount needed for examination is small, nano-diagnostic techniques are low-priced and straightforward. Lots of nanoparticles, both nonmetallic and metallic, have been utilized in the diagnosis of infectious illnesses. The most often utilized metallic nanoparticles for virus detection include gold, silver, iron oxide (magnetite), zinc oxide, and titanium dioxide. These nanoparticle-associated detection approaches rely on variations in the presence of target virus particles (specific virus antigens) or host antibodies produced in response to viral infection that are chromatic or electrochemical (Zhu et al., 2014). The principle of nanoparticle-based virus detection involves

TABLE 5.1 CRISPR-Cas-based diagnostic platforms and their characteristics.

Diagnostic platform	Cas protein involved	Approximate assay time	Preamplification method	Readout	Detection
NASBA	Cas9	2 hours amplification + 1 hour results	NASBA	Colorimetry	Zika virus
Cas-EXPAR	Cas9	1 hour	EXPAR	Fluorescence	<i>L. monocytogenes</i> ; DNA methylation
FELUDA	dFnCas9	1 hour	RT-RPA, RT-PCR	Lateral flow assay, smartphone app	SARS-CoV-2
SHERLOCK	Cas13	2 hours amplification +30 minutes – 3 hours results	RPA, RT-RPA	Fluorescence	Zika virus, dengue virus, HIV
SHERLOCK V2	Cas13	30 minutes amplification +30 minutes – 3 hours results	Fluorescence	RPA lateral flow assay	Zika virus, dengue virus
DETECTER	Cas12a	10 minutes amplification + 1 hour results	RPA	Fluorescence	HPV16, HPV18
HOLMES	LbCas12a	45 minutes amplification +15 minutes results	PCR	Fluorescence	Japanese encephalitis virus
HOLMES V2	AacCas12b	30 minutes amplification +30 minutes results	LAMP	Fluorescence	Japanese encephalitis virus, DNA methylation
STOPcovid	Cas12b	1 hour	RT-LAMP	Fluorescence, lateral flow assay	SARS-CoV-2
CARMEN	LwCas13a	20 minutes amplification + 3 hours results	PCR/RPA	Fluorescence	Drug-resistant HIV mutations, SARS-CoV-2, subtyping influenza strains, human-associated viruses

using nanoparticles that have been functionalized with biorecognition counterpart molecules, allowing for specific viral detection using a leading optical or electrochemical transducer framework. The interaction between the virus and the functionalized nanoparticles causes a measurable change in the physical or optical properties of the nanoparticles, such as changes in color or fluorescence intensity. This change in properties can be detected using various techniques such as UV-Vis spectroscopy, surface-enhanced Raman scattering (SERS), or magnetic resonance imaging. The signal generated by the nanoparticles can then be used to determine the presence and concentration of the virus in the sample (Nasrollahzadeh et al., 2020). The most frequent transducers employed in biosensor systems include the combined effect of plasmonic photothermal (PPT) and localized surface plasmon resonance (LSPR), colorimetric, fluorescence, field-effect transistor-based amperometric, and circle-to-circle amplification-based optogenetics. Moreover, nano-biosensors used with RT-LAMP/RT-PCR assays can improve RNA extraction, shorten analysis time, and/or remove the need for complex apparatuses (Aquino et al., 2022). In this section, we explore the use of nanoparticles in detecting various viruses, including HIV, hepatitis, influenza virus, and SARS-CoV-2.

4.1 Metallic and nonmetallic-based nanoparticles for the diagnosis of infectious illnesses

Nanoparticles, typically made of gold, silver, or magnetic materials, capture and detect viruses and are functionalized with specific biomolecules such as antibodies or aptamers. The detection process involves introducing the nanoparticles to a sample suspected of containing the virus. The nanoparticles bind to the virus, and this interaction causes a change in the optical or magnetic properties of the nanoparticles. Metal-based nanoparticles, for example, can interact with electromagnetic radiation and produce powerful signals (Nasrollahzadeh et al., 2020).

Metal and metal oxide nanoparticle combinations can be utilized to identify various viral infections. HBV, for example, was identified using nonfaradic electrochemical impedance spectroscopy with biotin-DNA probes and streptavidin-based magnetic nanoparticles (Shevtsov et al., 2017). Furthermore, iron magnetic particles were employed to isolate the influenza A virus H9N2 subtype, which was subsequently electrochemically detected using AuNPs (Saylan et al., 2019). Similarly, AuNPs and magnetic beads were utilized to colorimetrically detect and quantify HBV and hepatitis E virus (Sheikhzadeh et al., 2020).

Many illnesses have been identified using color change detection of Au nanoparticles associated with proteins, antibodies, and aptamers (Alhamid et al., 2024; Thongkum et al., 2019). Because of their unique chemical and optical properties, Au nanoparticles have been used in a variety of plasmonic biosensing methods, including Rayleigh scattering, LSPR, SERS, fluorescence enhancement, colorimetry-triggered quenching, and plasmon changes (Lee et al., 2018). For example, Au nanorods were utilized to detect single-base mismatch HIV-1 viral DNA using second-order nonlinear optical characteristics (Darbha et al., 2008). Furthermore, a similar hyper-Rayleigh scattering approach produced Au nanoparticles to detect HCV RNA (Griffin et al., 2009). In addition, paper-based POC nano diagnostic devices based on antigen-conjugated Au colloids for HBV detection (Gish et al., 2014), and multicolored Ag nanoparticles for Ebola virus detection were created (Yen et al., 2015). This nano-diagnostic device

was also used to detect COVID-19 by merging the PPT effect with LSPR sensing transduction (Qiu et al., 2020).

Fast, accurate, reproducible, and cost-effective SARS-CoV-2 identification can be achieved by employing nano-biosensors. To recognize DNA (genosensors), aptamers (aptasensors), or antibodies (immunosensors) in viral particles, biorecognition must be achieved. The detection of SARS-CoV-2 through different biorecognition molecules has been assessed, including sequences from ORF1 ab, the nucleocapsid phosphoprotein gene (N gene), RNA-dependent RNA polymerase (RdRp gene), the E gene or E protein, and the spike (S) protein, or using two targets. Coronavirus screening may be done using biosensing equipment on various clinical samples, including saliva, nasopharyngeal swab specimens, endotracheal sputum aspirates, and blood (Aquino et al., 2022).

Utilizing 2-D gold nano-islands coated with complementary DNA receptors, researchers could recognize the specific configurations of SARS-CoV-2 by nucleic acid hybridization. The thermos-plasmonic heat was generated on the device to boost sensing capability. Illumination at their plasmonic resonance frequency and the resulting local PPT heat improve the in-situ hybridization temperature and enable the exact differentiation of two similar gene sequences. This biosensor demonstrated exceptional sensitivity for the investigated SARS-CoV-2 sequences, with a substantially lower detection limit of 0.22 pM concentration, and allowed for precise target recognition in a multigene mixture (Qiu et al., 2020).

4.2 Conclusion

The advancement of micro and nanotechnologies in diagnosing viral infections has led to the commercialization of microfluidic-based tools to identify several worldwide viral infections. The fast growth of micro and nanotechnologies, along with advancements in the production of microchip-based devices, has resulted in significant improvements in the sensitivity, ease, transferability, and pace of collecting samples and detecting viral infections. However, the improvement of micro and nanotechnology enabling specific and quick assessment of viral load from a blood droplet, for example, in a single-use machine, poses a substantial trial for viral illnesses identification. Though micro and nanotechnologies permit the advancement of industrial microfluidic tools for HIV detection utilizing CD4⁺ T cell counts from blood drops, identifying viral illnesses using viral load counts is restricted by low sensitivity.

5. Weaknesses and technical challenges of different viral diagnosis methods

While viral diagnosis methods have made remarkable strides, they are not without limitations. This section examines the weaknesses and technical challenges associated with various viral diagnosis methods, shedding light on areas that warrant further research and development.

While highly sensitive, PCR-based methods require specialized equipment and expertise, making them less suitable for resource-limited settings (Poggio et al., 2021). Additionally, these methods might produce false negatives due to low viral loads in early infections or improper sample collection.

CRISPR-based diagnostics, although promising, face challenges in terms of off-target effects and efficiency (Chen et al., 2018). Ensuring high specificity and minimizing false positives are ongoing concerns that require careful design and optimization.

Sequencing-based methods, particularly whole genome sequencing, generate vast amounts of data that require sophisticated bioinformatics tools for analysis (Köser et al., 2013). Data interpretation and the identification of relevant variants demand specialized expertise.

Serological approaches can be confounded by cross-reactivity between antibodies targeting related viruses (Vasilakis et al., 2019). Accurate differentiation of recent infections versus past exposure is challenging, especially in regions with high seroprevalence.

Biosensors offer rapid detection, but their sensitivity might be compromised in samples with low viral loads (Laksanasopin et al., 2015). Achieving consistent performance across various sample types is a persistent challenge. While viral diagnosis methods have advanced significantly, they are not without limitations and technical challenges. Understanding these weaknesses is crucial for refining diagnostic approaches and improving their accuracy and reliability (Murad et al., 2023).

- **False Positives and Negatives:** All diagnostic methods risk producing false positives (indicating infection when none is present) or false negatives (failing to detect actual infections). The sensitivity and specificity of assays play a key role in minimizing these errors (Prince-Guerra et al., 2021).
- **Sample Quality and Preparation:** The accuracy of diagnostic methods heavily relies on the quality of the sample collected. Variations in sample collection techniques, storage, and transportation can impact the results (Kupferschmidt & Cohen, 2020; Yu, Yan, Wang, Yang, Wang, Tang, Gao, Wang, Ma, Xie, et al., 2020).
- **Genetic Diversity and Mutation Rates:** The genetic diversity and rapid mutation rates of certain viruses, such as RNA viruses, pose challenges in designing diagnostic assays that can target all strains accurately (Cotten et al., 2013).
- **Emerging and Unknown Viruses:** Diagnostic methods need to be flexible and adaptable to identify new and previously unknown viruses that may pose significant threats (Wang et al., 2002).
- **Resource Limitations:** Certain diagnostic methods, especially those involving advanced technologies, may require specialized equipment and skilled personnel, making them less accessible in resource-limited settings (Anfaara et al., 2018).

6. Future perspectives

The landscape of viral diagnosis is continually evolving, driven by advancements in technology, research, and our understanding of viral biology. As we look to the future, several promising trends and areas of development emerge that have the potential to shape the field of viral diagnosis. For instance, integrating artificial intelligence (AI) in viral diagnosis revolutionizes data analysis and interpretation (Mamoshina et al., 2016). AI algorithms can rapidly process vast sequencing datasets, identify relevant patterns, and predict viral evolution dynamics. Together, miniaturized and portable diagnostic device advancements will enable POCT, particularly crucial in remote and resource-limited settings (Wiriyaichaiyorn

et al., 2017). These devices will offer rapid results and improve early intervention. Moreover, real-time viral genomics monitoring can provide insights into pathogen behavior, aiding preparedness and response efforts. Viral diagnosis will contribute to the advancement of personalized medicine, tailoring treatments based on viral genetic information and patient profiles (Fenoglio et al., 2018; Rabaan et al., 2023c). Precision medicine approaches can optimize therapeutic strategies and minimize adverse effects.

Advancements in single-cell sequencing technologies are opening new avenues for viral diagnosis. By analyzing individual cells, researchers can uncover viral interactions, host responses, and heterogeneity within infected populations (Svensson et al., 2017). The integration of cutting-edge technologies, portability, and personalized approaches characterizes the future of viral diagnosis. These advancements will enhance our ability to swiftly identify viral pathogens and mitigate their impact on public health.

7. Conclusion

In the ever-evolving landscape of viral diagnosis, diverse methods and technologies have emerged, each with strengths, weaknesses, and unique contributions to clinical, pharmaceutical, and industrial applications. The field continues to advance from traditional approaches like serological assays to cutting-edge techniques such as CRISPR-based diagnostics and nanotechnology-enabled biosensors, driven by innovation and the pressing need to detect and combat viral infections.

The journey from the discovery of bacteriophage MS2's genome to today's high-throughput sequencing platforms and AI-driven diagnostics showcases the remarkable progress in understanding viral genetics, host-pathogen interactions, and the intricate dynamics of viral diseases. These advancements have improved our ability to identify viral pathogens accurately and enabled us to monitor viral diversity, track viral evolution, and design targeted interventions.

The prospects for viral diagnosis are exciting as we peer into the future. The integration of AI promises to enhance diagnostic accuracy and efficiency, while single-cell diagnostics and multiplex assays offer deeper insights into viral biology and enable comprehensive profiling. The concept of personalized medicine, tailored to an individual's genetic makeup and immune responses, holds the potential for more precise and effective viral interventions. Moreover, the global health community's growing emphasis on rapid response and global surveillance underscores the importance of robust diagnostic tools in identifying and mitigating emerging viral threats.

In closing, the chapters of this book, "Microbial Genomics: Clinical, Pharmaceutical, and Industrial Applications," collectively contribute to the ever-expanding body of knowledge in microbial genomics, showcasing how insights from genomics are transforming various sectors. The chapter on "Molecular techniques for viral diagnosis" highlights the pivotal role of genomics and innovative diagnostic methods in understanding viral infections, managing outbreaks, and safeguarding public health. By continuously pushing the boundaries of scientific knowledge and technology, we are better equipped to face the challenges of viral pathogens and respond swiftly and effectively in a rapidly changing world.

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Bacterial identification and diagnosis of bacterial infections through genomics, proteomics, nanotechnology, machine learning, and microelectromechanical systems

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1. Introduction

The rise of novel infectious agents, increased transmission due to human migration, and the development of antibiotic resistance have contributed to the fact that bacterial infections remain a leading cause of mortality worldwide (Pérez-Llarena & Bou, 2016; Roux-Dalvai et al., 2019). Identification of the bacteria in the biological sample is crucial for targeted remediation. Conventional identification procedures are time-consuming, wherein the bacteria are cultured, followed by additional lengthy steps of immunological or biochemical assays of various complexities and durations (Roux-Dalvai et al., 2019).

The two main categories of bacterial identification techniques are genotypic techniques, which profile an organism's genetic material (mainly its DNA) and phenotypic approaches,

which profile either an organism's metabolic characteristics or some component of its chemical composition. Genotypic approaches are advantageous over phenotypic methods as they are not affected by an organism's physiological condition, growth stage, or the makeup of its growth medium. However, phenotypic methods can provide more precise functional data that disclose what metabolic processes support the organism's survival, growth, and development. These may manifest, for instance, as a microbe's capacity to adapt and flourish on a specific substrate or its antibiotic resistance level (Tang et al., 1998).

To systematically classify organisms in an evolutionary or phylogenetic context, scientists have created categorization schemes from the first discovery of microbes (Emerson et al., 2008). Woese (1987) proposed that bacteria can be classified into two separate domains, the Bacteria and the Archaea, each as distinct from one another as they are from the Eukaryotes. This third domain includes all "higher forms" of life. This classification is based on molecular comparisons of evolutionarily conserved ribosomal genes. It is currently accepted among microbiologists and backed by additional molecular data. In addition, bacterial systematics is not fully standardized, and substantial debate exists regarding what qualifies as a bacterial species (Achtman & Wagner, 2008). Nevertheless, molecular systematics has provided a fundamental understanding for developing bacterial categorization methods. The timely identification, characterization, and classification of bacteria are essential in identifying biological threat agents in many fields. Recent developments in molecular methods in genomics and proteomics have provided compelling alternatives to traditional microbiological processes. These novel techniques may quickly provide multidimensional data sets with taxonomically pertinent molecular information about individual strains and whole populations (Logue et al., 2008).

This review is split into sections that briefly discuss phenotypic diagnostic methods and focus on the novel and genotypic bacterial identification methods.

2. Bacterial identification and characterization

2.1 Traditional diagnostic methods for bacterial identification

In clinical microbiology, bacterial identification is a systematic approach that combines several ways to identify (type) bacteria down to the species, subspecies, biotypes, and strain levels. The process dates back to the 19th century when culture media was first introduced, which has substantially developed since then (Bonnet et al., 2020).

The traditional methods for identifying bacteria are time-consuming, and findings from microscopic evaluation, growth in culture, biochemical tests, antimicrobial tests, and serology must be integrated to reach a result (Baron et al., 1996). These methods are still considered valuable despite having automated systems and more rapid ways of investigation. However, conventional techniques cannot be generalized to all pathogenic bacteria due to their diverse growth patterns under various conditions, such as temperatures, oxygen, and osmotic needs (Bonnet et al., 2020). They can only identify and characterize bacteria that can be grown in labs using synthetic media, cell cultures, or animal models. As a result, the goal of the investigation and the targeted bacterium will determine the proper methods and tests to be selected (Houpikian & Raoult, 2002). Traditional diagnostic methods are summarized in Tables 6.1 and 6.2.

TABLE 6.1 Summary of microscopic traditional diagnostic methods.

Wet mount	Gram stain	Acid fast stain
When stained, the visualization of bacterial cells is more accurate and readily apparent. However, most staining techniques expose the cells to chemicals and heat, which cause them to perish and lose their original size and shape. So, wet mounts can be done as a first step as it aims to view motile organisms while still alive to hold to their original shape (Norris & Swain, 1971).	Widely used differential staining method. This test helps to classify and differentiate microorganisms by dividing bacteria into Gram-positive and Gram-negative bacteria (Tripathi & Sapra, 2021).	This staining’s primary goal is to differentiate between acid-fast and non-acid-fast bacteria. Acid-fast bacteria are a group of organisms that contain abundant mycolic acids (lipid compounds) in their cell wall (Bayot and Sandeep Sharma, 2018).
For example, the most common application for bacterial identification is the potassium hydroxide (KOH) mount of vaginal secretions for bacterial vaginosis (Norris & Swain, 1971).	Typically, the thick peptidoglycan layer of the Gram-positive bacteria cell wall retains the crystal violet stain, giving them a violet-to-blue color. However, in Gram-negative bacteria, the thin peptidoglycan layer of their cell wall does not retain the crystal violet stain but captures the safranin counterstain giving them a pink color (Tille, 2017).	Due to the presence of these lipids, gram stains will not be effective, and other unique types of stain are required, such as Ziehl–Neelsen stain (Bayot and Sandeep Sharma, 2018).

Bacterial pathogens exist in a range of sizes that are too small for the unaided human eye to see, making a microscope a necessary tool in microbiology. Direct analysis of samples using light microscopy yields preliminary data on the morphology and biological group of the microorganisms causing the ailment (Goering et al., 2019).

2.2 Immunologic and serologic methods

Multiple factors can hinder the direct detection of an infectious agent using biochemical and cultural methods. An example is the inability or difficulty of growing *Treponema pallidum*, causing Syphilis, on artificial media (Edmondson et al., 2018). As a result, immunologic and serologic techniques gained more attraction throughout the years for their ability to rapidly detect pathogens without needing cultivation. These tests enable the assessment of a patient’s immune status against a suspected infectious pathogen by identifying a particular antibody or antigen to diagnose an acute infection (Boyles & Wasserman, 2015). Agglutination tests are commonly used in clinical laboratories for meningeal infections, brucellosis, and leptospirosis. The agglutination procedure requires a blood sample taken from the patient to be tested for any formation of clumps or antigen–antibody complexes (Goering et al., 2019).

Another rapid investigation is the enzyme-linked immunosorbent assay (ELISA), a quantifying assay. It measures and detects microbial antigens or antibodies in the patient’s sample (Goering et al., 2019). The assay is read and quantified after adding a substrate with a color indicator. A color change indicates the presence of a targeted antigen or antibody (Alhajj & Farhana, 2020). It can be used to detect multiple bacterial species, such as *Salmonella enterica* and *Escherichia coli* (Galikowska et al., 2011).

TABLE 6.2 Summary of traditional diagnostic methods.

Bacterial cultivation	Colony morphology	Biochemical tests
The sterile cultivating environment used to grow microorganisms in the lab is known as a medium. The medium might be liquid, semisolid, or solid in physical nature. And it can be made entirely of chemicals or organic materials. Once the microbes start proliferating and growing inside the media, they will produce what is known as a culture (Goering et al., 2019).	Colony morphological characteristics regard the appearance of the colony after it has grown on the agar plate (Tille, 2017).	Microorganisms go through different metabolic and biochemical reactions to maintain their cellular functions. Biochemical assays examine those enzymatic activities for bacterial identification. Examples are the oxidase and catalase tests (Fig. 6.1) (Al-Joda & Jasim, 2021).

Resistance and susceptibility to antimicrobial agents

Antimicrobial susceptibility testing aims to identify whether antibiotic therapy will be effective or unsuccessful once administered. The tests are conducted in the lab by adding antibiotics to bacterial cultures in a petri dish, in order to evaluate how a particular medicine or combination of pharmaceuticals affects the growth of an isolated organism (Baron et al., 1996).

Culture-based approaches have historically been the most exclusive way to diagnose bacteria and have long been regarded as the most accurate way to identify pathogens (Bonnet et al., 2020). However, some organisms cannot be easily detected by the standard culture techniques employed in most laboratories, such as anaerobes, because most specimens will be subjected to aerobic microbial culture unless expressly requested or indicated by the clinical presentation. Standard culture procedures can have lower specificity because they mostly rely on morphological and biochemical characterization for identification. Additionally, a small percentage of organisms in a multipathogen sample can be effectively cultivated. And that is due to multiple factors, most notably fastidious growth conditions, nonviability, or production of antimicrobial peptides from the other microbes in the sample that can inhibit or kill the targeted bacteria. These elements make it a challenge to diagnose and treat infections accurately (Baron et al., 1996).

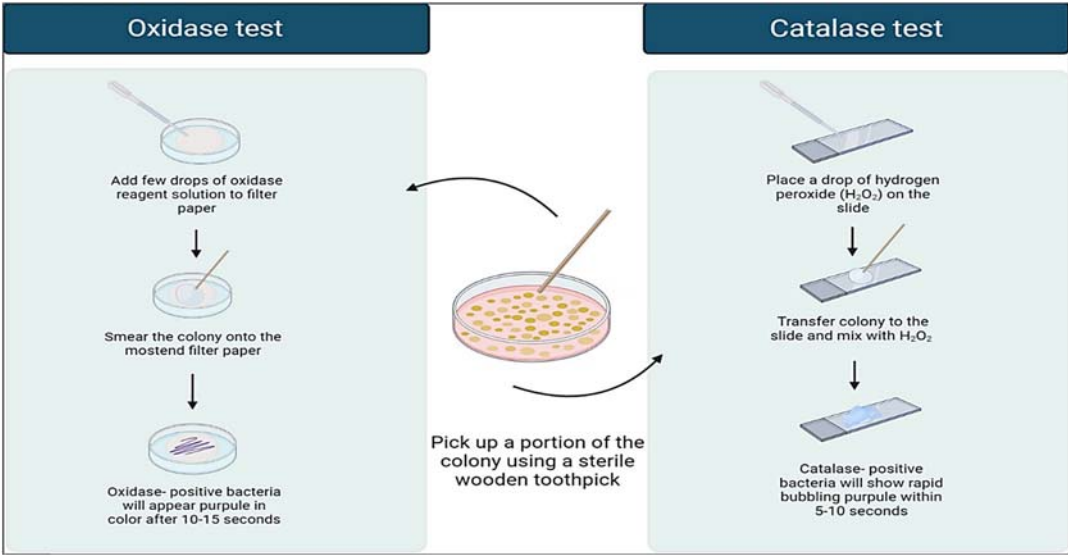


FIGURE 6.1 The figure highlights the steps for performing the oxidase and catalase test for bacterial identification. Based on Tille (2017). Created using Biorender.

3. Genome-based approaches

The methods for genotypic identification of microorganisms can be divided into two main sections:

- (1) Patterns or fingerprints-based techniques
- (2) Sequence-based techniques

Genotyping techniques are based on the sequencing of partial (16S small subunit ribosomal rRNA sequencing) or entire genomes (whole genome sequencing (WGS)) of the microorganisms. It can be used for complex samples containing multiple species. However, the cost and time required for sequencing technologies prevent their usage for bacterial identification in standard laboratories. Even though 16S rRNA sequencing is fast (identification results within 24 h) (Roux-Dalvai et al., 2019), it is reported to be significantly conserved across bacterial species. This compromises the precise identification of the strains. Recently, WGS has become very accessible and cost-effective. It offers previously unattainable insights into the bacterial type, evolutionary lineages, and analysis of the entire bacterial genome. This has revolutionized our approach and understanding of antibiotic resistance and epidemic investigations.

The current 16S rRNA amplicon sequencing method based on next-generation sequencing (NGS), in combination with genome search toolkit analysis, may provide a helpful tool for diagnosing bacterial infections in the bloodstream. This method is also a promising platform for clinical applications.

In fingerprint-based techniques, a succession of fragments from the chromosomal DNA of an organism is usually produced using pattern-based approaches. A profile or fingerprint specific to that organism and its very near relatives is created by separating these fragments according to size. The fingerprint data of known species can be compiled into a database, which can serve as a reference for identifying unknown strains. Two closely related organisms have similar fingerprints or profiles. In sequence-based techniques, the already characterized sequence of a specific gene or DNA segment is searched in the unknown strain. Initially, a database of known DNA sequences is created and used as a reference to identify unknown strains. The “degree of similarity” between the two sequences reveals their genetic closeness. Numerous computer algorithms have been developed to facilitate genetic comparison between the sequence and generate a phylogenetic tree from the findings (Ludwig & Klenk, 2015).

Each of the methods mentioned above has its advantages and disadvantages. While fingerprinting-based methods are good at identifying strain- or species-level relationships, they are less reliable when comparing organisms with higher taxonomic differences. In contrast, sequence-based methods, such as the 16S rRNA gene analysis, effectively establish broader phylogenetic relationships among bacteria at the genus, family, order, and phylum levels (Vandamme et al., 1996). A polyphasic approach is used to characterize novel bacterial species where a combination of different phenotypic methods is applied (Gillis et al., 2015). Current methodologies for identifying bacteria use a range of fingerprinting- or sequence-based techniques. These methods continually change to incorporate fresh approaches that boost sample throughput while increasing accuracy.

3.1 Fingerprinting-based methodologies

Over the years, various molecular techniques have been developed to identify and diagnose bacterial pathogens accurately. Two of the most used molecular techniques are Restriction Fragment Length Polymorphism (RFLP) and Loop-Mediated Isothermal Amplification (LAMP) (Magalhães et al., 2022).

RFLP is a molecular technique that uses restriction enzymes to cut DNA into fragments, then separate by gel electrophoresis. The resulting patterns of DNA fragments, called fingerprints, are unique to each bacterial species and can be used to identify and differentiate them. RFLP has been used to identify many bacterial pathogens, including *Mycobacterium tuberculosis*, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* (Chatterjee & Raval, 2019).

Several recent studies have reported using RFLP to identify bacterial pathogens in clinical and food samples. For instance, one study used PCR-RFLP to subtype *Listeria monocytogenes* isolated from a South African avocado processing facility (Strydom et al., 2013). Similarly, Elkady et al. (2022) used PCR-RFLP to determine the genetic diversity of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. However, despite its accuracy, RFLP has several limitations. The technique is time-consuming, requires large amounts of DNA, and may not be suitable for analyzing complex bacterial communities. Moreover, RFLP is less sensitive than other molecular techniques, such as PCR-RAPD, and may not detect low levels of bacterial pathogens (Tabit, 2016).

LAMP is a molecular technique that amplifies DNA under isothermal conditions using a set of four to six primers and a DNA polymerase. The method quickly produces a large amount of DNA and can detect bacterial and viral pathogens at low concentrations (Alhamid et al., 2022, 2023). LAMP has been used to identify many bacterial pathogens, including *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Helicobacter pylori*, and many more (Wong et al., 2018). For example, one study used LAMP to detect *Listeria monocytogenes* in ready-to-eat meat food samples, achieving a specificity and sensitivity of 100% (Fiore et al., 2023). Horiuchi et al. (2019) used LAMP to detect *Helicobacter pylori* from fecal specimens with a sensitivity of 10 times greater than that of PCR.

One of the main advantages of LAMP is its simplicity and speed (Wong et al., 2018). The technique does not require expensive equipment or specialized training, and results can be obtained within an hour (Matthew et al., 2022). Moreover, LAMP can detect multiple bacterial pathogens simultaneously, making it an ideal technique for screening large samples (Garg et al., 2022). The most popular genotypic approach for identifying bacteria is fingerprinting methods. Polymerase chain reaction (PCR) is used in repetitive element PCR (rep-PCR), amplified fragment length polymorphism (AFLP), and random amplification of polymorphic DNA to amplify numerous copies of short DNA fragments (Lin et al., 1996). These techniques utilize DNA polymorphisms within related species, which manifest due to differences in the evolutionary processes of the organisms. Multiplex PCR detects multiple microbes in a mixed sample (Tombuloglu et al., 2021, 2022). It employs distinct PCR primer sets for each organism within the mixed sample (Settanni & Corsetti, 2007). Riboprinting uses a sensitive probing technique to identify changes in gene patterns in different strains and species (Bruce, 1996). These methods are used for bacterial identification in taxonomic classification and systematics (Gevers et al., 2005; Vandamme 1996) for source tracking (Meays et al., 2004), authentication of isolates for archival purposes, and determination of microbial population structures and community studies (Singhal et al., 2015).

3.2 Sequencing-based methodologies

Multilocus sequencing is a recent and effective technique for identifying microbial species. It is conceptually similar to the 16S rRNA sequencing method but compares the sequences of several “housekeeping” gene fragments. This process is known as concatenation. Housekeeping genes produce proteins required for vital cellular functions. Gyrase B subunit (*gyrB*), RNA polymerase’s alpha and beta subunits (*rpoA* and *rpoB*), and *recA*, a gene encoding an enzyme essential for DNA repair, are some examples (Zeigler, 2003). Most cells have housekeeping gene loci that are often conserved across various species. Consequently, multigene PCR amplification of the same genes may be accomplished by designing general-purpose primers.

Some other sequencing methods are multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA). In MLST, 6–10 genetic loci are utilized, and suitable primers are used for each locus to enable PCR amplification and sequencing of the products (typically 400–600 base pairs) (Maiden et al., 1998). A database of sequences for the same organism is then compared with the concatenated sequences. The result offers a high-resolution identification of a particular strain, which may indicate tight evolutionary ties between other strains. This method is effective in epidemiological research, making it feasible to monitor the spread of severe bacterial diseases (Cooper & Feil, 2004). However, only a few common pathogens have been used for database generation in MLST, and each organism was subjected to a strict set of requirements for the PCR primers.

Multiple conserved protein-encoding gene segments are sequenced as part of MLSA. For comparative analysis, the genes are chosen more arbitrarily than MLST, and a smaller subset (≤ 6) of genes or loci is used (Gevers et al., 2005). Instead of tracing the evolution of specific strains, MLSA is often used to identify organisms while investigating species relationships within genera or families. However, it lacks the analytical power to identify the minute variations in sequence patterns crucial for epidemiologic research. MLSA is constrained by a lack of standards and a centralized database. For instance, MLSA was employed in only eight recent articles to identify a variety of bacterial phyla. A review of those papers revealed that each study used two to six distinct genes (Chelo et al., 2007; Richert et al., 2007).

Another emerging technology is microarrays, which can simultaneously identify microorganisms and provide ecological context for a given microbial community’s population structure and functional organization. The main idea behind how microarrays operate is to place probes for hundreds or thousands of genes on a substrate and then hybridize sample DNA or RNA to it. To quickly identify samples that hybridize with probes on the microarray, the DNA or RNA of the sample is tagged with a fluorescent reporter molecule. The small-subunit ribosomal gene has been the object of multiple versions of a “phylochip,” built for both particular and extremely large groupings of environmental bacteria (Wilson et al., 2002). Another example is the geochip, created to detect microorganisms participating in crucial biogeochemical processes such as metal transformations, pollutant degradation, and primary carbon cycling (He et al., 2007). Microarray technology is advancing quickly in the clinical setting, both for diagnostic reasons and for understanding the principles of disease pathology (Richter et al., 2006).

3.2.1 Identification of single-nucleotide polymorphism (SNP)

Single-nucleotide polymorphism (SNP) is a critical discriminative method for DNA tracing and genotyping that can identify a single nucleotide in a specific location that is present in a sizable enough portion of the targeted population, and it is especially helpful in outbreak situations for identifying, typing, and tracing the origin of bacterial organisms (Easterday et al., 2005; Rahman et al., 2022; Stephens et al., 2006; Van Ert et al., 2007). The PubMLST open-access database website provides over 130 population sequence datasets of different microbial species, integrating their phenotypic information available at the Public databases for molecular typing and microbial genome diversity (PubMLST) database (<https://pubmlst.org/>). Since foodborne bacterial infections continue to be a serious hazard to public health, they have been a focus of SNPs' research applications. SNP not only provides a rapid way of identification but also analyzes the phylogenetics and mutations to discover genetic diversity in the genetic resource. This could be applied to the medical field for quick and simple diagnosis and typing. The mutations can also be phylogenetic markers to categorize strains (Parsons et al., 2016). One of the applications was by Yokayama et al. (2018), who used gel electrophoresis and whole-genome sequencing to identify clusters of Shiga toxin-producing *E. coli* and identified 17 clusters from strains responsible for nine outbreaks. They could also use SNPs from the DNA backbone to subdivide the clusters further. In another study, 12 SNPs were derived from four housekeeping genes in the *E. coli* multilocus sequence database to determine where *E. coli* in food originated from. Then, they used these 12 SNPs for *E. coli* isolates from 12 countries (Liu et al., 2020). Variabilities in SNP were also measured in *Salmonella* infections from different food contamination incidents to check their phylogenetic relation and lineage (Wilson et al., 2016).

There are some available tools for SNP genotyping, such as the BioHansel tool, which uses a fast k-mer sequencing approach to map a pathogen's whole genome sequence data to canonical SNPs and allocate genotypes based on its profile. It provides genotyping algorithms for *Salmonella* serotypes, *Mycobacterium tuberculosis*, and other organisms. And it can promptly and precisely deliver phylogenetically instructive genotyping data to the typed specimen (Labbé et al., 2021). The source code is available at <https://github.com/phac-nml/biohansel>. The OmicsBox Genome Analysis module includes multilocus sequence typing in addition to quick and user-friendly analysis of recently sequenced genomes (Available at <https://www.biobam.com/>).

3.2.2 Metagenomics

Metagenome refers to the total number of genomes detected in a sample of interest (Handelsman et al., 1998). To get the metagenomic data, deep sequencing can be employed, where millions of tiny DNA fragments must first be sequenced, and then each sequence can be connected to recreate the genome using bioinformatics techniques (Dhawi & Alsanie, 2022; Papudeshi et al., 2017). This genome-based analysis has been utilized as a tool for the detection of pathogenic organisms in humans (Brown et al., 2018; Lim et al., 2014), plants (Gökdemir et al., 2022), and environmental samples (Tombuloglu et al., 2023). It also played a role in screening for the SARS-CoV-2 virus in sewerage water during the COVID-19 pandemic by sequencing the viral genome (Crits-Christoph et al., 2021; Landgraff et al., 2021, pp. 2021–03). What makes metagenomic sequencing more favorable when compared with traditional methods for bacterial identification is the reality that this sequencing method targets all

the genetic material in a given sample, whereas in conventional methods, the approach is typically restricted to bacteria that are well-known or suspected to be the pathogen (Chiu & Miller, 2019; de Vries et al., 2021; Gupta et al., 2019). However, this ability to analyze every genome in a sample can be an inconvenience, especially in a clinical setting where the specimen is collected from an infected person (the host). The human genome must be removed to ease the identification of the bacterial genome. This can be done by adding saponins to lyse the host cells, such as by diagnosing bacterial lower respiratory tract infections (Charalampous et al., 2019) and identifying the colonic microbiome (Bruggeling et al., 2021). Samples can also be treated with a mixture of saponins at a high NaCl concentration followed by DNase treatment with high-g force centrifugations to increase the selectivity of host cells for cell lyses and to markedly reduce the host DNA in a specimen (Aja-Macaya et al., 2023). Different methods to discover pathogenic organisms utilizing metagenomics have been established, such as the metagenomic NGS technique, which is recommended for genotypic identification of microbes, including organism identification, species identification, and highlighting metabolic processes (Gökdemir et al., 2022). Another protein-based method uses a metagenomic pipeline to diagnose and spot microorganisms (Pérot et al., 2022).

3.2.3 Whole-genome sequencing in bacterial identification and diagnosis

In recent years, the development of WGS technologies has revolutionized the field of microbiology, enabled rapid and accurate identification of bacterial species and strains (including those that are difficult to culture or identify by phenotypic characteristics), and provided valuable insights into their genetic characteristics and evolution (including virulence factors, antibiotic resistance genes, and other genomic features that may be relevant for diagnosis and treatment) (Quainoo et al., 2017).

One of the most important applications of WGS is identifying bacterial species and strains in clinical samples. WGS can be used to identify the causative agent of bacterial infections, including those that are difficult to diagnose using traditional methods, such as sepsis or meningitis. It can also be used to identify outbreaks of bacterial infections by comparing the genomic sequences of bacterial strains from different patients or sources (Yu et al., 2019). In addition, WGS can monitor the spread of antibiotic-resistant genes and identify potential new drug development targets (Punina et al., 2015).

Despite its many advantages, WGS has some challenges and limitations in bacterial identification and diagnosis. One major challenge is the interpretation of genomic data, particularly in cases where the clinical significance of certain genomic features is unclear. Another challenge is the standardization of sequencing and bioinformatics methods, which can affect the comparability of data across different studies and laboratories (Austin-Tse et al., 2022). However, WGS has a wide range of applications in clinical and public health settings and is likely to become increasingly important in the coming years as sequencing technologies continue to improve and become more widely available.

3.3 CRISPR, nanotechnology, biosensor

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system is a revolutionary genome editing tool recently adapted for detecting and identifying bacteria.

This system utilizes a unique pattern of DNA sequences that can be recognized and targeted by CRISPR-associated proteins (Cas proteins) for specific bacterial detection (Huang et al., 2022).

The CRISPR-Cas system has been used for bacterial identification based on the unique CRISPR spacers found in each bacterial genome (Rubio et al., 2023). In this system, CRISPR arrays are sequenced and compared with a reference database to identify the bacterial species (Pourcel et al., 2020). This CRISPR-based identification system has successfully identified various bacterial pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *E. coli* (Chakraborty et al., 2022).

By making use of the crRNA-based programmability of the CRISPR-Cas13a system and its RNase activity to target RNA recognition against *Staphylococcus aureus*, a bacterial sensing technology known as CCB (CRISPR-Cas13a based)-Detection has been created. CB-Detection can effectively detect gDNA within the detection range of 10^0 – 10^7 CFU/mL in food samples. A detection technology (named “APC-Cas”) has been developed that uses a three-step amplification to generate strong fluorescence signals, allowing for identifying tiny levels of a bacterial pathogen without the need for isolation. APC-Cas detects *S. enteritidis* utilizing a combination of allosteric probes based on nucleic acids and CRISPR-Cas13a with an aptamer domain. It is used to identify and quantify *Salmonella enteritidis* cells (1 – 10^5 CFU) in a wide range of samples, including milk, with sensitivity and accuracy equivalent to or better than those of classical real-time PCR (Bhattacharjee et al., 2022).

The CRISPR-based techniques for bacterial identification and diagnosis can potentially revolutionize the field of microbiology. They are fast, accurate, and cost-effective, making them ideal for use in clinical settings. In addition, developing portable CRISPR-based detection devices could allow for rapid and onsite identification of bacterial pathogens.

4. Proteomics methods

Since the completion of the human genome project and the sequencing of other prokaryotic and eukaryotic genomes, there has been an increased interest in examining proteins to identify and quantify expressed proteins. The proteome reflects the real-time condition of a cell concerning its environment, whereas the genome is fixed. Advances in mass spectrometry technology have enabled effective protein quantification, identification, and analysis. It permits quick and high-throughput examination of the biomolecular fingerprints that a bacterial strain produces. Along with advances in genomics, proteomics-based bacterial identification and characterization methods have also become more popular.

While genotypic information is valuable in identifying an organism and establishing its evolutionary relationship, phenotypic information helps understand the physiological and functional activities at the protein level. Phenotypic methods that determine the activity of specific enzymes, such as catalase or oxidase, or metabolic functions, such as the ability to degrade lactose, are useful in bacterial identification. Novel mass spectrometry-based proteomics tools help quickly detect biomolecules produced by an organism and provide an excellent complement to established microbiological and genomics-based methods for bacterial identification, classification, and phenotypic characterization.

According to the Uniprot database (Swiss-prot), more than 29,000 proteins are produced from genetic information when taking *Escherichia coli* as a microbial cell. Both gel-based and gel-free protein separation techniques helped further the microbial proteome's characterization and differentiation. With LC-MS/MS technologies, peptide amino acid sequences are more often discovered. After digestion, the ionized peptides are identified and further fragmented after being isolated by high-performance liquid chromatography from the whole proteome. The peptide sequences are determined by comparing the tandem spectra with the predicted peptide fragmentation spectra. For instance, [Kookan et al. \(2014\)](#) showed that it is possible to physically break apart bacterial cells and remove the proteins, which can then be separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After in-gel digestion, the target bacterial protein bands are checked using LC-MS/MS ([Cheng et al., 2016](#)).

Our knowledge of the microbial world has increased due to proteomic investigations. Since the U.S. Food and Drug Administration approved two MALDI-TOF-MS-based technologies, MS and proteomics have gained a substantial impetus in bacterial research and applications. MALDI-TOF-MS, electrospray ionization–mass spectrometry (ESI-MS), surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, one- or two-dimensional SDS-PAGE, or a combination of the aforementioned techniques along with bioinformatics have immensely advanced the proteomics research ([Al-Qadiri et al., 2006](#)).

4.1 Mass spectrometry methods

Mass spectrometry is used to identify and quantify novel substances and elucidate their molecular structure and chemical characteristics. When J. J. Thomson (1899) utilized it for the first time to gauge the mass-to-charge ratio of electrons, mass spectrometry began to take shape in the late 19th century. It can be used in physical measurement, chemical characterization, and biological identification.

However, in mass spectrometry, samples are converted to a vapor phase before analysis, which restricts its application in the characterization of biological samples. The invention of the soft ionization approach in mass spectrometry enables the examination of biological molecules (i.e., MALDI-TOF-MS and ESI-MS). Soft ionization has also enabled the analysis of bigger biological molecules, analyte sampling, and direct ionization from native material, including entire cells ([Fenn et al., 1989](#)). Since its first application for bacterial identification in 1975, mass spectrometry has generated protein profiles specific enough to identify antibiotic-resistant bacteria and their molecular markers. It has also assisted in resolving time-constraint issues imposed by conventional bacterial identification and characterization methods. [Fig. 6.2](#) shows the various methods used in mass spectrometry for microbial identification and characterization.

MS/MS-based methods can offer gene expression information down to the level of individual amino acids. This makes it possible to detect pathogenic delineators and genotypic characteristics expressed quickly and accurately, such as proteins linked to virulence and antimicrobial resistance ([Lima et al., 2013](#); [Radhouani et al., 2012](#); [Sauer & Kliem, 2010](#)). Thus, tandem MS can further advance the analytical workup of infectious bacteria.

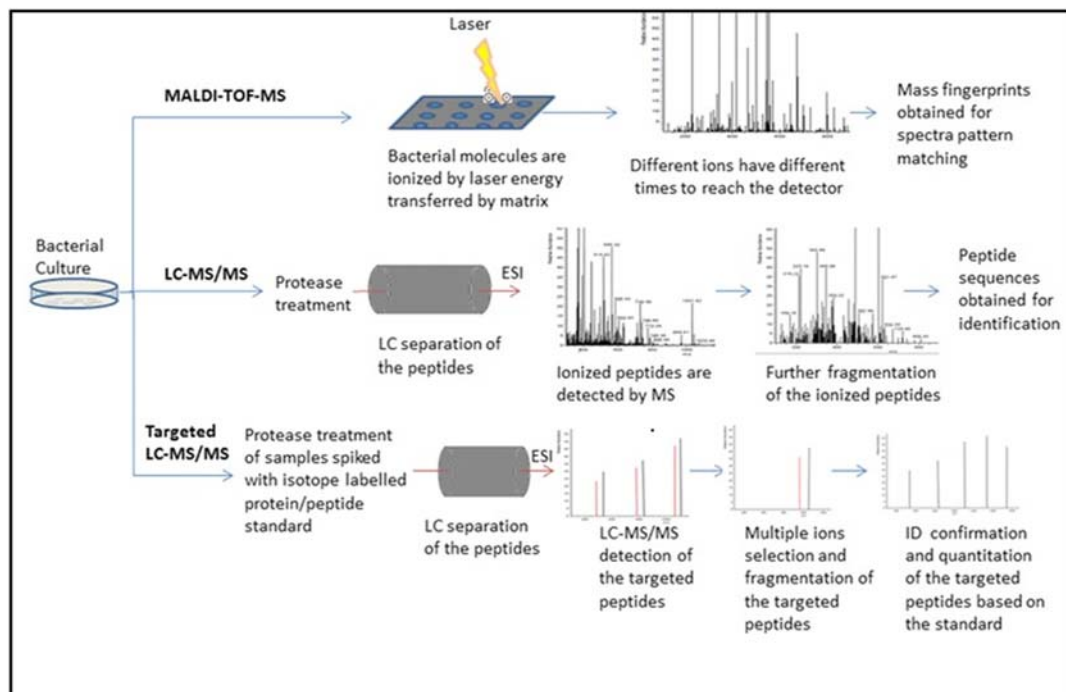


FIGURE 6.2 Common platforms for bacterial identification and characterization (Cheng et al., 2016).

Bottom-up tandem MS, when peptide-based sequences generated using proteolytic are identified by calculating the mass-to-charge ratio of the molecular ions, fragmenting the peptides, and comparing their spectra to a database (Karlsson et al., 2015).

4.1.1 MALDI-TOF-MS

Mass spectrometry employs the soft ionization technique known as MALDI, which enables the analysis of biomolecules and biopolymers such as proteins, peptides, sugars, and large organic molecules. Such compounds are fragile and fragmented when ionized by more conventional techniques such as ESI. MALDI-TOF-MS can analyze whole bacterial cells directly and produces relatively straightforward, repeatable spectral patterns over a wide mass range. These spectra can be used to characterize and identify bacterial species by comparing the spectra fingerprints of known and unknown species (Wang, Zhang, et al., 2022; Wang, Hsieh, et al., 2022; Wang et al., 2022; Wang, Hong, et al., 2022). Numerous studies have demonstrated the effectiveness of MALDI-TOF-MS for the rapid characterization of bacterial isolates. Holland et al. (1996) first reported using MALDI-TOF-MS to quickly identify entire bacteria (Holland et al., 1996). Thereafter, many bacteria have been characterized, including *Staphylococcus* species, *Mycobacteria* species, several extremophilic bacteria, and archaea.

The bacteria *Staphylococcus aureus* colonizes the human skin and can cause infection. Due to improper antibiotic usage, *S. aureus* is now resistant to antibiotics of the methicillin family. In the early 1960s, methicillin-resistant *S. aureus* (MRSA) was first observed at a European

hospital. Since then, hospitals and clinical environments have been no longer safe from the threat posed by MRSA, which has led to the deployment of methods that can quickly distinguish MRSA from methicillin-sensitive *S. aureus* (MSSA). A MALDI-TOF-MS approach for the detection, genotyping, and differentiation of MRSA and MSSA was created by Edward-Jones and colleagues in 2000. This procedure applies a sample collected from a single bacterial colony on a slide. The material is given the proper matrix, which MALDI-TOF-MS subsequently examines. MRSA and MSSA produce separate spectral peaks that enable quick differentiation and allow for targeted treatment of *S. aureus* infections (Fig. 6.3).

By matching the masses of biomarkers from the unknown creature with the proteome database or by comparing the peptide mass fingerprinting (PMF) of the unknown organism with that in the database, microorganisms can be identified. When performing PMF matching, the MS spectra of unknown samples are compared with those of known microbial isolates stored in the database. Microbes are often identified at the species level using a mass range m/z of 2–20 kDa, primarily reflecting ribosomal proteins and a few housekeeping proteins.

Sample preparation for MALDI-TOF-MS depends on the source of sampling and cell wall composition. Researchers for various microorganism groupings have assessed different sample preparation techniques. Direct cell profiling, which allows for the direct identification of some bacteria by MS, is used for other microbes, for which whole cell lysates or crude cell extracts are generated. In indirect cell profiling, a single colony of bacteria is selected and spotted directly onto the sample plate, and then the matrix solution is applied immediately. Using direct cell profiling and MALDI-TOF-MS, Gram-negative bacteria such as *Neisseria* spp., *Yersinia* spp., and *Vibrio* spp. were detected (Singhal et al., 2015).

Rapid, reliable, and affordable approaches are needed in clinical microbiology laboratories to identify possible pathogens in clinical samples so that the proper antibiotic medication can be started immediately. Microbiologists have recently comprehended the potential of MALDI-TOF MS in standard microbiological laboratories. Numerous studies have demonstrated the value of MALDI-TOF MS for the early detection of bacteria in various materials, including blood cultures, cerebrospinal fluids, respiratory tract infections, stool samples, and urinary tract infections. Numerous studies have demonstrated that MALDI-TOF-MS can better detect bloodstream infections faster and more accurately than traditional diagnostic techniques. When MALDI-TOF-MS-based identification techniques were compared with

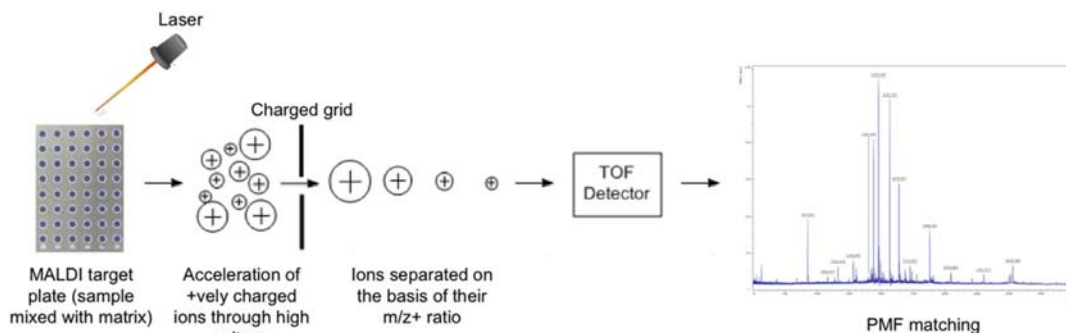


FIGURE 6.3 Schematic diagram showing the workflow in an MALDI-TOF-MS (Singhal et al., 2015).

traditional methods for diagnosing urinary tract pathogens, it was shown that MALDI-TOF-MS needed minimal processing time. It recognized bacteria from urine samples with more than two uropathogens (Singhal et al., 2015).

It is crucial to quickly identify pathogenic microorganisms to guarantee the safety and quality of food and drink items. It has been demonstrated that MALDI-TOF-MS is effective for the early identification of bacterial threats that might pollute drinking water. MALDI-TOF-MS has also been used in food microbiology for various tasks, including the characterization and identification of lactic acid bacteria in fermented foods. By combining the bacteria from a positive blood culture with the enzyme-substrate, MALDI-TOF-MS has also been recently reported to be able to identify carbapenemase or -lactamase activity, a sign of antibiotics hydrolysis (antibiotics). If the substrate hydrolyzes and the MS detection reveals a mass shift, antibiotic resistance may be predicted (Cheng et al., 2016; Singhal et al., 2015).

Less progress has been made in applying MALDI-TOF-MS in virology than in bacteriology or mycology. This could result from viruses having a relatively low protein content, viral proteins having a higher molecular weight (>20,000 Da), and possible carryover of cell-substrate detritus. Several studies have also demonstrated the effectiveness of MALDI-TOF-MS for detecting numerous pathogenic viruses in clinical samples, including enteroviruses, herpesvirus, hepatitis viruses, and human papillomaviruses (HPVs) (Piao et al., 2012; Yi et al., 2011). According to Yi et al. (2011), a PCR-based MS approach was used (Piao et al., 2012) to identify high-risk HPVs, a significant contributor to human cervical cancer. To create a PCR-Mass test that concurrently identified eight different viruses linked to human enteric diseases, Piao et al. (2012) coupled multiplex PCR with MALDI-TOF-MS. According to a recent study by Calderaro et al. (2014), MALDI-TOF-MS proved to be a useful, quick, and affordable method for identifying distinct poliovirus serotypes from varied clinical samples.

Antibiotic susceptibility testing and choosing an appropriate medication depending on the type of bacteria being treated are essential for successfully managing bacterial disease. In a study, MALDI-TOF-MS successfully detected bacterial isolates from human urine samples, providing dependable information down to the bacterial species level (Anwer et al., 2022). Given the success of this method, top-down proteomic recognition of certain peptides and factors that influence the virulence of pathogenic bacteria has experienced further improvements (Demirev et al., 2005; Fagerquist et al., 2010; Maus and Bisha, 2020). In top-down analysis, mature intact proteins' structure, mass, and posttranslational modifications (PTMs) can be determined and analyzed without the need for preceding proteolytic digestion (Catherman et al., 2014). This technology is relevant to bacterial recognition and diagnosis as rapid identification and characterization of bacterial pathogens are essential for the effective treatment of time-sensitive infections and public health crises (Angeletti and Ciccozzi, 2019; Sandrin et al., 2013). Characterization of infectious bacteria should have a focus on bacteriophages and plasmids, which play an important role in the horizontal transmission of virulence and antimicrobial information that can affect host/bacterial survivability (Angeletti & Ciccozzi, 2019; Sandrin et al., 2013).

4.1.2 ESI-MS

In bacterial characterization, ESI-MS has the potential to be crucial, particularly for examining cellular components. The lysed cells can extract and evaluate the proteins the bacteria

expressed. Proteins, carbohydrates, and lipids inside and outside cells may be analyzed using this method. The ability of ESI-MS to perform tandem mass spectrometry, in which the target protein can be fragmented for a second mass analysis that provides protein fragment sequence information or a peptide fragmentation fingerprint, is a significant advantage. This fingerprint can identify the target protein in a database search. Comparatively, identification utilizing only molecular weight data from a single MALDI-TOF-MS study has dramatically improved protein identification accuracy. According to research by Krishnamurthy and Ross from 1996, the total analytic time needed to identify bacteria in samples with certainty takes less than 10 min, with repeatable findings. The T5000 Biosensor System, newly created by Ibis Biosciences, uses PCR and ESI-MS technologies to detect and accurately characterize bacterial strains (Sampath et al., 2007). The combination of MALDI-TOF-MS and MLST analysis employing *Neisseria meningitidis* illustrates how mass spectrometry is used to study nucleic acids for bacterial identification (Honisch et al., 2007). These attempts exemplify how combined genotypic and proteomics technologies offer an even more potent tool for bacterial identification.

Another benefit of ESI-MS is its ability to identify target bacteria in mixed samples. Specific intracellular biomarkers for distinct microorganisms can be separated with adequate certainty due to the higher resolution of ESI-MS. Identifying and characterizing an unidentified bacterial species for protein analysis will be possible by comparing the experimentally determined protein profile with the profile data in a proteomics database (Chao et al., 2007). The roles and mechanisms of the potential virulence factors can then be further elucidated using phenotypic investigations such as mutagenesis, traditional biochemical techniques, and structural biology.

4.2 Surface-enhanced laser desorption/ionization (SELDI)

Complex biological material, including blood, serum, plasma, lymph, entire cells, cell lysates, urine, and cellular secretion products, can hamper direct MS analysis. Therefore, extensive sample purification and preparation processes are required before an MS examination. Liquid chromatography, electrophoresis, centrifugation, and membrane dialysis are a few examples of conventional sample purification techniques that are labor-intensive and need larger sample quantities. Additionally, samples can be compromised due to precipitation, dilution, or nonspecific binding. SELDI combines MALDI-TOF-MS and Protein Chip arrays as a MALDI target plate. It was developed by (Hutchens & Yip, 1993). With the use of SELDI technology, it is possible to quickly generate a protein expression profile from a range of biological samples, enabling the quick characterization of bacteria and the evaluation of the variability in their expression patterns. Based on the inherent characteristics of the proteins themselves, these various chemical and biological surfaces enable differential capture of proteins. Based on the mass-to-charge ratio and binding affinity of the proteins in the complex protein mixtures to the chip surface, the SELDI mass spectrometer generates spectra of the mixtures. Differentially expressed proteins may be identified from these protein profiles by comparing peak intensities.

Few studies have examined how well SELDI may be used to locate and identify bacterial pathogens and virulence factors (Seo et al., 2004). This study shows that SELDI technology offers an alternate method for examining bacterial proteomes to previous methods, ultimately

enabling bacterial identification based on comparing protein profiles and patterns. The use of SELDI technology to identify between four subspecies of *Francisella tularensis*, the cause of tularemia in humans, illustrates how it has been used. The only subspecies of *F. tularensis* found in North America is the most contagious of the four subspecies. The ability of SELDI-TOF-MS to produce distinct and repeatable protein profiles for each subspecies, enabling the subspecies to be identified from one another, was demonstrated by Lundquist and colleagues (Lundquist et al., 2005) in 2005.

Various parameters influence bacterial spectra repeatability, even though mass spectrometry offers a significant promise for using spectral profiles to identify bacteria. The repeatability of protein profiles can be affected by sample preparation, matrix choice, and instrument quality and performance (Wunschel et al., 2005). Both the growth medium and the stage of the cells' growth must be considered since, just as crucially, the cell's physiological condition may also impact the outcomes of mass spectral analysis. Mazzeo and colleagues (Mazzeo et al., 2006) concluded that changing growth conditions did not impact *Escherichia coli*'s mass spectra. However, it did affect the bacterial protein profile using MALDI-TOF-MS technology to investigate and distinguish foodborne pathogens. Similarly, Walker et al. (2002) showed that culture media alters mass spectra profiles, particularly when added blood, as in Columbia blood agar. Many researchers have developed standardized methods for MALDI-TOF-MS of entire cells due to many variables. Liu et al. (2007) created a universal sample preparation technique to characterize Gram-positive and Gram-negative bacteria using MALDI-TOF-MS (Liu et al., 2007).

4.3 Gel-based bacterial characterization and identification

Bacteria can be classified based on the proteins found in their cells. Protein electrophoresis on PAGE has become more popular for bacterial classification and identification in recent years. SDS-PAGE is a method that may be used to separate whole-cell proteins based on their molecular weight. It can also identify more complex taxonomic connections, particularly at the species and subspecies levels (Vandamme et al., 1996). Researchers may identify specific germs by comparing bacteria's migratory patterns with reference gel patterns stored in a database. However, SDS-PAGE analysis is not very helpful for quick identification of bacteria, especially for field and point-of-care applications, since it is slow and labor-intensive and because the application requires exact growth conditions that generate quite significant volumes of sample material. With isoelectric focusing (IEF) and SDS-PAGE, two-dimensional gel electrophoresis (2-DE) enables high-resolution separation of up to several thousand spots in a single gel examination. O'Farrell (1975) invented the 2-DE technique to separate intricate combinations of biological proteins (O'Farrell, 1975). According to each protein's isoelectric point in the first dimension, proteins are separated by IEF electrophoresis in 2-DE, followed by a second-dimension SDS-PAGE separation based on the relative molecular weight of each protein. After the second-dimension separation, the gel can be stained with common or delicate staining agents to visualize and examine protein spots. Scanning further, examining, and saving protein gel patterns or 2-DE maps from recognized microorganisms in a reference database are possible. By running a 2-DE gel and creating a 2-DE map from the unknown sample, an unknown species can be identified by comparing it with 2-DE maps in the reference database.

The most frequent applications of 2-DE as a stand-alone method are analyzing protein mixtures, isolating proteins of interest for identification, and comparing differential expression patterns of various sample types. With mass spectrometry, 2DE is significantly improved for more complex proteomics analysis. For instance, using SDS-PAGE, Redmond, and associates (Redmond et al., 2004) recovered the proteins from the exosporium of *Bacillus anthracis* spores. Then, they used delayed extraction to evaluate the isolated proteins by MALDI-TOF-MS. They discovered many proteins connected to the *B. anthracis* exosporium. The whole proteomes or subproteomes (or both) for several other bacteria, such as *E. coli*, *Bacillus subtilis*, *S. aureus*, *Pseudomonas aeruginosa*, and *Helicobacter pylori*, have also been made public using similar techniques. Researchers can efficiently compare and identify unknown bacteria if they can access a collective proteomics database with complete 2-DE maps and mass spectra of recognized bacteria.

In 2014, Penzlin et al. developed Pipasic. This tool examines proteomes from microorganism communities that uses expression level weighting and protein similarity estimation to correct recognition and spectral counting-based quantification to calculate a given species' presence and abundance in a sample (Penzlin et al., 2014) (<https://mybiosoftware.com/tag/pipasic?amp=1>). While in 2017, Fischer et al. developed DiTASiC, a new method for assessing and quantifying taxa in metagenomics. It creates a novel statistical framework to integrate abundance variance and infers abundance distributions for differential testing, sensitive to strain level. It incorporates a linear generalization for shared read counts, gathers abundance estimation uncertainties, and infers abundance distributions. Abundance estimations were highly accurate, including levels as low as substrain levels.

Also, the Bacterial and Viral Bioinformatics Resource Center website (BV-BRC) is created by the National Institute of Allergy and Infectious Diseases and the National Center for Biotechnology Information (NCBI) (<https://www.bv-brc.org/>).

5. Nanotechnology in bacterial pathogen detection

A crucial step in controlling pathogenic infections, treating bacterial diseases, and environmental safety is the sensitive, selective, and quick detection of these pathogens. For this reason, nanotechnology is the framework for developing different biosensors (immune-based sensors, aptasensors, bacteriophage-based sensors, array-based sensors, and optoelectronic noses). These nanosensors allow for the rapid detection of pathogens despite the scarcity of organism load in the sample (Hajipour et al., 2021).

5.1 Immune-based nanosensors

In this type of nanosensor, nanoparticles (NPs) (usually silver or gold) are modified by adding bacterial pathogen-specific antibodies to their surface. Identification is made possible when the specific antibodies on the NPs bind to protein receptors on the bacterial surface, and this binding can be detected by changes in the surface plasmon resonance (SPR) properties and colorimetric response (Wilson, 2008). For instance, various bacteria can be detected by aggregating antibody-conjugated gold NPs following binding with a particular bacterium.

When linked to these pathogens, gold nanoparticles functionalized with antibodies against *Legionella pneumophila* and *Escherichia coli* changed their localized SPR (LSPR) color from red to purple (You et al., 2020).

5.2 Aptasensors

Aptamers, short sequences of nucleic acids or proteins, can change their shape through folding. They can recognize and bind to specific proteins or bacterial epitopes. The binding can be detected by sensitive nanomaterial known as aptasensors (using surface-enhanced-Raman-scattering (SERS)- and fluorescent-based sensors) (Zhou et al., 2020). Different types of aptamers depend on the aptasensor used and the bacterium in question. An example can be seen where fluorescent chitosan nanoparticles loaded with an aptamer specific for *E. coli*, successfully targeted and bound to the organism. The binding was then identified and stratified based on the intensity of fluorescent feedback (Zhao et al., 2020).

5.3 Bacteriophage-based sensors

Given bacteriophages' ability to produce proteins for cellular receptors on bacterial surfaces, they can differentiate between and identify dead bacterial cells and those viable to grow in (Flores et al., 2011). Different bacteriophages and their receptor-binding proteins (RBPs) can be produced using genetic engineering. For example, from genetically modified phage M13, RBPs for the genus Inovirus were synthesized to target pathogenic bacteria such as *Pseudomonas aeruginosa* and *Vibrio cholerae*. Furthermore, the phage was then functionalized with a Thiol group and labeled with a fluorescent dye to prepare it for binding to the bacterial surface. After binding, gold nanoparticles are added and can attach to the thiol groups, turning the sample purple and indicating bacterial recognition. This detection process of pathogenic bacteria usually takes less than an hour (Peng & Chen, 2018). Furthermore, Bai et al. (2019) modified TF2, TF6 proteins derived from bacteriophage tail ϕ AB2 and ϕ AB6, which were used to detect the pathogen *Acinetobacter baumannii*, the proteins were labeled with hexahistidine to make purification easier, then TF2- and TF6-immobilized alumina-coated magnetic nanoparticles ($\text{Fe}_3\text{O}_4@\text{Al}_2\text{O}_3$ MNPs) were synthesized. The nanoparticles were added to a bacterial solution where the bacteria were successfully attached to the nanomaterial, extracted from the solution using a magnetic field, and identified through mass spectrometry.

5.4 Array-based sensors

In this method, no biorecognition elements are added to identify the organism. The benefit is gained from the enzymes the pathogenic bacteria produce, such as urease, which is not naturally found in humans (Santopolo et al., 2019). In this method, rapid detection of urease-producing bacteria was possible. The urease enzyme works by converting urea into NH_3 , which alters the pH of the sample, allowing binding to the positively charged gold NPs, resulting in a visible color change. The attachment can even be more rapid when bovine serum albumin at acidic pH is used in the sample. Furthermore, this nanomediated urease-positive/negative bacteria detection method takes about 40 min.

5.5 Optoelectronic nose

This technique has a special approach to bacterial detection that can be compared with the sense of smell. In this method, colorimetric sensor arrays are backed up with data connecting changes in color to a corresponding chemical (chemically responsive colorants). An example can be seen in pH-sensitive dyes and vapochromics (Li & Suslick, 2020). Using these colorimetric sensors based on nanoparticle arrays, 10 bacterial strains were identified in human urine and drinking water by applying the sample on a nanoparticle-coated paper. Ultimately, when the bacteria bind, they release volatile metabolites, which are then analyzed and result in a distinctive colorimetric arrangement for each strain. The time to reach bacterial identity following this method was about 10 min (Bordbar et al., 2020).

5.6 The role of lab-on-a-chip (LOC) in bacterial identification and diagnosis

In recent years, microfluidic-based lab-on-a-chip (LOC) technology has emerged as a promising tool for rapidly and accurately identifying bacterial pathogens (Daniel et al., 2022). Microfluidics-based LOC platforms are miniaturized systems that integrate multiple functions on a single chip, such as sample preparation, amplification, and detection. The advantages of LOC systems over traditional methods include reduced sample volume, faster processing times, increased sensitivity, and portability (Jagannath et al., 2022).

Microfluidic devices can perform sample preparation functions such as filtration, concentration, and lysis (Kim et al., 2009). Regarding amplification, PCR is a commonly used amplification method that has been adapted to microfluidic platforms. PCR-based microfluidic devices can achieve rapid and specific amplification of bacterial DNA from clinical samples (Daniel et al., 2022).

Bacterial detection is highly sensitive and specific and uses various detection methods, including fluorescence, electrochemical, and mass spectrometry (Lei et al., 2012). For example, a microfluidic device used electrochemical detection to identify *Escherichia coli* O157:H7 with high sensitivity and specificity (Song et al., 2021). Also, sputum samples carrying *Mycobacteria* were found using a paper-based microfluidics technology. The technique allowed to save the sputum sample and clean out nonmycobacteria (Molloy et al., 2021).

Using microfluidic-based LOC platforms for bacterial identification offers several advantages over traditional methods. These include reduced sample volume, faster processing times, increased sensitivity, and portability. However, several challenges remain, including the need for specialized instrumentation, optimization of assay conditions, and standardization of protocols (Xie et al., 2022).

6. Machine learning in bacterial identification and diagnosis

Machine learning (ML)-based approaches involve training algorithms on large datasets of bacterial genetic and phenotypic characteristics to predict the identity or diagnosis of an unknown bacterium. Several ML-based approaches have been developed for bacterial identification and diagnosis, including supervised, unsupervised, and deep learning techniques (Qu et al., 2019).

Supervised learning involves training an algorithm on a labeled dataset of bacterial characteristics to predict the identity of an unknown bacterium. Several supervised ML techniques have been developed for bacterial identification and diagnosis, including random forests, support vector machines, and neural networks (Rawson et al., 2021). One recent study used a supervised ML approach to identify and diagnose bacterial infections from blood cultures (Pai et al., 2021). On the other hand, unsupervised learning involves training an algorithm on an unlabeled dataset of bacterial characteristics to identify patterns or clusters that can aid in bacterial identification and disease diagnosis (Goodswe et al., 2021). One example of an unsupervised ML technique is clustering, which involves grouping bacteria based on shared characteristics (Shi et al., 2022).

Deep learning, however, involves training algorithms on large datasets of complex data, such as images or genetic sequences, to make predictions (Alharbi & Rashid, 2022). Several deep learning techniques have been developed for bacterial identification and diagnosis, including convolutional neural networks (CNNs) and recurrent neural networks (RNNs) (Wang, Zhang, et al., 2022; Wang, Hsieh, et al., 2022; Wang et al., 2022; Wang, Hong, et al., 2022). Two authors developed a deep-learning approach to differentiate between viral and bacterial pneumonia solely from chest X-rays. The authors trained the algorithm on a dataset of 10,128 chest X-rays and achieved an accuracy of 99.85% in distinguishing two types of pneumonia (Li & Li, 2022).

7. Microelectromechanical systems (MEMS) in bacterial identification and diagnosis

Microelectromechanical systems (MEMSs) have emerged as promising tools for rapidly and sensitively detecting bacteria. MEMS devices are small, portable, and capable of rapid analysis, making them ideal for point-of-care diagnostics.

MEMS-based devices for bacterial identification rely on detecting specific biomolecules or changes in the physical properties of bacteria. One example is using microcantilevers functionalized with antibodies to detect bacterial antigens. When the antigen binds to the antibody, it causes a deflection in the cantilever, which can be detected using a readout system. This approach has been used to detect various bacteria, including *E. coli* and *L. monocytogenes* (Etayash et al., 2016).

Another approach is to use MEMS-based impedance sensors to detect changes in the electrical properties of bacterial cells. When a bacterial cell binds to the sensor's surface, it changes the system's impedance, which can be measured and used to identify the bacteria (Radke & Alocilja, 2004). This approach has been used to detect bacteria, including *Staphylococcus aureus* and *Salmonella* (Liu et al., 2018; Ward et al., 2018).

MEMS-based devices have also been used for the rapid diagnosis of bacterial infections. One example is microfluidic devices that isolate and culture bacteria from patient samples. These devices use microchannels and valves to control the flow of fluids, allowing for the selective capture and growth of bacteria. This approach has been used to rapidly diagnose urinary tract infections (UTIs) caused by *E. coli* (Oluwafemi et al., 2017).

The latest advances in MEMS technology have enabled the development of devices that can detect specific biomolecules, changes in physical properties, and bacterial DNA. These devices can improve patient outcomes by enabling early and accurate diagnosis of bacterial infections.

8. The role of microfluidics in bacterial identification and diagnosis

Microfluidics is a multidisciplinary field that involves the manipulation of small volumes of fluids in channels with dimensions ranging from micrometers to nanometers. Microfluidic devices have emerged as a promising alternative for bacterial identification and diagnosis due to their ability to perform rapid, automated, and multiplexed assays with small sample volumes (Pattanayak et al., 2021).

One approach for bacterial identification using microfluidics involves the detection of bacterial biomolecules, such as DNA, RNA, and proteins. Microfluidic devices can be designed to perform nucleic acid amplification and detection assays, such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), in a compact and portable format (Wang, Zhang, et al., 2022; Wang, Hsieh, et al., 2022; Wang et al., 2022; Wang, Hong, et al., 2022). For example, a recent study demonstrated using a microfluidic device to detect *Salmonella* in salmon rapidly and chicken samples using LAMP and CRISPR/Cas12a detection (Luo et al., 2022).

Another approach for bacterial identification using microfluidics is based on bacteria's physical and chemical properties. Microfluidic devices can be designed to sort and analyze bacterial cells based on their size, shape, surface charge, or other characteristics (Pérez-Rodríguez, García-Aznar & Gonzalo-Asensio, 2022). For instance, a study from 2017 summarized the use of a microfluidic device to sort and identify bacterial cells based on their surface charge using a combination of dielectrophoresis and fluorescence detection (Fernandez et al., 2017).

In addition to bacterial identification, microfluidics has been used for bacterial diagnosis by monitoring the response of bacterial cells to specific stimuli. For example, microfluidic devices have been used to measure the growth and metabolism of bacteria in real-time by monitoring changes in their optical or electrical properties. A recent study reported using a droplet microfluidic device to measure the antibiotic susceptibility of bacterial cells and populations rapidly and cost-effectively (Postek & Garstecki, 2022).

In conclusion, microfluidics has emerged as a promising bacterial identification and diagnosis tool, offering rapid, sensitive, and specific assays with small sample volumes. Recent advances in microfluidic technology have enabled the development of portable and easy-to-use devices for bacterial identification and diagnosis, with potential applications in clinical, environmental, and food safety settings. As the field of microfluidics continues to evolve, we can expect to see further progress in developing microfluidic devices for bacterial identification and diagnosis.

9. Raman spectroscopy in bacterial identification and diagnosis

Raman spectroscopy is a nondestructive, label-free, and fast technique that has shown promise in identifying and characterizing bacterial strains. It is based on the interaction between a sample and a monochromatic laser beam, resulting in the scattering of photons at different wavelengths. The scattered photons provide information on the vibrational modes of the sample molecules, which can be used to identify and characterize the sample (Wang et al., 2021).

Raman spectroscopy can provide information on bacterial cells' chemical composition, including specific biomolecules such as proteins, lipids, and nucleic acids. It has been used to differentiate between bacterial strains, including pathogenic and nonpathogenic strains (Lorenz et al., 2020; Wang, Zhang, et al., 2022; Wang, Hsieh, et al., 2022; Wang et al., 2022; Wang, Hong, et al., 2022). The technique has also been used to identify antibiotic-resistant strains, which are critical in developing effective treatments (Al-Shaebi et al., 2022; Nakar et al., 2022).

One of the advantages of Raman spectroscopy in bacterial identification is its speed. The technique can provide results in minutes, compared with traditional culture-based methods that take several days. Raman spectroscopy has also been highly accurate, with studies reporting sensitivity and specificity values of over 90% (Zhang et al., 2022).

Recent developments in Raman spectroscopy have improved its applicability in bacterial identification and diagnosis. One of these developments is the use of microfluidic devices, which allow for the analysis of small sample volumes and can be used for high-throughput analysis. Microfluidic devices have been used to analyze bacterial cells from clinical samples, including blood and urine, with high accuracy (Prada et al., 2022).

Another development is the use of machine learning algorithms for data analysis. Raman spectroscopy generates large amounts of data, which can be challenging to analyze manually. Machine learning algorithms can be used to analyze Raman spectra and identify patterns that are characteristic of specific bacterial strains. This approach has been shown to improve the accuracy of bacterial identification using Raman spectroscopy (Qi et al., 2023).

10. Conclusions

The identification and characterization of bacteria will continue to be significantly aided by cutting-edge genomics, proteomics, and nanotechnologies in the 21st century. The rapid identification of harmful bacteria substantially affects the precise diagnosis of illnesses and prompt treatment of diseases. The identification of crucial protein targets will be made possible by bacterial characterization, which will also help clarify the mechanisms governing microbial pathogenesis and pave the way for developing vaccines, diagnostic tools, and treatments for infectious disorders.

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Fungal identification and diagnosis

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1. Fungi: An overview

Fungi (true fungi, kingdom fungi) are heterotrophic, single-celled or filamentous, eukaryotic organisms with their cell wall mostly consisting of chitin and glucans. They comprise the third most speciose eukaryotic kingdom with an estimated 2.2–3.8 to 6+ million species worldwide (Baldrian et al., 2022; Hawksworth & Lücking, 2017). Fungi exhibit diverse nutritional modes and multiple functions in nature. Numerous fungi are pathogens of humans, plants, and animals, and many are saprotrophic and decomposers, recycling nutrients in the soil, wood, and aquatic habitats through their powerful enzymatic machinery. They also form highly specialized cross-kingdom associations and symbionts with plants, algae, insects, and bacteria, as well as with yeasts and other fungi. Numerous fungi also thrive asymptotically within the bodies of other organisms.

Fungi are predominantly aerobic, except for the phylum *Neocallimastigomycota*, which comprises gut-residing anaerobic fungi. Owing to their wide range of functions and tremendous metabolite diversity, fungi thrive in nearly every imaginable habitat and may occupy extreme niches, some still remaining unexplored. As a result, they have a significant impact on the environment and human life.

Approximately 150,000 fungal species have been formally described, systematically classified into 19 phyla and 79 classes (Table 7.1). Slime molds (myxomycetes and allies) and oomycetes (*Oomycota*), traditionally studied by mycologists, are fungus-like organisms

TABLE 7.1 Phyla and classes of fungi.

Phylum	Class
<i>Aphelidiomycota</i>	<i>Aphelidiomycetes</i>
<i>Ascomycota</i>	<i>Archaeorhizomycetes</i> , <i>Arthoniomycetes</i> , <i>Candelariomycetes</i> , <i>Coniocybomycetes</i> , <i>Dothideomycetes</i> , <i>Eurotiomycetes</i> , <i>Geoglossomycetes</i> , <i>Laboulbeniomycetes</i> , <i>Lecanoromycetes</i> , <i>Leotiomycetes</i> , <i>Lichinomycetes</i> , <i>Neoelectomycetes</i> , <i>Orbiliomycetes</i> , <i>Pezizomycetes</i> , <i>Pneumocystomycetes</i> , <i>Saccharomycetes</i> , <i>Schizosaccharomycetes</i> , <i>Sordariomycetes</i> , <i>Taphrinomycetes</i> , <i>Xylobotryomycetes</i> , <i>Xylonomycetes</i>
<i>Basidiobolomycota</i>	<i>Basidiobolomycetes</i>
<i>Basidiomycota</i>	<i>Agaricomycetes</i> , <i>Bartheletiomycetes</i> , <i>Agaricostilbomycetes</i> , <i>Atractiellomycetes</i> , <i>Classiculomycetes</i> , <i>Cryptomycocolacomycetes</i> , <i>Cystobasidiomycetes</i> , <i>Dacrymycetes</i> , <i>Exobasidiomycetes</i> , <i>Malasseziomycetes</i> , <i>Microbotryomycetes</i> , <i>Mixiomycetes</i> , <i>Moniliellomycetes</i> , <i>Pucciniomycetes</i> , <i>Spiculogloeomycetes</i> , <i>Tremellomycetes</i> , <i>Tritirachiomycetes</i> , <i>Ustilaginomycetes</i> , <i>Wallemiomycetes</i>
<i>Blastocladiomycota</i>	<i>Blastocladiomycetes</i> , <i>Physodermatomycetes</i>
<i>Calcarisporiellomycota</i>	<i>Calcarisporiellomycetes</i>
<i>Caulochytriomycota</i>	<i>Caulochytriomycetes</i>
<i>Chytridiomycota</i>	<i>Chytridiomycetes</i> , <i>Cladochytriomycetes</i> , <i>Lobulomycetes</i> , <i>Mesochytriomycetes</i> , <i>Polychytriomycetes</i> , <i>Rhizophyidiomycetes</i> , <i>Rhizophlyctidomycetes</i> , <i>Spizellomycetes</i> , <i>Synchytriomycetes</i>
<i>Entomophthoromycota</i>	<i>Entomophthoromycetes</i> , <i>Neozygitomycetes</i>
<i>Entorrhizomycota</i>	<i>Entorrhizomycetes</i>
<i>Glomeromycota</i>	<i>Archaeosporomycetes</i> , <i>Glomeromycetes</i> , <i>Paraglomeromycetes</i>
<i>Kickxellomycota</i>	<i>Asellariomycetes</i> , <i>Barbatosporomycetes</i> , <i>Dimargaritomycetes</i> , <i>Harpellomycetes</i> , <i>Kickxellomycetes</i> , <i>Ramandelabermomycetes</i>
<i>Monoblepharomycota</i>	<i>Hyaloraphidiomycetes</i> , <i>Monoblepharidomycetes</i> , <i>Sanchytriomycetes</i>
<i>Mortierellomycota</i>	<i>Mortierellomycetes</i>
<i>Mucoromycota</i>	<i>Endogonomycetes</i> , <i>Mucoromycetes</i> , <i>Umbelopsidomycetes</i>
<i>Neocallimastigomycota</i>	<i>Neocallimastigomycetes</i>
<i>Olpidiomycota</i>	<i>Olpidiomycetes</i>
<i>Rozellomycota</i>	<i>Rudimicrosporea</i> , <i>Microsporidia</i>
<i>Zoopagomycota</i>	<i>Zoopagomycetes</i>

From Wijayawardene, N.N., Hyde, K.D., Al-Ani, L.K., Tedersoo, L., Haelewaters, D., Rajeshkumar et al. (2020). Outline of fungi and fungus-like taxa. *Mycosphere*, 11, 1060–1456. <https://doi.org/10.5943/mycosphere/13/1/2>.

with their natural places in *Amoebozoa* and *Straminipila*, respectively. These are not treated any further in this chapter.

Fungi have a relatively compact genome, sizing very differently, from 9 Mb up to 890 Mb (Table 7.2). Most fungi have a haploid genome, with their nuclei being haploid during the entire or the majority of the life cycle. This feature has rendered fungi amenable candidates for genetic research. Fungi share a considerable number of their genes with humans and other

TABLE 7.2 Comparison of genome size in some fungi and other organisms.

Species	Type of organism	Genome size (Mb)
<i>Agaricus bisporus</i>	Agaric basidiomycete	30.2
<i>Amanita muscaria</i>	Agaric basidiomycete	40.70
<i>Aspergillus niger</i>	Filamentous ascomycete	34.85
<i>Candida caseinolytica</i>	Yeast ascomycete	9.18
<i>Cryptococcus vishniacii</i>	Yeast basidiomycete	19.69
<i>Dendrothele bispora</i>	Corticoid basidiomycete	130.65
<i>Ganoderma</i> sp.	Poroid basidiomycete	39.52
<i>Gymnosporangium confusum</i>	Rust	893.2
<i>Mucor circinelloides</i>	Mucoromycete	36.6
<i>Neurospora discreta</i>	Filamentous ascomycete	37.3
<i>Saccharomyces cerevisiae</i>	Yeast ascomycete	12.07
<i>Homo sapiens</i>	Human	3000
<i>Drosophila</i>	Insect	165
<i>Escherichia coli</i>	Bacteria	4
<i>Phytophthora sojae</i>	Oomycete	82.60

Adapted from Mohanta, T.K., Bae, H. (2015). The diversity of fungal genome. *Biological Procedures Online* 17(1): 1–9. <https://doi.org/10.1186/s12575-015-0020-z>; Tavares, S., Ramos, A.P., Pires, A.S., et al. (2014). Genome size analyses of reveal the largest fungal genomes. *Frontiers in Plant Science* 5: 422. <https://doi.org/10.3389/fpls.2014.00422>.

eukaryotes, making them a suitable platform for biomedical research and recombinant vaccine development. Whole-genome sequences from more than 1600 fungal species have been obtained (Li et al., 2021). In comparison, the fungal mitochondrial genome (mitogenome) ranges from 19 to 200 Kb. Up to now, a full mitogenome sequence has been made available for about 560 species. Recently, some species-specific features have been reported for the fungal mitogenome, suggesting that it is a marker for identification (Kulik et al., 2020).

Fungi exhibit complex biology and have been the subject of research in various disciplines, e.g., in biology and life sciences, agriculture, plant pathology, medical and clinical mycology, veterinarian mycology, forensic, and biotechnology. Various fungal species have served as model organisms in numerous fields, including but not limited to developmental biology, genetics, medical pathology, bioremediation, medical toxicology, and medicine (Table 7.3).

Compared to the enormous fungal diversity, about 700 species are harmful to human health (Mendonça et al., 2022). Only a limited number of species are responsible for fungal infections in humans, yet they may cause serious, even life-threatening diseases and need a prompt diagnosis. Candidiasis, mucormycosis, aspergillosis, cryptococcosis, and some other mycoses threaten especially immunocompromised individuals. Moreover, approximately 20% of the described fungal species are phytopathogens, thereby responsible for 70%–80% of plant diseases worldwide, causing enormous economic loss (Kulik et al., 2020).

TABLE 7.3 Some model organisms from fungi.

Species	Fungal group	Field of research
<i>Agaricus bisporus</i>	Agaric basidiomycete	Genetics, reproduction, bioconversion (e.g., carbon sequestration studies)
<i>Amanita muscaria</i>	Agaric basidiomycete	Medical toxicology
<i>Aspergillus fumigatus</i>	Filamentous ascomycete	Medical pathology
<i>Candida albicans</i>	Yeast ascomycete	Medical pathology, medicine
<i>Coprinopsis cinerea</i>	Agaric basidiomycete	Developmental biology, genetics
<i>Cryptococcus neoformans</i>	Yeast basidiomycete	Medical pathology
<i>Neurospora crassa</i>	Filamentous ascomycete	Classical genetics, epigenetics
<i>Penicillium notatum</i>	Filamentous ascomycete	Medicine, antibiotic
<i>Phanerochaete chrysosporium</i>	Corticoid basidiomycete	Bioremediation
<i>Saccharomyces servisiae</i>	Yeast ascomycete	Developmental biology, molecular genetics
<i>Trichoderma</i>	Filamentous ascomycete	Integrative fungal taxonomy, fungal biology

1.1 Identification challenges

Species is the most fundamental unit in biology, biodiversity measurements, and bioprospecting. Identification of fungi at the species level is a crucial step toward their appropriate recognition and utilization. Identifying and properly naming fungi can be challenging due to their largely convergent morphology and complicated lifestyles. While fungi exhibit vast morphological diversity, their tissue compartmentalization and structures are not as highly specialized and sophisticated as in multicellular plants and animals. Moreover, the isolation of fungi is complicated by the fact that the majority of fungi tend to be inconspicuous and substrate-dwelling and reside in hidden habitats. Many fungi barely produce visible morphological structures and are difficult—indeed, often impossible—to cultivate in the laboratory.

The range of morphological features in fungi varies, as does the body size of the species, from single-celled, microscopic species to taxa with complex fruiting bodies, some reaching a meter or more. While fungi have many useful morphological traits, fungal evolutionary studies show that several features have evolved in parallel in distantly related species. Morphological features may also be polymorphic in the same species, rendering sole reliance on morphology undesirable.

Fungi may reproduce sexually, asexually, or both. In a large number of fungi, previously called “hyphomycetes,” sexual reproduction is not yet evident. These fungi seem to maintain genetic variation and speciation via complex mechanisms such as parasexuality. Correct relationship assignments and accurate identification of asexual species strongly rely on molecular sequence data.

2. Phenotypic identification of fungi in a modern view

Phenotypes are an essential source of evidence for biodiversity assessment, taxonomic decisions, and understanding ecological and evolutionary processes. Phenotypic information is quickly accessible and can be processed at relatively low costs. Therefore, it is widely used by both researchers and citizen scientists. On the one hand, phenotypic information provides a starting point for molecular analyses and serves as reference data to validate molecular results.

Identification is one of the crucial steps in processing a fungal specimen, along with retrieving a spore print, fixing the material for long-term storage, and bringing it into culture (Cléménçon, 2009; Wu et al., 2004). For successful identification and preservation of the specimen, it is also advisable to follow the guidelines for collecting fungi (Lodge et al., 2004). Obtaining fungal culture is recommended, to enable long-term access to high-quality DNA and metabolites. Guidelines for the isolation and maintenance of cultures are provided by (Cléménçon, 2009) for macrofungi, and by Crous et al. (2019) with a focus on microfungi.

One should also remember that scientific (Latin) names of fungi, of Latin or Greek origin, often reflect the peculiar phenotypic character of a taxon. Understanding these names can facilitate taxon identification; conversely, paying attention to a particular taxon's characters can help to remember its name and meaning.

2.1 Use of different human senses for macroscopic identification of fungi

For macrofungi, i.e., those with fruiting bodies roughly larger than 1 cm, four of five human senses can be used for taxon identification. These senses are sight, touch, smell, and taste. For microfungi, i.e., those with fruiting bodies under 1 cm and those not forming fruiting bodies but instead other sporulation forms or just mycelia, at least sight allows distinguishing some genera on the macroscopic scale (Crous et al., 2019). The principal types of potentially discriminative phenotypic characters, as perceived by four human senses, are described below.

2.1.1 Color

The following aspects of color are relevant for identification: the color of the intact, i.e., “healthy” fruiting body, the color of any injured part (color of the context and exuded liquid), and the color upon drying. The fruiting body or its parts can change substantially in color with age. Therefore, color at different developmental stages should be considered (Cazabonne, 2021). Color change upon the application of chemical reagents (macrochemical reaction) is important for the identification of stromatic *Ascomycota* (Stadler et al., 2014), selected mushroom-forming groups of the *Basidiomycota* (e.g., the genera *Russula* and *Ramaria*), and also some polypores (Lodge et al., 2004; Ryvarden & Melo, 2014). The phenomenon of bioluminescence provides some diagnostic value as well; it is known in 109 macrofungi, all belonging to the white-spored saprotrophic *Basidiomycota*, except one in *Ascomycota* (Ke & Tsai, 2022).

For mushroom-forming fungi, one has to be careful not to confuse the lamella's actual color with the spores' color. Therefore, the color of the spores en masse is best evaluated from spore prints made upon collection (Cléménçon, 2009). However, spore deposition other than

hymenophore parts of the fruiting body, e.g., cap surface, the upper part of the stipe, or the substrate around the fruiting body, can hint at the spore color (Cazabonne, 2021; Ryvarden & Melo, 2014).

Light conditions and hygrophanicity (color change in mushroom tissue as it loses or absorbs water) can affect color perception. Therefore, one should try to estimate the color under daylight or artificial light with similar characteristics. The latter can be even more consistent. It is, therefore, advisable to record all identification criteria linked to colors immediately in the field (Lodge et al., 2004).

The classical way to describe the color of the fungus is via adjectives. However, standardized color charts help ensure the comparability of taxonomic treatments. The most commonly used ones are listed by Cazabonne (2021). Particular mycological societies or journals may suggest specific color charts (Laessøe & Petersen, 2019, p. 1708). In addition to human judgment, an automated color acquisition from a digital image using specific software is possible (Krah et al., 2019). Substantial image/data curation is expected in this case.

2.1.2 Texture

Although the role of touch for identification may seem overly simple, it does allow a multi-component estimation of the properties of the fruiting body's texture. These properties include at least "roughness, smoothness, stickiness, slipperiness, oiliness, coarseness, and graininess." There is also the aspect of hardness, although it reflects "pressure resistance [rather] than texture perception" (Cazabonne, 2022). The results of touch perception may point to some particular plectology of the fruiting body, e.g., the presence of sphaerocysts in *Russula* or the presence of skeletal and/or binding hyphae in polypores. A glossary of texture terminology is still missing. In addition, climate conditions and the dryness/wetness of a fungal specimen should be factored in when estimating texture parameters.

2.1.3 Smell

Tens of smells of fungi can be recognized by humans. Besides its general diagnostic value for mycologists, the smell may help mushroom hunters identify poisonous or inedible mushrooms. Fungal smells are usually described by their resemblance to other natural or human-made substances. There seems to be more subjectivity in identifying and classifying smells than in the colors and textures of fungi. The reason is the striking variability of the smell sense between humans (Boniface, 2020). Furthermore, weather and fruiting body conditions may strongly affect the smell and its perception by humans. Perhaps this is the reason for the rarity of standard literature cataloging fungal smells. A catalog by Spoerke (1994), may serve as a standard. Lists of odors recognized by fungal citizen scientists in the USA (Luther, 2009, pp. 4–5; Winkler, 2010) and European German-speaking mycological communities (Bachmeier, 2006) are also available on the web. However, these sources are not easily findable and citable, and their long-term availability is an open question.

2.2 Standardized processing of macroscopic traits

We recommend scoring as many characters as possible from fresh fungal material and using standardized annotation sheets. These notes help with subsequent taxon identification (Lodge et al., 2004). During the identification process, along with traditional dichotomous

keys, innovative formats may enhance identification success, especially when it comes to fungal groups that are less familiar to the collector. Polytomous keys designed as graphical identification wheels allow efficient, flexible navigation between fungal groups down to the genus level (Laessøe & Petersen, 2019, p. 1708).

2.3 Microscopy: general advice

This part will focus on light microscopy, which is far more commonly applied than electron microscopy to investigate fungi. The latter can be helpful, e.g., for studying spore ornamentation, but is more challenging to apply (Suwannasai et al., 2012). Recent improvements in rehydration techniques may facilitate the use of scanning electron microscopy for diagnostic purposes (Koch et al., 2021).

For light microscopy, it makes sense to distinguish between “immediate” microscopy and microscopy based on dried fungal material; the latter is far more common. Valuable guidelines on fixation and sectioning material with a microtome and preparing permanent slides are found in (Clémentçon, 2009).

Immediate microscopy works most efficiently on small and reasonably thin slices when sectioning fruiting bodies under a dissecting microscope. The double razor blade method is especially useful for thin sections. Most fungal textures can be dissected well in fresh conditions, and only some (e.g., mucilaginous) may benefit from dry material. The proper microscopic examination of dry fungal collections requires remoistening in water or specific liquids (see below). Squashing material may be justified for a morphometric investigation such as spore measurements but should be avoided when studying plectological arrangements (Clémentçon, 2009).

2.4 Light microscopy: mastering mounting media

The choice of mounting media can dramatically affect the assessment of qualitative and quantitative traits in fungi. Therefore, good knowledge of the physical and chemical properties of the reagents is necessary for their correct application, as a sole medium, in combination, or one after another. The following groups of mounting media may be recognized: alkaline solutions without dyes, acid solutions without dyes, staining solutions for squash mounts, and iodine solutions (Clémentçon, 2009). The fundamental properties and considerations for applying the most common reagents of each group are provided below.

2.4.1 Alkaline solutions

Alkaline solutions rehydrate dried materials effectively. Ammonia (1%–25% water solution) does it more gently, while 1%–10% potassium hydroxide (KOH) or sodium hydroxide (NaOH) do it more intensively. The latter may lead to swelling and even dissolvment (*Polyporales*) and color changes of some hyphal walls, e.g., in *Thelephorales* (Köljalg, 1996, p. 213; Ryvarden & Melo, 2014). Glycerol-NaOH-methyl cellosolve is an improvement in alkaline solutions recommended for effective softening and penetration and reduced alkali content (Clémentçon, 2009). Glycerol (20%) in glycerol-NaOH also improves the viscosity and clarifies the medium properties.

2.4.2 Acid solutions

Acid solutions allow for clearer observation of hyphal textures. Lactoglycerol is preferred over the more toxic and less convenient lactophenol. Concentrated sulfuric acid stains the heteroplerous hyphae of some *Russulales* (Cléménçon, 2009).

2.4.3 Staining solutions for squash mounts

Staining solutions for squash mounts target various components of fungal cells. Solutions with Congo red as the main component primarily stain cell walls, septa, and dolipores with a proper red color. Congo red water solution is more stable and efficient than ammonia solutions. However, using the solution in glycerol-ethanol-boric acid or the reagent SDS-Congo red suppresses cell content staining. It allows observation of cell wall elements. Cotton blue is another reagent targeting cell walls. Although traditionally dissolved in lactophenol, a solution of lactic acid is recommended for work safety reasons. For quicker penetration of the stain into the fungal material, heating the slide to the boiling point is useful (Cléménçon, 2009; Niemelä, 2016, p. 430). After immersing Cotton blue in lactic acid, transfer to lactoglycerol is necessary to obtain a transparent background for the material treated with cotton blue. Brilliant cresyl blue and Toluidine blue work like cotton blue.

Staining not of the cell wall but of cell content is possible in several ways. Phloxine and Eosin Y stain cell content indifferently. Sudan III and Sudan IV stain lipids and other hydrophobic substances. Solutions of aldehydes (piperonal, vanillin, and benzaldehyde) in sulfuric acid indicate proteins. Carmine and acetocarmine stain nuclei, siderophilous granules, and the deuterooplasm (Cléménçon, 2009).

2.4.4 Iodine solutions

Iodine solutions color the starch-like polysaccharides of the cell wall and, in some cases, the cell content. Iodine reaction is crucial for identification in many fungal groups and is frequently brought up early in identification keys (Baral, 2009; Larsson & Ryvarden, 2021, p. 266). Reaction with iodine may serve as a shared taxonomic character at order-, family-, and genus-level in *Basidiomycota* (Hibbett et al., 2014; Vizzini et al., 2020). Staining in hues of blue (called amyloidity) or hues of brown (dextrinoidy) is regarded as a positive reaction. Additionally, hemi-amyloidity as a third type of reaction is recognized, characterized by a change from red to blue after treatment with KOH for ascus apical rings in some *Ascomycota* (Baral, 2009; Huhtinen, 1989). Dextrinoidy of the cell wall (due to glycine betaine) should not be confused with the similar reaction of the cytoplasm, for which glycogen is responsible (Blackwell et al., 2001; Cléménçon, 2009).

Melzer's, Lugol's, and Baral's reagents may be used to check the iodine reaction. Two schools—namely those that specialize in studying *Basidiomycota* or *Ascomycota*—favor either Melzer's or the two other reagents. Applying Melzer's to *Basidiomycota* fungi provides clearer preparations in which the iodine reaction is more straightforward and more consistently observed (Cléménçon, 2009; Leonard, 2006). Melzer's behavior applied to *Ascomycota* is quite the opposite. It does not improve the quality of the observed picture, does not perform consistently, and can cause shrinkage of essential diagnostic elements such as asci and ascospores. Therefore, Lugol's and Baral's methods are strongly recommended for *Ascomycota* (Baral, 2009; Cléménçon, 2009).

Challenged by the variability of the amyloid reaction in *Basidiomycota*, a standardized protocol was proposed that sets the conditions for rehydrating the material and time of exposure to the heated Melzer's reagent, followed by observation of the new drop of Melzer's under fixed optical and lighting settings of the microscope (Alvarado et al., 2018).

An additional consideration for applying iodine reagents is a thorough wash-out (with water) of alkaline solutions used as a pretreatment (Vizzini et al., 2020). Furthermore, it may be helpful to check the iodine reaction under a dissecting microscope when placing the material in a small drop of iodine-containing reagent (Cléménçon, 2009; Leonard, 2006).

2.5 Light microscopy: measuring sizes

Features of the disseminated propagules—the spores—are of the highest priority among phenotypic characters in fungal identification. Their detectability and usual presence en masse, together with limited size and shape variation (within a given sample), make spores a convenient tool for taxon identification.

The small size of fungal spores, commonly under 50 μm and often under 10 μm (Golan & Pringle, 2017), poses a challenge for capturing them correctly. Measurements should be taken from microscope slides under $\times 1000$ -magnification. The classical way is to use an eyepiece scale bar with a 1- μm -grid, where linear size, typically length and width, is estimated subjectively with an accuracy of 0.1 μm . The more technically sound method (and more comfortable for mycologists) is measurement by a microscope camera. Pointing to the digital image's pixels infers the linear size (and potentially more complex measurements) of the object (perimeter and area size as converted to micrometers). In addition to commercial programs supplied by camera producers, solutions are developed within the mycological community (Fannechère, 2020). These programs usually assume that the researcher first measures the length and then the width of a particular spore. It is often possible to apply more general biological measuring tools such as ImageJ (Schindelin et al., 2012). However, one needs a post-hoc programming effort to differentiate the measurements into “length” or “width” categories and ensure that they belong to the same spore. A solution in the R language is available from Ordynets, Keßler, and Willemsens (2021).

Although spores of a target fungus are ideally measured randomly, there is a chance of including some abnormal or alien spores that can undesirably affect the summary of the measurements. It is more efficient to simultaneously account for outliers in spore length and width. This can be achieved if the parameters (i) length multiplied by width and (ii) volume are additionally calculated (Wilk, 2012). Then values (i) followed by (ii) can be checked to represent the outliers in the spore sample at a given probability level, e.g., 95%, using one of the available outlier tests (David et al., 1954; Grubbs, 1950; Verma & Quiroz-Ruiz, 2006). Upon detection, outliers can be excluded from the sample. This procedure usually results in a better fit of the spore measurements to the normal distribution (Ordynets et al., 2018; Wilk, 2012).

Spore size variation within a particular specimen is usually presented separately for length and width, as the interval into which 90% of nonoutlier measurements fall, while 5% of the smallest and 5% of the largest nonoutlier measurements are included in parentheses. An R script for generating an automated summary of this format from the data is available from

Ordynets and Denecke (2018). For presenting variation in spore size at the species level, a highly recommended way is to calculate tolerance intervals, i.e., hypothetical intervals within which 90% of all existing individuals' specimen mean values lie at the given probability level (e.g., 90%). This calculation is possible for sets of at least three specimens and provides a narrower estimate of the interval upon adding additional specimens (Parmasto et al., 1987; Raitviir, 1972). An R solution for calculating the tolerance interval with the method of (Howe, 1969) is available from Ordynets (2018). To be able to assess spore size data at individual, populational, species, and other taxonomic levels in the long term, it is recommended to publish raw measurements along with the results of the study (Ordynets et al., 2018). This data type does not take up much space and preparation effort but can facilitate comparative morphometric studies in mycology.

2.6 Geometric morphometrics to aid fungal identification

Size, expressed as linear measurements, is routinely used as a numerical character for fungal identification. It is not just the case with spore shape, which is, in most cases, treated as a qualitative trait. Dozens of terms are used based on common geometric shape categories (e.g., globose or cylindrical) or similarity to some natural or cultural objects (e.g., ovoid or filiform). The picture becomes even more complicated when subcategories are arbitrarily introduced, e.g., via adding the prefix “sub-” or the qualifiers “slightly” or “almost”. Qualitative approaches do not allow a comprehensive assessment of trait variation at different scales (individual, population, and species). Using a template based on the length-to-width ratio is an improvement over a textual shape description (Kušan et al., 2014; Niemelä, 2016, p. 430). Even so, rich information about the spore outline is still not fully exploited.

In cases where traditional morphometric analysis fails to distinguish some genetically distinct fungal species, quantitative analysis of spore shapes may help to improve automated species identification (Ordynets, Keßler, & Langer, 2021; Fig. 7.1). Apart from specific approaches for diatom algae, there are only sparse instructions for processing geometric information from microscopic objects (Infantino et al., 2016) (but see Fodor et al., 2015). For this reason, Ordynets, Keßler, and Willemsens (2021) created a protocol that can be applied to fungal spores as well as other microscopic objects. This protocol uses software developed for the shape analysis of macroscopic objects (Iwata & Ukai, 2002) and additionally involves general-purpose software for statistical analysis and visualization. For the future, it is recommended to work in a single environment with all analysis steps written as code. Running analyses from code will simplify data management and enhance research reproducibility. R functions written by Claude (2008) and further developed by Bonhomme et al. (2014) and Stela and Monleón Getino (2016) are promising in this regard.

While Ordynets, Keßler, and Langer (2021) focused on smooth microscopic objects, fungal spores in general may be supplemented by additional projections like warts or ridges, or distinct spines. It remains unexplored how correctly ornamentations could be captured by the elliptic Fourier method, which tends to downweight such elements (Fodor & Hâruța, 2020). The ornamented spores of fungi may become an interesting object for accommodating

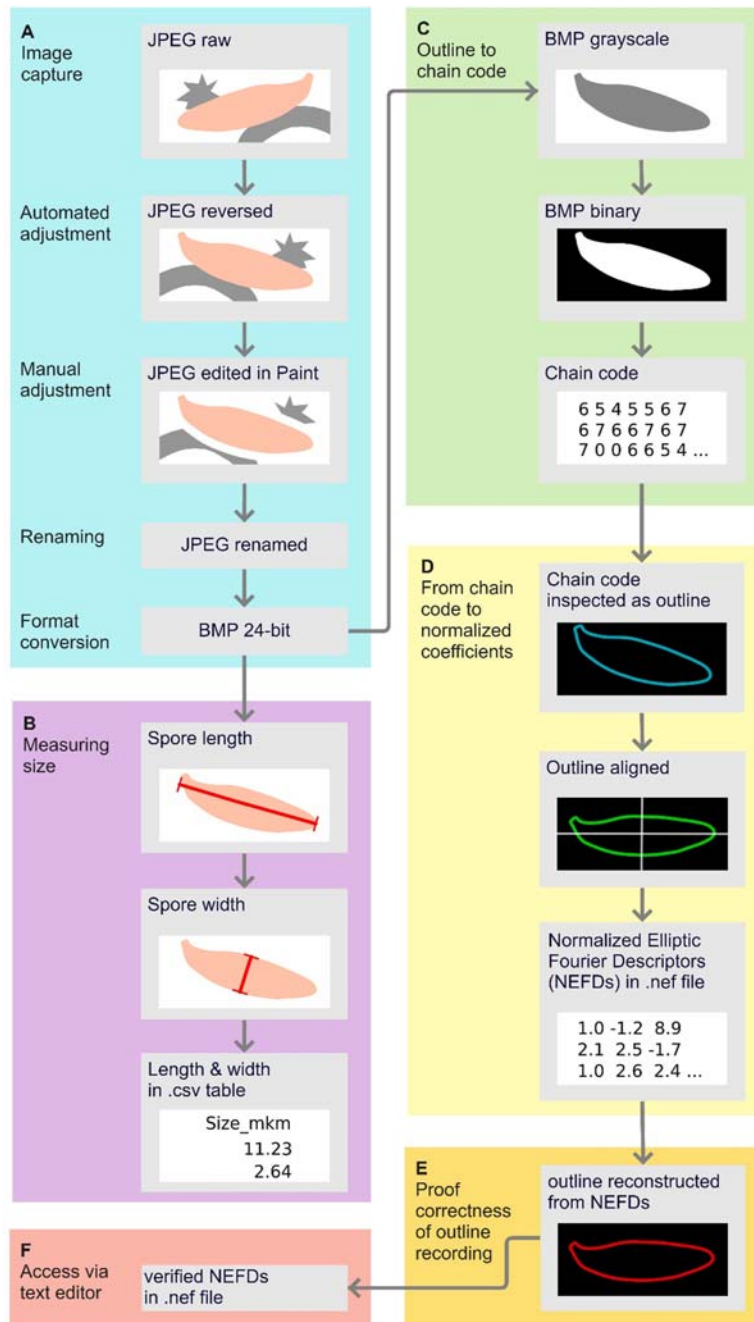


FIGURE 7.1 Workflow for partly automated extracting the size and shape information from a fungal spore. (A) Image capture and preprocessing; (B) acquiring linear measurements; (C) acquiring chain codes; (D) transforming chain codes to normalized elliptic Fourier descriptors; (E) checking quality; and orientation of the outlines; (F) option to open and manage outline descriptors in a text editor. Colored fields show the main phases of analysis. Different programs and levels of automatization are possible for each phase. Examples of programs used at each step can be found in [Ordynets, Keßler, and Willemsens \(2021\)](#). Republished with modifications from the latter under a CC BY license, original copyright 2021.

landmark-based and semilandmark-based methods of geometric morphometrics for microscopic objects (Bocxlaer & Schultheiß, 2010). Furthermore, these methods are extendable to three-dimensional analysis. The latter is becoming feasible even for objects smaller than 10 µm due to nano-computed tomography (Liu et al., 2018).

2.7 Statistical considerations for spore morphometrics

Several factors can strongly affect the results of estimating the size and shape of microscopic structures in fungi and should be addressed by a researcher to provide reliable results.

Meioisporae (basidiospores) of *Basidiomycota* show asymmetry along their long axis, which leads to differentiating two spore projections (also called faces or views): frontal and lateral. This latter method is usually more diagnostic for determining shape. The size (especially width) and shape of the spores can be meaningfully compared only for spores on the same projection (Duhem, 2010; Köljalg, 1996, p. 213).

The maturity of the measured spores is best assessed when working with spores deposited on a spore print (Duhem, 2010; Matočec, 1998). Measuring at least 30 spores per specimen gives an adequate estimate of spore variation at the specimen level and an adequate mean estimate (Parmasto et al., 1987). If the measurement distribution still deviates from normal, nonparametric statistics should be the choice for comparative analyses (Kušan et al., 2014).

2.7.1 Material fixation and preparation

Material fixation and preparation are known to affect spore size in *Ascomycota*. Living spores are usually larger than rehydrated spores. Usage of so-called lethal reagents (Melzer's, KOH, cotton blue) causes more substantial spore shrinkage than nonlethal reagents such as water or Lugol's. In ascospores and asci, shrinkage can occur at different intensities in length and width, affecting shape estimation. Detailed documentation of the preparation technique should accompany published measurement results (Huhtinen, 1989; Kušan et al., 2014; Matočec, 1998).

3. Incorporating molecular data in fungal identification

3.1 Molecular identification, DNA barcoding, and metabarcoding

The cryptic and inconspicuous nature of much of fungal life made mycologists embrace molecular data with open arms when PCR and DNA sequencing became available (White et al., 1990, pp. 315–322). Mycological examples of what we today call DNA barcoding were published in the first half of the 1990s, typically targeting the fungal component of ectomycorrhizal root tips. Despite the laborious and error-prone manual steps involved in PCR and DNA sequencing at the time and the almost complete lack of taxonomic reference sequences, these pioneering studies provided valuable ecological and taxonomic insight into a fungal symbiosis and other processes involving fungi.



FIGURE 7.2 The spacers ITS1 and ITS2, together with the intercalary 5.8S gene constitute the ribosomal ITS region. The neighboring genes nSSU and nLSU are conserved enough that PCR primers of varying degrees of specificity can be designed. The average length of the ITS region is some 550 bases in the fungal kingdom, but numerous exceptions in the form of fungi with dramatically shorter or longer ITS regions are known. Ordering of the genes and genetic markers in the figure from the most to the least conserved would produce the list 5.8S, SSU, LSU, ITS2, and ITS1. The ITS2 subregion is more conserved in length than is the ITS1, making the former less prone to taxonomic PCR biases in metabarcoding.

The nuclear ribosomal internal transcribed spacer (ITS) region was initially identified as a useful marker for molecular identification and species/genus-level phylogenetic inference (Fig. 7.2; Hillis & Dixon, 1991; Gardes & Bruns, 1993). Still today, the ITS region remains the default choice for molecular identification of fungi—at least for an initial assessment—and is considered the primary fungal DNA barcode (Schoch et al., 2012). It clearly offers many advantages. It is long enough to offer a significant amount of information, yet—in most fungi—short enough to be amenable to sequencing in a single round of Sanger sequencing. It is present in multiple copies per cell such that PCR tends to be straightforward even from 20-year-old herbarium samples. Its highly variable—and thus informative—spacers ITS1 and ITS2 are flanked by the much more conserved small subunit (SSU/18S) and large subunit (LSU/28S) genes, respectively (Figs. 8.1 and 8.5), which makes it straightforward to design PCR primers of varying degrees of specificity. Finally, since the ITS region is by far the most commonly sequenced genetic marker of fungi, a relatively rich corpus of taxonomic reference sequences is available.

While the ITS region is reasonably species-specific across the fungal tree of life, it does not lend itself to oversimplification of species boundaries. There is no single ITS similarity cut-off value below which two specimens are certain to belong to the same species and above which they are certain to belong to separate species. Blindly clustering ITS sequences at a 97% sequence similarity cut-off in the hope of demarcating operational taxonomic units (OTUs) at the species level is certain to produce unsatisfactory results from a taxonomic point of view (Lücking et al., 2020). Some species are known to have interspecific ITS variability greater than 3%, such that a 97% clustering step would represent an artificial increase in the number of species. More worryingly, perhaps, is that many fungi are significantly less than 3% distant from other species in terms of the ITS region. Indeed, in a number of fungal genera, there may be no differences in ITS regions at all across several demonstrably different species. In these cases, a 97% cut-off for demarcating species would lead to an artificially deflated view of the fungal community at hand and the loss of taxonomic precision. This is particularly true of several genera with a direct bearing on human endeavors, notably *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* (Abarenkov et al., 2016). This has led to the development of PCR primers for other genes for select taxonomic and functional groups of fungi. Thus, translation elongation factor 1-

α (*tef1 α*) has transpired as a relevant gene in the molecular identification of fungi of, e.g., medical and veterinary concerns (Meyer et al., 2019).

Identification of *Glomeromycota* species is traditionally done by the ribosomal small subunit (SSU/18S) (Davison et al., 2015), and the ribosomal large subunit (LSU/28S) is often used when targeting aquatic fungi (Wurzbacher et al., 2010). Similarly, a range of different genes is routinely employed to tease out species boundaries in various groups of, e.g., plant pathogenic fungi (Boonmee et al., 2021) (e.g., Crous et al., 2022). If the precise taxonomic identity of some particular fungal sample is of importance to the reader, we advise them to start by analyzing the ITS region. Depending on what the preliminary results show, the reader may then want to examine the literature to see what genetic marker(s) are used for species-level exploration in that particular group of fungi.

Fungal molecular ecology has evolved into a research field in its own right. Researchers in the field have gradually synthesized a nuanced understanding of how environmental fungal communities are best qualified and quantified. Thus, prior to the onset of a molecular ecology study involving fungi, the reader is advised to consult the rich literature on the many facets of molecular ecology, including aspects of sampling and field survey, PCR and sequencing, and ecological and bioinformatics-related analyses (Nilsson, Anslan et al., 2019, 2022; Tedersoo et al., 2022). Unlike metabarcoding for prokaryotes and some eukaryotic groups, the SSU and LSU genes are not commonly used when targeting fungal communities. Up until recently, the relatively short stretches of DNA produced from these genes were simply not informative enough to reach the fungal species level. Instead, by far the most commonly targeted genetic marker in high-throughput sequencing and metabarcoding studies of fungal communities is the ITS region. It is critical to note, however, that the choice of PCR primers for ITS-based fungal metabarcoding is a particularly important step. While minor mismatches between primers and the target DNA are often not a concern in single-specimen PCR runs, such mismatches will penetrate metabarcoding PCR runs with potentially hundreds of different species in the PCR tube. Here, templates that match the primers perfectly will be preferred, as will short templates. The result of poorly chosen PCR primers is likely to be far-reaching taxonomic bias, notably the artificial absence of entire lineages of fungi. Minimizing the variation in the length of the target marker—such as by opting for the ITS2 spacer of the ITS region—and choosing PCR primers developed specifically for metabarcoding are key. Primer overviews and recommendations can be found in Tedersoo et al. (2022).

3.2 Sequence quality control for molecular identification

Regardless of what sequencing technology was used to generate a sequence of interest, it is imperative to subject the newly generated sequence to at least basic sequence quality control beyond that offered by the sequencing facility. Countless technically compromised or incorrectly annotated sequences have been—and are being—submitted to the public sequence databases, sometimes with the effect of inviting further downstream submissions suffering from the same complication. Fortunately, basic sequence quality control is straightforward and requires little else than an Internet connection and perhaps local tools for computing and viewing multiple sequence alignments (Hyde et al., 2013). Careful examination of the outcome of a BLAST search (Altschul et al., 1997) in the INSDC (Artia et al., 2021) or UNITE

(Nilsson, Larsson, et al., 2019)—considering all of the BLAST “Descriptions,” “Graphical Summary,” and “Alignments” views—is typically enough to rule out the most severe complications. Doing a local multiple sequence alignment of the query sequence and the 10–20 topmost BLAST matches may be convenient for sufficient visualization of the BLAST results in some cases. As visualized in Fig. 7.3, things to look out for are sudden deterioration in alignment quality (a putative chimeric union, or nonsense sequence data), poor alignment quality

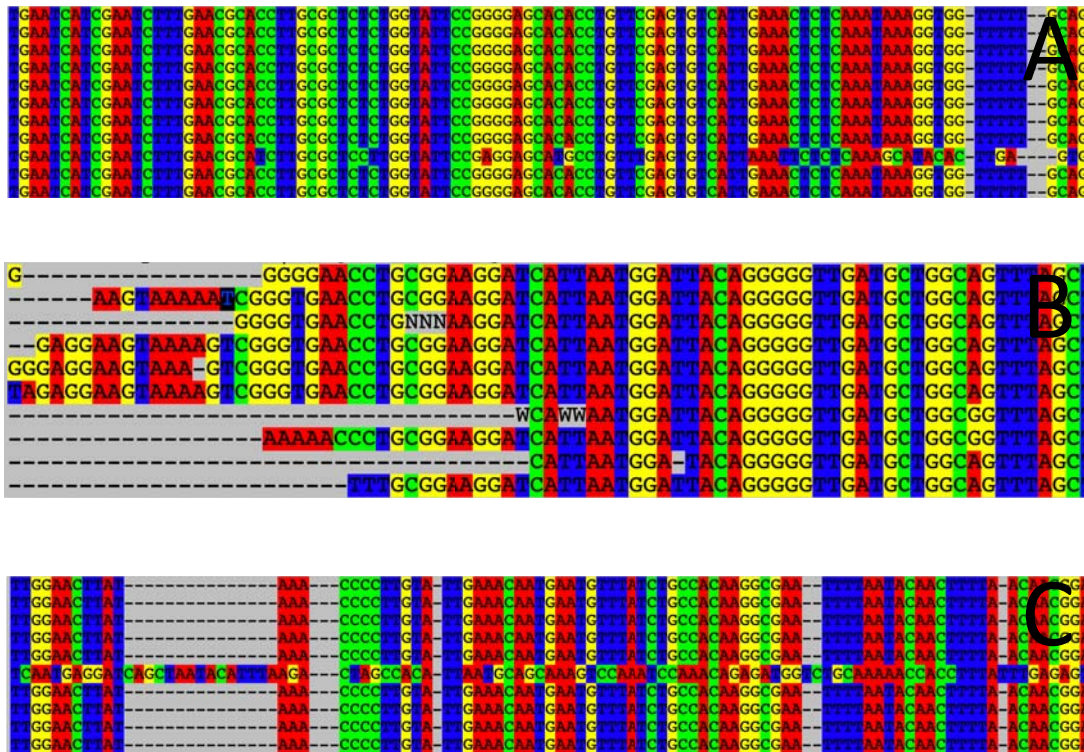


FIGURE 7.3 Examples of warning signs that the user needs to pay attention to in the context of molecular identification and other efforts involving sequence alignment. (A) A sequence that aligned perfectly well in the first half of the alignment (left side of A, not fully shown) abruptly starts to contrast with the other sequences. While a sudden reduction in read quality could perhaps explain this, the primary suspect is a chimeric union. A closer examination and separate BLAST searches will be needed to disentangle the exact cause. This case is a chimera between an *Amanita* and a *Hydnum*. (B) Many public DNA sequences are poorly trimmed for read quality in their distal ends, as the Fig. shows. Sometimes the poorly trimmed part stretches far into the sequence—and occasionally encompasses the entire sequence. Bases that mismatch due to suspected poor trimming should be excluded from match statistics, something that BLAST is not in a position to fully do. Manual intervention (a posteriori trimming) is needed in cases like this. (C) A sequence that produced robust BLAST statistics and that yet cannot be aligned at all is certain to be in the reverse complementary orientation (or all the other sequences in the alignment are). Modern alignment editors support the reorientation and realignment of such sequences. Alternatively, a sequence of a nontarget genetic marker was unintentionally included in the alignment through a backfiring keyword search, perhaps for the reason that its INDSC annotation was incorrect, or that the keyword matches to unintended genetic markers. SeaView v4.7 (Gouy et al., 2010) was used to visualize the multiple sequence alignments.

(often) at the beginning and/or end of the sequence (putatively insufficient trimming of distal ends), and sequences that do not seem to belong to the multiple sequence alignment in the first place, even though they produce promising BLAST statistics (potentially reverse complementary sequence data).

Examining the BLAST hit list for taxonomic clues is another key step: Is the proposed taxonomic affiliation compatible with any prior knowledge or expectations? So-called taxon-specific primers sometimes amplify other groups as well, occasionally even across kingdoms. For this reason, ITS-powered metabarcoding efforts of fungal communities should always strive to use Eukarya-wide taxonomic reference databases, and the taxonomic results should always be assessed by someone versed in fungal taxonomy. Typically, automatically inferred taxonomic names of OTUs can be improved by manual BLAST searches of, e.g., a representative sequence from each of the, say, 50 largest OTUs or most common amplicon sequence variants (Callahan et al., 2017). Finally, it is important to understand that BLAST performs local alignment. Thus, simply knowing and reporting the percentage of similarity between the query sequence and the best BLAST match says very little on its own. BLAST coverage—the proportion of the length of the query sequence involved in the BLAST match—should always be examined and reported. After all, 98% similarity but covering only 50% of the length of the query sequence translates into less than 50% global similarity—not a robust foundation for any lasting scientific conclusion.

3.3 Molecular reference databases and the naming of newly generated sequences

The INSDC is the largest and most up-to-date resource for fungal DNA sequences. With some exceptions, it houses all DNA sequences generated to date by the scientific community. That said, it partly suffers from a lack of standardized vocabulary for, e.g., gene names, and there are limited possibilities for third-party sequence annotation. The INSDC is a good place to start the examination of the taxonomic affiliation of newly generated sequences, although the reader should make it a habit to scrutinize all such results critically. Several other eukaryotic and fungal databases mirror parts of the INSDC to address specific needs. SILVA (Quast et al., 2012) and RDP (Cole et al., 2014) specialize in long SSU and LSU sequences for phylogenetic and, increasingly, metabarcoding purposes. The ISHAM database is an ITS/*tef1 α* database tailored for the identification of fungi of medical and veterinary interest. The UNITE database mirrors all reasonably full-length eukaryotic ITS sequences in the INSDC and offers extended functionalities such as third-party annotation, unambiguous identifiers digital object identifier (DOIs) for all species hypotheses known from sequence data, and formatted reference database files for a range of metabarcoding and molecular identification resources.

Some observations on what to keep in mind when using sequence similarity searches in public sequence databases to identify and name newly generated fungal sequences are provided in Abarenkov et al. (2022). In brief, the taxonomic annotation step is not readily amenable to satisfactory computerization. It should ideally be done manually—or at least double-checked manually—by someone with more than a fleeting familiarity with the intricacies of mycological taxonomy and nomenclature. While a phylogenetic analysis of the query sequence and the 20–30 topmost, unique BLAST matches is probably the best way to assess the taxonomic affiliation of a newly generated sequence, a regular BLAST search

is likely to be enough for most purposes. It is crucial to examine lists of proposed names with some degree of skepticism and never simply adopt the topmost name as the taxonomic identity of the newly generated sequence. Indeed, there is no shortage of under- or misannotated fungal sequences in public databases (Lücking et al., 2020). Sequence annotation tends to be about striking a balance between over- and under-annotating a sequence. Only rarely do BLAST searches in the INSDC provide unequivocal and crystal-clear identification to species level. Making use of the data in the largely type-derived NCBI RefSeq Targeted Loci ITS Project (GenBank identifier PRJNA177353; Schoch et al., 2014) may help to clarify the picture. In many cases, the best one can hope for is a genus-level annotation. Finding out where to draw the line between a species, a genus, and a family-level annotation borders on the impossible. A rough rule of thumb for the ITS2 subregion was provided by Tedersoo et al. (2014). These authors used the global similarity thresholds of 90%, 85%, 80%, and 75% identity for assigning operational taxonomic units to the genus, family, order, and class level, respectively. Many cases will still be unclear, and we argue that it is preferable to annotate a sequence at the parental lineage, such as “*Nectriaceae*” as opposed to a more tentative “*Fusarium*.”

It is important to recognize that fungal taxonomic reference databases are woefully incomplete even for the ITS region. Some 42,000 fungal binomials are represented by at least one more or less full-length ITS sequence in the INSDC. Type-derived sequences are available for less than a quarter of these names. These numbers pale in comparison to the ~150,000 described species of fungi and certainly to the estimated 6+ million extant species of fungi (Baldrian et al., 2022). Anyone doing molecular identification of fungi should thus expect to frequently find themselves stranded at the genus, family, or order level (or sometimes above) when it comes to Latin names. While this may be enough for at least some ecological conclusions (see the FUNGuild tool by Nguyen et al. (2016)), we would like to stress that such a finding represents an important taxonomic conclusion too: this species does not appear to have been sequenced before and thus merits further attention. Some of these species have been described but were never sequenced. A larger number of them will be undescribed and belong to more or less well-known fungal genera such as *Cortinarius* or *Fusarium*. But chances are—at least if environmental substrates are sequenced—that many of these species will be so-called dark taxa. These are fungal groups that, to the best of our understanding, do not seem to form fruiting bodies or other tangible morphological structures, and they have yet to be successfully cultivated in the lab. This precludes them from formal description under the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018). Species without formal names are unfortunately treated very superficially—if at all—in many parts of the sciences. They are routinely excluded from taxonomy efforts, phylogenetic inference, and nature conservation.

We urge the reader to always identify all fungi to species level to contribute to the accumulation of knowledge for described and undescribed species alike. Described species are best identified by their Latin binomial. Species for which no Latin binomial is available, we argue, should be tagged with and identified by the DOI of the corresponding species hypothesis in UNITE (Fig. 7.4). That way, precise communication of taxonomic identity can be accomplished even in the total absence of formal names. In addition, accurate characterizations of the taxonomic nature of fungal communities can be communicated in print and in digital form across time and space. Such data are likely to speed up the further pursuit and formal description of those species, processes that can thus be carried out in the context of all available molecular data generated so far for those lineages.

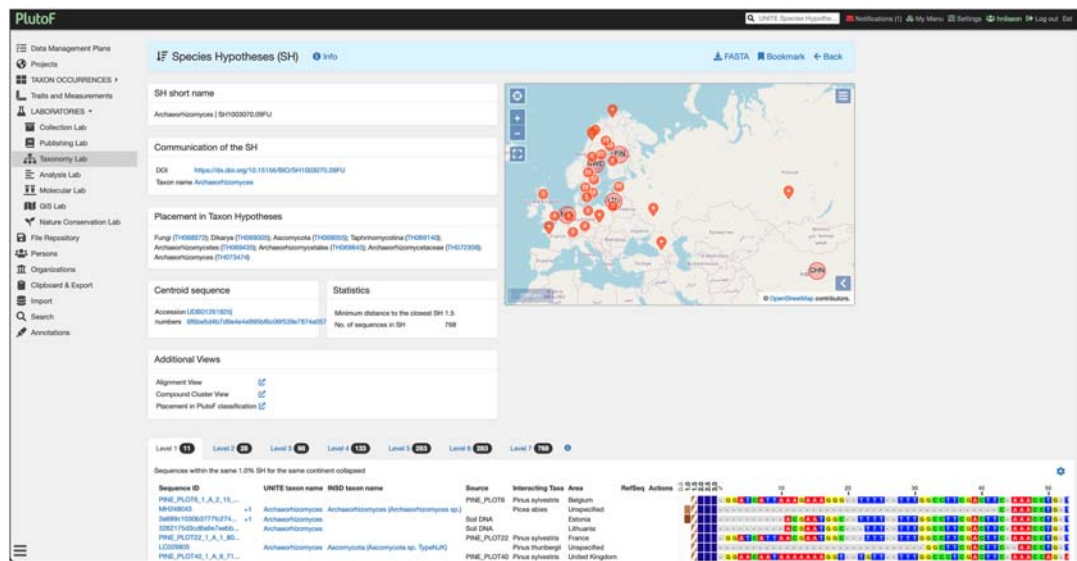


FIGURE 7.4 The UNITE species hypothesis DOI 10.15156/BIO/SH2701435.08FU (<https://app.plutof.ut.ee/unite/view/4824893>) is known from nearly 800 sequences, none of which have a more resolved Latin name than "*Archaeorhizomyces* sp.". Were it not for the DOI, there would have been no way to refer to this species in an unambiguous way across studies and datasets. Due to the use of the DOI, occurrences of this species are straightforward to compile across studies. The compiled data show that the species has been recovered from *Pinus*- and *Picea*-related soil and mycorrhiza samples in a total of 19 countries in Europe and Asia. As far as known, the genus *Archaeorhizomyces* does not form tangible, macroscopic morphological structures, and it has proved exceedingly difficult to cultivate in the laboratory, such that the genus largely falls outside the ambit of the nomenclatural code governing the naming of fungi.

4. Rapid fungal identification methods

Fast and accurate identification of fungi is crucial for various applications, especially in plant disease outbreaks, quarantine, clinical and forensic mycology, fungi producing mycotoxin in human food and animal feed, applied mycology, and eventually for nontaxonomist users dealing with fungi. In the case of invasive fungal infections, early and accurate diagnosis is a decisive factor in optimum antifungal treatment, managing antimicrobial resistance, and best practice disease therapy. This lowers hospitalization costs and mortality. Point-of-care diagnosis is highly relevant in health-related aspects.

Annual mortality in humans caused by fungal pathogens is estimated as high as about 1.7 million deaths (Kainz et al., 2020). Moreover, a rise in fungal infections has been reported in conjunction with the COVID-19 pandemic. Therefore, conventional identification methods need to be complemented with innovative approaches to expedite appropriate treatment. Due to their multitude of advantages, rapid identification methods may shift the paradigm of routine fungal identification.

4.1 Rapid molecular methods based on nucleic acid

4.1.1 PCR-based methods

During the past two decades, polymerase chain reaction (PCR) has been the fundamental basis for molecular identification and phylogeny methods in fungi. It uses at least a pair of primers to amplify a specific, appropriately sized region of the genome depending on the taxon in question and the taxonomic level at which the identification is required. The ITS region serves this purpose for the majority of species-level inquiries. Besides universal primers, numerous taxon-specific primer sets have been introduced for a number of fungal groups to enable more accurate amplification and detection.

In addition to the routine PCR methods, additional amplification assays have been developed such as real-time PCR (RT-PCR), multiplex RT-PCR, quantitative PCR (qPCR), and molecular inversion probe.

Nowadays, numerous commercially available products based on various PCR assays (e.g., RT-PCR and multiplex RT-PCR) are available for the identification of various *Candida* spp., *Aspergillus* spp., *Rhizopus* spp., *Mucor* spp., *Pneumocystis jirovecii*, and some other fungi, with a reasonable assay time of only a few hours (Kidd et al., 2020; Mendonça et al., 2022). Several species-specific qPCR methods have also been attempted for plant pathogenic fungi such as *Magnaporthe oryzae*, *Botrytis cinerea*, *Fusarium* spp., and *Rhizoctonia solani* (Kulik et al., 2020).

4.1.1.1 dPCR

Digital PCR (dPCR) is a new approach so far applied in critical care medicine for early and rapid diagnostics of a number of bacterial, fungal, and viral infections. Principally, it is classified among RT-PCR approaches, with the advantages of being more sensitive and reproducible without reliance on a standard curve (Merino et al., 2022). It applies a microfluidics technology to partition the PCR mastermix into microdroplets, and the final product is quantified by fluorescence. Currently, dPCR has limited usage, mainly due to its high cost and need for expertise.

4.1.1.2 Long-read amplicon technologies

Various ribosomal markers have different evolutionary rates, hence their capability to discriminate among fungal taxa. Moreover, a given marker may behave in a taxon-specific manner, which may compromise the unambiguous taxon assignment and identification. For instance, although the ITS region is the widely accepted gold standard for routine molecular fungal identification, there are more than a few empirical studies showing that the ITS region sometimes has an insufficient or unsuitable discriminatory signal at the species level. While DNA (meta)barcoding approaches rely on short-read ribosomal amplicons, long-read technologies have been developed to offer more data and higher confidence in species delimitation and characterization. One approach uses platforms such as Pacific Biosciences and Oxford Nanopore and targets the full fragment of fungal ribosomal tandem repeat (rDNA), covering 18S, ITS (including ITS1, 5.8S, ITS2), 28S, and IGS, with a length of about 3–6 (–10) Kb (Wurzbacher et al., 2019) (Fig. 7.5). Long-read ribosomal amplicon sequencing has been shown to be a rapid and powerful tool in real-time identification of clinical fungal

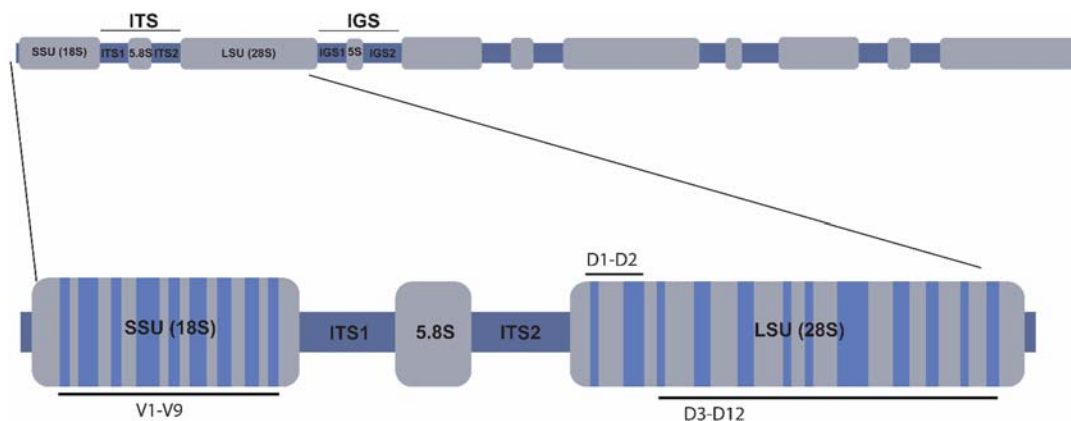


FIGURE 7.5 Nuclear ribosomal tandem repeat in fungi. *ITS*, internal transcribed spacer; *IGS*, intergenic spacer; *SSU*, small subunit; *LSU*, large subunit; *V1–V9*, variable parts in *SSU*; *D1–D12*, variable parts in *LSU*. Partitions lengths are not to scale.

taxa down to the species level (D'Andreano et al., 2020). However, high-quality DNA availability is an important factor in successful long-read amplification.

4.1.1.3 Endogenous reference gene

Endogenous reference gene methods refer to the screening of a large number of previously available sequences of a target fungus to recognize a specific gene, coupled with qualitative and quantitative PCR with no need for subsequent sequencing. However, the technique is constrained by the prior availability of numerous gene sequences of the target species. So far, it has been applied in food and crop plant research and for a number of edible mushrooms such as *Flammulina velutipes*, *Tricholoma matsutake*, and *Pleurotus ostreatus* (Wei et al., 2022).

4.1.2 Isothermal-based methods

In isothermal-based approaches, the whole amplification process is done in one thermal cycle. A number of isothermal-based techniques include loop-mediated isothermal amplification (LAMP), helicase-dependent amplification, rolling circle amplification (RCA), and recombinase polymerase amplification. Among these, LAMP has been more widely applied to various bacteria, viruses, and numerous ascomycetes and basidiomycetes.

4.1.2.1 LAMP

LAMP is a powerful tool to overcome the challenges of PCR methods in terms of costs, time, and power supply and has gained wide attention in the identification of microorganisms. It is also a promising approach according to the criteria of WHO for “ideal” diagnostic methods. The technique uses *Bst* DNA polymerase and four (or six) primers targeting six (or eight) genomic regions. The LAMP reaction can be completed in about 2 h, and the product can be detected by electrophoresis, visually by the naked eye, specific colorimetry, lateral flow dipsticks (LFD), or smartphone-based tools.

Several LAMP-based technologies have also been developed, such as LAMP combined with hyper-branched RCA, LAMP with enzyme-linked immunosorbent assay, real-time LAMP (RT-LAMP), quantitative real-time LAMP, and LAMP with digoxigenin-labeled pan-fungal primers.

So far, LAMP has been successfully used for the discrimination of various fungi, including phytopathogens, fungi in clinical samples, lethal amanitas, edible or poisonous mushrooms, and processed mushroom blends. An important limitation of LAMP is the sensitivity of its multiple primer designs (Niessen, 2015).

4.2 Other rapid methods

4.2.1 MALDI-TOF MS

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is an advanced method first developed for the rapid identification of bacteria and yeasts, using extensive in-house libraries of proteomes. It surveys the protein profile (mainly ribosomal RNA proteins) of a given microorganism and is now also increasingly applied to the detection of filamentous fungi, particularly in clinical samples. In principle, laser irradiation is used to dissociate protein crystals to produce ions with different mass to charge ratios, from which the mass spectra are taken.

Due to the challenges of sample preparation and protein extraction for MALDI-TOF MS, modifications have been proposed recently to modify the constraints in fungi (Honsig et al., 2022; Ning et al., 2021). Yet, the application of MALDI-TOF MS is currently limited to a few laboratories mainly due to its need for highly skilled personnel, specific in-house libraries, and well-characterized reference strains with readable compound spectra.

4.2.2 FT-NIR spectroscopy combined with ResNet

Fourier transform infrared (FTIR) spectroscopy combined with appropriate chemometrics has been used to some extent for detecting species-specific spectral fingerprints. Recently, Fourier transform near-infrared spectroscopy combined with residual convolutional neural network models has been successfully used as a rapid and effective approach to distinguish edible boletes with 100% accuracy (Chen et al., 2022). The technique was suggested as an effective approach to discriminate against various mushroom species as well as fraud in the food industry.

4.2.3 eNose

Electronic nose is an emerging technology used to detect, screen, and discriminate a number of fungal genera and species in microbiology research, the food industry, clinical medicine, and plant pathology. It is a sensor system based on the detection of volatile compounds from a sample. Currently, several commercial portable instruments have been designed with various sensitivities to the volatilome of dried, cultured, or clinical fungal samples. About 50 species of ascomycetes (e.g., *Aspergillus*, *Fusarium*, *Penicillium*, etc.), basidiomycetes (e.g., agarics, wood-decaying polypores, and corticioids, etc.), and oomycetes (e.g., *Phytophthora*) have been targeted so far to various degrees of success (Mota et al., 2021). However, an important prerequisite for this technology is to provide an aroma reference library from a wider range of fungal samples to enable more accurate detection algorithms,

especially for mixed samples. Moreover, controlling the physiological condition and storage state of the tested sample is important to deduce the best stage for the production of the fungal volatile substances and to manage the possible interference of volatile compounds from the growth medium or the substrate.

4.2.4 Artificial intelligence

Identification tools based on artificial intelligence are still on their way toward specification and progress before becoming widely greeted by the mycology community. Low specificity and limited taxon coverage are the main drawbacks. Exemplar progress may however be the artificial intelligence-based tool developed for the speciose and notoriously difficult basidiomycete genus *Hebeloma*. Hebeloma.org has been introduced as a unique machine-learning-based identification tool, combining numerous characters from the type materials and thousands of reference specimens to increase the discriminatory power of its algorithm, with detailed macroscopic and microscopic features, ecology, geography, as well as DNA sequence data parametrized into a “species identifier” tool (Bartlett et al., 2022).

Moreover, in vivo confocal microscopy combined with artificial intelligence has been suggested as a promising tool for prompt detection of fungal keratitis (Bakken et al., 2022).

In addition to the above methods, several on-site and portable detection devices, biosensors, and kits have been developed, allowing fast and specific diagnosis of a given pathogen. The specificity of the kits is still under debate, as some are reported to give rise to false positive detection. A number of biosensors, including nanobiosensors, have emerged for the detection of phytopathogenic fungi as well as human fungal pathogens (Ghormade, 2022). Metabarcoding and metagenomics approaches are used for unraveling species identities in complex mixtures.

Nanobiotechnology opens windows to minimize the detection platforms, while maximizing organismal specificity. For instance, the advanced “T2 *Candida* panel” has recently been developed with the ability to discriminate the five most common *Candida* species in less than 5 h, with 98% specificity and full sensitivity (Joshi & Shenoy, 2022). It should be said, however, that in most clinical pathogen diagnoses, culturing has been certified as the irreplaceable “gold standard.”

5. Integrative fungal identification

The integrative or polyphasic approach is primarily classified among species delineation concepts but has also become a useful approach in the identification of controversially distinguished fungal species. Numerous phenotypic, molecular, chemical, biochemical, serological, and combined detection and identification methods have been introduced. Satisfactory identification results usually stem from complementary approaches. For instance, despite its time- and labor-intensive nature and low sensitivity, culturing is still considered highly valuable, the “gold standard,” in clinical fungal diagnosis. This is considered a primary requirement for a “proven” diagnosis, with most other methods leading to a “probable/possible” diagnosis (Mendonça et al., 2022). A combination of culture-based and DNA-based detection of fungi has also been recommended as complementary methods to characterize fungal communities in a given environment. This is because numerous fungal species are fastidious about nutrient media, while DNA repositories may lack sufficient data from reference strains.

During species divergence, discontinuation happens in some traits, be they morphological, physiological, reproductive, geographical, ecological, or genetic characteristics, or a combination of them. Evolutionarily, a discontinuity occurs sequentially, or for some traits, simultaneously. Yet, traits may not evolve in the same sequence in all species. Two species may diverge genetically but still have lagging morphological divergence, as is the common case with “cryptic” species. Moreover, in cases where morphological differentiation precedes genetic divergence, e.g., due to epigenetic factors, single-marker molecular identification may not help alone. In other cases, character discontinuation may be so meager that it can only be discerned by experts and highly experienced individuals. Some cryptic species may indeed be “young,” in the sense of having diverged recently, or may still be in the process of speciation. As character discontinuation may be ambiguous, taking more than one trait into consideration would increase the chance of reliable identification. For this reason, and to achieve the most reliable identification, it is recommended to use the largest number of characters together. DNA barcoding with two (ITS and *tef1 α*) or three genetic markers (ITS, *tef1 α* , and *rpb2*) is likely to give more accurate identifications than does single-marker DNA barcoding, although the fungal reference databases are woefully incomplete for less commonly sequenced markers such as *tef1 α* and *rpb2*.

Ideally, species identification needs to be pragmatic and applicable to a wide range of people in the scientific community as well as society. It should also stand apart from, and not be affected by, the varying species delimitation concepts. This means that regardless of what criterion and concept the “species” is based on, identifying it should clearly result in a single, unique name (scientific name). Normally, species erected on a polyphasic basis have a greater chance of being resighted and detected by others. Therefore, it is recommended that an integrative approach be taken from the very stage at which a new species is introduced to increase the chance of its detection and identification via multiple lines of evidence (Fig. 7.6). As far as time is concerned, it is expected that technological advancements, machine learning, and artificial intelligence techniques will help to expedite integrative fungal identification and diagnosis in the future. Evidently, the integrative approach opens windows for interdisciplinary dialog between experts.

The best example of a successful integrative approach to fungal identification is probably *Trichoderma*. The fungal genus *Trichoderma* is taxonomically difficult and contains some species of high economic, industrial, and clinical importance. Cai and Druzhinina (2021) presented authoritative guidelines for the distinguishment of more than 370 *Trichoderma* species using comparative ecophysiology, DNA barcoding (with the three genetic markers ITS, *tef1 α* , and *rpb2*), and phenotyping. For this, *Trichoderma* has been suggested as a model for integrative fungal taxonomy.

Finally, all detection and diagnostic methods, from the basic to the most advanced, rely on “known” diversity, that is, previously obtained knowledge about the species. The known fungal diversity is perhaps 5% of the estimated number of extant fungal species, of which only some 1% have any representative sequence(s) in GenBank. As a consequence, in many screening efforts, novelties and unknown species are highly likely to be encountered. This necessitates the involvement of experienced taxonomists in all fungal screening efforts. This is to properly characterize the taxa, especially unknown entities not fitting the identification tools.

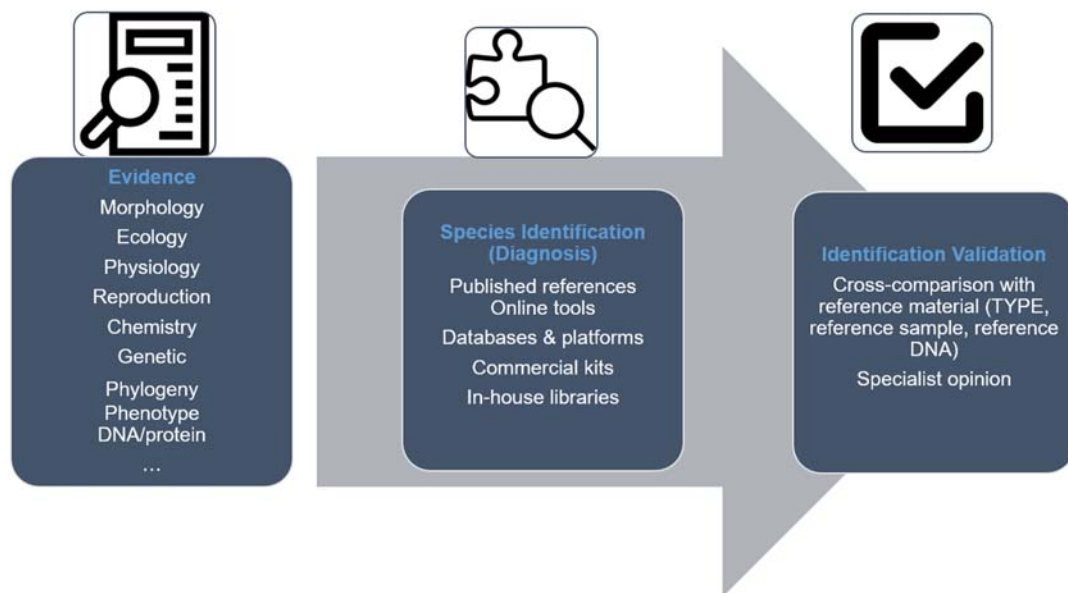


FIGURE 7.6 Simplified recommended process for the identification of fungal species. The process starts with documentation of available/possible evidence (characters) (the left panel). Based on availability and the level of user experience and expertise, various identification sources may be consulted (the middle panel). A final validation assures an accurate identification (the right panel). Note that the species identification tool used must be precisely reported in the respective publication so that the quality of identification can be critically assessed.

6. Key points

- Mastering a broad spectrum of mounting media, supported by knowledge of plectology and cytology, can essentially facilitate the identification of fungal species via light microscopy. Description sheets and measuring protocols for many fungal groups require further standardization efforts.
- For spores as diagnostically important structures of fungi, numerical descriptors provide more comprehensive information on shape than categorical assignments do. Using the former can lead to a gain in automated species identification. Due to the small size of fungal spores and related imaging difficulties, the protocols for digitizing spore shapes are still in the pioneer stage and can be substantially improved.
- For fungal spores, the limits of variation at different levels (individual, populational, or species) are roughly known but need an update for linear size and are yet to be primarily estimated for numerical shape descriptors. These tasks can be better addressed by publishing morphometric measurements as well-accessible and reusable open data.
- DNA sequence data and sequence analysis form very powerful tools for the identification of fungi. These approaches should be thought of as semi-automated where user intervention is needed to verify, e.g., sequence quality, sequence similarity statistics, and proposed taxonomic affiliations.

- If the precise taxonomic identity of some particular fungal sample is of importance to the reader, we advise them to start by analyzing the ITS region. Depending on what the preliminary results show, the reader may then want to examine the literature to see what genetic marker(s) are used for species-level exploration in that particular group of fungi.
- For molecular identification, solely reporting the percentage of similarity between the query sequence and the best BLAST match says very little on its own. BLAST coverage—the proportion of the length of the query sequence involved in the BLAST match—should always be examined and reported.
- Many advanced identification methods are constrained by insufficient species coverage and well-curated databases. Moreover, rapid methods with reliance on chemical content/image (such as FISH, FTIR, spectroscopy, etc.) are influenced by the age and other eco-physiological properties of the fungal sample. As a result, it is wise to interpret their results with care, and the protocol should be set up so that there is enough sampling.
- All projects and efforts that aim to identify fungi should seek to include a mycologist with taxonomic expertise right from the onset of the project or effort.

World wide web links

Functional and trait assignments of fungi

<https://github.com/traitecoevo/fungaltraits>
www.funguild.org

International Code of Nomenclature for algae, fungi, and plants

<https://www.iapt-taxon.org/nomen/main.php>

International Commission on the Taxonomy of Fungi

<https://www.fungaltaxonomy.org/>

Catalog of Life

<http://www.catalogueoflife.org>

Database with names of lichens, lichenicolous fungi and nonlichenized ascomycetes

<http://liasnames.lias.net>

Official fungal nomenclatural repositories

<https://www.indexfungorum.org/>(<http://www.speciesfungorum.org/>)

<http://www.mycobank.org/>

<https://nmdc.cn/fungalnames/>

State of the World's Plants and Fungi

<https://www.kew.org/science/state-of-the-worlds-plants-and-fungi>

Mycoses Study Group—Education and Research Consortium

<https://msgerc.org/>

International Society for Human and Animal Mycology

<https://www.isham.org/>

European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections

Cooperative Group

<https://www.eortc.org/>

Makroaufmaßprogramm

<https://ruedig.de/tmp/messprogramm.htm>

Der Geruch der Pilze

<http://tintling.com/pilzbuch/merkmale/geruch/geruch.html>

Geruch und Geschmack

<https://myclopedia.ch/pilze/8660.htm?q=geruch>

Pilzgerüche oder wie riechen Schwammerl aus dem Bayerwald

<https://www.123pilze.de/DreamHC/Download/2006Pilzgerueche.htm>

Piximetre

<http://piximetre.fr/>

UNITE database for molecular identification of fungi

<https://unite.ut.ee/>

BLAST: Basic Local Alignment Search Tool

<https://blast.ncbi.nlm.nih.gov>

The International Nucleotide Sequence Database Collaboration (INSDC)

<https://www.insdc.org/>

Acknowledgments

The support received from the Iran National Science Foundation (INSF) project no. 4000655 is acknowledged.

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Introduction to genomics-based pharmaceutical applications

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1. Introduction

The discipline of genomics has revolutionized biomedical research and pharmaceutical development in recent years. Genomics has yielded invaluable insights into the genetic mechanisms underlying disease and opened up new avenues for targeted therapeutic interventions. For the development of effective treatments, it is essential to comprehend the genetic basis of diseases. Targeting specific symptoms or pathways without a comprehensive understanding of the underlying genetic factors, traditional drug discovery and development processes frequently relied on trial and error. Nonetheless, with the advent of genomics, scientists are now able to analyze an individual's genetic composition and identify specific genetic variants associated with disease. Researchers are now able to identify genetic variants that contribute to disease susceptibility, progression, and treatment response as a result of the advent of high-throughput DNA sequencing technologies. This wealth of genetic information has paved the way for the development of personalized medicine, in which treatments can be tailored to an individual's genetic profile.

Moreover, genomics has been instrumental in the development of pharmacogenomics, the study of how an individual's genetic makeup affects their response to medications. By identifying genetic markers that predict drug efficacy and adverse reactions, pharmacogenomics seeks to improve treatment outcomes, reduce adverse events, and reduce prescribing errors.

This chapter will explore the numerous genomics applications in pharmaceutical research and development. Not only have genomic studies improved our comprehension of diseases

at the molecular level, but they have also shed light on the intricate relationship between genetics, environment, and drug response. In addition, we will discuss the influence of genomics on drug repurposing and the process of finding novel therapeutic applications for existing drugs. Researchers can identify common genetic signatures or molecular pathways that transcend traditional disease boundaries by analyzing genomic data from patients with various diseases. This strategy can potentially expedite the development of effective treatments for diseases that presently lack specific targeted therapies.

Moreover, we will discuss the ethical considerations surrounding pharmaceutical applications based on genomics, including privacy, data security, and equitable access to genomic information and therapies. These factors are crucial for guaranteeing the implementation of genomics in pharmaceutical practices in a responsible and equitable manner. Last but not least, we will discuss the role of bioinformatics in providing sequencing technologies and pipelines that aid in variant calling and annotation.

2. Genomics revolutionizing the field of pharmaceuticals

2.1 Pharmacogenomics

Pharmacogenomics studies on how an individual's genetic variations affect their response to drugs. It involves analyzing an individual's DNA to identify genetic variations affecting drug metabolism, efficacy, and toxicity. The goal of pharmacogenomics is to develop personalized medicine that is tailored to an individual's genetic makeup (Cascorbi, 2018). Pharmacogenomic testing involves analyzing specific genes known to affect drug metabolism, efficacy, and toxicity. These genes include cytochrome P450 enzymes (CYPs), which are responsible for drug metabolism; transporters, which are responsible for moving drugs in and out of cells; and receptors, which are responsible for mediating drug effects (Haidar et al., 2022; Theken et al., 2020).

Pharmacogenomic testing can predict how an individual will respond to a particular drug (Wake et al., 2019). For instance, if a patient has a genetic variation that affects the metabolism of a particular drug, leading to a decrease in its excretion rate or an increase in its accumulation, those patients may require a lower dose or a different medication altogether. Likewise, if a patient has a genetic variation that affects the efficacy of a particular drug, resulting in a decrease in drug sensitivity or lower expression of the receptor, those patients may require a higher dose or a different medication altogether (Haidar et al., 2022; Wake et al., 2019). Moreover, pharmacogenomic testing can also be used to predict the risk of adverse drug reactions. For example, if a patient has a genetic variation that increases their risk of developing liver toxicity from a particular medication, they may need closer monitoring or alternative treatment options (Theken et al., 2020).

There are many examples of pharmacogenomics in practice. One example is warfarin, a blood thinner used to prevent blood clots. Warfarin has a narrow therapeutic index, meaning that the difference between a therapeutic dose and a toxic dose is small. The optimal dose of warfarin varies widely among individuals due to genetic variations that affect its metabolism. Pharmacogenomic testing can identify patients requiring lower

or higher doses of warfarin based on their genetic makeup (Kaye et al., 2017). This can lead to improved treatment outcomes and a reduced risk of adverse drug reactions. Another example is the use of abacavir, an antiretroviral medication used to treat HIV infection. Abacavir is associated with a hypersensitivity reaction in approximately 5% of patients who take it. This reaction is caused by a genetic variation in the HLA-B gene (Wang et al., 2022).

2.2 Personalized medicine

Person-to-person variability in responsiveness to diagnostic tools, treatment approaches, and prevention strategies have abrogated the traditional perception of clinical interventions to be “one-size-fits-all” (Juengst et al., 2012). Tailoring medical treatment to an individual’s unique genetic profile in what is known as personalized medicine has allowed the clinical practice to be more patient-centered and helped predict an individual’s response to drugs (Suwinski et al., 2019). With the advancement in pharmacogenomics, personalized medicine has also considered other individuals’ unique characteristics, including their lifestyle, environment, and medical history. Indeed, the foundation of personalized medicine lies in integrating genomics into clinical practice, allowing for the identification of genetic variations associated with drug responses and the development of personalized treatment plans. It is undisputed that genomics has played a critical role in developing personalized medicine. With the advent of next-generation sequencing (NGS) technologies, it is possible now to sequence an individual’s entire genome or specific regions of interest in a rapid, affordable, and high-throughput manner (Morganti et al., 2019). This information can be used to identify genetic variations associated with drug responses, disease susceptibility, and therapeutic outcomes.

2.2.1 Genomic biomarker identification

Biomarker identification has been a field of intense study in recent years, with researchers investigating a wide range of markers that can be used to diagnose and monitor diseases. One area of particular interest has been the identification of genetic biomarkers, specific genetic variations associated with a particular disease or condition. These biomarkers have enormous potential for improving the ability to detect and treat diseases and have significantly impacted clinical practice. Genomics has played a key role in this process, allowing researchers to identify genetic biomarkers with unprecedented speed and accuracy quickly and accurately (Douglas & Russell, 2016). As our understanding of the human genome has grown, so has our ability to identify genetic variations associated with disease. This has led to the discovery of a vast array of genomic biomarkers, which are used in various applications.

One of the most important uses of genomic biomarkers is in the early diagnosis of disease (Bonifácio, 2020). Many diseases are most effectively treated if detected early when they are still in their early stages and have yet to cause significant damage. By identifying genetic biomarkers associated with specific diseases, it is possible to develop tests that can detect the presence of these diseases at an early stage, allowing for prompt

intervention and treatment (Bonifácio, 2020; Douglas & Russell, 2016). For instance, one of the most common uses of genomic biomarkers is in early cancer detection. There is now a wide range of genomic tests that can be used to detect the presence of various types of cancer, often long before any symptoms are present (Xiao et al., 2022). By detecting cancer at an early stage, treating it more effectively and increasing the chances of a full recovery became possible.

Genomic biomarkers are also being used to monitor disease progression. Once a disease has been diagnosed, it is important to track its progression and monitor the effectiveness of any treatments. Genomic biomarkers can be used to develop tests that track the presence and activity of disease-related genes, allowing doctors to monitor the course of the disease and adjust treatment as necessary (Poudineh et al., 2018). Perhaps most intriguingly, genomic biomarkers are being used to predict treatment outcomes (Piñero et al., 2020). By identifying genetic variations associated with how a patient is likely to respond to a particular treatment, doctors can develop personalized treatment plans tailored to the individual patient. This approach has already proven to be highly effective in a wide range of applications, from cancer treatment to lifestyle interventions. Genomic biomarkers could play a crucial role in identifying individuals who are at high risk of developing certain conditions as well (Giwerzman et al., 2022). By identifying individuals who have a genetic predisposition to a particular disease, it is possible to implement preventive measures or early interventions that can help to reduce the risk of developing that disease (Ginsburg et al., 2021). For instance, individuals who are at high risk of developing heart disease may be advised to make lifestyle changes such as eating a healthy diet, quitting smoking, and exercising regularly.

It is undisputed that genomic biomarkers have revolutionized the ability to diagnose, monitor, and treat disease. By identifying genetic variations that are associated with specific conditions, researchers have been able to develop tests that can detect diseases at an early stage, monitor disease progression, and predict treatment outcomes. With continued research and development, it is expected that genomic biomarkers will become an even more important tool in the fight against disease.

2.2.2 Pharmacokinetics and drug metabolism

Pharmacokinetics studies how drugs are absorbed, distributed, metabolized, and eliminated from the body. These processes are essential for understanding the effectiveness and safety of drugs in the body (van den Anker et al., 2018). The most common enzymes responsible for drug metabolism are CYPs, which are found mainly in the liver and other organs in the body (Zhao et al., 2021). Indeed, understanding pharmacokinetics and drug metabolism is crucial for determining an individual's response to a particular drug. Genomics has helped to unravel the complexities of drug metabolism and provided insight into an individual's drug response based on genetic variations in enzymes responsible for drug breakdown and clearance. Intriguingly, genomics has uncovered various genetic variations in CYP enzymes that can significantly affect drug metabolism and pharmacokinetics (Tornio & Backman, 2018). The pharmacokinetics of drugs can be influenced by a complex interplay between environmental factors, genetics, and drug-drug interactions. For instance, genetic variations in enzymes responsible for drug metabolism can lead to a wide range of drug responses, such as differences in absorption rates, activity levels, and drug clearance rates

(Huang et al., 2021). Understanding these variations can help to predict drug efficacy, make dosage adjustments, and minimize adverse reactions. In fact, the genetic variations associated with drug metabolism and pharmacokinetics are known as pharmacogenetic variants, and they are vital in predicting individual drug responses (Drent et al., 2018). Additionally, they help identify potential drug-drug interactions and guide therapeutic decisions (Drent et al., 2018; Huang et al., 2021).

As previously mentioned, pharmacogenetic testing can help identify individuals with genetic variations in CYP enzymes, which in turn could assist in selecting the most appropriate drug and dose for the individual. For instance, genetic variations in CYP2D6 enzymes can affect the metabolism of many drugs, including antidepressants, antipsychotics, and the analgesic codeine (Huang et al., 2021). Individuals with a decreased activity level of this enzyme may require a lower drug dosage or may need to switch to an alternative medication with a different metabolic pathway. By contrast, individuals with a rapid metabolizing activity level may require a higher dosage for therapeutic benefit (Drent et al., 2018; Huang et al., 2021). Another example is the drug warfarin. It is metabolized by CYP2C9 enzymes, and genetic variations in these enzymes can significantly influence warfarin sensitivity. Individuals with a decreased activity level of CYP2C9 may require a lower dosage of warfarin, while those with a rapid metabolizing activity level may require a higher dosage (Cronin-Fenton & Damkier, 2018; Drent et al., 2018; Huang et al., 2021).

Genomics can provide insights into not only the dose adjustment of drugs but also into the potential for adverse drug reactions. Individuals with specific genetic variants may be at a higher risk of toxic effects or adverse drug reactions, potentially leading to severe or life-threatening consequences. For instance, individuals with a genetic variation in the CYP2D6 enzyme may be at a higher risk of developing opioid-induced respiratory depression when exposed to the painkiller codeine. This knowledge can assist physicians in selecting an appropriate analgesic for the individual and monitoring adverse reactions.

Hence, understanding genetic variations in enzymes responsible for drug metabolism can provide valuable insights into an individual's drug metabolism and response, helping physicians to tailor drug therapies to an individual's needs, minimize adverse reactions, and optimize drug efficacy. Additionally, the identification of pharmacogenetic variants can assist in dose adjustment, predicting drug responses, and identifying potential drug-drug interactions to improve patient outcomes.

2.3 Drug discovery and development guided by genomic data analysis

The discovery and development of new drugs is a complex and resource-intensive process that involves years of research and testing. However, recent advancements in genomics and genomic data analysis have ushered in a new era of drug discovery. Genomic data analysis is a rapidly growing field that is transforming our understanding of genetics and disease. It involves using statistical algorithms and machine learning techniques to identify genes or gene pathways associated with a particular disease (Serral et al., 2021). As the cost of genome sequencing decreases and the availability of genomic data increases, the need for advanced computational techniques to analyze and interpret this data has become increasingly critical (Pareek et al., 2011). Hence, understanding the genetic basis of diseases will allow, as scientists are increasingly turning to the study of genes

and their expression, to develop new and more effective therapies to identify potential drug targets and develop novel therapies that directly modulate the underlying genetic mechanisms.

One of the primary ways in which genomic information is contributing to drug discovery is through the identification of potential drug targets (Pareek et al., 2011; Chen et al., 2020). Genes that play a role in disease pathways and cellular processes can be targeted by drugs to modulate their activity or function, leading to a therapeutic effect (Avelar et al., 2020). Through the use of genomic techniques such as genome-wide association studies (GWAS), researchers can identify genes that are associated with a particular disease and prioritize them as potential drug targets. For instance, GWAS studies have identified genetic mutations that increase the risk of certain cancers or neurodegenerative disorders. This identification of high-priority targets can help guide the development of targeted therapies that address the underlying genetic basis of the disease (Dehghan, 2018; Hayes, 2013).

2.3.1 Targeted therapy guided by genomics

Using genomics in drug development has also led to a greater understanding of disease pathways and their underlying genetic mechanisms. By analyzing gene expression data from disease tissue samples and comparing them to healthy tissues, researchers can identify genes and proteins that are involved in disease progression and develop drugs that target these specific proteins (Ionkina et al., 2021). This approach often leads to more precise and effective therapies, as they are targeted to specific disease pathways rather than broad areas of the body. For example, targeted therapies have revolutionized the treatment of certain cancers, where genomic analysis has revealed specific genetic mutations that drive tumor growth (Lee et al., 2018; Pan-Cancer, 2020). Drugs that target these mutations can halt or slow down tumor progression, leading to improved patient outcomes. Genomic data analysis has revealed that certain genes and proteins play a key role in the development of cancer, such as the HER2 gene in breast cancer (Pan-Cancer, 2020; Arriola et al., 2008). This knowledge has led to the development of targeted therapies that focus on inhibiting the activity of this gene and its associated pathway.

2.3.2 Gene therapy guided by genomics

Genomics is also being utilized in the development of gene therapies, which involve the insertion or modification of genes in a patient's cells to treat or prevent disease (Steffin et al., 2019). Gene therapies can potentially cure genetic diseases or provide long-term treatments for chronic diseases by targeting the underlying genetic cause (Steffin et al., 2019; Maule et al., 2020). Using genomic techniques such as CRISPR/Cas9, scientists can accurately and precisely edit or insert genes into a patient's DNA, opening up new possibilities for future therapies (Zhang et al., 2014).

2.3.3 Drug repurposing guided by genomics

Another application of genomic data analysis is drug repositioning, also known as drug repurposing. It is identifying new uses for existing drugs (Pushpakom et al., 2019). This approach is more cost-effective and time-efficient than traditional drug development and can help to bring new therapies to patients sooner (Pushpakom et al., 2019; Roessler et al., 2021). Genomic data analysis can play a crucial role in drug repositioning by identifying genetic variations associated with specific diseases and cross-checking these against known

drug targets (Lesmana et al., 2022). This approach can reveal new potential uses for existing drugs and prevent the need for lengthy and expensive clinical trials.

3. Genomics applications in the pharmaceutical fields

3.1 Preclinical drug testing

In addition to the contribution of genomics to drug discovery in recent years, genomics is now extensively used in preclinical drug testing to evaluate the effectiveness and safety of potential drug candidates, accelerating the drug development process and reducing costs (Spreafico et al., 2020). Genetic screening of model organisms has allowed researchers to identify potential drug targets with a high degree of accuracy (Bond et al., 2013). For example, in 2017, a group of researchers from MIT used CRISPR-based gene-editing techniques to target a specific gene (NLRP3) that had been identified through genetic screening as a potential therapeutic target for gout (Wan et al., 2021; Schmid-Burgk et al., 2016). Through their study, the researchers confirmed that NLRP3 is indeed a promising drug target, and they also identified a previously untested compound (CY-09) that effectively inhibited NLRP3 activity in vitro (Fan et al., 2021). The research suggests that therapeutic strategies targeting NLRP3 could be viable for treating gout and other inflammatory diseases.

Another example of using genomics in preclinical drug testing is evaluating drug efficacy and toxicity. By analyzing gene expression patterns in response to different drug candidates, researchers can determine which genes are affected by the drugs and how they respond (Möbus et al., 2022). This information can then identify the most effective drug candidates and eliminate those with potential toxicity issues, thus improving overall drug safety and efficacy.

3.2 Gene therapy

Gene therapy is a promising field of medicine that aims to treat or even cure genetic diseases by using genetic material to replace defective or missing genes (Wirth et al., 2013). Genomics plays a pivotal role in the development of gene therapies. By understanding the genetic defects underlying certain diseases, researchers can design and deliver therapeutic genes to correct or compensate for these abnormalities, offering potential cures for genetic disorders (Wirth et al., 2013; Tang & Xu, 2020). Identification of disease-causing mutations marks one of the main applications of genomics in gene therapy. Advances in sequencing technology have enabled researchers to identify genetic mutations associated with a wide range of diseases, from rare genetic disorders to more common illnesses like cancer (Xuan et al., 2013). By understanding the specific genetic mutations that contribute to disease, researchers can design targeted gene therapies that address the underlying cause of the illness.

Another way genomics can be applied to gene therapy is through the development of more precise delivery methods. The genetic material used in gene therapy must be delivered to the appropriate cells in the body, where it can be incorporated into the cell's DNA and

produce a therapeutic effect. Advances in genomics have helped researchers develop more precise delivery methods that can target specific cells or tissues in the body, increasing the therapy's effectiveness and reducing the risk of side effects (Giuliano et al., 2019). Moreover, genomics can also help researchers identify potential side effects of gene therapies. Gene therapies involve the delivery of genetic material to the body, lead potentially lead to unintended effects if the treatment is not delivered correctly. By analyzing the patient's genetic makeup, researchers can identify any genetic variations that may increase the risk of adverse reactions to the gene therapy (Han et al., 2020).

The treatment of inherited retinal degenerations (IRDs), a group of genetic disorders that cause progressive vision loss, represents an excellent example of genomic application in gene therapy. In this approach, gene therapies aim to replace the mutated genes that cause IRDs with a functional copy of the gene (Hu et al., 2021). In parallel, genetic testing is used to identify the specific genetic mutation responsible for the IRD, which guides the design of the gene therapy (Han et al., 2020; Hu et al., 2021). Intriguingly, delivery methods such as viral vectors and nanoparticles are used to deliver the therapeutic gene to the appropriate cells in the eye, where it can restore vision (Patel et al., 2019). Another example is the treatment of cystic fibrosis (CF), a genetic disease caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene (Gillen & Harris, 2012). Gene therapies for CF aim to restore the function of the CFTR gene by delivering a functional copy of the gene to the lungs (Gillen & Harris, 2012; Geurts et al., 2021). Identification of the specific CFTR mutation that a patient has, which guides the design of the gene therapy, is also obtained from genetic testing. However, in cases of CF, researchers are developing delivery methods such as nanoparticles and nebulizers that can deliver the therapeutic gene to the lungs (Cortez-Jugo et al., 2022).

While gene therapy holds great promise, there are still challenges associated with its application. In addition to the complexity of delivering genetic material to the appropriate cells in the body, there are also concerns about the potential side effects of gene therapy. These include the potential for the immune system to attack the therapeutic gene and the integration of the therapeutic gene into unintended locations in the genome.

3.3 Genomic-based cancer diagnostics

Cancer is a complex and heterogeneous disease that affects millions of people worldwide. It is caused by genetic alterations in normal cells that lead to uncontrolled growth and dissemination of abnormal cells (Roy & Saikia, 2016). The diagnosis and treatment of cancer have traditionally relied upon the analysis of histopathological features and the patient's clinical presentation. However, these methods can be subjective and may not accurately reflect the underlying genetic changes driving cancer growth (Roy & Saikia, 2016; Biller & Schrag, 2021). Hence, genomic profiling of tumors has emerged as a powerful tool for cancer diagnostics and treatment decision-making. This technology allows for detecting genetic alterations responsible for cancer development and progression (Chakravarty & Solit, 2021). These alterations can be used to identify targeted therapies that selectively inhibit the growth of cancer cells and spare normal cells from toxic side effects.

The application of genomic-based cancer diagnostics is critical in the context of personalized medicine (Litton et al., 2019). In cancer treatment, personalized medicine involves identifying the genetic alterations driving cancer growth and selecting therapies targeting those

alterations (Morganti et al., 2019). This approach has shown the potential to improve outcomes for cancer patients by reducing toxicity and increasing treatment efficacy. Genomic profiling can be performed using various techniques, including NGS and microarray analysis. NGS allows for the sequencing of a tumor's entire genome or exome, providing a comprehensive view of all genetic alterations present (Morganti et al., 2019; Pauli et al., 2017). Microarray analysis, on the other hand, allows for detecting specific genetic alterations associated with cancer, such as gene amplifications or deletions (Tao et al., 2017).

One of the main benefits of genomic profiling is the identification of potential resistance mechanisms (Kadioglu et al., 2021). Cancer cells are known to resist targeted therapies over time, leading to disease progression and treatment failure (Roy & Saikia, 2016; Biller & Schrag, 2021). Genomic profiling can identify the genetic alterations responsible for resistance, allowing for the development of new therapies that can overcome these resistance mechanisms (Kadioglu et al., 2021). Genomic profiling can also be used for cancer diagnosis, particularly in cases where traditional diagnostic methods are inconclusive (Biller & Schrag, 2021). For example, some types of cancer are difficult to diagnose using histopathological features alone. Genomic profiling can provide additional information confirming a diagnosis and guiding treatment selection. In addition to its use in diagnosis and treatment selection, genomic profiling can also be used to monitor cancer progression and response to therapy. By analyzing the genetic alterations present in sequential tumor samples, clinicians can assess whether a therapy is effective and adjust treatment as needed (Chakravarty & Solit, 2021).

Notwithstanding the many benefits of genomic-based cancer diagnostics, there are still challenges associated with the technology. One of the main challenges is the interpretation of genomic data. Identifying potentially actionable genetic alterations requires careful analysis and interpretation, which can be time-consuming and technically challenging. Additionally, many genetic alterations are poorly understood and may not have targeted therapies available. Another challenge is the cost of genomic profiling. While the cost of sequencing has decreased significantly in recent years, it is still relatively expensive compared to traditional diagnostic methods, which may limit access to genomic-based cancer diagnostics for some patients.

3.4 Drug repurposing

The application of genomics in drug development has not only led to the discovery of new drug targets but also the repurposing of existing drugs for new therapeutic indications (Spreafico et al., 2020). Genomics-based drug repurposing involves the identification of molecular targets and pathways relevant to different diseases, not just the ones for which the drug was initially developed. This approach has several advantages because it can significantly reduce the time and cost of developing new drugs (Russell et al., 2013; Spreafico et al., 2020). By analyzing the shared genetic and molecular pathways between seemingly unrelated diseases, researchers can discover new uses for drugs that have already been approved by regulatory authorities for other indications.

One of the most well-known examples of genomics-driven drug repurposing is the case of the drug thalidomide. Thalidomide was originally developed in the 1950s as a sedative and antiemetic drug but was subsequently withdrawn from the market due to severe teratogenic effects (Ridings, 2013; Vargesson & Stephens, 2021). However, in the early 1990s, thalidomide was reintroduced to treat leprosy based on its anti-inflammatory properties. Its use in leprosy

led to its repurposing for several other indications, including multiple myeloma and some autoimmune diseases. In 2006, thalidomide was approved for treating multiple myeloma, demonstrating the potential of genomics-based approaches to identify new therapeutic uses for existing drugs (Vargesson & Stephens, 2021). Another example of genomics application in drug repurposing is in the treatment of cancer. Researchers have discovered shared genetic mutations and molecular pathways that can be targeted with existing drugs by analyzing the genomic data from different cancer types. For instance, in 2012, the FDA approved the use of a drug called vemurafenib, that was originally developed to target BRAF mutations in melanoma, for the treatment of metastatic melanoma. Subsequent genomic studies revealed that vemurafenib was also effective in targeting BRAF mutations in other cancer types, such as non-small cell lung cancer and colorectal cancer (Garbe & Eigentler, 2018).

Genomics-driven drug repurposing has also been used to treat rare diseases without effective treatments. For example, in 2012, researchers discovered that an existing drug called miglustat, which is approved for the treatment of Gaucher disease, could also be repurposed for the treatment of Niemann-Pick disease. This rare lysosomal storage disorder causes neurodegeneration, and there are currently no effective treatments available. However, studies have shown that miglustat can improve neurological symptoms and cognitive decline in patients with Niemann-Pick disease, demonstrating the potential of genomics-based approaches to identify new therapeutic uses for existing drugs (Pineda et al., 2018).

In conclusion, genomics' incorporation into pharmaceutical research and development has ushered in a new era of precision medicine. The ability to analyze genetic information has revolutionized drug discovery, personalized medicine, pharmacogenomics, and drug repurposing. This chapter seeks to provide a comprehensive overview of the exciting applications and implications of genomics in the pharmaceutical industry, emphasizing its potential to revolutionize healthcare and improve patient outcomes.

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Antiviral history and viral targets for antiviral therapy

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1. History of viruses and antiviral therapy

The history of virology is fascinating and could be divided into different eras. Due to their parasitic behavior, viruses greatly impact the development and history of their hosts. When closely examined, almost all organisms have viral parasites; as such, even the tiniest living entities have a major impact on other life forms. The historical impact of viral diseases on human health has prompted virologists to make remarkable efforts to research, comprehend, and eliminate these pathogens. The virologists have significantly advanced scientific research by illuminating novel principles governing life processes. The microbial world containing bacteria, fungi, and protozoa was fully discovered in the late 19th century (Woese & Goldenfeld, 2009). The eminent German pathologist and anatomist Friedrich Gustav Henle (1809–85) discovered Henle's loop (a long U-shaped portion of the tubule that conducts urine within each nephron of the kidney of reptiles, birds, and mammals) and was the great-grandfather of the virologist Werner Henle, who proposed, as early as 1840, the presence of tiny biological entities smaller than bacteria, as they were too small to be seen using the light microscope, that could be the causative agents of unknown diseases. However, his theories were rejected because there was no evident proof for the existence of such

submicroscopic biological entities. During that era, three significant breakthroughs in microbiology came together to pave the way for the conception of a submicroscopic agent that would later be known as a virus. The disproving of spontaneous generation theory was the first breakthrough at that time when the French biologist Louis Pasteur (1822–95), the father of microbiology, employed the swan-neck bottle experiment to demonstrate this theory. He made evident throughout the course of his fermentations' research that "distinct kinds of microorganisms are linked to various types of fermentations," and he expanded this idea to disease mechanisms where there is a unique microorganism that is responsible for a specific disease (Bordenave, 2003). Using this as a foundation, the German microbiologist Heinrich Hermann Robert Koch (1843–1910), a student of Jacob Henle, proved for the first time that *Bacillus anthracis* (Gram-positive, rod-shaped spore-forming bacteria) was the etiological agent of Anthrax disease in 1876 (Milzbrand-Krankheit, 2010). Moreover, he proved that *Mycobacterium tuberculosis* (curved rod-shaped bacteria with a waxy coat containing mycolic acid), also known as Koch's bacillus, was the causative agent of human tuberculosis disease in 1882. The third major breakthrough in the science of microbiology was developed by the British surgeon Joseph Lister (1827–1912), who revolutionized medical microbiological techniques through the use of "antiseptic techniques" in surgery (Jessney, 2012). Continually, Robert Koch developed solid media and pure culture techniques for isolating and propagating one type of microorganisms and visualization of microbes using staining methods to distinguish between different microbes under the microscope (Kaufmann & Schaible, 2005). Robert Koch acknowledged some of Jacob Henle's original concepts in four statements, which are known as Koch's postulates or "germ theory of disease" to determine if the organism was the etiological agent of a disease. These statements could be summarized as follows: The microorganism must be found in a diseased organism, not a healthy one; the microorganism must be obtained in pure culture from the diseased organism; once introduced into a healthy living organism, it should cause the same disease; and the microbe must be reisolated from the inoculated host organism (Bordenave, 2003; Woese & Goldenfeld, 2009). Notably, Koch's postulates are still relevant today.

By the late 19th century, these ideas took hold as the prevailing paradigm of the microbiology field (Rivers, 1937). Pasteur, Koch, and Lister were considered "pioneers of modern medicine" as they created a unique experimental strategy for medical purposes. The idea of a virus wasn't even conceived until these concepts collapsed and failed to detect the etiological agent of certain fatal diseases. The story of viruses began at Wageningen, Holland, roughly in 1879 when the German agricultural chemist Adolf Eduard Mayer (1843–1942) started to study diseases associated with "tobacco" (it is the common name for the members of the genus *Nicotiana*) (Schiavinato et al., 2020). Mayer observed dark and light spots on the leaves of tobacco during his studies, and these spots inspired him to name this infection "tobacco mosaic disease." He was the first to transmit viral diseases in plants when he applied Koch's postulates through the inoculation of healthy tobacco with diseased plant juice from diseased tobacco leaves (Bos, 2000). Koch's postulates could not be supported because neither fungal nor bacterial cells could be reliably cultivated or found in this diseased juice; however, these investigations proved the contagious nature of this disease. Mayer continued his attempts to understand the biological nature of the infectious agent that produces the spots on tobacco's leaves, but he finally failed to identify it (Mayer, 1882). It is noteworthy to mention that Pasteur used viruses to develop the rabies vaccine in 1885, but he didn't look

into the peculiar characteristics of the infectious agent at this time (Hoenig et al., 2018). With tobacco research, the Russian botanist Dmitri Ivanofsky (1864–1920) spent 3 years (1887–1990) studying the same disease to find out the contagious agent that could induce infections in healthy plants. In addition to the same procedures employed by Mayer, Ivanofsky used Chamberland filter (a filter made by Charles Chamberland from unglazed porcelain to purify water from bacterial cells) to filtrate the diseased plant extract, but he failed to cultivate the infectious agent from the filtrate, and thus his suppositions did not meet Koch's postulates (Kruger et al., 2000). In 1892, he reported that "the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles" (Ivanowski, 1968). His thoughts were constrained by the fact that unfilterable bacteria were the causal agent, or maybe the filter was not efficient to catch them. He finally introduced the idea of the existence of nonliving organisms or toxins that cause the disease, as they were unculturable during his investigations. Although Ivanofsky failed to introduce the infectious agent to the scientific community, his experiments significantly offered a way to define the infectious agent as a virus. The majority of scientists were prevented from adopting a distinct perspective for their results due to the orthodoxy of the time and the clear success of Koch's postulates. In line with Ivanofsky's experiments, another attempt to define the identity of the causative agent of tobacco mosaic disease was carried out by the Dutch microbiologist Martinus Willem Beijerinck (1851–1931), who demonstrated that the filtered leaf extract of tobacco could be diluted and retain its infectivity after growth and reproduction in a developing plant tissue. The autonomous reproducibility of the agent only in a growing plant tissue made it impossible to be a toxin (as proposed by Ivanofsky). These findings paved the way for the discovery of an obligatory parasite that reproduces only in living tissues, smaller than the smallest known organisms at that time (bacteria). Beijerinck revolutionized the science of microbiology by defining the causative agents as "contagium vivum fluidum," the Latin term for contagious living liquid, which is considered the first expression to describe the virus (Beijerinck, 1898). Exploring the nature of "filterable agents" (the useable description of viruses at that time) was the state-of-art at that time, where scientists were eager to know if the "contagium vivum fluidum" was really in a liquid state or had a definitive cellular structure like any other organism. In 1898, the German bacteriologists Friedrich Loeffler (1852–1915), together with Paul Frosch (1860–1928), were the first to isolate filterable agents from animals, the causative agent of foot and mouth disease (Brown, 2003). Some time later, the American physician Walter Reed (1851–1902) and his Cuban team discovered the first filtrable agent to cause a human disease, yellow fever disease, in 1901 (Lustig & Levine, 1992). In 1917, the French-Canadian microbiologist Félix Hubert d'Hérelle (1873–1949), who devised the "Plaque Assay," discovered another obligate parasite that infects bacterial cells (Summers, 2017). Beijerinck then coined the term "virus" (a Latin word means poison or slimy liquid) to refer to any infectious agent that does not fulfill Koch's postulates (King, 1977). In 1939, the tobacco mosaic virus (TMV) was visualized by the German entomologist Gustav Adolf Kausche and his colleagues, who could identify, for the first time in history, the virus under the electron microscope and finally depicted its structure (Kausche et al., 1939).

The development of antiviral therapy, started around the early 1950s, has been one of the notable scientific accomplishments of the last century. It was associated with the discovery of anticancer therapies such as the pyrimidine analog, idoxuridine, where it also showed to

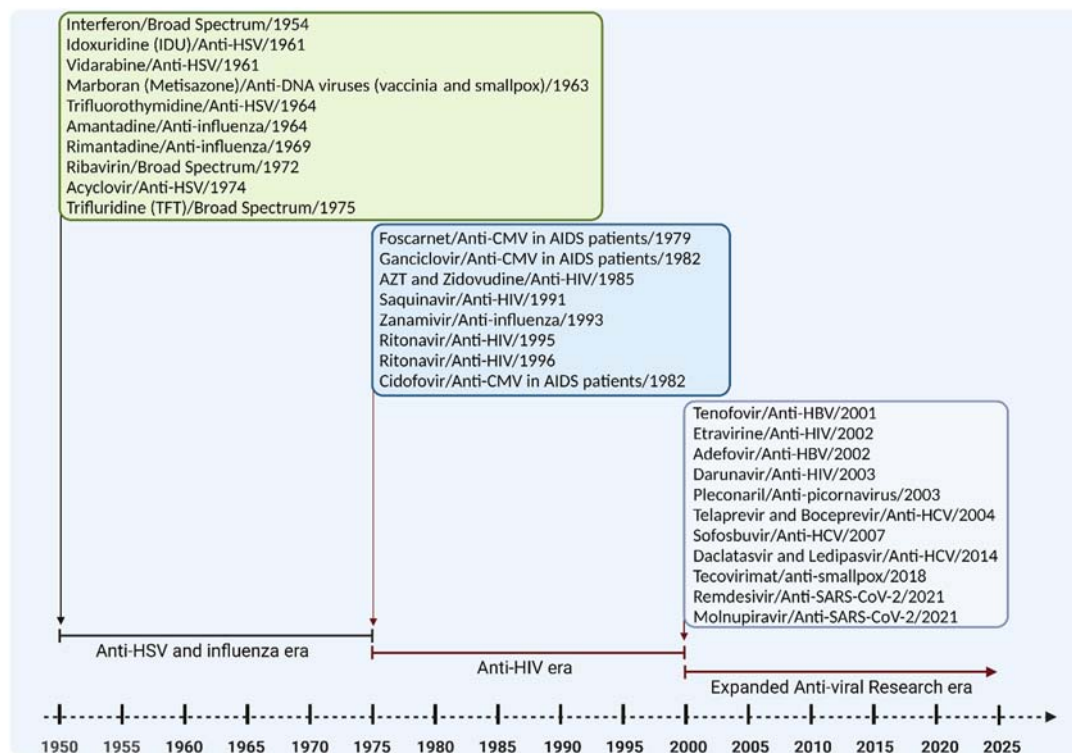


FIGURE 9.1 Important landmarks and era in the history of antiviral drug development. Created using Biorender.com.

inhibit the viral DNA replication of herpes simplex virus (HSV) (De Clercq, 1997). The emergence of the first antiviral medication for humans, idoxuridine, and its subsequent approval in 1963 to treat human keratitis associated with HSV infection marked the start of a new phase in the development of antiviral medications (De Clercq, 1997). Global attention has been paid to antiviral research since the emergence of the human immunodeficiency virus, which causes acquired immunodeficiency syndrome (AIDS) (De Clercq & Li, 2016). A continuous flow of efficacious therapeutics is being created as one of the major controls and prevention strategies against the expanding variety of viral diseases (Fig. 9.1).

2. Viruses' composition and specific viral targets

Viruses are pervasive parasitic agents of bacteria, plants, and animals that must have a host to survive. Viruses can appear in various forms. The two categories of enveloped and nonenveloped viruses, or those that do or do not possess a lipid-bilayer membrane, are the ones that are most broadly distinguished. The molecular organization of viruses determines how they are further classified as simple rod-shaped, icosahedral, or complex head and tail

viruses. The viral genome may be DNA or RNA, single-stranded (ss) or double-stranded (ds), positive or negative polarity, which is a unique feature among organisms, and segmented versus nonsegmented genomes. The viral genome is contained within and shielded by a protein or proteolipid coat called an envelope. Since it detects a vulnerable host, it attaches to the host cell receptor, and upon attachment, the virus itself or its genome enters the host and manipulates the host cell to create novel viral components for its progeny to be assembled and released from the infected cell (Fields BN: *Fields' Virology*, 2007).

Most virus–host protein interactions have only been studied in a few strains that are widely used in laboratory settings. For instance, influenza viruses comprise a plethora of types, subtypes, and strains with different pathogenicity, transmissibility, and pandemic potential (Mostafa et al., 2018). They comprise a major class of human respiratory pathogens, responsible for several morbidities and mortalities worldwide (Mostafa et al., 2018).

Following infection of permissive mammalian host cells, viral constituents and viral-replication intermediates trigger multiple signal transduction pathways and innate immune responses. To discuss recent advances in our understanding of cellular cascades and innate immune responses induced by influenza A virus (IAV) infection, we introduce an insight into the replication cycle of IAV.

2.1 Replication cycle of IAV

The replication cycle of IAV (Fig. 9.2) is distinguished into four stages: (1) Virus entry into the host cell and genome release; (2) replication and transcription of viral genome; (3) post-translational processing and virus assembly; (4) genome packaging and virus release.

2.1.1 Virus entry and genome release

The IAV hemagglutinin (HA) glycoprotein starts the replication cycle by recognizing and binding to specific sialyloligosaccharide receptors on the host cell surface, namely α 2,3- and α 2,6-galactose sialic acid (SA) linkages. The α 2,6-SA linkage is preferentially recognized by mammalian-type IAV, whereas the avian-type IAVs recognize the α 2,3-SA linkage. Notably, porcine-specific IAV recognize equally both linkages (Shaw and Palese, 2013; Skehel & Wiley, 2000). Upon virus-receptor binding, the attached viral particle undergoes receptor-mediated endocytosis to invade the host cell as an encapsulated endosome. In the late stage of endocytosis, the pH of the endosome is lowered (pH = 5–6) and this subsequently mediates a conformational change in HA1/HA2. While the HA1 receptor-binding domain is maintained, the N-terminus of the HA2 comprising the fusion peptide is exposed. The exposed fusion peptide inserts itself into the endosomal membrane, leading to direct contact and fusion between the viral envelop and endosomal cell-derived membrane (Huang et al., 2003; Skehel & Wiley, 2000). Meanwhile, the increased proton concentration resulted in a passive inflow of protons into the viral core via the matrix 2 (M2) ion channel viral protein (Pinto & Lamb, 2006). The low pH within the virions dissociates the viral ribonucleoprotein (vRNP) complexes from the matrix 1 (M1) viral protein, releasing the vRNPs into the host cell cytoplasm (Pinto & Lamb, 2006; Shaw and Palese, 2013). To start the viral genome replication and gene transcription, the vRNPs are translocated into the nucleus of the host cell by the help of the cellular

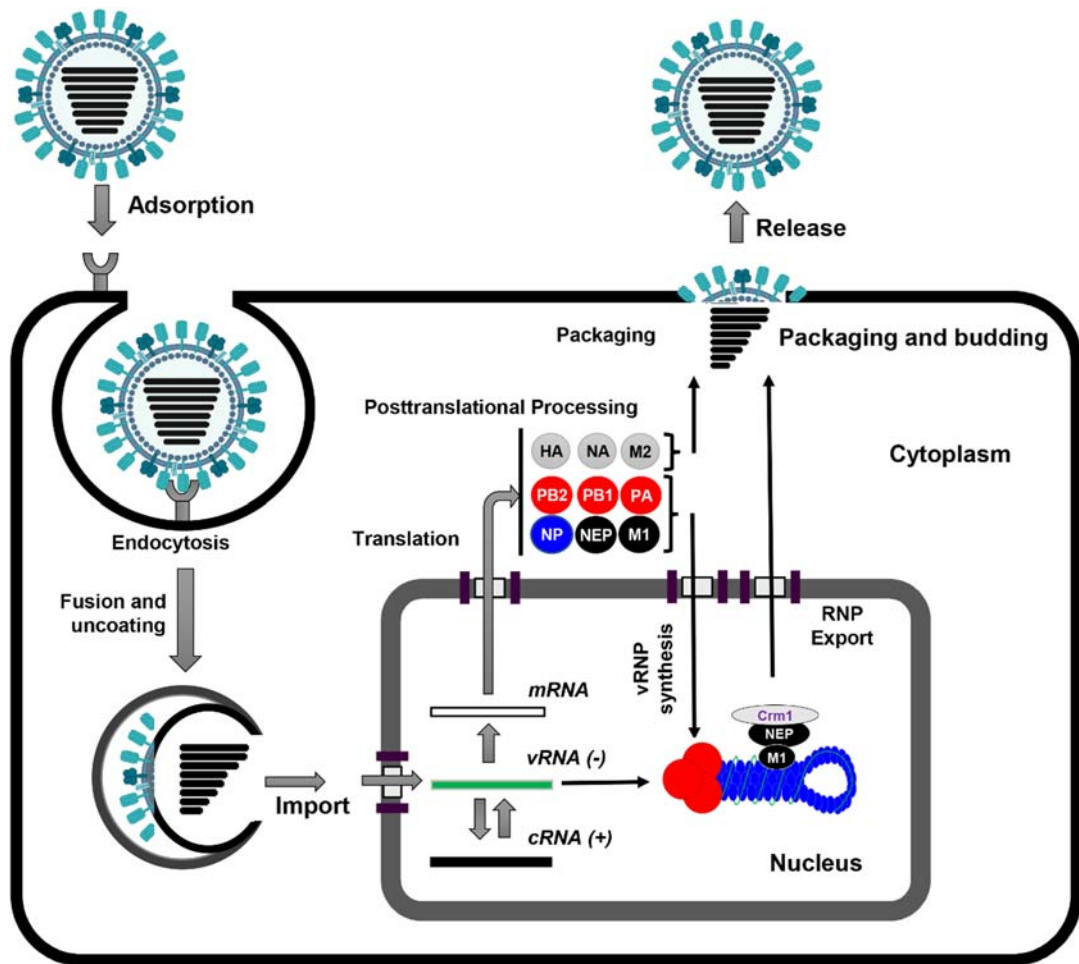


FIGURE 9.2 Replication cycle of IAV: The virion adheres to the host cell, recognizes, and binds to the sialic acid receptor on the host-cell surface. Using receptor-mediated endocytosis, the attached viral particle is taken into the cytoplasm of the host cell using an endocytosis-mediated mechanism. The pH of the endosome as well as the virion core become low (Milzbrand-Krankheit, 2010), leading to fusion of the viral and the endosomal membranes and release of the vRNP into the cytoplasm. The vRNPs are imported into the nucleus to start the replication of the viral genome and the transcription of the viral genes. The newly synthesized viral mRNAs of the viral segments are then translocated to the cytoplasm to start viral protein synthesis. The viral envelop proteins (HA, NA, and M2) are subjected to posttranslational modifications and then transported to the host cell membrane. Another set of viral proteins (PB2, PB1, PA, NP) are imported back into the nucleus to generate the new vRNPs. Newly synthesized vRNPs are exported from the nucleus to the cytoplasm using a Crm1-dependent nuclear export process mediated by the viral NEP and M1 proteins. After the assembly of the eight viral vRNPs at the host cell surface where the viral surface proteins and M1 are arranged, mature virions bud from the cell surface and are released, a process mediated by the viral NA protein, to begin another replication cycle. Created using Biorender.com.

nuclear import machinery (importin- α and - β) by interacting with the different nuclear localization signals on the vRNP-forming viral proteins, mainly by interaction with the viral nucleoprotein, NP (Boulo et al., 2007).

2.1.2 Replication and transcription of the viral genome

Unlike other RNA viruses, the replication of IAV genome depends on the nuclear functions of the host cell. The imported negative sense vRNAs of the eight viral segments act as templates for the synthesis of the corresponding capped and polyadenylated mRNA transcripts. The replication of the vRNAs is initiated by generating a positive sense complementary RNA copy (cRNA), which is subsequently used as a template to synthesize new vRNA (Shaw and Palese, 2013). The transcription and replication functions of the IAV genome are mediated by the viral RNA-dependent RNA polymerase (RdRp) complex made of the PB2, PB1, and PA polymerases, included in vRNP complexes.

To prime the viral transcription of the vRNA, the viral RdRp steals the cap structure of premature cellular mRNAs (Plotch et al., 1981). The PB2 subunit of the RdRp recognizes and binds to the 5' cap structure (m^7GpppX) of cellular mRNAs; the PA cleaves the PB2-bound cap structure approximately at 10–13 nucleotides from their 5'-ends by the virtue of its endonuclease activity (Nayak et al., 2009; Scheiffele et al., 1999). This process, which is carried out by PB2 and PA to pinch off the 5' cap structure of cellular mRNAs, is known as "cap-snatching." It is initiated by the binding of the catalytic subunit of RdRp (PB1) to the 5'-end of vRNA. The cap structure is required for efficacious binding of ribosomes to the newly synthesized viral mRNAs. Following the cap-snatching act, the chain elongation of the viral mRNA is carried out by the PB1 subunit and terminates through a steric hindrance caused by several uridine residues (5–7U) located about 16 nt before the 5'-end of each vRNA (Perez & Donis, 2001).

The opportunistic influence of IAV on the nuclear machineries of the host cell is not limited to cap-snatching, but also extends to take advantage of the host splicing machinery to expand its coding capacity through the generation of spliced forms of the viral matrix (M) and nonstructural (NS) mRNA transcripts (Inglish et al., 1980; Shih et al., 1998). After capping, the splicing process of the premature mRNAs is carried out by the spliceosome, a complex of small nuclear RNAs associated with a set of specific proteins, which coordinate together to remove the introns between splice donor and acceptor sites. The premRNA associates with an exon junction complex (EJC) to regulate the destiny of the spliced mRNA transcripts. The EJC affects the localization, stability, and translation of the intronless mRNA transcripts (Le Hir & Andersen, 2008; York & Fodor, 2013).

Contrary to the host cell, which utilizes specific polymerases for mRNA-adenylation, the capped unspliced/spliced viral mRNAs are polyadenylated by the same viral polymerases used for transcription (Shaw and Palese, 2013). Notably, the synthesis of mRNAs for the NP and NS segments prevails early in replication. However, the production of mRNA encoding the HA, NA, and M segments becomes more dominant at a late stage. The abundance of mRNA transcripts for the viral polymerase subunits (PB2, PB1, and PA) is comparatively low during the replication cycle except at the earliest times (Yamanaka et al., 1988). The capped and polyadenylated mRNA transcripts of the viral genome are exported into the cytosol where they are translated into the corresponding viral proteins using the translation machinery of the host cell.

It was proposed that the accumulation of the newly synthesized soluble NP in the nucleus, not in a complex with vRNPs, marks the turning point to switch from the transcription activity of the parent vRNPs (mRNA synthesis) to replication (vRNA $\rightarrow$ cRNA $\rightarrow$ vRNA) (Shaw and Palese, 2013). However, it was shown that the higher amounts of NP have no impact on the replication of the viral genome (Mullin et al., 2004). Others favor the concept that the transcription-to-replication switch is controlled by the shortage of the stabilizing vRNP proteins (NP and the polymerases) at early stage of replication leading to the degradation of unprotected cRNA and more accumulation of the protected capped/polyadenylated mRNA (Vreede et al., 2011). Later, the newly synthesized viral NP and polymerases help to stabilize the cRNA by encapsidation at a late stage and thereby promoting replication (Vreede & Brownlee, 2007; Vreede et al., 2004).

The nuclear export protein (NEP) is also suggested to regulate the transcription/replication activities due to the observation that the accumulation of this viral protein is associated with an accretion in transcription and an upregulation in the replication process (Robb et al., 2009). In addition, NEP is important to generate small viral RNAs (svRNAs), 22 to 27 nt in length, corresponding to the 5' end of each vRNA segment. These svRNAs are involved in the initiation of vRNA synthesis, but not cRNA synthesis. Consequently, the polymerase switches to replication mode when these svRNAs are available (Perez et al., 2010). The transcription-to-replication switch is also assumed to occur due to the accumulation of newly synthesized free polymerase complex components, which favor cRNA/vRNA production over the generation of mRNAs (Jorba et al., 2009). Unlike transcription of the IAV genome, the RdRp utilizes the secondary structure of the partially complementary noncoding regions of the vRNA as an internal promoter to initiate the synthesis of cRNA (Flick et al., 1996). Subsequently, the vRNA is replicated using the newly synthesized cRNA as a template to produce more vRNA copies (Shaw and Palese, 2013).

2.1.3 Posttranslational processing and virus assembly

Following the synthesis of the viral proteins and virus replication, the viral envelope HA, neuraminidase (NA) and matrix 2 (M2) proteins are proceeded through the endoplasmic reticulum for posttranslational glycosylation (except M2) and oligomerization into trimer (HA) and tetramers (NA and M2). Subsequently, these proteins are transferred into the Golgi apparatus where palmitoylation of cysteine residues occurs (HA and M2) in the transGolgi network (Shaw and Palese, 2013). The mature HA, NA, and M2 proteins are then clustered in the lipid rafts on the host cell membrane (Braakman et al., 1991; Doms et al., 1993). The NEP and matrix 1 (M1) proteins are also imported to the nucleus, where M1 bridges the NEP to interact with the nascent vRNPs. The nuclear export of the formed vRNP-M1-NEP complexes to the cytosol through the nuclear pore complex is mediated by interaction with the cellular Crm1 nuclear export machinery in an NEP-dependent manner (Boulo et al., 2007; Bui et al., 2000; Neumann et al., 2000; O'Neill et al., 1998; Paterson et al., 2012). The nuclear export of vRNPs was shown to be induced by membrane accumulated HA via protein kinase C-dependent activation of the Raf/MEK/ERK signal transduction cascade (Marjuki et al., 2006) and supported by virus-induced apoptotic caspase-dependent widening of the nuclear pores (Mühlbauer et al., 2015).

2.1.4 Genome packaging and virus release

The packaging of the vRNPs into apical protein-containing membranous vesicles begins when the frame of the progeny viral particle is structured. Substantial amounts of M1 molecules in the cytoplasm interact with themselves, the surface viral envelop proteins (HA, NA, and M2), and the lipid rafts of host cell membrane, inducing the formation of virus-like particles as AN outward curvature of the plasma membrane (Burleigh et al., 2005). Initially, two models were proposed to explain the packaging of viral genomic segments into virions: the random incorporation model (Bancroft & Parslow, 2002; Enami et al., 1991), and the selective incorporation model (Smith & Hay, 1982). The former model predicts that viral genomic segments are randomly packaged into virions; whereas, the second model predicts that packaging signals present in each viral segment control precisely the segments to be packaged into the virions. Recent electron microscopy and biochemical evidence favor the selective incorporation model over the random incorporation model (Fujii et al., 2003; Haralampiev et al., 2020). Following packaging, the M1 protein, associated with the packaged vRNPs, interacts with the cytoplasmic tail of HA, NA, and M2 proteins (Chen et al., 2008). This interaction acts as a budding signal (Chen et al., 2008; Nayak et al., 2004, 2009) to release the newly generated viral particles, with the viral NA protein resolving the binding between the SA-containing receptors on host cell membrane and the HA protein of newly synthesized virions (Shaw and Palese, 2013).

Molecular biology and virology, as well as the advancement of experimental and computational approaches, have all accompanied the important shift of adequate knowledge of virus structures and its replication cycle inside the host cell. A family of medications known as antiviral therapies is used to treat viral infections. There are two main techniques for developing these antiviral medicines: (1) Targeting the viruses themselves, or (2) affecting host cellular pathways and/or proteins required for viral replication (Kausar et al., 2021). Several antiviral medications (Woese & Goldenfeld, 2009) that directly target the viruses include inhibitors of virus attachment and entry; inhibitors of the uncoating process; and viral enzyme inhibitors including viral polymerase, protease, reverse transcriptase, and integrase inhibitors (Tables 9.1 and 9.2).

2.2 Inhibitors of viral attachment and entry processes

A host cell receptor allows viruses to adhere to the host cell membrane. Some viruses have unique surface structures that can identify and attach to specific cellular receptors, which are dispersed across the host cell membrane. The next step is a conformational change in the binding site by specific enzymes to further permit the viral entry into the host cell. This conformational change is a common factor that increases the binding affinity and facilitates virus entry into the host cell. Some antiviral drugs inhibit virus attachment to the host cell receptors (Table 9.2). Development of novel antiviral medications now heavily emphasizes the principle of inhibiting virus entry into the host cell. The first Food and Drug Administration (FDA)-approved antiviral drug acting by inhibiting the viral particles of HIV to attach and enter the host cell was enfuvirtide (Fuzeon or T-20) (Hardy & Skolnik, 2004). This drug is very similar to the viral envelope protein called gp41 which recognizes CD4⁺ of the T cells. It hinders gp41's attachment by blocking the CD4⁺, binding site. The virus-

TABLE 9.1 Antiviral drugs and the corresponding viral targets in an ascending order according to their discovery/license date.

Antiviral	Target/virus	Class	Year	Refs.
β -Thiosemicarbazone	Inhibit virus replication/ vaccinia virus (VACV)	Thiosemicarbazone	1950	Hamre et al. (1950) and Quenelle et al. (2006)
Interferon (IFN)	Inhibit virus replication/ hepatitis B (HBV)	Nucleoside analogue	1957	Mao et al. (2011) and Zein (1998)
IDU (idoxuridine)	Inhibit virus replication/ herpes simplex virus (HSV)	Nucleoside analogue	1959	Hanna and Wilkinson (1965) and Mills (2017)
Hydroxybenzyl benzimidazole	Inhibit virus replication/ picornaviruses (Coxsackie and Echo)	Benzimidazoles	1961	Klein et al. (2000) and Siegl and Eggers (1982)
Guanidine	Inhibit virus replication/ Picornaviruses (Coxsackie and Echo)	Amino- carboxamidine	1961	Klein et al. (2000) and Nikolaeva and Galabov (1999)
Marboran	Inhibit virus replication/ variola virus (VARV)	Thiosemicarbazone	1963	Bauer et al. (1963) and Prichard and Kern (2005)
Trifluoro-thymidine (TFT)	Inhibit DNA replication/ HSV	Nucleoside analogue	1964	Kaufman and Haw (2012) and Kaufman and Heidelberger (1964)
Amantadine	M2 ion channel blocker/ IAV	Tricyclic amine	1964	Davies et al. (1964) and Hay (1989)
Ribavirin	Inhibit RNA replication/ Respiratory syncytial virus (RSV)	Nucleoside analogue	1972	Hruska et al. (1980) and Leyssen et al. (2008)
Adenine arabinoside (vidarabine)	Inhibit DNA replication/ HSV	Adenosine analogue	1976	Pavan Langston and Buchanan (1976) and Suzuki et al. (2006)
Acyclovir (acycloguanosine)	Inhibit DNA replication/ HSV	Acyclic guanosine analogue	1977	Collins (1983) and Suzuki et al. (2006)
2,3-Dihydroxypropyl- adenine (DHPA)	Inhibit viral replication/ broad spectrum (VACV, HSV, measles virus (MeV), and vesicular stomatitis virus (VSV))	Nucleoside analogue	1978	De Clercq et al. (1978) , Temple et al. (1979) , and Warmke and Lee (1978)
Phosphonoformic acid (PFA)	Inhibit DNA polymerase activity/HSV	Pyrophosphate analogue	1979	De Clercq et al. (1979) and Norén et al. (1983)
Bromovinyldeoxyuridine (BVDU/brivudine)	Inhibit viral replication/ varicella-zoster virus (VZV) and HSV	Nucleoside analogue	1979	Clercq and Descamps (1981) and de Clercq et al. (1980)

TABLE 9.1 Antiviral drugs and the corresponding viral targets in an ascending order according to their discovery/license date.—cont'd

Antiviral	Target/virus	Class	Year	Refs.
Ganciclovir	Inhibit DNA replication/ Cytomegalovirus (CMV)	Acyclic guanosine analogue	1982	Matthews and Boehme (1988) and Ussery et al. (1988)
Azidothymidine (AZT/ zidovudine)	Inhibit viral replication/ human immunodeficiency virus (HIV)	Nucleoside analogue	1985	Furman and Barry (1988) and Mitsuya et al. (1985)
Didanosine (ddI) and zalcitabine (ddC)	Inhibit mitochondrial DNA (mt DNA) polymerase/HIV	Dideoxynucleoside (ddN) analogs	1986	Benbrik et al. (1997) and Johnston and Hoth (1993)
Cidofovir (HPMPC or Vistide)	Inhibit DNA replication/ African swine fever virus (ASFV), human adenovirus, VACV, HSV, and human cytomegalovirus (HCMV)	Acyclic nucleotide analogue	1987	Clercq (2010) , De Clercq (2002) , and Li, Yue et al. (2021)
Famciclovir	Inhibit viral DNA polymerase/HSV	Nucleoside analogue	1989	Hodge (1993) and Vere Hodge et al. (1989)
Saquinavir (Invirase)	Inhibit protease enzyme/ HIV	Aspartic acid derivative	1990	Debouck and Metcalf (1990) and Pereira and Vale (2022)
3 TC (lamivudine)	nucleoside analog-reverse transcriptase inhibitor (NRTI)/HIV	Dideoxynucleoside (ddN) analogue	1991	Anderson and Rower (2010) and Van Leeuwen et al. (1995)
Rimantadine	M2 ion channel blocker/ IAV	Cyclic amine	1993	Hay (1989) and Shen et al. (2015)
Tenofovir (PMPA)	nucleoside analog-reverse transcriptase inhibitor (NRTI)/HIV	Acyclic nucleotide analogue	1993	De Clercq (2009) and Iannuzzi and Kleist (2021)
Zanamivir (Relenza)	Neuraminidase (NA) enzyme inhibitor/IAV	Guanidines	1993	Gubareva et al. (2000) and Von Itzstein et al. (1993)
Oseltamivir (Tamiflu)	Neuraminidase (NA) enzyme inhibitor/IAV	Cyclohexene carboxylate ester/ prodrug of the Oseltamivir carboxylate active form	1997	Gubareva et al. (2000) and Hayden et al. (1997)
Adefovir dipivoxil	Inhibit DNA polymerase/ HBV	Nucleotide analogue	2002	Dando and Plosker (2003) and Segovia et al. (2012)
Peramivir (Rapivab)	Neuraminidase (NA) enzyme inhibitor/IAV	Guanidines	2009	Hata et al. (2014)
Baloxavir marboxil	Cap-dependent endonuclease inhibitor/ polymerase acidic endonuclease inhibitors	Prodrug of the active baloxavir acid active form	2018	Hayden et al. (2018) and Locke et al. (2019)

TABLE 9.2 Specific virus-targeting antiviral drugs.

Antiviral agent	Trade name	Chemical class	Virus(es)	Drug target	Status	Refs.
T-20 (Enfuvirtide)	Fuzeon	Synthetic polypeptide	HIV	Fusion	FDA-approved	Yuxian He et al. (2008) and Zhu et al. (2015)
Virus inhibitory peptide (VIRIP)	N/A	Natural peptide	HIV	Entry	Clinical trials (phase III)	Müller et al. (2018) , Münch et al. (2007) , and Quiñones-Mateu and Schols (2011)
Maraviroc (UK-427,857)	Selzentry or Celsentri	Cyclohexane and triazole derivative	HIV	Entry	FDA-approved	Kuritzkes et al. (2008)
RAFIs	N/A	Nucleoside derivatives	Enveloped viruses (HCV, HSV, IV and ASFV)	Fusion	In vitro	Colpitts et al. (2013) , Hakobyan et al. (2016, 2018) , and St Vincent et al. (2010)
Rimantadine	Flumadine	Cyclic amine and alpha-methyl derivative of amantadine	Hepatitis A virus (HAV) and IAV	Uncoating/replication	In vitro for HAV/FDA-approved for influenza virus	Caceres et al. (2022) , Galvão et al. (2014) , and Sasaki-Tanaka et al. (2022)
Amantadine	Gocovri	Synthetic tricyclic amine	IAV	Uncoating	FDA-approved	Caceres et al. (2022)
Pleconaril	Picovir	Natural heterocyclic compound	EV-71 and rhinovirus	Viral capsid/uncoating	In vitro, preclinical (for EV-71), and clinical trials (phase III for rhinovirus)-/ (rejected from FDA approval for)	(Zhang, Zhou et al. (2012), Jun Wang et al. (2022), and Pevear et al. (2005)
Acyclovir	Sitavig, Xerese and Zovirax	Nucleoside analog	HSV and VZV	DNA polymerase	FDA-approved	Rajalakshmi et al. (2010) , Schalkwijk et al. (2022) , and Shiraki (2018)
Cidofovir	Vistide	Nucleotide analog	HSV, CMV, poxviruses, adenovirus, VZV, EBV, papilloma virus, and polyomavirus	DNA polymerase	FDA-approved (for CMV)	Graciela Andrei and Snoeck (2010) , De Clercq (2002, 2003) , Tichý et al. (2011) , and Upadhyayula et al. (2013)

Ganciclovir	Cytovene	Nucleoside analog	HSV and CMV	DNA polymerase	FDA-Approved (For CMV)	Gane et al. (1997) , Upadhyayula et al.(2013) , and Wood and Crumacker (1996)
Famciclovir	Famvir	Nucleoside analog	HSV, HBV, and VZV	DNA polymerase	FDA-approved (for HSV and VZV)	Mondal (2007) , Semaan and Parmar. (2022) , and Simpson and Lyseng-Williamson (2006)
Penciclovir	Denavir	Nucleoside analog	HSV	DNA polymerase	FDA-approved	Schmid-Wendtner and Korting (2004) and Tomba et al. (2021)
Valacyclovir	Valtrex	Nucleoside analog	HSV and VZV	DNA polymerase	FDA-Approved	Andrei and Snoeck (2021) and Rajalakshmi et al. (2010)
CMV423	N/A	Nonnucleoside	HCMV and HHV-6 and HHV-7	DNA polymerase	In vitro and preclinical	Bolle et al. (2004) and De Clercq (2003)
Telbivudine	Sebivo or Tyzeka	Nucleoside analog	HBV	DNA polymerase	FDA-approved	Amarapurkar (2007)
Adefovir dipivoxil	Hepsera	Nucleoside analog	HSV, HBV, and retroviruses	DNA polymerase	FDA-approved (for HBV)	De Clercq (2003) , Qaqish et al. (2003) , and Upadhyayula et al. (2013)
Tenofovir	Viread	Nucleoside analog	HBV and retrovirus	DNA polymerase	FDA-approved (for HBV)	De Clercq (2003) and Wassner et al. (2020)
Entecavir	Baraclude (BMS)	Nucleoside analog	HBV	DNA polymerase	FDA-approved	Rivkin (2005)
Ribavirin	Rebetol, Ribasphere, RibaPak, Copegus, and Virazole	Nucleoside analog	HCV, adenovirus, IV, parainfluenza, and RSV	RNA polymerase	FDA-approved (for HCV)	Chan-Tack et al. (2009) , De Clercq and Li (2016) , and Riner et al. (2009)
Aryl benzoyl hydrazide analogs	N/A	Hydrazine derivatives	IAV	RNA polymerase	In vitro	Liu et al. (2022)

TABLE 9.2 Specific virus-targeting antiviral drugs.—cont'd

Antiviral agent	Trade name	Chemical class	Virus(es)	Drug target	Status	Refs.
Remdesivir	Veklury	Nucleotide analog	Ebola virus (EBOV), RSV, Hendra virus, SARS-CoV, MERS-CoV, and SARS-CoV-2	RNA polymerase	FDA-approved (for SARS-CoV-2)	Rubin et al. (2020) and Santoro and Carafoli (2021)
Amprenavir	Agenerase	Hydroxyethylamine sulfonamide derivative	HIV	Proteases	FDA-approved	Adkins et al. (1998) and Highleyman (1999)
Atazanavir	Reyataz	Aza-dipeptide analog	HIV	Proteases	FDA-approved	Choi et al. (2020)
Darunavir	Prezista	Carbamate derivative	HIV	Proteases	FDA-approved	Ghosh et al. (2006)
Indinavir	Crixivan	Synthetic hydroxy-aminopentane amide	HIV	Proteases	FDA-approved	Ghosh et al. (2001) and Folkers (1996)
Nelfinavir	Viracept	Aryl sulfide	HIV and human adenoviruses	Proteases	FDA-approved (for HIV)	Georgi et al. (2020) and James (1997)
Ritonavir	Kaletra, Norvir, and Viekira Pak	L-Valine derivative	HIV, SARS-CoV, MERS-CoV and SARS-CoV-2	Proteases	FDA-Approved (for HIV)	Chandwani and Shuter (2008) and Magro et al. (2021)
Saquinavir	Invirase	Aspartic acid derivative	HIV and SARS-CoV-2	Proteases	FDA-Approved (for HIV)	Bello et al. (2020) , McLean et al. (2009) , and Pereira and Vale (2022)
Tipranavir	Aptivus	Sulfonamide-containing dyhydropyrone	HIV, Zika virus (ZIKV), West Nile virus (WNV), and SARS-CoV-2	Proteases	FDA-approved (for HIV)	Flexner et al. (2005) , Indu et al. (2020) , and Stefanik et al. (2020)
Dolutegravir	Dovato, Juluca, Tivicay, and Triumeq	tricyclic carbamoyl pyridone analog	HIV	Integrases	FDA-approved	Dow and Bartlett (2014) and Miller et al. (2015)

Elvitegravir	Genvoya and Stribild	Quinoline monocarboxylic acid	HIV	Integrases	FDA-approved	Pandey (2014)
Raltegravir	Isentress and Isentress HD	Pyrimidone	HIV	Integrases	FDA-approved	Serrao et al. (2009)
Oseltamivir	Tamiflu and Ebilfumin	Cyclohexene-carboxylate ester	IAV and IBV	Neuraminidases	FDA-approved	O'Hanlon and Shaw (2019) , Shin et al. (2017) , and Sugaya et al. (2007)
Zanamivir	Relenza	Sialic acid-analogue	IAV and IBV	Neuraminidases	FDA-approved	Burnham et al. (2013) , Gubareva et al. (2000) , and O'Hanlon and Shaw (2019)
Peramivir	Rapivab	Guanidines	IAV and IBV	Neuraminidases	FDA-approved (for acute uncomplicated influenza in patients <18 years)	Alame et al. (2016) and Cies et al. (2019)
Laninamivir	Inavir	Acetamide	IAV and IBV	Neuraminidases	Clinical trials (phase III)	Dronova et al. (2021) , Higashiguchi et al. (2018) , and Ishiguro et al. (2018)
Tecovirimat	Tpoxx	imide	Human smallpox, monkeypox, and cowpox disease in adults and pediatric patients	Inhibitor of the orthopoxvirus VP37 envelope wrapping protein	FDA-approved	Frenois-Veyrat et al. (2022) , Grosenbach et al. (2018) ⁸ , and Sherwat et al. (2022)

inhibitory peptide (VIRIP), a naturally occurring, very effective HIV inhibitor, was discovered to inhibit HIV entry by binding with the gp41 fusion protein, and it has been demonstrated that slight alterations of amino acid sequences may significantly boost VIRIP's antiviral efficacy (Münch et al., 2007). Maraviroc (UK-427,857) is another antiretroviral drug that hinders the entry of HIV to its host cell by blocking the cellular receptor CCR5 (Dorr et al., 2005). The chemically synthesized rigid amphipathic fusion inhibitors, which mimic the phospholipid bilayer of cellular membranes, can lower the viral titers of various enveloped viruses such as hepatitis C virus (HCV), HSV, IAV, and African swine fever virus by interfering with the fusion process (Colpitts et al., 2013; Hakobyan et al., 2018; St.Vincent et al., 2010). The cholesterol uptake's cellular receptor "Niemann-Pick C1-like 1 (NPC1L1)" has been demonstrated to be one of the cellular factors that facilitate the entry of HCV viral particles into the host cell, which makes it one of the possible targets for antiviral therapy through antibody-mediated blocking or NPC1L1 gene silencing (Sainz et al., 2012).

2.3 Inhibitors of uncoating process

Acidity of the phagosome/endosome causes cellular and viral proteases, by different means, to become functional in the cytoplasm. Viral nucleoproteins are then released after the proteolytic digestion of the virus capsid or envelope (for nonenveloped or enveloped viruses, respectively). The adamantanes: Amantadine and rimantadine work by inhibiting the M2 ion channel protein of IAV, limiting viral uncoating and consequent release of IAV vRNA into the host cell (Table 9.2). They work by inhibiting its function as a proton ion channel, preventing the virus core from becoming acidic, which is necessary for the viral HA to become active. Rimantadine received its approval for therapeutic use against IAV in 1993, after amantadine in 1964 (Shen et al., 2015). Furthermore, the rise in lysosomal pH made amantadine to inhibit the activity of the viral envelop (E) protein ion channel and interfere with the host cell proteases' activity, preventing severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) from penetration and replicating into host cells (Butterworth, 2021). The anti-picornaviral drug, Pleconaril, works by binding with hydrophobic structures on VP1 protein in the capsid causing structural modifications that affect receptor binding affinity and impair the viral uncoating (Romero, 2001; Sun et al., 2015).

2.4 Viral enzymes inhibitors

2.4.1 Inhibitors of viral DNA and RNA polymerases

Several antiviral medicines were found to be effective against the viral polymerases and impair their replication processes inside the host cell. This class of antivirals is commonly known to belong to nucleotide and nucleoside analogs such as acyclovir, cidofovir, ganciclovir, famciclovir, penciclovir, and valacyclovir. They work by preventing the virus from replicating and spreading to new neighboring cells by selectively inhibiting the viral thymidine kinase (TK), which helps in the phosphorylation of these drugs into their monophosphate forms. As a result, the virus becomes unable to create its own DNA (Zenchenko et al., 2021). One specific activation by a viral TK enzyme into acyclovir mono-phosphate (ACVMP), which is then converted by host cellular kinases to acyclovir di- and triphosphate,

respectively, the active form of acyclovir, highlights significantly the selective activity of Acyclovir against HSV and varicella zoster virus, ending up with competitively inhibiting the viral DNA polymerase after incorporation of ACVMP where it irreversibly binds to DNA template and terminate the chain elongation (Hirsch et al., 1996, pp. 431–466; Reardon & Spector, 1991). Additionally, the antihuman cytomegalovirus (HCMV) compound 2-Chloro-3-pyridin-3-yl-5, 6, 7, 8-tetrahydroindolizine-1-carboxamide (CMV423) demonstrated extremely strong in vitro efficacy (Snoeck et al., 2002), and it also exerts low-level activity against human herpes viruses (HHV6 and HHV7) (Bolle et al., 2004). It works by affecting a phase of the viral replication that comes before the DNA polymerase phase and may occur concurrently with the manufacture of the immediate early antigen. Telbivudine, another example of the viral polymerase inhibitors, is a thymidine nucleoside analogue that effectively combats hepatitis B virus (HBV) infection, and it has been approved by the FDA to treat chronic HBV diseases in 2006 (Paintsil & Cheng, 2009, pp. 223–257). Additional nucleoside analogs that work as viral DNA polymerase inhibitors include adefovir, tenofovir, and entecavir. After replacing viral nucleosides, they effectively inhibit the HBV's DNA polymerase activity, resulting in viral DNA chain termination (Leung, 2005; Woo et al., 2010).

On the same hand, several drugs have been found to be effective against the polymerase activity of RNA viruses, such as ribavirin, a purine nucleoside derivative, which prevents the formation of guanosine triphosphate, hindering viral mRNA capping and impairing the functions of viral RdRp. Ribavirin proved its broad-spectrum antiviral efficacy against several RNA and DNA viruses, including IAV and influenza B viruses (IBV), parainfluenza, Respiratory Syncytial Virus (RSV), HIV, paramyxovirus, arenaviruses, and HCV (Chan-Tack et al., 2009; Fernandez et al., 1986; Gilbert & Knight, 1986; Graci & Cameron, 2006; Leyssen et al., 2005; Snell, 2001; Vanderlinden et al., 2016). Its antiviral activity against HCV is mediated by the direct targeting of the RNA sequences (NS5B) which encode the RdRp and/or by inducing a mutation in the vRNA, which consequently may result in hyper-mutating viral strains (Feld & Hoofnagle, 2005; Te et al., 2007). Several Aryl benzoyl hydrazide analogs showed high potential to act as RdRp inhibitors against different IAV strains when tested in vitro, most likely by targeting the PB1 polymerase subunit of the RdRp (Liu et al., 2022). Recently, the FDA approved Remdesivir (a nucleoside analog) as an antiviral to treat SARS-CoV-2 infection; it works by interfering with viral RNA polymerase (Table 9.2) (Kokic et al., 2021).

2.4.2 Inhibitors of viral protease

This class of medications works by inhibiting the cellular and/or viral proteases enzymes that are involved in different steps in the viral replication cycle. Since proteases are necessary for the development of fully infectious virus particles, they are potential targets for antiviral therapies. Amprenavir, atazanavir, darunavir, indinavir, nelfinavir, ritonavir, saquinavir, and tipranavir are among the medications identified by the “NAVIR” suffix that work against viruses by inhibiting viral proteases. Viral proteases support the viruses' ability to replicate either by enhancing the building up of structural proteins required for assembly and morphogenesis of virions or by proteolytic cleavage of high molecular weight viral polypeptide precursors into their active forms (Patick & Potts, 1998). Different studies have shown the efficacy of these protease inhibitors against various types of viruses, not only against DNA viruses such as CMV and HSV (Jarvest et al., 1996, 1997; Patick & Potts, 1998) but also against

RNA viruses such as picornaviruses, HCV, HIV, Middle East respiratory syndrome coronavirus (MERS-CoV), RSV, SARS-CoV, and SARS-CoV-2 (Banerjee et al., 2021; Berman & Kwo, 2009; De Palma et al., 2008; Ghosh et al., 2008; Gucht et al., 2017; Jung et al., 2018; Ma et al., 2022; Mahdi et al., 2020; Mellott et al., 2021). Recently, Paxlovid, which is a combination of two synergistic protease inhibitors called nirmatrelvir and ritonavir developed by Pfizer, has been approved by the FDA as an oral treatment for severe or critical COVID-19 cases (Table 9.2) (Owen et al., 2021).

2.4.3 Inhibitors of viral integrase

Strand transfer is one of the phases required for HIV to be integrated into the host genome by the enzymatic activity of the viral integrase enzyme. HIV integrase activity is inhibited by specific inhibitors (antiretroviral drugs) which hinder HIV replication in the host cell (Anstett et al., 2017). Viral-targeted approaches to stop and prevent HIV from multiplying in the human host cell are the focal points in antiretroviral therapies (Sierra & Walter, 2012). Several studies have shown the power of using integrase inhibitors such as dolutegravir, elvitegravir, and raltegravir to control HIV infection and to treat AIDS (Table 9.2) (Kobayashi et al., 2011; Shimura & Kodama, 2009; Summa et al., 2008; Underwood et al., 1999).

2.4.4 Inhibitors of neuraminidase (NAIs)

The last step in a virus replication cycle is the release of newly assembled virions from the host cell either through a lytic phase or a lysis phase. The lytic phase causes cell death where the host cell membrane bursts to open the door for the virions to exit, and polioviruses are an excellent example of lytic viruses. On the contrary, the lysis (temperate) phase allows the host cell to survive, where the viral particles leave the host via budding to form the cell membrane. IAV and IBV favors lysis phase, where the virus particles exit the host cell via enzymatic activity of the viral NA found on the surface of viral particles. NA disrupts the interactions between SA-containing receptors on the host cell membrane and the viral surface HA protein, which permit the release of the new viral progeny (McNicholl & McNicholl, 2001). NA's sialidase activity is mediated by amino acid residues that are both selective and highly conserved (active site). Thus, Neuraminidase inhibitions (NAIs) compete with the viral NA in the surface of IAV and IBV by blocking the cleavage of SA residues on budding newly produced virus particles, hence preventing the release of new virus particles from infected host cells (Bassetti et al., 2019). Currently, NAIs (Table 9.2) are the most used medications for both IAV and IBV. The only two FDA-approved antiinfluenza NAI medications are oseltamivir (Tamiflu) and zanamivir (Relenza) (Caceres et al., 2022). In addition, peramivir (Rapivab) and Laninamivir (Inavir) are effective NAIs, but their use is limited in certain countries (Mawatari et al., 2019).

3. Natural products as antiviral agents

Medicines made from natural ingredients have very deep roots in history. Because of their effectiveness and safety, people continue to use plant resources to develop remedies that are used to cure diseases. The one-drug-one-target or “mono-target drug” approach in drug

development is marked by multiple failures and significant lack of certainty. Unsuccessful trial to repurpose the combination of lopinavir/ritonavir to treat COVID-19 is the most recent example of the difficulties in creating extremely selective monotargeted medicines (Klean-drova & Speck-Planche, 2021). A Bulk of methods, both computational (virtual screening) and experimental (wet screening), have concentrated on finding medications that block particular targets. However, clinically effective antiviral medications can be made from natural-based agents that target both viral and host cellular factors, which are called “multi-target drugs.” Conventional medicine has relied on plants to treat human diseases and infections for centuries. The discovery of penicillin in 1928 by Alexander Fleming has been advantageous to the whole world. The development of significant bacterial metabolites from *Streptomyces* in the has led to the discovery of novel antibiotics that show the ability to target several strains of pathogenic bacteria and fungi. Microorganisms have become major players in the development of novel nature-based medicines ever since the discovery of penicillin. More than 20,000 biologically active substances produced by microorganisms as secondary metabolites are now available On the market as antimicrobials, antivirals, anticancer drugs, and antiinflammatory agents, among others. Fungal products account for about 42%, while *Actinomyces* products account for about 32% of the total production (Bule et al., 2019).

3.1 Fungi-derived antivirals

Fungal species produce a substantial number of bioactive substances, including antivirals, even though the majority of natural products come from terrestrial species. Luotonin A and nortopsentin were among the first fungal-derived products to have antiviral activity against TMV (Hao et al., 2020; Ji et al., 2018). Xanthones derived from *Aspergillus iizukae* also showed broad-spectrum antiviral activity against different DNA and RNA viruses, including IAV and HSV (Kang et al., 2018). Several other fungal extracts have also been shown to possess antiviral activities against HSV and vesicular stomatitis virus (Selim et al., 2018). Cyclosporin-72, which is another fungus-derived substance, has been shown to have high antiviral potential against SARS-CoV-2 in animal models (Sauerhering et al., 2022). The cyclosporin-72 derivative NIIM 811 has also been shown to have broad-spectrum antiviral potential against HIV, HCV, and SARS-CoV-2 (Han et al., 2022; Liu et al., 2020). Table 9.3 comprises some fungal-derived secondary metabolites with antiviral activities.

3.2 Bacteria-derived antivirals

Lactic acid bacteria (LAB) has been shown to control viral infections and/or prevent replication of a variety of viruses such as HSV, gastroenteric, IAV, IBV, and some other respiratory viruses (Ren et al., 2020). Exopolysaccharides are natural products produced by LAB. With a wide range of uses in the agricultural, pharmaceutical, and food industries, molecules of microbial exopolysaccharides have revealed a plethora of helpful and beneficial health effects. They exerted high antiviral activity against different viruses as they work by hindering the viral adsorption onto the host cell membrane by either targeting the host cell structures or the virion to control viral infection (Liu et al., 2004). Sulfated polysaccharides have been

TABLE 9.3 Microbial-derived metabolites with antiviral activities.

Antiviral	Microbe	Virus	Refs.
Formycin A and B	<i>Streptomyces candidus</i>	VV, VSV, IAV, and IBV	Eichberg et al. (2022) , Giziewicz et al. (1975) , Ko et al. (2017) , and Takizawa et al. (2022)
Siastatin B	<i>Streptomyces</i> culture	HBV and IAV	Greimel et al. (2003) , Nishimura (1995) , and Nishimura et al. (1988, 1993)
Statins	<i>Aspergillus terreus</i>	IAV, HIV, and SARS-CoV-2	Bifulco et al. (2021) , Chauvin et al. (2013) , Mehrbood et al. (2014) , Minz et al. (2020)
Myriocin	<i>Isaria sinclairii</i>	HCV and IAV	Miyake et al. (1995) , Umehara et al. (2006) , and Wang et al. (2015)
Fumonisin B1	<i>Fusarium</i> sp.	HCV and IAV	Gewaid et al. (2020) and Hidari et al. (2006)
Cyclosporine A	<i>Trichoderma polysporum</i>	HCV, HIV, IAV, and SARS-CoV-2	Anjum et al. (2012) , Fenzia et al. (2022) , Glowacka et al. (2020) , and Ma et al. (2016)
Rubrolide S	<i>Aspergillus terreus</i>	IAV and SARS-CoV-2	Quimque et al. (2020) and Tonghan Zhu et al. (2014)
Violapyrones (VLPs)	<i>Streptomyces youssoufiensis</i>	IAV and HSV	Ameen et al. (2022) , Mayer et al. (2022) , and Yi et al. (2029)
Salinomycin	<i>Streptomyces albus</i>	HSV, IAV, HCoV-OC43, and SARS-CoV-2	Jang et al. (2018) , Ju et al. (2021) , and Kapoor et al. (2013)
Cladosin C, Xoglyantrypine, norquinadoline A, desoxynortryptoquivaline, desoxytryptoquivaline, tryptoquivaline and quinadoline B	<i>Cladosporium sphaerospermum</i>	IAV	Guangwei Wu et al. (2014) and Peng et al. (2013)
Sorbicatechols A and B	<i>Penicillium chrysogenum</i>	IAV	Peng et al. (2014)
Diketopiperazine derivatives	<i>Streptomyces</i> sp.	IAV and SARS-CoV-2	Alhadrami et al. (2022) and Wang, Xi et al. (2013)
Truncateol O	<i>Truncatella angustata</i>	HIV and IAV	Zhao et al. (2018)
Cordycepin	<i>Cordyceps</i> sp.	HIV, Murine leukemia virus, Epstein Bar virus (EBV), IV, and SARS-CoV-2	Chellapandi and Saranya (2022) and Ryu et al. (2014)

TABLE 9.3 Microbial-derived metabolites with antiviral activities.—cont'd

Antiviral	Microbe	Virus	Refs.
Pseudomonadota	<i>Pseudomonas fluorescens</i> , <i>Pseudomonas mandelii</i> , and <i>Pseudomonas taiwanensis</i>	IAV	Morimoto and Sato (2016)
Lactic Acid bacteria and bacteriocins	<i>Lactobacillus</i> spp.	HSV, RSV, HIV, IAV, and SARS-CoV-2	Al Kassaa et al. (2014), Conti et al. (2009), Erol, Kotil, Ortakci et al. (2022), Erol, Kotil, Fidan et al. (2021), Jung et al. (2021), Klebanoff and Coombs (1991), and Nagai et al. (2011)
Exopolysaccharides	<i>Lactobacillus bulgaricus</i> and <i>Haloarcula hispanica</i>	Encephalomyocarditis virus (EMCV), human adenovirus (HAdV), HSV, IAV, and SARS-CoV-2	Biliavska et al. (2019), Gugliandolo et al. (2015), Nagai et al. (2011), Xu, Wang et al. (2022), and Yim et al. (2004)
Actinomycetota (actinomycetes' metabolites)	<i>Actinomycetes</i> spp.	HSV, IAV and SARS-CoV-2	Al-Bustany et al. (2022), Moghannem and Abd-elwahab (n.d.), Muhammad et al. (2022), and Yin et al. (2021)
Butenolide derivatives	<i>Streptomyces</i> sp.	HCV, IAV and SARS-CoV-2	Chung et al. (2016), Fangfang Li et al. (2018), and Nazir et al. (2022)
Nitrosporeusine A and B	<i>Streptomyces nitrosporeus</i>	IAV	Aigang Yang et al. (2013) and Tian et al. (2017)
Stachyflin and acetylstachyflin	<i>Stachybotrys</i> sp.	IAV and SARS-CoV-2	Bharadwaj et al. (2021), Minagawa et al. (2002), and Sahoo et al. (2021)
DAS181 (Fludase)	<i>Actinomyces viscosus</i>	IAV	Belser et al. (2007) and Malakhov et al. (2006)

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shown to prevent the attachment of virus into cells and to have antiviral effects against a variety of viruses, including the HSV, HBV, IAV, and HCMV (Mukherjee et al., 2019; Rahbar Saadat et al., 2019; Ray et al., 2021). Recently, murine sulfated polysaccharides showed their high antiviral potential to treat SARS-CoV-2 infections (Salih et al., 2021). Table 9.3 comprises some bacterial-derived secondary metabolites with antiviral activities.

3.3 Plant-derived antivirals

Both developed and developing nations have a long history of using herbal treatments, and they continue to do so for a variety of reasons, including their low cost, fewer side effects, and high levels of therapeutic efficacy. Plants serve as a reservoir for several biologically active compounds, including antiviral agents. Some of them have broad-

spectrum antiviral activity with minimal or no adverse side effects on human health. Nearly 60% of the world's population uses herbal medicines and traditional medical procedures, according to a survey by the World Health Organization. The majority of traditional remedies are crude plant extracts, including tinctures (ethanolic or methanolic plant extracts), decoctions (boiled plant component extracts), herbal baths, herbal infusions, and plant syrup. Plants provide a huge opportunity for drug discovery and development since the products of metabolic reactions inside plants or secondary metabolites, which are called "phytochemicals," have a wide range of biological activities including antiviral potential to target viruses at different stages of their replication cycle. Traditional uses of phytochemicals for disease treatment include their ability to inhibit viral genome replication and/or gene transcription. The majority of them inhibit viral infection either at the point of viral entry into the host cell or hinder viral replication inside the host cell. Several studies have shown that the presence of many types and classes of phytochemicals, including alkaloids, flavonoids, coumarins, terpenes, tannins, lignans, and phenolic compounds, are the main components that give medicinal plants their therapeutic effects, including antiviral activities. For instance, Veregen, a combination of leaf extract of green tea and combination tea catechins, was the first FDA-approved plant-derived treatment for the treatment of genital warts caused by human papilloma virus (Miyoshi et al., 2020). *Glycyrrhiza glabra* (or licorice) is another herbal treatment that possesses antiviral efficacy against HCV infection (Abraham & Florentine, 2021). It has been used for this purpose in Japan for over 20 years.

3.3.1 Terpenes and terpenoids

The chemical class terpene constitutes the majority of plants' phytochemicals with more than 30,000 compounds. They are simply composed of five carbon isoprene molecules with double bonds (unsaturated hydrocarbons) (Davis & Croteau, 2000, pp. 53–95; Kennedy & Wightman, 2011). Monoterpenes, which have two isoprene molecules, are the simplest and most basic terpene structure. According to the number of terpene units found in the terpene structure, they are divided into different chemical groups such as: monoterpenes (having only), sesquiterpenes (having three isoprene units), and diterpenes (having four isoprene units) (Buckle, 2015, pp. 37–72). However, terpenoids are simply terpenes that have multiple functional groups added to them in different places. Terpenoids, often referred to as isoprenoids, play a vital role in drug development as they have a broad spectrum of pharmacological activities including analgesic, anticancer, antiinflammatory, antimicrobial, antiparasitic, and antiviral effects (Cox-Georgian et al., 2019, pp. 333–359; Zielińska-Błajet et al., 2021). A large number of terpenes exerted their antiviral action on different viral infections, including HSV, HCV, HIV, Porcine epidemic diarrhea virus, IAV, and SARS-CoV-2 (Table 9.4) (Diniz et al., 2021; Dong et al., 2018; Grishko et al., 2014; Kim et al., 2015; Muhseen et al., 2020; Paduch et al., 2007; Perveen, 2021). Additionally, several other viruses, including retroviruses like simian immunodeficiency virus (SIV) and the murine leukemic virus, have been shown to be inhibited by terpenes and terpenoids (Chang et al., 1999; Ghildiyal et al., 2020). Terpenes and terpenoids are multitarget antiviral agents as they can target several steps of the viral infection cycle for many viruses (Mohammadi Pour et al., 2019).

TABLE 9.4 List of some Terpenes and Terpenoids with reported antiviral activities.

Compound	Plant source	Viruses	Refs.
Saponin	<i>Tieghemella heckelii</i> , <i>Panax ginseng</i> , <i>Glycyrrhiza glabra</i> , and <i>Vernonia amygdalina</i>	HIV, IAV, IBV, and SARS-CoV-2	Alexyuk et al. (2019), Gosse et al. (2002), Hayashi et al. (2000), Ogunyemi et al. (2021), Song, Qiu et al. (2014), and Yin et al. (2013)
Lupeol	<i>Bombax ceiba</i> and <i>Alnus japonica</i>	HSV, HIV, DENV, IAV, and SARS-CoV-2	Ambrose et al. (2022), Sharma et al. (2020), and Tung et al. (2010)
Thymol	<i>Thymus vulgaris</i>	HSV, IAV, and SARS-CoV-2	Knight et al. (1977), Kowalczyk et al. (2020), Kulkarni et al. (2020), Rolta et al. (2021), and Sharifi-Rad et al. (2018)
Camphor	<i>Cinnamomum camphora</i>	IAV and SARS-CoV-2	Malabadi et al. (2021), Sokolova et al. (2014), and Zarubaev et al. (2015)
Limonene	<i>Zanthoxylum myriacanthum</i> var. <i>pubescens</i>	HSV, yellow fever virus, IAV, and SARS-CoV-2	Astani and Schnitzler (2014), Gómez et al. (2013), Senthil Kumar et al. (2020), Najar et al. (2022), and Jingjing Yang et al. (2022)
Pinene	<i>Sideritis</i> spp. and <i>Cannabis</i> spp.	Human adenovirus (HAdv), HSV, and SARS-CoV-2	Astani and Schnitzler (2014), Qiu et al. (2022), and Roviello and Roviello (2022)
Nerol	<i>Cymbopogon citratus</i> and <i>Catharanthus roseus</i>	HSV	Vilhelmova-Ilieva et al. (2021)
Nerolidol	<i>Dalbergia parviflora</i> and <i>Brassavola nodosa</i>	HSV, RSV, and SARS-CoV-2	Chen, Wu et al. (2020) and Silva et al. (2020)
Farnesol	<i>Illicium verum</i> and <i>Vachellia farnesiana</i>	HSV and SARS-CoV-2	Astani et al. (2011) and Silva et al. (2020)
Phytol	<i>Premna seratifolia</i>	HSV	Ebenezer et al. (2021)
Linalool	<i>Lavandula spica</i> L. and <i>Ocimum basilicum</i>	Adenovirus (Adv), HSV, and IAV	Chiang et al. (2005) and Choi (2018)
Terpinolene	<i>Melaleuca alternifolia</i> and <i>Curcuma longa</i> L.	IAV	Garozzo et al. (2009)
Camphene	<i>Myristica fragrans</i>	IAV, EBOV, hanta virus, and HSV	Hachlafi et al. (2021) and Sokolova et al. (2021)
Terpineol	<i>Abies sibirica</i>	IAV and SARS-CoV-2	Garozzo et al. (2011) and Kulkarni et al. (2020)
Phellandrene	<i>Eucalyptus phellandra</i>	Dengue virus (DENV)	Silva-Trujillo et al. (2022)
Humulene	<i>Cyperus rotundus</i> L.	HSV and SARS-CoV-2	Bachar et al. (2021)
Pulegone	<i>Mentha pulegium</i> L., <i>Hedeoma pulegioides</i> L. and <i>Minthostachys verticillata</i>	HSV, pseudorabies virus, and SARS-CoV-2	Chattopadhyay (2006), Primo et al. (2001), and Zeljković et al. (2022)

(Continued)

TABLE 9.4 List of some Terpenes and Terpenoids with reported antiviral activities.—cont'd

Compound	Plant source	Viruses	Refs.
Geraniol	<i>Rose ottos</i> and <i>Cymbopogon martinii</i>	HSV	Hassan et al. (2018)
Eugenol	<i>Ocimum gratissimum</i> L.	Feline calicivirus, IAV, EBOV, and HSV	Benencia and Courrges (2000), Dai et al. (2013), and Lane et al. (2019)
β -caryophyllene	<i>Lippia alba</i> and <i>Ocimum campechianum</i> Mill	Newcastle disease virus (NDV), HSV, ZIKV, and IAV	Astani et al. (2011), Hassanin et al. (2020), and Yarovaya et al. (2022)

3.3.2 Alkaloids

Alkaloids are one of the secondary metabolites found in higher plants, and they are characterized by the presence of at least one nitrogen atom attached to a ring structure. There are more than 12,000 naturally occurring plant alkaloids that are divided into many different chemical groups according to their structure, including imidazoles, indoles, isoquinolines, purines, piperidines, pyrrolidines, pyrrolizidines, quinolizidines, and tropanes (Thawabteh et al., 2019; Ziegler & Facchini, 2008). Alkaloids are alkaline in nature, hence the name, and they are biologically active as they possess antiasthma, antiinflammatory, antioxidant, antidiabetic, antibacterial, anticancer, and antiviral properties (Herman & Herman, 2012; Nassiri, 2013). Regarding their antiviral activities, a large number of alkaloid compounds (about 43) exerted antiviral effects against IAV, which are thought to be due to the stimulation of interferons by the immune system (Moradi et al., 2018). Some alkaloids increase the activity of macrophages, allowing them to phagocytose the virus and stop the viral infection. At various phases of the viral replication cycle, alkaloids prevent IAV from replicating inside the host (Moradi et al., 2018). Several studies reported the antiviral effects of different groups of alkaloids against a variety of influenza and coronaviruses, among other viruses (Table 9.5).

3.3.3 Flavonoids

The color and scent of flowers, the ability of fruits to attract pollinators and hence facilitate fruit dispersion, the ability of seeds and spores to germinate, the growth and development of seedlings, and other biological properties of plants are all attributed to the presence of flavonoids (Griesbach, 2010). There are around 3000 different types of flavonoids, which are a class of polyphenolic chemicals that are extensively distributed across the Plantae kingdom and help the plant to oppose biotic and abiotic stresses. Based on their molecular makeup, flavonoids can be categorized into a number of classes. There are four major flavonoid categories including: Flavones (e.g., apigenin and baicalein), flavanones (e.g., naringin, hesperitin, and eriodictol), catechins, and anthocyanins (e.g., cyanidin, malvidin, and delphinidin) (Agrawal, 2011; Panche et al., 2016). Flavonoids have several biological activities including antioxidative, antiinflammatory, anticancer, anticarcinogenic, antimicrobial, and antiviral effects, which is explained by their ability to influence important cellular enzyme functions (Brodowska, 2017; Carletti et al., 2014; Karak, 2019). Antiviral activities of flavonoids against

TABLE 9.5 List of some Alkaloids with reported antiviral activities.

Compound	Plant source	Virus	Refs.
Hyoscyamine	<i>Hyoscyamus niger</i> and <i>Atropa belladonna</i>	Japanese encephalitis virus (JEV) and SARS-CoV-2	Bhattacharjee et al. (2022) and Skariyachan et al. (2021)
Homonojirimycin (HNJ)	<i>Commelina communis</i>	HIV and IAV	Dhavale and Matin (2004) and Zhang et al. (2013)
Dendrobine	<i>Dendrobium nobile</i>	IAV and SARS-CoV-2	Jiaranaikulwanitch et al. (2022), Li et al. (2017), and Rafli et al. (2022)
Thalimonine	<i>Thalictrum simplex</i>	IAV and SARS-CoV-2	Garg and Roy (2020) and Romeo et al. (2021)
Sophaline D	<i>Sophora alopecuroides</i>	IAV and SARS-CoV-2	Garg and Roy (2020) and Ti et al. (2021)
Papaverine	<i>Papaver somniferum</i>	IAV, IBV, and SARS-CoV-2	Aggarwal et al. (2020) and Valipour et al. (2022)
Quinine	<i>Cinchona</i> sp.	IAV and SARS-CoV-2	Latarissa et al. (2021) and Marois et al. (2014)
Caffeine	<i>Coffea</i> sp.	HSV, IAV, and SARS-CoV-2	Elzupir (2022), Murayama et al. (2008), Romero-Martínez et al. (2021), and Yamazaki and Tagaya (1980)
Ephedrine	<i>Ephedra sinica</i>	IAV and SARS-CoV-2	Hyuga et al. (2016) and Yu et al. (2021)
Reserpine	<i>Rauwolfia serpentina</i>	SARS-CoV-2	Choudhry et al. (2020) and Kuo et al. (2021)
Brucine	<i>Strychnos nux-vomica</i> L.	HIV and SARS-CoV-2	Alsulimani et al. (2022) and Syahdi et al. (2012)
Colchicine	<i>Colchicum autumnale</i>	DENV, ZIKV and SARS-CoV-2	Drosos et al. (2022), Richter et al. (2019), and Vitiello and Ferrara (2021)
Eserine (physostigmine)	<i>Physostigma venenosum</i>	Influenza virus (IV) and SARS-CoV-2	Llanes et al. (2021) and Sánchez-Bernal et al. (1998)
Berberine	<i>Berberis vulgaris</i> , <i>Enantia chlorantha</i> , and <i>Cortex Phellodendri</i>	IAV, HSV, HCMV, and NDV	Cecil et al. (2011), Kim et al. (2016), Luganini et al. (2019), Ohemu et al. (2018), and Song, Si et al. (2014)
Atropine	<i>Atropa belladonna</i>	Parainfluenza, HAV, HSV, IAV, NDV, Vaccinia virus (VACV), adenovirus, and Japanese encephalitis (JEV)	Alarcón et al. (1984), Biziagos et al. (1987), Yamazaki and Tagaya (1980), and Özçelik et al. (2011)
Scopolamine	<i>Hyoscyamus niger</i> and <i>Atropa belladonna</i>	Parainfluenza, HSV, and JEV	Kumar et al. (2020) and Özçelik et al. (2011)

(Continued)

TABLE 9.5 List of some Alkaloids with reported antiviral activities.—cont'd

Compound	Plant source	Virus	Refs.
Oliverine	<i>Polyalthia oliveri</i>	HSV	Montanha et al. (1995)
Lycorine	<i>Clivia miniata</i> and <i>Lycoris radiata</i>	ZIKV, IAV, DENV, HCV, and SARS-CoV	Chen et al. (2015), Huini Chen, Wu et al. (2020), He et al. (2013), Li et al. (2005), and Wang et al. (2014)
Emetine	<i>Cephaelis ipecacuanha</i> A.	HIV, HCMV, EBOV, ZIKV, SARS-CoV, MERS-CoV, and SARS-CoV-2	Bleasel and Peterson (2020), Choy et al. (2020), Mukhopadhyay et al. (2016), Valadão et al. (2015), and Yang et al. (2018)

several viruses, such as RSV, HSV, adenovirus, HIV, coronavirus, influenza, and parainfluenza have been reported (Table 9.6) (Alzaabi et al., 2022; Kaul et al., 1985; Ling et al., 2020; Liu et al., 2018; Lyu et al., 2005; Russo et al., 2020; Wei et al., 2004; Xu et al., 2000). Flavonoids work against viruses by targeting multiple stages in the viral replication cycle (multi-target antivirals) (Kaul et al., 1985; Mohammadi Pour et al., 2019).

3.3.4 Coumarins

Coumarins are a class of phenolic phytochemical compounds called benzopyrones (made up of benzene rings fused to pyrone rings). They were initially discovered in *Coumarouna odorata*, a plant which is commonly known as “Cumaru.” More than 1300 coumarins have been isolated from natural sources, primarily green plants, and they are present in many different types of plants as well as fungi and microbes (Borges et al., 2005). Coumarins offer a number of appealing properties, including low molecular weight, simple structure, high bioavailability, high solubility in most organic solvents, and low toxicity, which, along with their diverse biological activities, secure them a major place as lead compounds in drug research and development (Hoult & Payá, 1996; Zhu & Jiang, 2018). Moreover, the ability to develop new coumarin-based compounds and research their potential for the treatment of various infectious diseases, including viral infections, is made possible by the discovery that coumarin nucleus may be easily synthesized and designed (Annunziata et al., 2020; Penta, 2015, pp. 1–182). Biological activities of coumarins include antiinflammatory, antibacterial, anticancer, neuroprotective, antidiabetic, antioxidant, anticoagulant, and antiviral properties (Annunziata et al., 2020; Hassan et al., 2016). Several studies demonstrate the ability of different coumarins, and their derivatives, as multitarget antiviral agents (Table 9.7). The mechanisms of antiviral activity of coumarins include targeting viral replication, viral proteins, and viral structures (Hassan et al., 2016; Mohammadi Pour et al., 2019).

3.3.5 Lignans

Lignans are naturally occurring phenolic phytochemicals produced by plants and are commonly known as phytoestrogens. Plant lignans are phenylalanine-derived polyphenols known as syringaresinol, secoisolariciresinol, sesamin, pinoresinol, or lariciresinol. Lignans have antioxidant properties and have the ability to bind to estrogen receptors in breast cells; thus, they are called phytoestrogens (Wcislo & Szarlej-Wcislo, 2014). These phenolic

TABLE 9.6 List of some flavonoids with antiviral activities.

Compound	Plant source	Viruses	Refs.
Silybin (silymarin)	<i>Silybum marianum</i> (L.) Gaertn	HCV, HBV, DENV, Mayaro virus (MAYV), Chikungunya virus (CHIKV), IAV	Lani et al. (2015) and Liu, Hu et al. (2019)
Pinocembrin	<i>Glycyrrhiza glabra</i> L.	CHIKV, DENV, and ZIKV	Lee et al. (2019)
Apigenin	<i>Matricaria chamomilla</i>	Enterovirus-71 (EV-71), HCV, CHIKV, and ASFV	Hakobyan et al. (2016)
Baicalein	<i>Scutellaria baicalensis</i> Georgi	NDV, IAV, and DENV	Ding et al. (2014), Jia et al. (2016), and Moghaddam et al. (2014)
Naringenin	<i>Citrus paradisi</i>	HCV, DENV, ZIKV, and SARS- CoV-2	Mendes et al. (2020) and Tutunchi et al. (2020)
Hesperitin	<i>Citrus aurantium</i>	HSV	Amoros et al. (1992)
Eriodictyol	<i>yerba santa</i>	SARS-CoV-2	Deshpande et al. (2020)
Catechins	<i>Acacia catechu</i> L.,	HSV, RSV, IAV, IBV, and HCV	Hakobyan et al. (2016) and Kaul et al. (1985)
Cyanidin	<i>Sambucus nigra</i>	IAV, IBV and SARS-CoV-2	Choudhary and Pan (2020), Shrestha et al. (2022), and Torabian et al. (2019)
Malvidin	<i>Lathyrus latifolius</i> and <i>Vaccinium myrtillus</i>	IAV and IBV	Choudhary and Pan (2020)
Delphinidin	<i>Ribes nigrum</i> , <i>Viola</i> spp. and <i>Delphinium</i> spp.	IAV, IBV, WNV, DENV, and ZIKV	Knox et al. (2001) and Vázquez-Calvo et al. (2017)
Epigallocatechin (–) EGC	<i>Camellia sinensis</i>	IAV and IBV	Imanishi et al. (2002)
Quercitrin	<i>Mangifera indica</i> and <i>Rheum nobile</i>	JEV, DENV, and EV-71	Hakobyan et al. (2016)
Isoquercitrin	<i>Mangifera indica</i> and <i>Rheum nobile</i>	ZIKV	Gaudry et al. (2018)
Sorbifolin	<i>Pterogyne nitens</i>	HCV	Shimizu et al. (2017)
Myricetin	<i>Solanum lycopersicum</i>	ZIKV	Zou et al. (2020)
Kaempferol	<i>Aloe vera</i> , <i>Sambucus</i> <i>nigra</i> and <i>Rosmarinus</i> <i>officinalis</i>	Pseudorabies virus, DENV, JEV, IAV, and IBV	Care et al. (2020), Li, Wang et al. (2021), Yang et al. (2014), and Zhang, Wu et al. (2012)
Genistein	<i>Flemingia vestita</i> and <i>Flemingia macrophylla</i>	Lassa virus (LASV) and EBOV	Hakobyan et al. (2016)

TABLE 9.7 List of some Coumarins with antiviral activities.

Compound(s)	Plant Source(s)	Virus(es)	Refs.
Fesumtuorin A, B, bicoumarin, Fesumtuorin C, Spirobicoumarins, fesumtuorin D, E, F, G and H	<i>Ferula sumbul</i>	HIV	Zhou et al. (2000)
GUT70 (tricyclic coumarin)	<i>Callophyllum brasiliense</i>	HIV	Kudo et al. (2013)
Pyranocoumarins nordentatin and clausarin	<i>Clausena excavate</i>	HBV	Huang et al. (1997)
4-Hydroxycoumarins, (warfarin, 4-HC tetramer), Pyranocoumarins (Khellactone, Calanolide), furanocoumarin, 3-phenylcoumarins, and 4-Phenylcoumarins	Coumarin derivatives	HIV	Hassan et al. (2016)
Neoflavanoids	4-Phenylcoumarin derivatives	HIV	Olmedo et al. (2017)
Mesouol and isomesouol	<i>Marila pluricostata</i>	HIV	Márquez et al. (2005)
Eleutheroside B1	Coumarin derivative	IAV	YuTao Wang et al. (2017)
Bis (triazolothiadiazinyl coumarin)	Coumarin derivative	IAV	Pavurala et al. (2018)
Glycyrol	<i>Glycyrrhiza uralensis</i>	IAV	Ryu et al. (2010)
Resveratrol	Coumarin derivative	DENV	Zainal et al. (2017)
7-(4-(4-methyl-imidazole))-coumarin, 7-(4-benzimidazole-butoxy)-coumarin and 7-[6-(2-methylimidazole) hexyloxy] coumarin	Coumarin derivatives	Spring viremia of carp virus (SVCV)	Liu, Wang et al. (2019) and Liu, Jassey et al. (2019)
Isofraxidin	<i>Sarcandra glabra</i> and <i>Siberian ginseng</i>	IAV	Jin et al. (2020)
Glycycoumarin and Licopyranocoumarin	<i>Xi-bei licorice</i>	HIV	Hatano et al. (1988)
Suksdorfii	<i>Lomatium suksdorfii</i>	HIV	Lee et al. (1994)
Oxypeucedanin, oxypeucedanin hydrate, Isoimperatorin, byakangelicol and Byakangelicin	<i>Angelica apaensis</i>	HIV	Qiong et al. (2008)
Glycycoumarin, Glycyrin and Glycyrol	<i>Glycyrrhiza uralensis</i>	HCV	Adianti et al. (2014)
Chalepin	<i>Ruta angustifolia Pres</i>	HCV	Wahyuni et al. (2014)
Coumarin A and Coumarin B	<i>Mammea americana</i>	CHIKV and DENV	Gómez-Calderón et al. (2017)

compounds have a variety of biological properties, including antioxidant, anticancer, anti-inflammatory, antihepatotoxic, antidepressant, antineurodegenerative, antibacterial, and antiviral effects (Cui et al., 2020; Polimati et al., 2022; Zálešák et al., 2019). They offer a variety of naturally existing pharmacophores, and they can also be produced through chemical

TABLE 9.8 List of some Lignans with antiviral activities.

Compound	Plant source	Viruses	Refs.
Honokiol	<i>Magnolia</i> sp.	DENV	Fang et al. (2015)
3-Hydroxy caruillignan C (3-HCL-C)	<i>Swietenia macrophylla</i>	JEV, HIV, HBV, and HCV	Choudhary and Pan (2020), Ghildiyal et al. (2020), and Wu et al. (2012)
Bombasinol	<i>Bombax ceiba</i>	HBV	Wang, Lin et al. (2013)
Rhinacanthin E and Rhinacanthin F	<i>Rhinacanthus nasutus</i>	IAV	Kernan et al. (1997)
Rhinacanthone, Rnc, Rnd, Rnn, Rnq and Rne and Heliobuphthalmin	<i>Rhinacanthus nasutus</i>	IAV, human rhinovirus (HRV), and coxsackievirus	Ngoc et al. (2019)
Podophyllin	<i>Podophyllum peltatum</i>	Human papilloma virus (HPV)	Kaplan (1942)
Phyllamycin B and Retrojusticidin B	<i>Phyllanthus myrtifolius</i>	HIV	Chang et al. (1995)
Helioxanthin	<i>Heliopsis scabra</i> and <i>Taiwania cryptomerioides</i>	HBV	Li et al. (2005)
6-Deoxyglucoside derivatives of diphyllin	<i>Justicia</i> sp.	ZIKV, DENV, Tick-borne encephalitis virus, WNV, JEV, EBV, and HIV	Plescica et al. (2022) and Xu, Li et al. (2022)
7,8-Secoholostylone B, 7,8-Secolignans, and Marphenols A and B	<i>Schisandra wilsoniana</i>	HIV	Zhang et al. (2010)
Schinegllignans A and B	<i>Schisandra neglecta</i>	HIV	Duan et al. (2011)

synthesis. Several antiviral researches reported the antiviral potential of lignans against a variety of viruses such as HIV, dengue virus, HBV, HCV, HSV, RSV, mumps virus, Coxsackie virus, SARS-CoV, SARS-CoV-2, and IAV (Table 9.8) (Allam et al., 2021; Cui et al., 2020; Fang et al., 2015; Jardim et al., 2015; Kernan et al., 1997; Li et al., 2019; Meng et al., 2018; Song et al., 2013; Sun et al., 2006; Tanaka et al., 2004; Wen et al., 2007; Huiqin Xu et al., 2019). These studies proved that lignans can also target various phases of viral replication, representing multitarget antiviral agents (Mohammadi Pour et al., 2019).

4. Perspectives and conclusions

Viruses can infect, replicate, and cause damage to host cells by hijacking the cellular processes of the host with the use of structural and nonNS viral proteins and their interactions

with the cellular targets. Due to their rapid and occasional merciless spread, viral infections, especially zoonotic ones, show high mortality rates due to the absence of any host defense lines and the lack of effective treatments, in most cases. Consequently, viral infections represent a continuous public and global human health threat. Antiviral medications are drugs that control viral infections and subsequent transmission or spread by targeting specific viral components and/or virus-depending cell-based targets to hinder the replication of viral genome, blocking entry to host cells, or inhibiting viral protein synthesis or viral assembly. The range of antivirals available in the pharmaceutical market has been addressed mainly for persistent or latent viruses, with limited trials to develop effective antivirals against emerging and reemerging viral infections. This is probably related to the lack of investments for the development of new antivirals. Herein, we discussed the history of antiviral research and highlighted the current antiviral approaches and alternative plant-derived antiviral compounds with related pharmacological mechanisms. Despite the huge development in the field of antiviral research, the development of novel antiviral agents is among the global priorities as the emerging and reemerging of viral infections with high morbidity and mortality rates continues to be a human threat. This is further aggravated by the ability of viruses to mutate and generate drug-resistant variants, especially for widely circulating RNA viruses with limited antiviral options. To this point, additional studies have to be conducted to investigate other effective and novel synthetic and plant-derived antiviral lead compounds, with synergistic effects for a more favorable treatment with a capability to modulate immunity and reduce the cost, toxicity, and viral resistance, as well as defining their virus-specific targets and related pharmacological mechanisms of action.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

Conceptualization of the book chapter, A.M. and L.M-S; writing—original draft preparation, A.H., S.H.M., A.M.K., L.M-S and A.M.; writing—review and editing, A.H., S.H.M., A.M.K., L.M-S and A.M. All authors contributed to the book chapter revision, read, and approved the submitted version.

Funding

This work was supported by Egyptian National Research Center (NRC)-funded projects (TT110801 and 12010126 to AM). Research on influenza in L.M-S. laboratory was partially supported by the R01AI145332 and R01AI141607 grants from the National Institute of Health; by the Center for Research on Influenza Pathogenesis and Transmission, one of the

National Institute of Allergy and Infectious Diseases-funded Centers of Excellence for Influenza Research and Response (CEIRR; contract # 75N900014); and by the American Lung Association. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Host- and genomics-based antiviral approaches

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1. Cell signaling and virus-dependent cell-based targets

1.1 Cellular cascades and innate immune responses activation upon influenza A virus (IAV) infection in mammals

Human-adapted IAVs can efficiently replicate in the upper and lower respiratory tracts of humans reaching viral peaks approximately 48 h postinoculation into the nasopharynx (Taubenberger & Morens, 2008). Severe IAV infections are those associated with the infection of the lower respiratory tract by developing acute respiratory distress syndrome (ARDS) and respiratory failure, followed by death (Taubenberger & Morens, 2008; Wonderlich et al., 2017). Severe IAV infections are often accompanied by exaggerated innate immune response, which is induced and initiated upon recognition and binding of pattern recognition receptors (PRRs) to uncommon biological molecules carrying pathogen-associated molecular patterns (PAMPs) (Takeuchi & Akira, 2010). This robust innate immune response, known as “cytokine storm,” is mainly induced by the internalized viral particle as well as their uncoated viral genome to mediate viral clearance and to overcome the ability of IAV proteins to employ a variety of viral-induced cellular proteins and pathways for their own benefit (Liu, Zhou, & Yang, 2016). For better viral genome replication and gene transcription, IAVs control the

function of important cellular proteins to suppress type-I interferon (IFN-I) production, inhibit cellular mRNA maturation and translation, and facilitate expression and translation of its own viral proteins (Lyles, 2000). IAV takes advantage of the host cell following infection by dominating a variety of fundamental intracellular signaling cascades such as Protein Kinase C (PKC)/Protein Kinase dsRNA-activated (PKR), Toll-like receptor (TLR)/retinoic acid-inducible gene I (RIG-I), Phosphoinositide 3-Kinase (PI3K)/Protein Kinase B (PKB or Akt), 2'-5' oligo (A) synthetase (OAS)/RNase L, P38 mitogen-activated protein kinase (P38 MAPK)/extracellular signal-regulated kinases (ERK)/c-Jun N-terminal kinase (JNK), Nuclear factor kappa B (NF- κ B) canonical and noncanonical pathways, the Janus kinase-signal transducer and activator of transcription (JAK-STAT), death receptor signaling tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas Cell Surface Death Receptor (Fas) and its physiological ligand (FasL) pathways (Gaur et al., 2011; Ludwig, 2011).

Following the recognition of the viral PAMPs by PRRs, downstream signaling molecules are activated. In IAV, the PRRs include transmembrane-localized TLRs and the cytosolic RIG-I-like receptors, which include the helicases RIG-I, MDA-5 (melanoma differentiation-associated gene 5) and LGP2 (Hou et al., 2011; Satoh et al., 2010). The PAMP ligand for RIG-I-like receptors are specific viral single-stranded (ss)RNA structures with 5'-triphosphate termini (5'ppp), short double-stranded (ds)RNA, long double-stranded (ds)RNA, or the dsRNA-binding protein PACT (Kato et al., 2008; Kok et al., 2011; Pichlmair et al., 2006). However, the cytosolic myeloid differentiation primary response 88 (MyD88) is the signal adapter protein for all TLRs (except TLR-3) (Pichlmair et al., 2006; Tawaratsumida et al., 2014; Yoneyama & Fujita, 2009). The TLR-3- and TLR-7-signaling pathways are TRIF- and MyD88-dependent and subsequently activate the IFN regulatory factors (IRF) 3 and 7, respectively (Fig. 10.1). IRF3 and IRF7 dimers, associated with transcriptional coactivators, such as CBP and p300, translocate to the nucleus to bind to IFN-stimulated response elements (ISRE), leading to proinflammatory cytokine production (Siednienko et al., 2011).

Unlike MDA-5 that preferentially binds to long dsRNA, activation of RIG-I following its binding to short dsRNA is regulated by ubiquitination of different residues on its two N-terminal caspase-recruiting domains (CARD) by the ubiquitin ligases TRIM25 and RIPLET, resulting in RIG-I oligomerization (Fig. 10.2) (Oshiumi et al., 2009; Zeng et al., 2010). MDA5 and the ubiquitinated RIG-I are involved in activating the mitochondrial antiviral signaling (MAVS) protein that triggers the phosphorylation and nuclear localization of the essential transcription factors, including IRF3, AP-1 (activated by c-Jun N-terminal kinase (JNK)/ATF-2) and NF- κ B, which are required to mediate the transcription of IFN-I genes (Ludwig et al., 2002; McWhirter et al., 2005). In addition to this, MyD88-dependent signaling of TLRs plays a pivotal role in the antiviral response and induction of IFN- α against IAV by activating IRF7 (Koyama et al., 2007).

ISGylation is a ubiquitin-like posttranslational modification of proteins that occurs through conjugation of the protein substrate to ISG15, which is first identified ubiquitin-like protein. ISGylation plays an important role to regulate immune function from multiple perspectives (Zhang et al., 2021). For instance, STAT1 ISGylation maintains continuous phosphorylation and stimulation of downstream signaling, which ultimately ends up with a robust IFN-I response (Ganesan et al., 2016). However, ISG15 modification of RIG-I negatively modulates its level and function in IFN-I production and subsequent cellular IFN-stimulated responses (Jeon et al., 2010). During IAV infection, ISGylation triggers

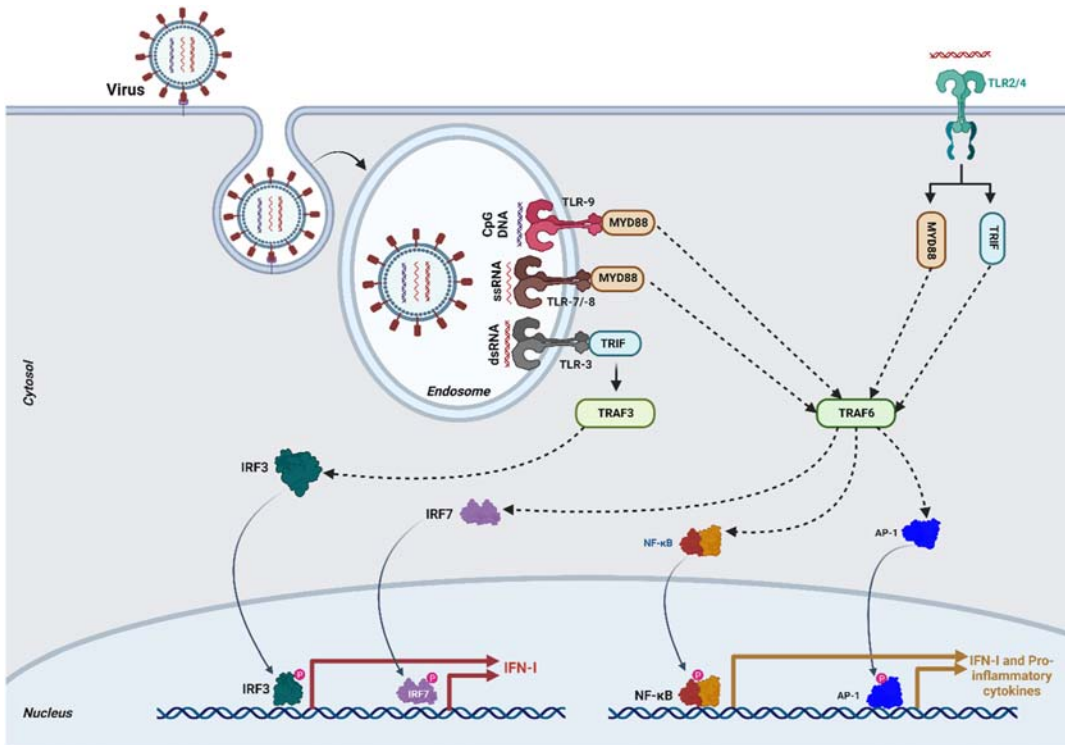


FIGURE 10.1 Modulation of TLRs following viral infection. TLR mediates the various antiviral immune responses following viral adsorption to the host cell, activating miscellaneous signaling pathways and inducing the production of antiviral IFN-I, proinflammatory cytokines, and chemokines. For more description, see the text. The figure has been assembled with [birender.com](https://www.birender.com/).

antiviral activities in IAV-infected cells through ISG15 modification of the K41 residue in the N-terminal and RNA binding-domains of its IFN-I antagonist, the nonstructural 1 (NS1) protein. ISGylation of this residue disrupts the RNA binding efficiency and nuclear localization of IAV NS1 protein through competitive interaction with importin- α , a component of the nuclear-transport machinery (Zhao et al., 2010). Herc5 attenuates IAV by catalyzing ISGylation of NS1, which impedes its ability to promote its function-dependent homodimerization (Zhao et al., 2010).

The OAS family and ribonuclease L (RNase L) work together in the cytosol to degrade the invading viral (v)RNA. Upon binding to the intracellular dsRNA sensor, OAS becomes enzymatically active, converting ATP into 2'-5'-oligoadenylate, which functions as a second messenger to activate latent RNase L. Activated RNase L degrades viral and cellular ssRNAs, which inhibits protein synthesis and viral growth (Fig. 10.3) (Ibsen et al., 2014).

To effectively reduce viral replication following IAV infection, the viral dsRNA binds also to the dsRNA-activated PKR, a serine/threonine kinase that phosphorylates the α -subunit of eukaryotic translation initiation factor 2 α (EIF2 α), resulting in inhibition of cellular and viral protein synthesis (Iwasaki & Pillai, 2014; Sadler & Williams, 2007). In addition, PKR has a role

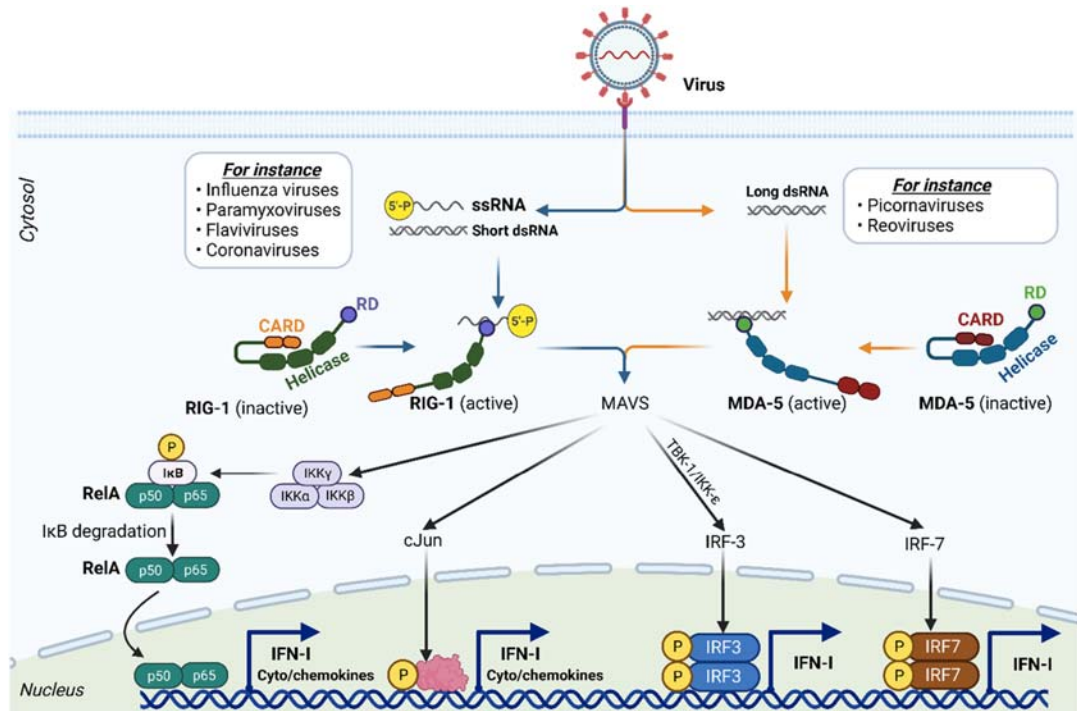


FIGURE 10.2 Viral genome accumulation activates retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Following activations, the cards of active RIG-1-like receptors interact with the mitochondrial antiviral-signaling protein (MAVS) to recruit a panel of downstream signaling molecules, activating canonical IRF-3, IRF-7, c-Jun, and canonical NF-κB pathways. This activation is associated with the upregulatory induction of IFN-I and proinflammatory cytokines and chemokines. For detailed information, see the text. The figure has been assembled with birender.com.

in induction of TNF- α , IL-8, and RANTES through p38 MAPK activation (Gaur et al., 2011). NF-κB is present as a complex with its inhibitor IκB. PKR regulates the NF-κB pathway through activation of the IκB inhibitor of NF-κB (Fig. 10.3) (Oeckinghaus & Ghosh, 2009).

PKC, an upstream molecule of Raf and essential for the activation of the Raf/MEK/ERK pathway, is activated late in infection upon accumulation of IAV hemagglutinin (HA) on lipid rafts at the cell membrane of the host cell to promote the viral ribonucleoprotein (vRNP) complex export to the cell membrane for packaging. PKC is known to play a crucial role in IAV and other enveloped viruses entry (Toker, 1998).

In addition, the PI3K signaling pathway is activated upon IAV infection to support efficient virus replication. PI3K represents a family of 3 main classes of enzymes (I, II, and III), which are fundamental for the regulation of a variety of cellular functions (Ayllon et al., 2012). Class I PI3K enzymes are mostly involved in IAV infection and composed of a catalytic p110 subunit and a regulatory p85 subunit. The regulatory subunit of PI3K p85 contains 2 Src homology domains, SH2 and SH3 (Backer, 2010). Following activation, the catalytic p110 subunit converts phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), which in turn leads to phosphorylation and activation of

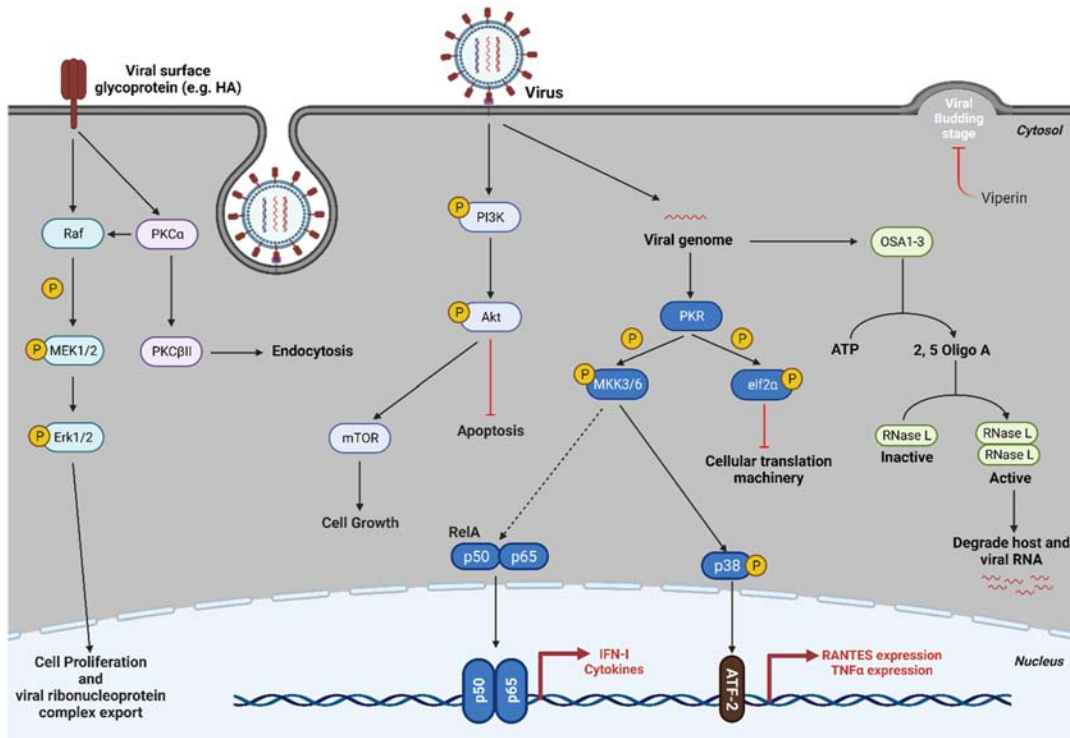


FIGURE 10.3 Virus-induced cell signaling of fundamental intracellular signaling cascades such as PKC, PI3K/Akt, OAS/RNase L, PKR, P38/JNK-MAPK, NF- κ B (canonical). For more description, see the text. The figure has been assembled with [brender.com](https://www.brender.com/).

a number of downstream kinases through binding to the SH2 domain of the p85 subunit, including Akt (Backer, 2010). Subsequently, activated Akt upon phosphorylation (p-Akt) modulate different downstream signaling pathways including cell cycle progression through targeting of rapamycin (mTOR) activation, and apoptosis suppression by inactivating essential proapoptotic factors, including BAD and caspase-9, by phosphorylation (Fig. 10.3) (Gaur et al., 2011; Showkat et al., 2014).

NF- κ B, a family of transcription factors, is activated by both canonical and noncanonical pathways. In the canonical NF- κ B pathway and following the binding of ligand to a cell surface receptor, typically a member of the TLR superfamily, the cytoplasmic downstream subunit of the receptor recruits certain adapters such as TRAF, which in turn recruit the IKK complex (Nemo/IKK α /IKK β). This recruitment leads to the phosphorylation and degradation of the NF- κ B inhibitor, I κ B, that release the active RelA (p50/p65) heterodimer (Oeckinghaus & Ghosh, 2009). However, the noncanonical NF- κ B signaling is induced by a small number of stimuli and is mainly activated upon stabilization of the protein kinase NIK. NIK, together with its downstream IKK α kinase, induces the activation of p52/RelB transcription factor by phosphorylating p100, a cytoplasmic inhibitor of NF- κ B (Razani et al., 2011).

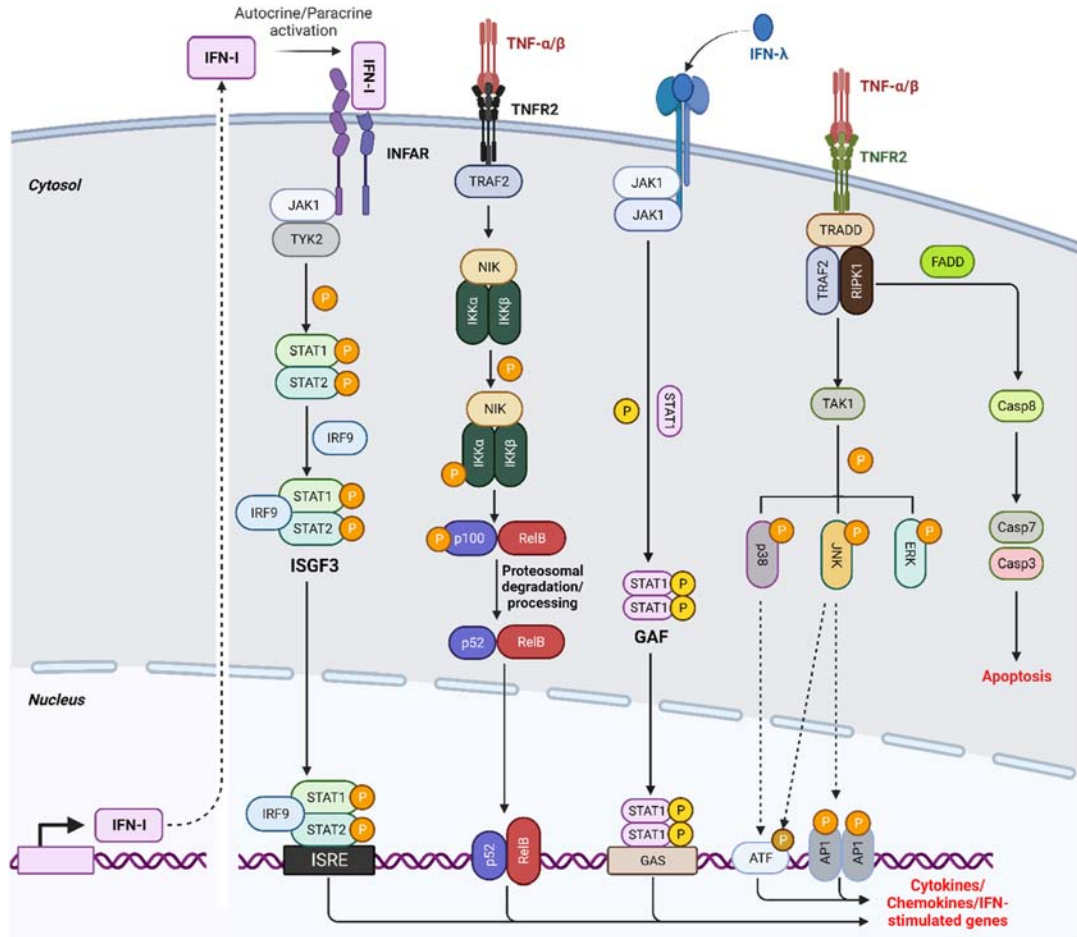


FIGURE 10.4 Autocrine and paracrine IFN-stimulated signaling cascades and pathways. Following secretion of IFN-I/III and TNF, binding to specific receptors on the surface of the same (autocrine) or neighboring (paracrine) cells induce downstream stimulation of the NF- κ B (non-canonical), JAK/STAT and P38/JNK-MAPK pathways. For more description, see the text. The figure has been assembled with [birender.com](https://www.birender.com/).

Following their expression, IFN-I (α/β) and IFN-III (λ) (secreted by T helper cells) induce a variety of signal transduction pathways by targeting different signaling receptors on the same (autocrine) or neighboring (paracrine) cell (Fig. 10.4). Secreted IFN- α/β binds to a heterodimeric transmembrane receptor, composed of the subunits IFNAR1/2, to catalyze the dimerization of the IFNAR1 and IFNRA2 chains of the receptor. This dimerization stimulates the auto-phosphorylation of the receptor-associated tyrosine-protein kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). Subsequently, JAK1/Tyk2 phosphorylates the downstream substrates STAT1 (signal transducer and activator of transcription 1) and STAT2 (signal transducer and activator of transcription 2). To form the essential heterotrimeric transcriptional factor complex IFN-stimulated gene factor 3 (ISGF3), active/phosphorylated STAT1 and

STAT2 engage IRF9 in the cytoplasm and translocate into the nucleus to bind to ISRE-containing promoters and stimulate the transcription of several hundreds of IFN-stimulated genes (ISGs), such as PKR, OAS, and myxovirus resistance 1 (Mx1) to control viral infections (Au-Yeung et al., 2013). In a related context, IFN- γ , an important regulator of immune responses, which is produced by activated T and NK cells, plays a pivotal role in the host defense against viral and bacterial pathogens (Chen & Kolls, 2013). IFN- γ binds to the IFNGR1/2 receptors to induce the autophosphorylation of the receptor associated kinases JAK1 and JAK2. Unlike the IFN-I-mediated JAK/STAT signaling pathway, the IFN- γ -mediated JAK/STAT signaling drives solely the phosphorylation of STAT1 (Li et al., 2007). Active/phosphorylated STAT1 molecules homodimerize forming the gamma activation factor (GAF) that translocates into the nucleus to induce the upregulation of different ISGs through its association with the IFN- γ responsive *cis* element gamma activation sequence (GAS) (Tau & Rothman, 1999).

Interferon-Induced Protein with Tetratricopeptide Repeats (IFIT) proteins family of nonenzymatic functions are stimulated by IFIT inducers, especially IFN-I and IFN-III. Currently, four IFIT proteins were characterized in the cytoplasm of human cells: IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60), and IFIT5 (ISG58). Human IFIT genes are localized and clustered on human chromosome 10 (Zhou et al., 2013). Following IFN stimulation of IFIT proteins, IFIT1 and IFIT2 directly bind eIF3 to control the transcription of the invading viral genes. Moreover, either the cytoplasmic IFIT1/IFIT2/IFIT3 complex or IFIT5 alone senses and breaks down 5' triphosphate dsRNAs (5' ppp-dsRNA) (Zhou et al., 2013). IFIT3 bridges TBK1 to MAVS in mitochondria, to subsequently synergize the activation of IRF3 and NF- κ B (Jacobs & Coyne, 2013; Liu et al., 2011). IFIT2 interacts with MITA (mediator of IRF3 activation) or STING (stimulator of interferon genes) and induces apoptosis through BAX/BAK activation. IFIT1 negatively regulates the cellular antiviral response by disrupting the interaction between TBK1, MAVS, and MITA (Daffis et al., 2010; Li et al., 2009).

Several reports have showed that a variety of upregulated ISGs are beneficial as antiviral biomarkers to discriminate between viral and bacterial infections including IFI6-16 (Marazzi et al., 2012), IFI27 (Pommerenke et al., 2012), and Viperin (RSAD2) (Tang et al., 2017). Viperin was shown also to disrupt lipid rafts to interfere with IAV budding (Fitzgerald, 2011).

In response to viral assaults, the IFN-stimulated tripartite motif (TRIM) proteins contribute in orchestrating PRR-mediated signaling cascades. To date, TRIM56 (Liu, Li, et al., 2016), TRIM25 (Gack et al., 2007), TRIM21 (Foss et al., 2015), TRIM19 (PML) (Chelbi-Alix et al., 1998), TRIM22 (Di Pietro et al., 2013), TRIM28 (Wolf & Goff, 2007), TRIM79 α (Taylor et al., 2011), and TRIM5 (Towers, 2007) were characterized as intrinsic host restriction factor to viral infections. TRIM25 promotes RIG-I ubiquitination and activation to sense dsRNA and subsequently induce IFN-I expression (Gack et al., 2007). Additionally, a recent study has shown that the nuclear TRIM25 specifically targets IAV vRNP complexes to block RNA chain elongation (Meyerson et al., 2017). However, TRIM22 inhibits IAV indirectly by polyubiquitinating of viral nucleoprotein (NP) and targeting it for proteasome-dependent degradation (Di Pietro et al., 2013). Being adjacent as paralogs on chromosome 11 in humans, TRIM22 and TRIM5 α restrict IAV and human immunodeficiency virus (HIV) in a similar way, respectively (Di Pietro et al., 2013). TRIM19, commonly known as the promyelocytic leukemia

(PML) protein, localizes to the mammalian nuclear compartments as a main constituent to form the nuclear bodies (NBs) with other proteins, including SP100 (Nisole et al., 2005). Over-expression of TRIM19 can negatively influence IAV replication in vivo (Chelbi-Alix et al., 1998). In addition, PML-NBs can regulate the recruitment and activation of p53, enhancing apoptosis as a response to stress conditions (Krieghoff-Henning & Hofmann, 2008). In an E3 ligase-independent manner, TRIM56 can specifically impede intracellular vRNA synthesis to restrict IAV replication (Liu, Li, et al., 2016). Unlike other TRIMs, TRIM21 acts a cytosolic crystallizable fragment (Fc) receptor that can be specifically recruited by IgG-, IgM- and IgA-associated pathogens in the cytosol to start its neutralization/engulfment at early times postinfection (Foss et al., 2015). TRIM21 helps in a different way to intracellularly engage neutralizing antibodies to coat the invading nonneutralized pathogen (Foss et al., 2015). In addition, TRIM21 supports IRF3 activation through interference with RBCK1-mediated ubiquitination and degradation of IRF3 (Yang et al., 2009).

Tumor suppressor p53 plays a key regulatory role in different biological processes including cell cycle arrest through the transactivation of the expression of its target gene p21 (Clark et al., 2008). In addition, p53 targets RIG-I to regulate cell survival and proliferation (Hsu et al., 2012). Human guanylate-binding protein 1 (hGBP1), an IFN-inducible protein, plays an important antiviral role. The p53 positively regulates hGBP1 transcription through its direct binding to the p53 response element (p53RE) in the human GBP1 promoter region (Zhu et al., 2013).

Apoptosis, programmed cell death, is one of the host defense mechanisms at early stage of infection to minimize the replication of invading virus (Hale et al., 2008). Nevertheless, IAV uses apoptosis to facilitate its own replication and pathogenicity (Iwai et al., 2013). Up on induction with viral dsRNA, the PKR and OAS/RNase L have been reported to trigger apoptosis following IAV infection (Castelli et al., 1997; Takizawa et al., 1996; Zhou et al., 1998).

In addition to TLRs- and RIG-I-like receptors, IAV activates receptors of the NLR family to initiate the formation of the NLRP3 inflammasome complex (Sarvestani & McAuley, 2017), which in turn prompts the inflammatory form of cell death, namely pyroptosis, by enhancing the processing and secretion of IL-1 β and IL-18 (Tate et al., 2016). Unlikely, the NOD-like receptor family CARD domain containing 5 (NLRC5) is negatively regulating IFN activity through direct interaction with RIG-I, MDA5, IKK α /IKK β (NF- κ B pathway) to block their activation and associated downstream cascades (Loo & Gale, 2011).

The NF- κ B- and ISGF3-mediated induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient IAV propagation (Wurzer et al., 2004). Death receptors (DRs) and their ligands regulate innate and adaptive immune responses against pathogens by supporting either cell death or survival. TRAIL, TNF- α , and Fas ligand are well-characterized DR ligands (Guicciardi & Gores, 2009). The binding of these ligands to the DR activates a cascade of specific proteolytic enzymes, namely caspases (Casp3, Casp7, and Casp9), leading to apoptosis (Lamkanfi & Kanneganti, 2010).

Eventually, to control essential virus-dependent cell-signaling pathways and regulate antiviral innate immune responses, a panel of active agents have been applied in preclinical and clinical studies (Table 10.1).

TABLE 10.1 Examples of immunoregulatory and cell-based antivirals.

Compound	Signaling pathway/target	Primary indication	Virus(es)	Status	References
PD-0325901, AZD-6244, AZD-8330 and RDEA-119,	Raf/MEK/ERK signaling pathway	Anticancer	IAV	Phase-I	Haasbach et al. (2013)
ATR-002	Raf/MEK/ERK signaling pathway	Anticancer	IAV	Phase-I	Hamza et al. (2021)
Cyclopentenone prostaglandins	Induction of cytoprotective heat-shock proteins, modification of viral glycoprotein maturation and control of NF-KB activation.	Antiinflammatory, anticancer, and antineoplastic	HIV, IAV, Polioviruses, VSV and HSV	In vitro	Santoro (1997) , Straus and Glass (2001)
CSC27 (manassantin B)	MTORC2–PKC/ AKT-signaling pathway and STING/TBK-1/IRF3 signaling pathway	N/A	Epstein–Barr virus (EBV) and coxsackievirus	In vitro	Song et al. (2019)
Cirsimaritin	NF-KB downregulation	Anticancer and antimicrobial	IAV	In vitro	Yan et al. (2018)
Caffeic acid phenethyl ester (CAPE), resveratrol, Bay11-7082, and parthenolide	NF-KB inhibition	N/A	SARS-CoV	Preclinical	DeDiego et al. (2014)
IRF3	NF-KB inhibition	Inducing IFN and antiviral genes	Sendai virus	Preclinical	Popli et al. (2022)
Ivermectin	Importin α/β inhibitor	Antiprotozoa	HIV and DENV	In vitro	Wagstaff et al. (2012)
Go 6983, bisindolylmaleimide I, enzastaurin, and sotrastaurin	Inhibition of protein kinase C (PKC)	N/A	SARS-CoV-2	In vitro	Huang et al. (2022)
Rapamycin	Inhibition of PI3K/Akt/mTOR pathway	Immunosuppressant	MERS-CoV and SARS-CoV-2	In vitro	Fattahi et al. (2022) , Patocka et al. (2021)
Everolimus	Inhibition of PI3K/AKT/mTOR signaling pathway	Indicated for renal angiomyolipoma and tuberous sclerosis complex (TSC)	Cowpox, IAV and DENV	Preclinical	Murray et al. (2012)

(Continued)

TABLE 10.1 Examples of immunoregulatory and cell-based antivirals.—cont'd

Compound	Signaling pathway/target	Primary indication	Virus(es)	Status	References
MK-2206	Inhibition of PI3K/AKT/mTOR signaling pathway	N/A	SARS-CoV-2	In vitro	Appelberg et al. (2020)
Rapamycin and PP242	Inhibition of PI3K/AKT/mTOR signaling pathway	N/A	Porcine reproductive and respiratory syndrome virus (PRRSV)	In vitro	Liu et al. (2017)
Metformin	Inhibition of PI3K/AKT/mTOR signaling pathway	Antidiabetic	SARS-CoV-2	In vitro	Pedrosa et al. (2023) , Ramaiah (2020)
SB203580 and SB202190	Inhibition of the p38/MAPK cell signaling pathway	N/A	Junín virus (JUNV)	In vitro	Brunetti et al. (2022)
AG879	Inhibition of nerve growth factor receptor (NGFR), human epidermal growth factor receptor 2 (TrkA/HER2) signaling	N/A	IAV, Sendai virus, HSV, mouse hepatitis virus, Pichinde virus and rhesus rotavirus	In vitro	Kumar et al. (2011a, 2011b)
Tyrphostin A9 (A9)	Inhibition of platelet-derived growth factor receptor (PDGFR) pathway	N/A	IAV, Sendai virus, HSV, mouse hepatitis virus, Pichinde virus and rhesus rotavirus	In vitro	Kumar, Sharma, et al. (2011)
Vemurafenib	Raf/p38/JNK MAPK pathways	Antitumor	IAV	In vitro	Holzberg et al. (2017)
U0126	Raf/MEK/ERK MAPK signaling pathway	N/A	IAV, IBV, PEDV, Borna disease virus (BDV), mouse hepatitis virus, JUNV and HSV	In vitro and preclinical	Cai et al. (2007) , Colao et al. (2017) , Kim and Lee (2015) , Ludwig et al. (2004) , Planz et al. (2001) , Rodríguez et al. (2014)
Gefitinib	Inhibition of epidermal growth factor receptor (EGFR)	Antitumor	Human cytomegalovirus (MHCMV) and poxvirus	In vitro	Herget et al. (2004) , Langhammer et al. (2011)

TABLE 10.1 Examples of immunoregulatory and cell-based antivirals.—cont'd

Compound	Signaling pathway/target	Primary indication	Virus(es)	Status	References
Hypericin	Inhibition of protein kinase C (PKC)	Antitumor	Murine cytomegalovirus (MCMV), Sindbis virus, and HIV	Phase-I	Gulick et al. (1999) , Hudson et al. (1991)

2. Genomics-based antiviral pharmaceutical applications

The traditional targets for antivirals are viral proteins that are pivotal for virus infectivity, genome replication, gene transcription, and budding from infected cells. However, due to the high viral evolution rate, viral escape and resistant mutants emerged against these pathogen protein-targeted antivirals, and many of these antivirals lost their activity ([Hayden & de Jong, 2011](#)). Also, drug development is a very long and costly process that will be a step behind in competing with the rapid pace of virus evolution, especially RNA viruses. Therefore, new antiviral targets, other than viral proteins, are substantial for keeping combating the continuous emergence of viral variants and reemerging viruses. Genomics-based antivirals are novel approaches that target either the viral or host genome in lieu of viral proteins in combating viral diseases. The significant advances over the past decade in high-throughput deep sequencing and molecular biology techniques expand and illuminate our understanding of the role of genetics in the control and prevention of viral diseases ([Munk et al., 2011](#)).

2.1 Viral genomic-based antivirals

Recently, several viral genomic-based approaches have been explored and investigated for combating viral diseases, including ribonucleases (RNases), short interfering RNA (siRNA), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems, among others. Herein, we highlight the antiviral activity of each of the aforementioned approaches against viral diseases.

2.1.1 RNases

RNases are hydrolytic enzymes that catalyze vRNA molecules conversion into smaller components by cleaving the phosphorous-oxygen bonds specifically after the pyrimidine residues thereby degrading vRNA and inhibiting viral replication ([Mohammadi Pour et al., 2019](#)). Also, RNases are contributing to the induction of IFN-I production and apoptosis as countermeasures against viral infections (see previous section). RNase L, a member of the RNases family, is an endoribonuclease key factor involved in IFN-regulated and dsRNA-activated antiviral immune response against different viruses including picornaviruses ([Han et al., 2007](#)), coronaviruses ([Zhao et al., 2012](#)), rotaviruses ([Zhang, Jha, et al., 2013](#)),***

Zhang, Song, et al., 2013nd IAV (Min & Krug, 2006). Through the OAS/RNase L signal pathway (Fig. 10.3), RNase L is activated by 2–5' oligoadenylates (oligo A) and cleaves cytoplasmic RNA and vRNA. Another RNase member, RNase A superfamily, showed antiviral activity against HIV (Lee-Huang et al., 1999). Nonetheless, viruses undergo defensive and evasive mechanism to inhibit or block the antiviral activity of RNases. For instance, the N-terminal of the RNA-binding domain of IAV NS1 protein binds dsRNA and inhibits the 2'-5'oligo A synthetase/RNase L pathway (Cooper et al., 2015). Also, RNases were shown to have cell cytotoxic effect if not retained in appropriate doses (Makarov & Ilinskaya, 2003). Likely, to evade mammalian RNase inhibitors and retain its catalytic activity inside eukaryotic organisms, small bacterial ribonuclease binase (12.2 kDa) has been used as an in vitro and in vivo antiviral against foot-and-mouth disease virus (FMDV), IAV, influenza B virus (IBV), reovirus, coronaviruses, and rabies (Müller et al., 2017; Shah Mahmud et al., 2016, 2018; Ulyanova et al., 2020).

2.1.2 *siRNA*

RNA interference (RNAi) or posttranscriptional gene silencing (PTGS) is one class of small RNAs (sRNAs), which are polymeric RNA molecules that are less than 200 nucleotides in length and usually noncoding. RNAi is a conserved biological process whereby mRNA is degraded and results in sequence-specific suppression of gene expression (Qureshi et al., 2018). In brief, the long dsRNA is cleaved by an endoribonuclease enzyme (DICER) to form siRNA, which is then incorporated in other proteins to form the RNA-Induced Silencing Complex (RISC) that eventually enables the binding of siRNA to the complementary mRNA and induced its cleavage (Gish et al., 2011). Indeed, siRNA showed antiviral activity against different viruses including HIV (Singh, 2008), IAV (Ge et al., 2003), severe acute respiratory syndrome coronavirus (SARS-CoV) (Meng et al., 2006), hepatitis B virus (HBV) (Konishi et al., 2003), and West Nile virus (WNV) (Kumar et al., 2006). Intriguingly, in 2018, the Food and Drug Administration (FDA) approved the first siRNA-based drug followed by three more drugs so far by which the siRNA-based drugs started to be implemented in combating viral diseases (Mehta et al., 2021). Nevertheless, the major obstacle for the application of siRNA is drug delivery and cell penetration due to the inability of naked siRNA to freely diffuse through cellular membranes, in addition to its poor stability. A great breakthrough in siRNA delivery has been achieved through nonviral (e.g., plasmid, liposomes, exosome nanoparticles, etc) (Tong et al., 2023; Yan et al., 2022) and viral vectors (Ghosh et al., 2020; Lundstrom, 2020) for in vitro and in vivo purposes (Fig. 10.5).

2.1.3 *CRISPR-Cas systems*

CRISPR-Cas is a defensive adaptive immune response of bacteria and archaea against virus and phage infections. Researchers have been able to harness this natural defense mechanism to develop novel antiviral therapies. For example, CRISPR-Cas can be used to target and destroy specific viral DNA or RNA sequences within infected cells, thus preventing the virus from replicating and spreading. CRISPR-Cas system is classified into two major classes (Class 1 and Class 2) with each of them having unique Cas protein effectors: Types I, III, and IV for Class 1; and Types II, V, and VI for Class II. Among the two classes of the Cas systems, class II Cas endonucleases have been extensively studied for combating DNA (Cas9 and Cas12) and RNA (Cas13) viruses. Generally, the CRISPR-Cas system is made of two main components:

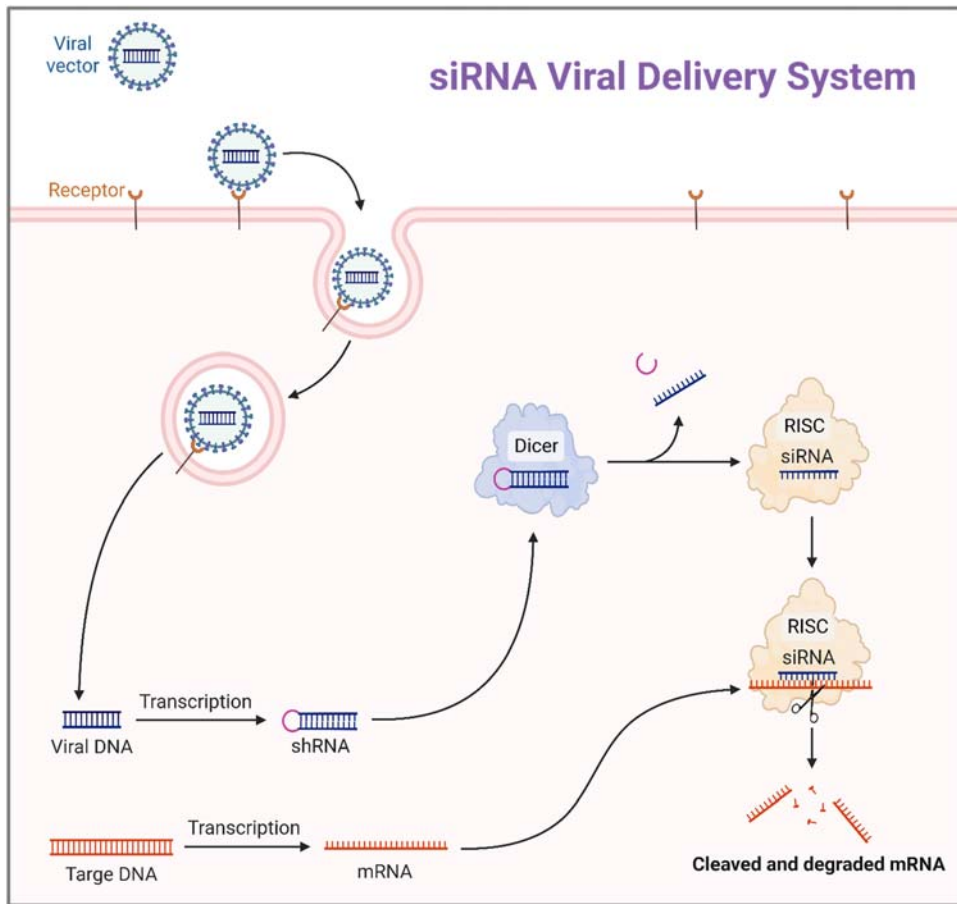


FIGURE 10.5 Schematic diagram of a generic viral vector. The helper DNA, delivered through a virus vector, is transcribed into short hairpin RNA (shRNA). The shRNA is then cleaved by an endoribonuclease enzyme (DICER) to form siRNA that interacts with RISC forming RNA-RISC complexes to facilitate the binding of siRNA to the complementary mRNA (genomic or viral) and induce its degradation. For additional information, see the text. The figure was assembled by [biorender.com](https://www.biorender.com).

CRISPR repeat-spacer arrays that transcribed into CRISPR RNA (crRNA) and *trans*-activating RNA (tracrRNA) together with CRISPR-associated (Cas) genes that encode for endonucleases (Koonin & Makarova, 2009).

The type II CRISPR-Cas9 system is one of the well-established approaches in treating viral diseases through its main protospacer adjacent motif (PAM) interacting domain. Once the Cas9 detects its PAM-specific nucleotide sequence, which is downstream to the target viral DNA sequence and when the target DNA is complementary to the guide RNA (gRNA), the Cas9 starts to cut the viral DNA genome leading to the stop of virus replication (Fig. 10.6). Similarly, Cas12 has antiviral activity against DNA viruses with some differences

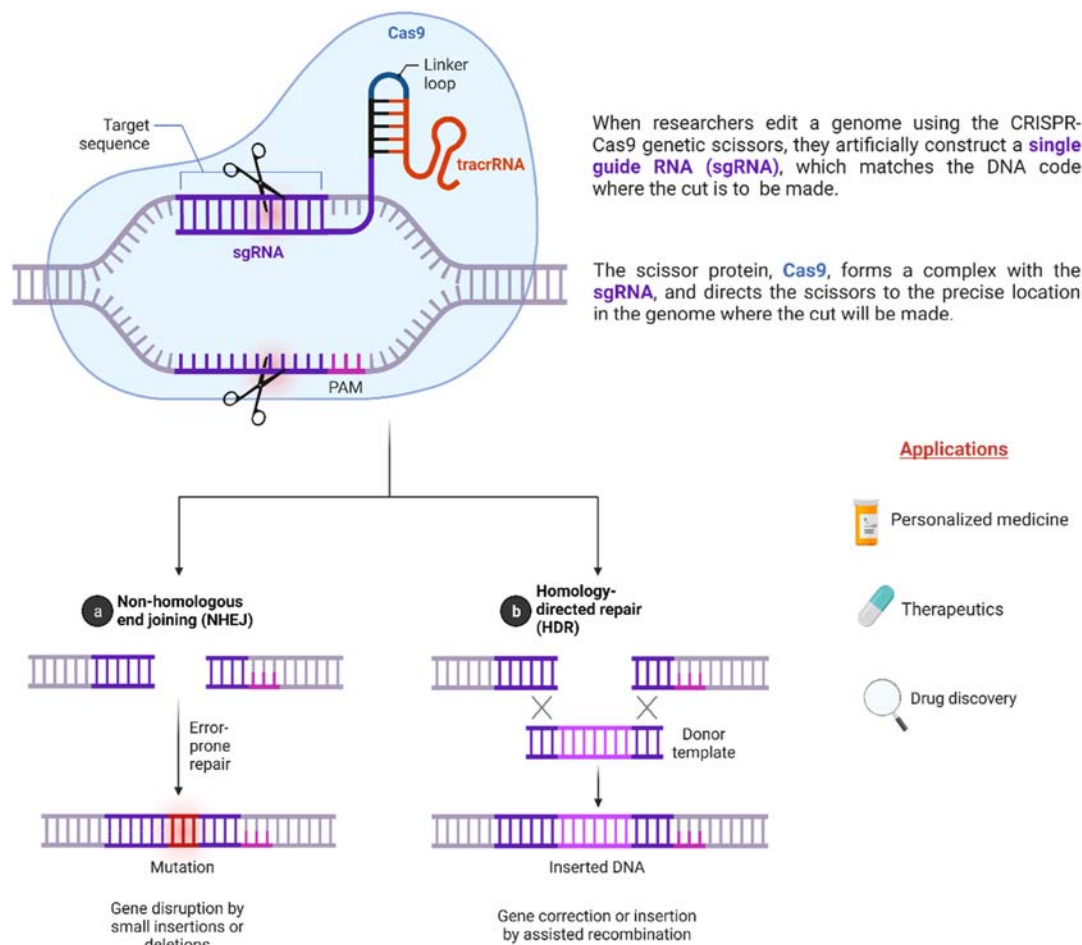


FIGURE 10.6 Mechanism of action of CRISPR-Cas9. The Cas9 first recognizes unique G-rich nucleotide sequences downstream to the target DNA sequence that is complementary to the synthetic gRNA. Then, Cas9 starts to cut the DNA sequence upstream to the PAM sequence. For genome editing, the Cas9 either follows the nonhomology-directed repair (A) or homology-directed repair (B). For additional information, see the text. The figure has been assembled with [birender.com](https://www.birender.com/).

in the mechanism of action compared with Cas9. Cas12 cuts the DNA downstream to the PAM sequence and results in sticky end cuts, while Cas9 exerts its effect upstream to the PAM and resulted in sticky ends DNA (Xu & Li, 2020). Unlike Cas9 and Cas12, Cas13 has antiviral activity against RNA viruses, which binds to the RNA using the protospacer-flanking sequence (PFS) domain instead of the PAM domain. Indeed, Cas13 has been used against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and IAV (Blanchard et al., 2021). Furthermore, the mechanism of CRISPR-Cas system (Xu & Li, 2020) and its application against infectious viral pathogens have been summarized in Fig. 10.6 and Table 10.2, respectively.

TABLE 10.2 List of CRISPR-Cas systems applications against viral diseases.

Cas	Target gene	Virus	Genome	References
Cas9	p30 gene (CP204L)	African Swine Fever Virus (ASFV)	dsDNA	Hubner et al. (2018)
	ICP0, ICP4, and ICP27 genes	HSV		Roehm et al. (2016)
	UL7 genes			Xu et al. (2016)
	UL15, UL27, UL29, UL30, UL36, UL37, UL42, UL5, UL52, UL8, UL54, UL9, US3, and US8			van Diemen et al. (2016)
	UL8, UL29, and UL52			Karpov et al. (2019)
	UL8 and UL29 genes			Yin et al. (2021)
	TLR	HIV	(+) ssRNA	Ebina et al. (2013)
	TLR-U3			Hu et al. (2014)
	5'-LTR and Gag gene			Kaminski, Bella, et al. (2016)
	LTR-U3			Kaminski, Chen, et al. (2016)
	LTR7, LTR8, and structural region (env5, vif2, rev3, gag8, pol6, and pol7)			Wang et al. (2018)
	LTR, gag, and pol			Yin et al. (2016)
	EBNA1, OriP	EBV	dsDNA	van Diemen et al. (2016)
	UL54, UL44, UL57, UL70, UL105, UL86, and UL84	HCMV		
Cas12a	Ei-1 gene	<i>Bombyx mori</i> nuclear polyhedrosis virus (BmNPV)	dsDNA	Dong et al. (2020)
Cas13a	PB1 and PB2 genes of influenza	IAV	(-) ssRNA	Blanchard et al. (2021)
	Conserved regions of H1N1			Freije et al. (2019)
	Replicase and nucleocapsid genes of SARS-CoV-2	SARS-CoV-2	(+) ssRNA	Blanchard et al. (2021)
	RdRp (ORF1ab) and N gene			Abbott et al. (2020)
	Replicase and transcriptase (ORF4b) and S gene			Nguyen et al. (2020)

2.2 Host-genomics antiviral applications

In addition to the viral genome as a potential antiviral target, high-throughput sequencing analyses have launched a new field of antiviral targets by elucidating the correlation between viral disease susceptibility and host genomics. As virus infection cannot be established without hijacking the host machinery throughout the whole virus replication cycle, targeting

those replication-critical host factors will halt the progression of virus replication. The major advantage of this approach is that the host genome is substantially stable, which is easy to be targeted compared with the fast-evolutionary viral genome.

Genome-wide association studies (GWASs) and whole-exome sequencing (WES) are highly advanced throughput tools that unveiled the host genetic diversity and further the susceptibility to viral diseases (Chapman & Hill, 2012; Verma & Sharma, 2018). For instance, the high expression of tyrosine kinases (TYK2) was significantly correlated with an increase in the severity of the 2019 coronavirus disease (COVID-19), thus, TYK2-targeted drugs (JAK inhibitor Baricitinib) could represent a potential therapeutic for the treatment of COVID-19 (Pairo-Castineira et al., 2021). Additionally, INAR2, JAK1, JAK2, TNF, IL6, TMPRSS2, and ILIR1 genes have been identified as potential targets for COVID-19*** (Liu, Yang, et al., 2021). Inhibition of human RACK1 protein showed a similar effect to the antih herpes drug acyclovir (Ullah et al., 2019). Also, the IFITM genes were correlated to the susceptibility to IAV infection (Zhai et al., 2015). Xu et al. defined the criteria for choosing antiviral host targets with all approved gene targets for variable viral diseases (Xu et al., 2021).

3. Drug repurposing for antiviral use

Unexpected epidemics and the risk of occasional global pandemics caused by highly pathogenic viruses are usually associated with unprecedented morbidities and mortalities due to the lack of prophylactic vaccines or efficient therapeutic antiviral medications. To this point, drug repurposing approaches based on in vitro, preclinical studies and medical observations have overcome the need of testing for safety, pharmacokinetic, and pharmacodynamic studies in preclinical and clinical phases and to compensate the unavailability of fast and accurate virus control options. Repurposing a medication is also known as therapeutic switching. This way allows finding new applications for FDA-approved drugs or clinically used chemicals. It is effective for creating innovative pharmacological or therapeutic drug candidates. Drug repositioning improves medicine development since drug discovery is costly, time-consuming, complicated, and highly risk. The repurposing reduces drug development time, cost, efficiency, and failure risk compared with traditional methods (Dotolo et al., 2021). Humans are at high risk of both viral epidemics and pandemics. These aggravated viral events are mostly confined to six viral families, including Arenaviridae, Coronaviridae, Filoviridae, Flaviviridae, *Nairoviridae*, and Orthomyxoviridae. Members in these viral families have reemerged in recent years to cause or potentially trigger an epidemic or pandemic in humans (Letko et al., 2020).

The COVID-19 pandemic is the most recent evidence of the threats posed by potential natural or resurgent human and zoonotic viruses to human health. Some of the viruses under question include, among others: Chikungunya Virus (CHIKV), Crimean-Congo Hemorrhagic Fever Virus (CCHFV), Dengue Virus (DENV), Ebola Virus (EBOV), Henipaviruses (Nipah, Hendra), Human Immunodeficiency Virus (HIV), IAV, Lassa Virus (LASV), Middle East Respiratory Syndrome (MERS-CoV), Rift Valley Fever Virus, SARS-CoV, WNV, Yellow Fever Virus (YFV), and Zika Virus (ZIKV) (Neumann & Kawaoka, 2023). In December 2019, a novel coronavirus, SARS-CoV-2, emerged in the city of Wuhan in China and has rapidly spread around the globe, causing the COVID-19 pandemic (Zhu et al., 2020). As of April 2023,

more than 762 million cases have been confirmed globally, including around 6.8 million deaths. To save funds and make better use of existing medicines, drug repurposing or repositioning represents an attractive type of drug development (Dotolo et al., 2021), because pre-clinical and clinical safety studies have already been conducted, and this aids in the discovery of novel medicines for widely spreading viral illnesses at a lower cost and in a shorter period of time. Most well-repurposed drugs were found either by coincidence or through the use of targeted pharmacological insights or experimental screening platforms (de Oliveira & Lang, 2018). Examples of repurposed drugs to control recently emerging epidemics and pandemics with Emergency Use Authorization (EUA) are listed in Table 10.3.

One of the recent antiviral drugs that received EUA by the FDA to treat the devastating consequences of the COVID-19 pandemic is the ribonucleoside Molnupiravir. This drug is approved for oral administration in a prodrug form that is converted inside the cell into Molnupiravir triphosphate (MTP, active form) that can be incorporated by viral RNA-dependent RNA-polymerases (RdRp) during viral mRNA synthesis, resulting in viral mutations and lethal mutagenesis (Fig. 10.7).

As a mutagenic ribonucleoside antiviral agent, there is an unapproved risk that MTP can be metabolized by the human host cell and incorporated into the host DNA, leading to mutations. However, the FDA concluded that, based on the available genotoxicity data and the 5-day duration of the treatment, molnupiravir has a low risk for genotoxicity.

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Chloroquine (CQ)	- Systemic-lupus erythematosus, rheumatoid arthritis and different skin conditions treatment. - Antimalaria; lysosomotropic CQ accumulates in acidic digestive vacuole inside red blood cells, where CQ binds to hemes to form a toxic product resulting in cell lysis and ultimately parasite cell autodigestion.	- SARS-CoV-2 - SARS-CoV - HCV - DENV - ZIKA - CCHFV - MERS-CoV	- Chloroquine increases lysosomal pH, which in turn affects SARS-CoV-2 receptor linkage and interaction with glycosylation. - Inhibit viral replication - As a lysosomotropic agent, CQ blocks the membrane fusion between virus and lysosomes to inhibit virus entry	Chatre et al. (2018) , Khandia et al. (2018) , Liu, Du, et al. (2021) , Sezer et al. (2022)
Hydrochloroquine (HCQ)	Rheumatoid arthritis, Sjorgren's syndrome, and juvenile idiopathic arthritis treatment.	SARS-CoV-2	Effective in reduction of viral loads	Jorge et al. (2018) , White et al. (2020)

(Continued)

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.—cont'd

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Ivermectin	- Strongyloidiasis and onchocerciasis treatment - Antiviral properties against RNA viruses.	- HIV - Dengue - Influenza - SARS-CoV-2	- Inhibits viral replication. - Inhibits more than 85% of enzymatic activity of 3 CL protease that has important role in generating functional and non-structural viral proteins.	Kory et al. (2021) , Mody et al. (2021)
Interferons α/β (IFNs- α/β)	- They are proteins, cytokines important for immune response to act on innate and adaptive immunity. - They are cell signaling proteins that are expressed in response to viral infections, tumors, and many other infectious agents. - Immunotherapy for cancer. - Approved for antiviral (HCV, HBV) and multiple sclerosis treatment; IFN induces the production of interferon-stimulated genes through JAK-STAT pathway	- SARS-CoV-2 - SARS-CoV - MERS-CoV - HCV - HBV - HIV - EBOV - DENV - ZIKV	- Regulates inflammatory factors that shows a promising antiviral activity against SARS-CoV-2. - Inhibition of multiple targets - IFN exerts the broad-spectrum antiviral activity by inducing ISG production, which may inhibit each step of the viral life cycle	Al Ghamdi et al. (2016) , Nakhband et al. (2021) , Pires de Mello et al. (2018a, 2018b) , Ströher et al. (2004)
Fluvoxamine	- It belongs to class of other antidepressants as: selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs). - Obsessive-compulsive disorder treatment	SARS-CoV-2	Its mechanism of action as an antiviral is uncertain, but was examined as a possible treatment for its antiviral and antiinflammatory effects.	Reis et al. (2022)
Molnupiravir	Oral antiviral drug	SARS-CoV-2	- RdRp inhibitor - Reduce the virus's ability to replicate.	Hashemian et al. (2022) , Zarenezhad and Marzi (2022)

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.—cont'd

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Sofosbuvir	— Drug used for hepatitis C (HCV) treatment	— SARS-CoV-2 — HCV — HIV	— The first oral antiviral drug that is directly effective in attenuating viral RNA and SARS-CoV-2 in the nasopharynx, which is well tolerated and with good safety. — RdRp inhibitor — Suppressing some families of positive-strand RNA viruses.	Eslami et al. (2020)
Ebselen	— Organoselenium, antioxidant compound, exhibiting antiinflammatory and antiviral activities	— SARS-CoV-2 — HCV — HIV — Zika — IAV	— MPRO (main protein) inhibitor.	Jin et al. (2020) , Sharun et al. (2020)
Favipiravir	— Antiinfluenza drug	— Yellow fever viruses (YFV) — Ebola — SARS-CoV-2 — LASV — JUNV — CCHV — RVFV — Rabies — RSV	— RdRp inhibitor — Decrease levels of IL-6 and other lymphocytes which prevent cytokine storm	Furuta et al. (2013) , Oestereich et al. (2014, 2016) , Qomara et al. (2021) , Sarkar et al. (2020) , Sleeman et al. (2010) , Yamada et al. (2016)
Ribavirin (RBV)	— Antiviral drug	— SARS-CoV-2 — SARS-CoV — MERS-CoV — HCV — RSV — HBV — HEV — ZIKA — LASV — Ebola (EBOV)	— RdRp inhibitor — Inhibits DNA and RNA virus reproduction. — RNA capping. — Increases viral RNA instability	Gong et al. (2021) , Ortega-Prieto et al. (2013)
Remdesivir	— Antiviral activity against paramyxoviruses, flaviviruses and coronaviruses.	— Ebola (EBOV) — JUNV — SARS-CoV-2 — MERS-CoV — SARS-CoV — RSV — HCV — NiV	— RdRp inhibitor — Remdesivir drug inhibits viral replication and reduces the viral load	Jacobs et al. (2016) , Pedersen et al. (2019) , Sheahan et al. (2017) , Warren et al. (2016)

(Continued)

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.—cont'd

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Galidesivir	– EBOV antiviral	– SARS-CoV-2 – IAV (H1N1) – SARS-CoV – EBOV – ZIKA – RSV – LASV – RVFV – DENV – WNV – DENV – YFV – TBEV – SUDV – MARV	– RdRp inhibitor	Eyer et al. (2017, 2019) , Julander et al. (2017) , Warren et al. (2014)
Lopinavir/ Ritonavir	– HIV treatment	– SARS-CoV – SARS-CoV-2 – MERS-CoV – HIV	– Protease inhibitor	Choy et al. (2020) , De Wilde et al. (2014) , Masse et al. (2007)
Rupintrivir	– HRV treatment	– HRV – Echovirus-6 – CVB2 – EV71/695 F – HCoV-229E – TGEV – BOC – Norovirus/ Norwalk	– Protease inhibitor	Binford et al. (2005) , Kim et al. (2012) , Rocha-Pereira et al. (2014) , Zhang, Song, et al. (2013)
Chlorpromazine (CPZ)	– Antipsychotic; CPZ antagonizes dopamine receptors to exert antipsychotic activity	– SARS-CoV – MERS-CoV – SARS-CoV-2 – HCV – DENV – ZIKA – CSFV – CCHFV – SFV – EBOV	– Virus entry inhibitor – CPZ and other dopamine receptor antagonize clathrin-mediated endocytosis which is required for some virus entry	Blanchard et al. (2006) , De Wilde et al. (2014) , Khandia et al. (2018)
Sunitinib/erlotinib	– Antitumor; inhibiting tumor cell growth by inhibiting receptor tyrosine kinase (RTK) pathways	– SARS-CoV-2 – HBV – HCV – DENV – ZIKA – EBOV	– Virus entry inhibitor – Sunitinib/erlotinib inhibits RTKs including AAK1 or EGFR, which is involved in	Bekerman et al. (2017) , Gan et al. (2020)

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.—cont'd

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Camostat	— Chronic pancreatitis and postoperative reflux esophagitis; inhibiting transmembrane protease, serine 2 (TMPRSS2)	— CHIKV	intracellular vesicle trafficking that is used by some virus to enter cells	Hoffmann et al. (2020) , Hosoya et al. (1992)
		— SARS-CoV — MERS-CoV — SARS-CoV-2 — IAV — IBV	— Virus entry inhibitor — Camostat is an inhibitor to TMPRSS2 which is required for entry of coronaviruses or influenza	
Statins	— Hypercholesterolemia; statins are HMG-CoA reductase inhibitors to reduce cholesterol biosynthesis	— DENV — ZIKA — HCV — HIV	— Virus replication inhibitor — Statin inhibits replicase formation of flaviviruses, reverse transcription of HIV; impairs EBOV glycoprotein processing	Españo et al. (2019) , Martínez-Gutierrez et al. (2011) , Werner et al. (2014)
Digoxin	— Heart failure; the cardiac glycoside can block the Na ⁺ /K ⁺ ATPase ion pump activity to raise the intracellular Ca ²⁺ level to improve the cardiac failure	— HSV — HIV — HBV — JEV — CHIKV — SINV — RRV — RSV — LCMV — LASV — JUNV — reovirus — VSV — EBOV — MERS-CoV	— Virus replication inhibitor — Digoxin impedes the immediate-early or early gene expressions of herpes viruses; impairs JEV or EBOV RNA replication or virus entry; may also inhibit coronavirus entry; inhibits arenaviruses whose replication depends on Na ⁺ /K ⁺ ATPase activity	Grosso et al. (2017) , Guo et al. (2020) , Iwasaki et al. (2018) , Su et al. (2008)
Imatinib	— Antitumor; imatinib inhibits tyrosine kinase c-abl to block tumor cell proliferation	— SARS-CoV — MERS-CoV — SARS-CoV-2 — EBOV — DENV — VacV	— Virus assembly/release inhibitor — Imatinib decreases the budding or release of EBOV, VacV, and DENV; imatinib also inhibits CVB entry	Chen et al. (2020) , García et al. (2012) , Han et al. (2021)

(Continued)

TABLE 10.3 Repurposed FDA-approved drugs for antiviral emergency use.—cont’d

Drug	Original indication	Repurposing to (virus)	Mechanism of action	References
Nitazoxanide	– Antiparasite; nitazoxanide interferences with the pyruvate: Ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction to disrupt parasite energy metabolism	– IAV (H1N1/PR8) – SeV – SARS-CoV2 – RSV – HCV – HBV – HIV – rotavirus – CHIKV – MHV – JEV – ZIKV	– Nitazoxanide inhibits morphogenesis of IAV and rotavirus; post-entry steps of SeV and RSV; HBV DNA replication; viral protease activity of ZIKV or coronavirus; induce innate immune genes	Cao et al. (2015, 2017) , Wang et al. (2020)

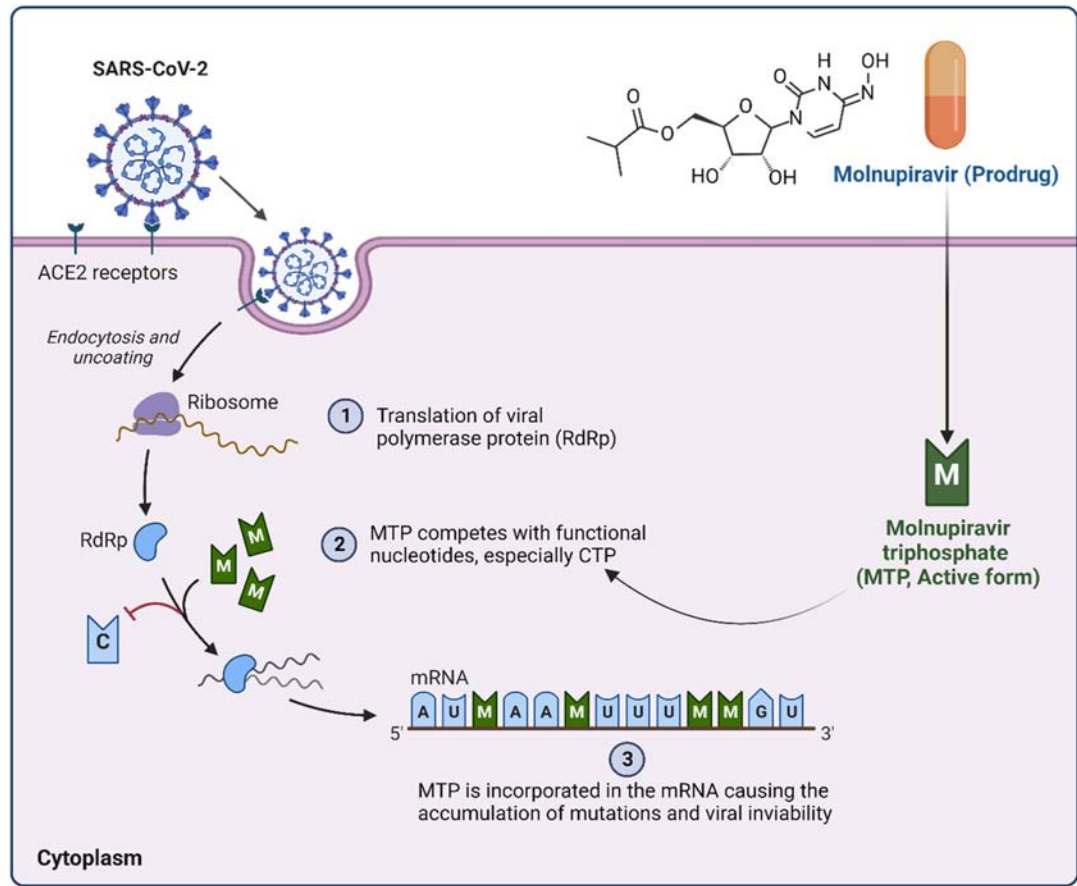


FIGURE 10.7 Mechanism of action of the mutagenic ribonucleoside antiviral agent molnupiravir. The active form of molnupiravir is formed intracellularly to compete with the natural dNTPs, especially CTP, introducing lethal mutations to the viral genome. The figure has been assembled with [birender.com](#).

4. Perspectives and conclusions

The emergence and reemergence of life-threatening viral infections that eventually led to seasonal epidemics and devastating pandemics of high economic and public health impact in humans have created an immediate demand for nonclassical and effective antiviral approaches to combat emerging viral infections. Being a pathogen that replicates only inside the living cells of the host, viruses completely depend on essential host cell elements, machinery, and pathways to efficiently replicate their genomes and transcribe/translate their corresponding structural and auxiliary viral proteins. To this point, several antiviral therapies were clinically approved or are currently in clinical trials to counteract these essential pathways and/or cellular machineries.

Traditionally, the conventional antiviral drugs were designed to target viral proteins including surface glycoproteins, viral polymerases, or proteases. However, with the rapid evolution of viruses and their ability to acquire adaptive nonsynonymous mutations led to the rapid emergence of drug-resistant viruses, limiting the usage of these antiviral drugs in acute and chronic viral infections. With the progress of the science and comprehensive understanding of the viral replication cycles, and its depending consequences on cellular proteins and viral genome levels, new cell-based and genome-based approaches were implemented to control virus replication and/or severity.

Herein, we review the available host-based and genome-based antiviral therapeutics that are already available to combat emerging and remerging viral infections, taking the advantage that the genetic variability rate(s) among hosts is assuredly less than viruses. This supports the fact that the host-based antiviral drugs are less likely to acquire resistance markers and to become ineffective against the treatment of viral infections, including potential subsequent variants. On the same hand, drug repurposing approaches have been intensively implemented, especially during epidemics or pandemics as recently illustrated with the COVID-19 pandemic, due to the unavailability of effective antiviral prophylactic vaccines or therapeutic drugs. By studying the mechanism by which these repurposed FDA-approved drugs could affect viruses, most of these drugs have showed either host-based or genome-based nonspecific mode of antiviral action to block viral replication and diminish their pathogenicity.

On the other hand, the major drawback of genome- or host-targeted antiviral drugs is related to cellular impairments and toxicity due to nonspecific targeting of essential cellular pathways and/or cascades. Therefore, optimal characterization of the host target, outlining the mechanism of action of the candidate drug and precise identification of off-target effects are real challenges to the implementation of genome- and host-targeted therapy.

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Antibiotic resistance and target-based technologies for drug development

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1. Introduction

The discovery of broad-spectrum antibiotics was initiated with penicillin, the first of the β -lactams, which paved the way for the “Golden Age” (1940–62) of antibiotic discovery. During this period, a plethora of novel natural compounds were identified, such as phenylpropanoids (chloramphenicol), polyketides (tetracycline), aminoglycosides (streptomycin, gentamycin), macrolides (erythromycin), glycopeptides (vancomycin), and streptogramins (quinupristin and dalbapristin [Synercid]), followed by synthetically derived, nalidixic acid, and second-generation β -lactams (cephalosporins) (Wright, 2012). In the early 1970s, a third class of β -lactams (carbapenems such as imipenem) was also found. These three classes of β -lactam antibiotics are structurally different in the second non- β -lactam ring motif containing sulfur heterocycle and five-membered carbocycle (Fernandes et al., 2013). In the 1990s, new methods such as recombinant DNA and protein structure determination have emerged with a potential of new candidate discovery. These advanced techniques of recombinant DNA technology and molecular modeling facilitated the creation of vast chemical libraries and expedited the design and screening of drugs. These approaches were successfully implemented across multiple medical domains, including the modern discovery of antibacterial medications (Pina et al., 2009).

Antibacterial drugs have greatly improved our ability to control bacterial diseases, significantly reducing illness and death by being a crucial part of modern medicine. However, despite their importance, society undervalues antibacterial drugs in terms of cost. Over the past century, antibiotics have put solid pressure on microorganisms, leading those with antibiotic-resistance mechanisms to survive and spread. As a result, multidrug resistance is now prevalent among bacterial pathogens, affecting all types of antibiotics (Wright, 2012).

2. Development of antimicrobial agents for drug-resistant strains

Antibiotic drug resistance is the ability of the bacteria to survive and multiply despite exposure to drug levels that would typically control or eliminate them. Bacteria can overcome the effects of antibiotics by adapting and creating obstacles. They can develop resistance in various ways, such as genetic changes due to rapid reproduction. These changes can affect the enzymes, proteins, or other target molecules that the drug typically targets in the bacteria, ultimately resulting in a diminished ability of the drugs to prevent or eliminate the mutated bacteria. Through horizontal gene transfer, bacteria can share genetic material with other bacteria, even across different species. It involves the transfer of genes that encode for drug resistance mechanisms. Bacteria can acquire plasmids, small, circular DNA molecules that carry genes capable of producing enzymes that can break down or modify antibacterial drugs, making the drugs ineffective (Munita & Arias, 2016).

There is compelling evidence that infectious diseases have accounted for a significant portion of all illnesses causing mortality worldwide. Several studies have shown that microorganisms are the main cause of many infectious diseases that had plagued humans for centuries, chemotherapy was developed as the primary treatment strategy, targeting the organisms responsible for various pathogenic conditions including throat infections, gastritis, tuberculosis, bacterial meningitis, cellulitis, Lyme disease, and tetanus. In 1910, the first antimicrobial agent, salvarsan, was developed by Ehrlich as a remedy for syphilis. Later, in 1935, sulfonamide, synthetic compound was screened by Domagk and other researchers against bacterial infections with limited safety and efficacy. Finally, in 1928, Fleming research found that *Staphylococcus aureus* growth was inhibited in an area surrounding a contaminated blue mold. This fungus came from the *Penicillium* genus and produced substances that could prevent the development of other microorganisms. This discovery led to the creation of the antibiotic penicillin, which was used clinically in the 1940s. Penicillin was incredibly effective and safe and rescued the lives of many infected soldiers during World War II (Hashimoto and Chuokoron-Shinsha, 2000; Powers, 2004).

Following this discovery, new classes of antimicrobial agents were developed, leading to a golden age of antimicrobial chemotherapy. Streptomycin, an aminoglycoside antibiotic, was obtained from the soil bacterium *Streptomyces griseus* in 1944. Additional antimicrobial agents, such as chloramphenicol, tetracycline, macrolide, and glycopeptide (e.g., vancomycin), were discovered in soil bacteria. A quinolone antimicrobial drug called nalidixic acid was synthesized (Aminov, 2017; Saga & Yamaguchi, 2009). Each class of antimicrobial agents has significantly improved, resulting in a broader range of protection against various microbes and enhanced effectiveness. Fig. 11.1.

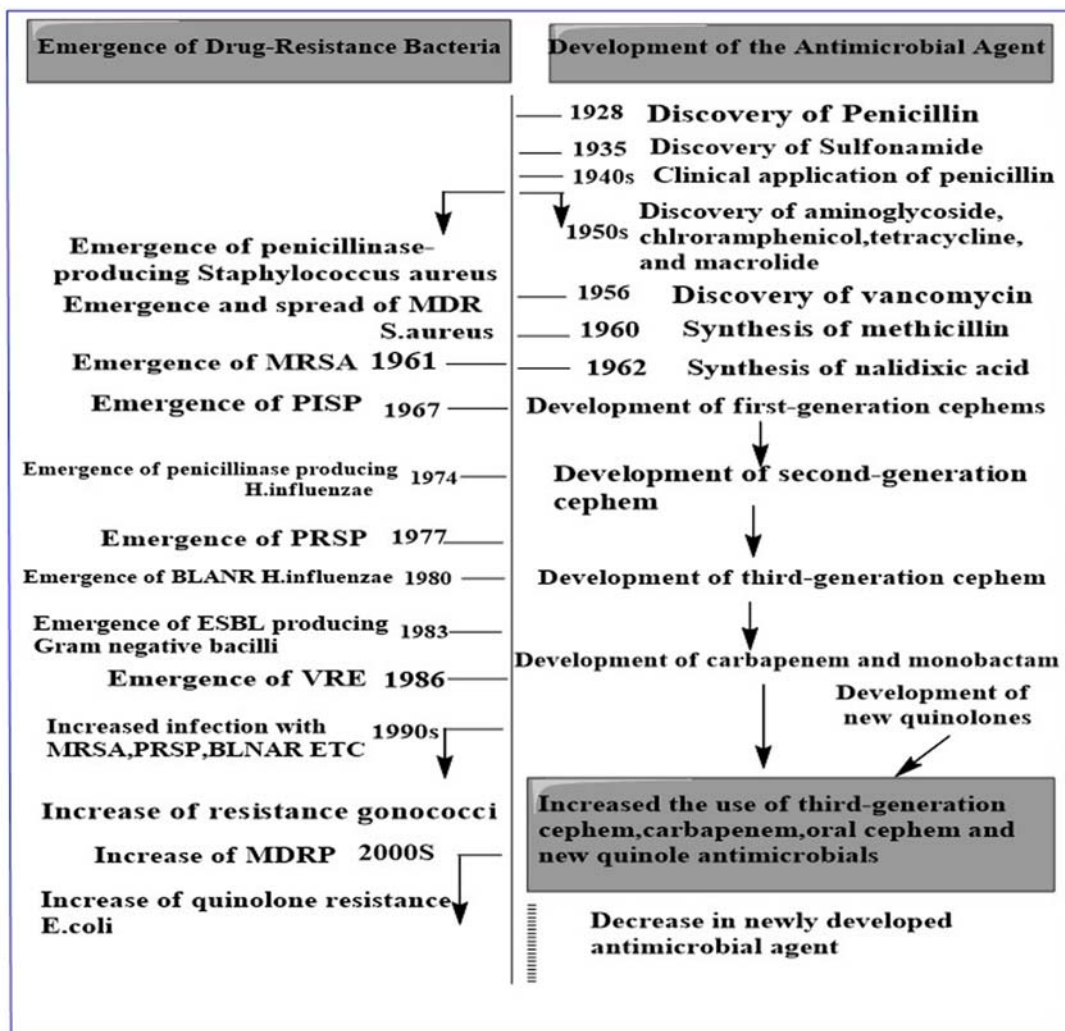


FIGURE 11.1 Represents the evolution of antibacterial drugs, based upon natural and synthetic origin.

2.1 Mechanism of antibiotics action

The mechanism of action of all antibiotics currently in clinical use was extensively studied following large-scale clinical trials and safety assessment studies. Most antibiotics mediate their actions by inhibiting bacterial cell walls or blocking protein synthesis (Gottlieb & Shaw, 2013). In line with this, quinolones inhibit DNA synthesis, whereas, sulfonamides inhibit the synthesis of metabolites required for the synthesis of DNA. The inhibition process of various antibiotics has been illustrated in Fig. 11.2 Antimicrobial agents are categorized based on their mechanism of action. The main agents can prevent cell wall synthesis, disrupt

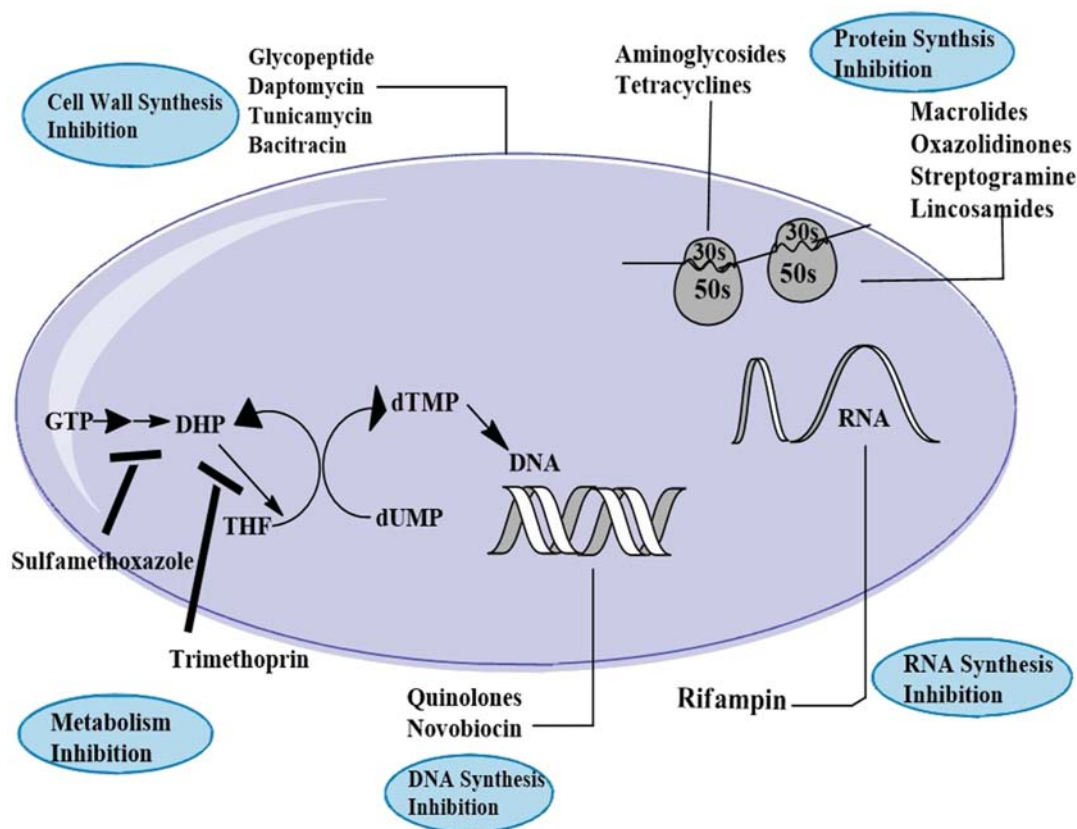


FIGURE 11.2 Represents how various broad-spectrum antibiotics target the bacterial cell.

the cell membrane, hinder protein and nucleic acid synthesis, and obstruct metabolic pathways in bacteria. With such diverse mechanisms and potential of antibiotics, still it is difficult to manage and control harmful resistant organisms (Etebu & Ariekpar, 2016, p. 90–101).

2.2 Classes of antibiotics and SAR

Various classes of antibiotics are derived from natural or synthetic sources that specifically target their target molecules associated structure–activity relationships. The structure–activity relationship (SAR) approach effectively establishes connections between the chemical structure (or properties related to structure) and the biological activity (or target property) of compounds being studied (Etebu & Ariekpar, 2016, p. 90–101; Kumar Verma et al., 2020). Some of the classes of antibiotics are mentioned in Table 11.1. For developing potent antimicrobial agents, SAR studies are essential. These studies have investigated the correlation between the chemical structure of antibiotic compounds and their potential to inhibit bacterial growth or eradicate bacteria. Scientists acquire valuable insights into the requisite structural characteristics for effective antibiotic action by altering specific molecule regions and examining the resulting activity changes (Bhat et al., 2023).

TABLE 11.1 Classification of antibiotics and their targets (Zhao et al., 2017; Heide, 2014; Houghton et al., 2010; Mukherjee & Boshoff, 2011; Spřek & Rezanka, 2017; Tipper, 1985; Vara et al., 2021).

Antibiotic classes	Source of origin	Examples	Target(s)
Aminocoumarins	Actinomycete-based natural products	Novobiocin Declovanillobiocin Novclobiocin10 Clorobiocin	DNA gyrase
Aminoglycosides	Actinomycete-based natural products/ semisynthetically derived	Streptomycin Amikacin Kanamycin Plazomicin Gentamicin Capreomycin	Ribosome
β -lactams	Fungal-based natural products/ semisynthetically derived	Sulbenicillin Temocillin Penamecillin Pheneticillin Ticarcillin Metampicillin Pivmecillinam Pivampicillin	Cell-wall biosynthesis
Diaminopyrimidines/ trimethoprim derivatives	Synthetic	Trimethoprim Iclaprim Brodimoprim <i>Epiroprim</i> Tetroxoprim	Folate metabolism
Lincosamides	Actinomycete-based natural products	Lincomycin Clindamycin Pirlimycin	Ribosome
Lipopeptides	Bacterial natural products	Daptomycin	Cell membrane
Nitroimidazoles	Synthetic	Metronidazole Secnidazole Tinidazole Pretomanid	Damage DNA

Understanding antibiotics' structure–activity relationship (SAR) is crucial in determining their bacterial enzyme or protein interaction. This knowledge is the foundation for developing new antibiotics that are more potent, effective against a broader range of bacterial strains, less hazardous, and more resistant to bacterial resistance (Bhat et al., 2023). SAR studies are crucial in identifying structural changes that can effectively combat bacterial resistance mechanisms. The daunting challenge of antibiotic resistance poses a significant obstacle in treating bacterial infections, as bacteria have developed mechanisms to evade the effects of antibiotics. However, by comprehending the SAR of existing antibiotics, structures of existing antibiotics can be modified to overcome resistance or create new antibiotics that can efficiently combat drug-resistant bacteria (Wiese & Pajeva, 2001).

Advanced computational techniques such as molecular modeling, virtual screening, and machine learning algorithms are employed to study SAR and predict the efficacy of antibiotics against MDR microorganisms. These methods enable a quicker and more efficient screening, enhancing potential drug candidates. Computational methods, including molecular modeling, virtual screening, and machine learning algorithms, are being utilized to analyze SAR and predict the efficacy of antibiotics against MDR microorganisms. These approaches facilitate the rapid screening and optimization of potential drug candidates (Roy, 2015, p. 1–703).

3. Sources of antibacterial agents

There are two primary sources for antibiotic discovery and development, classified as natural products and synthetic compounds. Natural products have traditionally provided unique roadmap for the development many drugs, through chemical modifications.

3.1 Natural sources

Antibacterial drugs in use today are derived mainly from natural sources. Antibiotics have been synthesized from small natural sources such as penicillin or large peptides such as teicoplanin. They have complex structures and tailoring of functional groups at different positions further enhances their potential to target pathogens with minimum side effect to host effectively (Newman, 2019). Natural sources have long been valuable for discovering and developing antibacterial drugs. Different sources of antibiotics include:

3.1.1 Bacteria-derived antibiotics

Streptomycin, derived from the bacterium *Streptomyces griseus*, is an antibiotic that has been used among the earliest successful treatments for tuberculosis. It is still used in combination therapies against chronic conditions. Streptomycin has the ability to block the synthesis of bacterial proteins (Shehzad et al., 2013). Daptomycin, derived from *Streptomyces roseosporus*, is an antibiotic that effectively combats Gram-positive bacteria, including MRSA. Daptomycin's mechanism of action involves the disruption of the bacterial cell membrane (Segreti et al., 2006).

3.1.2 Fungus-derived antibiotics

Penicillin, is a well-known antibiotic derived from the *Penicillium* fungus. It revolutionized the treatment of bacterial infections by inhibiting the cell walls in bacteria. Echinocandins are another antifungal medication that primarily combats fungal infections but also have antibacterial properties. Their mechanism of action involves fungal cell wall inhibition, making them an effective treatment option (Karwehl & Stadler, 2016; Szymański, 2022).

3.1.3 Plant-derived antibiotics

Berberine, a natural compound found in *Berberis* species, scientifically proven to possess potent antibacterial properties against various pathogens, including *Helicobacter pylori* and Methicillin-resistant *Staphylococcus aureus* (MRSA) (Khan et al., 2022). Quercetin, a flavonoid in vegetables, fruits, and herbs, has potent antibacterial properties that can effectively combat various bacteria, including *Escherichia coli* and *Staphylococcus aureus* (Jaisinghani, 2017).

3.1.4 Marine-derived antibiotics

Vancomycin, an antibiotic derived from the bacterium *Amycolatopsis orientalis* found in soil, is primarily used to treat severe infections caused by Gram-positive bacteria, including MRSA. Its mechanism of action involves inhibiting bacterial cell wall synthesis (Dhanda et al., 2019). Bryostatin is another antibiotic that is found in marine organisms, particularly bryozoans. Previously it has been studied as a cancer-fighting agent, with antibacterial properties

against MRSA (Lee et al., 2017). The aforementioned descriptions demonstrate that natural sources can be exploited to manufacture antibacterial drugs. It is imperative to search natural products and their derivatives, as this is a critical approach for uncovering new compounds with potent antibacterial properties.

3.2 Synthetic sources

Antibacterial drugs are typically synthesized in a laboratory or manufactured through chemical tailoring of various compounds. Synthetic sources have proven to be effective in creating antibacterial drugs. Organic compounds have low molecular weight that can be synthesized chemically to display antibacterial properties (Galloway et al., 2009). Recent examples include bedaquiline. Bedaquiline, an FDA-approved synthetic small molecule, effectively treats multidrug-resistant tuberculosis by targeting ATP synthase, an enzyme essential to the survival of *Mycobacterium tuberculosis* (Han et al., 2020). Another antibiotic, Delafloxacin, is a highly effective synthetic antibiotic that can combat a broad spectrum of bacterial strains, including MRSA. Delafloxacin impedes the bacterial enzymes responsible for DNA replication, inhibiting their growth and spread (Ocheretyaner & Park, 2018). Amoxicillin-clavulanate is another effective synthetic-based antibiotic. Augmentin is an effective combination antibiotic comprising amoxicillin, a synthetic penicillin, and clavulanate, a β -lactamase inhibitor. It is highly beneficial in treating various bacterial infections, including skin and respiratory tract infections (Ahmad et al., 2019, p. 1–21).

3.3 Semisynthetic sources

Semisynthetic antibacterial drugs are medications derived from natural sources that have undergone chemical modifications to enhance their potency, stability, and efficacy against bacterial infections. It involves adding particular chemical functional groups to the original compound, significantly improving its pharmacological properties (Qadri, 2022). Penicillin and its derivatives were some of the earliest semisynthetic antibacterial drugs created. They evolved from the fungus *Penicillium*'s natural compound, penicillin. Scientists modified penicillin's structure to generate semisynthetic penicillins, such as ampicillin, methicillin, and amoxicillin. These drugs exhibit a broader range of antibacterial activity and are more resistant to enzymatic degradation (Hamilton-Miller, 2008). Linezolid is an exceptional semisynthetic antibiotic expertly engineered to combat Gram-positive infections, particularly MRSA. By modifying the naturally occurring compound, eperezolid is a potent antibiotic that effectively obstructs bacterial protein synthesis, making it a highly effective treatment option.

3.4 Peptides as a source of antibiotics

Peptide antibiotics, also known as antimicrobial peptides, are potent amino acid chain compounds with antibacterial properties. These compounds can be found naturally in various organisms, including bacteria, plants, fungi, and animals, or can be synthetically manufactured. Due to their unique mechanisms of action and broad-spectrum activity, peptide antibiotics are considered promising antibacterial medication sources. They can be produced chemically or through recombinant DNA technology, making them adaptable to

combating bacterial infections (Hancock & Chapple, 1999; Kang et al., 2014). Some of the peptide-based antibacterial drugs include Daptomycin and Teixobactin. Daptomycin, a member of the lipopeptide group, is an antibiotic that can treat skin and bloodstream infections caused by Gram-positive bacteria, particularly MRSA. Its mechanism of action involves disrupting the bacterial cell membrane, preventing proliferation (Kang et al., 2014). Teixobactin is a highly effective peptide antibiotic with a unique mechanism of action. Teixobactin binds to lipids II and III of peptidoglycan, interfering with bacterial cell wall synthesis. This leads to disruption of the bacterial cell wall and impaired peptidoglycan synthesis. Teixobactin has demonstrated impressive efficacy against a range of drug-resistant bacteria, including *Mycobacterium tuberculosis* and MRSA (Müller et al., 2017). By refining and enhancing synthetic compounds, researchers can develop more potent and stable treatments, leading to successful outcomes in the fight against bacterial infections.

4. Obstacles in the development of new antibacterial drugs and their proposed solutions

Developing new antibacterial drugs can be challenging due to bacterial resistance, development investment, limited research, scientific challenges, and complicated regulatory processes (Coates et al., 2002; Kunin, 1993). Here is a detailed explanation of these challenges and proposed potential solutions.

4.1 Bacterial resistance

The development of antibiotics has been hindered by lack of potent candidates and bacterial resistance. Over time, bacteria have been mutated which adversely affects the effects of antibiotics, rendering them less potent or utterly ineffective by utilizing various survival strategies, such as antibiotic inhibition or destruction (Sun et al., 2013).

Antibiotics can become ineffective through various means (Christaki et al., 2020) including:

1. Enzymes such as β -lactamase inactivating β -lactam antibiotics,
2. Transportation out of cells through efflux mechanisms such as tetracyclines subject to tetM efflux pumps
3. Target mutations that decrease the effectiveness due to modification of sequence d-Ala-d-Ala to d-Ala-d-Lac rendering vancomycin less effective.
4. Overproduction of the target such as DHFR, bypassing the metabolic pathway to remove the essentiality of the target as with peptide deformylase in *Streptococcus pneumoniae* or *Haemophilus influenzae*
5. Decreased antibiotic uptake, such as *Pseudomonas aeruginosa* losing its D2 porin, causing of antibiotic resistance.

Unfortunately, this has resulted in a rise of multidrug-resistant bacteria, leading to incredible difficulty in treating infections. To deal with this challenge, several strategies can be employed, such as developing new types of antibiotics that target various bacterial mechanisms, making it harder for bacteria more sensitive and less resistant. The discovery of synthetic and natural-product-based compounds and using differential sensitivity screening

methods can identify potential leads (Singh & Barrett, 2006). The second approach is combination therapies, which involve using multiple antibiotics with different mechanisms of action to reduce the likelihood of resistance development. Antibiotic combinations that target various crucial cellular processes can work synergistically to inhibit the growth of bacteria. One antibiotic may hinder the synthesis of cell walls, while another might inhibit the synthesis of proteins. Their combined effect is more potent than their individual antibacterial effect. Combinations can prevent the establishment of resistance by attacking several resistance pathways such as metabolism, influx, and efflux of antibiotics (Cottarel & Wierzbowski, 2007).

Bacterial cell membrane permeability can be increased of antibiotics by tailoring existing compounds, which makes bacteria susceptible and have a more significant impact of drugs. Combinations can interfere with quorum sensing (QS) and biofilm formation, which makes bacteria more susceptible to antibiotic treatment. One antibiotic might, for instance, prevent QS while the other explicitly targets bacterial growth. Combinations can suppress the enzymes that provide bacteria antibiotic resistance. For instance, one drug may prevent the beta-lactamase enzymes from breaking down beta-lactam antibiotics, while another antibiotic targets the bacterial cell (Cottarel & Wierzbowski, 2007). Overall, combination therapy is a potent method of coping with antibiotic resistance. It is possible to improve treatment effectiveness, circumvent resistance mechanisms, and widen the spectrum of activity by combining several antibiotics with various modes of action (Cottarel & Wierzbowski, 2007).

4.2 Insufficient investment in research and development

Antibiotic development is an expensive and protracted procedure that frequently lasts more than a decade. However, compared with other therapeutic fields, antibiotics do not exhibit a good economic return. As a result, many pharmaceutical firms have cut back on their funding for antibiotic research. To encourage pharmaceutical companies for investing in antibiotic research and development, governments and regulatory agencies can offer financial incentives such as tax breaks, grants, and market exclusivity. Public–private partnerships between academia, industry, and government can help overcome financial barriers, leading to sustained investment in this priority area (Theuretzbacher, 2012). It is also vital to explore alternative funding models, such as public funding for antibiotic research, to ensure sustained investments in the form of Market Entry Rewards. Governments could implement a reward system for pharmaceutical companies that create new antibacterial medications (Daniel et al., 2018). The reward would be determined by the drug's effectiveness in treating medical conditions and its potential to combat antibiotic-resistant infections, thus contributing to the promotion of public health, which are in accordance with UN SDGs goal 3 of health and human well-being.

4.3 Regulatory obstacles

The regulatory procedure for approving antibiotics is frequently extensive and complicated. Navigating the regulatory structure, including preclinical and clinical trials, takes several years. Government agencies need to opt advance methods to streamline this process. Regulatory agencies can create expedited antibiotic courses, considering their urgent need

and unique challenges. Adaptive trial designs that permit approvals can accelerate the process of clinical trials for new antibiotics. The approval process can be expedited by implementing harmonized guidelines from regulatory agencies worldwide, reducing redundant efforts (Kunin, 1993; Theuretzbacher, 2012).

4.4 Scientific hurdles

Scientific obstacles for antibiotic development include finding novel therapeutic targets, optimizing treatment efficacy and safety, and comprehending the intricate relationships between bacteria and their host (Jackson et al., 2018). Possible solutions include advanced technologies such as proteomics, genomics, and high-throughput screening can help researchers to identify and validate potential drug candidates and their pathways that can disrupt bacterial survival and virulence (Pulido et al., 2016). By implementing personalized medicine approaches, such as therapeutic drug monitoring, antibiotic dosing can be optimized to minimize the risk of toxicity and virulence. A multidisciplinary strategy by researchers, healthcare professionals, policymakers, and pharmaceutical firms is necessary to address these issues (Velez & Sloand, 2016). To overcome these obstacles and ensure the emergence of efficient antibacterial drugs to fight emerging infections, collaboration, consistent funding, and innovation in research and development are essential.

5. Innovative approaches for antimicrobial drug discovery

5.1 Natural products-based drug discovery methods

Compounds with chemical, biological, and therapeutic benefits can be found in natural products derived from plants, animals, and microbes. Over half of the small therapeutic molecules have been derived from natural products, their derivatives, which are modified through chemical ways and synthesized natural product compounds. For mandated purposes, it is feasible to diversify natural products and their respective analogs. Natural product discovery was accomplished by isolating the desired chemicals using a conventional bioassay (Bucar et al., 2013). Application of a nonreductionist strategy that investigates the synergistic effects of extract components and their modes of action is required when combining existing biological methods (such as proteomics, genomics, etc.), computational approaches, and automation with advanced technologies. For instance, to accurately exploit new drugs, the Computational Analysis of Novel Drug Opportunities platform has been developed to analyze and evaluate the polypharmacological effects of numerous drug molecules (Thomford et al., 2018). Many other different target identification methods have been summarized further Table 11.2.

5.1.1 Chemical-based approach for untapped natural sources

Many bacterial and fungal species remain undiscovered, with less than 5% of fungal species and only 1% of bacterial species being identified to date (Gunatilaka, 2006). This indicates a wealth of untapped natural resources, including marine microorganisms and bryophytes, a recent focus of natural product research (Li et al., 2014). Cultivable organisms can be induced

TABLE 11.2 Novel approaches for drug development.

Method	Mode of action	Limitations	References
DARTS	Determine the target proteins based on the stability of the label-free natural product-bound proteins.	Not appropriate for cell lysates with less concentration of target proteins	(Dejonghe and Russinova, 2014; McFedries et al., 2013)
SPROX	Identifies the amount of methionine oxidized in a mixture of the potential therapeutic molecule, proteins, and an oxidizing agent. It is proceeded by Mass spectrometry analysis of peptides.	Applicable to highly concentrated proteins	(Liu et al., 2017; McFedries et al., 2013)
SILAC	An improved version of SPROX for more target proteins.	Applicable to proteins with attached methionine group	(Tran et al., 2014)
CETSA	Based on how their interactions alter in response to various temperature shifts and modifications, this method measures the stabilization of the target and ligand-bound proteins. Later, a western blot method is applied to identify the separated protein. It is effective for locating several antibacterial and anticancer drugs.	During western blot analysis, nonspecific antibodies may produce false positive results. Proteins without binding sites are not detectable.	(Molina et al., 2013)
TPP	It is an upgraded version of CETSA that has enhanced thermal stability for measuring target proteins. Off-target TPP detection can be used to monitor the adverse effects of potential drug molecules.	Expensive and complicated to operate	(Savitski et al., 2014)

to biosynthesize various secondary metabolites under certain laboratory conditions, producing diverse natural compounds. These compounds may possess bioactivity and could be helpful in drug development. Notably, cultivable microorganisms have been found to produce natural compounds containing a “decalin” motif with significant antibacterial, antifungal, and anticancer properties (Li et al., 2014).

5.2 Biological-based approaches

There are several methods to modify the production of secondary metabolites, including genetic and epigenetic manipulation. These methods involve bioengineering of the microbial gene clusters, such as activating biosynthetic gene clusters (BCG gene clusters), manipulating transcription regulators, or replacing the natural promoter with a more influential one. Enzymes from other organisms can also create complex metabolites with different functional groups. However, this approach can be costly and may result in low transformation yields or the production of nonspecific products. Studies have recently uncovered that actinobacteria and filamentous fungi genomes contain a plethora of cryptic biosynthetic gene clusters (BGCs) that hold immense potential for producing intricate metabolites (Olano et al., 2009).

By altering the conditions of microorganism culture, the expression of BGCs can be affected, leading to the production of different metabolites. These pathways can be manipulated through changing cultivation conditions, cocultivation, and chemical manipulation, epigenetics for the discovery of new bioactive compounds that can be isolated and purified (Knight et al., 2003). Changes in culture media and physical parameters can significantly impact the production of bioactive secondary metabolites. By introducing even, a small amount of scandium, a rare earth element, to *Streptomyces* cultures, the growth conditions can be altered to increase the production of antibiotics, including the antituberculosis drug streptomycin. As a result, there is a notable impact on metabolite production, potentially aiding in biosynthesis. A new substance called berkeleylactone A was discovered by combining *Penicillium fuscum* and *P. camemberti*. DNA methyltransferase or histone deacetylase, can change the outcome of biosynthetic processes (Stierle et al., 2017). Adding the DMT inhibitor 5-azacytidine to the *Penicillium* variable causes it to produce vitamin A, which can be toxic to HCT-116 cells (He et al., 2015).

5.3 Synthetic chemistry-based innovative approaches

Target-oriented drug synthesis is highly effective for creating new drugs using recently discovered molecules with varying biological activities (He et al., 2017). This method is particularly advantageous because it eliminates the limitations associated with natural resources, which often have low yields. Numerous approaches have been developed to produce analogs of natural products, either through de novo synthesis or one-step multicomponent synthesis. This process simplifies the natural product's structure and generates new scaffolds with the desired biological activity (Li & Lou, 2018). By conducting further medicinal chemistry research, natural product scaffolds' drug efficacy and pharmacokinetic properties can be significantly enhanced (Rodrigues et al., 2016).

5.4 Target-oriented drug discovery

5.4.1 Peptide-based drug discovery and reverse pharmacology approach

Peptides research is rapidly growing field in clinical trials. The therapeutics market for these small biomolecules is set to reach an impressive \$45,475 million by the end of 2024 (Mimmi et al., 2016). One of the many advantages of peptides is their superior safety profile, with lower toxicity and safer metabolites as compared with other small drug molecules. They also have a high target affinity as well as cost-effective for manufacturing and storage (McGregor, 2008). The antimicrobial peptides (AMPs) found in plants, isolated from roots, seeds, leaves, flowers, and fruit are particularly noteworthy for their dendritic cell activity, chemoattractant function, and antimicrobial effects. AMPs disrupt pathogen membranes or create membrane pores in various microbial targets, such as bacteria, bacteria, parasites, and enveloped viruses. Peptides are a promising avenue for future therapeutic development (Liu, 2011).

Reverse pharmacology is a highly focused and effective method for identifying potential disease treatments. This approach involves screening peptide libraries against specific

biological targets using cutting-edge technologies such as phage display. Developed in 1985 by George Smith, this innovative technique utilizes genetically modified DNA phages, such as the M13 and filamentous phages, to express and display various peptides on their surface (Scott & Smith, 1990). With this powerful technology, it is possible to clone up to 2800 copies of DNA segments, each of which encodes unique peptides, making it a reliable and promising tool for discovering new treatments for a wide range of diseases (Cabilly, 1999).

5.4.2 Antibody-oriented drug discovery

Antibodies can specifically attach to a target and are highly beneficial for medicinal purposes, especially in controlling and treating infections. For example, Raxibacumab is a highly effective antibody that combats zoonotic diseases caused by *Bacillus anthracis*. Its mechanism of action suppress the interaction between bacterial toxins and the mammalian receptor, thus preventing infections (Mazumdar & Raxibacumab, 2009). Although antibody-based drug discovery needs much attention, but researchers have made rapid progression in this field.

An antibody-based drug TGN1412 was developed for immunotherapy, but unfortunately, it doesn't specifically target bacteria. In 2006, a phase I clinical trial targeted CD28, protein of immune cells. Unfortunately, the trial resulted in severe adverse effects on human volunteers (Casucci, 2012). This event highlighted the safety testing for developing antibody-based drugs. A monoclonal antibody named MEDI4893 has been developed by MedImmune/AstraZeneca. The antibody specifically designed to target the alpha-toxin of *Staphylococcus aureus* and currently being researched for its potential to prevent *S. aureus pneumonia* in high-risk patients (Vaughan et al., 2017). As of the latest update, MEDI4893 is in phase II clinical trials.

5.4.3 Automated de novo design

De novo ligand design was employed in the 1990s to produce high-affinity ligands by utilizing protein–ligand interactions and the already accessible 3D protein structures. Unfortunately, results were unsatisfactory since it generates complex ligands with poor affinity and necessitates a lot of humanitarian aid and knowledge. The de novo philosophy underwent significant alterations in the late 1990s, and new ligands were discovered. However, despite this progress, the rates are still subpar compared with other available virtual screening methods (Rognan, 2012).

To produce novel chemical entities from the stored databases, several automated compound generators, such as completely autonomous or semiautonomous de novo design, are receiving more attention. The most crucial element in these approaches is the use of a powerful chemical reaction on a limited number of building blocks to produce high yields of various products with a complex oligomeric structure. Technical advancements that can be introduced to help the automated system include parallelization, stereoselective synthesis, and automated purification (Schneider, 2018). A remote-controlled robotic synthesis is a cutting-edge, successful method for designing of new drugs. Thus, the production of Csp3-rich macrocyclic and polycyclic compounds is an example of automated drug discovery. A C–C bond and cyclization processes were used to link numerous building blocks to create Csp3 drug-like molecules (Li et al., 2015).

5.5 Molecular docking

The USCA Kuntz Group invented the docking strategy in the 1980s by utilizing HTS devices, ligands are automatically screened for and synthesized. The virtual screening predicts the binding posture and affinity strength between the ligand and the protein, particularly based on their 3D structures and physicochemical properties. Thus, this technique reduces the pool of ligands that must be screened. Using a mathematical model called the scoring function (SF), molecular docking technology investigates potential binding modes of the ligand to the target protein, also known as binding poses. The last one uses a computed score in which millions of potential ligand candidates are selected and scored for their best posture and binding free energy to forecast the stability of the ligand-protein bound. The prospective medication is tested and produced utilizing HTS based on findings (Ashtawy & Mahapatra, 2014).

6. Quorum sensing and drug discovery

The process of QS is a highly regulated gene expression system that relies on small signaling molecules known as autoinducers (AIs). These AIs interact with specific receptor proteins, triggering targeted gene expression. The concentration of AIs and the bacterial population's density are crucial factors in the functioning of the QS system. Over 50 bacterial species employ AIs for various purposes, including virulence, toxin production, antibiotic biosynthesis, metabolite synthesis, and exoenzyme production. Biofilms, physical structures that attach to the cell surfaces, pose a significant challenge in the development of medication and its efficacy and are a prime factor in the bacterial pathogenicity and its survival. Biofilm bacteria exhibit more excellent antibiotic resistance than their planktonic counterparts (Eberhard et al., 1981). Nevertheless, biofilm formation can be reversed, and the two types of QS regulation—positive and negative—respectively promote and inhibit biofilm formation. Suppressing the QS system is a promising strategy for combatting bacterial pathogenicity and treating infectious diseases. The potential of QS systems in developing new medicines is currently being explored apart from conventional antibiotics and drugs (Yan & Wu, 2019).

6.1 Mechanisms impeding quorum sensing

Multiple strategies exist for disrupting QS, the bacterial communication system responsible for coordinating their actions (Zhao et al., 2015);

1. One practical approach involves utilizing small molecules to inhibit the synthesis or function of autoinducers, the signaling molecules used by bacteria. This method can decrease bacterial virulence by preventing the production or detection of autoinducers.
2. Another viable option involves interfering with the signaling cascade triggered by autoinducers binding to their receptors. Such interference can be achieved by inhibiting the enzymes or pathways involved in the cascade or blocking the receptors.
3. Lastly, combining quorum-sensing inhibitors with antibiotics can mediate the effectiveness of antibiotics against bacterial growth or virulence. In particular, combining an

antibiotic with a quorum-sensing inhibitor has demonstrated synergistically in lowering the production of biofilms and increasing bacterial susceptibility to antibiotics.

6.2 Quorum-sensing inhibitors

Here are a few quorum-sensing inhibitors that have paved the way for the introduction of new antibacterial drugs.

6.2.1 Halogenated furanones

A class of chemicals called halogenated furanone, generated from marine algae, has been found to prevent bacteria from sensing quorums. They have demonstrated potential in preventing biofilm formation and lowering pathogenicity in various bacterial species, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Chang, 2019).

6.2.2 Aminoimidazole derivatives

Researchers recently developed derivatives of the 2-aminoimidazole that block the LuxR family of receptors to prevent QS. These agents have demonstrated effectiveness against the development of biofilms and pathogenicity in bacteria, including *Pseudomonas aeruginosa* and *Vibrio cholera* (Huigens et al., 2009).

6.2.3 Inhibitors of LuxS

Many bacteria use the signaling-molecule autoinducer-2 (AI-2) for interspecies communication, and LuxS is an enzyme involved in the production of AI-2. Due to their ability to interfere with QS in various bacterial species, LuxS inhibitors have been explored as possible antibacterial agents. Derivatives of boronic acid have been investigated as LuxS inhibitors. These substances can interact with LuxS's active site, blocking the enzymatic activity and lowering the synthesis of AI-2. Boronic acid derivatives have successfully combat QS and pathogenicity in bacteria such as *Pseudomonas aeruginosa* and *Vibrio harveyi* (Fernandes et al., 2023).

6.2.4 Peptide-based inhibitors

QS in bacteria is mainly targeted by peptides synthesized from naturally occurring antimicrobial peptides. These peptides may hinder the binding of autoinducers to their receptors, and they may also impede the quorum-sensing-related downstream signaling pathways. The *Enterococcus faecalis*-derived peptide (EDF), a type of peptide-based inhibitor, is one example. In numerous bacteria, including *Vibrio cholerae* and *Escherichia coli*, EDF has been identified to block the formation of the quorum-sensing signal molecule (AI-2). EDF alters bacterial behavior by impairing AI-2 synthesis, interfering with the QS mechanism. It is worth noting that despite the promising results of quorum-sensing inhibitors in laboratory experiments, translating them into effective clinical drugs has been challenging. Successful development of therapies targeting QS requires careful consideration of different factors, such as the intricate nature of bacterial communication networks and the possibility of resistance development. Nonetheless, QS remains an active area of research to combat bacterial infections (Jesin, 2019).

7. Nanotechnology and antibacterial drug development

Nanoscience and nanotechnology have seen some of the fastest growth during the last decade. Nanotechnological techniques are becoming increasingly crucial because of bacterial antibiotic resistance and difficulties in synthesis of novel compounds. Effective drug delivery techniques such as solid-lipid nanoparticles, dendrimers, polymeric micelles, liposomes, and polymeric micelles have been research worldwide. Due to their great durability in biological environments and potential to serve as targets for antibiotic drugs in infectious zones, polymer-based nanoparticles have attracted the most interest among these delivery technologies (Gumustas et al., 2017; Shehzad et al., 2020, p. 173–190).

7.1 Polymer-based nanoparticles for antimicrobial drug delivery

Designing polymer-based nanocarriers as possible antimicrobial drug delivery systems in treating intracellular bacterial infections has become more important in recent years. This approach is promising in achieving the best possible therapeutic outcomes against many pathogenic bacteria. Recently, polymer-based nanocarriers as drug-delivery systems have been used for numerous antibiotics. According to multiple reports, polymer-based nanocarriers may be broadly categorized into three classes as antimicrobial drug delivery systems: polymeric micelles, polymeric-type dendrimers, and polymeric nanoparticles (Imbuluzqueta et al., 2010).

7.2 MAO of nanoparticles with respect to antibacterial activity

Different nanomaterials have displayed antibacterial properties. However, it is still unclear how exactly they work to kill germs. A number of physicochemical reactions are involved ranging from direct physical contact with the microbial body to chemical cell destruction. There are numerous processes that have been put out to represent the function of various nanomaterials in biological applications, the underlying mechanism for these processes include:

1. the interaction of nanomaterials with cell membranes
2. the production of ROS that harms cells
3. the absorption of free ions from the nanomaterials solution that damages cells (Ul-Islam et al., 2014).

Instead of employing targeted antibacterial effect, nanomaterials may use a variety of potential processes (Ul-Islam et al., 2014). Following is a detailed explanation of each of these three hypothesized processes, along with research-based examples.

7.2.1 The interactivity of nanoparticles with cell membrane

According to reports, NPs can cross bacterial cell wall and modulate downstream signaling upon interaction with their surface receptors. For instance, Ag NP penetration had left holes in the surface of *E. coli* (Smetana et al., 2008). A similar process has been proposed for ZnO NPs that reach the cell envelope and break down the cell membrane. In another study, ZnO NPs extend the lag phase of the microbial cell cycle after attaching to

the cell membrane (Brayner et al., 2006). Precise mechanism of NPs interaction with cell membrane is still under investigation. One potential explanation is the electrostatic contact brought by the difference in charge between the negatively charged cell surface and the positively charged particle. According to a more comprehensive theory, the NPs (Ag) connect to the cell wall's sulfur-containing proteins, causing the cell wall to rupture and increase the permeability of the cell membrane. The reason for this interaction is sulfur-containing proteins on the cell membrane surface and their high affinity for Ag (Raffi et al., 2008). Bioluminescence study has revealed how protein membrane degradation develops in response to Ag exposure. Cell death results from the discharge of cytoplasmic substances from the perforated and porous membrane to the surrounding media (Hwang et al., 2008).

7.2.2 ROS-mediated bactericidal effects

ROS are reactive oxygen-containing molecules that are by-products of respiration's metabolism. ROS is produced as a by-product of metabolic reactions and stress, yet cells have antioxidant defense mechanisms to control or eliminate ROS. However, oxidative stressors are produced when ROS levels become too high. Additionally, ROS degrades mitochondrial membranes and disrupts its activities by attacking membrane lipids. The antibacterial activity of NPs is mediated by the formation of ROS. Previous studies have demonstrated the photo-generation effect of ROS on the surface of metal oxides. These oxides and hydroxides act as a precursor for producing additional ROS. It has been demonstrated that the photocatalytic generation of H_2O_2 from the ZnO surface may effectively stop bacterial growth (UI-Islam et al., 2014).

7.2.3 Uptake of free ions from nanoparticles

NPs that are suspended or dissolved frequently produce highly reactive metallic ions that are incredibly hazardous to bacteria. Cell death is eventually caused by interaction of ions with respiratory and transport proteins through thiol groups. Therefore, the absorption of these metallic ions is the potential mechanism of the bactericidal effects of nanomaterials. Depending on the characteristics and charge of the metallic ions, the bactericidal effectiveness varies. The antibacterial ability of metals with different charges exists in the following order: $\text{Ag}^+ > \text{Hg}^{2+} > \text{Cu}^{2+} > \text{Au}^{2+} > \text{Zn}^{2+} > \text{Ca}^{2+} > \text{Na}^+$. $\text{Ag}^{3+} > \text{Ag}^{2+} > \text{Ag}^{1+}$ (UI-Islam et al., 2014). Aforementioned mechanisms cause NPs to demonstrate antimicrobial action. Nanomaterials have been found to exhibit substantially more potent antibacterial activity than bulk materials, regardless of the specific mechanism for a given bacterium or nanomaterial. This has dramatically increased the use of nanoparticles across many industries, particularly the health sector.

8. Conclusion

In conclusion, the most significant source of innovative drug scaffolds is the natural world, making this a practical choice in the search of new antibiotic molecules. The natural product drug discovery will be facilitated and improved by advancements in bacterial culture techniques, molecular biology, and metagenomics, which will reduce these limiting procedures. Whole-bacterial cell assays must be a part of screening protocols to address the problem of

bacterial permeability and efflux early in the discovery phase. Currently, pharmaceutical firms are developing training programs in conjunction with advanced drug development techniques to ensure the development of effective and secure therapeutic compounds.

Successful target-based technology is required for the automated designing of therapeutic molecules from various naturally occurring chemicals or synthetically varied molecules. The computerized medication design process reduces costs and saves much time. The function of QS in this context has recently attracted much attention. Many recent developments in drug discovery were based on reducing bacterial pathogenicity induced by interaction and communication with other nearby bacterial communities. It is crucial to remember that QS is also challenging since bacteria are constantly evolving and may acquire resistance to newly introduced medications. In addition to the latest methods, nanotechnology has significantly increased the bactericidal potency of antibacterial medications. Finally, drug research is still expanding, leading to the development of new pharmaceuticals that benefit the living planet.

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Discovery and development of new antibacterial drugs

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1. Introduction

Pathogenic bacteria have certain features that enable them to infect hosts. These features include:

- 1. Adherence factors:** Attachment projections such as pili, fimbriae, and adhesins enable pathogenic bacteria to attach to host cells. For example, *Escherichia coli* (*E. coli*), a common pathological agent of urinary tract infection, attaches to uroepithelial cells by pylonephritis-associated pili. In gonorrhea, *Neisseria gonorrhoeae* attaches to mucosa epithelial cells by type IV pili and an external membrane adhesion, Opa (Donnenberg, 2000; Todar, 2004; Wu et al., 2008).
- 2. Toxin production:** Many exotoxins are developed by pathogenic bacteria, which include cytotoxin, enterotoxin, and neurotoxin. *Corynebacterium diphtheriae*, the pathological agent of diphtheria, produces a heat-sensitive cytotoxin that inhibits polypeptide extension and thus inhibits protein synthesis (Awasthi et al., 2013; Klose, 2001). *Vibrio cholerae*, the common pathogen of cholera, produces an enterotoxin that activates the

adenylate cyclase enzyme in intestinal mucosa cells, resulting in increased intracellular cAMP and the production of water and ions into the small intestine lumen. Tetanus is a neurotoxin produced by *Clostridium tetani*; the poison prevents the release of γ -aminobutyric acid, thus causing spastic paralysis. Gram-negative bacteria can produce endotoxin rather than exotoxins. Endotoxins are lipopolysaccharides that can induce inflammatory responses and are important in sepsis.

3. **Invasins:** include various extracellular enzymes or proteins that enable bacteria to invade hosts. For example, *Staphylococcus aureus* produces a wide range of invasins, including hyaluronidase, which lyses hyaluronic acid in connective tissue, DNases, which break down DNA, hemolysins, which destroy red blood cells, and staphylokinase, which converts plasminogen to plasmin, which digest fibrin clots (Schutte et al., 2021; Toprak et al., 2012; Vandenesch et al., 2012).
4. **Capsule:** Bacterial capsule are responsible for the virulence of some bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitidis* by helping them resist phagocytosis of the host immune system. Some bacterial pathogens can evade host defenses, including phagocytosis, complement, and immune response, through many mechanisms. For example, *Streptococcus pyogenes* produces a hyaluronic acid capsule that shields most of the antigenic proteins of bacteria from the host immune system. Also, the human connective tissue contains hyaluronic acid, which limits antibody responses against the *Streptococcus pyogenes* capsule. *Haemophilus influenzae* can modify its lipopolysaccharide (principal target of complement) by attaching sialic acid to its O antigens (Todar, 2004).

2. Antibacterial treatment

Bacterial pathogenesis is an interaction of bacterial virulence factors and the host immune response, and the actual mechanism involved differs from one organism to another. A bacterial pathogen may not use all the virulence factors mentioned above to cause disease in its host.

Antibacterial agents or antibiotics are considered the main method used to treat a bacterial infection, and they affect the pathogens by either inhibiting their growth (static effect) or killing them (cidal effect). Since the discovery of penicillin (the first antibiotic) in 1928, many antibacterial agents have been discovered (Khameneh et al., 2019).

2.1 The mechanisms of action of antibacterial agents (Newton, 1965; Yu et al., 2023)

- a. **Inhibition of cell wall synthesis:** The most important example of these drugs is the β -lactams that bind and inhibit penicillin-binding proteins, which catalyze the formation of peptidoglycan cross-links in the bacterial cell wall, which causes bacterial damage.
- b. **Inhibition of protein synthesis:** The drugs, including aminoglycosides, tetracyclines, macrolides, and chloramphenicol, act on the ribosome and interfere with protein synthesis at different stages. Aminoglycosides interfere with protein synthesis by binding to

the A-site on the 16S ribosomal RNA of the 30S ribosome. Tetracycline blocks the binding of the t-RNA-amino acid to the ribosome. Erythromycin binds to the 23S rRNA molecule (in the 50S subunit) of the bacterial ribosome and blocks the exit of the growing peptide chain. Chloramphenicol binds to the 23S rRNA of the 50S bacterial ribosomal subunit, inhibiting the peptidyl transferase activity and the protein chain's elongation.

- c. **Inhibition of nucleic acid synthesis:** The drugs include fluoroquinolones and rifamycins. Fluoroquinolones act by inhibiting DNA gyrase. Fluoroquinolones also inhibit topoisomerase IV. Rifampin inhibits bacterial RNA polymerase.
- d. **Inhibition of metabolic pathways:** The drugs sulfonamides and trimethoprim. Sulfonamides are chemical analogs of para-aminobenzoic acid and competitively inhibit the dihydropteroate synthetase enzyme. Trimethoprim inhibits the dihydrofolate reductase (DHFR) enzyme. These enzymes are essential in the production of folic acid in bacteria, which is required for nucleotides and necessary for DNA synthesis.
- e. **Interference with cell membrane integrity:** The drugs include polymyxin B, which acts by binding to the bacterial cell membrane and altering its permeability, leading to leakage in the cell.

2.2 Anti-virulence therapeutic strategies

Antivirulence agents are a group of drugs that work against the pathogenicity components of bacteria rather than the bacteria themselves. Although the main goals of conventional antibiotics are to eradicate or stop bacterial growth, they can also result in the emergence of antibiotic resistance. An alternative strategy is provided by antivirulence agents, which disarm the bacteria and lessen their capacity to harm the host (Clatworthy et al., 2007; Gill et al., 2015).

Specific compounds or mechanisms used by bacteria to induce infections, elude the immune system, and cause the pathogenesis of bacteria are known as virulence factors. Toxins, adhesins, biofilm development, and other elements linked to pathogenicity can be among these factors. Antivirulence substances prevent the synthesis or operation of these components, lowering the bacteria's pathogenic potential (Meena et al., 2019).

The benefit of antivirulence drugs is that they do not subject bacteria to intense selective pressure to evolve resistance. Resistance is less likely to develop when virulence factors are targeted rather than bacterial growth. This strategy also makes it easier for the host's immune system to get rid of the infection because the bacteria are disarmed (Dickey et al., 2017).

Several methods can be used to develop antivirulence compounds:

1. **Inhibiting toxin production:** Antivirulence agents can target the genes or processes involved in toxin formation, lowering their expression or neutralizing their effects.
2. **Bacterial adhesins enable bacteria to adhere to host cells or surfaces, promoting infection.** Agents that interfere with adhesion can stop bacteria from colonizing the host and establishing infections.
3. **Quorum sensing disruption:** Quorum sensing is a system of communication utilized by bacteria to coordinate group behavior, such as the development of biofilm or toxins. Bacterial pathogenicity can be hampered by inhibiting quorum sensing.

4. Targeting the development of biofilms: Biofilms are bacterial populations enclosed in a protective matrix. Bacteria in biofilms are more difficult for the immune system to clear. Agents that prevent or disrupt biofilm formation can enhance the effectiveness of conventional antibiotics (Dickey et al., 2017; Gill et al., 2015).

2.2.1 Gene editing-based antibacterial approach

The cutting-edge gene editing technique CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) enables researchers to precisely alter the DNA. It is a natural defense mechanism that bacteria and archaea have that aids in defending them against viral infections and bacteriophages (Li et al., 2020). There is emerging research investigating the development of CRISPR-based antibacterial agents (Greene, 2018).

Using CRISPR-Cas systems as a tool to specifically target and eradicate particular harmful bacteria is one method. In order to create programmable antimicrobials that can recognize and cleave the DNA of particular bacterial strains, researchers are looking into the usage of CRISPR-Cas systems. This would effectively destroy the bacteria. This strategy gives us the possibility of highly specialized and focused antibacterial therapies (Nath et al., 2022).

Another method involves controlling bacterial gene expression and deleting crucial genes in bacteria using CRISPR interference (CRISPRi). Researchers can use CRISPRi to prevent the expression of genes essential for bacterial survival and growth, which could result in the creation of novel antibacterial medicines (Schultenkämper et al., 2020).

Although these strategies are still in the experimental stage and face a number of difficulties, such as delivery mechanisms and off-target effects, they offer promising new directions for researching diverse antibacterial treatments. It's crucial to keep in mind that CRISPR-based antibacterial approaches are still in the development stage and have not yet been widely used in clinical settings (Murugaiyan et al., 2022).

3. Bacterial resistance

Bacterial resistance to current antibiotherapy is a persistent issue that receives recognition on a global scale. There is a recurring need for new antibacterial medications, and recent research and development efforts have led to a few new medications or medication combinations that have been suggested for use in clinics (Andrei et al., 2019; York, 2020).

Bacteria develop many mechanisms to resist the action of antibacterial drugs. Generally, these mechanisms include (Davies et al., 2010; Nikaido, 2009; Serral et al., 2021):

- Alteration of the target protein.
- Enzymatic inactivation of the drug.
- Preventing drugs from reaching their target.
- Inhibit influx.
- Development of genes for less susceptible target proteins from other species.
- By passing the target.

Sometimes a bacteria may possess more than one resistance mechanism at the same time, a condition called multidrug resistance. Recently, many bacteria have shown this condition, for

example superbugs such as *Staphylococcus aureus*, *Mycobacterium tuberculosis* (Mtb), *Klebsiella pneumoniae*, and *Clostridium difficile*. Multidrug resistance of superbugs is considered the major threat to public health, as it reduces treatment options and increases morbidity and mortality.

4. Antibiotic discovery in the pre-genome era

The discovery of penicillin was followed by the discovery of enormous antibiotics from microbes, especially from members of the actinomycetes and fungi. From the 1960s on, an increase in bacterial resistance to these antibiotics and their spread required the search for new antimicrobial agents. At the same time, semisynthetic modifications of existing antibiotics were discovered by the scientists. Semisynthetic antibiotics proved their efficiency (they are more potent and less susceptible to inactivating enzymes that cause resistance). Also, some of these drugs showed activity against a broad spectrum of bacteria, and toxicity problems were very minimal. By the early 1980s, progress in the discovery of semisynthetic antibacterial agents was almost depressed, and drug resistance in bacteria was still rising (Church et al., 2021).

5. Bacterial genomes and genome sequencing

5.1 Bacterial Genomes

The genome of an organism refers to all genes contained in the DNA of its chromosome(s). The bacterial genome is usually present in a circular DNA molecule which is supercoiled and localized within the nucleoid of the cell. There are some exceptions: Some bacteria have two or more chromosomes and some contain linear chromosomes. *Vibrio*, *Burkholderia*, *Leptospira*, and *Brucella* species contain two or more chromosomes, while *Borrelia burgdorferi* has a linear chromosome.

On average, a typical bacterial genome has about 2500 genes. The genome of bacteria encodes all the biochemical functions that are necessary for the survival of the bacteria. Also, pathogenic bacteria may carry genes responsible for virulence, and noncoding regions are located in the bacterial genome. Bacterial genes may be organized into operons, which are defined as a group of genes located adjacent to one another and whose functions are similar. For example, the lactose operon in *E. coli* contains three genes involved in the conversion of lactose, a disaccharide into monosaccharide units: Glucose and galactose.

Bacterial genomes are dynamic and are expressed in various genetic events. The evolution and spread of antibiotic resistance are significantly influenced by the bacterial genome. To develop new approaches to effectively battle resistance, it is essential to understand the bacterial genome and the mechanisms underlying antibiotic resistance. This comprises the creation of novel antibiotics and the identification of alternative therapeutic modalities (Normark et al., 2002; Banin et al., 2017).

Through genetic mutations in their DNA, bacteria can develop resistance. The bacterial genome can spontaneously mutate, changing certain genes responsible for antibiotic targets

or drug metabolism. Antibiotic resistance may result from these alterations, rendering microorganisms less vulnerable to their actions. These resistant bacteria may become the predominant strain over time (Levy, 1998; Toprak et al., 2012).

Bacteria could have specific genes that can confer resistance to certain antibiotics. These resistance genes may be found on mobile genetic elements like plasmids or transposons or within the bacterial genome. These genes produce enzymes that render antibiotics inactive, alter the antibiotic's target location, or actively pump the antibiotic from the bacterial cell, among other processes that can confer resistance (Jiang et al., 2018; Partridge et al., 2018).

Bacteria are capable of transferring genetic material horizontally within the same species or even across different species. The rapid spread of resistance within a bacterial population is caused by this process, which makes it possible for resistance genes to be transferred from one bacterium to another. The three main methods of horizontal gene transfer are conjugation (direct DNA transfer between cells), transformation (environmental uptake of naked DNA), and transduction (gene transmission via bacteriophages) (Dutta et al., 2002; Jian et al., 2021).

5.2 Genome Sequencing and Insights into Development of Antibiotics

One of the most important applications of genome sequence data is the discovery of antibiotic targets for the development of new antibiotics, considering the current trend of increasing antibiotic resistance, especially multiple drug resistance in superbugs.

Resistance frequently happens when newly designed antibiotics are chemically similar to previous ones that are already ineffective. Therefore, ideally, new antibiotics should have new alternative mechanisms of action, which is the goal of the genome sequencing approach to discover new antimicrobials. Current antimicrobial agents target a small fraction of the bacterial genome, indicating good expectations for the discovery of new antibacterial drugs.

There are essential attributes of good drug targets in the genomes of bacteria (Glass et al., 2002):

Firstly, appropriate drug targets must be essential for viability or required for disease;

Secondly, they must be specific to bacteria or at least significantly different from orthologous genes in humans;

Thirdly, for broad-spectrum antibiotics, the targets must be present in pathogenic bacteria.

It is important to mention that though antibiotic targets have been discovered through genome sequencing efforts, no antibiotics have reached the marketplace via this route. Progress towards the development of lead compounds that inhibit the antibiotic targets has been very slow. Using the example of the pharmaceutical company GlaxoSmithKline, 67 high throughput sequencing antibacterial targets were investigated between 1995 and 2001, and only five resulted in lead compounds. Part of the problem may be attributed to the fact that chemical libraries have been biased toward meeting Lipinski's "rule of five," a chemical algorithm used to predict drug-likeness. The problem with Lipinski's "rule of five," is that many existing antibiotics do not conform to it, and therefore its applicability to antibiotics is not conclusive. Another major issue is that, though several conserved antibiotic targets have been discovered through genome sequencing, antibiotics that bind to the targets may

not be able to penetrate the bacteria or may be removed by efflux. There is also the problem of a high susceptibility of single targets to mutational resistance. A good example is peptide deformylase, a genomics-derived target. Though suitable lead compounds that inhibit this enzyme have been developed, they have a high tendency to generate mutants, and therefore antibiotic development related to this target has not progressed beyond clinical trials ([Guzmán et al., 2008](#)).

6. RNA as a target for drug design

Ribonucleic acids are the middlemen of protein synthesis in the cell. They feature varying structures that include bulges, turns, pseudoknots, and loops, which enables them to form tertiary structures much like proteins do or serve as ligand binding sites. RNA are attractive targets for drug design because of the presence of these tertiary structures, and, while there's not much genetic variation in their transcription, there are posttranscription alterations that do introduce variations ([Rastogi et al., 2008](#)).

Many antibacterial formulations target bacterial rRNA with the goal of reducing protein translation and thereby inhibiting bacterial growth. This is a result of the complicated tertiary structures that they contain, causing them to be irreparable if altered. Bacterial ribosomes, complexes of RNA and other proteins, are formed of two subunits, one large and one small, each of which contains one or more of these three moieties: 5S, 16S, and 23S. Most prokaryotes have a 30S small ribosomal ribonucleic acid, or SSU, and a 50S large ribosomal ribonucleic acid, or LSU, both combining to form a 70S ribosome ([Melnikov et al., 2012](#)).

6.1 Mechanisms

Most antibiotics available target bacterial rRNA and not ribosomal proteins, like, for example, neomycin that prevents the binding of aminoacyl-tRNA synthetase with its anticodon on the tRNA molecule. mRNA targeting is not as common in drug design.

6.2 rRNA targeting antibiotics

Medications can target either the SSU or LSU of the ribosome, and as follows are some mechanisms associated with them:

6.3 Small ribosomal ribonucleic acid (SSUs)

There are two common sites for drug targeting in the SSU: the decoding center (A-site), and the P and E sites. The A-site ensures the pairing of each mRNA codon with its corresponding t-RNA anticodon; the P-site holds the peptidyl chains during translation, and the E site is the last destination for tRNA predissociation. Drugs that target the P and E sites are also referred to as translation initiation inhibitors. Aminoglycosides (e.g., streptomycin) target the A-site of the rRNA. Differences in that site between prokaryotic 16S and human 18S cause selectivity. They are toxic, and bacteria develop resistant strains.

Drug design should aim to alter sugar moieties to overcome these resistances. Some examples of SSUs:

Streptomycin: causes translation errors and slows translocation by binding to nucleotides in helices h44, h18, h27, and the ribosomal protein uS12 in the 16S moieties while not being bound to the RNP complex. It can also bind to 30S subunits and protect their decode centers from dimethyl sulfate attacks in the presence of magnesium ions, protecting the 16S moiety from separating from the 30S subunit.

Spectinomycin: commonly used to treat gonorrhea, which binds to 16S subunits in many Gram negative strains and prevents the translocation of peptidyl-tRNA from the A-site to the P-site via blocking elongation factor G, leading to faulty protein synthesis and death; this inhibition of translation may not correlate to the strength of binding to the A-site. Spectinomycin can also bind with multiple nucleotides via hydrogen bonds; its binding site has been confirmed through X-ray crystallography to be the end of h34 of 16S rRNA.

Tetracycline, by influencing the binding of the tRNA anticodons to the A-site or specifically Ac-Phe-tRNA to the P-site in some reports, tetracycline inhibits said binding. When no ribonucleoprotein is present, can bind to ribosomal proteins since they show high affinity for it, especially protein S7. It mainly targets 16S and 23S RNA.

6.4 Large ribosomal ribonucleic acid (LSUs)

Many antibiotics that act on LSUs target the peptidyl transferase center, where peptide bonds are created, as well as the NPET (nascent peptide exit tunnel). For example:

Lincomycin and Clindamycin: Both lincosamides prevent peptide bond formation by targeting the PTC in domain V of 23S rRNA. Clindamycin is more lipid-soluble and thus penetrates cell membranes easier, thus making it more potent than Lincomycin.

Chloramphenicol: inhibits protein synthesis by binding to the 50S LSU at the central loop of domain V of the 23S rRNA, which causes peptidyl transferase activity to be inhibited.

Erythromycin and Carbomycin A belong to the macrolide class of antibiotics, formed of 12–16 macrocyclic lactone rings and one or more side chains, usually sugars. They bind to the NPET, preventing peptide bond formation while they remain bound.

6.5 tRNA targeting antibiotics

Puromycin another aminonucleoside antibiotic, causes premature chain termination during translation because part of the molecule resembles the 3' end of an aminoacylated tRNA. It does not possess any selectivity and thus is now used as a tool for studying protein synthesis.

Mupirocin a.k.a. pseudomonic acid A, is the exclusive drug for targeting aminoacyl tRNA synthetase (aaRSs) and serves as a prototype for the development of similarly acting aaRS inhibitors. It inhibits isoleucyl-tRNA synthetase (IleRS) specifically. It inhibits fungal growth of dermatophytes and bacterial growth of multiple bacterial strains of staphylococcus and streptococcus, as well as some archeal members, but not mammalian cells. However, it has no effect on IleRS2, present in some eukaryotic bacteria.

6.6 mRNA targeting antibiotics

Some novel drugs are being produced usually in conformations that provide binding sites for small molecules, such as the iron response element responsible for iron homeostasis or the amyloid precursor proteins of Alzheimer's disease, or the AU-rich element that regulates mRNA translation.

Possible novel targets for drug discovery are allosteric controllers of mRNA regulation, like the enzymes involved in thiamine biosynthesis in *E. coli* can bind to thiamine without protein cofactors, or the riboswitches present at the 5' untranslated region of mRNAs can also serve as regulators of mRNA gene expression themselves. It should be noted that mRNAs in prokaryotes and eukaryotes are vastly different, owing mainly to a difference in lifespan, as prokaryotic mRNA lacks a 5' cap and 3' polyA which cause its easy degradation by endonuclease, and owing to prokaryotic mRNA not needing processing or transport prior to translation by ribosomes, while eukaryotes require premRNA molecules to be spliced into mature mRNA.

6.7 Riboswitches

Antibiotic-resistant superbacteria are a prime target for novel antibiotics, and bacterial riboswitch RNAs are an appealing target. The regulation of bacterial gene expression depends on their ability to bind to cognate metabolites (Lee et al., 2021).

Riboswitches are structures responsible for gene expression present in the UTRs (untranslated regions) of the 5' end of mRNAs. There are three reasons riboswitches are attractive drug targets:

1. Predominantly present in bacteria while absent in eukaryotic cells, the only exception being TPP (thiamine pyrophosphate) riboswitches
2. Predisposed to only binding to small molecules (however, this presents a problem where drugs exhibit poor selectivity)
3. They respond to common second messengers and metabolites that are associated with mRNAs encoding essential proteins.

Riboswitches are comprised of two types of domains: Aptamers and expression platforms. Expression platforms are RNA segments downstream of or embedded into aptamer domains that are responsible for ligand-binding signal transduction. Meanwhile, aptamers are nucleic acids selected to specifically bind to certain ligands in vitro, and they were identified by subjecting up to a quintillion (10¹⁵) RNA and DNA molecules to iterative rounds of selection and amplification of certain functional groups. To bind to the aptamer domain, a ligand must contain a certain substructure like how a lock and key mechanism works, and naturally occurring riboswitch aptamers have few such substructures that they interact with (Fig. 12.1). Some riboswitches terminate translation when ligated (OFF-switches) while others allow gene translation when bound (ON-switches) (Berens et al., 2015; Roth et al., 2007).

Riboswitches regulate gene expression in one of several ways: alternative splicing through ligand binding; transacting; transcriptional attenuation, where the aptamer domain competes with a terminator-stem loop for folding and accessibility; ligand-binding-induced self-cleavage that causes mRNA destabilization and subsequent destruction by RNase.

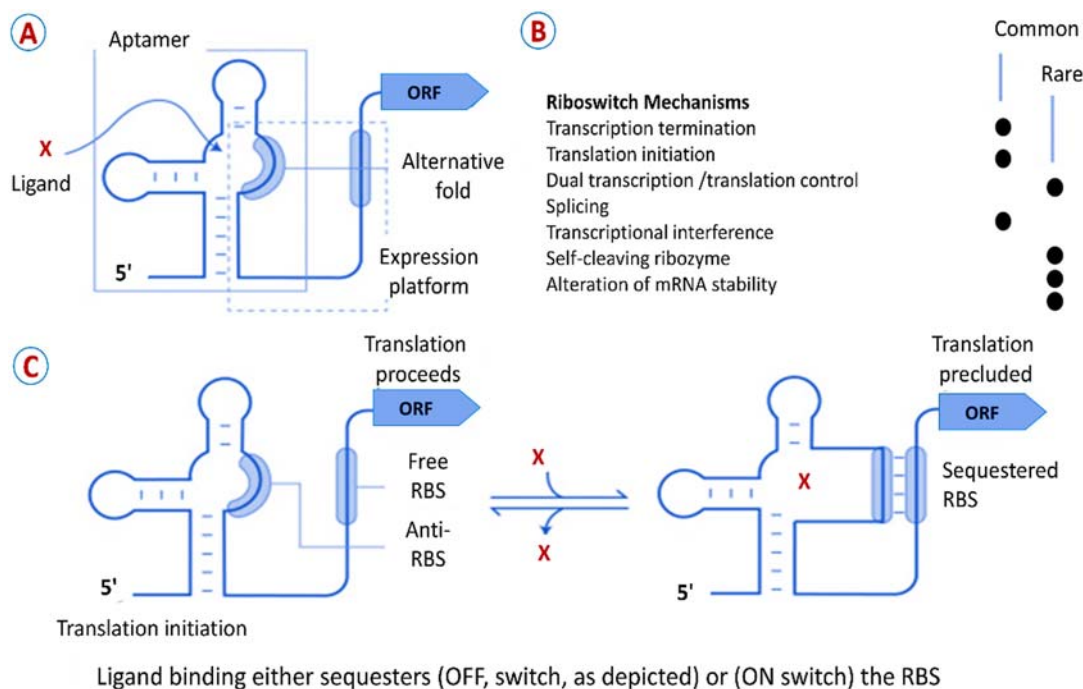


FIGURE 12.1 (A) Generic diagram of a riboswitch unit; (B) mechanisms of riboswitch-mediated gene regulation; (C) unbound riboswitch allowing translation; (D) Riboswitch bound to ligand disallowing translation (Deigan et al., 2011).

Across over 6000 bacterial species, 28 experimentally verified riboswitches exist, and they are categorized based on the folds of their aptamers and their respective cognate ligands; the ligands may be one of many types of biomolecules including amino acids, phosphorylate sugars, nucleic acids, ions, and coenzymes. The variation in the ligands' prevalence allows for selectivity in drug design to also vary between targeting by broad-spectrum or narrow-spectrum antibiotics.

6.8 Riboswitch targeting compounds

Roseoflavin: Inhibits the growth of gram-positive bacteria by binding to the FMN riboswitch (flavin mononucleotide), which regulates the synthesis of all riboflavin within these cells. Mutations to the FMN aptamer domain exist in resistant strains as they disrupt roseoflavin binding, but they also reduce bacterial survival overall because of a weaker regulatory response to riboflavin as well.

Pyriethamine (PT): A pyridinium-ring analog of the thiazine-based thiamine, toxic to both bacteria and fungi. It is phosphorylated in vivo to PTPP (pyriethamine pyrophosphate), which, due to structural similarity to TPP (thiamine pyrophosphate), binds to the TPP riboswitches in said organisms. PT when bound to the aptamer stabilizes the conformation poorly, while

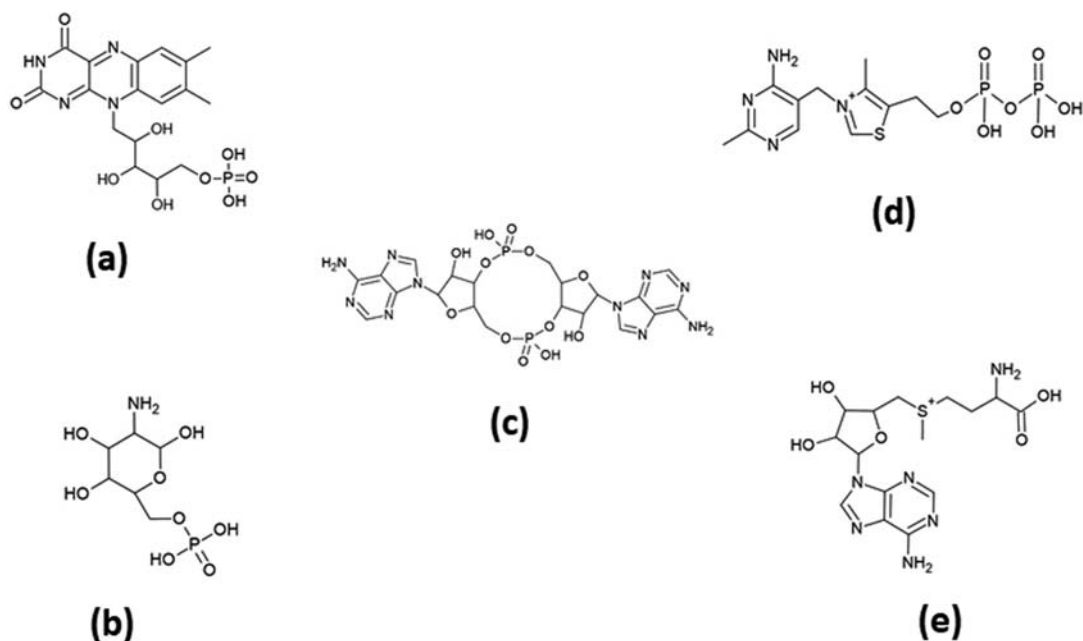


FIGURE 12.2 (A) FMN, (B) glmS, (C) c-di-AMP, (D) TPP, (E) SAM. Examples of riboswitches prevalently present across human bacterial pathogens are the cobalamin, cyclic diadenosine monophosphate (c-di-AMP), glutamine-fructose-6-phosphate (glmS), S-adenosyl methionine-I (SAM-I), flavin mononucleotide (FMN), glycine, lysine, and flavin mononucleotide (FMN) riboswitches (Breaker, 2018).

PTPP stabilizes it to a high degree. However, like roseoflavin, some bacteria exhibit mutations that reduce PT binding to the TPP domains but in return exhibit abnormal TPP metabolic gene regulation. The specificity of action PTPP to the TPP riboswitch is due to the RNA molecule changing conformation during ligation to form hydrogen bonds with the pyrimidine ring, while in contrast, it does not bind whatsoever to the thiazole ring of TPP (Fig. 12.2).

L-aminoethylcysteine and DL-4-oxalysine: These are lysine analogs that bind to the lysC riboswitch in *Bacillus subtilis* and repress the expression of a lysC-regulated reporter gene. A possible mechanism is the inhibition of lysyl-tRNA synthetase by AEC, leading to the inhibition of protein synthesis. Some strains of *B. subtilis* and *E. coli* are resistant to AEC and DL-4-oxalysine due to mutation.

7. Genetic approaches to facilitate antibacterial drug development

Many classes of antibacteria in clinical use today originate from the outstanding period of antimicrobial disclosure, which had its most useful period from 1940 to 1960 (Davies, 2006).

Most bacterial infections may be treated within a few weeks with a few pills, usually without any side effects, due to these drugs. Therefore, it is easy to forget that infections caused by bacteria have a large impact on human well-being and remain a leading cause of fatalities in developing countries.

The emergence and spread of drug-resistant bacterial contamination coincided with the lack of novel antibacterials identified after 1960. When resistant to not just one, but several medications, such contaminations work to undermine the basis of existing medical treatment (Davies, 2006).

A great example of how therapy has succeeded in developed countries while consistently failing in developing nations is tuberculosis (TB). Treatment failure contributed to the resurgence of a disease that was thought to have been cured but is now a serious threat to public health. The first-line treatment for TB consists of drugs that were first discovered during the glorious era of antimicrobial disclosure. These drugs helped to significantly reduce TB infections in Europe and North America, along with social reforms, while failing in Africa and Asia. Soon after the development of TB chemotherapy, drug-safe Mtb appeared and quickly developed into an extremely drug-safe Mtb, undermining global TB control initiatives. As a result, since the preantibiotic era, the need for innovative medicines to cure this severe infection has not changed (Waksman, 2021).

7.1 Genetics and Classical Whole-Cell Screening

Finding hits that obstruct bacterial growth is the first step in whole-cell screening (WCS)-based antibacterial medication discovery. This inhibitor-first approach laid the groundwork for the great period of antibacterial drug discovery and continues to be the primary course of action approach that has brought antibacterial drugs to the facility (Dharra et al., 2017).

This keeps in mind all the first-in-class drug competitors currently being developed for the treatment of TB. Since phenotypic screening has proven to be more effective than target-driven approaches across a range of disorders, the unmatched achievement pace of inhibitor-first strategies is nothing new for the development of antibacterial medications. The development of traditional WCS is aided by a few factors. These screening processes pay particular attention to substance architectures that can pass the key antibacterial medication improvement test of being able to enter the bacterial cell membrane. Additionally, WCS provides a chance to identify factors that obstruct the growth of various systems, including those that draw in many objectives. The disadvantage is that the system for all hits is still not fully established (Lechartier et al., 2014; Swinney et al., 2011).

Although understanding a drug competitor's mode of action (MOA) is not required to obtain administrative approval for use in people, understanding how a compound uses its biological potential during improvement is desirable for a number of reasons. First off, many WCS hits work in an ambiguous manner (for example, as cleansers or alkylating specialists) and cannot therefore be converted into pharmaceuticals. Second, not every hit that is damaging to the host needs to be disposed of because a compound's antibacterial effect does not always result in poisonousness. When a substance's antibacterial effect and poisonousness both result from the suppression of the same goal, such as a substance that is rationed in both the microorganism and the host. Such combinations are unsuitable for further turns of events. Compound modifications, however, can occasionally eliminate a compound's poisonousness without impairing its antibacterial action when the instrument of poisonousness differs from that of bacterial development constraint. Third, after identifying the focus, a closer look at the compound's design action relationship (SAR) can be made. Fourth, and perhaps most importantly, a compound's

in vivo potency is determined by its antibacterial MOA. Recently discovered pyrimidine-imidazoles serve as an illustration of this; they were effective against *Mtb* in vitro and had appropriate pharmacokinetic characteristics, but they had no effect on *Mtb*-infected mice. The presence of glycerol, which is used in many common fluid media but is not a physiological carbon source, was necessary for the mobility of these molecules. As a result, there was no method to further develop these pyrimidine-imidazoles because the lack of in vivo strength was component-based (Payne et al., 2007; Pethe et al., 2013; Roemer et al., 2013).

Given that screening medium may, at best, only mimic some of the settings experienced during a disease, there is a great chance that other compounds with MOAs that naturally have no in vivo power (referred to as novip drugs) will be discovered. More often than not, they create a physiological environment that is very different from that found inside the host because many standard media contain nutrients that *Mtb* cannot obtain from the host in the given structure (e.g., iron), cannot obtain from the host at all (e.g., biotin) (Sassetti et al., 2003; Woong Park et al., 2011), and needs nutrients that *Mtb* is clearly prepared to rummage from the host (e.g., NAD precursors) (Boshoff et al., 2008; Rodriguez et al., 2006).

These discrepancies may cause some modifications (such as those in nothing) to either result in the transmission of *Mtb* in vitro or not during contamination. Alternately, you could prevent development when there are contaminations but not in normal media (as when alterations are anticipated to hunt for iron in the host); screening against intracellular *Mtb* is one thoughtfully simple method to reduce the risk of discovering novip chemicals. However, such screening techniques can also reveal characteristics that are needed ex vivo but not when exposed to contamination. Additionally, *Mtb* causes a dynamic and complicated infection that manifests in a wide variety of intracellular and extracellular regions. While some of these areas may restrict the microorganism's development but allow it to be diligent, others may allow it to reproduce. As a result, it seems challenging to design screening scenarios that accurately reflect the pathophysiological context of a *Mtb* contamination in individuals who are generally ok to completely incline toward novip structures. Therefore, a thorough understanding of a compound's MOA is the strongest defense against MOA-based whittling down (Boshoff et al., 2008; Christophe et al., 2009; Dartois et al., 2013; Muñoz-Elías et al., 2006; Rodriguez et al., 2006).

7.2 Genetic Strategies for Studying the MOA of Bioactive Small Molecules' RNA Profiling

The quantity of full RNA produced at any particular time determines the broad RNA profile of a cell. Following the development of DNA microarrays (Schena et al., 1995), the interpretation of these profiles became evident, and they may now be accounted for with even greater precision by sequencing all RNA extracts (Nagalakshmi et al., 2008). The anesthetic dyclonine was used to demonstrate the use of wide-RNA profiling for illustrating the MOA of bioactive particles (Hughes et al., 2000). Hughes et al. collected *Saccharomyces cerevisiae* (*S. cerevisiae*) RNA profiles that were brought on by a variety of inherited or artificial medications. This resulted in a reference profile, against which the RNA profile started by dyclonine may be compared. Dyclonine generated modifications similar to those triggered by changes in the ergosterol pathway, according to design matching estimates. This implied that dyclonine inhibited sterol production, and subsequent investigations supported this notion that dyclonine's target was the sterol isomerase Erg2p. Since then, various more antimicrobial combinations' targets and MOA have been revealed using this

example matching process (Kohanski et al., 2010; Schnappinger, 2015; Smith et al., 2010; Wecke et al., 2011). Undoubtedly, *Mtb* microarrays were initially used to focus on how isoniazid affected the RNA profile of *Mtb* (Fig. 12.3) (Wilson et al., 1999). Five years later, in a landmark work, Boshoff et al. (2004) revealed hundreds of compound-prompted RNA profiles. These profiles were used to investigate various antimycobacterial small molecules, such as capreomycin (Fu et al., 2007), isoniazid (Karakousis et al., 2008), PA824 (Manjunatha et al., 2009), an inhibitor of menaquinone synthesis (Dhiman et al., 2009), β -lactams (Slayden et al., 2009), vancomycin (Provvedi et al., 2009).

The two main obstacles to RNA profiling are interrelated. These profiles, as it were, reveal the organic pathway(s) that lead to the medication, but they fail to differentiate the direct purpose of a molecule. This is partially because controlling different proteins can result in similar RNA profiles, for instance when the proteins are required for the same metabolic pathway or when they form a complex. Second, the profiles are often instructive for chemicals that commence with a mark similar to mixes with a recognized MOA (along with other example matching approaches). For substances with a wholly new MOA, it is challenging to make explicit forecasts because there are typically no suitable reference profiles available (Fig. 12.3). This disadvantage can be overcome by advancing the reference abridgment with RNA profiles from hereditarily characterized mutants. Tragically, there are far too few of these profiles available for MTB to use this recommendation in conjunction with the

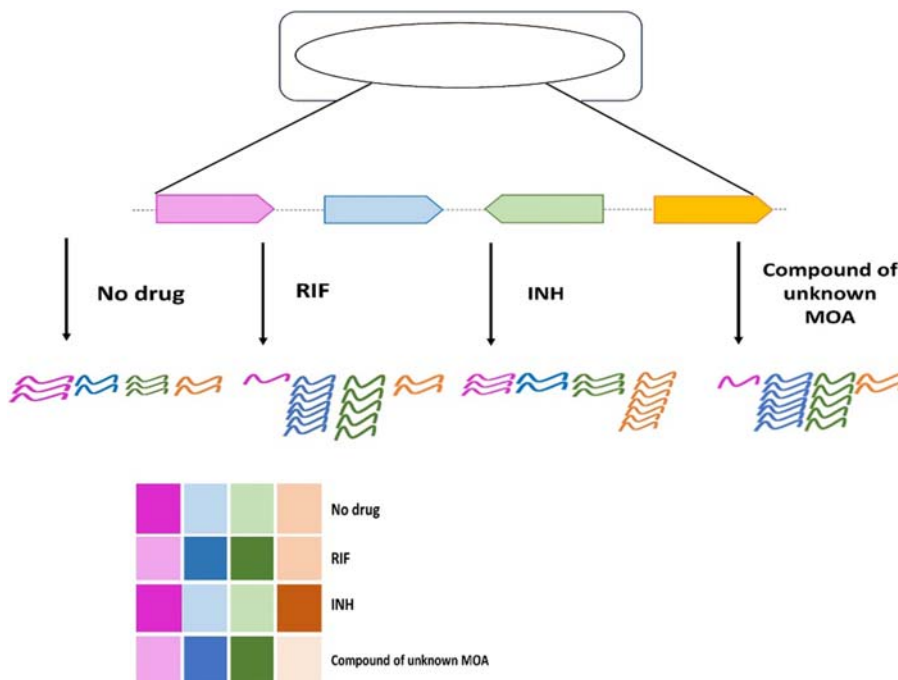


FIGURE 12.3 RNA profiling. RNA molecules produced by four genes of the *Mtb* genome with and without drug treatment are represented as colored lines. The color of the squares depicted below the RNA molecules reflects their abundance, with darker colors identifying more abundant RNAs. The fictional data here would suggest that the compound of unknown MOA functions in a manner similar to RIF (Schnappinger, 2015).

analysis of antimycobacterial chemicals. In any case, broad RNA profiles for relatively small bacterial genomes are made up of thousands of individual estimates. This intricacy allows for the creation of unique markers for the vast majority of bioactive small atoms while making it difficult to identify the most illuminating RNAs. The amount of data produced by RNA profiling is huge, but even more importantly, the natural responses to developmental problems are frequently complex. This rule is applicable to RNA profiles obtained during periods of time focus or during pharmacological fixations that affect the overflow of many RNAs. Such profiles' nebulous pressure reactions may conceal a compound-explicit response. The analysis of bioactive atoms by RNA profiling frequently benefits from cutting treatment time and drug focus. Additionally, the already complex system is multiplied by a factor of many thanks to the ability of comprehensive RNA studies to detect minute changes in any one characteristic in conjunction with a transcriptional reaction that is fundamentally unique to bacteria. In this way, it can be difficult to obtain very similar RNA profiles on two seemingly unrelated tests. In order to produce illuminating RNA profiles, it is essential to depict every single test detail, apply strict normalization, and avoid procedural variations in quality articulation (which might occur, for example, while centrifuging live Mtb) (Freiberg et al., 2005; Hughes et al., 2000; Mnaimneh et al., 2004).

7.3 Selection and Identification of Resistance Mutations

The strains chosen for their ability to grow when exposed to bedaquiline (BDQ; formerly known as TMC207 and initially published as R207910) provided the primary evidence that BDQ prevents Mtb growth by impeding ATP synthase. These BDQ-safe bacteria' entire genome sequencing revealed point mutations in *atpE*, which codes for ATP synthase subunit C. Hereditary and pharmacological investigations revealed that BDQ restricts AtpE to inhibit ATP production, establishing ATP synthase as the primary target (Andries et al., 2005; Koul et al., 2007). Selective obstructive therapy followed by whole genome sequencing (Fig. 12.4) has identified mutations that permit development while under the effect of a few other tiny particles with antimycobacterial activities. Models take into account modifications to the properties of breath (*qcrB*) (Abrahams et al., 2012a; Pethe et al., 2013), unsaturated fat blend (*inhA*) (Hartkoorn et al., 2012), protein blend (*aspS*) (Ioerger et al., 2013), protein emission (*eccB3*) (Ioerger et al., 2013), polyketide biosynthesis (*pks13*) (Ioerger et al., 2013; Wilson et al., 2013), mycolic corrosive vehicle (*mmpL3*) (Grzegorzewicz et al., 2012; Ioerger et al., 2013; La Rosa et al., 2012; Remuiñán et al., 2013; Tahlan et al., 2012), and arabinogalactan blend (*dprE1*) (Christophe et al., 2009; Magnet et al., 2010; Riccardi et al., 2013). High-value pharmacological targets can be easily distinguished by changes in opposition. Since the entire genome is reviewed, selecting and identifying these alterations can be done quickly and thoroughly with no need to improve target-specific measures. Nevertheless, this method has its drawbacks and fails if a resistant strain cannot be found. In addition, one of the few mechanisms by which changes can provide resistance is the modification of the objective responsible for the development of an inhibitory effect on a small particle. However, the basis of obstruction can be shaped by strategies like synthetic inactivation, efflux, or the inability to transform a dormant prodrug into its active offshoot. The knowledge of the transformation frequencies that cause opposition by these instruments proves to be very useful in determining the value of a compound.

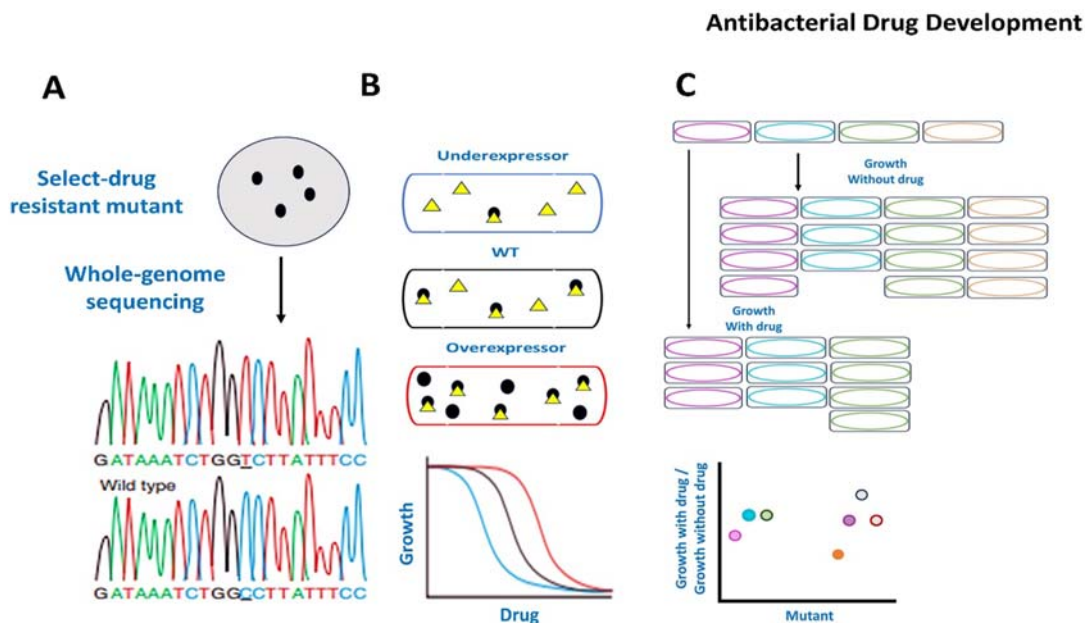


FIGURE 12.4 Forward and reverse genetic approaches to investigate small molecule MOAs. (A) Selection of resistant mutants. Mutants that are resistant to the compound are selected on agar plates supplemented with the compound of interest. The mutations present in these resistant isolates are identified by whole-genome sequencing. (B) Under/overexpression. Reducing or increasing the expression of a candidate target (black circles) can either decrease or increase the MIC of a compound (yellow triangle) inhibiting the target. (C) Phenotyping pooled mutants. Mutants are labeled with a genetic barcode and their fitness is analyzed in a competitive growth experiment. This principle can be applied to deletion mutants, underexpressors, overexpressors, or a mix of these strains. The orange-label mutant shows increased sensitivity to the tested drug; the black-labeled mutant (which is only shown in the graph) shows decreased sensitivity. A significant recurrence of opposition implied by such systems renders the compound wholly substandard. However, a compound's MOA is rarely described using this information. Nevertheless, obstacle change provides one of the most straightforward methods of describing a compound's MOA in circumstances where a transformation may be planned for a real purpose (Schnappinger, 2015).

7.4 Linking Small Molecules to Candidate Targets by Increasing or Reducing Gene Activity

A protein must actually interact with a bioactive small particle's MOA in order to be directly engaged with it. By demonstrating a direct correlation between the protein's overflow and the minimal inhibitor fixation predicted to block development (MIC) (Fig. 12.4), a candidate protein's capacity for such an interaction inside living microorganisms can be indirectly confirmed. This type of experiment has helped to explain the MOA of INH (isoniazid), which was used to block either InhA or KasA, one of the two catalysts involved in the production of unsaturated fatty acids. The susceptibilities of strains overexpressing these proteins to INH and the KasA inhibitor thiolactomycin (TLM) were tested. The MIC of INH increased with overexpression of InhA, but the MIC remained unchanged. Overexpression of InhA increased the MIC of INH; however, it didn't change the MIC of TLM;

overexpression of KasA had the contrary impact (Larsen et al., 2002). These findings supported the idea that InhA and KasA are, respectively, crucial targets of INH and TLM. With *Mycobacterium smegmatis* (*M. smegmatis*), comparative overexpression studies have frequently been carried out, in which the MOA of various inhibitors was connected to at least one target, including D-cycloserine to alanine racemase (Alr) and D-alaD-ala ligase (Ddl) (Feng et al., 2003), platensimycin to KasA and KasB (Brown et al., 2009). Overexpression in *mycobacteria* has mostly been used to evaluate the performance of certain candidate target proteins for compound action, but it can also provide a method to identify emerging targets for a molecule with unknown MOA. To achieve this, libraries of overexpression clones are purposefully examined for those that promote further development while the inhibitor is present. The lipoprotein escort, LolA, was identified as a competitor focus for the clever antibacterial chemical, MAC12343, in such a screen, which made use of an organized library of overexpression clones for all basic *E. coli* qualities. According to biochemical analyses, MAC12343 binds LolA and interferes with lipoprotein transport (Pathania et al., 2009). For *Mtb*, comparable overexpression libraries are being created and should be accessible soon. The constant detachment of efflux syphons is a drawback of overexpression screens, particularly when using arbitrary libraries. By decreasing the intracellular centralization of the chemical under investigation, these syphons increase blockage, and their disengagement doesn't reveal a drug's MOA. A target's association with a protein family in which multiple members are encoded in the same genome might also make it challenging to understand MIC movements.

When one relative is the physiological goal, overexpression of any member of such a protein family will, in any case, increase the MIC if the inhibitor restricting site is being watched. This has been discussed in relation to methionine aminopeptidases (MapA/B) (Olaleye et al., 2010), ketoacyl-ACP synthases (KasA/B) (Brown et al., 2009), and inosine-50-monophosphate (Demon) dehydrogenases (GuaB1/2/3) (Usha et al., 2011). In some of these instances, the MIC may have increased because the overexpressed protein sequestered the inhibitor from its physiological target. Target underexpression should decrease the MIC just as target overexpression should increase it (Fig. 12.4). This was unquestionably shown for a few conditional *M. smegmatis* knockdown strains.

Underexpression of gyrase subunit A (GyrA, focus of quinolones (Sugino et al., 1977), Alr (Caceres et al., 1997), DHFR [focus of trimethoprim] (Schweitzer et al., 1990), RNA polymerase subunit B [RpoB, focus of RIF] (Lancini et al., 1969), and protein biotin ligase (BirA, focus of BioAMS (Duckworth et al., 2011)) improved *M. smegmatis* susceptibility to ciprofloxacin, D-cycloserine, trimethoprim, RIF, and BioAMS, separately (Duckworth et al., 2011). Comparative observations were made with *Mtb*, where inadequate genetic inactivation of the biotin-blend catalysts DHFR or BioA increased the organism's susceptibility to their respective inhibitors. For the vast majority of these strains, it was also shown that they were generally unequivocally sharpened to inhibitors of the somewhat exhausted protein (Abrahams et al., 2012b; Shi et al., 2011).

Other findings confirmed that halfway depletion of an in vitro fundamental protein frequently results in compound-explicit MIC moves and can therefore provide information on the MOA of a bioactive small particle. But just like in the case of overexpression, underexpression concentrates alone are insufficient to ensure that the underproduced protein is the MIC-determining target of an inhibitor, as underexpression could also lower the MIC by

enhancing other compounds in similar pathways to synthetic restraint. Working with them has shown that underexpression, in any case, provides an excellent tool for identifying future targets; this is obvious from work with *S. cerevisiae* (Abrahams et al., 2012b; Donald et al., 2009; Giaever et al., 1999; Lum et al., 2004; Ollinger et al., 2012; Yin et al., 2004).

In *S. cerevisiae*, underexpressor development is typically direct and frequently done with the cancellation of one high-quality duplicate. Almost all *S. cerevisiae* characteristics, including those essential for typical development, are available in these heterozygous erase strains. This mutant collection has been extensively used in drug-initiated haploinsufficiency profiling (HIP) to focus on the MOA of bioactive small atoms. The effect of sublethal drug fixations on the general rates of development of various strains is estimated by HIP (Fig. 12.4). These studies essentially confirmed that the strains of well-known targets of antimicrobial specialists are among those that are typically resistant to the development hindrance caused by the respective drug, and they were then used to identify new competitor targets for well-established drugs. This included the rRNA-handling exosome, which HIP predicted to be related to the MOA of the anticancer drug 5-fluorouracil and was confirmed by various trials. HIP helped identify the guanine monophosphate synthase (Kammiller et al., 2008; Rodriguez-Suarez et al., 2007), a greasy corrosive desaturase, and poly(A) polymerase as targets of new inhibitors in the clever bacterium *Candida albicans* (Jiang et al., 2008). Tests similar to HIP have been conducted in bacteria where antisense RNAs can successfully reduce the quality of speech (Donald et al., 2009; Huber et al., 2009). Unfortunately, antisense RNAs have only rarely proved successful in *Mtb*. Currently, methods like transcriptional or proteolytic hushing, which require homologous recombination during freak development, are used to estimate the age of a wide range of *Mtb* underexpressors (Ehrt et al., 2005; Giaever et al., 2004; Lum et al., 2004).

WCS hits should primarily concentrate on the fundamental quality components because they are development-inhibiting by definition. All things considered, changes in unimportant characteristics typically counteract the movement of a compound. Several studies, including those that used the *E. coli* Keio assortment, provided evidence for this. Each of the over 4000 strains in this library has had one quality deleted. Two hundred eightythree of the four thousand strains tested responded noticeably better to at least one of the 14 novel antimicrobial than the wild type (Liu et al., 2010; Tamae et al., 2008). While some strains were simply tuned to substances with a similar MOA, others were rendered more ineffective by some kinds of antiinfection medicines (Girgis et al., 2009). You can group a compound's MOA with techniques like RNA profiling by using the drug responsiveness profiles of the most recent strains. The discriminative influence of these profiles can be enriched with changes that decline antitoxin (Girgis et al., 2009). *S. cerevisiae* homozygous cancellation strains are equivalent to *E. coli* mutants. For intensities whose main target isn't a protein, synthetic profiling of such strains (homozygous cancellation profiling, Jump) has been extremely helpful. For instance, a Jump of 4700 *S. cerevisiae* strains study showed that health deserts brought on by the loss of traits involved in DNA repair or in endurance during DNA damage can be useful for DNA-damaging specialties. Actually, these mutants' profiles could distinguish between intensities that cause interstrand cross-joins and those that don't, making them easily detectable for synthetic agents that damage DNA by a variety of methods (Lee et al., 2005). Hereditary communication research provides data that can be used to further our understanding of jump trials. According to Dixon et al. (2009), two qualities are said

to communicate if the aggregate of their twofold mutant deviates from the typical aggregates of the two single quality strains. Numerous such digenic connections have been identified in *S. cerevisiae* (Costanzo et al., 2010).

8. Facilitating target-based drug discovery

Typically, target-based techniques begin by looking for inhibitors. Unfortunately, the majority of inhibitors identified in numerous high-throughput screenings (HTSs) against bacterial compounds showed negligible activity against living germs (Payne et al., 2007). Target-based strategies will eventually have their own benefits. In general, MOA reads are easier to understand than WCS hits from an objective-based approach. For instance, it was recently discovered that a bisubstrate inhibitor (BioAMS) meant to deactivate the protein biotin ligase (BirA) was inhibiting the growth of Mtb. The review, led by Duckworth et al. (2011) examined how BioAMS affected *M. smegmatis* and Mtb protein development and biotinylation. True to form, development inhibition coincided with the depletion of biotinylated proteins and the effect of BioAMS (but not of INH or ethambutol) against Mtb and *M. smegmatis* directly associated with BirA articulation. Together, these distinct findings supported BirA's status as BioAMS's primary goal (Duckworth et al., 2011). The ability to explore a much larger substance space than what can be covered by phenotypic screens is the second major advantage of target-based techniques, especially if piece-based approaches are included (Scott et al., 2012). This is crucial because the majority of the particles in a typical WCS library have physicochemical features that are distinct from those of recognized antibacterials.

9. The power and limits of genetic target validation

A target-based drug revelation program will be inevitable if a poor target is chosen, but it may not become obvious until years of effort and significant financial investment have been made. In this sense, it is vital to distinguish appropriate targets. The ideal protein would meet the following criteria: (1) It should be evenly distributed among the pathogen(s) in question; (2) it should be impossible to stop with a single atom; (3) it should be sufficiently distinct from human homologs to allow the development of low-harmful inhibitors; (4) it should be compatible with biochemical HTS; and (5) it should be anticipated for bacterial development (or endurance) such that its partial inactivation will have a beneficial effect on the health of a patient. Showing that an objective satisfies this multitude of models requires a little particle inhibitor that is protected for use in people (i.e., a medication). In this way, developing medications to combat novel targets always has the additional risk of blocking a target that hasn't been fully approved. This additional risk is applicable to inhibitors identified via WCS or biochemical screens and is one of the factors contributing to the rapid weakening of constructs with completely unique MOAs. Druggability assessments evaluate potential limiting regions using computational or chemical methods; grouping conservation within microscopic organisms and between bacterial species as well as human homologs can be surveyed with genome succession data. Hereditary traits significantly influence how possible targets are

evaluated and the ability to determine the importance of a possible target for development (Barelrier et al., 2011; Fauman et al., 2011; Pérot et al., 2010).

Transposon mutagenesis is the source of this data's full hotspot. Transposon site hybridization (Junk) characterized many of the properties that Mtb anticipates to normally acquire in vitro, in macrophages, or in mice's spleens. Junk makes use of DNA microarrays to simultaneously map and analyze the phenotypic effects of many transposon additions. Recent advancements in these analyses have been made by Tn-seq, which uses cutting-edge DNA sequencing rather than microarrays and provides a single-nucleotide goal and a more powerful reach. Furthermore, compared to other hereditary methodologies, Tn-seq has contributed more to the phenotypic presentation of superior skills in Mtb. However, for two reasons, they are not the best to accept quality goods as targets for therapeutic development. First off, while both Waste and Tn-seq may identify essential traits in vitro, neither methodology can produce strains for these attributes that can be studied in multiple contexts. Genetic screens can separate novip targets (targets whose inactivation kills Mtb under a certain in vitro setting but doesn't prevent persistence or even development in critters or people) similarly to how WCS can identify novip chemicals (Rengarajan et al., 2005; Sassetti et al., 2001, 2003).

Therefore, it is crucial to evaluate how well a quality item performs in a variety of conditions, including those that encourage nonreplicating perseverance. These studies use conditional knockdown strains because the characteristics that Mtb needs to develop under typical lab conditions are some of the most tempting targets for therapeutic development.

With the advancement of synthetically controlled genetic switches for mycobacteria, the generation of contingent knockdown strains in Mtb became possible. The majority of mycobacterial switches that have been developed depend on moving the quality of interest downstream from a modified advertiser so that its record can be controlled by an antibiotic medication repressor and a tetracycline subsidiary (Blokpoel et al., 2005; Boldrin et al., 2010; Ehrt et al., 2005; Klotzsche et al., 2009). These organizational structures were successful when used to examine a few aspects of Mtb (Schnappinger et al., 2014), however they rely on optional occurrences, most notably cell division, to drain the target protein. As a result, they are less able to focus on the significance of quality attributes for the endurance of nonreplicating Mtb. With administrative frameworks that directly increase protein turnover, investigations of nonreplicating bacteria can be carried out more effectively. This can be done in Mtb by changing the region that it encodes the DAS-4 debasement tag's layout (Kim et al., 2011). Contingency is achieved by regulating the protein (SspB) that starts the corruption tag from escaping. The double control (DUC) switch, which was recently developed, combines transcriptional and proteolytic methods of regulation to achieve effective and powerful quality inactivation in growing and nonreplicating Mtb. The DUC modification was used in studies of the NAD synthetase (NadE), which revealed this protein to be essential for the survival of both replicating and nonreplicating bacteria. In addition, during intensive and ongoing contaminations, its inactivation was sufficient to immediately eradicate culturable microscopic organisms from mouse lungs and spleens. NadE's necessity for Mtb's adaptability under all experimental settings strongly suggests that this protein is also anticipated for growth and endurance under the vast majority of environments that Mtb encounters in humans. It still needs to be demonstrated whether it enables the development of tiny atom inhibitors that are safe for usage by people (Kim et al., 2013).

Waste and Tn-seq do not provide estimations of the level at which a quality item should be deactivated in order to stop development. The aggregates reported by Junk/Tn-seq are commonly inferable to inclusions that result in an invalid allele, despite the fact that a transposon inclusion's impact on quality capacity can vary depending on the inclusion site. Rarely, if ever, can tiny particles completely inactivate a protein in a living microscopic creature. Therefore, the phenotypes produced by total erasure are less useful for evaluating a different goal for drug development than those produced by its incomplete exhaustion. A protein that should be inactivated by 90% (or, on the other side, toned down would be preferable) is a more enticing goal than one that should be inhibited by 90% before development is hampered, all other things being equal. *M. smegmatis* was the first mycobacterium to exhibit the vulnerability to fractional consumption of numerous basic proteins. For instance, degradation caused by the HIV protease depleted *M. smegmatis* of 97% of its DHFR and Alr, although this exhaustion just slowed down growth. It's interesting to note that even slight RpoB depletion was sufficient to completely halt development. Unfortunately, only a few numbers of proteins, including BioA and PptT, which are anticipated for the combination of biotin and polyketides individually, have the level of functional inactivation necessary to hinder the development of Mtb. As both catalysts anticipate being drained by 90% to effect growth, Mtb isn't particularly resistant to fractional inactivation of either one. To identify proteins that need to be exhausted, frameworks for contingent quality inactivation should be applied to the depiction of extra qualities 90% to stop the emergence of Mtb (Leblanc et al., 2012; Woong Park et al., 2011; Xiao et al., 2011).

10. Concluding remarks

None of the methodologies included in this review existed during the outstanding time of antimicrobial discovery, and antibacterial medication revelation can be sought after without them. Nonetheless, current genetics can assist with further developing a cycle that has delivered few novel sub-atomic elements during the last 50 years. One significant commitment would be to assist with decreasing late-stage steady loss (i.e., disappointment in clinical preliminaries). The expense of presenting a new medication to the market differs between US \$500 and 2000 million. Late-stage wearing down can consequently channel the assets of monetarily ugly regions, similar to antibacterial medication improvement, and make them explode completely over a significant stretch of time. The two primary drivers of late-stageawakening are absence of security and adequacy in individuals. Foreseeing clinical viability will be uncertain. Notwithstanding, if we comprehend the MOA of the medication competitors that are being tried in the center, it ought to be conceivable to keep away from preliminaries with synthetically unique intensifies that fall flat for the equivalent unthinking reasons. The hereditary methodologies examined here have assisted with describing the MOA of a few antibacterial mixtures. Nonetheless, the MOA of numerous antibacterials is perplexing, and new insights are as yet acquired into the MOA of medications that have been in clinical use for quite a long time. No single methodology will therefore be adequate to characterize the MOA of most antibacterials. Concentrates on that join a few approaches, for instance, underexpression, overexpression, opposition determination, and biochemical

studies vow to be the best. Drug applicants with novel MOAs are appealing in light of the fact that they could get to the next level in TB chemotherapy generally (e.g., by reducing its span). Be that as it may, they bear a higher chance of late-stage disappointment assuming that they inactivate targets that have not yet been clinically approved. Measuring the impacts that can be accomplished by inactivating such clever targets (e.g., by hereditary means) in various examinations, including creature models, appears to be reasonable. Second, hereditary qualities can likewise add to mediate improvement by giving single-quality underexpressors that assist with expanding the responsiveness of WCS; what's more, inclination hits toward an ideal objective. Underexpressors can, moreover, be utilized in an optional examination to assess biochemical HTS hits. This recognizes intensifies that enter the bacterial cell envelope ineffectively (and are not dynamic against wild kind but dynamic against an underexpressor) from those that don't enter at all (and are dynamic neither against wild type nor the underexpressor). Such mixtures could be beginning stages for restorative science endeavors to work on compound adequacy against wild-type microorganisms, which could grow the synthetic space into the area that is successful against microscopic organisms and underrepresented in most screening libraries (Adams et al., 2006; Chakraborty et al., 2013; Kola et al., 2004; Paul et al., 2010).

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Potential targets for the development of antifungal drugs

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1. Introduction

Fungi are eukaryotic organisms such as yeasts, molds, and mushrooms. This class of microorganisms' unique cell wall composition comprises chitin and polysaccharides and a sterol-based cytoplasmic barrier, mainly ergosterol. Depending on their morphology, fungi that cause illness in humans might include yeasts, molds, and biphasic (dimorphic) fungi (Ikeh et al., 2017). Over 1 million individuals are expected to die each year from infections caused by fungi, especially those linked to the species *Aspergillus*, *Candida*, and *Cryptococcus*. Although antifungal medications are available, underdiagnosed infections are difficult to eliminate, and their mortality is significant (Gonzalez et al., 2017).

Human mycoses have been categorized into three classes based on the acquisition route, i.e., exogenous, endogenous, and exo-endogeneous. The examples of all three categories are dermatomycosis, candidiasis, and cryptococcosis, respectively. Mycosis can also be divided into two groups based on location: superficial and deep invasive. The specified fungal infection locations for a superficial type are superficial, cutaneous, and mucocutaneous, and the most common infections are onychomycosis, keratomycosis, and otomycosis, which infect the nails, ears, and eyes, respectively (Ikeh et al., 2017). Fungi are localized and proliferate in profound tissues and organs (such as the pulmonary system, spleen, bones, brain, liver, and blood). These infections are challenging to identify, have a poor prognosis,

and have a complicated clinical history. *Aspergillus* and *Candida* fungi are the most frequent pathogens (Ikeh et al., 2017; Sawant & Khan, 2017; Su et al., 2018).

This chapter discusses improvements in conventional antifungal therapy and identifies new targets for the development of novel antifungal drugs. These novel targets included modifying cellular efflux, the process for ergosterol biosynthesis, molecular chaperones such as heat shock protein-90 (Hsp90), cellular structures including biofilms, the cell wall of hyphae, and mitochondria. It also included strategies such as targeting metabolic pathways and enzymes and developing antifungal vaccinations. This study aims to thoroughly examine the limitations of the antifungal medications that are already on the market and the potential for developing new antifungal medications using revolutionary, innovative methods.

2. Classification and mechanism of antifungal drugs

The six primary groups of currently accessible antifungal drugs are azoles, allylamines/thiocarbamates, morpholines, polyenes, pyrimidine analogs, and echinocandins (Campoy & Adrio, 2017). These antifungal drugs affected the biosynthetic pathway of ergosterol, the fungal cell wall, or the DNA/RNA of the fungus.

2.1 Azoles

The most prominent antifungal class is azoles (Roemer & Krysan, 2014). Azoles exhibit antifungal action by inhibiting the cytochrome P450-dependent 14-lanosterol demethylase (Cyp51), an enzyme encoded by the ERG11 gene. Obtusifolione, which has significant antifungal properties, accumulates in the ergosterol biosynthesis process owing to specifically blocking the activity of NADPH-dependent 3-ketosteroid reductase (ERG 27). ERG 27 converts the 3-ketosteroid obtusifolione to obtusifoliol in *C. neoformans* (Prasad et al., 2016). Drug efficacy had been hampered by inducing drug resistance through the increasing concentration of azoles (Poeta, 2016). The creation of novel azole substances, however, continues to be a focus of modern antifungal medication research. For the treatment of invasive aspergillosis and mucormycosis, isavuconazole was approved in 2015 (Liu et al., 2018). When compared with voriconazole, isavuconazole demonstrated highly effective and safe due to its broad spectrum of antifungal action, high water dissolved state, and safety (Flick et al., 2017). Another brand-new azole, albaconazole, was tested in clinical settings and showed strong antifungal activity (Liu et al., 2018). Squalene epoxidase (ERG1 gene) converts squalene to 2,3-squalene epoxide, was inhibited by allylamines (for example, terbinafine) and thiocarbamates (for example, tolinaftate) (Campoy & Adrio, 2017; Prasad et al., 2016). The Δ^8 - Δ^7 isomerase (ERG2) and the Δ^{14} -reductase (ERG24) that regulate the biosynthetic pathway of ergosterol were inhibited by morpholines such as amorolfine, and in this case, it showed promising antifungal activity (Campoy & Adrio, 2017). However, most of these medications have been employed to treat dermatophyte fungal infections (Prasad et al., 2016). The actinomycete-derived polyenes damage the fungal plasma membrane by complexing with ergosterol, increasing the permeability of the membrane, allowing essential cytoplasmic components to seep out, and eventually killing the fungal cell (Kathiravan et al., 2012).

2.2 Polyenes

Only three classes of polyenes were used in clinical settings, which include amphotericin B (AMB), natamycin, and nystatin (Campoy & Adrio, 2017). However, polyenes convert lanosterol to ergosterol, which is one of their main disadvantages (Prasad et al., 2016). Furthermore, considerable toxicity was also produced by itraconazole and fluconazole (FLC) (Roemer & Krysan, 2014). Lipid formulations of AMB might lessen nephrotoxicity, especially at larger dosages or after extended administration duration (Falci et al., 2015). A substantial novel polyene component called N-dimethylaminoacetylpartricin, a 2-dimethylaminoethylamide diascorbate salt (SPK-843) was discovered by scientists. It showed greater efficacy than micafungin in vivo, and SPK-843 demonstrated less renal toxicity than AMB or liposomal AMB (Espinel-Ingroff, 2009). The phase III clinical trial was conducted to check the efficacy, safety, and pharmacokinetics of SPK-843 in the prevention and management of cryptococcosis or aspergillosis infections (Mijaljica et al., 2022). The synthesized structural analogs of the nucleotide cytosine were the pyrimidine analogs 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) (Mijaljica et al., 2022). 5-FC has the ability to transform inside target cells into 5-FU, which therefore has an antifungal effect. Through its action on thymidylate synthase, 5-FU is integrated into RNA, disrupting protein biosynthesis. It also hindered DNA production and division of the nucleus (Kathiravan et al., 2012). Some *Candida* and *Cryptococcus* strains were susceptible to 5-FC. Most filamentous fungi didn't possess thymidylate synthase, which resulted in extremely limited antifungal spectrum activity (Campoy & Adrio, 2017). The antifungal properties of echinocandins, such as micafungin, caspofungin, and anidulafungin, were recently found (Prasad et al., 2016). They were cyclic hexapeptide lipopeptides with various lipid side chains that were partially synthetic amphiphilic lipopeptides. Echinocandins prevented 1,3-D-glucan synthase (GS) from producing 1,3-D-glucan, a crucial part of the fungal cell wall, by inhibiting the enzyme's catalytic subunit (McCarthy et al., 2017). However, a disadvantage of prolonged treatment was the daily intravenous administration of therapeutically available echinocandins [12]. Recent large-scale screening of compound databases derived from natural products or synthesized compounds led to the discovery of numerous novel GS inhibitors, including pyridazinone, enfumafungin, and piperazine propanol (Liu et al., 2018). Several novel antifungal agents are under development. Regarding the nature of their modes of action, these compounds were comparable with commercially available antifungal treatments, but they offered advantages over the present medications in terms of conquering resistance to antifungal agents and preventing side effects [12]. It's encouraging that some of these drugs may soon be accessible for therapeutic usage. These compounds were primarily divided into five groups: The first group is CYP51 inhibitors that were only found in fungi, such as VT-1129, VT-1161, and VT-1598, they showed promising inhibitory activity for fungal CYP51 when compared with mammalian cytochrome P450 (CYP450) enzymes (Warrilow et al., 2014, 2016). The second group is AX001, which hindered the development of glycosylphosphatidylinositol-anchored proteins by preventing inositol acyltransferase (Espinel-Ingroff, 2009; Nishikawa et al., 2016). The third group includes SCY-078 and CD101, which are inhibitors of glucan synthase, whereas CD101 had a longer half-life, i.e., greater than 80 h and SCY-078, when administrated through the oral route due to excellent absorption from the gastrointestinal system (James et al., 2017). The fourth group is F901318, a substance that inhibited fungal pyrimidine biosynthesis. It was

substantially more effective than the human counterpart against *A. fumigatus* dihydroorotate dehydrogenase (Oliver et al., 2016); the fifth group is T-2307, which causes the breakdown of the mitochondrial membrane potential in fungi, which was preferentially absorbed by fungi as opposed to mammalian cells (Nishikawa et al., 2016).

2.3 Echinocandins

A group of antifungals known as echinocandins has cyclic nonribosomal hexapeptides with a lipophilic side chain. These filamentous fungi-produced substances have been exploited to create semisynthetic derivatives, including caspofungin, micafungin, and anidulafungin, which are primary antimycotics used in treating invasive mycosis. Their mode of action relies on blocking the activity of 1,3-D-glucan synthase, which consequently prevents the production of 1,3-D-glucan, an important polysaccharide found in cell walls (Hüttel, 2021). The prevalence of *Candida* species, such as echinocandin-resistant strains, has increased. 1,3- β -D-glucan synthase 1 (FKS1) and 1,3- β -D-glucan synthase 2 (FKS2) gene mutations are linked to the resistance. The precise mechanism of resistance is a mutation in two areas of the FKS1p subunit of (Ikeh et al., 2017; Su et al., 2018) d-glucan synthase, which ultimately results in the replacement of serine 645 for proline, phenylalanine, and tyrosine and alters the target spot for echinocandins (Pristov & Ghannoum, 2019).

2.4 Amines

Amines are primarily employed to treat fungal infections topically (Zhang et al., 2007). Naftifine, butenafine, and amorolfine are some examples of amines. By attacking squalene epoxidase, allylamines prevent the formation of lipid ergosterol produced by fungi. The stereospecific epoxidation of squalene is catalyzed by squalene epoxidase, an important flavin adenine dinucleotide-dependent enzyme of the ergosterol and cholesterol biosynthesis pathways called compound 2,3-(S)-oxidosqualene (Nowosielski et al., 2011). This family of antifungals has minimum effect on mammalian cholesterol production and is selective for fungal enzymes. While terbinafine, the analog of naftifine, also has oral action, naftifine exclusively has topical activity (Birnbaum, 1990).

3. Antifungal targets and strategies

The finding of antifungal drugs has made significant development in the last few years. However, only a small portion of compounds now undergoing clinical investigations will ultimately be licensed for use in humans; it is vital to identify and investigate more antifungal compounds in the future (Fernandes et al., 2021). Our understanding of fungal cells' structural and functional elements has greatly supported the development of potent antifungal drugs to cure fungal infections. Biofilms are one of the significant virulence factors, so the thorough investigation of the structure and functioning of biofilms is crucial for developing novel antifungal drugs. Studies suggested that a variety of fungal cell structures, including the cell wall and cell membrane, as well as metabolic processes (such as the glyoxylate cycle, pyrimidine

synthesis, cytochrome P450 enzymes, iron metabolism, heme biosynthesis, and acetate metabolism), signaling pathways (such as mitogen-activated protein kinase (MAP kinase), phosphoinositide-dependent protein kinase 1 (PDK1), and calcium signaling), and gene expression, could be targeted in the development of novel antifungals (McCarthy et al., 2017).

4. Therapeutic interventions against fungal drug resistance

The prevalence of resistance to antifungal agents is currently rising. Research conducted in Bari, Italy, found that the percentage of fluconazole-resistant *C. albicans* strains obtained from their hospital increased from 2.3% in 2015 to 14.2% in 2018 (Bavaro et al., 2022). Recently, the multidrug-resistant yeast *Candida auris* spread worldwide and posed a serious global hazard (Chakrabarti & Sood, 2021). According to research, 34.8% and 23.3% of *C. auris* isolates are resistant to amphotericin B and voriconazole, whereas 79.6% of *C. auris* strains are resistant to fluconazole, respectively (Hu, 2009). The fungal infections with the highest rates of resistance to antifungal agents are usually recognized to be those belonging to the genera *Candida*, *Aspergillus*, *Cryptococcus*, and *Pneumocystis* (Arastehfar, 2020; Hu, 2009). Several factors may contribute to antimicrobial resistance in the fungus kingdom. Reduced medication effectiveness, altered drug targets, and metabolic bypasses are some potential causes of antifungal resistance (Sanglard, 2016). Some innovative compounds may unravel these pathways, which can be exploited to create novel treatment approaches based on boosting the antifungal activity of already-approved medications. Both ergosterol production pathways and fungal efflux pumps have been identified as targets in recent studies to prevent medication resistance (Holmes et al., 2016). Prospective targets for creating antifungals that can suppress resistance mechanisms include cell efflux, ergosterol production, and Hsp90. These mechanisms have been discussed under the following headings. A list of drugs that can overcome resistance has been summarized in Table 13.1.

4.1 Modulating cell efflux as an antifungal approach

Increased expression of the ATP-binding cassette transporter genes *Candida* drug resistance 1 and 2 (CDR1 and CDR2) corresponds to one of the antifungal mechanisms that result in *Candida* strains resistant to azole antifungals (De Micheli et al., 2002). By blocking the pumps and resensitizing resistant fungi to azole medications, azole resistance can be overcome. The development of novel antifungal drugs targeting fungal efflux pumps serves as the foundation for an azole-enhancing combination treatment (Holmes et al., 2016). Apigenin and apigetrin had a good impact among seven flavonoids when examined for CDR1 down-regulations (Ivanov, 2022). Astragalin also suppressed CDR1 (Ivanov et al., 2020). In fluconazole-resistant *C. albicans*, kaempferol downregulates the CDR1 and CDR2 genes (Shao et al., 2016). Polyunsaturated fatty acids can diminish fluconazole efflux from *C. krusei* cells, whereas berberine hydrochloride, a traditional Chinese drug with antibacterial properties, decreases intracellular efflux of fluconazole by downregulating CDR1 (Yong et al., 2020). In *C. Albicans*, benzoxazole derivatives may prevent the production of ergosterol and inhibit cell efflux (Staniszewska et al., 2021).

TABLE 13.1 Antifungal agents for specific fungal strains and their respective structural targets.

Fungal strain	Targeted structure or process	Antifungal agent	References
<i>C. albicans</i> ATCC10231	Metabolic pathways	AMP-17	Yang et al. (2022)
	Cell membrane	ApoB-derived peptides	Dell’Olmo et al. (2021)
	Biofilm, hyphae	NCR PeptideFragments	Szerencsés et al. (2021)
<i>Aspergillus fumigatus</i> kexb	Endo-protease	AF.KEX1	Rayens et al. (2021)
<i>C. albicans</i> LABMIC 0125	Biofilm, cell membrane	Antimicrobial peptideCtn (Arastehfar, 2020 ; Bavaro et al., 2022 ; Birnbaum, 1990 ; Chakrabarti & Sood, 2021 ; Dell’Olmo et al., 2021 ; Fernandes et al., 2021 ; Holmes et al., 2016 ; Hu, 2009 ; Hüttel, 2021 ; James et al., 2017 ; McCarthy et al., 2017 ; Nishikawa et al., 2016 ; Nowosielski et al., 2011 ; Oliver et al., 2016 ; Pristov & Ghannoum, 2019 ; Sanglard, 2016 ; Warrilow et al., 2014, 2016 ; Yang et al., 2022 ; Zhang et al., 2007)	Aguiar et al. (2020)
<i>C. albicans</i> (ATCC90028)	Biofilm, cell membrane	Antimicrobial peptideCtn (Arastehfar, 2020 ; Bavaro et al., 2022 ; Birnbaum, 1990 ; Chakrabarti & Sood, 2021 ; Dell’Olmo et al., 2021 ; Fernandes et al., 2021 ; Holmes et al., 2016 ; Hu, 2009 ; Hüttel, 2021 ; James et al., 2017 ; McCarthy et al., 2017 ; Nishikawa et al., 2016 ; Nowosielski et al., 2011 ; Oliver et al., 2016 ; Pristov & Ghannoum, 2019 ; Sanglard, 2016 ; Warrilow et al., 2014, 2016 ; Yang et al., 2022 ; Zhang et al., 2007)	Aguiar et al. (2020)
	Production of phospholipase and exopolysaccharide	Morin	Abirami et al. (2020)
	EPS production	Usnic acid	Nithyanand et al. (2015)
<i>C. albicans</i> (475/15)	Biofilm, Hyphal growth, and CDR1	Apigenin, Apigetrin, Luteolin, Quercetin, Quercitrin, Isoquercitrin, Rutin	Ivanov (2022)
	Cell membrane, Biofilm, and EPS,	<i>Artemisia absinthium</i> extract	Ivanov et al. (2021)
	Hyphal growth and structural integrity of cell membrane	Astragalin	Ivanov et al. (2020)

TABLE 13.1 Antifungal agents for specific fungal strains and their respective structural targets.—cont'd

Fungal strain	Targeted structure or process	Antifungal agent	References
<i>A. niger</i> N402	Hyphal growth and biofilm	CamphorEucalyptol	Ivanov (2021)
	Biofilm, EPS production, and mitochondria	Rosmarinic acid	Ivanov (2020)
	Cell membrane and metabolic activity	ApoB-derived peptides	Dell'Olmo et al. (2021)
<i>C. neoformans</i> H99	Capsule formation	Aqueous seed extract from Allamandapolyantha	Bresciani (2020)
	Biosynthesis of Capsule and biofilm and melanin production	Eltrombopag	Ko et al. (2020)
	Mitochondrial and vacuolar function	Mefloquinederivatives	Montoya et al. (2020)
<i>Saccharomyces cerevisiae</i> 24657	Blocking of respiratory chain complexes	Arylamidine T-2307	Yamashita et al. (2019)
<i>C. albicans</i> (SC5314)	Ergosterol, cell efflux	Benzoxazole derivatives	Staniszewska et al. (2021)
	Biofilm viability	Blue light	Bapat and Nobile (2021)
	Mitochondria	Chiloscyphenol A	Zheng et al. (2018)
	Hyphal growth, biofilm, and phospholipase production	Dioscin	Rajasekar (2021)
	Biofilm and hyphae	Curcumin sophoro lipid nano complex	Yang et al. (2018)
	F-box protein Fbp1, ligase subunit	Heat-killed fbp1D cells	Wang et al. (2019)
	Functions of Mitochondria and Vacuole	Mefloquinederivatives	Montoya et al. (2020)
	Isocitrate lyase in glyoxylate pathway	Mohangamide A	Bae et al. (2015)
	Glyoxylate cycle blocker	Monoterpenoidperillyl alcohol	Ansari et al. (2018)
	Multivalent	mvPC	Tarang et al. (2020)
	Fructose-1,6-bisphosphatealdolase	Phenylhydrazones	Han et al. (2017)
	Hyphal growth, biofilm formation	Shikonin	Yan et al. (2019)
	Biofilm and hyphae	Sophorolipids	Haque et al. (2016)

(Continued)

TABLE 13.1 Antifungal agents for specific fungal strains and their respective structural targets.—cont'd

Fungal strain	Targeted structure or process	Antifungal agent	References
<i>A. fumigatus</i> R21	F-box protein Fbp1, ligase subunit	Heat-killed fbp1D cells	Wang et al. (2019)
<i>C. tropicalis</i> CBS 94	Biofilm and hyphae	NCR PeptideFragments	Szerencsés et al. (2021)
<i>C. auris</i> strains(CAU-01, CAU-03,CAU-05, CAU-07,and CAU-09)	Adhesins	NDV-3A	Singh et al. (2019)
<i>C. tropicalis</i> (ATCC 750)	Secreted aspartyl protease	Recombinant Sap2	Shukla and Rohatgi (2020)
<i>Candida glabrata</i>	Carbonic anhydrase	Thiazolidinones	Güzel-Akdemir et al. (2020)
<i>Sporothrix globosa</i>	Mitochondrial function	ToAP2D Peptide	Yan et al. (2021)
<i>A. fumigatus</i> (Af 239)	Ergosterol production	α -bisabolol	Jahanshiri et al. (2017)

4.2 Targeting ergosterol biosynthesis and ERG11 expression

Ergosterol is the most prevalent sterol in yeast. Sterols integrate membrane heterogeneity, block water absorption, and maintain the structural integrity, stiffness, and fluidity of the plasma membrane, all of which are crucial for the preservation of fungal cells. Ergosterol biosynthesis pathway is shown in Fig. 13.1 Azole medications that target lanosterol 14-demethylase are now available on the pharmaceutical market. Treatments for ergosterol biosynthesis include allylamines, which are squalene synthase (ERG1p) inhibitors, and polyene medicines, which alter the distribution of ergosterols on the membrane (Lv et al., 2016). The ergosterol production pathway is also implicated in the resistance and fungal cell survival and function can be targeted. Increased copy number of chromosome 5 (where ERG11 is located) or mutations that confer gain-of-function in the transcription promoter of Sterol Uptake Control Protein 2 (UPC2) can result in the overexpression of ERG11 transcripts, which reduces azole sensitivity. Azole-resistant clinical fungal strains frequently have ERG11 mutation (Flowers et al., 2015). The expression of ERG11 was downregulated by apigenin, rutin, and palmitic acid (Prasath et al., 2020). Other investigations used tormenting acid (Bvumbi et al., 2021), benzo dioxane compounds (An et al., 2022), honokiol and magnolol (Trifan et al., 2021), cold atmospheric plasma (Ebrahimi-Shaghaghi et al., 2021), and benzo dioxane derivatives to target ergosterol production.

4.3 The potential of Hsp90 as a therapeutic target in fungal diseases

In eukaryotes, Hsp90 functions as a molecular chaperone to control cellular signaling. Although targeting Hsp90 is seen as a potentially effective antifungal mechanism, it is also

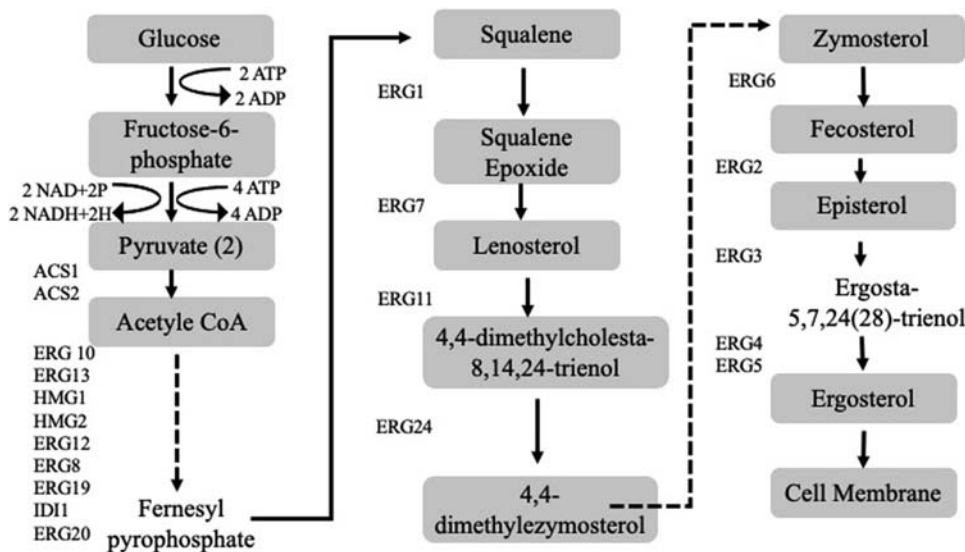


FIGURE 13.1 Ergosterol biosynthesis pathway; *solid lines* in figure show one-step catalysis of reaction, whereas *dotted lines* show the involvement of various catalytic steps.

implicated in drug resistance of both fungal biofilms and cells under the circumstances of planktonic growth (Robbins et al., 2011). The most encouraging action was found for ganetespib when four commercial Hsp90 inhibitors—AUY922, ganetespib, PU-H71, and CH5138303—were tested for their ability to suppress the growth of fungi (Yuan et al., 2021). Though, this substance failed to exhibit antifungal characteristics in the microdilution experiment (MIC >64 g/mL) and exhibited synergistic effects when paired with fluconazole, *in vivo*. When coupled with fluconazole, it lowers cell efflux and suppresses the expression of the azole resistance-related genes CDR1, CDR2, ERG11, and MDR1 (Yuan et al., 2021). By impairing Hsp90 action and cell wall integrity in fungal infections, the sesquiterpene quinone puupehenone amplifies the effects of caspofungin. The Hsp90 inhibition is also the basis for vorinostat and azoles' synergistic actions against *Aspergillus* species (Tu et al., 2020).

5. A multifaceted approach to tackle fungal virulence

5.1 Targeting biofilm and hyphae

Targeting the hyphae and biofilm communities of fungi that are entrenched in the extracellular matrix is one of the novel methods for the development of antifungal drugs. The removal of biofilms is a difficult task since fungal infections can develop these structures to avoid being destroyed by immune cells such as neutrophils and monocytes (Kernien et al., 2018). Fungi use a variety of mechanisms to enhance biofilm resistance, including high cell density in the matrix, nutrient, and growth restriction, the presence of persistent cells, the expression of antifungal resistance genes, and a spike in the number of sterols on

the fungal cell membrane (Silva, 2017). The extracellular matrix is made up of proteins, carbohydrates, lipids, and extracellular DNA, which contributes to its increased biofilm resilience (Pierce, 2017). Numerous pathogenic fungi can change their shape, a characteristic that is frequently closely related to virulence. Numerous diseases often and successfully live in mammalian animals by changing their morphology. The main fungal diseases may develop in yeast, pseudo-hyphae, and hyphae morphologies, among others. Many human fungal infections are controlled by the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) route and the molecular chaperone Hsp90 routes (Li & Nielsen, 2017).

The capacity of the most common human fungal pathogen, *C. Albicans*, is the transformation between yeast forms, pseudo-hyphae and hyphae during infection and illness, which makes it unique. The hyphal form serves as the biofilms' framework and is associated with the fungal capacity to cause illnesses (Bar-Yosef et al., 2017). A variety of compounds, including those derived from natural sources, have been investigated as a potential source for development of antifungal drugs because of their antibiofilm properties (Smiljković et al., 2019). Recent research has highlighted possible biofilm inhibitory agents among a variety of diverse products as shown in Table 13.1.

It is necessary to create new, stronger antifungals that target morphological changeover since many pathogenic fungi's dimorphic traits may be crucial to their pathogenesis and virulence (Table 13.1). By slightly reducing the amount of exopolysaccharide (EPS) in the matrix of biofilms, the plant polyphenol Rosmarinus acid can inhibit the growth of biofilms (Ivanov, 2020). Similarly, *C. Albicans* produces less EPS when exposed to a nonpsychoactive Phyto cannabinoid generated by Cannabis sativa, and usnic acid, a lichen secondary metabolite (Nithyanand et al., 2015). An extract from the plant *Artemisia absinthium* exhibited antibiofilm properties as confirmed by crystal violet assay. A decrease in EPS synthesis may have contributed to this antibiofilm activity. According to studies for crystal violet absorption and nucleotide leakage, the disruption of the membrane's functionality is the mechanism through which absinthium exerts its antimicrobial action (Ivanov et al., 2021). Similarly, *Ononis spinosa* extract showed antibiofilm activity against three different strains of *Candida*, through interference with membrane integrity. Antifungal action was dependent on damage to the cell wall, the antimicrobial peptide VLL-28, derived from an archaeal transcription factor can inhibit fungal capacity to build biofilms (Roschetto et al., 2018). When combined with photosensitizing substances, photodynamic treatment can effectively disrupt the biofilms produced by *C. auris* (Bapat & Nobile, 2021). The phytochemical nanocomplex of curcumin and sophorolipid affects *C. albicans* biofilm and hyphae involving the downregulation of genes related to virulence and resistance such as hyphal regulatory genes SAP4, HWP1, and HYR1, as well as RAS1 and ERG11 (Rajasekar, 2021). Catechol prevents *C. albicans* from forming biofilms and hyphae. It also prevented virulence factors from secreting hydrolases and decreased EPS levels in the biofilm matrix. Catechol treatment results in a considerable downregulation of the RAS1, HWP1, and ALS3 genes, which are involved in the pathogenesis of fungi (Jothi et al., 2021). The biological significance of several flavonoids, including quercetin and its glycosylated derivatives (quercitrin, isoquercitrin, and rutin), flavone aglycones (luteolin and apigenin), flavone glycosylated derivatives (apigenin), and flavone aglycones (luteolin and apigenin), was investigated to examine the morphological shift between the growth of yeast and hyphae along with the potent antibiofilm activity (Ivanov, 2022). The flavonoids showed modest efficacy in terms of suppressing fungal hyphal

development, with apigenin and apigenitrin having the strongest effects. Isoquercitrin was shown to have the most antibiofilm efficacy at minimum inhibitory concentrations (MICs) (76% inhibition), whereas apigenin and isoquercitrin treated in MIC/2 could both suppress the growth of biofilm by more than 60% (Ivanov, 2022). According to several research findings on antihyphal potential, quercetin is more effective at concentrations up to 200 g/mL (Singh et al., 2015) and 64 g/mL (Gao et al., 2016), whereas concentrations of 75 g/mL only slightly inhibited growth (Ivanov, 2022). In another investigation, astragalin at 0.075 mg/mL caused a slight decrease in the number of hyphal cells after 4 h of treatment (Ivanov et al., 2020). Morin was able to obstruct the development of biofilms, phospholipase, and exopolysaccharides in *C. albicans* (Abirami et al., 2020). Like how dioscin does, it exhibits a variety of antifungal actions, including the reduction of biofilm growth and formation, adhesion, and extracellular secreted phospholipase (Yang et al., 2018).

Camphor (0.125 mg/mL) and eucalyptol (23 mg/mL) were tested for their antihyphal efficacy against a clinical isolate of *C. albicans* (Ivanov, 2021). Both substances have antibiofilm capabilities as well as the ability to significantly reduce the number of hyphal cells and impede hyphal transformation. Another research found that 0.01% camphor completely inhibited *C. albicans*' hyphal growth and decreased the level of expression of the hypha-specific gene ECE1 (extent of cell elongation) (Manoharan et al., 2017). A phenolic substance called biatriosporin D was discovered in an endolichenic fungus called *Biatrispora* sp. It hinders the development of biofilm, hyphal adhesion, and other virulence factors in *C. albicans*. The antihyphal potential was highlighted because it can be attained at considerably lower doses of Biatriosporin D than its MIC value.

The in vivo *C. elegans*, *C. albicans* infection model validated the compounds' promising antifungal activity. Biatriosporin D controls the Ras1-cAMP-Efg1 pathway to prevent hyphal development by using antihyphal mechanisms (Zhang & et al., 2017). Emodin (1,3,8-trihydroxy-6-methyl anthraquinone), a natural substance derived from *Rheum palmatum* rhizomes (Janeczko et al., 2017), has also been found to decrease fungal hyphal development in *C. albicans*. Shikonin, a crimson pigment obtained from *Lithospermum erythrorhizon* (Yan et al., 2019), and sophorolipids, glycolipid biosurfactants derived from *Strmerella bombicola* (Haque et al., 2016), both have the antihyphal capability. Haque et al. stated that the interference with the expression of the hypha-specific genes Hyphal wall protein 1 (HWP1), Actin-like protein 1 (ALS1), Actin-like protein 3 (ALS3), Extracellular matrix protein 1 (ECE1), and Secretory pathway protein 4 (SAP4) accounts for the inhibitory impact of sophorolipids on hyphal development (Haque et al., 2016). Eucarobustol E was discovered to inhibit genes associated with ergosterol production and to have antifungal action against *C. albicans*. This substance lowers the hydrophobicity of the cellular surfaces of the biofilm cells and prevents the yeast-to-hypha transition (Liu et al., 2017). The antimicrobial peptide C-terminal fragment of crotalidicin (Ctn) derived from the rattlesnake *Crotalus durissus terrificus* venom has shown antifungal efficacy toward *C. albicans*. The application of the peptide was effective against planktonic or biofilm growths of both the reference strain and fluconazole-resistant *C. albicans*. A variety of experiments were used to study membrane rupture and contact underlying the antifungal mechanism of peptides (Aguiar et al., 2020). Other peptide derivatives, such as the cysteine-rich (NCR) peptides NCR335 and NCR169 variants, might inhibit the growth of hyphae on the surface of the studied *Candida* strains (Szerencsés et al., 2021).

Antimicrobial peptides offer a wide range of potential uses in both human and veterinary medicine, particularly as antifungals (Bruni et al., 2016). Comparing *C. neoformans*' morphogenesis to other *Candida* species, it differs from other human fungal pathogens. These fungi induce a considerable increase in cell size to alter the shape in vivo. These cells can reach a size of 40–50 microns in vivo, compared with 4–6 microns in vitro. The immune system is unable to remove these “titan cells” because of their huge size as their structure includes capsule as well (Zaragoza, 2019). The antifungal efficacy of *Xylosma prockia* leaf ethanolic extract and its fractions was initially tested against *Candida neoformans* and *Candida gattai* (Folly et al., 2020). According to morphometric analysis, ethyl acetate fraction causes lower surface-to-volume ratios in *C. gattii* and *C. neoformans* and decreased ergosterol levels in the fungal cell membrane. The inhibitory impact of *Bacillus safensis* effectively prevented the production of fungal capsules and numerous other crucial *C. neoformans* virulence factors such as a reduction in total cell size (Mayer & Kronstad, 2017). Since it decreases the thickness of the capsule in *C. neoformans*, Cyperone, derived from the rhizome of *Cyperus rotundus*, may be utilized for tampering with *Cryptococcus* morphology (Horn & VEDIYAPPAN, 2021).

The thrombopoietin receptor agonist eltrombopag was modified to function as an anti-*Cryptococcus* agent. Since it inhibits cryptococcal virulence factors such as capsule and bio-film development, melanin synthesis, and growth capacity at 37 C, this medication may be used as an antivirulence agent. Its methods of action include the modification of transporter genes, lipid biosynthesis genes, and genes implicated in the calcineurin pathway (Janeczko et al., 2017). Additionally, Allamanda polyantha aqueous seed extract may affect *Cryptococcus* morphology by preventing capsule formation and reducing cell size (Bresciani, 2020).

5.2 Mitochondria-targeted approaches to combat fungal infections

Pathogenic fungi depend on their mitochondria for cell development and long-term survival, proposed as possible targets for the creation of new antifungal drugs (Li & Calderone, 2017). The activity of *C. albicans* mitochondria has been markedly decreased by rosmarinic acid treatment. Rosmarinic acid (0.1 mg/mL) decreased mitochondrial activity by more than 50% as confirmed by cell viability assay. Berberine, a natural substance, builds up in fungal mitochondria and hinders the function of Complex I and the membrane around the mitochondria. Additionally, this substance can take over the overexpressed Mdr1p and decrease *C. albicans*' susceptibility to antifungal medications. The use of berberine was also shown to be effective in vivo since it increased the duration of survival of mice with blood-borne spread of multidrug-resistant candidiasis caused by overexpression of Mdr1p (Tong et al., 2021). By using a variety of methods, such as the buildup of reactive oxygen species and the collapse of mitochondrial membrane potential, papaya, *Carica papaya*, and seed extract demonstrated inhibitory efficacy toward *C. albicans* (Zhang & Chen, 2017). The natural small-molecule chiloscypenol A, which was isolated from Chinese liverworts, has antifungal properties as evidenced by its fungicide properties in both mature *C. albicans* biofilms and at minimal inhibitory concentrations of 8–32 g/mL. Chiloscypenol *Caenorhabditis elegans* was tested in vivo for a fungicidal activity. This substance results in increased adenosine triphosphate (ATP) generation, intracellular reactive oxygen species (ROS) buildup, aggregated distribution of Tom70, hyperpolarization of the mitochondrial membrane potential, and it also

affected the integrity of cell membranes. All these results were validated by different characterization techniques such as confocal microscopy, transmission electron microscopy (TEM), calcein-leakage tests, and flow cytometry (Zheng et al., 2018). To achieve its antifungal action, Kalopanaxsaponin A, a triterpenoid saponin from the stem bark of *Kalopanax pictus*, increases the formation of ROS. Repetitive ROS interferes with respiratory chains and consequently causes mitochondrial malfunction.

The disruption through permeability of the cell membrane is another mechanism by which kalopanaxsaponin A exerts its fungicidal effect (Ying Li et al., 2020). In subinhibitory doses, mefloquine derivatives prevent the expression of fungal virulence-related genes such as *C. albicans* filamentation and *C. neoformans* capsule formation/melanization. These derivatives' mode of action is based on the disruption of both mitochondrial and vacuolar activities (Montoya et al., 2020). Arylamidine T-2307 can specifically interfere with mitochondrial function in yeast through the suppression of respiratory chain complexes. This substance inhibits respiratory chain complexes III and IV in *Saccharomyces cerevisiae* and *Candida albicans*, which causes yeast cells to produce less intracellular ATP. T-2307 has a limited impact and is a selective inhibitor of bovine respiratory chain complexes. Peptide *Sporothrix globosa* was susceptible to a shorter peptide's (ToAP2D) antifungal activity, which resulted in *Sporothrix globosa* growth inhibition, increased apoptosis, and fungal cell deformation and rupture. Treatment with ToAP2D raised the amounts of ROS and decreased the levels of mitochondrial membrane potential (Yan et al., 2021).

5.3 Targeting metabolic pathways and enzymes

Innovative antifungal approaches might be developed by focusing on several metabolic pathways. These pathways include trehalose metabolism, lipid biosynthesis, N-acetylglucosamine metabolism, and other critical fungi cell functions (Wijnants et al., 2022). As they are essential for fungal growth and pathogenicity, many enzymes found in fungal cells are also among the possible targets for therapeutic development. A variety of compounds have been investigated as metabolic pathways and/or fungal enzyme inhibitors, a list of these inhibitors and pathways are summarized in Table 13.1. RNA degradation, propionate metabolism, and fatty acid metabolism are only a few of the critical metabolic pathways that the antimicrobial peptide AMP-17 disrupts in *C. albicans* (Yang et al., 2022). Peptides produced from apolipoprotein B (ApoB) also exhibited antifungal activity. The peptides have disrupted the structural integrity of the *C. albicans* cell membrane and delayed the metabolic process of *Aspergillus niger* spores (Dell'Olmo et al., 2021). Enzyme class II fructose-1,6-bisphosphate aldolase (FBA-II) is absent in mammals and higher plants but may offer selectivity. Novel FBA-II inhibitors were tested against *C. Albicans*, with phenylhydrazones to confirm the inhibitory potential. The experiments used to examine enzyme inhibition revealed that these compounds had promising action with an IC₅₀ value of less than 10 μ M (Han et al., 2017).

Thiazolidinones could hinder the activity of carbonic anhydrase enzyme (KI values 0.1–10 μ M against the *Candida glabrata* enzyme) as confirmed by molecular dynamics, enzyme inhibition tests, and molecular docking (Güzel-Akdemir et al., 2020). Vanillin and monoterpenoid perillyl alcohol were tested for their potential to block the glyoxylate cycle against

C. albicans. α -bisabolol inhibits the activity of fungus 24-sterol methyltransferase. At the maximum dosage of 9 mM, it decreases the expression of ERG6 by 76.14% and lowers ergosterol production (26.31%–73.77%) in a dose-dependent manner (Jahanshiri et al., 2017). Homoserine dehydrogenase blocker is known as (S)-2-amino-4-oxo-5-hydroxypentanoic acid. It was discovered that it functions as an enzyme-assisted suicide inhibitor of Hom6p after being isolated from a *Streptomyces* species. It was found that RI-331 had activities against *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*, but had no impact on *Aspergillus* species (Kuplińska & Rząd, 2021).

5.4 Targeting fungal cell wall to combat fungal infections

In comparison to human cells, the fungal cell wall has a distinct structure, making it a desirable target for the development of antifungal medications (Cortés et al., 2019). Although the composition of the fungal cell wall varies depending on the species, the fundamental scaffold structure is formed by the polysaccharide (Ikeh et al., 2017; Su et al., 2018) glucan that is covalently bonded to chitin (Curto et al., 2021). Chitin synthase enzymes CHS1–CHS3 in *S. cerevisiae* and CHS8 in *C. albicans* produce chitin, and their corresponding genes are CHS1, CHS2, CHS3, and CHS8, respectively. Enzymes for chitin production are generated in the cytoplasm and subsequently transferred to the cell membrane (Schmidt et al., 2003). Compared with the development of medications that target α 1,3-glucan production, cell wall mannoproteins have a smaller impact as prospective antifungal medicines. Glycosylphosphatidylinositol (GPI)-modified proteins, such as glycosylhydrolase, glycosyltransferase, or transglycosidase activity, are important elements of the cell wall mannoproteins. These enzymes are involved in the production of cell walls and are thus appealing antifungal targets since blocking their biosynthesis and localization to the cell wall. It might have a negative impact on virulence and cell wall biosynthesis (Ibe et al., 2021).

Nikkomycin Z prevents the chitin structure from forming, a step that is crucial for fungi but not for humans (Larwood, 2020). The most current study provided an overview of natural products that aim to produce (1,3)-D-glucan and chitin, two components of the fungal cell wall. The glycolipids, acidic terpenoids, and cyclic lipopeptides are (1,3)-D-glucan synthases inhibitors (Curto et al., 2021). Commercially available echinocandins have been shown to inhibit (1,3)-D-glucan synthases, and research on new inhibitors is still ongoing today. As a first-in-class antifungal triterpenoid one of the well-known examples is ibrexafungerp (formerly SCY-078 or MK-3118), it prevents the formation of α 1,3-D-glucan in the cell wall of fungi. There is little cross-resistance between echinocandin- and ibrexafungerp persistent strains because the target locations for ibrexafungerp and echinocandin on the α 1,3-D-glucan synthase are different (Jallow & Govender, 2021; Shahzad et al., 2020). The 3-substituted amino-4-hydroxycoumarin derivatives were shown to be able to lower the amount of chitin by Ge et al. (2016). Antifungal medicines (a pyridine 2-amine-based molecule (APX001A), phenoxyacetanilide (gepinacin), OGT2468 (derivatives of rhodamine-3-acetic acid), and M720 may suppress the formation of GPI anchors. They are effective against *Fusarium*, *Aspergillus*, *Candida*, and *Scedosporium* species at low concentrations. *C. albicans* and *C. auris*, which are caspofungin-resistant, can be successfully treated with APX001A (Ibe et al., 2021). Alpha-glucosidase, an enzyme necessary for maintaining a healthy cell wall

and the pathogenicity of *Candida albicans*, was inhibited by magnoflorine (50 g/mL) to more than 50% extent (Kim et al., 2018). This mechanism also exhibited antibiofilm potential.

6. Antifungal vaccines to combat fungal infections

Preventive vaccination is a long-term approach used to lessen the frequency of Candida-related infections. It might be one of the treatments for individuals who struggle with a high risk of candidiasis. The vast variety and flexibility of fungi, the existence of immunological tolerance, as well as the difficulties in progressing protective memory responses in patients with compromised adaptive immunity, provide hurdles for the development of selective vaccines (Tso et al., 2018). Studies conducted in vivo on mice suggest that vaccinations are effective in protecting against as species of *Candida*, *Cryptococcus*, and *Aspergillus*: vaccines composed of live-attenuated and dead fungi, raw extracts, recombinant subunit compositions, as well as vaccines containing nucleic acid (Oliveira et al., 2021). The in silico work investigated the Candida proteome and determined the most immunodominant Human leukocyte antigen class I (HLA class I), Human leukocyte antigen class II (HLA class II), and B cell epitopes using an immunotherapeutic peptide vaccine-based method. To boost immunogenicity, scientists have chosen the most promising epitopes and produced a multivalent recombinant protein against *C. albicans* (mvPC) with an additional synthetic adjuvant (RS09). Given that currently available annotated reference sequences of 22 *C. albicans* strains are homologous to the chosen mvPC epitopes, it is possible to obtain better coverage and a stronger protective response. By identifying multiple epitopes, the multivalent character of mvPCs may offer improved defense against complicated Candida antigens (Alshwyeh et al., 2022; Tarang et al., 2020). Bioinformatics has been used in other studies to find *C. Auris* adhesin proteins that have sequence and structural similarities.

After receiving the NDV-3A vaccination, mice developed anti-Als3p antibodies that recognized *C. auris* in vitro, disrupted the fungus' capacity to form biofilms, and improved the destruction of the fungus by macrophages. This vaccine is based on the N-terminus of the Als3 protein and is made with alum. Similarly, NDV-3A vaccination significantly increased immune responses against fungi and shielded immunosuppressed mice against *C. auris* infection (Singh et al., 2019). Recombinant secretory aspartyl protease (rSap2) from *Candida parapsilosis* was used to immunize mice, increases survival after systemic *C. Tropicalis* infection. This vaccination produces greater titers of Sap2-induced antibodies that help fight systemic candidiasis, as well as humoral and cellular immunity (Shukla & Rohatgi, 2020). *Aspergillus Fumigatus* vaccine has also been explored. A single vaccination with the AF.KEX1 vaccine and the Titermax squalene: water adjuvant can produce enough AF.KEX1 antibodies. It was shown that the fungal load was significantly decreased in the lungs of mice given the AF.KEX1 vaccine related, and levels of protection were found to be between 75% and 100% protection (Rayens et al., 2021). The F-box protein Fbp1, a subunit of the SCF(Fbp1) E3 ligase, which controls host-Cryptococcus interactions and promotes fungal virulence, served as the foundation for the development of the Cryptococcus vaccine. It is suggested that heat-killed fbp1 yeast cells may increase immunogenicity, protect against future infection with the virulent parental strain. Heat-killed fbp1 cells also gave considerable cross-

protection against a variety of invasive fungal infections, including *C. neoformans*, *C. gattii*, and *A. fumigatus*, as well as partial immunity against *C. albicans*, for mice lacking CD4⁺ T cells.

7. Novel antifungal therapeutics

There are several in vivo models for studying fungal infection and consequently, anti-fungal medicines accessible today. *Caenorhabditis elegans* serves as one of the in vivo models. It is fully sequenced genome, availability of genetic mutations, ease of laboratory upkeep, absence of ethical constraints, and potential for liquid tests for high-throughput antifungal screening are some of its benefits. Conversely, the drawbacks include its inability to withstand temperatures of 37 C and the lack of an adaptive immunological response (Madende et al., 2020).

Toll-deficient flies may be used to examine the pathophysiology and therapeutic action of the developing pathogen *C. auris*. *Drosophila melanogaster* and mice were also employed as a fungal infection model for the evaluation of antifungal drugs that target biofilms (Hassan et al., 2022; Xin et al., 2019). According to a recent study, there are new antifungal medicines under clinical development. The innovative candidates focus on several structures, including cell membranes, glucan production, and cell wall (Rauseo et al., 2020). Fosmanogepix, one of the unique options, has just been reviewed (Shaw & Ibrahim, 2020). This medicine, is the N-phosphonoxyethylene prodrug of Manogepix, which was investigated for the treatment of invasive fungal infections caused by Candida, Aspergillus, and uncommon molds. It works by inhibiting GPI-anchored wall transfer protein 1 (Gwt1). Manogepix is effective against resistant strains such as echinocandin-resistant Candida and azole-resistant Aspergillus. A variety of models, including those for disseminated infection in mice and rabbits, have demonstrated the in vivo effectiveness of fosmanogepix (Nadeem, n.d.; Shaw & Ibrahim, 2020).

Olorofim is another agent being researched right now. This drug is being tested in clinical studies for the treatment of invasive mold infections and belongs to the orotomide class of antifungals. Dihydroorotate dehydrogenase, a crucial enzyme in the production of pyrimidines, is the target of olorofim. It is effective against a variety of fungi, including those that are amphotericin B and azole-resistant; however, it does not affect yeasts or the Mucorales (Wiederhold, 2020). Considering the number of clinical trials that are now being done, there is still a need for the development of new antifungals by using innovative methods to combat resistant fungal strains.

8. Conclusions and future perspectives

The increasing prevalence of fungi infections across the world is a serious threat to the pharmaceutical business and a significant concern for medical professionals. Fungal infectious agents have developed immunity against the mode of action of currently available antifungal drugs. One of the main issues and the driving force behind the advancement of research into

the development of antifungal drugs is antifungal resistance. We are aware of the variety of adverse effects brought on by various antifungal medications in addition to the growing futility of the present antifungal arsenal. All the aforementioned points emphasized the investigation of novel antifungal mechanisms for the development of new medications. A variety of pharmaceuticals, both natural and synthetic compounds, have been screened to prevent fungal growth and subsequent infection progression. The antifungal mechanisms can be linked to several elements such as the development of fungi, their metabolic processes, or pathogenicity through interfering with their metabolic processes by impairing their mitochondrial function or their cell wall, harmful fungal strains' growth can be reduced. The interference with fungal efflux pumps, ergosterol production, or Hsp90 protein may be one of the antifungal effects reducing the fungal capacity to build resistance to antimicrobial therapy. Antifungal approaches could also be modified by targeting the complicated structure of biofilms. Antibiofilm factors also relate to the thickness of the extracellular matrix and its constituents, such as EPS, as well as the capacity of yeast cells to produce hyphal structures, in addition to their effects on biofilm biomass and the survivability of fungal cells. Similarly, the influence on the morphology of other fungal diseases might be exploited in addition to the production of yeast hyphae. Numerous compounds have been attempted, and all these features have been well explored. As the new antifungal research moves forward, more investigations using in vivo infection models and subsequent clinical trials are required. Nevertheless, several items all around us show promise in fending against the growing threat posed by fungi.

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Introduction to genomics-based industrial applications

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1. History of genomics in biotechnology

Genomics has witnessed many breakthroughs since discovering the DNA and its double helix structure. The recent monumental advances in next generation sequencing technologies resulted in a rapid decrease in sequencing costs. This has enabled the sequencing of the entire genomes of organisms ranging from prokaryotes to eukaryotes. The massive amount of generated genomic data coupled with the availability of enormous computing power have facilitated the development of state of the art bioinformatics tools for analyzing and interpreting large genomic data. Subsequently, these developments are paving the way for discoveries and numerous genomics applications in industries ranging from medicine, agriculture, the environment, and more. In the upcoming years, it is anticipated that the impact of genomics in the industry will continue to expand.

Genomics, as a disciple, is becoming a prominent player in biotechnology, where many novel insights in genomics were driven by innovative biotechnology approaches for studying and analyzing biological systems. In principle, biotechnology can be formally defined as “the integration of natural sciences and engineering sciences to achieve the application of organisms, cells, parts thereof, and molecular analogs for products and services” (*Biotechnology*. IUPAC Goldbook, 2014). The term *biotechnology* was first coined in 1919 by Károly Ereky to use living organisms, such as bacteria or yeast, to transform raw resources into products (Ereky, 1919). Biotechnology can be used to describe diverse techniques for modifying living organisms that date back to ancient history. For instance, animal domestication and plant cultivation, while adopting selective breeding for improving livestock and crops, can be regarded as an example of a biotechnology process. A modern definition of biotechnology

refers to utilizing biological processes, organisms, or systems to create products to improve human lives (Bhatia, 2018, p. 1).

Discoveries in genomics and microbiology in the past century have provided valuable insights for the developments of biotechnology applications. During World War I, there was a great demand for acetone production in the UK, to manufacture explosives. In 1917, Chaim Weizmann designed a novel industrial process using a pure microbiological culture of *Clostridium acetobutylicum* to convert corn starch, a readily available product, into acetone in industrial production (Springham et al., 1999). A few years later, in 1928, Alexander Fleming discovered the fungus *Penicillium*, which led to the purification and development of the first antibiotic drug, penicillin, by Howard Florey, Ernst Boris Chain, and Norman Heatley. By 1940, penicillin became accessible for medicinal use to treat human bacterial infections (Thieman & Palladino, 2008). It was not until 1971 when Paul Berg conducted his successful experiment on gene splicing, creating the first man-made recombinant DNA. This innovative and pioneering technology led to Berg winning the Noble Prize in Chemistry in 1980. The technology enabled researchers to extract genetic material from an organism and introduce it artificially into the genome of another organism, where the alien genetic material will be replicated and expressed. Boyer and Cohen were some of the first to utilize this technology in 1972 by introducing genetic material into a bacterium, resulting in the successful transfer and reproduction of the genetic material in the host bacterium. Berg is regarded as the father of genetic engineering, and through his gene-splicing technology, modern biotechnology started to flourish. In 1980, the US Supreme Court decided that a genetically altered microorganism might be copyrighted in response to the case of *Diamond v. Chakrabarty*. This decision was monumental in increasing business value and the commercial feasibility of a biotechnology business model (United States Supreme Court, 1980, pp. 79–139). In his case, and while working for General Electric, Ananda Chakrabarty synthesized a bacterium belonging to the genus *Pseudomonas* with the ability to break down crude oil. This presented a great opportunity for using genetically engineered microorganisms to solve one of the major environmental challenges in disaster discovery by using these microorganisms to clean up oil spills. It is worth mentioning that Chakrabarty's approach involved moving the whole organelle between *Pseudomonas* bacterial strains rather than manipulating specific genes.

In the context of genomics, genetic engineering is one of the main techniques used in modern biotechnology. Genetic engineering alters an organism's genetic makeup to produce desired products. This can be achieved by inserting genes from one organism into another, developing new traits, or changing existing ones. Cell and tissue culture is another technique widely used in biotechnology where researchers can grow cells and tissues in the lab for various biomedical studies and applications. For example, tissue culture can be used in fermentation, an ancient method to produce food such as cheese, pickles, and alcoholic beverages. On a large scale, industrial fermentation refers to utilizing microorganisms to manufacture products, including chemicals, enzymes, and proteins. In recent decades, genetic engineering techniques have been used with tissue culture to modify the genetic makeup of microorganisms to decrease manufacturing costs and increase yield.

2. The colors of biotechnology

The industrial applications of genomics in biotechnology are diverse. Some examples include developing pharmaceutical drugs, genetically modified (GM) drought and salinity-resistant crops, innovative biomaterials to fill cracks in cement, or producing cost-effective color dyes for food or clothes. Another important application is to address various environmental concerns, such as producing biodegradable plastic and microorganisms that can degrade nondegradable plastic or clean up oil or chemical contamination sites. Due to the vast applications of biotechnology nowadays, different domains of applications have been assigned colors for classification. Blue biotechnology explores marine resources for various industrial applications, such as producing bio-oils with photosynthetic microalgae (Biotech, 2009, pp. 26–31; Aldridge, 2009). Moreover, green biotechnology refers to the domain that exploits advancements in genomics for agricultural benefits. One example of green biotechnology is designing genetically engineered plants or crops that survive specific environmental conditions, such as the presence or absence of certain chemicals or conditions. Red biotechnology encompasses medical and pharmaceutical applications for healthcare. Some examples of red biotechnology are novel disease diagnostics and tests, vaccines, antibiotics, and hormone development. Industrial applications of biotechnology are referred to as white biotechnology. One example of white biotechnology is genetically engineering a microorganism to produce a specific chemical or utilize enzymes to break down pollutants or hazardous chemical compounds. One of the advantages of white biotechnology is that it typically uses fewer resources than conventional industrial manufacturing processes (Frazzetto, 2003). Yellow biotechnology classifies biotechnology uses in the food industry. A classic example is fermentation for making cheese and other food. Another example of yellow biotechnology includes processes for controlling harmful insects and the study of insects' genetic material to characterize genes or gene products (Advances in Biochemical Engineering/Biotechnology, 2013). Environmental applications are referred to as gray biotechnology, and their main focus is the development of approaches to maintain biodiversity, disaster recovery, and the removal of pollutants. Brown biotechnology addresses how deserts and dry environments are managed. This includes the development of improved seeds that can withstand the harsh environmental conditions seen in dry locations, such as drought or salinity. Brown biotechnology, in many aspects, intersects with green biotechnology, especially in applications concerning agricultural techniques and resource management in arid lands and deserts. The legal, moral, and philosophical aspects of biotechnology are categorized as Violet's biotechnology. Lastly, the development of biological weapons and biowarfare are labeled as dark biotechnology, in which microorganisms or toxins are utilized to cause diseases and threaten the lives of humans, animals, or crops. This classification of biotechnology applications is not mutually exclusive. Fig. 14.1 lists the different colors of biotechnology applications with examples. Many modern applications span different color spectrums of biotechnology, such as green and brown biotechnology.

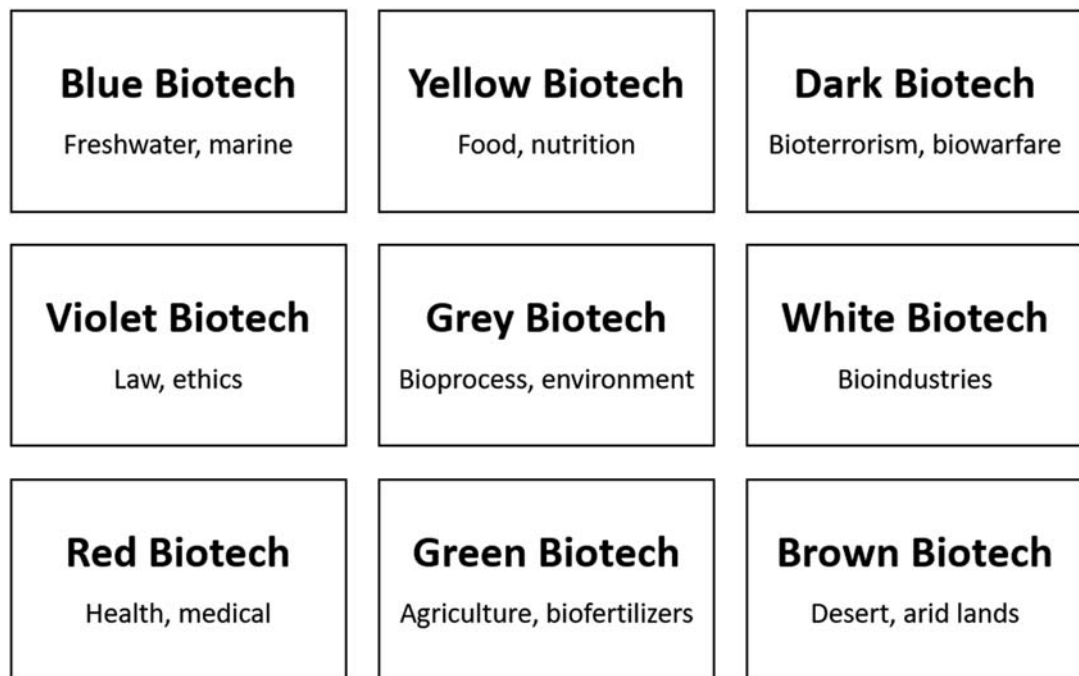


FIGURE 14.1 Colors classifying the major areas of biotechnology applications.

3. Classification of genomics-based applications

While many diverse applications are expanding by the day, genomics-based industrial applications in biotechnology can be classified into four main categories; medical and pharmaceutical applications, agricultural and food applications, nonfood industrial applications, and environmental applications.

3.1 Genomics-based applications in medicine

Modern applications of genomics in biotechnology are used extensively in the medical field in areas such as pharmacogenomics, drug discovery and repurposing, and diagnostics of diseases. In 2021, oncology accounted for about 40% of the global pharmaceutical and biotech industry's total business value, followed by neurology and rare diseases representing the next two significant applications ([Top Global Pharmaceutical Company Report" \(PDF\). The Pharma 1000, 2021](#)).

One of the prominent applications of genomics in medicine is the design and development of traditional pharmaceutical drugs. Advancements in genomics have enabled the manufacturing of small-molecules cost-effectively. One of the first examples of genetically engineered drugs was in 1978 by Genentech, where researchers synthesized humanized insulin by extracting its gene and joining it with a plasmid vector, then inserting it into *Escherichia*

coli. This novel process of synthesizing insulin, which is primarily used to treat diabetes, opened the door for new and relatively cheap processes of manufacturing insulin in comparison to the old approach of extracting insulin from the pancreas of animals such as pigs or cows (Bains, 1987). In the genomics era, emerging therapeutics in gene therapy have witnessed significant breakthroughs over the past few decades. Ever since the Human Genome Project was completed, scientists and researchers have gained tremendous insights into genomics and genetic variations, thereby starting to enable the prevention or treatment of previously untreated diseases through the adoption of gene therapy.

As the name suggests, pharmacogenomics is the intersection between pharmacology and genomics. It aims to study how genomics affects the response to drugs individually. That is, based on the genetic variations present in humans, researchers study how these genetic variations may result in a positive or negative effect on the response to prescribed drugs (Ermak, 2013). Pharmacogenomics is a vital component of research that directly associates with the promise of precision and personalized medicine, shifting from a “one size fits all” approach in medicine to a more tailored individualized approach (Squassina et al., 2010). By investigating drug toxicity and efficacy with respect to patients’ genetic variation, pharmacogenomics aims to optimize patient treatments by increasing efficacy and decreasing potential adverse drug reactions (Becquemont, 2009).

The rapid drop in sequencing costs has significantly accelerated the use of genetic testing in the clinic and other applications. Genetic testing is used for different purposes, including determining a person’s ancestry or helping elucidate the molecular diagnosis of genetic diseases. In diagnostics, genetic testing is vital in facilitating the diagnosis of rare genetic diseases by identifying candidate disease-causing genetic variations. Moreover, genetic testing is useful in studying the overall genome of an individual to identify genetic variations reported with increased risks of certain diseases or conditions, or flag certain variations that may cause certain adverse drug reactions to certain drugs. Genetic counseling is frequently provided to patients in conjunction with genetic testing in a genetics clinic since genetic testing may raise ethical or psychological issues.

3.2 Genomics-based applications in agriculture

One of the main applications of genomics in agriculture is the introduction of GM crops. In GM crops, genetic engineering techniques alter the genome of different plants and crops to introduce new traits that are not naturally present in the plant. The technology behind GM crops aims to eradicate world hunger and contribute to future food security by enhancing the nutritional value of various crops or expanding agricultural landscapes. Some examples of GM crops include developing crops resistant to pests, herbicides, pesticides, diseases, various environmental conditions such as drought or salinity, or crops with reduced rot and increased nutritional value. Farmers have increasingly utilized GM crops in the past few decades, resulting in around 10% of the world’s cropland being planted with GM crops by 2010 (James, 2011). The adaptation of GM crops have been focused on high-value crops, including soybean, corn, rice, canola, and cotton seed oil. These GM crops have been designed to be rich in nutrient profiles, resistant to diseases and herbicides, or have longer shelf lives. GM crops may also have a variety of positive ecological effects. It has been

demonstrated that insect-resistant crops reduce pesticide use (Brookes & Barfoot, 2017). Despite the large adoption of GM crops, there is an ongoing debate on the potential health risks of consuming GM crops compared to nonGM crops. In this regard, different countries have different laws and regulations governing GM products. Some countries forbid or restrict the consumption of GM products, while others allow them with varying degrees of regulation (Lynch & Vogel, 2001).

3.3 Genomics-based applications in industry

Using genomics for industrial processes, such as industrial fermentation, is known as industrial biotechnology. It involves producing industrially useful products from microbial cells or parts of cells, such as enzymes, in industries manufacturing chemicals, detergents, textiles, and more. The development of GM organisms (GMOs), have increased the variety of uses and commercial viability of industrial biotechnology, resulting in tremendous strides in recent decades. For instance, some industrial applications aim to reduce greenhouse gas emissions and shift away from a petrochemical-based economy by using renewable raw materials to create a variety of chemicals and fuels. Given its economic and long-term benefits to the manufacturing industry, synthetic biology is regarded as one of the fundamental pillars of industrial biotechnology. Synthetic biology and biotechnology work together to produce environmentally friendly and cost-effective products using bio-based production methods rather than fossil fuel-based ones (Clarke & Kitney, 2020).

3.4 Genomics-based applications in the environment

The two most prominent applications of genomics in the environment that aim to minimize environmental waste and design environmentally safe processes are biofiltration and biodegradation (Pakshirajan et al., 2014). Environmental biotechnology can have both beneficial and negative effects on the environment. The terms applications and implications can be used to distinguish between the positive impact of biotechnology (such as the use of bioremediation to clean up an oil spill or hazardous chemical leak) and the negative ones (such as the transfer of genetic material from GMOs into wild strains) (Vallero, 2010, pp. 1–742). In other words, environmental biotechnology can be used to clean up ecological trash, but it can also negatively affect the environment, such as by causing the loss of biodiversity or the inability to confine dangerous microorganisms.

4. Regulation of genomics-based applications

Despite their many advantages in addressing various problems, genomics-based applications and biotechnology present ethical and societal challenges, such as concerns regarding genetic engineering and intellectual property rights. Therefore, the usage and application of biotechnology in numerous businesses and fields are continuously being discussed and regulated. The regulation of genetic engineering refers to the methods used by governments to evaluate and control the risks associated with using genetic engineering technology, as

well as producing and releasing GMOs. There are international variations in GMO regulation, with the US and Europe having some of the most pronounced differences (Gaskell et al., 1999). Different regulations apply depending on how a country intends to use genetic engineering products. For instance, authorities in charge of food safety typically do not assess a crop that is not intended for consumption as food (The History and Future of GM Potatoes, 2010). Furthermore, the European Union has issued different regulations for authorization for domestic cultivation and authorization for import and processing. Interestingly, only a few GMOs have approved EU cultivation, while many more have received approval for import and processing (Wesseler & Kalaitzandonakes, 2011). The production of GMOs has brought up the debate on the coexistence of GM and nonGM crops. The incentives for growing GM crops vary depending on the coexistence regulations (Beckmann et al., 2011).

5. The future of genomics-based applications

Healthcare, agriculture, and biotechnology are just some areas in which genomic applications have already transformed. However, the potential of genomic technology for stimulating innovation and opening up new opportunities is still largely unrealized. In healthcare applications, genomics could be leveraged to create even more precise diagnostic systems, enabling earlier and more efficient disease treatment (Lewis, 2023). Genomic technologies could also be applied to agriculture to create more sustainable farming methods, such as precision farming, which uses data analytics to maximize crop yields and minimize waste (Cheng et al., 2023). Utilizing the cutting-edge knowledge of synthetic biology, genomic technologies have the potential to completely transform the area of biotechnology when used in industrial settings (Voigt, 2020). This could lead to breakthroughs in developing new therapies and vaccines, as well as new materials and energy sources. On the other hand, recent advances in gene editing and synthetic biology will allow for the industrial manufacturing of complex biological products like proteins and antibodies. Bio-manufacturing is, therefore, also anticipated to profit from genomic technology. Furthermore, a promising area for future innovation is the application of genomes in drug discovery and repurposing (Pushpakom et al., 2019). Genomic technology can find new pharmacological targets, provide more targeted treatments, or modify existing drugs. In addition, researchers can identify patient subgroups who are more likely to respond positively to a specific treatment by analyzing genomic data from large patient populations, thereby enabling precision medicine. Genomic technologies are bringing the promise that the development of novel medications that are more efficient and have fewer adverse effects may finally become feasible (Lu et al., 2023).

In summary, genomic technologies will become more impactful in numerous industries in the next few years. Research advances, emerging markets, and novel technologies will drive innovation and open new business prospects. Even though many obstacles and restrictions prevent the broad adoption of genomic technologies, such as data privacy and regulatory issues, overcoming these obstacles is essential to maximizing the potential of genomics in the industry (Bonomi et al., 2020; Gürsoy et al., 2022; “Walking the Tightrope between Data Sharing and Data Protection,” 2022; Wan et al., 2022).

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Tailed phage machinery: toward advanced biocontrol

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1. Introduction

1.1 Bacteriophage structure

Bacterial viruses or bacteriophages (often shortened to phages) are very efficient nano-machines designed to infect their hosts with exquisite specificity and high efficacy. Felix d'Herelle in 1917 proposed the term “bacteriophage” from the two words “bacteria” and the Latin word “phagein”, which means “devour or eat.” Phages represent the most abundant biological entity on Earth, with an estimated number of 10^{31} particles in the biosphere; most (96%) are tailed phages (Brüssow & Hendrix, 2002; Hendrix et al., 1999). Phages are responsible for reducing the bacterial population by 4%–50% (Wommack et al., 2009). Coinciding with the huge number of phages in the biosphere, they are also diverse in their structural and functional character, such as genome sizes that range from 4–600 kb. Tailed phages are characterized by a double-stranded DNA (dsDNA) genome encompassed in a proteinaceous polyhedral head or capsid that most frequently adopts an icosahedral shape. Attached to the head is the phage tubular tail, which is considered to be the host-recognizing and genome delivery part of the molecular machine. Given this unique structure, tailed phages are easily recognized with electron microscopy techniques (Ackermann, 1998).

As mentioned above, the range of tailed phage genome sizes is substantial, but the minimal genome requirement of tailed phages encodes the genes for DNA packaging, DNA replication, transcription regulation, and lysis, as well as the structural genes for the head, tail, and tail fibers. It has been noted that with increasing genome size, virion morphology becomes more complicated and phages possess more genes dedicated to increasing their repertoire

of possibilities that target host cellular activities (Brüssow & Hendrix, 2002). With this combined understanding, the classification of phages using a number of criteria regarding structure and genome annotation has taken place.

1.2 Classifying tailed bacteriophages

Recently, the International Committee on Taxonomy of Viruses approved the new taxa proposed in 2021. Significant changes to the taxonomy of bacterial viruses were made by the introduction of the binomial system of nomenclature. Families *Podoviridae*, *Siphoviridae*, and *Myoviridae* as well as the order *Caudovirales* were abolished and replaced by the class *Caudoviricetes* to group all tailed bacterial and archaeal viruses with icosahedral capsids and a dsDNA genome (Turner et al., 2023). To avoid the confusion with the classification in the published papers and review, old classification will be used in this chapter.

According to the old viral classification, tailed phages are grouped into the order *Caudovirales*. This group is subdivided into five families, namely (Brüssow & Hendrix, 2002): *Myoviridae*, which are characterized by contractile long tails in addition to tail fibers (Hendrix et al., 1999), *Siphoviridae*, which are characterized by long noncontractile tails (Wommack et al., 2009), *Podoviridae*, characterized by short noncontractile tails (Ackermann, 1998), *Ackermannviridae*, such as *Salmonella virus Vi1*, that has a myovirion morphology with different head and tail dimensions and genome organization, and (Turner et al., 2023) *Herelleviridae*, that has myovirion morphology with a genome size ranging between of 127–157 kb and the bacterial phylum *Firmicutes* as hosts (Barylski, Enault, et al., 2020; Barylski, Kropinski, et al., 2020) (Fig. 15.1).

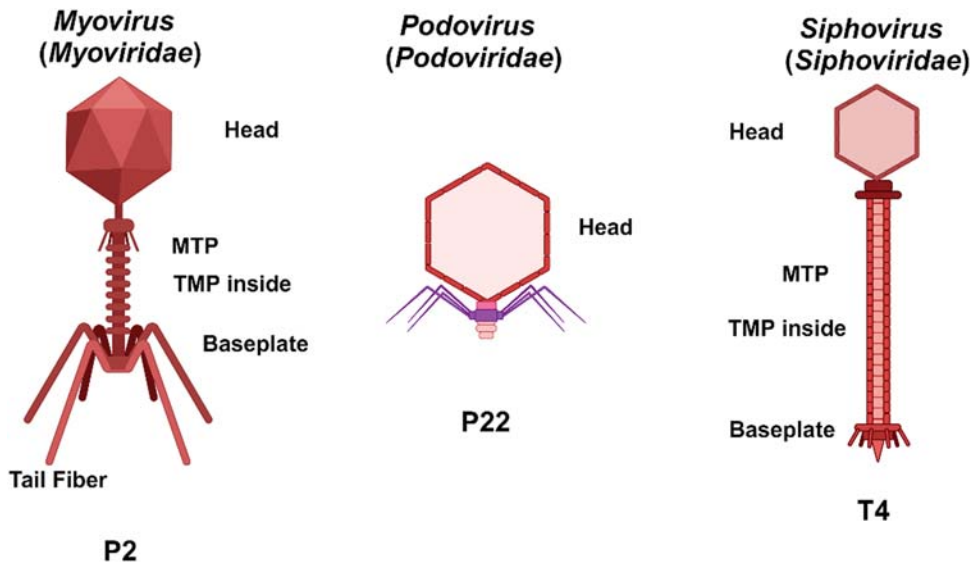


FIGURE 15.1 Structure of *Caudovirales* phages. Structure of phages belong to family *Myovirus* (*Myoviridae*) (e.g., T4), family *Podovirus* (*Podoviridae*) (e.g., P22), and family *Siphovirus* (*Siphoviridae*) (e.g., P2). MTP, major tail protein. TMP, tape measure protein.

It is worth mentioning that phage classification based on genome sequences becomes far more complex than classification using phage morphology alone. For example, nucleotide sequence similarity between *Siphoviridae* and major tail tube proteins is often very low (Rohwer & Edwards, 2002). However, there is a high degree of conservation of function-associated genes in regions encoding morphogenesis modules, such as capsid or tail tube proteins, when comparing phage genomes from *Siphoviridae*, *Podoviridae*, and *Myoviridae* families (Veesler & Cambillau, 2011). This “modular nature of phage genomes” is considered a very important feature because it has allowed accurate gene annotation for uncharacterized genes despite low sequence similarity. For example, this modularity feature was instrumental in the building of the genetic maps of P22 and λ phages, which belong to two different families, *Podoviridae* and *Siphoviridae* families, respectively (Botstein, 1980). In separate studies, in spite of the fact that the genomic analysis of EPS7 showed similarity to a T5 phage, its receptor-binding module and gene arrangement showed similarity to a *Siphoviridae* phage, BF23 (Hong et al., 2008; Mondigler et al., 2006). Again, *Siphoviridae* phages λ and phage TP901-1/SPP1 have similar structural gene modules at equivalent genome locations that encode tail components in spite of the fact that they target different bacterial groups, gram-negative and gram-positive bacteria, respectively (Veesler & Cambillau, 2011). This canonical gene synteny can be described in the following ORF order scheme: the tail terminator, major tail protein, two chaperones, tape measure protein (TMP), baseplate hub (Dit), tail fiber, and the baseplate/tip peripheral proteins (Pell et al., 2009; Xu et al., 2004). Modular phage genome organization is conserved between the same phage family, but begins to slightly vary between different families, as shown in Fig. 15.2 for *Staphylococci* phages (Deghorain & Van Melder, 2012).

During the classification and examination of possible gene functions, a number of things have become apparent. As mentioned earlier, comparing phage genomes alone is not enough for gene annotation due to the low similarities between phage genomes. Still, the nature of the modular structure of gene arrangements can provide a very useful tool to predict the function of any gene when comparing it to other phage genomes. In addition to this, another very characteristic feature of phages is their use of particular protein folds. Extensive structural analysis of several phage virion components from the *Myoviridae*, *Podoviridae*, and *Siphoviridae* families has been carried out over the last several years. This structural analysis has provided a strong platform to compare different phage building blocks. It has led to the conclusion that there is a limited number of folding possibilities for structural phage proteins (detailed more below) that can augment further classification efforts.

2. Bacteriophage assembly

Phage assembly can be divided into four main phases: (1) Head formation and DNA packaging, which includes capsid nucleation, prohead/procapsid formation with the help of scaffolding proteins, DNA packaging, scaffolding protein removal, and procapsid maturation into mature capsid, and (2) the attachment of tail proteins to the mature capsids. These two features are expanded on in more detail below to provide insight into the general mechanism of bacteriophage assembly.

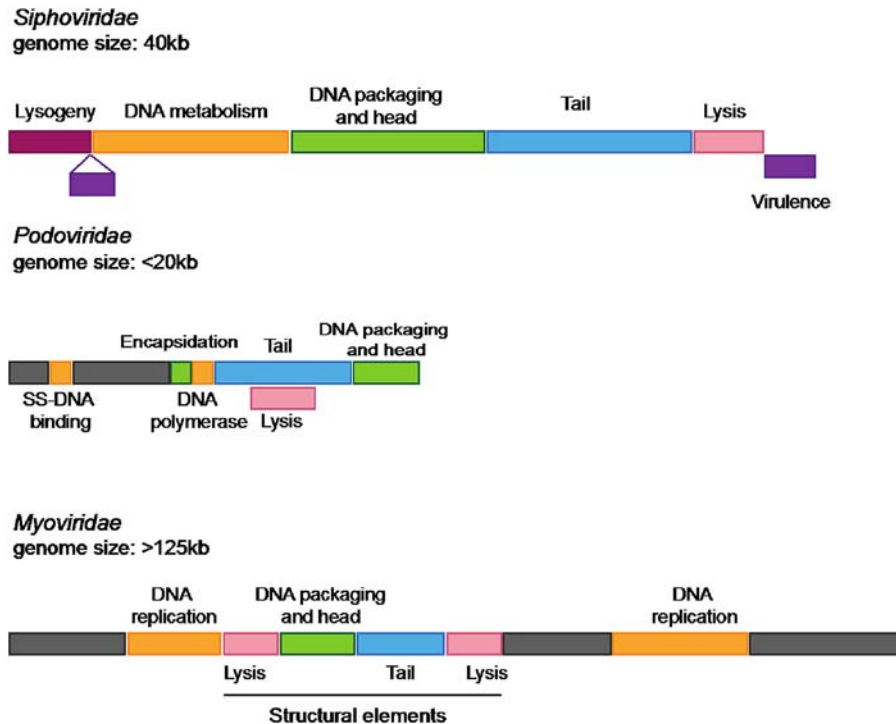


FIGURE 15.2 Modular phages genome synteny of *Staphylococci* phages. *Siphoviridae* genomes are represented in five functional modules, including lysogeny, DNA metabolism, DNA packaging, capsid morphogenesis, tail morphogenesis, and cell host lysis. Virulence genes are generally found downstream of the lysis module, or inserted between the lysogeny and DNA metabolism modules. *Podoviridae* genomes differ because their lysis module and tail morphogenesis modules are overlapping. DNA metabolism genes are also located in a region of genes of unknown function upstream of the tail module. *Myoviridae* genomes have DNA metabolism genes that are distributed into two modules. Figure adapted from Deghorain, M., Van Melder, L. (2012). The staphylococci phages family: An overview. *Viruses*, 4(12), 19994915, 3316–3335. <https://doi.org/10.3390/v4123316>.

2.1 Head formation and DNA packaging

2.1.1 Capsid/shell formation

The HK97 fold has been labeled as the “amazing fold” and is present in most capsid proteins in the *Caudovirales*, including *Myoviridae* (Fokine et al., 2005), *Podoviridae* (Jiang et al., 2006), and *Siphoviridae* (Effantin et al., 2006), and interestingly enough in herpes viruses (Baker et al., 2005), as well as in bacterial icosahedral encapsulins (Sutter et al., 2008). Due to the abundance of tailed phages, the HK97 fold is considered the most abundant fold in the biosphere. It is also worth mentioning that this conservation of the HK97 fold among tailed phages is not only found in the capsid proteins, but is also found in proteins of the maturation pathway that leads to formation of the mature phage heads (Baker et al., 2005; Bamford et al., 2005).

The HK97 fold recognizes conserved elements. These have been labeled as the axial domain (A-domain), peripheral domain (P-domain), extended loop (E-loop) and

N-terminal arm, in addition to some less standard regions, such as “bells and whistles” (Fig. 15.2) (Duda & Teschke, 2019). These additional regions contain the D-loop and glycine-rich loop. Among the more conserved elements, the A-domain forms a triangular shape that represents the central wedge-shaped sectors of hexons and pentons of the capsid. It has a β -sheet-rich core surrounded by protruding α -helices along the edges that interact with adjacent A-domains from other capsid units (Fig. 15.2, panel B). The P-domain forms the periphery of hexons and pentons, while the E-loop is a flexible long β -sheet hairpin that forms contacts with other subunits following multimerization. Fig. 15.3.

With regards to phage assembly and the HK97 fold, there are still a number of outstanding questions. For example, how will the capsid protein monomers form into a mature head with its packaged phage genome (dsDNA)? Does this process require additional help for correct maturation or is self-directed assembly enough? Important to understanding this process is the knowledge that, when more than one capsid protein is present, the major capsid proteins (MCPs) can polymerize into aberrant structures and form insoluble aggregates (Prevelige & Fane, 2012; Zlotnick & Fane, 2010, pp. 180–202). However, icosahedral procapsid shells (immature heads) are self-assembled by copolymerization of MCPs and a scaffolding protein, which helps to dictate the final size and shape of the mature head (capsid or shell) due to its molecular chaperone properties (Casjens & Hendrix, 2015). All dsDNA viruses, including phages, adopt this conserved self-assembly procapsid method that results in icosahedral capsids composed of MCPs and the scaffolding protein that remains inside the capsid interior (Duda & Teschke, 2019; Prevelige & Fane, 2012; Rao & Feiss, 2015; Steven et al., 2005). As revealed by cryo-EM, scaffolding proteins assemble as dimers along the capsid interior (Chen et al., 2011; Dearborn et al., 2017). During the DNA packaging process, scaffolding proteins exit the shell as intact protein to start a new cycle of capsid formation (as noted for P22 phage) or are degraded by a phage protease (as described in the case of λ phage) (Elizabeth Medina et al., 2010; King et al., 1978).

An excellent model example to show the system of virus assembly is phage, λ , where the MCP is gpE and the scaffolding protein is gpNu3 (Casjens & Hendrix, 2015; Catalano et al.,

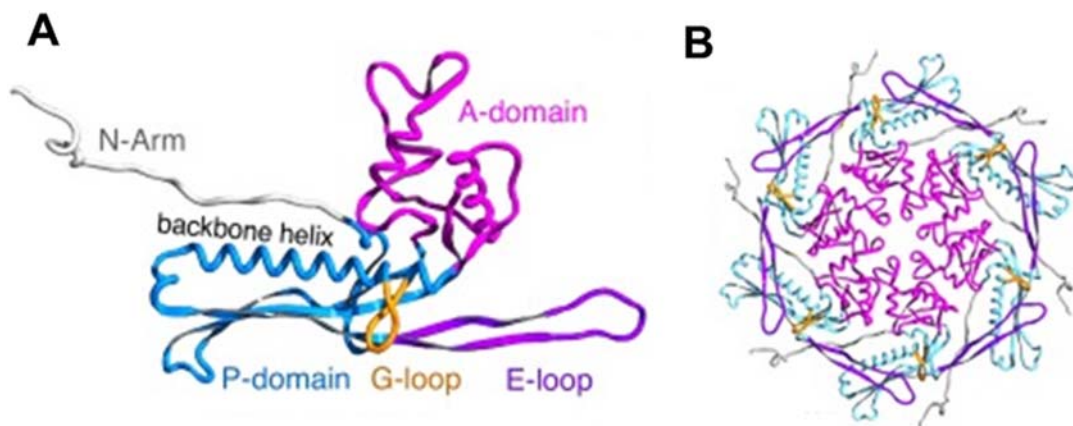


FIGURE 15.3 Phage capsid protein folding (A) HK97 fold with the major components labeled. (B) hexamers form a mature HK97 folded capsid (colored as in A) capsid following multimerization (Duda & Teschke, 2019).

2018). MCPs copolymerize with scaffolding protein to form MCP hexamers, then three hexamers form each of the 20 faces of the icosahedron, and MCP pentamers reside at 11 of the symmetry vertices. The 12th vertex contains the portal protein that helps in genome packaging. Hexamers and pentamers assemble into a mature capsid or shell, which includes 70–300 of the scaffolding protein molecules (Medina et al., 2010). In this process, one MCP is proposed to interact with a dimer of scaffolding proteins to ultimately assemble into a capsid. Any mutational changes in MCP affect the fundamental interactions between MCP monomer-scaffolding protein dimer formation, which ultimately causes abrogation of the initiation of high-fidelity capsid maturation (Davis, 2022).

2.1.2 Scaffolding protein

Structural analysis of scaffolding protein gpNu3 showed that it is an intrinsically disordered protein, meaning it has inherent flexibility and structural disorder (Dyson & Wright, 2005). This intrinsically disordered property of gpNu3 is higher than that of other characterized scaffolding proteins (Fu et al., 2007; Poh et al., 2008). In contrast to gpNu3, most the scaffolding proteins have a common helix–turn–helix structural motif, termed a “capsid binding” motif, as found in P22 and ϕ 29 scaffolding proteins (Morais et al., 2003; Sun et al., 2000). Purified gpNu3 efficiently chaperones MCP or gpE to form virus-like particles (VLPs) in vitro. In the absence of gpNu3 scaffolding protein, purified gpE protein displays a strong tendency to self-assemble into insoluble aggregates. In the presence of gpNu3, gpE remains soluble and assembles into VLPs. The function of gpNu3 has thus been summarized to control the yield of gpE capsid assembly and to ensure the complete assembly into an icosahedral capsid (Medina et al., 2011). The need for scaffolding protein for mature capsid assembly is not restricted to capsid formed by different capsid proteins. The capsid shell, which is composed of a single protein type, also requires scaffolding proteins for proper folding conformation. This single capsids shell protein usually has multiple “quasi-equivalent” conformational states that must be adopted to assemble into a mature icosahedral capsid by the aid of the scaffolding protein (Johnson, 2010; Lander et al., 2008).

Using simulation modeling, the successful self-assembly of capsid proteins into an icosahedron capsid was noted to require a template, inner core, or inner scaffold that helps to control the capsid assembly process to reach the optimum radius of the final capsid. Without this template, capsid proteins assemble into either smaller capsids or nonstructural icosahedrons (Li et al., 2018). All interactions between capsid proteins and scaffold proteins must be specific but transient to catalyze mature capsid assembly and scaffolding protein release from the assembly of capsid. This scaffolding protein release must occur without disruption of the already established assembled capsid. The “intrinsic disorder” property of the scaffolding protein may guarantee the structural plasticity that is required to correct capsid assembly and subsequent scaffolding protein release, as shown in many macromolecular assemblies (Dyson & Wright, 2005). In the era of machine learning and with the use of Kinetic Network Models, scientists may be able to uncover additional important kinetic details regarding the phage self-assembly process. Support for this idea comes from parallel work that used similar approaches to study self-assembly of macromolecules, like pyridinium bromide derivatives, that were used to facilitate the design of new nanomaterials and biotechnological applications (Liu et al., 2022; Zheng et al., 2017). One pertinent question that might be tackled with these tools is whether the mature capsid needs to be formed and ready for DNA packaging or is the

mature capsid ultimately formed only after DNA packaging? Some models propose that mature capsid with a central opening for DNA packaging are formed from MCP with the aid of scaffolding proteins and take place independent of DNA packaging (Dearborn et al., 2017). Other models propose that the formation of mature capsids takes place through protein rearrangements and structural transitions during the DNA packaging to form capsids with a central opening. Once the DNA is packaged inside the capsid, this central opening will be closed by the packed DNA and capsid further matured by conformational rearrangements (Gertsman et al., 2009; Ignatiou et al., 2019).

2.1.3 Chaperonin/cochaperonin

Some bacteriophages encode their own chaperonin that assists in the folding of phage protein(s). The first reported chaperonin was from (*Pseudomonas aeruginosa*) *P. aeruginosa* phage EL (Kurochkina et al., 2012), which has a different mechanism compared to other known chaperonins (Molugu et al., 2016). The ϕ EL chaperonin is proposed to have the unique activity of controlling the phage endolysin (Semenyuk et al., 2015). Some other phages are known to encode for cochaperonin. For example, phages T4 and RB49 have their own cochaperonin that functions with the bacterial host chaperonin to facilitate the proper folding of phage proteins (Ang et al., 2001). Gp31 is another cochaperonin encoded by T4 during the early stages of the phage life cycle to ensure the proper folding of MCP (Keppel et al., 2002). Similarly, RB49 has a gp31-like homolog, called CocO cochaperonin, that is required for phage assembly and proliferation (Ang et al., 2001; Keppel et al., 2002).

2.1.4 Genome packaging

Once the capsid is self-assembled enough to initiate genome packaging, DNA is driven into the capsid by a translocating motor powered by ATP hydrolysis. Two important phage proteins are needed for this process, the portal protein and the terminase, which has ATPase activity. Interestingly, the DNA packaging machinery of some phages requires prohead RNA (pRNA) in addition to these two proteins. This pRNA, noncoding phage RNA, forms a multimeric ring that helps to guide the TerL ring for the DNA packaging motor (Ding et al., 2011; Hill et al., 2016).

2.1.5 Portal protein

In tailed phages and herpes viruses, the portal protein has a cone-shape of radially arranged dodecamer subunits and is considered a key component of the viral immature head and one of the main components of the DNA packaging machinery (Fig. 15.1) (Trus et al., 2004; Valpuesta & Carrascosa, 1994). Portal proteins from different phages have low amino acid sequence similarities; however, they share a common core structure. Portal protein structures can be subdivided into the clip, stem, wings, and crown regions (Fig. 15.4). The clip has an α/β fold that intertwines the portal subunits to stabilize the overall portal oligomer structure. Clips also provide the interface for terminase interaction. It should be noted that mutational studies of the portal gene have led to properly assembled phage, but the mutation affects the infectivity of the assembled phages (Tavares, 2018). These results shed light on the importance of the portal protein during initiation of phage infection (Tang et al., 2011). The portal protein may play a vital role in both genome packaging as well as genome ejection during infection. Thus, the portal protein complex is a crucial component in 3 out of the 4

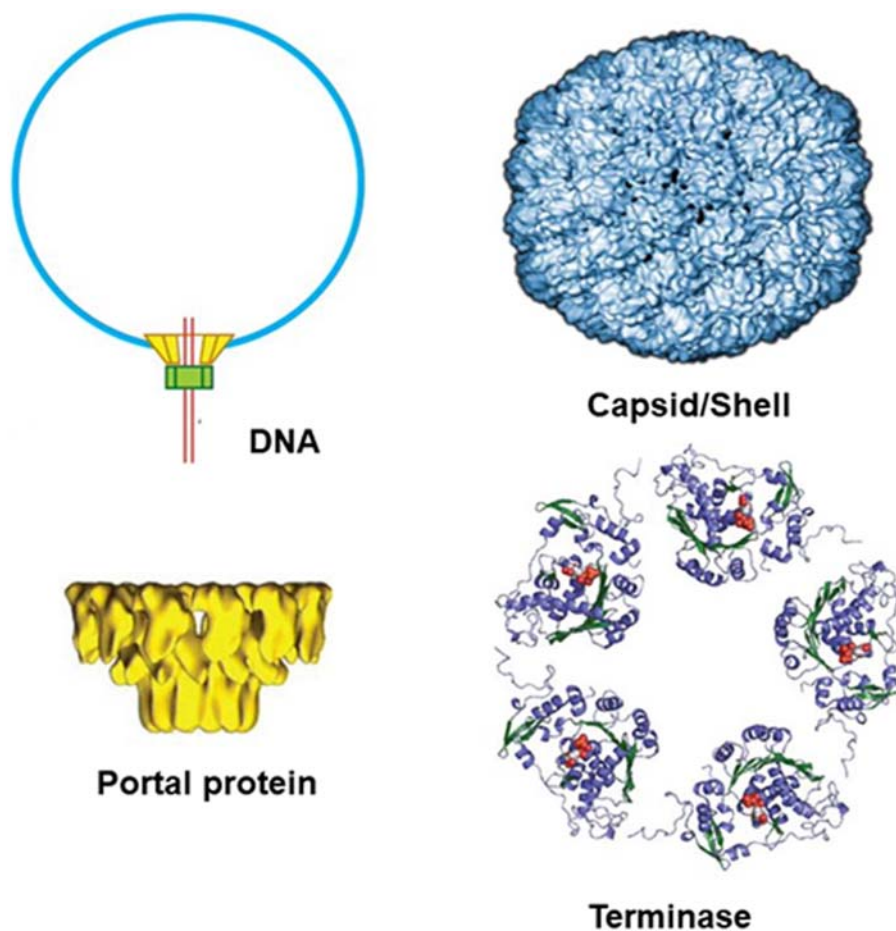


FIGURE 15.4 Phage DNA packaging machine. Left-up: The components of the DNA packaging machine. Left-down: Portal protein. Right-up: Capsid/shell/head, and terminase pentameric model (right-down). Adapted from Rao and Feiss (2008).

phases of phage assembly that were mentioned above. In phase 1, procapsid nucleation starts with the association of coat protein, scaffolding protein, and a single portal protein oligomer as a single ring, which then forms a dodecameric complex structure as reported for many phages and herpes viruses (Fu & Prevelige, 2009; Kato & Baschong, 1997; Motwani et al., 2017; Newcomb et al., 2005). Once the assembly process reaches the DNA packaging and maturation phase, phage DNA is selectively bound and translocated through the portal complex by terminase (Prevelige & Fane, 2012; Rao & Feiss, 2015). After complete genome packaging, the dodecameric portal protein complex undergoes conformational changes to terminate the DNA packaging process, and the assembly process proceeds to the next step where the portal complex works as head–tail connectors (Smith et al., 2001). As explained later, these conformational changes in the portal protein alter its affinity to the packaging

motor. In podoviruses, the portal protein interacts with plug proteins and the adaptors for tail proteins, while in myo- and siphoviruses, the portal protein interacts with connector proteins that are necessary for attachment of a preformed tail (Tavares et al., 2012).

The DNA packaging system recognizes a specific recognition site, *cos*, which is the cohesive end sequence found on many tailed phage dsDNA genomes, such as λ -like or P2-like phages (Casjens & Gilcrease, 2009). In these phages, the genomes form concatemers (multimeric head-to-tail polymers of phage DNA genomes), and terminase introduces staggered nicks at *cos* sites to regenerate the cohesive ends. Other tailed phages that lack cohesive sequences generate concatemers by homologous recombination or end-to-end recombination, as in P22/SPP1 and T4/T7, respectively. In such phages, terminase binds to a *pac* recognition site on the concatemer and makes the initiating cut at a nearby sequence. Once the genome packing commences, terminase generates the second, nonspecific cut, which produces a terminally redundant DNA according to a strict relationship between the capsid size and phage genome length (reviewed extensively elsewhere (Rao & Feiss, 2008). Fig. 15.5.

In a defining study on P22 portal protein, the portal protein was shown to work as a DNA ensor that measures the amount of genomic DNA packaged inside the capsid by specific structural determinants that allow the portal protein in the procapsid to sense the amount of packaged genome. The portal protein also sends signals to the large terminase subunit to end genome packaging. This study also demonstrated that the portal protein does not rotate during DNA packaging but instead undergoes a dramatic conformational change,

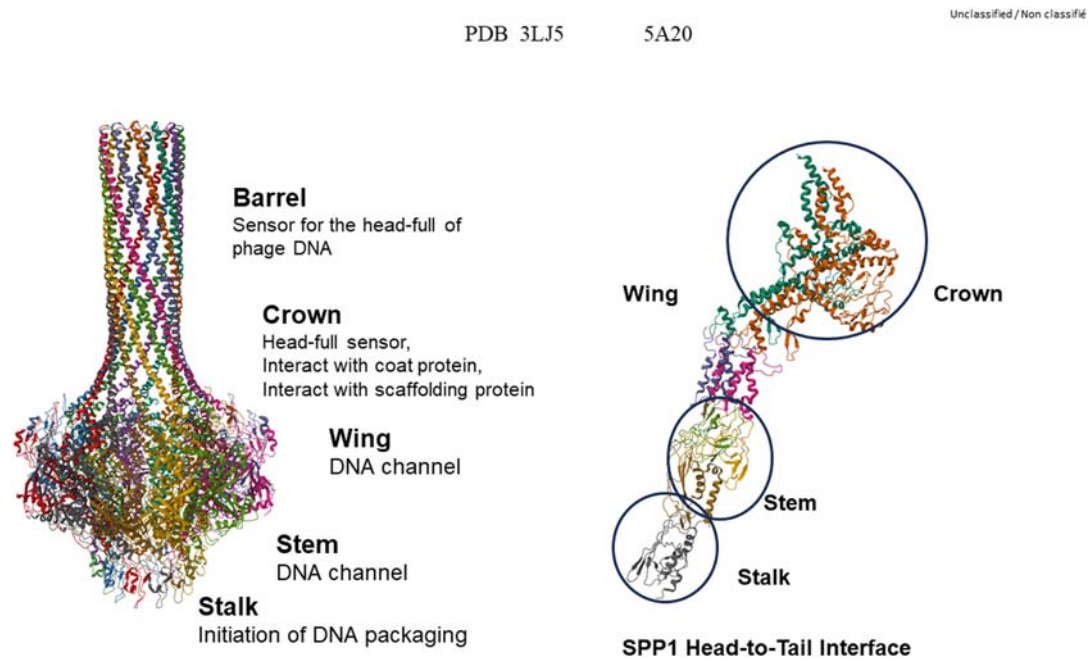


FIGURE 15.5 The portal protein. Left: Functional organization of the full length phage P22 portal protein complex (PDB: 3LJ5). Right: Protein domain organization in SPP1 head-to-tail protein (PDB: 5A20).

switching from a procapsid conformation (with high binding affinity to the large terminase subunit) to a mature capsid confirmation with a symmetric, highly folded structure that has low affinity for the packaging motor and is ready for tail attachment (Fig. 15.6) (Dedeo et al., 2019; Lokareddy et al., 2017). These conformational changes during transformation from procapsid to mature capsid are not restricted to just the portal protein, but also take place for the MCP, gpE, of λ phage (Wang et al., 2022). Similarly, a CryoEM study on SPP1 phage showed the conformational changes in capsid proteins and the portal protein during the capsid maturation and DNA packaging (Orlov et al., 2022). After DNA packaging, the capsid protein (gp15) was shown to bind via its C-terminus to the portal protein (gp6) oligomer, thereby positioning gp15 subunits for oligomerization. The gp15 protein then refolds its inner loops to create an intersubunit β -barrel to establish a connection with another capsid protein, gp16, resulting in the structural formula of gp6₁₂gp15₁₂gp16₆.

2.1.6 Genome recognition and translocation

The initial two steps of the phage genome packaging are genome recognition, followed by the process to insert the genome inside the procapsid. During this sequence of events, there are three recognition mechanisms, depending on the tailed phage, with two of them requiring the small subunit of terminase, TerS. As noted above, P2/P4-like phages using *cos* sites employ the terminase to recognize these sites on the concatemers and introduce staggered

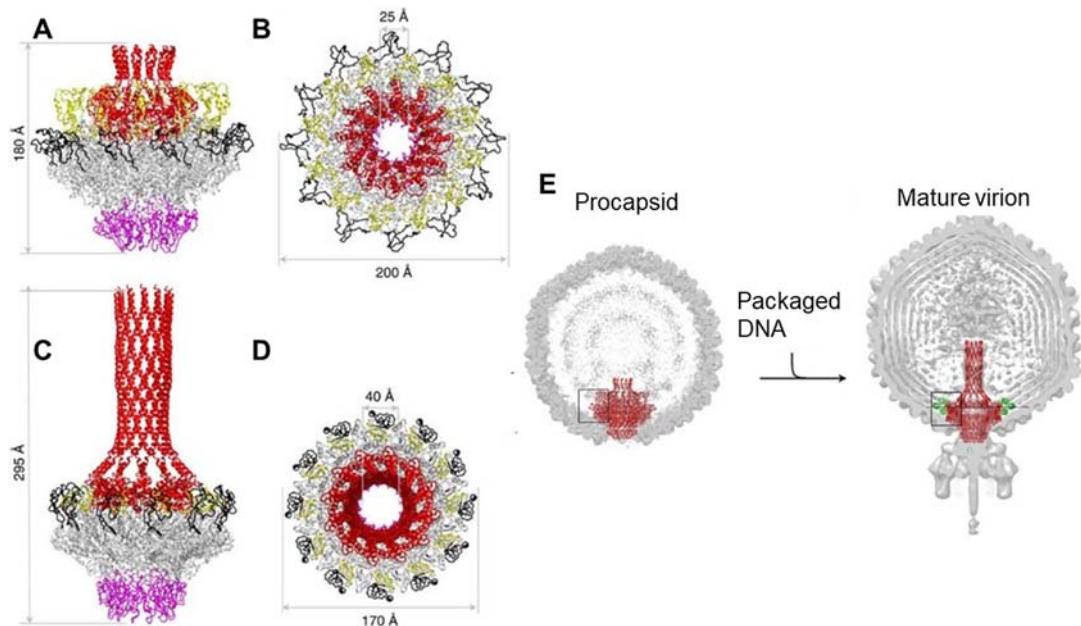


FIGURE 15.6 Conformational changes in the P22 phage portal protein during capsid maturation. Depicted is the portal protein structure in procapsid (A–B) versus mature capsid (C–D) and its maturation modeling (E). Adapted from Lokareddy, R.K., Sankhala, R.S., Roy, A., Afonine, P.V., Motwani, T., Teschke, C.M., Parent, K.N., Cingolani, G. (2017). Portal protein functions akin to a DNA-sensor that couples genome-packaging to icosahedral capsid maturation. *Nature Communications*. 8. 20411723. <http://www.nature.com/ncomms/index.html>, <https://doi.org/10.1038/ncomms14310>.

nicks to regenerate the cohesive ends, while other tailed phages (P22, SPP1, T4 and T7) that generate concatemers by recombinations (homologous or end-to-end recombination) have *pac* recognition sites instead. The third recognition mechanism does not require TerS, as in ϕ 29-like phages and adenoviruses, where there are no concatemers formed during the DNA replication. In this case, the viral genomes replicate as monomers by protein-primed DNA replication.

After genome recognition, terminase starts unidirectional DNA translocation into the procapsid until exactly one genome size (*cos*-containing and protein-primed phages) or slightly more (due to the position of the cut being more than one genome unit—about 104%–110% unit-length genome for *pac*-containing/headful phages) is inside the capsid. Filling the capsid with phage genome initiates enough pressure to trigger the second terminase cut that results in packaged DNA with 3%–10% terminal redundancy, reviewed in detail here (Casjens, 2011; Pajak et al., 2021; Rao & Feiss, 2015). Once the phage genome is packed inside the capsid, the mature phage capsid is formed and stabilized to enable it to resist the internal pressure exerted by the packaged phage genome (about 30 atm.), as well as the external harsh environment, such as exposure to UV-C, H₂O₂, and even proton beam exposure (Molineux & Panja, 2013; Plano, 2022). These stabilization mechanisms are numerous, including many proteins' conformational changes, intercapsomer interactions, and isopeptide bond formation in some phages (Podgorski, 2022).

2.1.7 Terminase and packaging motor

Bacteriophage and herpesvirus terminases have an oligomer complex structure of heterodimeric units (Fig. 15.1). Each unit, or protomer, consists of small (TerS) and large protein (TerL) subunits in the form of (TerL-TerS)₂. The TerS subunit is about 17–23 kDa in size, and it has distinct functional domains consisting of an N-terminal helix-turn-helix DNA binding motif, a helical central self-assembly domain, and a C-terminal domain that binds to the TerL subunit in the holoenzyme. TerS is responsible for genome recognition through its DNA-binding activity and also modulates TerL activity (Oliveira et al., 2013; Rao & Feiss, 2015). TerS has a unique feature that works as a DNA-dependent stimulation for the ATPase activity of TerL (Roy et al., 2012). The TerL subunit is about 50–80 kDa in size, and it has three domains, consisting of an N-terminal domain or the packaging domain that has the canonical ASCE P-loop ATPase fold (ATP binding site is located at the interface of two adjacent subunits in the holoterminase), a lid domain and linker region, and a conserved C-terminal RNase H-like nuclease domain for cutting the dsDNA at the beginning and end of packaging (Pajak et al., 2021). Interestingly, some phages, such as ϕ 29, lack TerS or the endonuclease activity of TerL (Catalano & Morais, 2021). Only individual structural information for TerS and TerL is available since the proteins purify separately due to TerL-TerS heterotrimer instability in vitro. However, there is one exception, where structural analysis of phage λ terminase was possible upon the finding that its TerL-TerS heterotrimer is tabled in vitro.

After phage genome binding to the terminase to form a binary complex, this complex will bind to the portal vertex of a procapsid to construct the packaging motor complex during the maturation process. The portal proteins TerL and TerS form three stacked rings where the dsDNA genome is threaded through their common central channel. C-terminal procapsid binding residues of TerL, which is mostly a pentameric oligomer (although the structural

configuration [TerS₂-TerL]₄ was also proposed), are arranged in a parallel orientation to be in position for binding to the dodecameric portal vertex, and similarly to how TerS forms a ring-like structure (Fig. 15.7). ATP hydrolysis provides enough energy for the terminase packaging motor to translocate the DNA phage genome through the portal and into the procapsid shell. About 2bp per round of ATP hydrolysis are packaged in a mechanochemical coupling process that generates a force of about 60 pN (Chemla et al., 2005; Fuller et al., 2007).

Detailed elucidation of the phage DNA packaging motor formation and its translocation mechanism(s) has been discussed in many informative reviews (Casjens, 2011; Pajak et al., 2021; Rao & Feiss, 2015). From these combined studies, the “inchworm” translocation model has the most experimental support. In this model, the TerL nuclease and ATPase domains are both capable of binding DNA with conformational switching between “tense-relaxed,” “dynamic-static,” or planar-lock washer” conformations. The switching is driven by ATP hydrolysis and leads to sequential steps of DNA binding and release by the two domains, which results in translocation of the DNA into the procapsid (Rao & Feiss, 2015). Thus, the mechanochemical cycle has two distinct phases: “Dwell” long phase, where ATPase domains sequentially exchange ADP for ATP, followed by a “burst” short phase, where ATP hydrolysis and ~10 bp of DNA translocation occur (Dai et al., 2021). It was proposed that only four TerL subunits are associated with DNA movement, each translocating 2.5bp/complete translocation cycle, while the fifth subunit was proposed to maintain a “grip” on the DNA. The nucleotide-dependent DNA gripping and an end-clamp mechanism seem to regulate the packaging motor. From the data, it was demonstrated that the DNA rotates 14 degrees per 10-bp translocation burst. The degree of rotation and the translocation step size change in response to head filling during the last stages of packaging (Dai et al., 2021; Ordyan et al., 2018). In other words, the velocity of the packaging motor significantly decreases as genome

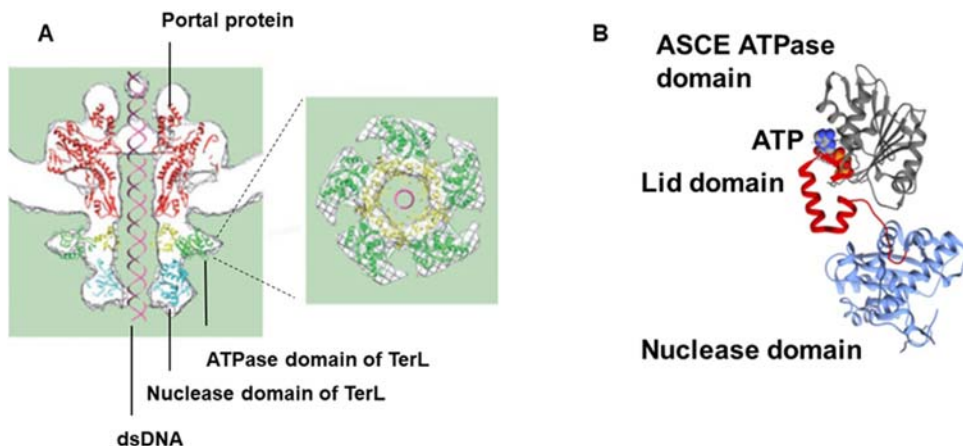


FIGURE 15.7 Structure of the DNA packaging motor and TerL protein. (A) Ribbon diagrams for the portal protein of SPP1 phage (red), the T4 TerL protein with its C-terminal nuclease domain (blue), and the N-terminal ATPase portion with subdomains I and II (yellow and green), with a top view of the central channel (right image). (B) Structural domains of sf6 TerL protein, ASCE ATPase, lid, and nuclease domains. Image depicted from Casjens (2011); Catalano and Morais (2021).

packaging nears completion (Liu, Chistol, Bustamante, et al., 2014; Liu, Chistol, Hetherington, et al., 2014). Changes between ATP binding and ATP hydrolysis are accompanied by many conformational changes between static-dynamic, planar-lock washer, or grip DNA-translocation. Studies have demonstrated that it is not necessary for all subunits to bind to ATP, since binding of four subunits to the ATP is enough to allow the packaging process to proceed (Dai et al., 2021).

Head-filling with packaged DNA appears to be the signal for termination of the packaging process and triggers the C-terminal domain of TerL to rotate ~ 90 degrees angle to position the nuclease domain to cut the DNA and allow the C-terminal domain of TerL to grip the DNA until the motor complex dissociates from the filled capsid (Mahler et al., 2020). The portal protein was shown to work as a DNA sensor that measures the amount of DNA packaged inside the head and signals for termination of packaging (Lander et al., 2006). As noted above, the portal protein has specific structural conformational changes from an asymmetric one at the beginning of DNA packaging that changes to a symmetric quaternary structure at the end of the packaging process. The protein in the asymmetric conformation has a high affinity for binding to TerL, while in the symmetric conformation the protein has a low affinity for the packaging motor and is stabilized by attachment of tail factors (Lokareddy et al., 2017). After dissociation of the packaging motor, it is not entirely understood how the phage remains packaged despite the high internal pressure from the genome. One study proposed that the portal ring tightly binds to the DNA as a “one-way valve” to prevent its release (Fang et al., 2012). Other phages have head-finishing proteins that assemble on the portal after genome packaging and form a platform for tail attachment (Feiss & Rao, 2012).

2.1.8 Connector/neck proteins

Once the genome is packed inside the capsid, the connector region starts to assemble on the head to provide a platform for the tail to attach to its capsid. The connector region is formed as two rings of head completion proteins (or neck) that attach to the portal protein. The first ring of the phage connector attaches directly to the portal protein and is formed of gp15 and gpW proteins in phages SPP1 and λ , respectively. The bottom ring of the connector is then formed of gp16 and gpFII proteins in phages SPP1 and λ , respectively, thereby acting as a plug to keep the DNA inside the capsid (Cardarelli et al., 2010; Lhuillier et al., 2009). In the case of T4 phage, the connector proteins are instead comprised of gp13, gp14, gp2, and gp4 (Veesler & Cambillau, 2011).

2.2 Parallel assembly (tail and tail fibers)

While capsid assembly and DNA loading take place, parallel assembly of other phage parts, such as the tail and tail fibers (depicted in Fig. 15.8 for T4 phage) decreases the time of the overall infection cycle. In phages with contractile tails, the first step in the tail assembly is the baseplate, where it works as a scaffold for inner tube and contractile sheath assembly and may require the presence of viral chaperones. For example, T4 phage tail assembly involves 19 different structural proteins and seven viral chaperones. A central hub of gp5 and gp27 forms first and then six heterooligomeric wedges, comprised of gp6, gp7, gp8, gp10, gp11, gp25, and gp53, bind to it to form the initial T4 baseplate. Short tail fibers (STFs), formed from gp9 and gp12, insert into the gaps between wedges, while the

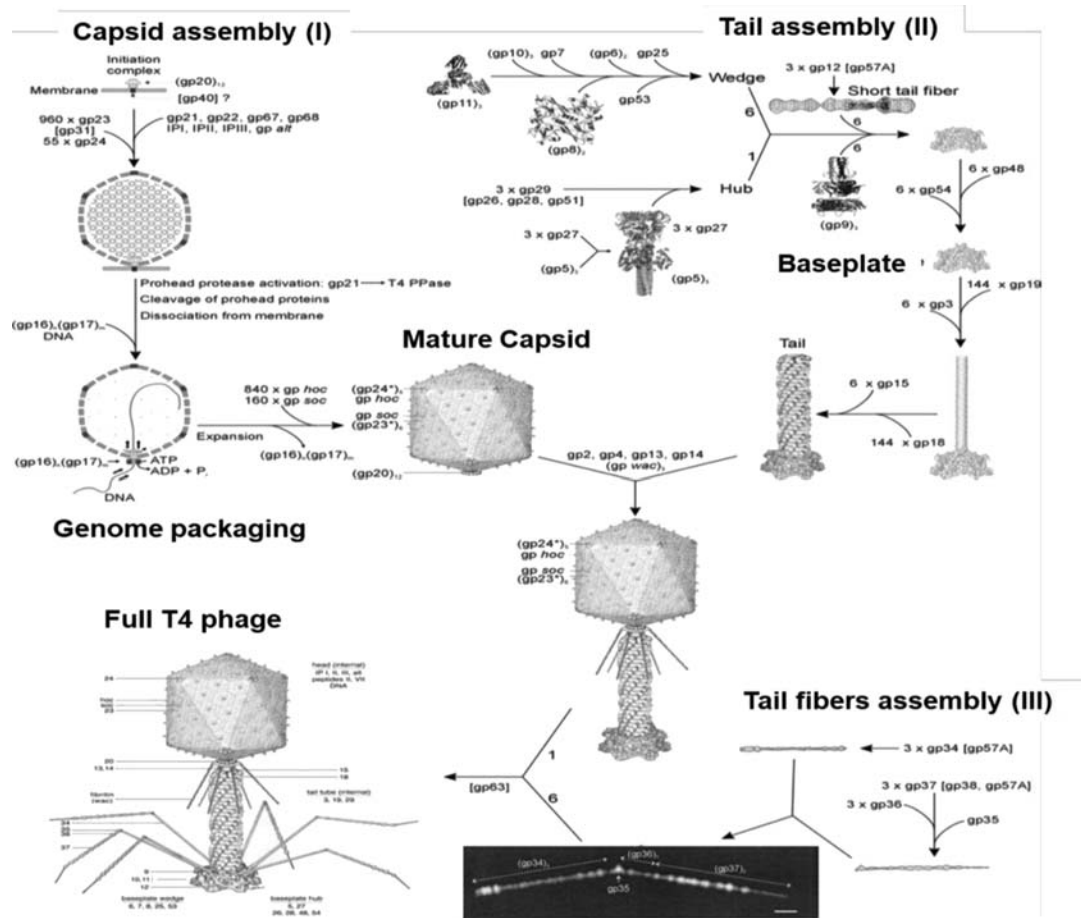


FIGURE 15.8 Parallel assembly of phage T4. The assembly of T4 phage is subdivided into three parallel pathways for capsid (I), tail (II), and tail fiber (III) assembly lines. Depicted from Leiman, P.G., Kanamaru, S., Mesyanzhinov, V.V., Arisaka, F., Rossmann, M.G. (2003). *Structure and morphogenesis of bacteriophage T4*. Cellular and Molecular Life Sciences. 60(11), 1420-682X, 2356-2370. Springer Science and Business Media LLC, <https://doi.org/10.1007/s00018-003-3072-1>.

wedges-hub interface is sealed by gp48 and gp54 (San Martín, 2013). After assembly, the baseplate serves as a scaffold for the formation of the inner tail tube that is composed of gp19. The regulation of the tube length is achieved with the help of the TMP gp29, which extends from the hub to the tube end where the tail capping protein gp3 binds (Katsura & Hendrix, 1984). The inner tube then works as a scaffold for the tail sheath assembled by gp18. Afterward, the tail terminator protein gp15 binds to gp3, and the last row of gp18 subunits for the tail sheath forms. At this point, the tail is ready to bind to the connector/neck proteins and leads to the formation of the mature capsid. Tail proteins (e.g., tail tube, TMP, and tail terminator) are all major structural tail components in *Myoviridae* and *Siphoviridae*, in addition to the sheath protein found in contractile tails (Kostyuchenko et al., 2005; Leiman et al., 2004).

The final stage of T4 mature phage assembly is the attachment of the assembled tail fibers to the baseplate (Fig. 15.8; Stage III). T4 tail fiber is kinked and has two main parts: The proximal part formed by gp34 protein and the distal part formed by gp35, gp36, and gp37 proteins. Both proximal and distal parts are assembled separately and then joined together before attaching to the baseplate. All tail fiber proteins form trimers, except monomeric gp35. Interestingly, despite the initial parallel assembly pathways, the later stage requires an order of attachment for the different preassembled parts. The tail will only bind to the capsid after capsid maturation through DNA packaging, and fibers cannot join the tail until the tail is bound to the DNA-filled capsid. For noncontractile-tailed phages, they have a similar assembly pathway. However, short-tailed phages, as seen in *Podoviridae*, have their tails assembled with the capsid as an outward part of the portal vertex. In other words, the capsid portal vertex serves as a base for *Podoviridae* tail assembly (Fokine & Rossmann, 2014).

3. Conformational changes during infection

During infection, T4 uses the long tail fibers (LTFs) to recognize lipopolysaccharide (LPS) and/or OmpC on the surface of a target *E. coli* cell. It was proposed that the initial step of binding to these receptors is a conformational change in the baseplate from a dome-shaped structure to an intermediate structure, which allows the release of STFs to attach to the cell surface. Binding of STFs induces further conformation changes in the baseplate to its final star-shaped structure that causes the sheath to contract and the tail tube to puncture the outer membrane of these bacteria. An adaptor protein, gp9, is used to attach the LTFs to gp7 of the baseplate. When there is no interaction with the host cell, there is a flexible interaction between gp9 and gp7. However, once the phage attaches to the host, Brownian motion of the phage particle has been proposed to cause these flexible regions to pull on domain III of gp7 (Hu et al., 2015). The interactions of gp9 with domain III of gp7 are likely to further disturb interactions among gp7, gp8, and gp10 at the vertices of the dome-shaped baseplate and trigger the periphery of the baseplate to change to a star-shaped conformation. Subsequently, STFs will attach to gp10, which is then followed by many changes that ultimately will cause gp6 to detach from the central hub. This then leads to the movement of gp25 and initiates contraction of the metastable tail sheath by displacement of the C-terminal domain of the tail sheath protein, gp18 (Yap et al., 2016). Afterward, STFs will attach to cell-surface receptors, which in turn will trigger the baseplate to change its conformation to the star-shaped configuration. These baseplate conformational changes cause the sheath to contract and drive the tail tube into the host outer membrane, across the periplasmic space (while localized degradation of the peptidoglycan layer with enzymes takes place), and ultimately to the inner membrane. Penetration into the host cytoplasm is accompanied by a dramatic local outward curvature of the cytoplasmic membrane as it fuses with the phage tail tip (Hu et al., 2015). Specifically, the data from T4 infection studies have demonstrated that the cytoplasmic membrane bulges from its normal plane by ~16 nm to fuse with the ejection tube and that this process does not require an energized membrane (Hu et al., 2015). However, opening a channel across the membrane for DNA transport does require a membrane potential (Boulanger & Letellier, 1988). For example, *E. coli* cells treated with an ionophore,

like *p*-trifluoro-methoxy-carbonylcyanide phenylhydrazone, before infection lead to disruption of the membrane potential and inhibition of phage DNA ejection (Hu et al., 2015). However, with a membrane potential, phage genomic DNA is ejected into the *E. coli* cytoplasm and ultimately allows the infection cycle to begin again.

4. Advanced applications

Phages have a wide range of applications that continue to be an area of expansion. However, there are classical applications, such as phage display, phage therapy, and biocontrol, that remain mainstays in this sector. In addition to the improvement and growth of these classical applications, emerging applications with great promise include biosensors, biofilm degradation, food safety, wastewater treatment, and others.

4.1 Phage engineering

Among the advantages of using phage is the availability of numerous methods for novel engineering and/or complete redesign for specific bioapplications. These methods include homologous recombination, bacteriophage recombineering of electroporated DNA, in vivo recombineering, CRISPR-Cas-mediated genome engineering, rebuilding/refactoring phage genomes in vitro, whole-genome synthesis and assembly from synthetic oligonucleotides, yeast-based assembly of phage genomes and cell-free transcription-translation systems, which have all been nicely reviewed elsewhere (Pires et al., 2016). With the aid of these design techniques, ongoing modification can enable the continual evolution of phages to overcome bacterial resistance to phages, enhance the bactericidal activity of antibiotics when coadministered (Edgar et al., 2012; Lu & Collins, 2009), improve the accessibility of phage to its targeted bacteria (Bhattarai et al., 2012), provide a system for lethal-gene delivery (Moradpour et al., 2009; Westwater et al., 2003), facilitate biofilm degradation or quorum sensing quenching by phage-harboring enzymes (Lu & Collins, 2007; Pei & Lamas-Samanamud, 2014), expand the host range of phages to broaden applicability (Yoichi et al., 2005), and many other applications. By improving our understanding of the details surrounding individual phage genomics and structural features, the genetic engineering of these phages has become a powerful platform to generate synthetic specialized phages with desirable characteristics to overcome the limitations of phage applications and may reduce the potential risks of horizontal gene transfer by phage.

4.2 Phage display

Even though phage display techniques mainly focus on the use of filamentous phages, they have become an important part of the bioapplication sector. Phage display technology was first introduced in 1985 as a novel expression vector that displays a foreign antigen as a fusion peptide with the phage capsid protein and hence displayed on the surface of a phage virion, but has since been adapted to be a powerful tool for the display of antibody fragments and more recently for research, diagnostic, and therapeutic peptides (Alfaleh et al., 2020; Ledsgaard et al., 2022; McCafferty et al., 1990; Smith, 1985). Depending on the inserted

peptide sequence, phage display can be used as a robust tool for antibody production, vaccination, and therapeutic applications, as shown in Fig. 15.9 (see “Phage Therapy” below). Different filamentous phages, such as fd, M13, and f1, are readily available for use in the development of libraries for peptide display (Kehoe & Kay, 2005). Using these processes, phage-derived monoclonal antibodies (mAb) have been shown to have advantages over murine-derived mAb, where the later can invoke allergic reactions and have a faster clearance rate from plasma (Legouffe et al., 1994). Another study reported in 2020 that phage-derived mAbs have now been part of 70 clinical studies, and some of them (14 studies) have already been approved for further use (Alfaleh et al., 2020). A final good example of one of these 14 would be nice to see their importance and specific application.

4.3 Phage for human and animal therapy

The first antibiotic was introduced in 1910, followed by penicillin in 1928, which led to drastic changes in modern medicine’s approach to infections and extended the average human lifespan by 23 years (Hutchings et al., 2019). However, antibiotic misuse has resulted in antimicrobial resistance (AMR) with an increasing number of untreatable pathogenic bacteria. It was predicted that the mortality due to AMR will reach 10 million patients a year by 2050 if nothing changes (Prescott, 2014). A comprehensive assessment carried out in 2022 estimated that mortality due to AMR in 2019 amounted to 4.95 million deaths (Murray et al., 2022). Unfortunately, it is not only antibiotic misuse that can cause AMR, but it has also been discovered that some human-targeted drugs (e.g., diacerein for osteoarthritis treatment,

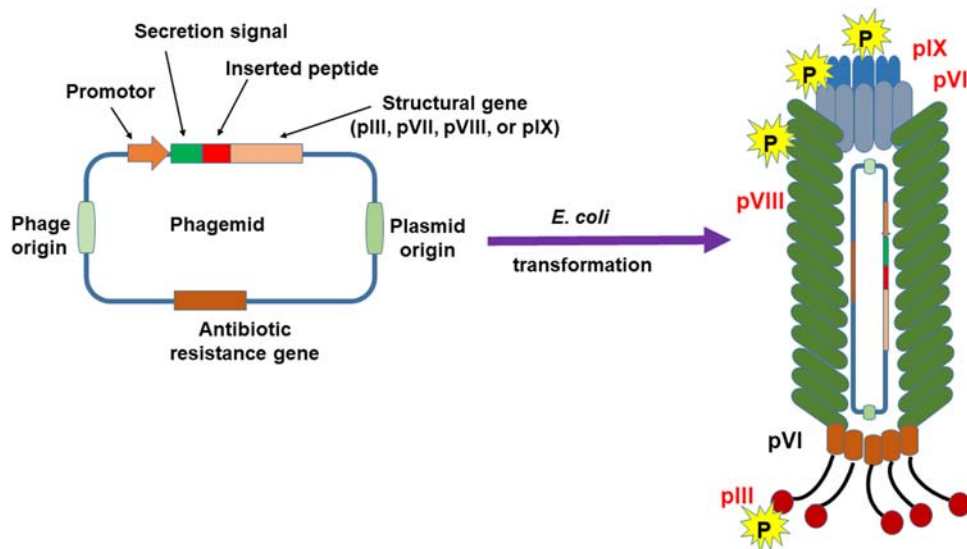


FIGURE 15.9 Phage display technique and its applications. The gene of target peptide is fused with the capsid protein gene or the gene for other structural proteins. After *E. coli* infection, the phage progeny will display the target peptide fused with capsid protein (or other structure proteins). Depending on the target peptide, phage display can be applied for antibody production, phage therapy, and other applications.

ethopropazine for Parkinson's disease treatment, and tamoxifen for breast cancer treatment) can initiate AMR within the human microbiome, as the bacteria that reside there are presumably using common resistance mechanisms against antibiotics and human-targeted drugs (Maier et al., 2018).

In the last 5 years, phage therapy has been used to successfully treat life-threatening multidrug-resistant bacterial infections (Eskenazi et al., 2022). With the increasing AMR crisis, these results have led to bacteriophage therapy receiving increasing attention as a prominent alternative to antibiotics. According to Precision Report, the phage therapy market size was valued at 176.75M USD in 2022 with an expectation that it would reach 328.99M USD by 2028 with a compound annual growth rate of 10.91% (Precision, 2022). Productive phage therapy programs are also now established in many countries, including the Republic of Georgia, Poland, the US, Belgium, France, and Sweden. Recently, the UK announced that it will begin to consider compassionate-use phage therapy requests through the National Health Service (Strathdee et al., 2023). Following this current trend, successful clinical trials of phage therapy for humans and animals continue to be reported and open up possibilities for further expansion and development (Lin et al., 2017; Ling et al., 2022; Osei et al., 2022; Strathdee et al., 2023).

Phage-based vaccines have also been explored in different studies. In these applications, phage-display and/or phage-packaged DNA vaccines are the two most prominent avenues being investigated. In phage-displayed vaccines, the antigenic peptide or protein are displayed on the surfaces of the phage and delivered to the target cells or tissues (see above section on Phage Display) (González-Mora et al., 2020; Santi et al., 2000). In phage DNA vaccines, the eukaryotic promoter-driven vaccine genes are inserted into the phage genome, which are carried by phages to the target cells to then be expressed and generate antigens for immune system recognition (de Vries et al., 2021). For example, Fa-Arun and colleagues (2023) were able to use genetically engineered phages as a vector for delivering chromosomal-targeting Cas9 constructs, which were attached to engineered P2/P4 phage chimeric tail fibers. Engineered phages showed Cas9-mediated killing of two important gut pathogens, *Shigella flexneri* and *E. coli* O157:H7 (Fa-Arun et al., 2023). While phage therapy has been safe and well tolerated in most of the studies to date, a comprehensive understanding of the interactions of phage and human/animal hosts is still in its infancy. Thus, with the continual progress/expansion of techniques and applications, there is also an urgent need for standardized assessments that outline phage therapy safety (Liu, Van Belleghem, et al., 2021).

4.4 Phage as a biocontrol agent

The ongoing surge in AMR is not only due to the misuse of antibiotics by patients in the healthcare and animal/livestock settings, but is also exacerbated by excessive use within agriculture. Again, using phage as a microbial biopesticide to replace antibiotics has been around for many years (e.g., first introduced in 1924 to prevent rot in cabbages (Mallmann & Hemstreet, 1924), but only recently have phage products been commercialized and obtained approval from the U.S. Environmental Protection Agency (Huang, 2022). One of the main

concerns that has led to this latency period is the highly variable results due to issues related to field condition consistency, including variable weather combined with the timing and method used for application (Svircev et al., 2018). As mentioned with phage therapy in the last section, there is an urgent need for standardized protocols to improve phage-based biopesticides and their outcomes (Boyetchko & Svircev, 2013, pp. 37–43). It is also worth mentioning that a number of the advantages to using engineered phages in agriculture also overlap with desirable outcomes for phage therapy (outlined above) and should help address issues in both these sectors simultaneously. For example, targeting plant-pathogenic bacterial biofilms, quorum sensing, lethal gene delivery, and broadening host range or stress tolerance will similarly be beneficial when targeting unwanted pathogens of plant tissues or crops by phages (Dy et al., 2018).

Phage application as a plant biocontrol method still has several challenges, such as the development of bacterial resistance to the applied phage or specificity (i.e., narrow host range of phages). However, the use of phage cocktails (i.e., preparations with more than one phage) could overcome these two challenges by including phages that infect different hosts in the cocktail and simultaneously making it more difficult for the host bacteria to develop resistance to multiple phages at once. As for problems with field application and longevity of the phage biocontrol agents, there have been a number of successes with using either phage formulations that include protective compounds or genetically modified phages to enhance phage tolerance to environmental stresses (e.g., UV, desiccation, ... etc) (Holtappels et al., 2022; Luo, 2022).

4.5 Phage use in food safety and wastewater treatment

The control of bacterial food contamination is normally carried out by chemical, physical, and biological techniques, but a substantial drawback of these traditional procedures is that they can affect the food quality (He et al., 2021). For example, the most widely used traditional procedure is thermal treatment that usually causes undesirable effects on the sensory and nutritional qualities of food (Ramos-Vivas et al., 2021). Irradiation has also been noted to change the food's flavor and color (Jia et al., 2021). Phage applications also hold promise for the elimination of bacterial pathogens in a number of commercial avenues, but may offer an alternative that results in less detrimental effects on food quality. To date, phage applications along the food supply chain (e.g., meat and dairy products, fruits and vegetables production) have been approved by the FDA in the United States as “generally recognized as safe” and in many other countries (Alomari et al., 2021; Huang, 2022). For example, *Salmonella* phages LPST10 and D1-2 are considered potential methods to control *Salmonella* contamination in dairy foods, lettuce, sausages, egg white, and yolk (Cao et al., 2022; Chenxi Huang et al., 2018; Zhiwei Li et al., 2020), and this has also been reviewed extensively elsewhere (Ge et al., 2022; Hyla et al., 2022; Vikram et al., 2022; Żbikowska et al., 2020).

Similar to food safety applications noted above, phages have been gaining increasing interest as biocontrol agents against different environmental problems arising from the wastewater process, such as reducing foam formation by microorganisms, fighting pathogenic bacteria, and reducing competition between unwanted bacteria and functionally important

microbial populations (Bolsan et al., 2022). The specific advantages of phage application in wastewater treatments include (Brüssow & Hendrix, 2002): Phage specificity and how they will not be harmful to beneficial bacteria (Hendrix et al., 1999); due to their nontoxic nature they are environmentally sustainable (Wommack et al., 2009); their high efficiency in bacteria growth control; and (Ackermann, 1998) they are self-replicating and therefore there is often no need for multiple doses (Ji, 2020; Runa, 2021). The number of phage applications in wastewater treatment is a thriving area and has been a subject of other exhaustive literature reviews (Bacteriophages as a Potential 360-Degree Pathogen Control Strategy) (Microorganisms, 2021; Bolsan et al., 2022; Ji, 2020; Liu, Li, et al., 2021).

4.6 Phage as biosensors

Early-stage detection of pathogenic bacteria is a crucial step in minimizing the risk of THE transmission of bacterial diseases. Early detection is especially important in food production, water treatment, and hospitals' facilities, where human contraction is frequently possible. Conventional microbiological and biochemical detection methods are laborious, require skilled individuals, and are time-consuming. Given the time it takes to produce results, this often means that more individuals have the potential of contracting the pathogen. In contrast, molecular diagnostic tools are very time-effective, but not cost-effective at producing similar results.

Phage-based biosensors now offer an alternative to these molecular and traditional methods while offering specificity, sensitivity, and cost-effectiveness (Machera et al., 2020). For example, *Staphylococcus aureus* (*S. aureus*) was efficiently detected using a biosensor that consists of mutated phage lysin LysGH15 and biolayer interferometry (BLI) technology, which is a novel label-free optical analysis technique that offers results in real time (Liu, 2022). While a C54A mutation resulted in the loss of the lytic activity of the phage lysin LysGH15, the mutant still retained the ability to bind and report the presence of *S. aureus*. This BLI-based biosensor has a highly sensitive detection limit of 13 CFU/mL and a binding/reporting time of 12 min (Liu, 2022). The literature on phage-based biosensors has been increasing steadily and is evidence of the growing interest in this area (Abril, 2022; Al-Hindi, 2022; Husain et al., 2021; Juusti, 2023).

4.7 Phage as a biofilm wiper

Bacteria can attach to surrounding surfaces and develop biofilms. Biofilm-associated cells (sessile cells) are different from their suspended counterparts (planktonic cells) by producing extracellular polymeric substances and have reduced growth rates and different gene profiles (Donlan, 2002). Biofilm formation has great importance for public health because of its role in certain infectious diseases and its importance in a variety of device-related infections. Biofilm formation has also become an important concern with regards to animal health, plant pathogenic bacteria, and biofouling of industrial applications (Elois, 2023). Exacerbating this problem is the fact that biofilm-growing bacteria are recalcitrant to treatment. The biofilm matrix protects bacterial cells against dehydration,

oxidation, antibiotics, ultraviolet radiation, and other agents (Flemming & Wingender, 2010). Phages are emerging as promising alternatives for treatment of these biofilm bacteria since many have enzymes that target the biofilm (termed biofilm depolymerases) (Cooper, 2023). When the phages degrade the biofilm material with these enzymes, they can mitigate the growth and spread of these colonies (Cornelissen et al., 2011; Hanlon et al., 2001; Harper et al., 2014). In addition to these biofilm degrading enzymes, phages also have a repertoire of other enzymes that specifically break down host barriers to enter, replicate, and lyse their bacterial hosts. This enzyme repertoire includes other depolymerases for degrading capsules, LPS or exopolysaccharides, virion-associated lysins for degrading the peptidoglycan layer to allow entry and exit of phage and their progeny (Abdelrahman, 2021; Sharma et al., 2018). Several studies have now shown the potential of using these phage-derived enzymes as alternatives to antibiotics (called enzybiotics) or for biofilm degradation (Abdelrahman, 2021; Chang et al., 2022; Chen, 2022; Ferry et al., 2021; Knecht et al., 2019; Ling et al., 2022; Nazir et al., 2023; Singh et al., 2022). One prominent example that shows the promise of using phage as a biofilm wiper is the use of a phage cocktail against *Proteus mirabilis*, causing catheter-associated urinary tract infections, when used in gel or liquid form to wipe the surface of the catheter, which showed very good phage destructive effect on the biofilm (Maszewska et al., 2018). Similarly, a phage cocktail against *P. aeruginosa* showed a 99.9% reduction in biofilm formation (Fu, 2010; Singh et al., 2022).

4.8 Antiphage

In contrast to the techniques mentioned above, the development of antibacteriophage drugs is also an attractive research topic for academics and industry. Not only can these drugs be used to support bacterial-based industries that require healthy bacterial cultures, but they can also be used as research tools to further understand/promote phage-based development industries. Ideal targets along these streams for the development of bacteriophage potent inhibitors have focused on key phage machinery assembly elements, such as DNA-packaging motors and the capsid assembly process (Muller, 2021; Pi et al., 2016).

4.9 Miscellaneous

Micropattern fabrications are used commonly in various applications, such as tissue engineering and sensors, due to their unique properties. By taking advantage of the self-assembly of M13 phages, a programmable color pattern has been developed by some phage researchers. This color micropatterning of barcode-like structures has a potential use in encryption of high-security information applications (Nguyen et al., 2021). Using genetically engineered M13 phage, it has been shown that phage can be used as a versatile nanoink for bioprinting of tissue-mimetic 3D structures (Lee et al., 2016). Along these same lines, engineered phage is beginning to be explored as a possible construction material for electronic devices (Cui, 2016). These novel applications are continuing to grow in number and represent the burgeoning interest in exploiting phage for financial gain. Fig. 15.10.

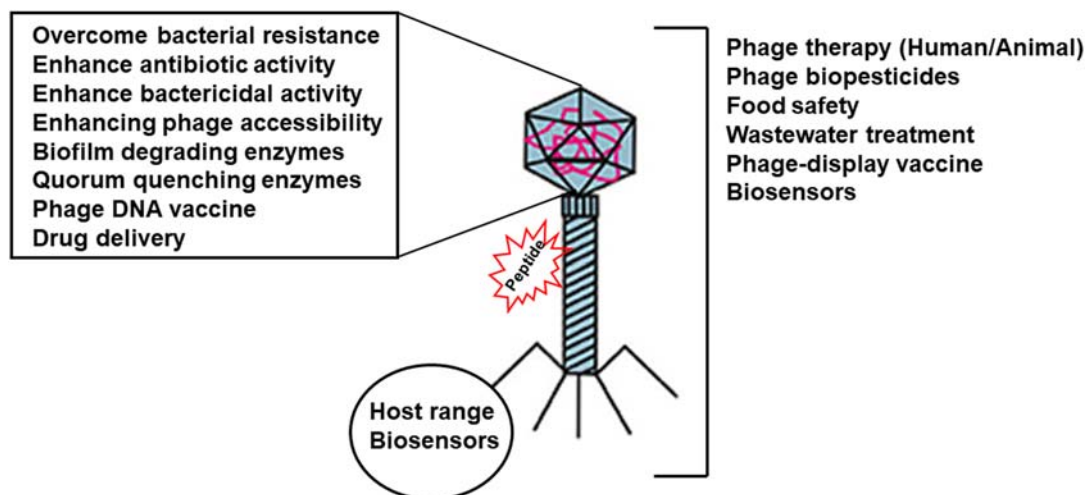


FIGURE 15.10 General applications of natural phages or engineered phages. The listed applications are next to the region of the phage that has been the target site of engineering for these phages.

5. Conclusions

Bacteriophages are amazing biological machines. Their structures, assembly, and machinery have been studied in detail and are leading to a better understanding of the molecular mechanisms they employ that are key to their lifecycle. This information has led to the recent development of novel phage therapy applications and has provided the basis for phage-based applications in other industries, as well (Fig. 15.9). While much of this work is still in its infancy, phage-based research holds great promise for the development of additional novel applications, but also at a fundamental level, to broaden our understanding of these biological entities.

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Advances in fungal biotechnology in agriculture

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1. Introduction

Fungal biotechnology increasingly uses a variety of techniques, including selectable markers (drug resistance, auxotrophic), transformation systems, cosmid and plasmid vectors for transformation, controllable promoters, and reporter genes (lacZ, GFP, GUS), to enable genetic modifications for better economic results. These molecular tools include deep EST sequences (>8000 unigenes), annotated genome sequences, recombination-based vectors, selectable markers, and recombination-based vectors (transposon-arrayed library and arrayed physical map as cosmid or BAC clones). We will go over some of the more recent tools and technologies in this chapter that can be used to promote fungi-based biotechnology programs for agriculture sustainability.

2. Fungi and pest control

2.1 Role of fungi in biological control

In the report of the Food Agriculture Organization of the United Nations (FAO) 2021, trespassing insects produce waste of \$70 billion in the world. These huge damages indicate the significance of biopesticides as a sustainable alternative for insect control. Numerous hazards are related to extending the application of chemical pesticides, mostly the development of resistance and the aggregation of chemical toxins from pesticides in the soil. Thus, it is

transmitted through the food chain, which undoubtedly exposes the world to the problem of food contamination. Therefore, the fundamentals for manufacturing sustainable crops through the save pest management method are being mostly applied recently.

Myco-biocontrol is an ecologically effective product due to its ability to decrease the insect pests by its reactions via the use of the benefits of natural biological enemies in their environment. Fungi are the main attackers on a great number of pest species, and they can manage pest populations normally. Using fungi as natural enemies in fungal biocontrol is not a modern strategy but existed from the late 18th century to the first decade of the century. Due to the risky effects of chemical insecticides like carcinogenic products, pest-resistance, and not environmental save, there has been a revival in the application of biological control agents as fungi.

Plant-incorporated protectants, which are substances produced by plants after genetic modification, natural substances (biochemical pesticides), and microorganisms (microbial pesticides) that naturally control pests, are the three categories of biopesticides that the US Environmental Protection Agency (2022) considers. These goods are crucial for integrated pest management. They have many benefits, including the protection of pollinators and non-target species, the preservation of biodiversity and the natural enemies of invading insects, the reduction of populations resistant to chemical pesticides, and the absence of toxic residues and toxicity for people (Essiedu et al., 2020).

A greater understanding of the relationships between plants, fungi, and insects was made possible by molecular biology and DNA sequencing developments, which also allowed for the collection and classification of organisms.

2.2 Entomopathogenic fungi

Understanding the physiology of the natural parasites of insects is crucial since they are the most numerous groups of animals and are responsible for most of the harm in forest management. Among these biological parasites are entomopathogenic fungi (EPF). These are heterogeneous groups that are divided into various systematized groups, a. The majority of EPF are harmful to insects and other arthropods. These exhibit a higher level of host infection efficacy and can therefore serve as a regulator for controlling the abundance of harmful insects, such as forest insects (pests) (Augustyniuk-Kram, 2012).

As of right now, fungi (true) are classified into four phyla: Basidiomycota, Ascomycota, Zygomycota, and Chytridiomycota. Deuteromycota, an artificial phylum, is also known to contain filamentous fungi that exist in anamorphic forms (asexual). The members of this phylum are actually basidiomycetes or ascomycetes that are either unable to reproduce sexually or whose sexual structures have not yet been identified (Alexopoulos et al., 1996). Additionally, teleomorphs for different genera of Deuteromycete (entomopathogenic) have been identified in a similar manner, and some of these teleomorphs can actually be entomopathogenic (e.g., *Cordyceps* and *Torrubiella* spp.) are recognized teleomorphs of *Paecilomyces* spp. (Samson et al., 1988).

The genera *Metarhizium* (Family Clavicipitaceae), *Beauveria* (Family Cordycipitaceae), *Cordyceps* (Family Cordycipitaceae), *Paecilomyces* (Family Trichocomaceae), and *Akanthomyces* (Family Cordycipitaceae) have the most investigated and marketable species (Litwin et al., 2020).

Fungal organisms have typically been classified using their respective ultrastructure (such as the structure of the septum and that of the cell wall) and morphology (such as the method of cnidogenesis) as the primary standards.

Using genomic studies (e.g., mtDNA restriction analysis) and an assessment of their digestive systems (e.g., synthesis of toxins and enzymes and nutrition use), it has been possible to more precisely group EPF into recognized scientific taxa. rRNA sequences, karyotyping, and length polymorphism allowed for the more precise classification of EPF into defined scientific taxa. Using genomic analysis (including karyotyping, rRNA sequencing, and mtDNA restriction length polymorphism) ([Khachatourians, 1991](#)).

2.3 Virulence mechanism

There are several phases that are involved in adherence and degradation by these fungi that infect insects: (a) Adhesion (b) germination (c) differentiation (d) penetration. Here, a diverse range of intrinsic and extrinsic factors work together to determine pathogenicity at each stage. It is necessary for the infection to begin the process of fungal spore attachment or adhesion to the host cell; typically, this is accomplished by the creation of mucilage. Other enzymes, lectins, and various sorts of contacts, such as electrostatic forces and hydrophobic interactions, also play a significant role in this process of infection in addition to mucilage ([Boucias et al., 1998](#)).

The insect's epidermis is hydrolyzed with enzymes following attachment, which controls the pathogenicity. enzymes; among these secreted enzymes, lipases, proteases, and chitinases are mentioned ([Smith & Grula, 1981](#)). These enzymes are created successively, depending on the substrate encountered.

Numerous elements, including nutrition, fatty acids, water, cuticle surface ions, host physiological condition, etc., have an impact on spore germination and behavior ([Hassa, 1989](#)). The prerequisites for effective germination include absorption of the edible nutrients and tolerance against the poisonous substance that is present on the surface ([Latge et al., 1987](#)). The creation of the appressorium, a specialized structure that adheres to the cuticle layer and creates a restricted bridge, or the germ tube itself, allows entry into the insect lumen through the cuticle layer ([Boucias & Pendland, 1982](#)).

The bridge subsequently creates a passageway for fungus hyphae to enter the insect's body ([Wraight et al., 1998](#)). There are two methods for achieving penetration: Mechanical and enzyme-assisted routes ([Leger et al., 1988](#)). The specific ingress mechanism varies depending on the species. During the penetration phase, a number of cuticle-degrading enzymes are created ([Gillespie et al., 1998](#)). The most likely candidates for mycoinsecticides in agriculture, horticulture, and forestry are entomopathogens.

When an arthropod's integument is penetrated, the cuticle breaks down because of mechanical pressure and the activity of numerous enzymes. Proteases and chitinases are the two major types of enzymes involved in this process ([Leger et al., 1988](#)). Proteinases, often known as proteases, are enzymes that function by catalyzing the breakdown of the peptide link between the amino acid residues of peptides and proteins (EC 3.4). Additionally, chitinases (EC 3.2.1.14) break down the glycosidic bonds between N-acetyl-glucosamine residues connected by [1-4]-type linkages of chitin. These enzymes play a crucial role in the infection process and are closely related to a fungal isolate's pathogenicity ([Andreis et al., 2019](#); [Mondal et al., 2016](#)).

2.4 Evolution of fungal entomopathogenicity by molecular biology or biotechnology

With the help of symbiosis theory and developments in molecular biology and DNA sequencing, it has been possible to collect and classify species and gain a deeper understanding of the relationships between plants, fungus, and insects. Due to the worrying environmental effects of the widespread use of synthetic substances, research interests have recently shifted to non-chemical pest control methods, which has brought EPF back into the spotlight.

Recently, new techniques have been used to increase the virulence of entomopathogenic fungus, including genetic engineering and several protease recombinations. The experimental transformation of *Metarhizium anisopliae* (*M. anisopliae*) involved the insertion of the aaIT gene. The *Androctonus australis* scorpion's aaIT gene, which is controlled by the mcl1 promoter, expresses a neurotoxin in its haemolymph (Wang & St Leger, 2007). Co-transformation of Pr1 and the aaIT gene from *A. australis* was used to genetically modify *Beauveria bassiana* (*B. bassiana*). The chit1 gene was used to characterize chitinase from *M. anisopliae* for the first time (Baratto et al., 2006). Chit1, a 42 kDa protein, and the chitinases from *Aspergillus album* and *Trichoderma harzianum* have been determined to share a significant amount of homology. In addition, the *M. anisopliae* chit2 gene produces a 42 kDa chitinase protein that is subject to catabolic suppression (Fang et al., 2009). The chit2 gene had the same signal transduction-based regulation mechanism as proteases (Boldo et al., 2010). Two distinct transcripts from RNA splicing were found by analyzing the chit2 gene expression profile; these transcripts encode two distinct proteins with two different biological functions (Xiao et al., 2012).

To evaluate the fungal entomopathogenicity of *B. bassiana*, which is a pathogen of hundreds of insect species and is commercially produced as an environmentally friendly mycoinsecticide. A phylogenomic investigation of the *B. bassiana* genome verified the polyphyletic nature of ascomycete entomopathogenicity while also demonstrating convergent evolution of insect pathogenicity. Additionally, a large number of species-specific virulence genes as well as gene family expansions and contractions that relate to host ranges and pathogenic tactics were discovered (Fan et al., 2007).

The results of a genetic analysis of *B. bassiana*, one of the most extensively researched and applied insect biocontrol agents. Bb and Cm are closely related and evolved as insect pathogens independently of the *Metarhizium* lineage, according to a comparison with the genome sequences of three other insect pathogens (Lee et al., 2018; Xiao et al., 2012). Therefore, the similar expansion of some gene families was assumed, such as proteases and chitinases. This reflected convergent evolution and was linked to functions required for insect pathogenesis (McKinnon et al., 2018).

The production of a recombinant Bbchit1 gene, created by fusing the Bbchit1 gene with the chitin binding domain (ChBD), under the regulation of the promoter with overexpression of chitinase, and decreasing the desiccation period of the infected insect, improved the virulence of *B. bassiana* for silkworm mouth *Bombyx mori* (*B. mori*) (Liu et al., 2017). After that, it was discovered that a hybrid protein created by recombining the CDEP-1 gene of *B. bassiana* with the ChBD fragment from *B. mori* had serine protease activity, increasing the ability of proteases to bind to chitin if insect cuticle was present. Due to the solubilization of protein components during insect cuticle degradation, this recombinant strain demonstrated enhanced pathogenicity over *Myzus persicae* larvae (Smith et al., 2016).

The genome also showed that *B. bassiana* is heterothallic, like the closely related *Cordyceps militaris*, even though its sexual stage is rarely seen. *B. bassiana* was found to be able to sense and adapt to various environmental niches by activating well-defined gene sets, according to a high throughput RNA-seq transcriptomic investigation. The goal of these studies was to help the advancement of *B. bassiana* as a reasonably priced, useful, and affordable mycoinsecticide.

2.5 Applications of entomopathogenic fungi as biopesticides and arbuscular mycorrhizal fungi in sustainable agriculture

By 2050, the world's population is projected to reach 9.1 billion people (Liu et al., 2017), hence efforts are being made to ensure sustainable agricultural output. However, these attempts are hampered by issues such as overuse and dependence on synthetic fertilizers and pesticides, climatic changes, inadequate land management, and widespread urbanization (Smith et al., 2016).

EPF have the potential to be used as biocontrol agents against herbivores, offering an environmentally sustainable alternative to traditional insect pest management strategies (West and Gwinn, 1993). In the right circumstances, EPF are recognized for their capacity to directly colonize an insect's body by piercing its cuticles, resulting in illness. More than 700 species from about 90 different genera have been identified as insect-pathogenic fungi thus far (Khachatourians & Qazi, 2008).

The most well-known strains from the genera *Beauveria*, *Metarhizium*, *Isaria*, *Hirsutella*, and *Lecanicillium* are included in this group (Khachatourians & Qazi, 2008). The most often researched fungi among them are *B. bassiana* (Balsamo-Crivelli) Vuillemin, *Isaria fumosorosea* (*I. fumosorosea*) Wize, *M. anisopliae* (Metschnikoff) Sorokin, and *Lecanicillium lecanii* (Zimmerman) Viegas (Chen et al., 2015; Li et al., 2011). Many EPF species, such as *I. fumosorosea* and *I. farinosa*, can infect several hosts without displaying any of the numerous negative effects linked to chemical pesticides (Gao et al., 2017). As a result, they are regarded as harmless to the environment.

Endophytic EPF's many characteristics ensure that, in addition to their traditional use as insecticides, they can also be embraced as helpful plant growth-promoting microbes, and they have so far demonstrated significant potential as biofertilizers. These endophytic fungus species are thought to offer an eco-friendly and effective alternative to systemic fertilizers in the pursuit of food security (Glick, 2014). Fungal endophytes are increasingly being used in organic farming systems to increase yields and safeguard plants from harm (Shrivastava et al., 2010). For their potential as indirect biocontrol agents in extensive agricultural applications, a number of endophytic fungus species have been highlighted (Lacey & Neven, 2006). By inoculating plants with modified fungal strains, biotechnology can be used to improve crops while lowering their toxicity to humans, livestock, and the environment. In order to genetically alter the genes of fungal endophytes, either harmful genes must be eliminated or new, advantageous genes must be added (Adeleke & Babalola, 2021). Then, endophytes might be utilized as stand-in hosts to genetically modify crops. The decrease in endophyte has been successfully genetically altered using this technique and used as a deterrent to herbivores (Murray et al., 1992).

A significant evolutionary adaptation to the land environment is the coevolution of mycorrhizae and plants. By collaborating symbiotically with several agricultural plants, arbuscular mycorrhizal fungi serve as a bioinoculant and contribute to sustainable agriculture (Begum et al., 2019; Kalamulla et al., 2022; Rashad et al., 2023, pp. 415–445). Following the “Green Revolution,” farmers rushed to adopt high-input farming techniques that involve synthetic inorganic fertilizers and agrochemicals, intensifying the production processes required to meet the world’s food demand without considering the effects of agrochemicals on the functioning of natural ecosystems. Degradation of soil quality and disruption of the performance of natural ecosystems are obvious, endangering environmental sustainability and possibly serving as a warning for future food security. The establishment of sustainable agriculture is a better solution to resolve this issue. Utilizing environmentally friendly methods in all aspects of farming is the main goal of sustainable agriculture (Ataei et al., 2021; Gnanaprakasam & Vanisree, 2022; John & Babu, 2021).

3. Fungal bioremediation of organic pollutants: Mechanisms, advantages, constraints and future perspectives

As a result of rapid industrialization and civilization, many persistent pollutants of inorganic and organic nature invade the terrestrial, aquatic, and atmospheric ecosystems. Consequently, these emerged pollutants cause many deteriorations to ecosystems’ balances thus raising many complicated human health issues, from immortality to even death. The conventional clean-up techniques implement physical, mechanical, thermal, and/or chemical techniques, with many technical limits on a large scale. Applications of conventional clean-up methods are expensive, energy-consuming, and environmentally disruptive techniques (Yavari et al., 2015). So, it is mandatory to implement alternative remediation techniques.

Fungi are considered a powerful remediation tool for many organic pollutants in different environments, including aquatic, terrestrial, and atmospheric. As fungi have significant potentiality to decontaminate wide pollutant spectrum such as polycyclic aromatic hydrocarbons (PAHs) (Aranda, 2016; Kadri et al., 2017), explosives (Anasonye et al., 2015), radionuclides (Wollenberg et al., 2021), pesticides (Bose et al., 2021; Maqbool et al., 2016), pharmaceuticals (Asif et al., 2017; Naghdi et al., 2018), although fungi handle different environmental organic pollutants, these pollutants share the same fungal bioremediation techniques. The fungal remediation techniques are categorized into two major techniques based on the fungal exploitation methodology, which are (a) fungal cell-based bioremediation and (b) fungal cell-free based bioremediation (Fig. 16.1). In fungal cell-based bioremediation, the fungal cells or mycelia are considered as the maestro controlling the remediation process. On the other hand, fungal cell-free based bioremediation depends on fungal mycelia exploitation as biofactories producing bio compounds, which in turn are used as remediator agents.

3.1 Fungal cell-based remediation technique

Regardless of fungal species or even genus, the fungi have intracellular and extracellular mechanisms for organic pollutants’ remediation. In fact, mycelia depend on the combination between these two cellular mechanisms, maximizing their remediation potentialities.

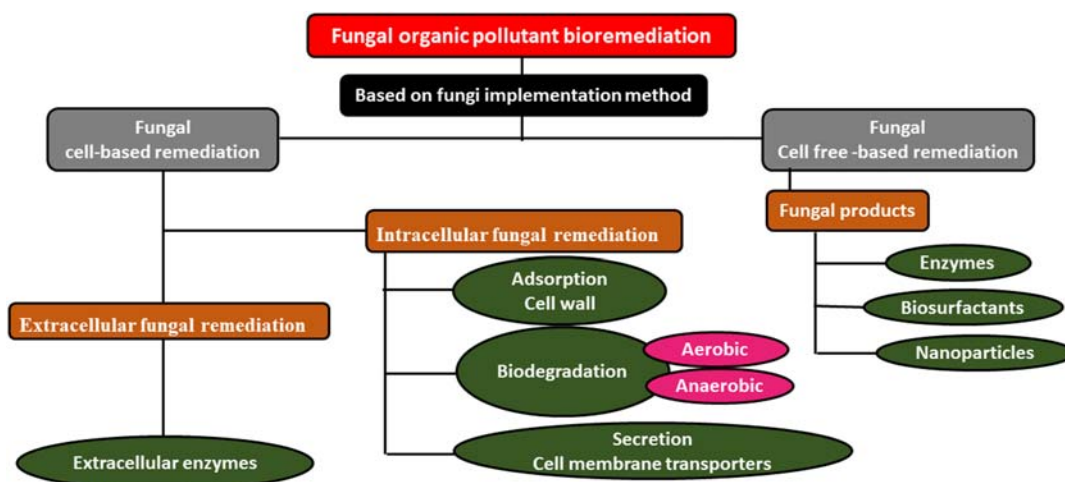


FIGURE 16.1 Diagram represents the possible workflow of the fungal-based bioremediation of the organic pollutants.

By intracellular fungal remediation mechanism, the organic pollutant passes through three major stages till its remediation, which are (a) passage through the cell barriers (cell wall of plants and fungi) followed by (b) pollutant detoxification and finally (c) cellular secretion (Sang & Hulvey, 2018, pp. 1–17). First, the organic pollutant passes through the cell's physical barriers, which are cell walls, to invade the cell and be processed by its intracellular remediation mechanism. Such a pollutant's cell wall passage takes place through the adsorption process.

Adsorption is a physical interaction between an extracellular compound and the chemical constituents, amino polysaccharides of chitosan or chitin, of the cell wall (Omoregie et al., 2018), leading to accumulation or concentration of such compound onto the cell wall with synchronized reduction in the environment (Dhankhar & Hooda, 2011). In fact, the pollutant adsorption to the cell wall takes place in both alive cell wall-surrounded organisms and dead biomasses as well (Kumar et al., 2020; Vahabisani & An, 2021). Therefore, the adsorption process has significant roles in pollution bioremediation using natural biosorbents. Many studies proved that the adsorption mechanism is not restricted to heavy metals (Medfu Tarekegn et al., 2020; Shrivastava & Gupta, 2022), but also with different types of organic pollutants such as PAHs (Vahabisani & An, 2021), dyes in industrial effluents (Maurya et al., 2006), pharmaceuticals (Jureczko & Przysaś, 2021) and pesticides (Denizli et al., 2005).

After pollutant adsorption to the cellular barrier, the pollutant undergoes the detoxification stage. Pollutant detoxification is defined as sets of biological reactions chemically converting the pollutant structure from toxic to less toxic. So, pollutant biotransformation is considered a mitigating mechanism for living organisms to adapt to environmental pollution (Sinha et al., 2009).

The extracellular fungal remediation mechanisms depend on extracellular fungal production of biological agents, facilitating pollutant dissimilation. These biological remedying agents can be classified into two groups that are (a) extracellular enzymes and

(b) extracellular non-enzymatic biological agents (Ghosh et al., 2023). There are different sets of extracellular fungal enzymes with significant potential for degradation of a wide spectrum of organic contaminants. The fungal extracellular enzymes including laccases (Riva, 2006), peroxidases (Bansal & Kanwar, 2013) and hydrolases (Imran et al., 2016) have wide applications for bioremediation approaches of organic pollutants as synthetic dyes (Ba & Vinoth Kumar, 2017), aromatic pollutants as PAHs (Asgher et al., 2018), herbicides and pesticides (Bilal et al., 2019). There are many fungi produced and secreted extracellular biosurfactants that improve the contaminated regions' bioremediation (Moussa et al., 2023). The biosurfactants are natural surface-active constituents with amphipathic structures that have both hydrophobic and hydrophilic parts (Satpute et al., 2010). Such structural criterion of the biosurfactants makes them potential natural adsorbents of different organic pollutants as crude petroleum oil (Felse et al., 2007), synthetic dyes (Perez-Ameneiro et al., 2015) and pharmaceuticals (Malkapuram et al., 2021).

Although fungi have significant bioremediation potentialities, their implementation on a large scale is still facing many challenges in bioaugmentation techniques. There are some complications to exploiting the fungi as remediating agents in situ decontamination. These obstacles are related to bioaugmentation issues. Fungal bioaugmentation is a treatment process that involves inoculation of a living organism into the contaminated environmental matrix to improve the bioremediation potentiality of the ecosystem (Tigini et al., 2019). However, there are possibilities for bioaugmentation failure, mainly in situ bioaugmentation (Kim et al., 2008).

3.2 Future prospective

To benefit from the advantages of fungal bioremediation and remove or, at least, decrease its implementation obstacles at a large scale, there is another fungal-based bioremediation approach, which is a fungal cell-free-based biodegradation approach. Such an approach uses the fungi as biofactors to produce bio-based remediation agents, which in turn can be applied in situ or ex situ, so known as the cell-free approach. After enzyme or biosurfactant production, these agents can be immobilized on solid supporting materials, avoiding the complications related to living cell implementation. Add to this, fungi are used to produce fungal nanoparticles of biopolymers, for example, enzymes, polysaccharides, and proteins mostly secreted extracellularly with nanoadsorptive and catalytic breaking down capabilities removing different hydrocarbon pollutants (Alao & Adebayo, 2022).

3.2.1 Bioinformatics for bioremediation improvement

Bioremediation is considered an eco-friendly restoration method of contaminated environments using living organisms such as bacteria and fungi and has different types (Kumar et al., 2022). Based on anthropogenic intervention, there are two types of bioremediation techniques: (a) natural attenuation and (b) bio stimulation. The natural attenuation is a bioremediation technique that takes place naturally or spontaneously without any anthropogenic intervention mitigating the harmful effects of pollutant(s) in the environmental matrix using the endogenous microflora of the contaminated site (Sokal et al., 2022, pp. 3–36). Like any technique, natural attenuation has its benefits and constraints, as this technique is a

spontaneous, eco-friendly, and cost-effective restoring technique. However, the natural attenuation exhibited limitations in terms of complete remediation of the pollutant(s) and long-term, significant reduction of pollutant concentrations, resulting in dramatic disasters in the polluted sites. The natural attenuation's limits are due to unoptimized abiotic and biotic factors in the contaminated environments.

Regarding the abiotic factors (Sokal et al., 2022, pp. 3–36) (Fig. 16.2), there are two subclasses that are related to the environmental conditions as temperature, pH, moisture content, and availability of nutrients and oxygen. While the other abiotic factors subclass is related to the pollutant's information, such as the pollutant's physicochemical properties and hence its bioavailability and pollutant concentration, in the biotic factors (Sokal et al., 2022, pp. 3–36) (Fig. 16.2) are related to the endogenous microflora of the contaminated site, which are the taxonomical identification of the microflora means the presence or absence of pollutant-degrading microbes, population densities of each microbial strain especially of the pollutant-degrading species (El Fantroussi & Agathos, 2005), and inter- and intra-species interactions (Kong et al., 2018; Li et al., 2022). For instance, microbe-microbe interactions are divided into six types based on the interaction's nature, which are (1) cooperation, (2) competition, (3) predation, (4) commensalism, (5) amensalism, and (6) neutralism. That means there are microbial interactions that are harmful to the pollutant-degrading microbes, resulting in reductions in their bioremediation capacity and efficiency in the polluted sites.

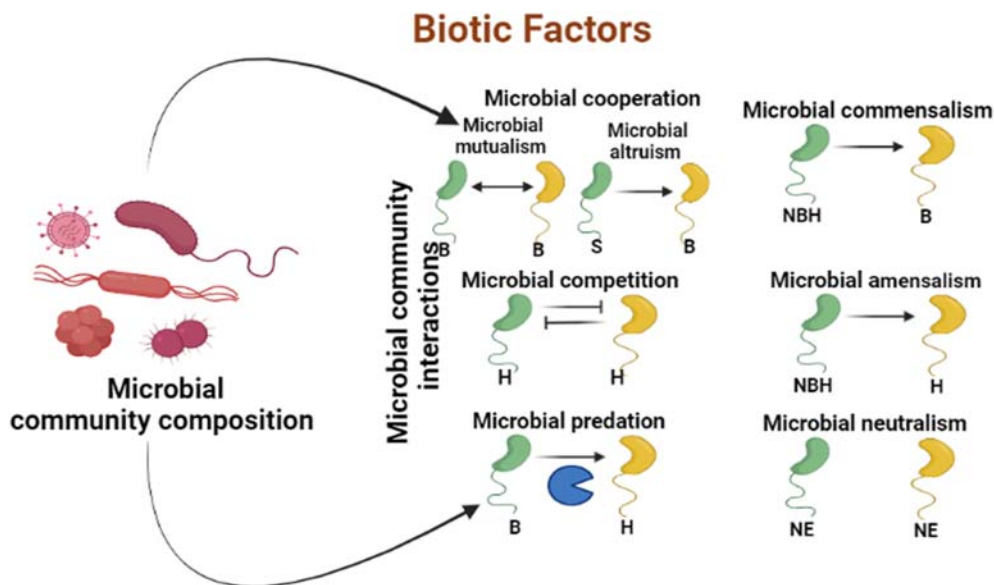
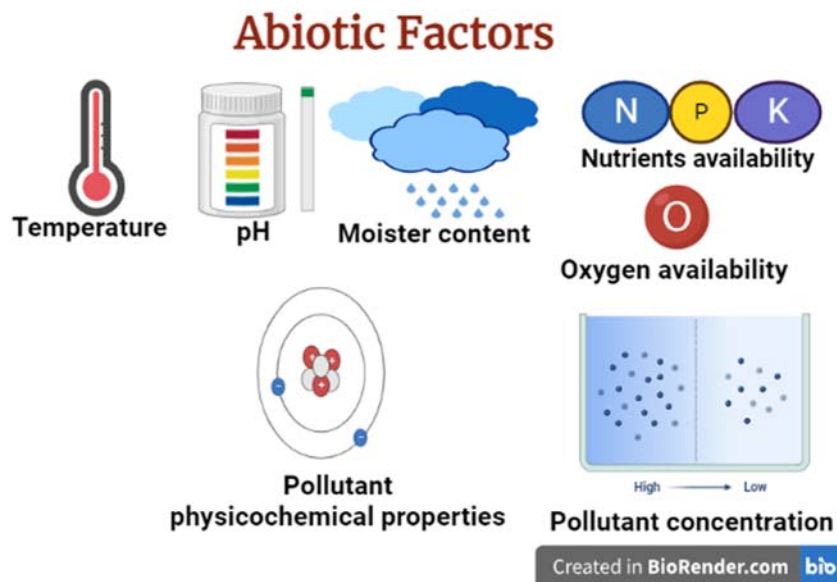


FIGURE 16.2 Abiotic and biotic factors affect the capacity and the potentiality of the natural attenuation of polluted environmental matrices. The taxonomic identification of the endogenous microflora of contaminated sites implies the presence or absence of pollutant-degrading microbes. The capital letters indicate the microbial status in each relationship: B, benefited; H, harmed; NBH, not benefited or harmed; NE, not affected, and S, sacrificial. Created with BioRender.com.



Construction of a synthetic microbial consortium for efficient bioremediation is considered a critical step in bioaugmentation success. The synthetic microbial consortium should consist of potent pollutant-degrading microbes with beneficial microbial inter- and intra-species interactions for efficient restoration. The determination of these potent pollutant-degrading microbes can be inspired by the contaminated sites themselves. So, bioinformatics application in this era has pipeline starting from exploring potent pollutant-degrading microbes to testing and verification of the postulated bioremediation model passing through omics techniques and different prediction algorithms for best bioremediation models (Fig. 16.3). The pipeline consists of major seven phases: (1) Selection of the study location that is contaminated with target pollutant; (2) sampling of the polluted environmental matrix in aseptic containers; (3) extraction of the targeted biomolecules, which are metagenome, transcriptome, proteome, and metabolome to identify the microbial consortium composition (microbial strains and their population densities) in the polluted site; (4) exploration of novel and similar pollutant degradation pathways using specific databases; (5) modeling of the most promising microbial strains combination for most efficient pollutant bioremediation; (6) verification of capacity and efficiency of the postulated microbial consortia; and (7) in situ verification of the constructed synthetic microbial consortia under uncontrolled field's condition. In fact, metadata such as metagenome, transcriptome, proteome, and metabolome and their integration together become essential keys for taking a close-up vision about the microbial composition and dynamics in the contaminated site and understanding how professionally manipulates the natural attenuation and enhances environment restoration. So herein, the participation of the metagenome, metatranscriptome, metaproteome, metabolome, and fluxome is discussed. Furthermore, how can these data be used for prediction of bioremediation capacity and efficiency.

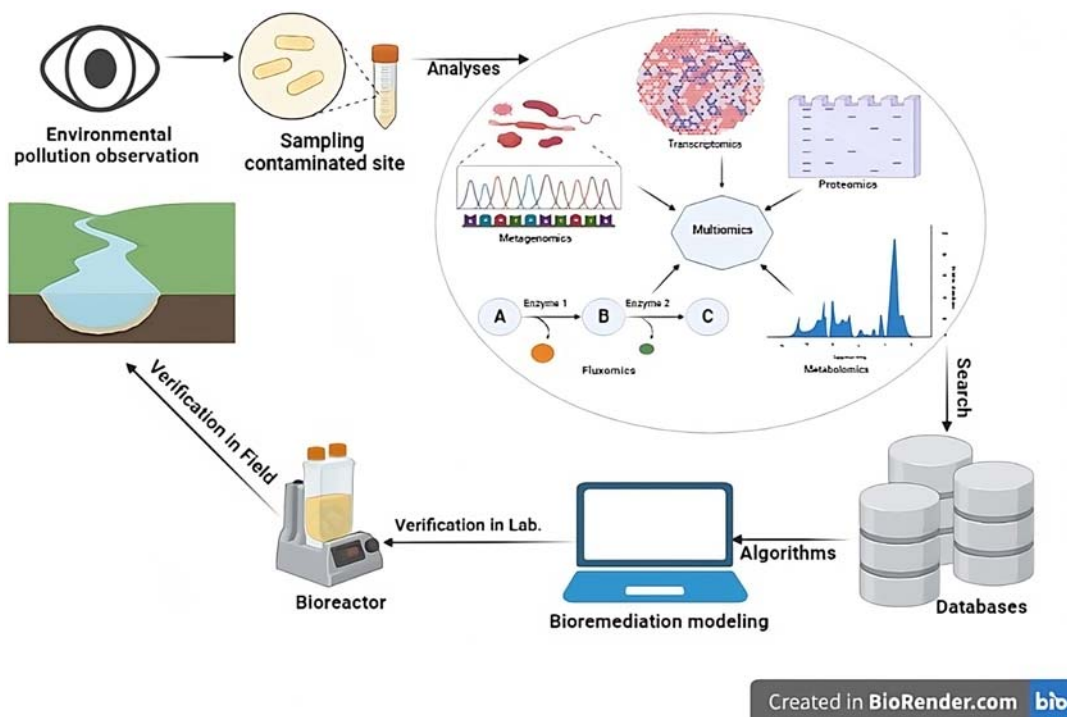


FIGURE 16.3 Bioinformatics pipeline for bioremediation enhancement. Created with BioRender.com.

Metagenomics means sequencing of the genetic material, DNA, of culturable and non-culturable microbial strains of an environmental sample (Grelewski-Nowotko et al., 2018; Jhariya et al., 2022; Moussa et al., 2016, 2017). Based on the DNA sequenced, there are two metagenomics methods, which are targeted and shotgun techniques (Jhariya et al., 2022). The targeted metagenomics is interested in screening of the target gene's sequence throughout the isolated microbial community such as 16S rRNA and 18S rRNA genes for bacterial and fungal species identification, respectively (Moussa et al., 2017; Techtmann & Hazen, 2016). The targeted metagenomics is achieved via polymerase chain reaction using specific primers for 16S rRNA and ITS and next generation sequencing (Moussa et al., 2017; Techtmann & Hazen, 2016).

The targeted metagenomics aims to illustrate the taxonomic identification and population densities of the isolated microbial strains from the studied contaminated site and compare those identified from non-contaminated locations (Noor et al., 2021). Based on microbial community composition, we can suppose the pollutant-degrading microbial strains are supposed to have the highest population densities in the contaminated location compared with non-contaminated ones. As the relatively higher microbial population densities under contamination compared with the uncontaminated locations means that these microbial strains can withstand the pollutant's toxicity through its biodegradation, while shotgun metagenomics gives insights about the microbial community functionality besides its taxonomical identification, such detailed insights are the result of getting the whole genome sequences of each

microbial strain. After genome annotation, pools of genes involved in pollutant degradation pathways are identified and hence can predict pollutant degradation pathways and their possible fate in the environment. Identification of possible pathways involved in pollutant degradation needs mainly annotated genes from metagenome analysis, biological pathways especially of hydrocarbon degradation such as Bio-catalysis/Biodegradation database (Ellis & Wackett, 2012) and Kyoto Encyclopedia of Genes and Genomes database (Jhariya & Pal, 2022), and computational servers for comprehensive functional analysis and accurate taxonomic classifications such as Metagenome Analyzer (Huson et al., 2016) and Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (Singh et al., 2009).

Meta-transcriptome means the whole cellular transcription profiles of each microbial strain under certain circumstances (Jhariya et al., 2022). In fact, the transcriptome illustrates details of the regulatory mechanisms, including stress-related genes (Liu et al., 2019). Although metagenomics can indicate which stress-related genes are reported through certain microbial communities, it cannot determine which of these stress-related genes are activated under certain pollution circumstances. On the other hand, the transcriptome illustrates the expressed stress genes specific to the studied environmental pollution. Meta-transcriptomics uses many bioinformatics tools such as Abyss-Explorer, TransAbyss, Scripture, Oases, and Cufflinks (Jhariya et al., 2022).

A metaproteome is the protein profile, protein types, and concentrations expressed from a microbial metagenome of a certain environmental matrix under certain surrounding conditions (Jhariya et al., 2022). Like the metaproteome, the metaproteome clarifies the interacting proteins of the microbial consortium involved in pollutant degradation pathways. Add to this, it's possible to explore novel proteins and pathways essential for pollutant degradation through the metaproteome.

Metabolomics is the identification and quantification of the entire metabolic profile of living organisms (Jhariya et al., 2022). As the metabolome illustrates, the metabolites are mains or byproducts of the biological pathways, indicating the possible fates of targeted pollutants undergoing degradation (Aguiar-Pulido et al., 2016).

Fluxome is interested in dynamics regulating the biological pathways constructing a cellular network flux to emphasize the cellular dynamics in pollutant's bioremediation (Datta et al., 2020; Jhariya et al., 2022). Therefore, understanding the fluxome of a microbial consortium under pollution is important to predict the rate-limiting steps throughout the degradation pathway and hence control the pollutant's degradation activities. The pollutant's degradation pathway dynamics are predicted via different algorithms, as the most widely applied one is the flux balance model (FBA) (Singh et al., 2014).

Although each "omics" approach clarifies details of the whole microbial community in a polluted site, the multiomics approach is the most powerful bioinformatics participation (Fig. 16.4). Multiomics is the integration of different metadata: Metagenomics, meta-transcriptomic, metaproteomic, metabolomics, and fluxomic, giving whole details about the microbial community taxonomical composition and functionality under pollution. In addition, structural bioinformatics in terms of the molecular docking tool is used to suppose certain degradation pathways from the suspected list provided by the metagenome, meta-transcriptome, and metaproteome, such that the molecular docking can predict the interaction possibilities between the targeted pollutant and the enzymes of different suspected degradation pathways based on the binding affinities' scores (BAS) of enzyme-pollutant complexes

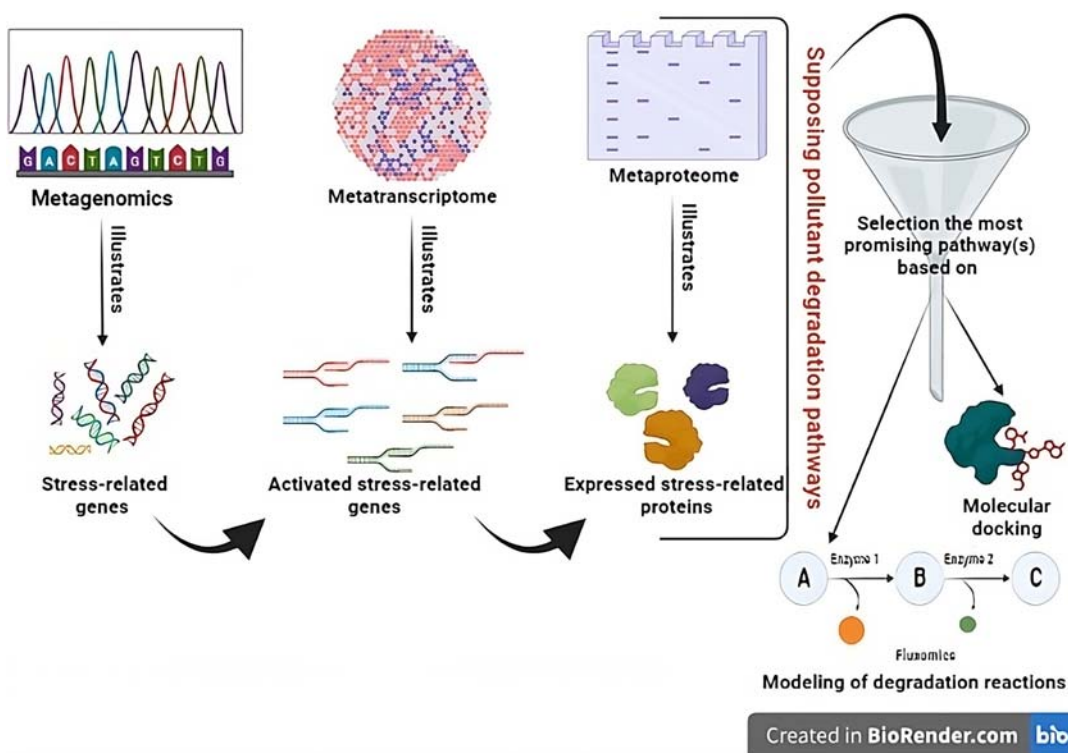


FIGURE 16.4 The diagram represents the multiomics function to select the possible promising pollutant-degrading fungi and hence the possible promising microbial consortium composition. Created with BioRender.com.

(Naqvi et al., 2019). As a result, the pathways with higher BAS are selected as promising candidate pollutant-degrading pathways. Using fluxome, we can understand these pathways' dynamics and hence control the pollutant's degradation activities by the microbial consortium.

4. Fungal genetic engineering

Numerous fungi can be found in a variety of habitats, including soil, biological waste, and plants. Filamentous fungi have attracted a lot of attention due to their outstanding capacity as cell factories for producing useful products and many degrading enzymes in nature. Numerous fungus species are employed in agriculture to manage plant pests and diseases (Fernández-Bodega et al., 2009; Huang et al., 2007). Fungi are significant producers of secondary metabolites and valuable proteins, which contribute significantly to the economy. A new age of functional investigations is beginning in mycology as a result of the proliferation and exploration of fungal genome sequence data (Michiels et al., 2005).

The development of genetic transformation technologies has made it possible for researchers to introduce foreign DNA into filamentous fungi and so produce the appropriate strains with enhanced target traits for agricultural uses. All major methods of genetic transformation for

fungi, including the protoplast-mediated transformation (PMT) method and the *Agrobacterium*-mediated transformation (AMT) method, which are common methods used for many years, the electroporation method and the biolistic method, which are less well-known but are particularly suitable in some specific circumstances, the emerging shock-wave-mediated transformation (SWMT) technology and the genome editing technology (Li et al., 2017).

4.1 Protoplast-mediated transformation (PMT) method

PMT is the most COMMONLY used fungal transformation method, which relies on the generation of significant quantities of suitable protoplasts. In order to prepare protoplasts, the cell wall must be removed, which is mostly accomplished via either enzyme or non-enzyme procedures (Li et al., 2017). Non-enzyme (physical) methods to prepare protoplasts, including grinding and supersonic wave shock. The limited yield of protoplasts and practical difficulties prevent them from being widely employed (Tan et al., 2006).

The fungal cell wall components vary among fungal species, but are comprised mainly of glucan, mannan, and chitin. Therefore, the idea behind enzyme techniques is to use some combined commercially available enzymes to remove the complex components of fungal cell walls to produce competent protoplasts for some common fungal species such as *Neurospora crassa* (Case et al., 1979), *Aspergillus* sp. (Azizi et al., 2013), *Penicillium* (Jun et al., 2007), *Trichoderma* sp. (Gruber et al., 1990; Penttilä et al., 1987), *Rhizopus* (Zhang, Sun et al., 2015), *Monascus* (Campoy et al., 2003), and *Saccharomyces cerevisiae* (*S. cerevisiae*) (Hutchison & Hartwell, 1967) (Table 16.1).

4.2 The *Agrobacterium*-mediated transformation method

Agrobacterium tumefaciens (*A. tumefaciens*) is a Gram-negative bacterium inhabiting the soil that mainly infects injured plants. Throughout the injury process, *A. tumefaciens* enters the plant through the wound, and incorporates part of its tumor-inducing plasmid (Ti plasmid) into the genome of the infected plant cells. The integrated DNA fragment known as transfer DNA or T-DNA has two repeats on either side (left and right borders) and contains genes that encode enzymes for the production of plant hormones that promote tumor growth (Citovsky et al., 2007).

The positive *A. tumefaciens* clone was used as a binary vector to integrate the target gene into the fungal genome between the left and right T-DNA borders. Since the first AMT was performed for *S. cerevisiae* transformation (Bundock et al., 1995), the technique has been proved to be stable and effective to many ascomycetes, such as *A. awamori* (Michielse et al., 2008), *A. fumigatus* (Michielse et al., 2005) and *A. giganteus* (Meyer et al., 2003), *T. reesei* (Zhong et al., 2007), *F. velutipes* (Cho et al., 2006), *M. circinelloides* (Nyilasi et al., 2005) and *M. purpureus* (Campoy et al., 2003). All those AMT commonly used plasmids carrying a hygromycin-resistance gene (HygB) as selective marker (Table 16.2).

4.3 The electroporation method

Electroporation transformation (ET) is a simple, quick, and effective transformation technique applying either square or exponential decay waveforms for gene transformation of various filamentous fungal species. Numerous investigators applied exponential decay

TABLE 16.1 Protoplast-mediated-transformation of several fungal species.

Fungal strain	Protoplast origin	Vector	Selective marker	Function of gene(s)	References
<i>Trichoderma reesei</i> QM 9414 and VTT-D-79125	Mycelium	pSa143	<i>amdS</i> gene of <i>Aspergillus nidulans</i>	Encoding acetamidase that hydrolyses acetamide to acetate and ammonium	Penttilä et al. (1987)
			<i>argB</i> gene of <i>Aspergillus nidulans</i>	Encoding omithine carbamoyl transferase (OTCase, EC 2.1.3.3) which catalyzes the formation of citrulline from omithine and carbamoyl phosphate	
<i>T. reesei</i> QM 9414	Mycelium	pRLM _{EX30}	<i>pyr4</i> gene of <i>Neurospora crassa</i>	Encoding orotidine-5'-monophosphate decarboxylase	Gruber et al. (1990)
			<i>pyrA</i> gene of <i>Aspergillus niger</i>	Encoding carbamoylphosphate synthase	
<i>T. reesei</i> QM 9414	Mycelium	pRLM _{EX30}	<i>E. coli</i> hygromycin B gene (hph)	Coding for phosphotransferase which inactivates the antibiotic by phosphorylation	Mach et al. (1994)
<i>N. crassa</i> 74A	Mycelium	pVK88	<i>qa-2⁺</i> gene	Encodes catabolic dehydroquinase (3-dehydroquinase hydro-lyase, EC 4.2.1.10)	Case et al. (1979)
<i>A. oryzae</i> NRRL 492	Mycelium	pILJ16	<i>A. nidulans</i> <i>argB</i> gene	Encoding omithine carbamoyl transferase	Hahm and Batt (1988)
<i>A. nidulans</i> strain FGSC237	Mycelium	pHY201	Wild-type copy of the <i>A. nidulans</i> <i>trpC</i> gene	Coding phosphoribosylanthranilate isomerase	Yelton et al. (1984)
<i>M. purpureus</i> NRRL 1596, <i>M. ruber</i> NRRL 1597 and <i>M. purpureus</i> IBCC1	Mycelium	pULJL43	<i>Sh ble</i> from <i>Streptoalloteichus hindustanus</i>	Phleomycin resistance gene encodes a protein that binds to phleomycin, inhibiting its DNA cleavage activity	Campoy et al. (2003)
<i>Aspergillus giganteus</i> IfGB15/0903	Germinated conidia	pAN7-1	<i>hygB</i>	Coding for phosphotransferase	Meyer et al. (2003)

Modified from Li, D., Tang, Y., Lin, J., & Cai, W. (2017). Methods for genetic transformation of filamentous fungi. *Microbial Cell Factory*, 16, 1–13.

waveform for the ET of *S. cerevisiae* (Neumann, 1992), *P. pastoris* (Wu & Letchworth, 2004), *P. urticae*, *A. oryzae*, *N. crassa*, and *L. maculans* (Chakraborty et al., 1991), *A. niger* (Ozeki et al., 1994), *A. nidulans* (Richey et al., 1989) and *T. harzianum* (Goldman et al., 1991, pp. 175–180), while square wave was used for *Rhizopus oryzae* ET (Li et al., 2017).

Table 16.3 summarized the electroporation-mediated transformation protocols performed on several fungal species. Chakraborty and Kapoor (1990) developed a direct electroporation

TABLE 16.2 Mediated-transformation of several fungal species.

Fungal strain	<i>Agrobacterium</i> sp.	Transformation material	Vector	Selective marker	Function of gene(s)	References
<i>A. awamori</i>	<i>A. tumefaciens</i> LBA 1100	Conidiospores	NA	Hygromycin B resistance gene (hph, hygB)	Coding for phosphotransferase which inactivates the antibiotic by phosphorylation	Michielse et al. (2008)
<i>A. fumigatus</i> B-5233	<i>A. tumefaciens</i> EHA 05	Conidia	pDHt/hph			Sugui et al. (2005)
<i>T. reesei</i> QM 9414	<i>A. tumefaciens</i> AGL-I	Protoplasts and conidia	pBI-hph			Zhong et al. (2007)
<i>M. circinelloides</i> ATCC1216b	<i>A. tumefaciens</i> AGL-I	Spores	pTiBo542			Nyilasi et al. (2005)
<i>A. giganteus</i> IfGB15/0903	<i>A. tumefaciens</i> LBA 1100	Germinated conidia	pURS750	Benomyl resistance gene (the <i>ben</i> gene) was cloned from <i>Neurospora crassa</i>	Encodes a β -tubulin, confers benomyl resistance in fungi	Meyer et al. (2003)
<i>F. velutipes</i>	<i>A. tumefaciens</i> AGL-1	Spores	pBG-gHg			Cho et al. (2006/2006)
<i>C. militaris</i> strain CM10 (GIM5.271)	<i>A. tumefaciens</i> AGL1	Spores	pAg1-H3			Lou et al. (2021)

Modified from Li, D., Tang, Y., Lin, J., & Cai, W. (2017). *Methods for genetic transformation of filamentous fungi*. Microbial Cell Factory, 16, 1–13.

TABLE 16.3 Electroporation-mediated-transformation of several fungal species.

Fungal strain	Waveform	Transformation material	Vector	Selective marker	Importance of gene(s)	References
<i>R. oryzae</i> AS3.819	NA	Protoplasts	pBS-hygro-IdhA	<i>hygB</i>	Coding for phosphotransferase which inactivates the antibiotic by phosphorylation	Zhang, Sun et al. (2015) and Zhang, Jiang et al. (2015)
<i>R. oryzae</i> AS 3.819	NA	Germinated spores	pBS-hygro-IdhA	<i>hygB</i>		
<i>A. niger</i> ATCC 20739	Exponential decay waveform	Germinated spores	pXba192 and pBXba2	<i>argB</i> ⁻		Ozeki et al. (1994)
<i>P. urticae</i> NRRL 2159A	Exponential decay waveform	Germinated spores	pCSN44	<i>hygB</i>		Chakraborty et al. (1991)
<i>L. maculans</i> "virulent"	Exponential decay waveform	Germinated spores	pCSN44	<i>hygB</i>		
<i>N. crassa</i> R-206A	Exponential decay waveform	Germinated spores	Bsqa	<i>hygB</i>		
<i>A. oryzae</i> ATCC 14895	Exponential decay waveform	Germinated spores	pBEN	Benomyl resistance gene (the <i>ben</i> gene) was cloned from <i>Neurospora crassa</i>	Encodes a β -tubulin, confers benomyl resistance in fungi	

Modified from Li, D., Tang, Y., Lin, J., & Cai, W. (2017). Methods for genetic transformation of filamentous fungi. Microbial Cell Factory, 16, 1–13.

system for germinating conidia of *N. crassa*. Also Chakraborty et al. (1991) transfer exogenous genetic material into the germinated conidia of *P. urticae*, *A. oryzae*, *N. crassa*, and *L. maculans*. Ozeki et al. (1994) enable the transformation of the germinal spores of *A. niger*. Zhang, Sun et al. (2015) applied this advanced ET to insert genes in protoplasts and germinated spores of *R. oryzae* isolates. While Lakrod et al. (2003) electroporated a mutant albino strain of *M. purpureus*. Table 16.3.

4.4 The biolistic method

The term "biolistic transformation" also refers to the gene gun or ballistics or particle bombardment. In the presence of both calcium chloride and spermidine, foreign DNA gets coated onto the surface of tungsten or gold micro-sized particles. Foreign DNA is made up

of a few terminal DNA bases and the coding region of a specific gene located between the promoter and terminator. Those particles are delivered into host cells and tissues under high pressure. When the foreign DNA reaches the nucleus and is incorporated into the host chromosomes, it realizes both stable and transient transformations (Gouka et al., 1997).

Particle bombardment is the most potent technique for genetic transformation methods. Operating a particle bombardment is simple and practical. However, the equipment and supplies needed for particle bombardment are pricey. For fungi, particle bombardment is effective enough for organisms that are challenging to culture or prepare from protoplasts. Particle bombardment has currently been used to successfully alter *A. nidulans* and *T. reesei* and other organisms (Barcellos et al., 1998; Herzog et al., 1996).

4.5 Shock-wave-mediated transformation method

SWMT is a novel method that applies a brief pressure disturbance and twisting force across cells via the principle of energy transformation and transmission to alter the permeability of cell membranes by creating a temporary cavitation, through which exogenous nucleic acid enters the cells (Ogden et al., 2001). Exogenous foreign nucleic acids and plasmids have been successfully introduced into some bacteria (Loske et al., 2011; Prakash et al., 2011). Recently, this method was efficiently employed for genetic transformation of *A. niger*, *F. oxysporum*, and *Phanerochaete chrysosporium* (Magaña-Ortíz et al., 2013).

4.6 Genome editing technology

The technology for genome editing has advanced quickly in recent years. The key technologies currently in use include clustered regularly interspersed short palindromic repeats (CRISPR) technology (Zhang, Sun, & Li, 2015), transcription activator-like effector nucleases (Tesson et al., 2011), and zinc-finger nucleases (Urnov et al., 2005) to achieve genome-wide gene disruptions, gene replacements, and precision editing. However, filamentous fungi are still in the early stages of using genome editing technology.

Due to their potential as biological control agents and their high enzyme production, researchers engineered strains of *Trichoderma* for desired applications and the future trends in agriculture (Wang, Li et al., 2022). Various strategies were employed in establishing CRISPR-Cas systems to introduce *Cas* nucleases and guide RNAs (gRNAs) into *Trichoderma* spp., as reported by (Chai et al., 2021; Hao & Su, 2019; Rantasalo et al., 2019; Vidgren et al., 2020; Vieira et al., 2021; Wang, Chen et al., 2022; Wu et al., 2019).

To prevent or control fungal diseases of plants, a variety of tactics are being used. With the advance in the CRISPR/Cas9 system, it is now possible to treat plant diseases with novel approaches by modifying the genomes of diverse fungal and oomycete species through mutagenesis of single to multiple CRISPR/Cas9 targets (Paul et al., 2021), as seen in Table 16.4. It is acclaimed that oomycete pathogens release many effector proteins into the tissues of their hosts to inhibit host defense mechanisms and facilitate colonization. Recently, the development of CRISPR/Cas9-mediated gene-editing technology facilitates the genetic identification of the virulence genes of oomycetes (Fang & Tyler, 2016; Gumtow et al., 2018).

TABLE 16.4 Fungal/oomycete genome editing of CRISPR/Cas9 and CRISPR/Cpf1 systems for plant resistance to fungal and oomycete pathogens.

Fungal pathogen	Plant species	Target gene	Encoding protein/function of gene	Methods	References
<i>Claviceps purpurea</i>	<i>Secale cereale</i> (rye)	CpTrpE, Cppyr4	a-subunit of anthranilate synthase/key enzyme of tryptophan biosynthesis orotidine 5'-phosphate decarboxylase/involved in pyrimidine biosynthesis	CRISPR/Cas9 and homologous recombination (HR) knock-out	Králová et al. (2021)
<i>Leptosphaeria maculans</i>	<i>Brassica napus</i> (rapeseed)	AvrLm7	Effector protein/recognized byhost R gene	CRISPR/Cas9	Zou et al. (2020)
<i>Peronophythora litchii</i>	<i>Litchi chinensis</i> (litchi)	PIPAE5	Pectin acetylesterase/consumehost pectin as carbon source	CRISPR/Cas9	Kong et al. (2019)
<i>Phytophthora sojae</i>	<i>Glycine max</i> (soybean)	PsSu(z)12	Core subunit of H3K27me3 methyltransferase/participate ingene silencing	CRISPR/Cas9	Wang et al. (2020)
<i>Phytophthora sojae</i>	<i>Glycine max</i> (soybean)	PsAvr3c	RxLR effector/promotes host susceptibility	CRISPR/Cas9	Huang et al. (2017)
<i>Phytophthora sojae</i>	<i>Glycine max</i> (soybean)	Avr4/6	RxLR effector/promotes host susceptibility	CRISPR/Cas9	Fang and Tyler (2016)
<i>Phytophthora palmivora</i>	<i>Carica papaya</i> (papaya)	PpalEPICs	Cysteine protease inhibitor/inhibition of cysteineprotease in host	CRISPR/Cas9 and <i>Agrobacterium</i> -mediated transformation	Gumtow et al. (2018)

Modified from Paul, N. C., Park, S.-W., Liu, H., Choi, S., Ma, J., MacCready, J. S., Chilvers, M. I., & Sang, H. (2021). Plant and fungal genome editing to enhance plant disease resistance using the CRISPR/Cas9 system. *Frontiers in Plant Science*, 1534.

5. Plants genetic engineering for fungal disease resistance

A sufficient supply of food is required to feed the global population. With an estimated 9.2 billion people on the planet in 2050, there will be a considerable augmentation in the demand for food. In addition to the expanding world population and climate changes, numerous developing, emerging, and introduced plant diseases also pose a threat to global food security (Bebber & Gurr, 2015).

Many plant diseases, including mildews, smuts, blights, rusts, and rots, are caused by fungal pathogens, which have a significant bad impact on agriculture by reducing their yields or even destroying them, causing famine. Although chemical and cultural controls have been employed to protect crops, these methods require ongoing expenses and time. Additionally, reports mention that fungi can adapt to new hosts and develop fungicide resistance due to their diverse fungal lifestyles and great genetic flexibility, which makes disease treatment very difficult (Paul et al., 2021; Yin & Qiu, 2019).

Therefore, researchers use genetic engineering to change the genetic make-up of plants to increase resistance to several microbial infections as an alternative to applying chemical agents. This approach has been achieved through traditional transgenic techniques or more advanced genome-editing methods. Despite their different policies, they continue to be useful and effective tools for crop development (Dong & Ronald, 2019).

5.1 Traditional transgenic techniques

There are numerous intricate systems that have developed in plants in response to pathogen infection. The host-microbe interactions gave researchers insights and ideas for genetically engineering susceptible crops for disease resistance (Dangl et al., 2013). Reprogramming essential defense hub regulatory genes by introducing proteins that can breakdown mycotoxins (Karlovsky, 2011) or halt cell-wall-degrading enzyme pathways can fine-tune defensive responses (Juge, 2006). According to Osusky et al. (2000), these can be accomplished by modifying plants to synthesize and produce antimicrobial peptides and/or other chemicals that directly inhibit colonization.

Transgenic plants that are resistant to fungi have been created using chitinases, glucanases, defensins, and other antifungal genes as defense-responsive genes (Table 16.5). The function of these genes, the various pathways involved in immune reactions following fungal infection, and methods of gene transfer employed to create plants resistant to fungi are also provided.

5.2 The antimicrobial (AM) proteins

Antimicrobial proteins (AMPs) are small proteins produced by prokaryotes and eukaryotes and have antimicrobial activity that efficiently inhibits the growth of viruses, microbes, and parasites. AMPs may give unicellular cells the ability to compete against other species on nutritional and ecological needs, while plants and other multicellular organisms use these AMPs as the first line of defense and innate immunity from pathogen invasion (Hegedüs & Marx, 2013). Plant-derived antimicrobial peptides and other compounds can reduce pathogenicity either by directly detoxifying the organism or by deactivating their virulence factors (Ahuja et al., 2012; Thomma et al., 2002).

The ability of AMP-encoded genes to be either constitutively expressed or rapidly transcribed by induction gives scientists the ability to produce several plant species with increased resistance against phytopathogenic fungi (Hegedüs & Marx, 2013).

5.2.1 Antimicrobial (AM) proteins chitinase

An effective tactic has been achieved to transform crops for phytopathogen resistance by inserting the chitinase gene, which causes an overexpression of chitinase that immediately degrades the chitin component of fungal cell walls (Ceasar & Ignacimuthu, 2012).

It was found that transgenic plants with chitinase genes of plant origins had strong resistance to fungal infection: tobacco chitinase gene (Chi) in peanut (Rohini & Rao, 2001), chitinase gene (RCC2) of rice inserted in *Chrysanthemum* (Takatsu et al., 1999), grapevine (Yamamoto et al., 2000), cucumber (Kishimoto et al., 2002) and trifoliate orange (Mitani et al., 2006), rice chitinase gene (OsChia, RC7 and chi11) to rice again (Datta et al., 2001; Kumar et al., 2004; Takakura et al., 2000), bean chitinase (Chi) gene to cotton (Tohidfar

TABLE 16.5 Transgenic plants with chitinases, glucanases, defensins, and other antifungal genes as defense-responsive genes.

Name of the gene	Source of gene	Methods of transformation	Vectors	Transformed plants	References
Chitinase (chi1)	<i>Rhizopus oligosporus</i>	<i>Agrobacterium</i> mediated	pBI121CH	Tobacco	Terakawa et al. (1997)
Chitinase (RCC2)	Rice	<i>Agrobacterium</i> mediated	pBI121-EN4-	Chrysanthemum RCC2	Takatsu et al. (1999)
Glucanase (SGN1)	Soybean	<i>Agrobacterium</i> mediated	pBI101.1	Tobacco	Cheong et al. (2000)
Chitinase (RCC2)	Rice	<i>Agrobacterium</i> mediated	pBI121-	Grapevine RCC2	Yamamoto et al. (2000)
Trichosanthin (TCS)	Snake gourd	<i>Agrobacterium</i> mediated	pC1301-HY	Rice	Ming et al. (2000)
Chitinase (OsChia)	Rice pistils	<i>Agrobacterium</i> mediated	pYOT175G	Rice	Takakura et al. (2000)
Chitinase (Chi)	Tobacco	<i>Agrobacterium</i> mediated	pBI121-pBTex	Peanut	Rohini and Rao (2001)
Chitinase (RC7)	Rice	Biolistic and PEG-mediated	pGL2RC7	Rice	Datta et al. (2001)
Chitinase (RCC2)	Rice	<i>Agrobacterium</i> mediated	pBI121_/RCC2	Cucumber	Kishimoto et al. (2002)
Chitinase-like-cDNA (Chs2)	American elm	Biolistic	KYLX71-pHS2; JS101	Creeping bent grass	Chai et al. (2002)
B-1,3-glucanase and chitinase genes	Pea	<i>Agrobacterium</i> mediated	pGlu; pChit	Potato	Chang et al. (2002)
Chitinase (CTS1-2)	<i>Saccharomyces cerevisiae</i>	<i>Agrobacterium</i> mediated	pART27: CTS	Tobacco	Carstens et al. (2003)
Ribosome-inactivating protein (MOD1); chitinase (RCH10)	MOD1 from maize; RCH10 from rice	Biolistic	pZRC72	Rice	Kim et al. (2003)
Stress-inducible-glucanase (Gns1)	Rice	<i>Agrobacterium</i> mediated	pBI333-35S-Gns1	Rice	Nishizawa et al. (2003)
Chitinase (RCH10); glucanase (ALG)	RCH10 from rice; ALG from alfalfa	Biolistic	pABT127; pZ100	Creeping bent grass	Wang et al. (2003)
Chitinase (chi11)	Rice	<i>Agrobacterium</i> mediated	pMKU-RF2	Rice	Kumar et al. (2003)
Cationic peptide (msrA3)	Synthetic preparation	<i>Agrobacterium</i> mediated	pDMSRA3-1217	Potato	Osusky et al. (2004)
afp gene	<i>Aspergillus giganteus</i>	<i>Agrobacterium</i> mediated	pBIN61	Olive (<i>Olea europaea</i> L.)	Narvaez et al. (2018)

Modified from Ceasar, S. A., & Ignacimuthu, S. (2012). Genetic engineering of crop plants for fungal resistance: Role of antifungal genes. *Biotechnology Letters*, 34(6), 995–1002. <https://doi.org/10.1007/s10529-012-0871-1>.

et al., 2005), rice chitinase gene (ricchi11) for producing fungal resistant taro (He et al., 2013), and rice chitinase gene (chi11) in finger-millet (Ignacimuthu & Ceasar, 2012).

5.2.2 Antimicrobial (AM) proteins glucanase

In transgenic research to create fungus-resistant plants, the glucanase gene has been assigned the highest priority due to the ability of β -1,3-glucanases to destroy the cell walls of some fungi (Ceasar & Ignacimuthu, 2012).

The soybean glucanase gene was transferred into tobacco (Cheong et al., 2000). Beta-1,3 and 1,4-glucanase genes (Gns1) of rice were reintroduced in rice by Nishizawa et al. (2003), to improve its disease resistance. While Akiyama et al. (2004) reinsert the rice gene OsGLN2. Additionally, β -1,3-glucanase gene (bglu) of potato was inserted into flax to boost resistance to the *Fusarium* infection (Wróbel-Kwiatkowska et al., 2004). The tobacco β -1,3-glucanase gene (GLU) was inserted in the tomato (Chen et al., 2006). Indian mustard was transformed with the tomato glucanase gene to combat the *Alternaria* leaf spot disease (Mondal et al., 2007).

5.2.3 The antifungal protein (AFP)

The mature antifungal protein (AFP) contains 51 amino acids with similar structure to the plant defensins and U-thionine (Campos-Olivas et al., 1995). Although ascomycetes have a growing number of small, cysteine-rich defensin-like AMPs discovered, only a limited number were characterized such as AFP and AFPNN5353 from *A. giganteus* (Binder et al., 2011), PAF and PgAFP from *P. chrysogenum* (Marx et al., 1995; Rodríguez-Martín et al., 2010), bubble protein from *P. brevicompactum* Dierckx (Seibold et al., 2011), ANAFP from *A. niger* (Lee et al., 1999), NAF from *P. nalgiovense* (Geisen, 2000), AcAFP and AcAMP from *A. clavatus* (Skouri-Gargouri & Gargouri, 2008) and NFAP from *N. fisheri* (Kovács et al., 2011). Those proteins exclusively have antifungal activity against filamentous ascomycetes and zygomycetes, opportunistic plant and animal pathogens (Meyer, 2008). In sensitive fungus, the AFP protein compromises the integrity of the plasma membrane and prevents chitin production (Leiter et al., 2017). In addition, AFP may penetrate the host cell and bind to its DNA, promoting its charge neutralization and condensation (Moreno et al., 2006).

The *afp* gene was expressed or overexpressed in some transgenic plants to enhance diseases resistance as in *Oryza* species (Moreno et al., 2005), *Triticum* (Li et al., 2008), tobacco (Park et al., 2002; Swathi Anuradha et al., 2008), apple (Bondt et al., 1996), tomato (Abdallah et al., 2010; Chen et al., 2006), pear (Lebedev et al., 2000), peanut (Swathi Anuradha et al., 2008), olive (Narvaez et al., 2018), *Cenchrus americanus* (Girgi et al., 2006) and others against a wide range of fungal pathogens (Table 16.6).

5.2.3.1 Defensin

Plant Defensin consists of small cationic peptides of 45–54 amino acid residues with six to eight cysteines in form of three to four intramolecular disulfide bonds that stabilize an anti-parallel β -sheet conformation surrounded by α -helical segment, known cysteine stabilized α/β motif (CS α/β), while Defensin-like antifungal AMPs of fungal origin lack the α -helical segment (Hegedüs & Marx, 2013). Accordingly, most plant defensins are effective against a variety of phytopathogenic fungi such as *F. graminearum* (Sagaram et al., 2011), *Botrytis cinerea* (Stotz et al., 2009), and *A. brassicicola* (de Zélicourt et al., 2007). Defensins and defensin-like AMPs may indirectly damage the target cells' plasma membranes by interacting with their lipid and/or protein components, causing their selective ion permeability, its temporal

TABLE 16.6 Some transformed plant with antifungal defensins derived from plants origin.

Name of the gene	Source of gene	Host plant species	Pathogenic fungi tested	References
Rs-AFP2	Radish	Tobacco	<i>Alternaria longipes</i>	Terras et al. (1995)
		Apple	<i>Fusarium culmorum</i>	Bondt et al. (1996)
		Tomato	<i>Alternaria solani</i> <i>Fusarium oxysporum</i> <i>Phytophthora infestans</i> <i>Rhizoctonia solani</i>	Parashina et al. (2000)
		Pear		Lebedev et al. (2000)
		Rice	<i>Magnaporthe oryzae</i> <i>Rhizoctonia solani</i>	Jha and Chattoo (2010)
Pea defensin	Pea	Canola	<i>Leptosphaeria maculans</i>	Wang et al. (1999)
D4E1	Synthetic	Tobacco	<i>Aspergillus flavus</i> <i>Verticillium dahlia</i>	Cary et al. (2000)
BSD1	Stamen	Tobacco	<i>Phytophthora parasitica</i>	Park et al. (2002)
BjD	Mustard	Tobacco	<i>Fusarium moniliforme</i> <i>Phytophthora parasitica</i>	Swathi Anuradha et al. (2008)
		Peanut plants	<i>Cercospora arachidicola</i> <i>Pheoisariopsis personata</i>	
Wasabi defensin	Wasabi	Rice	<i>Magnaporthe grisea</i>	Kanzaki et al. (2002)
alfAFP	Alfalfa	Potato	<i>Verticillium dahliae</i>	Gao et al. (2000)
		Tomato	<i>Ralstonia solanacearum</i>	Chen et al. (2006)
MsDef1Origin	<i>Medicago sativa</i>	Tomato	<i>Fusarium oxysporum</i>	Abdallah et al. (2010)

Adopted from Lacerda, A. F., Vasconcelos, E. A. R., Pelegri, P. B., Grossi de Sa, M. F. (2014). Antifungal defensins and their role in plant defense. *Frontiers in Microbiology*, 5. <https://doi.org/10.3389/fmicb.2014.00116>.

porosity, and/or the transport of active protein into the host cell, where these antifungals interact with the intracellular targets (Marx et al., 2008).

In addition to their role in defending against microbes, defensins also defend against parasitic plants and insects (de Zélicourt et al., 2007), regulate growth, development, and fertilization (Oomen et al., 2011; Stotz et al., 2009), and help plants resist abiotic stresses (Tamaoki et al., 2008).

5.2.4 Combined antimicrobial (AM) proteins

The main limitations of these research studies are the low degree of resistance that can be obtained with the insertion of just one gene and the limited set of pathogens against which resistance may be established (Dana et al., 2006).

To overcome this hurdle, attempts were performed to insert two different AMPs genes at the same time. (Kim et al. (2003) transformed rice plants with a rice basic chitinase gene (RCH10) and a modified maize ribosome inactivating protein gene (MOD1). The co-expression of these two genes in rice plants exhibited heightened resistance to *Rhizoctonia solani*. Also, the barley ribosome-inactivating protein (rip) and the bean chitinase (chi) genes

were used by [Li et al. \(2004\)](#) to develop transgenic soybean plants. In addition [Chen et al. \(2006\)](#), established transgenic tomato plants using both defensin (GLU-AFP) and a glucanase from alfalfa (alfAFP) and found a synergic effect of both proteins as antifungal molecules toward some phytopathogenic fungi. Moreover, transgenic wheat lines overexpressed the β -1,3-glucanase and α -1-purothioninandthaumatin-likeprotein1 (tlp-1) genes, which were produced for resistance to *F. graminearum* ([Mackintosh et al., 2007](#)). Further, a transgenic barley plant resistant to fungi was performed using the rice chitinase gene (chi11) in combination with the rice thaumatin-like protein (tlp) gene ([Tobias et al., 2007](#)).

5.3 Advanced genome-editing methods

Genome editing enables modifications of the plants by changing in their endogenous DNA via insertions, deletions, or substitutions at specific sites using DNA strands of different lengths ([Barrangou & Doudna, 2016](#)). This is why CRISPR/Cas9 genome editing, as opposed to conventional or molecular breeding techniques, could quickly create the needed disease resistance ([Paul et al., 2021](#)).

Three different approaches are used to implement the system: (1) AMT, which is frequently used to transform Cas and sgRNA constructs into the target plant genome ([Fister et al., 2018](#)); (2) polyethylene glycol (PEG)-mediated protoplast transformation; or (3) biolistic delivery of RNP complex into plant cells for DNA-free plant genome editing ([Svitashev et al., 2016](#)) ([Table 16.7](#)).

TABLE 16.7 Plant genome editing of CRISPR/Cas9 and CRISPR/Cpf1 systems for plant resistance to fungal and oomycete pathogens.

Fungal or oomycete pathogen	Plant species	Target gene	Encoding protein/function of gene	Disease resistance/ phenotype	Method	References
<i>Blumeria graminis</i> f. sp. <i>tritici</i> (powdery mildew)	<i>Triticum aestivum</i> (wheat)	TaMLO-A1, B1, D1	Transmembrane protein/negative regulator of defense response	Increased resistance to <i>Blumeria graminis</i>	Triple-knock-out using particle bombardment of Cas9 and selective marker plasmid	Wang et al. (2014)
<i>Botrytis cinerea</i> (gray mold)	<i>Vitis vinifera</i> (grape)	VvWRKY52	Transcription factor/positive regulator of defense response	Increased resistance to <i>Botrytis cinerea</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Wang et al. (2019)
<i>Erysiphe necator</i> (powdery mildew)	<i>Vitis vinifera</i> (grape)	VvMLO7	Transmembrane protein/negative regulator of defense response	No mutated plants regenerated	Protoplast transformation with Cas9/gRNA expression binary vectors	Malnoy et al. (2016)
<i>Magnaporthe oryzae</i> (rice blast)	<i>Oryza sativa</i> (japonica rice)	OsERF922	Ethylene responsive factor/negative regulator of defense response	Enhanced resistance to <i>Magnaporthe oryzae</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA	Wang et al. (2016)

TABLE 16.7 Plant genome editing of CRISPR/Cas9 and CRISPR/Cpf1 systems for plant resistance to fungal and oomycete pathogens.—cont'd

Fungal or oomycete pathogen	Plant species	Target gene	Encoding protein/function of gene	Disease resistance/ phenotype	Method	References
<i>Magnaporthe oryzae</i> (rice blast)	<i>Oryza sativa</i> (japonica rice)	Pi21	Negative regulator of defense response		expression binary vectors	Li et al. (2019)
<i>Oidium neolycopersici</i> (powdery mildew)	<i>Solanum lycopersicum</i> (tomato)	SIMLO1	Transmembrane protein/negative regulator of defense response	Enhanced resistance to <i>Oidium neolycopersici</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Nekrasov et al. (2017)
<i>Oidium neolycopersici</i> (powdery mildew)	<i>Solanum lycopersicum</i> (tomato)	SIPMR4	Callose synthase gene/negative regulator of defense response			Santillán Martínez et al. (2020)
Pathogenicity not tested	<i>Ocimum basilicum</i> (sweet basil)	ObDMR1	Homoserine kinase/phosphorylation of homoserine	No infection assay	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Navet and Tian (2020)
<i>Phytophthora parasitica</i> (black shank)	<i>Arabidopsis thaliana</i> (arabidopsis)	AtERF019	Ethylene responsive factor/negative regulator of defense response	Enhanced resistance to <i>Phytophthora parasitica</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Lu et al. (2020)
<i>Phytophthora tropicalis</i> (black pod)	<i>Theobroma cacao</i> (cacao)	TcNPR3	NPR protein family/negative regulator of defense response	Enhanced resistance to <i>P. tropicalis</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Fister et al. (2018)
<i>Plasmopara viticola</i> (downy mildew)	<i>Vitis vinifera</i> (grapevine)	VvPR4b	Chitinase/inhibition of fungal mycelium growth	Decreased resistance	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Li et al. (2020)
<i>Sclerotinia sclerotiorum</i> (stem rot)	<i>Brassica napus</i> L. (rapeseed)	BnWRKY70	Central regulator of salicylic acid and jasmonic acid signal pathways	Enhanced resistance to <i>S. sclerotiorum</i>	<i>Agrobacterium</i> -mediated transformation with Cas9/gRNA expression binary vectors	Sun et al. (2018)

Adapted from Paul, N. C., Park, S.-W., Liu, H., Choi, S., Ma, J., MacCready, J. S., Chilvers, M. I., & Sang, H., 2021. Plant and fungal genome editing to enhance plant disease resistance using the CRISPR/Cas9 system. *Frontiers in Plant Science*, 1534.

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Index

Note: 'Page numbers followed by f indicate figures, t indicate tables, and b indicate boxes.'

A

- Acid solutions, 180
- Acyclovir mono-phosphate (ACVMP), 234–235
- Adverse drug reactions (ADRs), 98
- AF.KEX1 vaccine, 375–376
- Agriculture, 16
 - fungal biotechnology
 - biological control, 427–428
 - entomopathogenic fungi, 428–429
 - entomopathogenicity evolution, 430–431
 - virulence mechanism, 429
 - viral genomics, 54
- Agrobacterium-mediated transformation method, 440
- Alkaline solutions, 179
- Alkaloids, 242, 243t–244t
- Amines, 364
- Aminoimidazole derivatives, 325
- Amoxicillin-clavulanate, 317
- Amphotericin B (AMB), 363–364
- Antibacterial drugs
 - antibiotic discovery, 337
 - antivirulence therapeutic strategies, 335–336
 - bacterial genomes, 337–338
 - bacterial resistance, 318–319, 336–337
 - discovery and development
 - bioactive small molecules RNA profiling, 345–347
 - drug design, RNA, 339–343
 - genetic approaches, 343–351
 - genetic target validation, 351–353
 - resistance mutations identification, 347
 - small molecule MOAs, 348–351
 - target-based drug discovery, 351
 - whole-cell screening (WCS), 344–345
 - evolution, 313f
 - genome sequencing, 338–339
 - mechanisms of action, 334–335
 - nanotechnology, 326–327
 - natural sources, 316–317
 - regulatory obstacles, 319–320
 - research and development investment, 319
 - scientific obstacles, 320
 - semisynthetic sources, 317
 - synthetic sources, 317
- Antibiotics
 - classification, 315t
 - discovery, 337
 - peptides, 317–318
 - structure-activity relationship (SAR), 314–315
- Antibody-oriented drug discovery, 323
- Antifungal drugs
 - antifungal vaccines, 375–376
 - biofilm and hyphae targeting
 - biatriosporin D, 371
 - biofilm resistance, 369–370
 - camphor and eucalyptol, 371
 - Candida albicans* infection model, 371
 - exopolysaccharide (EPS), 370–371
 - morphometric analysis, 372
 - classification, 362–364
 - fungal cell wall targeting, 374–375
 - fungal strains, 366t–368t
 - mechanism, 362–364
 - metabolic pathways and enzyme targeting, 373–374
 - mitochondria-targeted approaches, 372–373
 - resistance and interventions
 - Candida auris*, 365
 - cell efflux modulation, 365
 - ergosterol biosynthesis and ERG11 expression, 368
 - Hsp90 functions, 368–369
 - targets and strategies, 364–365
- Antifungal protein (AFP), 448–449
- Antimicrobial agents
 - antibiotic drug resistance, 312
 - antibiotics, 314–315, 315t
 - drug discovery methods
 - biological-based approaches, 321–322
 - chemical-based approach, 320–321
 - natural products-based, 320–321
 - novel approaches, 321t
 - synthetic chemistry-based innovative approaches, 322
 - target-oriented drug discovery, 322–323
 - mechanism of action, 313–314
 - penicillin, 312
 - quinolone, 312
 - streptomycin, 312

- Antimicrobial proteins (AMPs), 446–450
 - chitinase, 446–448
 - combined, 449–450
 - glucanase, 448
- Antimicrobial resistance (AMR), 105, 411–412
- Antimicrobial susceptibility test, 146t
- Antiphage, 415
- Antiseptic techniques, 219–220
- Antiviral drugs, 228t–233t
- Antivirals
 - natural products, 236–247
 - pharmaceutical applications
 - drug repurposing, 292–293
 - host-genomics antiviral applications, 291–292
 - repurposed FDA-approved drugs, 293t–298t
 - viral genomic-based antivirals, 287–290
- Antiviral therapy
 - development, 221–222, 222f
 - history, 219–222
- Apoptosis, 284
- Applied Biosystems sequencer, 7
- Aptasensors, bacterial pathogen detection, 160
- Arbuscular mycorrhizal fungi, agriculture, 432
- Array-based sensors, bacterial pathogen detection, 160
- Artificial intelligence (AI)
 - fungal identification and diagnosis, 194
 - viral diagnosis, 131–132
 - viral genomics, 57
- Aryl benzoyl hydrazide analogs, 235
- Augmentin, 317
- Autoinducers (AIs), 324
- Azoles, 362
- B**
 - Bacteria-derived antibiotics, 316
 - Bacteria-derived antivirals, 237–239
 - Bacterial Artificial Chromosome (BAC), 12
 - Bacterial genome, 337–338
 - architecture, 3–5
 - characteristics and organizational framework, 4f
 - DNA folding, 3
 - mobile genetic elements, 4–5
 - organization, 5
 - Bacterial identification
 - common platforms for, 154f
 - genome-based approaches
 - fingerprint-based techniques, 147–148
 - polyphasic approach, 147
 - sequencing-based methodologies, 149–151
 - 16S rRNA amplicon sequencing method, 147
 - whole genome sequencing (WGS), 147
 - immunologic and serologic methods, 145
 - machine learning, 161–162
 - microelectromechanical systems (MEMS), 162–163
 - microfluidics, 163
 - oxidase and catalase test, 146f
 - pathogen detection, 159–161
 - proteomics methods
 - gel-based method, 158–159
 - mass spectrometry methods, 153–157
 - phenotypic methods, 152
 - surface-enhanced laser desorption/ionization (SELDI), 157–158
 - Raman spectroscopy, 164
 - techniques, 143–144
 - traditional diagnostic methods, 144, 145t
 - Bacterial resistance, 336–337
 - Bacteriophage
 - antiphage, 415
 - assembly, 397–409
 - bacterial pathogen detection, 160
 - biocontrol agent, 412–413
 - biofilm wiper, 414–415
 - biosensors, 414
 - food safety, 413–414
 - head formation and DNA packaging
 - capsid/shell formation, 398–400, 399f
 - chaperonin/cochaperonin, 401
 - connector/neck proteins, 407
 - genome packaging, 401
 - genome recognition and translocation, 404–405
 - parallel assembly, 407–409, 408f
 - portal protein, 401–404
 - scaffolding protein, 400–401
 - terminase and packaging motor, 405–407
 - human and animal therapy, 411–412
 - infection, conformational changes, 409–410
 - micropattern fabrications, 415
 - modular phages genome synteny, 398f
 - natural/engineered phages, 416f
 - parallel assembly, 407–409, 408f
 - phage display, 410–411, 411f
 - phage engineering, 410
 - structure, 395–396
 - wastewater treatment, 413–414
 - Beauveria bassiana*, entomopathogenicity, 430
 - Bedaquiline (BDQ), 317, 347
 - Berberine, 316, 372–373
 - Biatrisporin D, 371
 - Bioactive compounds, 89
 - BioGRID database, 72
 - Bioinformatics, 106f
 - antimicrobial resistance prediction, 107

- comparative genomics, 106
- data management and sharing, 108–109
- genetic variation and drug resistance, 107
- phylogenetic analysis, 106–107
- surveillance and early detection, 107–108
- vaccine target prediction, 107
- Biolistic transformation, fungal species, 443–444
- Biosensors
 - phage-based, 414
 - viral diagnosis, 131
- Biosynthetic gene clusters (BCG gene clusters), 321–322
- Biotechnology
 - colors, 387, 388f
 - genomics-based applications
 - agriculture, 389–390
 - environment, 390
 - industry, 390
 - medicine, 388–389
 - regulation, 390–391
- Birnaviruses, 35
- Bisubstrate inhibitor (BioAMS), 351
- Blue biotechnology, 387
- Brown biotechnology, 387
- Bryostatin, 316–317
- C**
 - Camostat, 293t–298t
 - Cancer diagnostics, 212–213
 - Candida drug resistance 1 and 2 (CDR1 and CDR2), 365
 - Candida* Genome Database (CGD), 78
 - Capsid, 32–33
 - Capsule, bacteria, 334
 - Caudovirales phages, 396f
 - CAZymes, 91
 - Chaperonin/cochaperonin, 401
 - Chiloscyphenol A, 372–373
 - Chitinases, 429
 - Chitin binding domain (ChBD), 430
 - Chloroquine (CQ), 293t–298t
 - Chlorpromazine (CPZ), 293t–298t
 - Circovirus, 6
 - Class II fructose-1,6-bisphosphate aldolase (FBA-II) inhibitors, 373
 - Clustered regulatory interspaced short palindromic repeats (CRISPR-Cas) technology, 336
 - bacterial identification, 151–152
 - components, 288–289
 - CRISPR-Cas9 system, 289–290, 290f
 - fungal species, 444, 445t
 - plant genome editing, 450, 450t–451t
 - viral diagnosis, 128t, 131
 - COVID-19 diagnosis, 127
 - CRISPR-Cas12 and CRISPR-Cas13-based assays, 125–127
 - CRISPR-Cas9-based assay, 125
 - viral diseases, 291t
 - Coccidioides species, genome sequencing, 86
 - Combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN), 126–127
 - Comparative genomics, 3–5, 14–15, 106
 - Computational biology technique, 11
 - Coumarins, 244, 246t
 - COVID-19 pandemic
 - clustered regulatory interspaced short palindromic repeats (CRISPR-Cas), 127
 - diagnosis, 49
 - genomic surveillance, 46
 - global pandemic, 44
 - outbreak in China, 43–44
 - SARS-CoV-2
 - genomic analysis, 44
 - genomic origin, 43
 - new variants, 44
 - vaccine development, 45–46
 - CRISPR interference (CRISPRi), 336
 - Cryptococcus neoformans*, genome sequencing, 84, 86
 - Culture-based approaches, bacteria, 146t
 - Cystic fibrosis (CF), 212
 - Cystic transmembrane conductance regulator (CFTR) gene, 212
 - Cytochrome P450 enzymes (CYPs), 206
 - D**
 - Daptomycin, 317–318
 - Death receptors (DRs), 284
 - de Bruijn graph (DBG), 10–11
 - Deep learning, bacterial identification and diagnosis, 162
 - Defensin, 448–449, 449t
 - Delafloxacin, 317
 - De novo genome assembly, 10–11
 - De novo ligand design, 323
 - Digital PCR (dPCR), fungal identification and diagnosis, 191
 - Digoxin, 293t–298t
 - Dihydroorotate dehydrogenase, 376
 - DNA barcoding, 184
 - DNA chips, 101
 - Double-stranded RNA viruses, 35
 - Droplet digital polymerase chain reaction (ddPCR), 120
 - Drug development
 - antimicrobial agents, 312–315

- Drug development (*Continued*)
 - antimicrobial drug discovery
 - biological-based approaches, 321–322
 - molecular docking, 324
 - natural products, 320–321
 - synthetic chemistry-based, 322
 - target-oriented drug discovery, 322–323
 - quorum sensing (QS). *See* Quorum sensing (QS)
 - Drug discovery and development
 - drug repositioning, 210–211
 - gene therapy, 210
 - genomic information, 210
 - targeted therapy, 210
 - viral genomics, 52–53
 - Drug-initiated haploinsufficiency profiling (HIP), 350
 - Drug repurposing, 213–214, 292–293
- E**
- Ebola virus, genomic analysis, 38
 - Ebselen, 293t–298t
 - Echinocandins, 364
 - Electronic nose, fungal identification and diagnosis, 193–194
 - Electroporation transformation (ET), fungal species, 440–443, 443t
 - Emodin, 371
 - Endogenous reference gene methods, fungal
 - identification and diagnosis, 192
 - Enterococcus faecalis-derived peptide (EDF), 325
 - Entomopathogenic fungi (EPF)
 - as biopesticides, 431–432
 - molecular biology/biotechnology, 430–431
 - Entomopathogenicity evolution, 430–431
 - Envelope, 33
 - Environmental biotechnology, 390
 - Environmental microbiology and bioremediation, 15–16
 - Enzyme biotechnology, fungi sequencing, 89–91, 90t
 - Enzyme-linked immunosorbent assay (ELISA), 145
 - Eperezolid, 317
 - Epigenetic modifications, 6–7
 - Epigenomics, viral genomics, 57
 - Ergosterol biosynthesis, 368
 - Eucarobustol E, 371
 - Evolutionary dynamics, viral genomics, 57
 - Evolutionary genomics, 3
 - Exotoxins, 333–334
 - Exponential amplification reaction (EXPAR), 125
 - Extracellular fungal remediation, 433–434
- F**
- Favipiravir, 293t–298t
 - Fermentations, 219–220
 - Fingerprint-based techniques, 147
 - Fingerprinting-based method, bacterial identification, 148
 - Flavonoids, 242–244, 245t
 - 5-Fluorocytosine (5-FC), 363–364
 - 5-Fluorouracil (5-FU), 363–364
 - Fluvoxamine, 293t–298t
 - Fluxome, 438
 - FnCas9 editor-linked uniform detection (FELUDA), 125
 - Food microbiology, 16
 - Forensic virology, 54–55
 - Fosmanogepix, 376
 - Fourier transform near-infrared (FT-NIR)
 - spectroscopy, 193
 - Functional genomics, 3, 38
 - Functional metagenomics, 18
 - Fungal biotechnology
 - agriculture
 - biological control, 427–428
 - entomopathogenic fungi, 428–429
 - entomopathogenicity evolution, 430–431
 - virulence mechanism, 429
 - bioinformatics, 434–439, 437f
 - abiotic factors, 435–436, 435f
 - metabolomics, 438
 - metagenomics, 437
 - metaproteome, 438
 - meta-transcriptome, 438
 - multiomics approach, 438–439
 - natural attenuation, 434–435
 - synthetic microbial consortium, 436
 - genetic engineering, 439–450
 - organic pollutant bioremediation
 - conventional clean-up methods, 432
 - fungal cell-based bioremediation, 432–434
 - Fungal cell-based bioremediation
 - adsorption, 433
 - bioaugmentation, 434
 - extracellular fungal remediation, 433–434
 - intracellular, 433
 - mycelia, 432
 - Fungal genome
 - characteristics and organizational framework, 4f
 - chromatin organization, 6
 - epigenetic modifications, 6–7
 - noncoding RNA genes, 6
 - protein-coding genes, 6
 - size and density, 6
 - Fungal genome initiative (FGI), 72
 - Fungal genomics
 - fungi classification, 71
 - genome assemblies, 80t–82t
 - genome databases and used functions, 76t–77t, 83–84

- genome history, 72–78
 - genome sequencing
 - and bioactive compounds, 89
 - and enzyme biotechnology, 89–91
 - and human health, 84–87
 - whole genome sequencing, 85t–86t
 - sample genomes, 79t
 - Fungal Genomics Program, 91
 - Fungal identification and diagnosis
 - geometric morphometrics, 182–184, 183f
 - integrative/polyphasic approach, 194–195
 - macroscopic identification
 - color, 177–178
 - macroscopic traits processing, 178–179
 - smell, 178
 - texture, 178
 - microscopy, 179
 - molecular identification
 - DNA barcoding, 184
 - metabarcoding, 186
 - molecular reference databases, 188–189
 - sequence quality control, 186–188, 187f
 - morphological differentiation, 195
 - morphological features, 176
 - phenotypic identification, 177–184
 - rapid molecular methods, 191–193
 - species identification, 195
 - spore morphometrics, 184
 - Fungi
 - genome size, 174–175, 175t
 - model organisms, 175, 176t
 - phyla and classes, 173–174, 174t
 - FungiDB, 78
 - Fungi-derived antivirals, 237
 - Fungus-derived antibiotics, 316
- G**
- Galidesivir, 293t–298t
 - Gel-based bacterial characterization and
 - identification, 158–159
 - Gene editing-based antibacterial approach, 336
 - Genetic counseling, 389
 - Genetic engineering, 386
 - fungal, 439–444
 - fungal disease resistance
 - antifungal protein (AFP), 448–449
 - antimicrobial proteins (AMPs), 446–450
 - CRISPR/Cas9 and CRISPR/Cpf1 systems, 450, 450t–451t
 - plant diseases, 445
 - traditional transgenic techniques, 446
 - transgenic plants, 447t
 - Genetic testing, 389
 - Genome editing technology, fungal species, 444
 - Genome OnLine Database, 78
 - Genome scaffolding procedure, 11–12
 - Genome sequencing technologies
 - first-generation sequencing, 7
 - genome assembly, 9–12, 9f
 - next-generation sequencing, 7–8
 - third-generation sequencing, 8–9
 - Genome-wide association studies (GWASs), 210, 292
 - Genomic-based clinical applications
 - bioinformatics, 106–110
 - drug development, 98
 - genetic variants, 99
 - Human Genome Project, 97
 - next-generation sequencing (NGS) technologies, 97
 - pathogens
 - diagnosis and prediction, 100–103, 101f
 - genomic basis, 99–100
 - novel and emerging pathogen detection, 104–105
 - rapid and accurate diagnoses, 104
 - target identification, 97–98
 - Genomic biomarkers, 207–208
 - Genomic data, 2–3
 - Genomic profiling, cancer diagnostics, 213
 - Genomics, 1
 - Geochip, 149
 - Germ theory of disease, 219–220
 - Glucanase, 448
 - Green biotechnology, 387
- H**
- Halogenated furanones, 325
 - Helicase-dependent amplification (HDA)
 - disadvantage, 123
 - viral diagnosis, 122–123
 - Hepatitis C diagnosis, 49
 - Herelleviridae, 396
 - High-throughput chromosomal conformation capture (Hi-C) sequencing technique, 11–12
 - Horizontal gene transfer (HGT), 17
 - Host-genomics antiviral applications, 291–292
 - House-keeping genes, 149
 - Human Genome Project, 388–389
 - Human guanylate-binding protein 1 (hGBP1), 284
 - Human papillomavirus (HPV), 33–34, 49
 - Hydrochloroquine (HCQ), 293t–298t
 - Hydrolytic enzymes, 91
- I**
- Illumina sequencing, 36–37
 - Imatinib, 293t–298t
 - Immune-based nanosensors, bacterial pathogen
 - detection, 159–160

- Immunoregulatory and cell-based antivirals, 285t–287t
- Industrial applications, 184
- Industrial biotechnology, 390
- Influenza A virus (IAV)
infection, 277–284
replication, 224f
genome packaging, 227
posttranslational processing, 226
transcription, 225–226
virus assembly, 226
virus entry and genome release, 223–225
virus release, 227
- Inherited retinal degenerations (IRDs), 212
- Inhibitors of neuraminidase (NAIs), 236
- Insulin, 388–389
- Interferon-induced protein with tetratricopeptide repeats (IFIT) proteins, 283
- Interferons α/β (IFNs- α/β), 293t–298t
- Influenza virus
diagnosis, 49
genomic analysis, 42
- Invasins, 334
- Iodine solutions, 180–181
- Ion Torrent sequencer, 7–8
- ISGylation, 278–279
- Isothermal-based methods
fungal identification and diagnosis, 192–193
viral diagnosis, 120–124
- Ivermectin, 293t–298t
- K**
- Kalopanaxsaponin A, 372–373
- L**
- Lab-on-a-chip (LOC), bacterial pathogen detection, 161
- Lactic acid bacteria (LAB), 237–239
- L-aminoethylcysteine, 343
- Large ribosomal ribonucleic acid (LSUs), 340
- Light microscopy
fungal identification and diagnosis
acid solutions, 180
alkaline solutions, 179
geometric morphometrics, 182–184
iodine solutions, 180–181
staining solutions, squash mounts, 180
fungal spore size measurement, 181–182, 183f
- Lignans, 244–247, 247t
- Linezolid, 317
- Long-read amplicon technologies, fungal
identification and diagnosis, 191–192
- Long-read sequencing technologies, 18
- Loop-mediated isothermal amplification (LAMP), 148
fungal identification and diagnosis, 192
viral diagnosis, 120–121
- Lopinavir/Ritonavir, 293t–298t
- Luotonin A, 237
- LuxS inhibitors, 325
- M**
- Machine learning (ML)
bacterial identification and diagnosis, 161–162
viral genomics, 57
- Major capsid proteins (MCPs), 399
- Malassezia* globose, genome sequencing, 86–87
- Manogepix, 376
- Maraviroc, 227–234
- Marine-derived antibiotics, 316–317
- Mass spectrometry method, bacterial identification, 153–157
- Matrix-assisted laser desorption ionization e time of flight mass spectrometry (MALDI-TOF MS)
bacterial identification, 154–156, 155f
fungal identification and diagnosis, 193
- Maxam-Gilbert method, 7
- Metabarcoding, 186
- Metabolomics, 438
- Metagenomics, 3, 15
bacterial identification, 150–151
fungal bioremediation enhancement, 437–438
pathogen diagnosis and prediction, 102
viral genomics, 57
viral, 55
- Metallic and nonmetallic-based nanoparticles, viral
diagnosis, 129–130
- Metaproteome, 438
- Meta-transcriptome, 438
- Microarray technology
bacterial identification, 149
pathogen diagnosis and prediction, 101
- Microbial-derived metabolites, antiviral activities, 238t–239t
- Microbiology, 219–220
- Microelectromechanical systems (MEMS), bacterial
identification, 162–163
- Microfluidics, bacterial identification and diagnosis, 163
- Microscopy, 179–181
- Minlon USB sequencer, 8–9
- Molecular clock analysis, 37
- Molecular docking technology, 324
- Molnupiravir, 293t–297t, 298f, 298t
- Monoterpenes, 240
- M protein, 33
- Multilocus sequence analysis (MLSA), 149

Multilocus sequence typing (MLST), 149
 Multiomics approaches, viral genomics, 57
 Mutagenic ribonucleoside antiviral agent, 293, 298f
Mycobacterium tuberculosis, 219–220
 Myco-biocontrol, 428
 Myovirus, 396f

N

Nanopore sequencer, 8
 Nanotechnology
 antibacterial drug development, 326–327
 in bacterial pathogen detection, 159–161
 viral diagnosis, 127–130
 N-dimethyl amino acetyl partricin, 363–364
 NDV-3A vaccination, 375–376
 Negative-sense RNA viruses, 34
 Neuraminidase inhibitions (NAIs), 236
 Neurospora crassa genome, 71–72
 Next-generation sequencing (NGS), 7–8, 36, 97
 influenza virus evolution, 42
 pathogen diagnosis and prediction, 101–102
 Nikkomycin Z, 374–375
 Nitazoxanide, 293t–298t
 Nortopsentin, 237
 Nuclear export protein (NEP), 226
 Nuclear factor kappa B (NF- κ B), 279–281
 Nucleic acid, 32
 Nucleic acid amplification methods
 pathogen diagnosis and prediction, 100–101
 viral diagnosis
 helicase-dependent amplification (HDA), 122–123
 isothermal amplification methods, 120–124
 polymerase chain reaction (PCR), 118–120, 130
 rolling circle amplification (RCA), 123–124
 Nucleoid-associated proteins (NAPs), 5

O

Obtusifolione, 362
 Olorofim, 376
 Oncolytic virotherapy, 55
 Oomycete genomes, 73t–75t
 Optoelectronic nose, bacterial pathogen detection, 161
 Orthoreoviruses, 35
 Overlap-Layout-Consensus (OLC) algorithm, 10
 Oxford Nanopore Technologies, 36–37
 Oxidase and catalase test, 146f

P

Pacbio technology, 8
 Pathogen detection and surveillance, 15
 Pathogen diagnosis and prediction
 antimicrobial resistance testing, 105

disease outbreaks and transmission chain, 105
 drug resistance, 105
 genomics impact, 104f
 novel and emerging pathogens, 104–105
 personalized treatment strategies, 105
 rapid and accurate diagnoses, 104
 vaccine development and surveillance, 105
 Pathogenic bacteria, host infection, 333–334
 Penicillin, 316–317
Penicillium chrysogenum, genomic sequencing, 91
 Peptide antibiotics, 317–318
 Peptide-based drug discovery, 322
 Peptide-based inhibitors, 325
 Personalized medicine
 genomic biomarker identification, 207–208
 person-to-person variability, 207
 viral genomics, 57
 Personalized treatment strategies, 105
 Phage-based vaccines, 412
 Phage display technique, 410–411, 411f
 Phage DNA packaging machine, 402f
 Pharmaceutical applications
 drug discovery and development, 209–211
 genetic variants, 205
 genomics application
 cancer diagnostics, 212–213
 drug repurposing, 213–214
 gene therapy, 211–212
 preclinical drug testing, 211
 personalized medicine, 207–209
 pharmacogenomics, 206–207
 pharmacokinetics and drug metabolism, 208–209
 Pharmacogenomics, 206–207, 389
 Pharmacokinetics, 208–209
 Phylogenetic analysis, 42, 106–107
 PI3K signaling pathway, 280–281
 Plant-derived antibiotics, 316
 Plant-derived antivirals, 239–247
 alkaloids, 242
 coumarins, 244
 flavonoids, 242–244
 lignans, 244–247
 phytochemicals, 239–240
 terpenes and terpenoids, 240
 Plant-incorporated protectants, 428
 Plaque assay, 220–221
 Podovirus, 396f
 Polyenes, 363–364
 Polymerase chain reaction (PCR), 148
 fungal identification and diagnosis, 191–192
 pathogen diagnosis and prediction, 100–101
 viral diagnosis, 118–120, 130

Polymer-based nanoparticles, antimicrobial drug delivery, 326

Portal proteins, bacteriophage

assembly process, 401–403

clips, 401–403

conformational changes, 401–403

DNA packaging process, 401–403

P22 portal protein, 403–404, 403f

procapsid nucleation, 401–403

SPP1 phage, CryoEM study, 403–404

Positive-sense RNA viruses, 34

Posttranscriptional gene silencing (PTGS), 288

Precision medicine, 109

Preclinical drug testing, 211

Productive phage therapy programs, 412

Proteinases, 429

Protoplast-mediated transformation (PMT), fungal species, 440, 441t

Protospacer adjacent motif (PAM) interacting domain, 289–290

Pyrithiamine (PT), 342–343

Q

Quercetin, 316

Quorum sensing (QS)

autoinducers (AIs), 324

biofilms, 324

disruption, 335

inhibitors, 325

R

Raman spectroscopy, bacterial identification, 164

Real-time sequencing, viral genomics, 57

Recombinase polymerase amplification (RPA), viral diagnosis, 121–122

Red biotechnology, 387

Remdesivir, 293t–298t

Reoviruses, 35

Restriction fragment length polymorphism (RFLP), 148

Retroviruses, 35

Reverse pharmacology, 322–323

Reverse transcriptase polymerase chain reaction (RT-PCR)

COVID-19 pandemic, 119–120

viral diagnosis, 118–120

Reverse transcription loop-mediated isothermal amplification (RT-LAMP), 121

Ribavirin (RBV), 235, 293t–298t

Ribonuclease L (RNase L), 279

Ribonucleases (RNases), 287–288

Ribonucleic acid (RNA) viruses
double-stranded, 35

drug design

large ribosomal ribonucleic acid (LSUs), 340

mechanisms, 339

mRNA targeting antibiotics, 341

riboswitches, 341–342

rRNA targeting antibiotics, 339

small ribosomal ribonucleic acid (SSUs), 339–340

tRNA targeting antibiotics, 340

genome, 5

negative-sense RNA viruses, 34

positive-sense RNA viruses, 34

profiling, 346f

dyclonine, 345–346

obstacles, 346–347

Riboprinting, 148

Riboswitches, 341–343, 342f

RNA interference (RNAi), 288

Rolling circle amplification (RCA), viral diagnosis, 123–124

Roseoflavin, 342

Rosmarinic acid, 372–373

ROS-mediated bactericidal effects, 327

Rotaviruses, 35

Rupintrivir, 293t–298t

S

Saccharomyces Genome Database (SGD), 72

Sanger sequencing technology, 7, 36

Scaffolding protein, 400–401

Sequel II system, 8–9

Sequenced microbial genomes, 1–2, 2f

Shikonin, 371

Shock-wave-mediated transformation (SWMT),
fungal species, 444

Short interfering RNA (siRNA), 288

Shotgun sequencing, pathogen diagnosis, 102

Single-cell genomics, 18

Single-nucleotide polymorphism (SNP), bacterial
identification, 150

Siphovirus, 396f, 397

Small ribosomal ribonucleic acid (SSUs), 339–340

Small viral RNAs (svRNAs), 226

Sofosbuvir, 293t–298t

Sophorolipids, 371

Spatial genomics, viral genomics, 57

Spectinomycin, 340

Squalene epoxidase, 362

Staining solutions, 180

Staphylococcus aureus, invasins, 334

Statins, 293t–298t

Streptomycin, 312, 316, 340

Structural and functional annotation
gene prediction tools, 13

protein domains, 13
 regulatory elements, 13
 statistical model approach, 12
 Structure-activity relationship (SAR) approach,
 314–315
 Sunitinib/erlotinib, 293t–298t
 Supervised learning, bacterial identification and
 diagnosis, 162
 Surface-enhanced laser desorption/ionization
 (SELDI), 157–158
 Surface/spike proteins, 33

T

Tailed bacteriophages, 396–397
 Targeted genome, 2–3
 Target-oriented drug discovery
 antibody-oriented drug discovery, 323
 automated de novo design, 323
 molecular docking, 324
 peptide-based drug discovery, 322
 reverse pharmacology, 322–323
 Teixobactin, 317–318
 Terpenes and terpenoids, 240, 241t–242t
 Tetracycline, 340
 Thiazolidinones, 373–374
 Tobacco mosaic disease, 220–221
 Tobacco mosaic virus (TMV), 33, 220–221
 Topoisomerases, 5
 Toxin production, 333–334
 Transcriptomics sequencing, pathogen diagnosis,
 102–103
 Transposon mutagenesis, 352
 Tripartite motif (TRIM) proteins, 283–284
 Tumor necrosis factor-related apoptosis-inducing
 ligand (TRAIL), 284
 Tumor suppressor p53, 284

U

Ustilago maydis, genome sequencing, 86–87

V

Vaccine development
 and surveillance, 105
 viral genomics, 57
 Vancomycin, 316–317
 Viral diagnosis
 classical methods, 117–118
 clustered regulatory interspaced short palindromic
 repeats (CRISPR-Cas), 124–127
 immunological methods, 117–118
 modern molecular techniques, 117–118
 nanotechnology, 127–130
 nucleic acid amplification methods, 118–124

Viral DNA and RNA polymerases inhibitors,
 234–235
 Viral engineering, 57
 Viral genome
 antivirals
 CRISPR-Cas systems, 288–290
 ribonucleases (RNases), 287–288
 short interfering RNA (siRNA), 288
 characteristics and organizational framework, 4f
 circovirus, 6
 DNA genome, 5
 RNA genome, 5
 Viral genomics
 agricultural applications, 54
 applications, 49–55
 COVID-19 pandemic, 43–46
 DNA sequencing technology, 31–32
 drug discovery, 52–53
 emerging viruses, 55
 environmental monitoring, 53–54
 evolution and phylogenetics, 53
 forensic virology, 54–55
 functional genomics, 38
 genome assembly, 37
 genome decoding, 32–35
 genomes sequencing, 36–37
 genomic analysis, 35–43, 39t–41t
 host-virus interactions, 55
 long-read sequencing, 56–57
 metagenomics, 55
 molecular clock analysis, 37
 next viral outbreak prediction, 46–47, 47t–48t
 oncolytic virotherapy, 55
 phylogenetic analysis, 37
 public health policy, 55
 single-cell sequencing, 56
 vaccine development, 50–52
 virus structure, 32–33
 Viral infections
 diagnosis, 49
 epidemiology and surveillance, 49–50
 spread tracking, 49–50, 56
 Viral integrase inhibitors, 236
 Viral protease inhibitors, 235–236
 Viral ribonucleoprotein (vRNP), 223–225
 Virology history, 219–220
 Virulence mechanism, fungus, 429
 Viruses
 categories, 222–223
 enzymes inhibitors
 inhibitors of neuraminidase (NAIs), 236
 viral DNA and RNA polymerases, 234–235
 viral integrase, 236

Viruses (*Continued*)

- viral protease, 235–236
- influenza A virus (IAV) replication, 223–227
- molecular organization, 222–223
- uncoating process inhibitors, 234
- viral attachment inhibitors, 227–234
- virus-host protein interactions, 223
- Virus inhibitory peptide (VIRIP), 227–234

W

- White biotechnology, 387
- Whole-cell screening (WCS), 344–345
- Whole-exome sequencing (WES), 292

- Whole genome sequencing, 292
 - bacterial identification, 151
 - fungi
 - enzyme biotechnology, 90t
 - human fungal pathogens, 85t–86t
 - pathogenic plant-fungi, 88t
- Whole genome shotgun (WGS), 79–82

Y

- Yellow biotechnology, 387

Z

- Zika virus, genomic analysis, 38

Microbial Genomics: Clinical, Pharmaceutical, and Industrial Applications

Edited by Huseyin Tombuloglu and Amal Mahmoud

Microbial Genomics: Clinical, Pharmaceutical, and Industrial Applications focuses on the various genomic-based applications of microbial genomics in the clinical, pharmaceutical, and industrial fields. Coverage includes bacterial, viral, and fungal genomics, as well as their applications in viral, fungal, and bacterial identification and diagnosis and in choosing potential targets for the discovery and development of antiviral, antibacterial, and antifungal drugs. Written by experts in their respective disciplines, the chapters provide an introduction to microbial genomics and genomic-based clinical, pharmaceutical, and industrial applications, available software implementations, biotechnological advances, and other advanced topics.

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ACADEMIC PRESS

An imprint of Elsevier

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ISBN 978-0-443-18866-4



9 780443 188664