Comparative proteomics analyses of *Acinetobacter baumannii* strains ATCC 17978 and AB5075 reveal the differential role of type II secretion system secretomes in lung colonization and ciprofloxacin resistance

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**A R T I C L E   I N F O**

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**A B S T R A C T**

*Acinetobacter baumannii* is an emerging nosocomial pathogen with alarming antibiotic resistance profiles. A better understanding of the virulence and resistance mechanisms of this pathogen is necessary for identifying new methods to combat its infections in a more efficient way. In this regard, the type II secretion system (T2SS) of *A. baumannii* is an attractive target majorly secreting lipid-metabolizing enzymes and contributes significantly to its virulence. No attempts have been made to study the differential role, and the nature of T2SS secreted proteins among different strains of *A. baumannii*. In this study, we compare T2SS substrates and functions between *A. baumannii* strains ATCC 17978, and the MDR highly virulent strain AB5075. The functional categories of the T2-secreted proteins were analyzed, and the virulence potential of the tested strains was compared in vivo using a murine pneumonia model. Biofilm formation was compared using crystal violet assay in micro-titer plates. The contribution to antibiotic resistance was measured by determining the minimum inhibitory concentration (MIC) of different classes of antibiotic. Results indicate that the T2SS secretome gives a colonization advantage to AB5075 over ATCC 17978 but is more important for biofilm formation by the latter. Transposon insertional inactivation of the general secretory pathway protein D (gspD), which is a key component in the structure of the T2SS, significantly increased the MIC of AB5075 to ciprofloxacin. Our report is the first to describe the strain-dependent evolution of the T2SS secretome in relation to the virulence and antibiotic resistance attributes of Gram-negative species.

1. Introduction

*Acinetobacter baumannii* is increasingly recognized as a dangerous nosocomial pathogen. It causes a wide range of infections, such as ventilator-associated pneumonia and bacteremia, especially in immuno-compromised patients in intensive care units (ICUs) [1]. Due to its ability to persist in hospital settings, and rising rates of resistance to all classes of antimicrobials, the World Health Organization (WHO) regards it as the top priority pathogen for the development of new antibiotics [2]. The development of new antimicrobials requires a better understanding of the virulence attributes of *A. baumannii*, and how it employs them against the host, to identify novel therapeutic targets.

Secreted virulence factors play a very important role in manipulating the host’s defenses and establishing the pathogen in the infection site [3]. Targeting the secretion apparatus, as well as the secreted products could be an effective strategy to fight these pathogens. Secretion systems of Gram-negative bacteria are complex membrane structures that are one of the mechanisms used to transport bacterial products to the extracellular environment [4]. Six systems have been extensively studied in Gram-negative bacteria, and there are many trials to exploit them as therapeutic targets [5–7]. The majority of these systems have been characterized in *A. baumannii* [8].

The type II secretion system (T2SS) has recently gained attention in *A. baumannii*, and several studies characterized its genetic organization, secreted proteins, and contribution to the pathogenesis of this microbe [9,10]. Since lipid-metabolizing enzymes are the major products secreted by this system in *A. baumannii*, a high-throughput screen was developed based on this activity to discover small molecule inhibitors of T2SS [11]. Despite the apparent role T2SS plays in *A. baumannii* infections, it is not known if the T2 secretome is conserved across different strains of varying levels of antibiotic resistance and virulence. In this work, we compare between the T2SS secretome of *A. baumannii*
strain ATCC 17978, and the highly virulent strain AB5075, and the contribution of this strain-specific secretome to the virulence and antibiotic resistance of each of them.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*Acinetobacter baumannii* strains ATCC 17978 and AB5075 were used as the wild-type (WT) and the derivatives of each were generated in its specific WT background. Strains of *A. baumannii*, and *Escherichia coli*, used as cloning and expression hosts, were grown at 37 °C in LB broth with shaking at 180 rpm, or on LB agar. When needed, media were supplemented with kanamycin with a final concentration of 30 μg/mL (for the ATCC 17978 *gspD* mutant [9]), ampicillin with a final concentration of 100–500 μg/mL (for the ATCC 17978 *gspD* mutant complementation [9]), tetracycline with a final concentration of 5 μg/mL (for the AB5075 *gspD* mutant, as described below), or apramycin with a final concentration of 25 μg/mL (for the AB5075 *gspD* mutant complementation, as described below).

2.2. Construction of the *gspD* mutant and its genetic complementation

The construction of the *gspD* mutant in *A. baumannii* ATCC 17978 (*gspD::pDv*) was previously published by our group [9]. For AB5075, the *gspD* mutant (*gspD::Tn*) was obtained from a transposon mutagenesis library that was constructed by the Manoil Lab, University of Washington Seattle, in which each mutant maintains a tetracycline resistance marker gene [12]. The genetic complementation of *gspD* in *A. baumannii* ATCC 17978 was done by our group as previously published [9]. For AB5075 *gspD::Tn* complementation, primer pair AN196 (5′-ATTTgccggGAAATCgGAGTTgATCgGCTTAT-3′, Xmal site underlined), and AN174 (5′-AATTgcggCTACgGCgGTCT-3′, PstI site underlined), was used to amplify a 2277 bp fragment corresponding to the full *gspD* open reading frame (ORF), in addition to 15 bp upstream to include the ribosomal binding site (RBS). The PCR product was column purified, and then digested using Xmal, and PstI restriction enzymes (NEB) at 37 °C overnight. The digested PCR product was then ligated into plasmid pMUG120, which carries an apramycin resistance cassette [13], which has been digested using the same restriction enzymes, using T4 DNA ligase (NEB), for 2 h at room temperature. The ligation reaction (20 μL) was deactivated at 65 °C for 10 min.

The ligation was transformed into chemically competent DH5α *E. coli* cells. The cells were recovered in SOC medium (NEB), and plated on LB plates, supplemented with 25 μg/mL apramycin.

The obtained transformants were colony PCR-screened for the correct plasmid harboring the insert using primers AN180 (5′-GGAAGCT GTGGTAtGCGCgGT-3′), and AN181 (5′-AGGGCgTTACgACTTCTG AGT-3′), which bind on the sense, and antisense strands, respectively, outside of the multi-cloning site (MCS) of pMJG120. Plasmid pMJG120-*gspD* was prepared from the clone yielding the PCR product with the correct size, verified by DNA sequencing, and transformed into electrocompetent *A. baumannii* AB5075 *gspD::Tn*, to yield AB5075 *gspD::Tn/*gspD.

2.3. Extracellular protein fraction preparation and SDS-PAGE analysis

The extracellular protein fractions from both *A. baumannii* ATCC 17978, and AB5075 were prepared as previously described [9]. Briefly, 10 mL overnight cultures (18–20 h) were adjusted to an optical density at 600 nm (OD600) of 1.8, and centrifuged at 4800 × g for 10 min. Culture supernatants were filtered through 0.22 μm cellulose acetate membrane filters, followed by concentration using the Pierce™ Protein Concentrators, 9 K MWCO, 7 mL (Pierce, UK), or Corning® Spin-X® UF concentrator, 10 MWCO, 6 mL (Corning, USA), reducing the 10 mL to a final volume of 100 μL. Secreted fractions were analyzed by SDS-PAGE (4–15% gradient Mini-PROTEAN™ TGX™ Precast Protein Gel, Bio-Rad, USA), followed by silver staining using the PlusOne silver staining kit (GE Healthcare, USA) using the manufacturer’s mass spectroscopy-compatible protocol [14]. Briefly, the reagents were reconstituted as indicated by the manufacturer, omitting the gluteraldehyde from the sensitizing solution, and the formaldehyde from the developing solution. The gel was silver stained according to the following protocol: fixation (2 × 60 min), sensitizing (1 × 120 min), washing (5 × 8 min), silver reaction (1 × 60 min), washing (4 × 1 min), developing (1 × 2 min), stopping (1 × 45 min), washing (2 × 30 min).

2.4. Sample preparation for identification of the T2SS secretome through shotgun proteomics

An amount corresponding to 4 μg total protein of each of the *A. baumannii* ATCC 17978, and AB5075 (WT, *gspD* mutant) concentrated extracellular preps were run on a 12% SDS-PAGE gel (Bio-Rad, USA). The gel was stained briefly with silver stain, and gel sections were excised, rinsed and preserved in 500 μL of ultra-pure DNase/RNase-free distilled water (Invitrogen, USA) in micro-centrifuge tubes. The samples were then submitted for analysis at the Taplin mass spectrometry core facility at Harvard Medical School.

2.5. Mass spectrometry

Excised gel pieces were subjected to trypsin digestion as previously reported [15]. Identification of proteins was performed in the Taplin Mass Spectrometry Facility, Harvard Medical School using standard procedures described before [16].

2.6. Data analysis

To analyze the data sets, Excel (Microsoft) was used to calculate fold changes in the T2SS mutant, relative to the WT strain. The presence of protein secretion signals was detected using Phobius (http://phobius.sbc.su.se/index.html) [17], BlastP (http://www.ncbi.nlm.nih.gov/blast) [18] was used to identify common members across the T2SS secretome of ATCC 17978, and AB5075. The percentages of identity and similarity between the common T2SS substrates in both strains were calculated using the EMBOSS Needle global pairwise sequence alignment tool (http://www.ebi.ac.uk/Tools/pfa/emboss_needle/) [19]. The functional categorization of the secreted proteins was performed using the eggnoG-mapper tool (http://eggnogdb.embl.de/#/app/emapper) [20], matching them to the clusters of orthologous groups (COG) categories. For uncharacterized substrates, InterPro database (https://www.ebi.ac.uk/interpro/) [21], was used for the identification of protein families, and functional domains.

2.7. Determination of the minimum inhibitory concentration

The susceptibility of *A. baumannii* ATCC 17978, and AB5075, each with its corresponding *gspD* mutant, and complemented mutant was determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI documents M100-S24, and M07-A9) [22]. Briefly, stock solutions corresponding to 5120 μg/mL from each of amikacin, meropenem, colistin, cefazidime, and cefepime were prepared. A ready-made commercial injectable solution preparation of ciprofloxacin, corresponding to 2000 μg/mL was directly used as a stock solution. Twelve-fold dilutions of each antibiotic were prepared starting from a concentration of 512 μg/mL, or 100 μg/mL for ciprofloxacin. The wells of a 96 U-shaped well plate were filled with 75 μL of sterile, double-strength Muller Hinton broth (MHB), to which 75 μL of each antibiotic concentration were serially added. The OD600 of an overnight culture of each bacterial strain was matched to that of a 0.5 McFarland standard (0.09–0.1), diluted a further 200 fold in MHB, and then 15 μL of each strain were added to the wells of each row in the
96-well plate corresponding to the dilutions of a specific antibiotic. The positive control wells contained each of the tested strains, replacing the antibiotic by sterile saline solution. The negative controls contained the tested antibiotics, replacing the microorganism by sterile saline solution. For strain AB5075 gspD::Tn::ppgD, the MHB was supplemented with IPTG to a final concentration of 1 mM. The plates were incubated overnight for 24 h at 37 °C. The MIC was considered the lowest concentration that inhibited the visible bacterial growth. MIC values were considered different if they differed by more than a two-fold dilution [23].

2.8. Biofilm formation assay

A crystal violet biofilm assay was performed as previously described [24] with slight modifications. An overnight culture of *A. baumannii* ATCC 17978, and AB5075, each with its corresponding gspD mutant, and the complemented mutant were adjusted to match the OD_{600} of 0.5 McFarland and 120 μL of each strain were transferred in quadruplicates to the wells of a flat-bottom 96-well ELISA plate. For strain AB5075 gspD::Tn::ppgD, the LB broth was supplemented with IPTG to a final concentration of 1 mM. The plates were incubated for 24 h without shaking at 37 °C. The OD_{600} of the grown cultures was measured by an ELISA plate reader (Biotek) before being discarded, and wells washed three times with 200 μL saline. The wells were thoroughly dried before adding 150 μL 0.1% crystal violet in water, and incubating for 30 min at room temperature. The crystal violet was discarded, and the wells were washed thoroughly three times with 200 μL water. The wells were dried, then 150 μL 96% ethanol were added to solubilize the crystal violet in the biofilms, incubated at 4 °C for 20 min to minimize evaporation. The OD_{560} of crystal violet solutions was measured by the ELISA plate reader and divided by the OD_{600} of the grown cultures for normalization.

2.9. Murine pneumonia infection model

A competitive infection model was developed as previously described [25]. Two groups of eight-week-old black C57BL/6 female mice (Theodor Bilharz Research Institute, Egypt) were infected intranasally with 30 μL containing a bacterial dose of 2.3 X 10^{7} CFU of a 1:1 mixed inoculum of either AB5075 WT/gspD mutant, or ATCC 17978 WT/gspD mutant. Thirty-six hours post-infection, mice were sacrificed; lungs harvested, homogenized, serially diluted, and plated on plain LB agar plates, or LB supplemented with kanamycin (30 μg/mL), or tetracycline (5 μg/mL) for colony counts. Plain LB agar plates would grow both the WT and mutant strains, while LB supplemented with antibiotics would only grow the mutant strains in the sample. The difference between CFU counts from the same sample represented the count of WT cells [26]. The competitive index for each strain was calculated using the following equation (Mutant output/WT output)/(Mutant input/WT input) [27].

2.10. Statistical analyses

Analyses were performed using GraphPad Prism (version 6.0) (GraphPad Software, Inc.), applying the one-way ANOVA test. In the case of animal competitive infection, Wilcoxon signed rank test was applied. The *p* values ≤ 0.05 were considered significant.

2.11. Ethics statement

Animal procedures were approved by the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (approval No. MI1055) following the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Research (USA).

3. Results

3.1. The T2SS is active in *A. baumannii* AB5075

Our group has previously showed that the T2SS is functional in *A. baumannii* ATCC 17978 [9]. Here we used an SDS-PAGE analysis of secreted proteins in normalized, concentrated cell-free culture supernatants from both WT AB5075 and its gspD mutant. The samples showed a difference in secreted proteins in the supernatants of the WT and mutant, confirming the T2SS activity in AB5075 (Fig. S1).

3.2. The T2SS-dependent secretome in *A. baumannii* ATCC 17978 and AB5075

A list of all the identified peptides in each sample of the WT, and gspD mutants, in ATCC 17978, and AB5075, and the corresponding proteins was obtained following mass spectroscopic analysis. The list was refined to exclude 1 hit wonders, and any possible contaminants from the processing, or prior to sample submission. The proteins were then short-listed to include those with the highest number of peptides reads, showing at least a 4-fold decrease in the gspD mutant sample supernatants. T2SS-dependent potential candidates in both ATCC 17978 and AB5075 are listed in Tables S1 and S2, respectively.

3.3. Proteins secreted by the T2SS of *A. baumannii* AB5075 differ from those secreted by ATCC 17978 in predicted functions

In our previous study [9], we have characterized the T2SS secretome of *A. baumannii* ATCC 17978 and showed that it is predominated by lipid-metabolizing enzymes, part of this data is presented in (Table S1) in the current study. Here, we performed another secretome study, choosing strain AB5075 as a model of highly virulent, MDR *A. baumannii*, to compare it to ATCC 17978 as a standard laboratory strain of low virulence and antibiotic resistance. Twenty-eight proteins showed differential secretion between the WT and gspD mutant in ATCC 17978 versus eighteen proteins in AB5075, with a minimum of four-fold difference. To compare the two sets of proteins, first, we did a comparative analysis of the secretomes of the two strains with respect to their functional categories (Fig. 1). The classification of the predicted T2-secreted proteins in both strains is predominantly unknown. The next major categories represented in the ATCC 17978 secretome are amino acid transport, post-translational modification, protein turnover and chaperones. For AB5075, the predicted secreted proteins are equally distributed among the functional categories.

The top 5 proteins showing the highest fold difference between the AB5075 WT and the gspD mutant were gamma-glutamyl transferase (GGT) (109 fold), alpha/beta hydrolase family protein (46 fold), chorogenate esterase (18 fold), histidine phosphatase family protein (15 fold), and putative N-acetyltransferase YedL (15 fold) (Table S2). Both the gamma-glutamyl transferase, and the alpha/beta hydrolase family protein are common T2 substrates shared between the two *A. baumannii* strains, with a percent similarity of 95.6%, and 97.3%, respectively. In addition to these two proteins, eight other proteins were detected in the secretomes of ATCC 17978, and AB5075 (Table 1). Among these common substrates, the highest similarity was detected between the AB5075 phosphorlase family protein, and the corresponding ATCC 17978 putative MTA/SAH nucleosidase (100%), and the least similarity was observed between the AB5075 putative outer membrane protein A, and the ATCC 17978 CSLREA domain protein (54.1%). The rest of the detected proteins in the ATCC 17978, and AB5075 secretomes (18 proteins, and 8 proteins, respectively) are unique to each strain. Two proteins are exclusively encoded by the AB5075 genome, namely another N-acetyltransferase YedL (KGP66627), with a toxin-anthrax protective antigen domain, and a putative lipoprotein predicted to act as a lytic transglycosylase (Table 2). Proteins also exclusively encoded by the ATCC 17978...
genome included a putative metallopeptidase predicted to function as a collagenase, and an uncharacterized protein of an unknown function. Two other uncharacterized proteins were also unique to this strain, predicted to function as a serine protease, and a cell-wall surface anchor family protein (Table 2). It is worth mentioning that some proteins were enriched in the supernatant of the AB5075 gspD::Tn compared to the WT as seen in the SDS-PAGE gel (Fig. S1) and they also appeared in the proteomic analysis. These were mostly cytoplasmic proteins, ribosomal and/or translational proteins, including a selenocysteine-specific translation elongation factor (13 fold), adenylate kinase (8 fold), ribosomal protein S2 (6.5 fold), and an electron transfer flavoprotein domain protein (6 fold). This is probably the result of the possible leakage due to membrane destabilization as a result of the loss of the GspD component of the T2SS. Proteins over-represented in the mutant supernatant with the highest peptide abundance included translation elongation factor G, and a class A extended-spectrum beta-lactamase GES-11, yet with lower fold changes than the previously mentioned proteins (2.5, and 3.5 fold, respectively).

3.4. The T2SS secretome gives a higher lung colonization and proliferation advantage to A. baumannii AB5075 than to ATCC 17978

To test the contribution of T2SS to the virulence manifestations of both ATCC 17978 and AB5075, a pneumonia infection model was used, infecting the mice intranasally with a mixture of the WT and the gspD mutant of each strain. In previous work published by us [9], significantly higher numbers of the WT cells were recovered from the lungs of infected mice compared to the T2SS mutant in the strain ATCC 17978 when each strain was inoculated into an independent group of mice. In the current study, both ATCC 17978 and AB5075 were inoculated each with its respective gspD mutant in the same mouse applying the competitive index method. In both groups, the WT had a significantly higher fitness in the lungs of the mice than the gspD mutant as indicated by having a competitive index with a value less than one (Fig. 2). Comparing the values of the competitive index of ATCC 17978 and AB5075, the later one had an approximate 1.5 logs decrease in its competitive index as compared to ATCC 17978 (Fig. 2). These results indicate that proteins secreted by the T2SS in AB5075 have a higher contribution to the virulence of AB5075 than to the generally less virulent ATCC 17978.

3.5. The loss of T2SS has a higher impact on biofilm formation by ATCC 17978 than on AB5075

To test whether the higher ability of AB5075 to colonize the lungs of infected mice is through the contribution of T2SS to biofilm formation,
AB5075 indicates that the difference is statistically significant (ATCC 17978 \( p = 0.0156 \), AB5075 \( p = 0.002 \)) as determined by Wilcoxon signed rank test (* \( p \leq 0.05 \)) and (** \( p \leq 0.01 \)).

Fig. 2. The T2SS secretome gives a higher lung colonization and proliferation advantage to A. baumannii AB5075 than to ATCC 17978. Two groups of mice were infected intranasally with a mixture of an equal amount of the WT/gspD mutant of either ATCC 17978 or AB5075. Thirty-six hours post infection, lungs were harvested, homogenized, serially diluted and plated on plain LB agar plates, and on LB agar plates supplemented with kanamycin 30 \( \mu \)g/mL (ATCC 17978), or tetracycline 5 \( \mu \)g/mL (AB5075). Each dot represents the competitive index calculated for the retrieved WT and mutant cells from each mouse, and the horizontal line represents the geometric mean. The * presents the competitive index calculated for the retrieved WT and mutant cells and (**) presented represent the means of 2 independent experiments (each one done in quadruplicates), and the error bars represent the standard error. The asterisk (*) indicates that the difference is statistically significant (ATCC 17978 \( p = 0.0156 \), AB5075 \( p = 0.002 \)) as determined by Wilcoxon signed rank test (* \( p \leq 0.05 \)) and (** \( p \leq 0.01 \)).

Fig. 3. The loss of T2SS has a higher impact on biofilm formation by ATCC 17978 than on AB5075. Absorbance at 550 nm representing biofilm formation measured using the crystal violet assay for the WT, gspD mutant, and complemented mutant of each of A. baumannii ATCC 17978, and AB5075. The data presented represent the means of 2 independent experiments (each one done in quadruplicates), and the error bars represent the standard error. The asterisk (*) indicates that the difference is statistically significant as determined by one-way ANOVA, followed by the Newman–Keuls multiple comparison test (* \( p \leq 0.05 \)).

A comparative biofilm formation assay was used. Although there was no statistically significant difference, ATCC 17978 gspD::pDv generally formed remarkably weaker biofilms than the WT (Fig. 3). The biofilm formation ability was restored in the complemented ATCC 17978 gspD::pDv/gspD mutant to levels comparable to the WT. Interestingly, AB5075 gspD::Tn formed slightly better biofilms than its WT, however still not statistically significant. The complemented mutant gspD::Tn/gspD formed stronger biofilms than all the tested strains, including the WT of both strains. Despite the biofilms formed by the WTs of ATCC 17978, and AB5075 were comparable, ATCC 17978 gspD mutant formed significantly weaker biofilms than that of AB5075. These results indicate that although the biofilm formation ability within a strain was not affected by the presence of an intact T2SS, the loss of this system have a higher impact on the biofilm formation of ATCC 17978 than AB5075.

3.6. Susceptibility to fluoroquinolone antibiotics is affected by the loss of T2SS in A. baumannii AB5075

The susceptibility of the WT, and gspD mutants of ATCC 17978 and AB5075 to members of the major antibiotic classes was investigated to test the contribution of T2SS to antibiotic resistance in both strains. Both strains remain highly sensitive to the membrane-acting agent colistin, with MICs less than 0.125 \( \mu \)g/mL. The gspD mutant of both strains did not show shift (difference more than 2 fold) in MIC with the majority of the tested antibiotics (Table 3). A difference more than 2 fold occurred in three instances. In these 3 instances, the complemented mutants were tested to determine if this effect is T2SS-dependent or not. The first instance occurred in the ATCC 17978 gspD::pDv, which showed a 4 fold increase in the MIC of the aminoglycoside “Amikacin”, and this could be attributed to harboring of the aph1 gene encoding the kanamycin resistance on the pDrive plasmid inserted into the gspD ORF to generate the mutant. Complementing the mutant did not lower the MIC back to the WT level indicating that the observed difference is not T2SS-dependent. The second instance occurred in the ATCC 17978 gspD::pDv, which showed a 4 fold decrease in the MIC of the third generation cephalosporin Cefazidime, however again this difference was not compensated upon gspD complementation indicating that the observed difference is not T2SS-dependent. On the other hand, the third instance occurred in the AB5075 gspD mutant which showed an eight-fold increase in MIC value to the fluoroquinolone antibiotic ciprofloxacin. Interestingly, upon mutant complementation, the MIC value was restored to almost the WT-levels indicating that the observed shift in the MIC value is T2SS-dependent.

4. Discussion

So far, the T2SS of A. baumannii has been shown to contribute to the assimilation of lipids, serum resistance, and help its successful colonization to various host tissues [9–11]. In this study, we present an investigation of the differential role played by the T2SS in two different strains of A. baumannii. The comparison aimed at identifying whether the T2 secretome would vary between ATCC 17978 (low virulence/antibiotic susceptible) and AB5075 (highly virulent/MDR), and how this difference might reflect on tissue colonization, biofilm formation, and antibiotic resistance. Since we have previously published the T2SS secretome of A. baumannii ATCC 1798, we focus in this study on the description of the T2SS secretome in A. baumannii AB5075, with comparative references to ATCC 17978.

Primary SDS-PAGE confirmed the activity of T2SS in AB5075 as expected, evident by the visual changes in the secretion pattern between the WT and gspD::Tn mutant. This observation was followed by a comparative analysis of the T2-dependent secretomes between the two A. baumannii strains ATCC 17978, and AB5075. The T2 secretomes of the two strains differed in the numbers, and the nature of the proteins detected in the extracellular supernatants. More proteins were detected in the supernatants of A. baumannii ATCC 17978, and the secretome of ATCC 17978 gspD::pDv, which showed a 4 fold decrease in the MIC of the third generation cephalosporin Cefazidime, however again this difference was not compensated upon gspD complementation indicating that the observed difference is not T2SS-dependent. On the other hand, the third instance occurred in the AB5075 gspD mutant which showed an eight-fold increase in MIC value to the fluoroquinolone antibiotic ciprofloxacin. Interestingly, upon mutant complementation, the MIC value was restored to almost the WT-levels indicating that the observed shift in the MIC value is T2SS-dependent.

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strains is composed of uncharacterized proteins, the majority of which have domains of unknown functions. The phospholipase C enzyme LipAN has been one of those uncharacterized proteins, described in our previous work [9]. Similarity-based search across a number of databases allowed us to predict the functions of two of the uncharacterized proteins unique to the T2SS secretome of ATCC 17978, as a serine esterase, histidine phosphatase family protein, and putative N-acetyltransferase YedL. With the exception of the alpha/beta hydrolase, which is a predicted lipase, the specific functions of the other substrates in A. baumannii are still unknown. However, some of them have been previously described in a number of studies as secreted virulence proteins. The best studied among these proteins is the GGT, which was detected in the secretomes of both A. baumannii strains. The metabolic function of this enzyme is to catalyze the cleavage of the amides of y-glutamyl compounds, and glutathione, and transfer this y-glutamyl moiety to receptor groups like amino acids, peptides, and water [29]. It is crucial for maintaining the redox equilibrium inside the cell, through the glutathione cycle. GGT is an extensively-studied virulence factor of the gut pathogen Helicobacter pylori. It is secreted to the extracellular environment, causing apoptosis, premature cell death, and inhibition of CD4-positive immune cells of the host, which are crucial for bacterial elimination [30,31]. The GGT of A. baumannii has never been described before as a virulence factor. However, our results demonstrate that greater fold changes in the levels of GGT were observed with AB5075 than ATCC 17978. This might indicate that A. baumannii has evolved to secrete more of this enzyme and that it could be partially responsible for the higher virulence traits displayed by AB5075. Chlorogenate esterase is an enzyme secreted by the fungal pathogen Ustilago maydis, and Aspergillus niger [32,33]. A chlorogenate esterase HcaG has been described as a membrane-associated enzyme of Acinetobacter sp. strain ADP1 required for caffeate and quinate catabolism [34]. As expected, ten T2 substrates are shared between the two strains. Interestingly, the T2 secretome of AB5075 showed two YedL putative N-acetyltransferase, and one of those is exclusive for AB5075 (KGP66627) (Table 2). This protein possesses a domain that is homologous to the B. anthracis protective antigen (PA), which along with the lethal factor (LF) forms the lethal toxin (LT) of B. anthracis. The PA binds to the host cell receptors, forming a pore that allows the LT to translocate to the cytoplasm to manifest the effects of anthrax disease [35]. This is especially intriguing because; 1) this toxin-like protein was only detected in AB5075 supernatants, and 2) so far, no toxins have been characterized for A. baumannii, thus could account again for the virulence traits of this strain.

To test if the T2SS secretome has a greater contributor to the virulence of the A. baumannii strain AB5075 than to ATCC 17978, we carried out a pneumonia infection model in mice and calculated the competitive index between the WT and gspD mutant of each strain. The competitive index was remarkably higher for AB5075, indicating that its T2SS mutant has a greater disadvantage than the ATCC 17978 mutant compared to the WT of each in the infection site.

Since biofilm formation is one of the hallmarks of A. baumannii pathogenesis, we investigated if the difference observed in the infectivity of the T2SS mutants in the two strains could be attributed to a defect in their ability to form biofilms. We expected that AB5075 would form better biofilms than ATCC 17978. Indeed, WT AB5075 formed slightly better biofilms than ATCC 17978 on average, however we did not find a statistically significant difference between the WT of the two strains. Yet, ATCC 17978 gspD::pDv formed significantly weaker biofilms than the AB5075 gspD::Tn, which actually formed better biofilms than its AB5075 WT. This significant variation in the behavior of the gspD mutants of the two strains could be attributed to their T2SS secretome, and the different nature of the secreted proteins in each. The T2SS mutants of the two strains grew comparable to their WTs (data not shown), and therefore it is unlikely their biofilm formation behavior is attributed to a growth defect. However, since we do not know the true extent of the functions of the T2-secreted proteins in the two strains, it is hard to point out a reason without further characterization of these functions, and the regulation mechanisms of T2SS expression. Biofilm formation ability dramatically varied according to the secreted proteins in other T2SS-possessing Gram-negative species. In V. cholerae, biofilm formation was diminished in a T2SS mutant, since the system secretes biofilm proteins RbmC, RbmA, and Bap1, which promote bacterial aggregation and clustering, in culture supernatants [36]. In contrast, the loss of T2 substrates PlcB, and PlcN phospholipases of Pseudomonas aeruginosa led to a hyperbiofilm phenotype [37]. The same was observed with a mutation in its membrane-bound lytic transglycosylases, which are not T2 substrates in P. aeruginosa, but are predicted T2 substrates for AB5075 (Table 2) [38]. These hyperbiofilm phenotypes were attributed to the ability of phospholipases to break down the biofilm matrix, and a stress response to the damage of the cell envelope with the loss of transglycosylases. These reports suggest that the difference in biofilm formation by the two investigated A. baumannii gspD mutants could be explained by the variation in their T2SS secretomes.

The general antibiotic resistance profiles revealed by MIC determination to a number of selected antibiotics representing different classes confirmed the MDR nature of AB5075 in comparison to ATCC 17978. The only antibiotic resistance that was found to be dependent on T2SS (as it was compensated to by introducing the WT copy of the gspD ORF on a plasmid) was the resistance to the fluoroquinolone antibiotic ciprofloxacin, where a remarkable 8-fold increase in MIC was detected with the AB5075 gspD::Tn, which actually formed better biofilms than its AB5075 WT. This significant variation in the behavior of the gspD mutants of the two strains could be attributed to their T2SS secretome, and the different nature of the secreted proteins in each. The T2SS mutants of the two strains grew comparable to their WTs (data not shown), and therefore it is unlikely their biofilm formation behavior is attributed to a growth defect. However, since we do not know the true extent of the functions of the T2-secreted proteins in the two strains, it is hard to point out a reason without further characterization of these functions, and the regulation mechanisms of T2SS expression. Biofilm formation ability dramatically varied according to the secreted proteins in other T2SS-possessing Gram-negative species. In V. cholerae, biofilm formation was diminished in a T2SS mutant, since the system secretes biofilm proteins RbmC, RbmA, and Bap1, which promote bacterial aggregation and clustering, in culture supernatants [36]. In contrast, the loss of T2 substrates PlcB, and PlcN phospholipases of Pseudomonas aeruginosa led to a hyperbiofilm phenotype [37]. The same was observed with a mutation in its membrane-bound lytic transglycosylases, which are not T2 substrates in P. aeruginosa, but are predicted T2 substrates for AB5075 (Table 2) [38]. These hyperbiofilm phenotypes were attributed to the ability of phospholipases to break down the biofilm matrix, and a stress response to the damage of the cell envelope with the loss of transglycosylases. These reports suggest that the difference in biofilm formation by the two investigated A. baumannii gspD mutants could be explained by the variation in their T2SS secretomes.

Table 3
Minimum inhibitory concentration of selected antibiotics of A. baumannii strains ATCC 17978 and AB5075.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ATCC 17978 WT</th>
<th>ATCC 17978 gspD::pDv</th>
<th>ATCC 17978 gspD::pDv/pegpD</th>
<th>AB5075 WT</th>
<th>AB5075 gspD::Tn</th>
<th>AB5075 gspD::Tn/pegpD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Cefepime</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.0975</td>
<td>0.0975</td>
<td>0.0975</td>
<td>12.5</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Colistin</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

Cefepime 4 > 256 > 256 > 256 > 256 > 256
Ceftazidime 16 > 256 > 256 > 256
Colistin < 0.125 < 0.125 < 0.125 < 0.125 < 0.125 < 0.125
Ciprofloxacin 0.0975 0.0975 0.0975 12.5 100 25
Colistin < 0.125 < 0.125 < 0.125 < 0.125 < 0.125 < 0.125
Ceftriaxime 16 4 4 > 256 > 256 > 256
Amikacin 2 8 8 32 16 16

complemented strain, it is not possible that this mutant had acquired additional polar mutations in gyrA and topoisomerase genes. It is likely that the change in MIC observed is attributed to the altered expression of membrane components. Recently, a colony-variant phenotype has been reported for *A. baumannii* AB5075, in which two shapes of colonies appeared within a population of WT bacteria. This variation is dependent on the Ompr/EnvZ two-component system, which controls the expression of outer membrane porins in response to osmotic stress. Interestingly, the translucent variant is more susceptible to antibiotics [44]. The loss of an intact T2SS or its substrates might trigger a high-throughput screen for the identification of small molecule inhibitors, Front Cell Infect. Microbiol. 7 (2017) D190–D219.

In summary, our study shed lights on the differential roles of the T2SS secretome in *A. baumannii* strains, representative of varying degrees of virulence and antibiotic resistance. Further understanding of the evolution of the *A. baumannii* T2SS secretome is needed for potential therapeutic applications.

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Conflicts of interest
All authors declare that they do not have any competing interests.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2018.12.039.

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