ABSTRACT:
Saprophytic soil Bacillus subtilis strain NC was isolated from an industrial region, at Shubra Al-Khima, north Cairo, Egypt. A progeny strain was isolated and adapted to metal bioremediation under stress of Cr\(^{6+}\), Hg\(^{2+}\) and Fe\(^{3+}\) ions. Both parent (Pt) and progeny (Pg) were treated with separate nine heavy metals. According to their impact upon the bacterial viability, the heavy metals tested were assigned into three groups: Growth promoting elements (GPE) including Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\) and Mg\(^{2+}\); growth retarding elements (GRE) including Li\(^{+}\), and B\(^{3+}\); and growth inhibiting elements (GIE) including Sr\(^{2+}\), V\(^{5+}\) and Pb\(^{2+}\). Fortifying these groups with traces of Cr\(^{6+}\) (C), Hg\(^{2+}\) (A), and Fe\(^{3+}\) ions (F), separately, resulted in a dissimilar influence on the groups. For the Pg lines, the toxic effect of GRE was antagonized and that of GPE was synergized while the inhibitory effect of GRE was prominent for Pt line. Consumption of heavy metals in the bacterial growth (% of residual control) was the highest with GPE and the least was GIE fortified with C, A and F, indicating the lysis of cells and impairment of permeability control. Pt and Pg proteinomes were subjected to SDS-PAGE analysis. Electrophoretic profile analysis indicated the presence of novel bands expression of regulons in Pg smear emerging the possibility of induction of proteins/enzymes capable of adapting the high concentrations of heavy metals.

INTRODUCTION:
Rapid industrialization and urbanization have resulted in an elevated level of toxic heavy metals and metalloids entering the biosphere. The fate of toxic metallic cations in the soil environment (and its subsequent movement into ground water or uptake by plants) depends largely on the interactions of these metals with soil organic and inorganic components (Ochiai et al., 2007).

Metal ions, including Mn\(^{2+}\), Fe\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\), are both essential and potentially toxic. Therefore, homeostatic regulation of their intracellular concentrations is critical (Jakubovics and Jenkinson, 2001).

The occurrence of heavy metals in the soil results in the impairment of most plant processes and causes drastic drop in yield, in addition to the transposition of these heavy metals to the plant consumers. Mercury, chromium and iron deposits are the most hazardous heavy metals continuously added to the soil according to their customary exhaustion in many industries and human activities.

Detoxification of mercury by bacteria was investigated by Pilon-Smith and Pilon (2000) in their transformation of Arabidopsis thaliana with mercuric reductase (Mer A) and organomercurial lyase (Mer B) derived from Gram negative bacteria, where the double transgenic of the genes of both enzymes resulted in the highest tolerance to organic mercury up to 10 µM. The inconceivable accumulation of mercury in the soil, changes the flux of biologically available mercury in natural microbial communities where enzymatic activities underpin the biogeogical cycling of mercury with consequence human exposure to toxic forms of this element (Ogunseitan, 2002).

The use of chromium in metal refinishing and electroplating is extensively increased leading to high contamination of soil with the high soluble toxic forms of Cr\(^{6+}\) anions; CrO\(_4^{2-}\) and Cr\(_2O_7^{2-}\). Some bacteria can reduce chromate (Cr\(^{6+}\)) to insoluble and less toxic Cr\(^{3+}\), but many fundamental parameters to remediate chromate are not characterized (Keyhan et al., 2004).
In many bacteria, the ferric uptake regulator (Fur) protein coordinates the expression of iron uptake and homeostasis pathways in response to available iron (Andrews et al., 2003). Fur represses a small RNA, RyhB, which in turn, negatively regulates the expression of iron-rich enzymes such as succinate dehydrogenase, fumarase, and aconitase (Masse and Arguin, 2005). This allows the production of these enzymes to be activated in response to available iron.

Because of large surface area-to-volume ratio, microorganisms provide a large contact area which can interact with metals in the surrounding environment. They accumulate metals through various mechanisms including complexation, adsorption, precipitation, and active transport into the cell (Samuelson et al., 2000). Many microorganisms are used for bioremediation of soils from heavy metals: Trichoderma viride, T. Harzianum, Pseudomonas putida, P. syringae, P. florescence, Bacillus subtilis, Vibrio harveyi, E. coli, Schewanella putrefaciens, Deinococcus radiodurans, Staphylococcus aureus, Acinetobacter spp., and Sporosarcina urea (Keyhan et al., 2004).

In the present study, some saprophytic Bacillus isolates, capable to accumulate part of the deposited heavy metals, were obtained and subsequently identified as Bacillus subtilis strains. Heavy metals analysis for soil samples was performed. The successful growing Bacillus subtilis isolate was subjected to gradient concentrations of Hg⁺², Cr⁺⁶ and Fe⁺³ to obtain progeny isolates. This present study aimed to adopt the saprophytic Bacillus isolates able to accumulate, internalize and reduce the toxic heavy metals Hg⁺², Cr⁺⁶ and Fe⁺³ by inducing novel regulons of enzymatic machinery systems.

MATERIALS AND METHODS:

Chemicals:
Most chemicals were purchased from Sigma chemicals co. U.S.A., Aldrich co. U.S.A. and ADWIC Egypt. Chemicals of gel electrophoresis were from Promega co. U.K. Buffers for the enzymatic activities of mercuric reductase were obtained from Bio-Rad Laboratories, N.Y.

Soil samples and isolation of the bacteria used:
Seven soil samples were collected from the industrial area at Shubra Al-khima (Athawrah suburb) 25 Km north Cairo. Soil samples were collected from distributed sites, beside the field area where massive economical plants are established for the industry of glass, fiberglass ceramics, batteries fabrication, iron smelter, textiles, semiconductors, and others. Soils were aseptically divided into portions and sieved (3-mm mesh). Samples from each portion were heated (75°C, 10 min) and cultivated on a modified trypton LB medium (TLB) described by Simbahan et al. (2005). Chemical analysis of heavy metals in the soil samples were conducted at Micro Analytical center, Cairo Univ. Egypt using the flame atomic absorption spectrometry (FAAS) according to D’Haese et al. (1997).

Bacterial identification:
Bacterial isolates were routinely cultivated on TLB for identification tests and to select the Gram-positive spore forming rods. Strains producing colonies on TLB agar after heating of the soil inoculum were considered to have spores. Following morphological and biochemical schemes derived from Bergey's manual of systematic bacteriology; (Holt, 1986) bacterial isolates were defined morphologically and biochemically. The isolate was assigned to be B. subtilis NC strain.

Resistance of bacterial strains to heavy metals:
For induction of resistant progeny of the parent isolates, a grown culture was subjected separately and collectively to three heavy metal supplements (Cr₆⁺, Hg⁺² and Fe⁺³) in a gradient loading scheme (5, 10, 20, 30, 40, 50, 100µM) on liquid TLB with shaking for 48 hr. Then, the cultures were incorporated into the same fresh TLB - heavy metals containing media for another 3 cycles before plating on solid TLB - heavy metals containing media to isolate a clone of a successful growing progeny. Nine different heavy metals (Mn⁺², Co⁺², Zn⁺², Mg⁺², Li⁺, B⁺³, Sr⁺², V⁺⁵ and Pb⁺²) were added separately at a concentration of 50 µM to TLB broth cultures of both Pt and Pg isolates to induce their growth under the stress of these heavy metals. Traces (5µM) of Cr₆⁺, Hg⁺² and Fe⁺³ ions (chlorides) were added separately to each metal culture to persuade the enzymes machinery systems to achieve their highest productivity.

Growth, proteinome and nucleic acids analysis:
Bacterial growth (as dry weight) in all growing cultures were determined as described in Dawoud and Eweis (2006), while proteinome and nucleic acids were determined as described in Dawoud and Mawgoud (2008).

Proteinome characterization and enzyme assay:
Proteinome analysis on SDS-PAGE was conducted according to Laemmli (1970). Gel bands were analyzed using the computerized image analysis (Gel-Pro analyzer, version 4, Media Cybernetics, 2002). The HgCl₂ dependent oxidation of NADPH (tetrasodium salt) was measured by mercuric reductase assay as described by Simbahan et al. (2005).
RESULTS AND DISCUSSION:

Living organisms depend strongly on soil and freshwater sources for their subsistence. Through the course of millions of years of Earth’s history, these resources have acquired a well-defined chemical composition suitable for life (Martinez et al., 2004). Accumulation of trace amounts of foreign organic and inorganic species to these resources alters their pristine composition. These alterations make them unsafe for consumption by living organisms (Dong et al., 2003). Toxicity in aqueous and soil environments stems, in part, from an artificial influx of toxic metals generated by human industrial activity. Species of Pb, Hg, Cd, Cr, Cu, Fe, and Zn among others have been detected in such ecosystems and found to bioaccumulate in organisms in nanomolar to micromolar concentrations (Pandey et al., 2000). Effective bioremediation of the polluted sites requires knowledge of genetic pathways for resistance and biotransformation by component organisms within a microbial community (Hu et al., 2005). Some elements (e.g., Al, Cd, Hg, and Pb) seem to have no essential biological functions, but are taken up and accumulated by microorganisms, and occasionally they become toxic at their higher concentrations (Nriagu, 1990). The accumulations of these heavy metals, subsequently, intensify their hazardous impact, therefore, it is of great importance to drain heavy metals or, merely remove them, continuously before reaching their higher concentrations. Many of the heavy metals are important components of biological systems and serve several functions; they represent the prosthetic groups in many proteins and dictate the active site of the enzyme. They also act as cofactors for some enzymatic reactions and multidentate center for poryphrin molecules; in addition they act as redox centers for transferring electrons in microbial cells (Coppi et al., 2007).

Bacillus subtilis provides a model system for the investigation of metal ion homeostasis in gram-positive bacteria (Moore and Helmann, 2005). The metalregulatory proteins controlling iron, zinc, and manganese homeostasis of B. subtilis were identified and characterized the corresponding regulons by using transcriptional profiling and DNA-binding studies (Hantke, 2001). The largest class of metal-regulated genes in B. subtilis encodes three transport systems, which suggests the presence of multiple metal uptake pathways. However, the contributions of these metal-regulated operons to cell physiology have not been systematically assessed (Ollinger et al., 2006).

In the present study seven soil samples were collected from a field area in the industrial region, north Cairo, and analyzed for the quality and quantity of heavy metals dumped from the surrounding and nearby factories. Soils samples were subjected to a screening scheme for isolation of Bacillus subtilis strain naturally tolerant to such heavy metals pollution. Out of 247 colonies grown on TLB medium, 32 colonies survived after three replications on the same medium. Only 6 colonies were successfully grown and maintained from a single clone colony. Following the assigned procedures of Bergey’s manual for systematic bacteriology (Holt, 1986) for identification of Bacillus subtilis, two strains were demonstrated to be similar clones, while the other four colonies were preserved for further identification profiles. The identified candidate strain was assigned to be Bacillus subtilis strain NC, the parent isolate (Pt). Pt was subjected separately and collectively to three heavy metal supplements (Cr\(^{6+}\), Hg\(^{2+}\) and Fe\(^{3+}\)) in a gradient loading scheme (5, 10, 20, 30, 40, 50, 100µM) to induce the bacterial growth under heavy metals stresses. Few colonies with faint growth were obtained in application of the three ions together and their advanced growth was maintained on TLB medium supplemented with lower concentrations of the employed heavy metals. Two survival isolates were finally obtained and assigned to be the stressed progenies, one of which was selected for the present study (Pg). The influences of different application strategies of nine different heavy metals (Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), Mg\(^{2+}\), Li\(^{+}\), B\(^{3+}\), Sr\(^{2+}\), V\(^{5+}\) and Pb\(^{2+}\)) on the growth of both Pt and Pg isolates coincide and Pg isolates coincide with pure heavy metal elements synergized with faint growth were obtained in application of the three ions together and their advanced growth was maintained on TLB medium supplemented with lower concentrations of the employed heavy metals. Two survival isolates were finally obtained and assigned to be the stressed progenies, one of which was selected for the present study (Pg). The influences of different application strategies of nine different heavy metals (Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), Mg\(^{2+}\), Li\(^{+}\), B\(^{3+}\), Sr\(^{2+}\), V\(^{5+}\) and Pb\(^{2+}\)) on the growth of both Pt and Pg isolates coincide.
supplementation of growing cultures with metal ions ameliorated the toxic effect of salinity on *Bacillus subtilis*.

![Graph](image1.png)

**Fig. 1** Response of the growth of *Bacillus subtilis* NC isolates, Pt and Pg to the impact of different heavy metals stresses.

Induction of suitable regulon enzymatic systems in bacteria using different stresses was regarded by Keyhan et al. (2004). Budde et al. (2006) found that during the growth of *Bacillus subtilis* under stress of low temperature, its propagation in a minimal medium at 15 °C triggered the induction of 279 genes and repression of 301 genes in comparison to its growth at 37 °C. Antelmann et al. (2000) observed similar results in the stress of phosphate starvation response in *Bacillus subtilis*. On the other hand, it was postulated that the mode of action of heavy metals on the bacterial components may be traced in sugar transport, cell wall synthesis and construction, membrane function, carbohydrate metabolism, protein synthesis, and construction, membrane function, carbohydrate metabolism, protein synthesis, and construction, membrane function, carbohydrate metabolism, protein synthesis, and enzyme activation / inhibition. Moreover, combination of more than one heavy metal has a profound biological effect than a single element (Jjemba, 2004).

Proteinome and nucleic acids evaluation (Figs 2&3) revealed that pure heavy metal elements of GPE group (Mg²⁺, Zn²⁺, Mn²⁺ and Co²⁺) increased the amount of proteinome and nucleic acids in the parent *Bacillus subtilis* strain NC and that would be resulted from the stimulatory effect of these elements, mainly, on the regulon enzymatic systems and that was reflected in the biosynthesis of these main components. However, it was recorded that glutathione synthase and phosphoenolpyruvate carboxylase were activated by Mg²⁺ (Wedding and Black, 1988), where carboxylases are bounded preferentially to nitrogen and phosphoryl groups via Mg²⁺ ions and Mg-ATP is the substrate for plasma membrane bound ATPase. In addition, Mg²⁺ is used also for charge compensation and osmoregulation processes, while, inadequate supply of Mg²⁺ leads to ceases of protein synthesis (Yazaki et al., 1988). Zn²⁺ is essential elements in polymerization of amino acids into proteins where its shortage activates the RNA-degradation system by RNA-ase activity (Sharma et al., 1982). Zinc metalloproteins are involved in DNA replication, transcription and regulation of gene expression (Coleman, 1992). Zinc might bind to sulfhydryl group of cell membrane constituent or form tetrahedral complexes with cysteine residues of polypeptide chains and thereby protect membrane lipids and proteins against oxidative damage (Cakmak and Marschner, 1990). Mn²⁺ acts as cofactor for activation of more than 35 different enzymes (Sharma et al., 1991) involved in redox reactions, decarboxylation, hydrolytic reactions and biosynthesis of aromatic amino acids.

The proteinome and nucleic acids increased in the presence of small traces of Cr⁶⁺, Hg²⁺ and Fe³⁺ (Fig. 2) and that was reflected in the little amounts of heavy metals residuals (Fig. 3).

![Graph](image2.png)

**Fig. 2** Response of the proteinome of *Bacillus subtilis* NC isolates Pt and Pg to the impact of different heavy metals stresses.

![Graph](image3.png)

**Fig. 3** Residuals of different heavy metals after bacterial growth of *Bacillus subtilis* NC isolates; Pt and Pg.

These results suggest the occurrence of some activity in the regulating machinery of the enzymes. In this connection, Keyhan et al. (2004) observed that mixing various combinations of metals greatly affects the metal-enzymatic activities. The results (Figs 2&4) demonstrated different activation rate of combined heavy metals. Remarkably, the stressed Pg isolate exhibited more interaction with GPE than Pt, and that may be conferred as the existence of novel developed regulon enzymatic machinery and/or induction of mutant novel genes which facilitated the exploitation of those applied heavy metals. Data of GRE group (Li⁺ and B³⁺) revealed a decrease in proteinome content (Fig. 2) unparalleled with the nucleic acids production.
and that would reflect the mode of action of GRE which may interfere with protein synthesis through the nucleic acids transcription and translation to produce amino acids polymerization and protein. Application of C, A and F resulted in sharp declines in the proteinome and nucleic acids of Pt isolate. On the contrary, application minimized the toxic effect of heavy metals in the Pg isolate which appoint a sort of antagonism for the effect of heavy metals or the restoring of the enzymatic activity machinery systems.

The third group of heavy metals (GIE) appeared to be very destructive for both cell constituents and metabolic activities of the studied isolates. The inhibiting consequence was reflected in the sharp decline of dry weights, proteinome quantity and nucleic acids synthesis (Figs 1-4). Toxicity of heavy metals are well understood in their influence on the binding bonds in the tertiary and quaternary structures of enzymes distorting their active sites and denature the enzymatic configuration leading to the complete irreversible inhibition of enzymatic machinery systems (Summer, 1992).

The genes governing the metal resistance in bacteria are plasmid or chromosomal-born (Mahadevan et al., 2006). The former expectation assists the transferring recombination process of these plasmids via conjugation between acceptable species in the same micro niche habitat (Xiong et al., 2000). Although most resistance regulon systems function by energy-dependent efflux of toxic ions, some resistance engage enzymatic transformation such as mercurial resistance regulon system which involves mercuric reductase that converts soluble inorganic Hg\(_2^+\) to Hg\(_0\) where the latter eliminates as gas. In addition, there is mercurial lyase which cleaves the Hg-C bond of more toxic organo-mercurials to less toxic Hg\(_2^+\). Mercuric reductase was estimated with NADPH, tetrasodium salt (Fig. 5) which was more expressed in the traced amalgamated Mg\(_2^+\) culture of Pg isolate. The Pg proteinome was investigated for the presence of novel expressed protein bands. The electrophoretogram of the proteinome of both Pt and Pg isolates (Fig. 6) revealed the presence of new regulatory protein bands in Pg proteinome between 37.4 and 48.7 KD. Four intensive bands (36.1, 44.6, 57, 71.2 KD) compared to Pt smear were appeared, while many bands between 17 and 26.5 KD were disappeared. These changes are confirmed with the biochemical estimations and that support the hypothesis of the occurrence of novel enzymatic machinery system to cope with such elevated concentrations and varieties of applied heavy metals which induced the bacterial genome for a novel trend of metabolic processes. However, this point needs more exploration concerning the enzymatic regulatory proteins evolved in the present study.

**CONCLUSION:**

The Gram positive saprophytic *Bacillus subtilis* has a definite effect in the plant growth enhancement; where it could dissolve P of weathered rocks and mobilize useful minerals (as P\(^{3+}\), K\(^+\), Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), Cu\(^{2+}\)).
and Zn$^{2+}$) in minerals rock (Puente et al., 2004). It was used by Kahrui et al., (2005) as a cadmium sensor in smelting area containing Cd$^{2+}$, Pb$^{2+}$ and Zn$^{2+}$. Also, it could alleviate the adverse effect of salinity in the soil (Saleh et al., 2005). Also, Korsten et al., (1994) utilized B. subtilis as a field biocontrol agent spray to reduce the severity of Avocado black spot caused by Pseudocercospora purpurea. In the present study, the utilized heavy metals achieved different influences on the bacterial growth varied from growth promoting elements (Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$ and Mg$^{2+}$), growth retarding elements (Li$^{+}$, B$^{3+}$) and growth inhibiting elements (Sr$^{2+}$, V$^{5+}$ and Pb$^{2+}$). Combination of traces (5 μM) of Cr$^{6+}$, Hg$^{2+}$ and Fe$^{3+}$ with the growing cultures of Pt and Pg isolates enhanced the growth yields, and the synthesis of proteinome and nucleic acids particularly with Mg$^{2+}$ ions. The internalization of heavy metals was contentment with Pg isolate and this was confirmed by the decreased amounts of minerals residues acquired after the bacterial growth. The electrophoretogram of Pg proteinome display a great possibility for the presence of novel expression of enzymatic machinery systems responsible for the adaptation of the bacterium to such heavy metals stress and this was confirmed by the appearance of novel regulon bands of molecular weight range between 37.4 and 48.7 KD.

REFERENCES:
Trends in metal homeostasis and the regulation of metal responsive transcriptional regulators.


