Arsenate bioremediation by *Bacillus pumilus* through *cis*- acting of both arB and subtilisin genes

Elsayed E Hafez¹, Mohamed M Yacout², A A Abdelkhalek¹ and Abdelaal Shamseldin³*

¹Department of Plant Protection and Biomolecular Diagnosis (ALCRI) and ²Environmental Biotechnology Department (GEBRI)
City for Scientific Research and Technological Applications (SRTA) New Borg El-Arab, Alexandria, Egypt
²Genetics Department, Faculty of Agriculture, Alexandria University, Egypt

Received 29 May 2015; revised 10 November 2015; accepted 15 November 2015

Accumulation of arsenate in the natural environment causes serious environmental problems. Hence, the main goal of the present study was to isolate arsenic-resistant bacteria capable of removing arsenic from the environment. Screening of 30 bacterial isolates, isolated from arsenate-contaminated soil, revealed a highly resistant strain, which could tolerate up to 2 g L⁻¹ of arsenate. This strain was identified as *Bacillus pumilus* based on the partial sequences of 16S rRNA. Molecular tools, such as, differential display-PCR, cloning and sequencing were used to screen and identify genes that can be involved in arsenate bioremoval. Differential display for scanning the most abundant of induced/suppressed genes at different sodium arsenate concentrations of the bacterial genome indicated the significant induction of the two genes, arsenical pump membrane protein (*arB*) and subtilisin. It was noticed that the *arB* gene was up regulated at high arsenate concentrations, while subtilisin gene was induced at low concentrations. The results showed that *arB* gene and subtilisin were working together for reducing the arsenate effect through its hydrolysis by β-galactosidase enzyme. The study gives significant insight about the expected mechanism of both two genes for remediating arsenate-polluted ecosystems.

**Keywords:** Arsenate, arB/β-galactosidase, arsenate resistance gene, *Bacillus pumilus*, resistance, subtilisin

**Introduction**

The heavy metal and radionuclide pollution from nuclear power plants, mining industries, electroplating industries and agriculture runoff is a major cause of concern to public health, animals and ecosystem¹.² Metal contamination represents a long-standing, widespread and recalcitrant selection pressure with both environmental and clinical importance, which potentially contributes to the maintenance and spread of antibiotic resistance³.

Arsenic is a ubiquitous pollutant that can produce cancer and cause serious health problems in certain parts of the world⁴. Arsenic is one of the most prevalent toxic metals in the environment. It is mainly of geochemical origin in an insoluble form but also derives from anthropogenic sources⁴. In soluble forms, arsenic occurs as trivalent arsenite [As(III)] and penta valent arsenate [As(V)]. Arsenate, a phosphate structural analogue, can enter the bacterial cell *via* the phosphate transport system. Its toxicity is due to its interference in normal phosphorylation processes by replacing cellular phosphate⁴.⁵

Biological remediation techniques using living/dead cells or biosynthesized molecules have been examined⁶. Studies have shown that both plant and microorganism are able to accumulate metal ions *via* processes, such as, transportation across the cell membrane, biosorption onto cell wall, entrapment in extracellular capsule, precipitation, oxidation-reduction reaction and biosorption to extracellular polysaccharide⁷. Hyper accumulating plant species, such as, *Pityrogramma calomelanos* and *Pteris vitta*, were shown to accumulate arsenic in the form of arsenate at the leaf section⁸. Studies reported the ability of algae, fungi and bacteria to transform arsenite to arsenate and *vice versa* during their growth⁹.¹⁰ *Desulfomicrobium* sp. BenR-B has been shown to reduce arsenate to arsenite *via* enzyme arsenate reductase⁹.

The most common means of producing resistance to drugs and metals including arsenicals is *via* an extrusion mechanism, which lowers the amount of the metalloid to levels that are no longer toxic¹¹. It has
been demonstrated that arsenite enters the cells, at neutral pH, by aqua-glyceroporins (glycerol transport proteins) in bacteria, yeasts and mammals, and its toxicity lies in its ability to bind sulphydryl groups of cysteine residues in proteins, thereby inactivating them. Arsenite is considered to be more toxic than arsenate and can be chemically or microbiologically oxidized to arsenate. In some Gram-negative bacteria, arsenite is converted to arsenate by an arsenite oxidase, a periplasmic membrane-bound enzyme member of the dimethyl sulfoxide reductase that belongs to family molybdoenzymes. The toxic properties of arsenic are well known and have been exploited in the production of antimicrobial agents, such as, the first specific antimicrobial drug Salvarsan 606, in addition to the commonly used wood preservative chromate copper arsenate.

Arsenic resistance in Synechocystis sp. strain PCC 6803 is mediated by an operon consisted of 3 genes that are regulated by an unlinked arsR homolog. The operon includes an arsenite transporter gene, arsB—an arsH homolog without a clear function in arsenic resistance—and an arsenate reductase gene arsC, Lopez-Maury et al., whose product is referred as ArsCsyn. ArsCsyn belongs to a new type of hybrid arsenate reductases that, though related to thioredoxin-dependent arsenate reductases, use the GSH/glutaredoxin system for reduction. Our goals were directed to select the most effective bacterial strain for arsenate bioremoval and to identify the genes that may play role for arsenate bioremediation using different molecular biology approaches.

Material and Methods
Arsenic Analysis by Atomic Emission Spectrometer
Total arsenic was extracted from soil samples according to Saeki and the arsenic concentrations were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (PerkinElmer, Inc., USA).

Isolation of Arsenate Resistant Bacteria
Soil samples were collected from different sites of Borg El Arab region (Alexandria, Egypt) and were used for preparing soil dilution up to 10⁻⁷ in sterile distilled water. From 10⁻⁵ dilution, 200 µL was taken for inoculating onto mineral salt medium (MSM) agar plates amended with sodium arsenate (50 µg mL⁻¹) according to the method of Fisher et al. The MSM agar plates were incubated for 4 d at 30°C. Arsenate resistant bacterial strains (30 in number) were selected to test their ability for growth on the MSM broth with high concentration of arsenate (upto 2 mg mL⁻¹) by measuring the optical density at 600 nm (OD₆₀₀) after 3d. Strain that could grow on this high level of arsenate was kept in glycerol at −80°C for further studies.

DNA Isolation and Identification of Strain Based on 16S rRNA Sequences
The DNA of the arsenate resistance bacterium strain was extracted using DNA extraction kit (Qiagen) and subjected to PCR for amplifying 16S rRNA gene using the specific primers. Primers and conditions of PCR cycling were used based on the studies of Hafez and Elbestawy. The expected fragment of variable region of the 16S rRNA gene (350 bp) were purified using the same kit and the same primers used for amplification, and were used for sequencing by automated sequencer (Macrogen, South Korea). The sequences were analyzed using DNA BLASTN and submitted in the GenBank under accession number GQ917222.

Examination of Arsenate Resistance Gene Using Differential Display
RNA Extraction and Preparation
Total RNAs of the bacterium strain treated and non-treated with arsenate were extracted using RNAase kit according to manufacturer's instructions (Qiagen). The RNA was dissolved in DEPC-treated water, its concentration and purity was examined with spectrophotometer at OD₂₆₀ and loading on 1.2% agarose gel.

Reverse Transcription of RNA and cDNA Synthesis
A 2.5 µL from each RNA was mixed with 5 µL of 2× reverse transcription mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 20 mM dithiothreitol, 2.5 µL dNTPs (each at 4 mM), 1 µL oligo (dT) primer (Promega), 13 µL of RNAase free water and 1 µL (50 unit µL⁻¹) of murine leukemia virus reverse transcriptase and then incubated at 37°C for 1 h according to the procedure of

Differential Display-PCR
After cDNA synthesis, PCR was carried out to a 23 µL of Taq DNA polymerase reaction mixture containing: 10 mM Tris HCl (pH 8.3), 2.5 mM KCl, 4 mM MgCl₂, 2 µL from each primer (Table 1), 1 unit of Taq polymerase (AmpliTaq, Perkin-Elmer) and 2 µL of the synthesized cDNA. The mixture was cycled first in 97°C thermal
Table 1—List of primers used in the study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHR</td>
<td>TGCCTTTGATTCTAGTCATC</td>
</tr>
<tr>
<td>DFRf</td>
<td>CAAAAGCCCGAATACGATG</td>
</tr>
<tr>
<td>F3HF</td>
<td>AGAGAGGGGAAATGTAAGG</td>
</tr>
<tr>
<td>16S Forward</td>
<td>AGGTTTGATCTCGGTCGAG</td>
</tr>
<tr>
<td>16S Reverse</td>
<td>ACGGCTACCTGTTACGACTT</td>
</tr>
</tbody>
</table>

cycler (Perkin-Elmer-USA) programmed at 94°C for 5 min, 56°C for 5 min and 72°C for 5 min, followed by 40 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min. Reaction was then incubated at 72°C for 10 min for final extension. Prior to loading of 10 μL for gel electrophoresis, 2 μL of loading dye was added. Gels were run at 80 V in 0.5× TBE buffer and 1.5% agarose. After electrophoresis, gel was stained in 0.5 μg mL⁻¹ (w/v) ethidium bromide. Finally, gels were visualized and photographed using gel documentation system.

Cloning of Expected Fragments of Differentially Display of Arsenate Resistant Genes

The differential-PCR product of up regulated genes that may play an essential role for arsenate bioremediation was cut from the gel and was purified using a Qiagen quick gel extraction kit (Qiagen Inc., Germany). These purified DNA fragments were ligated into the pGEM-T vector (Promega Co., USA) and then transformed into Escherichia coli DH5α. The recombinant DNA plasmid was then directly sequenced using the automated sequencer (Macrogen Company, Korea) with M13 forward universal primer. DNA homology searches were carried out with the National Centre for Biotechnology Information (NCBI) databases, using the BLAST network service.

Nucleotide Sequence, Sequence Analysis and GenBank Accession Numbers

Analysis of DNA nucleotide sequence and deduced amino acid sequences was carried out using EditSeq-DNAstar Inc., Expert Sequence Analysis software, Windows 32 Edit Seq 4.00 (1989-1999) and ExPasy database on the internet. BLAST search for alignment of the obtained sequences compared with the published data were done using database of NCBI, under accession numbers GU295989-GU295990.

Phylogenetic Analysis for Arsenate Removal Genes Compared with Others from GenBank

Phylogenetic analysis was carried out using MEGA4 program (http://www.megasoftware.net).

Results

Isolation of Arsenate Resistant Bacterial Strains

We isolated 30 isolates that resisted the presence of different concentrations of arsenate in the medium (Fig. 1). However, for further studies, only one strain (EG1) was selected, which was resistant to the high concentration (upto 2 g L⁻¹) of arsenate (Fig. 1). This highly arsenate resistance strain was isolated from the soil samples contaminated with arsenate in the area of Borg-El Arab, North of Alexandria and this area is known as a big industrial production sector in Egypt. The growth activity of this strain decreased markedly with the increase of arsenate concentration as compared to the control; the reduction rate of the growth was about 47%.

Identification of Highly Arsenate Resistant Strain

The total DNA of active bacterial strain EG1, selected for arsenate bioremoval, was isolated and amplified by two specific primers for 16S rRNA gene. The amplified portion of 16S rRNA gene was sequenced and subjected to BLAST search against the available sequence of the GenBank. The sequence of EG1 strain gave high similarity (99%) with bacterial species Bacillus pumilus. Using the sequence, a phylogenetic tree was constructed to confirm the results of BLAST search and strain EG1 was identified as B. pumilus.

Up/Down Regulated Genes Scanned by Differential Display

Differential display showed 260 different mRNA that were scanned using the three arbitrary primers. Most of the scanned genes were present in both treated and control samples. However, few of the genes were found to be up and down regulated. The 4 up regulated genes were induced at high concentration of arsenate (1 & 2 mg mL⁻¹). These genes were
amplified using F3Hf primer, which gave the products of mol wt ranged 450 to 700 bp, while 2 up regulated genes were noticed at low concentration of arsenate (25 µg mL⁻¹). The later 2 up regulated genes were amplified using primers CHR and DFRf. The band size of both the genes had mol wt of 650 bp (Fig. 2).

Cloning, Sequencing and Phylogenetic Analysis of Identified Genes

The two highly up regulated genes were selected, cut from the gel and purified for cloning in E. coli DH₅α. The confirmations of new recombinants using mini prep were listed in supplementary file (†Suppl Figs S1 & S2). Sequencing the fragments of arsenate resistant genes could help us to identify the first up regulated genes as β-galactosidase (Gu295989) and the second identified as subtilisin (Gu295990). These two genes were theoretically having pI/Mw of 5.21/35.6 kDa and 5.36/12.7 kDa for araB and subtilisin, respectively.

Phylogenetic tree of the β-galactosidase gene with the other related genes obtained from GenBank revealed that the obtained β-galactosidase from our native arsenate resistant strain was similar to β-galactosidase from several Bacillus strains with high similarity (Fig. 3) and they evolved from the same ancestor. On the other hand, it gave high similarity with arsenical pump membrane protein. Phylogenetic tree in Fig. 4A shows the similarity of arsenical pump membrane protein (arsB1) gene from standard strains and β-galactosidase from native B. pumilus. The amino acid sequences of the β-galactosidase gene was aligned with arsenical pump membrane protein 1 (arsB1) gene isolated from B. pumilus and the results showed many conservative regions between the two aligned genes (Fig. 4B). The second up regulated gene was identified as subtilisin, as it was similar to subtilisin gene isolated and identified from several bacterial strains of Bacillus sp. The similarity of new subtilisin gene from arsenate resistant strain with B. pumilus was about 67% (Fig. 5).

Fig. 3—Neighbor-joining phylogenetic tree showing the evolutionary relationship between the β-galactosidase gene from native B. pumilus strain EG1 and others presented from standard strains in GenBank. [The numbers on branches represent bootstrap support for 1,000 replicates. Accession numbers of β-galactosidase genes from GenBank were included between brackets.]

Fig. 2—Differential display technique using 3 different arbitrary primers (CHR, DFRf & F3HF) to scan the up or down regulated genes of B. pumilus involved in bio-removal of arsenate. [Lane M, 1 Kbp DNA marker; Lane 1, Control; Lanes 2-10, Bacterium strain EG1 grown in medium containing different concentrations of arsenate (1 to 2 mg mL⁻¹)].
Fig. 4 (A & B)—Relationship between β-galactosidase gene and the arsenate resistant genes: A. Phylogenetic tree for β-galactosidase gene and arsenate resistant genes from different bacterial species; B. Amino acid sequence alignment between obtained β-galactosidase gene and arsenical pump membrane protein 1 (arsB1) gene, both genes isolated from B. pumilus. [The alignment was carried out using Clustal W 1.4 program; red blocks show the conservative regions between the two aligned genes.]

Fig. 5—Neighbor-joining phylogenetic tree showing the evolutionary relationship between subtilisin gene from B. pumilus and others presented in GenBank. [The numbers on branches represent bootstrap support for 1,000 replicates. Accession numbers of nucleotide sequences are included between brackets.]

Discussion

Worldwide arsenic is a pollutant that can produce cancer and cause serious health problems in different regions. Arsenic compounds are widely distributed everywhere, so isolation of arsenic resistance bacterial strains is a major interest for several scientists in the world\textsuperscript{20}. The well known resistant system to arsenate by bacterial strains can be performed by reducing arsenate to arsenite and removing the latter outside the cell, or transporting it through a vacuole\textsuperscript{4,5,21}.

Arsenate reduction to arsenite is catalyzed by arsenate reductase enzyme. The enzyme has been reported to be produced by three bacterial families. The first family is \textit{E. coli}, which produces ArsC, a protein uses glutathione (GSH)/glutaredoxin system as a reducing system and has a single catalytic cysteine. The second family is \textit{Staphylococcus aureus}, which generates arsenate reductase (also named ArsC). The last family is present only in eukaryotic organisms and was initially described in \textit{Saccharomyces cerevisiae} and \textit{Arabidopsis thaliana}\textsuperscript{21}.

In the present study, among 30 bacterial isolates which isolated from different contaminated sites and were screened for arsenate resistance, only one strain showed very high resistance to this metal, up to 2g L\textsuperscript{-1}. This strain identified as \textit{B. pumilus} based on partial sequencing of 16S rRNA (GQ917222). Similar results were obtained by Shivaji \textit{et al}\textsuperscript{22} who found that different bacterial species belonging to the genus \textit{Bacillus} were capable of bioremediating different kinds of heavy metals including arsenate. Chari \textit{et al}\textsuperscript{23} noted that strain of \textit{B. pumilus} S8-10 could survive in the heavy metal-contaminated environments and accumulated considerable amount of various heavy metals.

In the present study, we reported the identification of arsenate resistant gene, which was similar to the
most arsenate resistant genes in Bacillus strains, but at the same time this gene showed similarity with β-galactosidase gene. Thus, it shows that this gene may produce two different proteins using the epigenetic way. The first protein is the arsenate resistant protein, which is necessary for producing energy by the hydrolysis of hydrocarbons by β-galactosidase (the second gene).

Lopez et al. reported two genes of Synechocystis sp. that were associated in arsenate reduction. These two genes had one mechanism or pathway as they worked together and could not function separately. This confirms our results of the arsenate resistant gene in B. pumilus, which plays a role in arsenate reduction and carbohydrate hydrolysis using one post transcription process. In another study, Ordoenx et al. showed that arsenate permeases was also an essential genes for arsenate resistance in Corynebacterium glutamicum.

In the present study, we observed that subtilisin was produced in control sample and the cells that were grown in 50 and 100 µg mL⁻¹, but it was completely diminished in the other concentrations. The sequence analysis of the isolated genes revealed that it is a new gene because it showed similarity with the subtilisin not exceed more than 64%. Bacilli produce a variety of proteases, among which subtilisin is one of the most extensively studied of all bacterial proteins. More than 500 subtilisin-like serine proteases have been assigned to the superfamily of subtilases, which can be found in both prokaryotes and eukaryotes. Subtilisin plays an important role in polyaromatic hydrocarbons degradation. It also plays another role in protein modification. We suggest that subtilisin may play an important role in the synthesis of β-galacconase enzyme, which is essential for bacterial cell resistance against arsenate toxic effects. β-Galactonase enzyme plays an important role in the bacterial defense system against the pathogens toxins and secrerctions.

The up regulation of the subtilisin gene in bacteria treated with high arsenate concentration revealed that arsenate might suppress its expression but at the same time induce the arB gene. Miyaji et al. demonstrated that a zein-degrading bacterium assigned to B. pumilus produced two different types of extracellular proteases, which exhibited an ability to hydrolyze zein in an extreme alkaline condition.

Conclusion
In the present study, we screened the ability of a number of bacterial strains (30 isolates), isolated from the soil in the big industrial sector of Borg-El Arab, to degrade arsenate. Among these strains, a highly resistant strain EG1 able to tolerate up to 2g L⁻¹ arsenate was selected. This strain was identified as B. pumilus on the basis of 16S rRNA gene. Further, two genes, arB and subtilisin, were selected on the basis of differential display of genes involved in arsenate bioremediation. These genes oxidize the arsenate and produce the necessary energy from hydrolysis of carbohydrate by galactosidase enzyme. Thus, the present B. pumilus strain can be used for successful bioremediation of arsenic polluted environments.

Acknowledgement
The present work funded by Alexandria University, Alexandria, Egypt.

References
19 Shati A A, Elsaid F G & Haife E E, Biochemical and molecular aspects of aluminum chloride-induced neurotoxicity in mice and the protective role of Crocus sativus L. extraction and honey syrup, Neuroscience, 175 (2011) 66-74.