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# Isolation and *ars* Detoxification of Arsenite-Oxidizing Bacteria from Abandoned Arsenic-Contaminated Mines

**CHANG, JIN-SOO, IN-HO YOON, AND KYOUNG-WOONG KIM***

*Arsenic Geoenvironment Laboratory (National Research Laboratory), Department of Environment Science and Engineering, Gwangju Institute of Science and Technology (GIST), Gwangju 500-712, Korea*

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**Abstract** The ecosystems of certain abandoned mines contain arsenic-resistant bacteria capable of performing detoxification when an *ars* gene is present in the bacterial genome. The *ars* gene has already been isolated from *Pseudomonas putida* and identified as a member of the membrane transport regulatory deoxyribonucleic acid family. The arsenite-oxidizing bacterial strains isolated in the present study were found to grow in the presence of 66.7 mM sodium arsenate (V; $Na_2HAsO_4 \cdot 7H_20$), yet experienced inhibited growth when the sodium arsenite (III; $NaAsO_2$) concentration was higher than 26 mM. Batch experiment results showed that *Pseudomonas putida* strain OS-5 completely oxidized 1 mM of As(III) to As(V) within 35 h. An *ars*B gene encoding a membrane transport regulatory protein was observed in arsenite-oxidizing *Pseudomonas putida* strain OS-5, whereas *arsB*, *arsH*, and *arrA* were detected in strain OS-19, *arsD* and *arsB* were isolated from strain RW-18, and *arsR*, *arsD*, and *arsB* were found in *E. coli* strain OS-80. The leader gene of *arsR*, *-arsD*, was observed in a weak acid position. Thus, for bacteria exposed to weak acidity, the *ars* system may cause changes to the ecosystems of As-contaminated mines. Accordingly, the present results suggest that *arsR*, *arsD*, *arsAB*, *arsA*, *arsB*, *arsC*, *arsH*, *arrA*, *arrB*, *aoxA*, *aoxB*, *aoxC*, *aoxD*, *aroA*, and *aroB* may be useful for arsenite-oxidizing bacteria in abandoned arsenic-contaminated mines.

**Keywords:** *ars* Detoxification, arsenite-oxidizing bacteria, abandoned mine

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Arsenite-oxidizing bacteria play an important role in the ecosystems of abandoned arsenic-contaminated mines, as a multiplicity of *ars* mechanisms affect the transformation between soluble and insoluble arsenic forms and toxic and non-toxic arsenic forms [9, 15, 17]. The results of a previous study conducted by Saltikov *et al.* [25] suggested that ArsB and ArsC may be useful for bacteria that perform As(V)-respiring in environments where As concentrations are high, although neither is required for respiration. Results found for *Thermus aquaticus* and *T. thermophilus* suggest that the ecological role of arsenite oxidation is the detoxification of arsenic [6, 16].

Many indigenous arsenic-resistant bacteria have been isolated from soils and sediments that contain high levels of arsenic [7, 21, 34, 28]. Within these bacterial strains, the arsenic resistance system (*ars*) genes, which encode proteins that provide resistance through the oxidation of arsenite or the reduction of arsenate depending on the bacterial strain [4, 32], can be found in either the chromosome [2] or plasmids [1, 22, 30, 41]. Arsenic bioremediation has already been achieved with the use of $As^r$ (arsenic-resistant) bacteria, and several of the mechanisms involved in the detoxification of arsenic have been identified using the chromosomes and plasmid-encoded *ars* operons of *E. coli* [3]. These operons consist of several open reading frames (ORFs), including *arsR*, *-D*, *-A*, *-B*, *-C*, *-H*, *-O*, and *-T* [36] and other heavy-metal ORFs, such as *merR* [32]. The arsenic operons typically encode at least three genes (*arsR*, *B*, and *C*) [28]. The *arsR* gene is the first gene within the operon, and is only transcribed after the cells come into contact with As(III) or Sb(III) [38, 39]. When either of these metals binds to the ArsD protein, which represses the transcription of the *ars* operon, ArsD undergoes a conformational change and dissociates from the promoter region of the *ars* operon. The arsenate is then reduced to arsenite by ArsC and transported by ArsB. These first and secondary carrier proteins are located in the plasma membrane and push the arsenite from the cells. Thus, oxidizing by bacteria in subsurface environments is one of the most important processes in the geochemical heavy metal and redox [8, 18, 20].

A chromosomally encoded efflux system transports arsenic using either a single-polypeptide system (ArsB) or a two-

*Corresponding author
Phone: 82-62-970-2442; Fax: 82-62-970-2434;
E-mail: kwkim@gist.ac.kr

component system (ArsA and ArsB), which functions as a chemiosmotic transporter [11, 31]. The ArsB protein is a membrane protein that functions alone as a chemiosmotic arsenite transport protein [32], whereas the *arsA* gene encodes a unique ATPase that binds to ArsB [37]. The ArsAB pump is composed of six transmembrane segments and a catalytic subunit that functions in the reduction mechanism of the ArsA ATPase [1]. As a mettalloregulatory protein, ArsR [40], a regulatory gene of *ars*R, is overexpressed during the bioaccumulation of arsenic.

The arsenate-reductase genes of *Shewanella* strain ANA-3 (AY271310), *arrA* (*arrA*; putative product 804 aa residues) and *arrB* (*arrB*; putative product 234 aa residues), were previously identified by transposition in their known or periplasmic locations [15]. The function of the *arsC* gene is to support arsenic detoxification by bacteria *via* arsenite-oxidization or arsenate reduction in soil columns with 75 μM As(III) or 250 μM As(V), respectively, under nonsaturated conditions [14]. Although the function of the *ars*H chromosomal gene is unknown, it is likely required for resistance to both arsenite and arsenate in *Y. pseudotuberculosis* and is an *ars* gene [13]. Furthermore, the molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26, a member of the arsenic resistance system, has already been studied in *aroA*, *aroB*, *aroC*, and *aroD* [29].

Accordingly, this paper describes the isolation and characterization of numerous arsenic-resistant strains, their *ars* genes, and redox activities under facultative aerobic and anaerobic conditions. The resulting information suggests that the study of indigenous arsenite-oxidizing bacteria may lead to a better understanding of molecular geomicrobiology and can be applied to the bioremediation of arsenic-contaminated sites. This may also be the first study to investigate chromosomal *ars* mechanisms using *Pseudomonas putida* strains and *E. coli*. In the foreseeable future, the investigation of the modern biogeochemistry of abandoned mines using *ars* system ecological studies may inspire the development of integrated indigenous *ars* models for the processes that shape the arsenic contamination.

## MATERIALS AND METHODS

### Field Sampling and Isolation of Aerobic and Anaerobic Strains

One g of soil and 1 g of sediment were collected from ten sites at two abandoned silver and gold-mine areas (Myoung-bong and Duck-um) in the Republic of Korea. The experiments were all initiated within seven days after the sample collection. The soil and sediment samples were added to an MSB medium [33] containing sodium arsenite ($NaAsO_2$ [Sigma]) and sodium arsenate ($Na_2HAsO_4$ [Sigma]). After several transfers, the isolated colonies were assessed for arsenic(III) and arsenic(V), and every isolate selected. The As(III) and As(V) were used at concentrations from 0 mM to 66.7 mM and 133.4 mM, respectively, by the 7th pure culture. The arsenic-resistance experiments were carried out at a variety of arsenic concentrations [As(III): $NaAsO_2$; 0, 5, 10, 15, 20, and 26 mM. As(V): $Na_2AsO_4 \cdot 7H_2O$; 0, 5, 10, 15, 20, 26, 40, 53, and 66.7 mM] for all the isolates. All the arsenite-oxidizing and arsenate-reducing microorganisms were cultured at 30°C in an MSB medium (Stranier's Basal Medium, pH 7) with 1 mM D(+)-glucose and ethanol as the carbon sources. The strains routinely grew in the MSB, which was prepared as follows: 40 ml of solution A ($4.8\times10^{-4}$ mM $Na_2HPO_4 \cdot 7H_2O$ and $1.5\times10^{-4}$ mM $KH_2PO_4$), 20 ml of solution B ($3.0\times10^{-4}$ mM $C_{10}H_{12}N_2O_8Na_4 \cdot 2H_2O$, $2.7\times10^{-3}$ mM $ZnSO_4 \cdot 7H_2O$, $4.0\times10^{-4}$ mM $MnSO_4 \cdot H_2O$, $8.0\times10^{-6}$ mM $CuSO_4 \cdot 5H_2O$, $3.5\times10^{-3}$ mM $Co(NO_3)_2 \cdot 6H_2O$, $5.4\times10^{-2}$ mM $MgSO_4 \cdot 7H_2O$, $2.2\times10^{-2}$ mM $CaCl_2 \cdot 2H_2O$, $7.0\times10^{-6}$ mM $(NH_4)_6Mo_7O_{24} \cdot H_2O$, $3.0\times10^{-4}$ mM $FeSO_4 \cdot 8H_2O$, $1.0\times10^{-4}$ mM $C_{12}H_{17}C_{12}N_4OS$-HCl, $1.0\times10^{-4}$ mM Biotin, $1.7\times10^{-4}$ mM NaCl, and $1.8\times10^{-2}$ mM $KNO_3$), and 5 ml of $1.5\times10^{-4}$ mM $(NH_4)_2$. A $SO_4$ solution was also mixed and the volume adjusted to 1 l with distilled water, plus the media were all autoclaved at 121°C for 25 min prior to use. The bacteria were harvested (12,000 rpm, 20 min) prior to inoculation, and the isolates stored in 25% glycerol at −80°C. After an aerobic test with each pure culture, Petri dishes containing the media were placed in an anaerobic chamber (Coy Laboratory, MI, U.S.A.) at 30°C (gas mixture; 5%$CO_2$, 10%$H_2$, and 85%$N_2$). The isolated colonies were again assessed for their levels of sodium arsenite ($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), and a single isolate was selected. The same arsenic concentrations used in the aerobic tests were also used in the arsenic resistance experiments. The pH of the MSB was 7.0. For the anaerobic liquid cultures, the MSB media were first preincubated in the glove box of the chamber for at least 3 days. The arsenic-resistant bacteria were found to grow in the MSB with (1 mM) D(+)-glucose.

### Determination of Arsenic Speciation for Oxidation and Reduction Assays

Using a hand auger, a total of 5 g of soil and 5 g of sediment were collected from each mine in July 2003. The soil samples were air-dried and sieved through a 10-mesh screen (less than 2 mm), and 25 ml of distilled water added to each sample. After shaking, the water phase was filtered through a membrane PTFE filter (Whaitman 0.45-μm pore size, 13 mm), and 1 ml of $HNO_3$ and 3 ml of HCl were added to 0.25 g of the residue. This solution was then heated at 70°C for 1 h with shaking, while also adding distilled water to the solution. The samples were again filtered through a membrane PTFE filter (Whatman 0.45-μm pore size, 13 mm), and the arsenic concentrations measured using a hydride-generation atomic absorption spectrophotometer (HG-AAS, Perkin Elmer 5100) with a detection limit of

1 μl/l As [35]. In brief, the oxidation of arsenite to arsenate was monitored over the period of incubation. The arsenite concentration was determined based on removing the arsenate from the solution by passing it through a silica-based strong anion cartridge (LC-SAX SPE, Supelco) [12]. A cartridge method was then used to evaluate the arsenic speciation, where a 2-ml sample solution was allowed to flow through an anion-exchange (SAX) and strong cation (SCX) cartridge, which had been presoaked, with deionized water (1:1).

**Isolation of Genomic DNA**

Bacterial genomic DNA was prepared from the bacterial cultures using standard methods [27], and placed in a 1-ml microcentrifuge tube with the appropriate individual colony. The bacterial suspensions were composed of $10^8$ CFU/ml of each isolate, and incubated overnight at 30°C with intermittent shaking. The cultures were then placed in a 1.5-ml microcentrifuge tube with 1 ml of TES (10 ml Tris-HCl, 50 mM EDTA, 10% sodium dodecyl sulfate) and 10 μl of proteinase K (50 mg/l), and then allowed to react in a 55°C tremulous cistern for 10–12 h to digest the protein. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the reaction mixture, and then the solution was manually mixed for 3 min, centrifuged at 12,000 rpm for 15 min, and the supernatant removed. This process was repeated 3 times. The supernatant was then mixed with a 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The tubes were shaken slowly and the genomic DNA gently removed, cleaned with 75% ethanol, dried, and dissolved in TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH 7.2). The DNA purity was

**Table 1.** Oligonucleotide primers used for amplification of chromosomal *ars* operon genes.

| Amplified region[a] | Primer sequence | $T_m$[b] |
|---|---|---|
| SEL0904 Ars sense (universal) | 5'-ATC ATG GCT CAG ATT GAA CGC-3' | 55 |
| SEL1226 Ars Antisense (universal) | 5'-T ACC TTG TTA CGA CTT CTA CCT-3' | |
| AGL0609 ArsR sense | 5'-ATC CAG CTC TTC AAA ACC-3' | 53 |
| AGL0310 ArsR Antisense | 5'-GTT TTT CAG CTT CAT AC-3' | |
| AGL0725 ArsD sense | 5'-ATG TGC TGC AGT ACC GCC GT-3' | 60 |
| AGL0726 ArsD Antisense | 5'-TAT TAC CAC CAC AGC AAC-3' | |
| AGL0923 ArsA sense | 5'-ACC CAC GCT TAG CAA TAT CAT CGA-3' | 55 |
| AGL1230 ArsA Antisense | 5'-TGA AAG TCT TCA TAT AGG TCT TCC-3' | |
| AGL0929 ArsB sense | 5'-GTG GAA TAT CGT CTG GAA TGC GAC-3' | 57 |
| AGL0226 ArsB Antisense | 5'-GGT AAT TTT CGG CCC CAA ATC G-3' | |
| AGL1008 ArsAB sense | 5'-AAA ACT TCC ATT TCC TGC GCG ACG-3' | 60 |
| AGL1203 ArsAB Antisense | 5'-AAG TGA AAG AGA GAC GTA GCG CCA-3' | |
| AGL1117 ArsC sense | 5'-TGC GGC ACT TCG TGA AAC AC-3' | 57 |
| AGL0822 ArsC Antisense | 5'-AAG TAT ATC CAG AAC CAC TT-3' | |
| AGL0507 ArsH sense | 5'-ATG GAC CAG TTC CCA GAC-3' | 55 |
| AGL1103 ArsH Antisense | 5'-CTG ATT GGG GAT GGT GAA CA-3' | |
| AGL-*arrA* sense | 5'-CTT TGA AGA AAT TCA AAC GTA CG-3' | 59 |
| AGL-*arrA* Antisense | 5'-TCA AAG TTT CGC TGT AAA ACT CA-3' | |
| AGL-*arrB* sense | 5'-AAC ACG AAC GAC GGT ATT CAC TGG-3' | 59 |
| AGL-*arrB* Antisense | 5'-ATA CCT TGC TCT GTG GAT CAT CTA-3' | |
| AGL-*aoxA* sense | 5'-ATG GAA CAT CAA ACT AGT CG-3' | 59 |
| AGL-*aoxA* Antisense | 5'-ACA GAA TGT TGG ATT GAC G-3' | |
| AGL-*aoxB* sense | 5'-AGC ACT GGC GTA CTC GCA ACA TAT-3' | 59 |
| AGL-*aoxB* Antisense | 5'-ATT GAC GTA ACC AAC GAG CAT GAT-3' | |
| AGL-*aoxC* sense | 5'-TTT GAA CAA CGC ACA AGT CCA-3' | 59 |
| AGL-*aoxC* Antisense | 5'-TTA GAA AAA TGA AAG GAT TGT-3' | |
| AGL-*aoxD* sense | 5'-ATG AAA TAT GCC ATT GCT ATT-3' | 59 |
| AGL-*aoxD* Antisense | 5'-AAT ATC CAT TAC ACG ACA AGT-3' | |
| AGL-*aroA* sense | 5'-GT ATG TCA CGT TGT CAA AAC-3' | 59 |
| AGL-*aroA* Antisense | 5'-TTA TAG AAC GTT GGA CAG AC-3' | |
| AGL-*aroB* sense | 5'-ACT CTT CAC CTA TAT CGC CGA-3' | 59 |
| AGL-*aroB* Antisense | 5'-TTC TCG TAA CCG AAC ATG ACA-3' | |

[a]GenBank sequence data were obtained by alignment of sequences in Search Tool (BLAST; National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]).

[b]Tm, annealing temperature used in the PCR cycle.

measured using a spectrophotometer set at 260 and 280 nm. All the DNA had an absorbance ratio ($A_{260\,nm}/A_{280\,nm}$) ranging from 1.7 to 2.0.

**PCR Amplification of 16S rRNAs and *ars* Genes**

The oligonucleotides used in the PCR reaction were obtained from the Bioneer Corp., Republic of Korea, and the PCR carried out using an AccuPower PCR Premix (Bioneer, Republic of Korea). The sequences of the PCR primers for the 16S rRNA and arsenic resistance genes are shown in Table 1. The bacterial genomic DNA was prepared from the bacterial cultures using standard methods [27], and the universal primers used for the 16S rRNA detection were SEL0904 16S rRNA sense (universal) (5'-ATC ATG GCT CAG ATT GAA CGC-3') and SEL1226 16S rRNA antisense (universal) (5'-T ACC TTG TTA CGA CTT CTA CCT-3'). The large fragment of the 16S rRNA gene was approximately 1.5 kb in size. The primers used to amplify the *ars* genes were designed specifically for *arsR*, *arsD*, *arsA*, *arsB*, *arsAB*, *arsC*, *arsH*, *arrA*, *arrB*, *aoxA*, *aoxB*, *aoxC*, *aoxD*, *aroA*, and *aroB*, respectively. The PCR amplification (35 cycles of denaturation at 94°C for 5 min, annealing at 55°C for 1 min, and an extension at 72°C for 2 min, followed by an additional extension for 7 min at 72°C) was performed in a final volume of 50 µl containing 0.5 µg genomic DNA and 10 pmol of the primers. The PCR was conducted in a Mastercycler Gradient (Eppendorf, Germany), and the PCR products were identified by gel electrophoresis using 0.7% to 1.5% agarose gels. The samples were analyzed using an automated DNA sequencer (Model 3100; ABI PRISM Genetic Analyzer System Profile, Seoul, Republic of Korea). The accession numbers for the *ars* gene sequences were as follows: *arsR* - NC_002974, AB004659, M68624, and AY04276; *arsD* - DQ141542, DQ112333, AY046276, AY271, A004659, AY297781, and U13073; *arsB* - AY866406, NC_002974, J02591, M68624, and AJ271973; *arsAB* - AY866408, DQ112328, DQ112332, DQ112329, NC_005861, BX908798, AB004659, AF168737, and U38947-AB; *arsC* - DQ112328, NC_002974, AY004659, AY046276, and AJ271973; *arsH* - DQ112328, DQ141542, DQ141544, NC_002974, U58366, XM_748061, and AJ271973; *arrA* - AY283639, AY660883, AY660884, AY660885, AY660886, and AY271310; *arrB* - AY283639 and AY271310; *aoxA* - AY345225, AF509588, Z48565, DQ151549, AB020482, AY791695, and AB040418; *aoxB* - AY345225, AF5588, DQ151549, and AB020482; *aoxC* - AY345225, AF509588, and AB020482; *aoxD* - AY345225 and AF509588; *aroA* - AY345225, AY775558, and AY775559; and *aroB* - AY345225.

**Sequencing of 16S rDNA and Ars[r] Genes**

The nucleotide sequences were determined using the dideoxy-chain termination method, with a PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). The sequencing primers were purchased from Geno-Tech (Seoul, Republic of Korea). The oligonucleotide primers included in this investigation were used for the positions of the 16S rRNA gene, whereas the arsenic resistance system primers (Table 1) were designed according to previously published sequences. The sequencing was performed in a 6 µl volume, with 15 to 50 ng of the PCR product and 1 pmol of each primer. The samples were analyzed using an automated DNA sequencer (Model 3100; ABI PRISM Genetic Analyzer System Profile, U.S.A.), and the 16S rDNA sequencing results compared with sequences from the NCBI database. The BLAST algorithm integrated with the Vector NTI Suite v5.5.1 (InforMax, U.S.A.) was used to determine values for the sequence homologies. Database sequences with fewer than 1,500 nucleotides were excluded from the phylogenetic analysis.

**Arsenic Oxidation and Reduction Assays**

To test the ability of the strains to oxidize arsenite, the isolates were inoculated in 250-ml glass flasks with 60 ml of MSB and 75 ml/l sodium arsenite. For the batch tests, strain OS-5 ($10^7$/CFU) was inoculated into MSB media supplemented with 1,000 µM of sodium arsenite, and the concentrations of As(III) and As(V) in the culture media then determined. The experiments were performed in triplicate using six 60-ml Erlenmeyer flasks, and the bacteria incubated aerobically at 30°C for 200 h with shaking (180 rpm). The control experiments using sterile media (*i.e.*, un-inoculated) with 75 mg/l sodium arsenite were also incubated under the same conditions. Two-ml samples were taken periodically to measure the cell density and determine the arsenic speciation. These samples were centrifuged at 12,000 rpm for 10 min, decanted, and stored at 4°C prior to the arsenic analysis [35]. Thus, to monitor the oxidation of arsenite to As(V) over the period of incubation, the arsenite concentration was determined by measuring the amount of As(V) using a silica-based strong anion cartridge (LC-SAX SPE, Supelco) [12]. As such, the concentration of As(III) was measured using a hydride generation atomic absorption spectrophotometer (HG-AAS, Perkin Elmer 5100). All the analytical measurements were performed in duplicate. The As(V) concentration was then determined based on the difference between the total arsenic concentration and the measured As(III) concentration.

## RESULTS

**Isolation and Characterization of Indigenous Ars[r] Bacterial Strains**

High concentrations of arsenic were found in both the tailings and sediments from the Myoung-bong (strain OS-5 sample point; 26.90 ppm) and Duck-um (tailing; 54.7 ppm, sediment;

**Table 2.** Bacterial strains, their identification, *ars* genotypes, and survival during chronic exposure to arsenic under aerobic and anaerobic conditions. Each strain was analyzed for the presence of the *arsR, arsD, arsA, arsB, arsAB, arsC, arsH, arrA, arrB, aoxA, aoxB, aoxC, aoxD, aroA,* and *aroB* genes within its genomic DNA using a PCR, as described in Materials and Methods.

| Strain or isolate | Isolate accession no. | 16S rDNA Similarly (%) to known bacteria | Phenotype | *ars* Genotype[a] | Doubling time (h) | Dead time: mM; day (aerobic/anaerobic)[b] | Total As concentration in sample points |
|---|---|---|---|---|---|---|---|
| *Pseudomonas aeruginosa*[c] | | | As(III), As(V) | *arsB*$^+$ | 25 | | |
| Myoung-bong mine soil | | | | | | | |
| OS-5 | AY952321 | 93.27%/*Pseudomonas putida* | As(III) | *arsB*$^+$ | 18 | 1; 7/1; 14 | 26.90 ppm |
| Duck-um mine soil | | | | | | | |
| OS-19 | AY866406 | 99.92%/*Pseudomonas putida* | As(III) | *arsB*$^+$ *arsH*$^c$ *arrA*$^+$ | 25 | 6; 180/3; 7 | 54.70 ppm |
| Duck-um mine sediment | | | | | | | |
| RW-28 | DQ112333 | 99.64%/*Pseudomonas putida* | As(III), As(V) | *arsD*$^+$ *arsB*$^+$ | 20 | 8; 7/8; 7 | 83.90 ppm |
| OS-80 | ABB76686 | 95.84%/*E. coli* | As(III), As(V) | *arsR*$^+$ *arsD*$^+$ *arsB*$^+$ | 25 | 0; 7/0; 7 | None |

[a]*ars* Resistance relationship analyzed for *ars* genotype: +, positive PCR product generated.
[b]Arsenic concentration test: Aerobic tests - Arsenic conc.; survival (days)/Anaerobic tests - Arsenic conc.; survival (days).
[c]Control strains: *Pseudomonas aeruginosa* (Korean Collection for Type Cultures - strain KCTC1636; American Type Culture Collection - strain ATCC15522).

83.9 ppm) mines. Four indigenous bacterial strains were isolated from the two abandoned Au-Ag mines, and subsequently characterized at a manifold sampling site to determine their arsenic uptake (Table 2 and Fig. 1). The isolated strains were grown at 30°C, with doubling times of 15–25 h in MSB containing at least 6 mM sodium arsenite (III) or 14 mM sodium arsenate (V). Along with several other bacterial species, such as *Escherichia coli* and strains

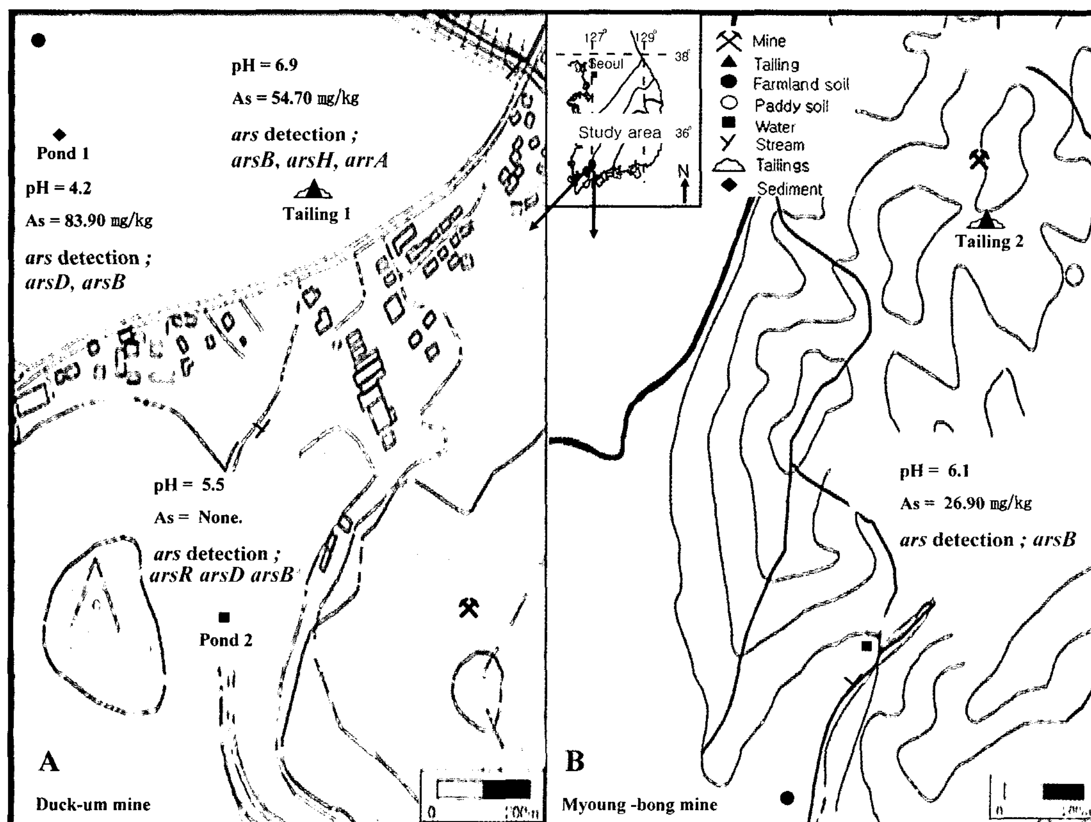


**Fig. 1.** Diversity, pH location, and *ars* features of two arsenic pond sites and two arsenic tailings located at Duck-um mine and Myoung-bong mine.

of gammaproteobacteria, *Pseudomonas putida*, an indigenous bacterium (phenotype; $Ars^r$), was isolated from the sediments collected at both sites, including a *Pseudomonas putida* strain capable of utilizing arsenite, arsenate, glucose, or ethanol. Several resistant strains from soil samples without arsenic were also identified as *Pseudomonas putida*, as shown in Table 2. These isolates were able to grow in the presence of 66.7 mM sodium arsenate (V), yet experienced inhibited growth when the arsenite (III) concentration was higher than 26 mM. Many of the organisms were tolerant to chronic arsenic exposure. For example, strain OS-19 was tested for 180 days to determine its tolerance range and found to be resistant to 6 mM over that period of time (Table 2). Furthermore, eight new arsenite-oxidizing bacteria and six new arsenate-reducing bacterial strains were discovered. One indigenous bacterial strain that utilized a high concentration of arsenite(III) (up to 1 mM under aerobic and anaerobic conditions) and glucose (1 mM/l), stain OS-5, was isolated from the soil obtained from the Myoung-bong mine and found to have a doubling time of 18 h.

**Characterization of *ars* Genes and Phylogenetic Analysis**
The diversity, pH 4.0 to 7.0, location, and *ars* features were assessed for two arsenic-contaminated pond sites and two arsenic tailings located in the Duck-um mine and Myoung-bong mine. The presence of *ars* genes within the chromosomes of each bacterial strain was evaluated using a PCR. The primers noted in Table 1 corresponded to the *arsR*, *arsD*, arsA, *arsAB*, *arsB*, *arsC*, *arsH*, *arrA*, *arrB*, *aoxA*, *aoxB*, *aoxC*, *aoxD*, *aroA*, and *aroB* genes. The strains differed greatly with respect to the genes they carried. For example, strains OS-5 (AY952321) and OS-19 (AY866406) encoded the *arsB* and *arsB*, *arsH*, and *arrA* genes, respectively, whereas the control strains, *Pseudomonas aeruginosa* and *E. coli*, encoded the *arsB* and the *arsR*, *arsD*, and *arsB* genes, respectively (Table 2 and Fig. 1). Interestingly, the gene encoding the *arsH* protein, with an unknown function, was found in one isolate, strain OS-19. The sequences for each *ars* gene have been deposited in the GenBank database under the accession numbers indicated in Table 2. The phylogenetic analysis of the complete 16S rDNA gene sequence (<1,486 nucleotides) from strain OS-5 (AY952321) is shown for the detection of *arsB* in Fig. 2. The tree was constructed from a matrix of pairs of genetic distances using the neighbor-joining tree method, and the phylogenetic data obtained by aligning the different sequences of arsenic-resistant bacteria using the BLAST Search Tool (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]). The arsenic resistance in *Pseudomonas putida* strain OS-5 was found to be mediated

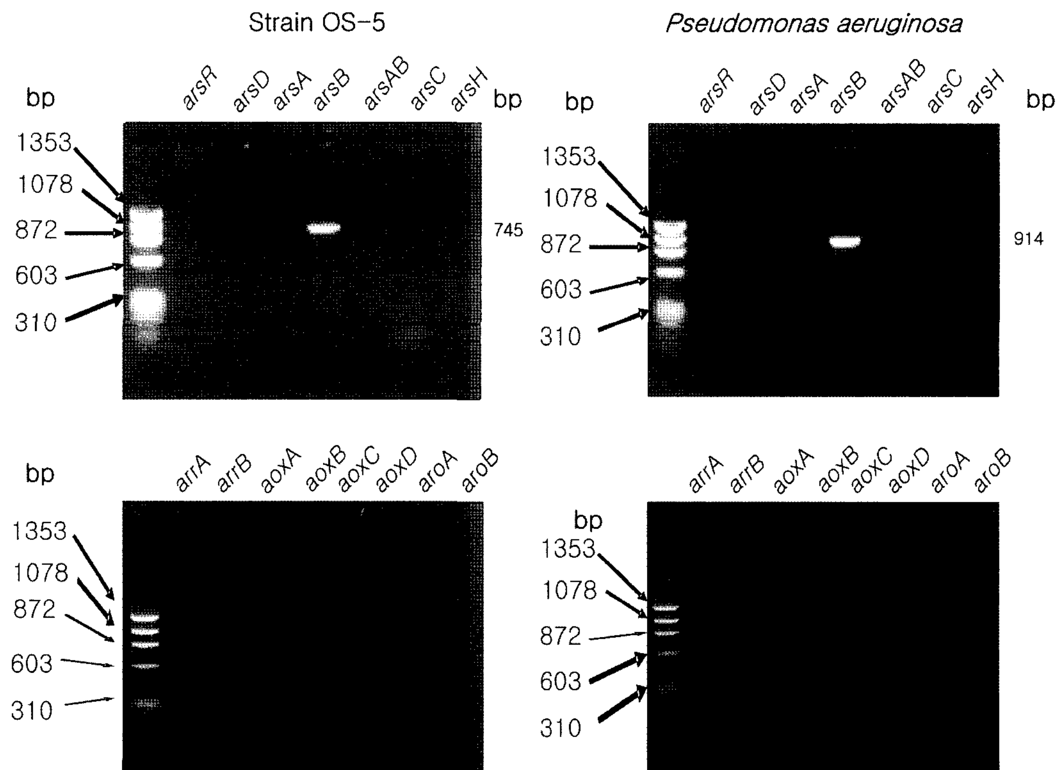


**Fig. 2.** Agarose gels (0.7 to 2.0%) showing PCR products amplified from genomes of several arsenite-resistant bacterial strains.
Lanes M: Lambda DNA/HindIII size mark (Promega, U.S.A.). The strains shown are OS-5 (AY952321) and *Pseudomonas aeruginosa* (KCTC 1636; ATCC 15522).

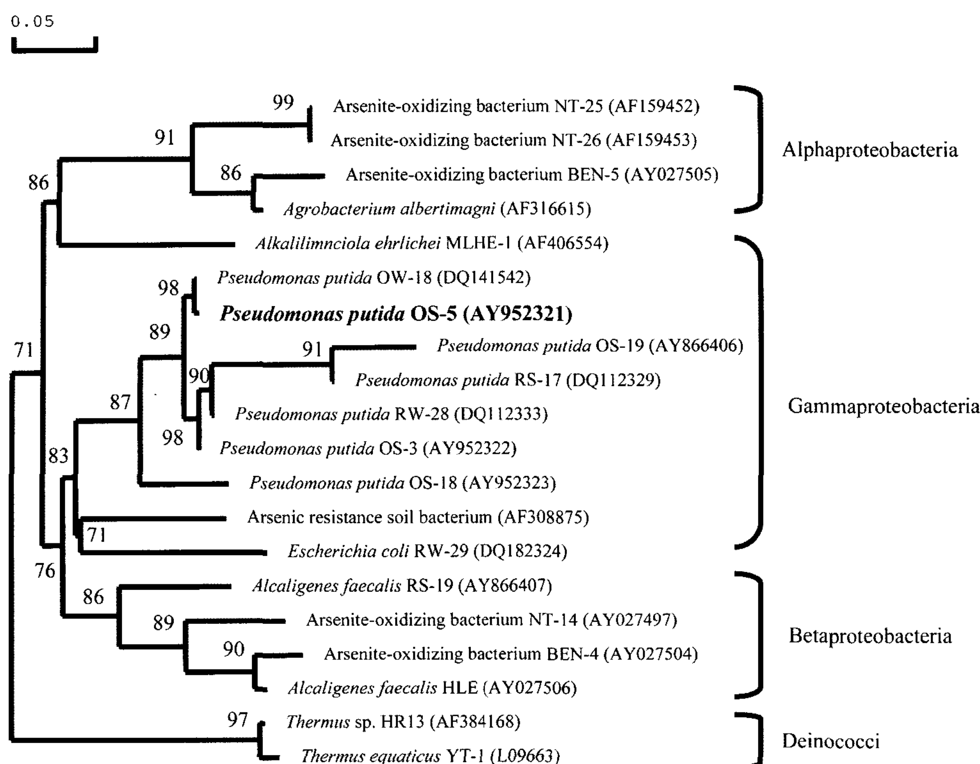


**Fig. 3.** Phylogenetic tree based on 16S rDNA sequence showing the position of arsenite-oxidizing bacterial isolates OS-5 (AY952321) and gammaproteobacteria.

The tree was constructed from a matrix of pair-wise genetic distances using the neighbor-joining tree method. The phylogenetic data were obtained by aligning the different arsenic-resistant bacterial sequences in the Search Tool (BLAST; National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) using standard parameters. The scale bar represents 0.05 substitutions per 100 nucleotides within the16S rDNA sequence.

by an efflux pump *arsB* gene, and the OS-5 nucleotide sequence analysis revealed that the *arsB* gene of *Pseudomonas putida* strain OS-5 had an approximately 93% similarity when compared with the 16S rDNA gene.

## Comparison with Other *ars* Genotypes

All the arsenite-oxidizing bacterial strains were found to be positive for at least one of the *ars* genotypes, yet differed with respect to the genes they contained (Table 2). Table 2 shows the genotypes for the selected strains and those for other bacterial strains previously reported to carry the same gene. The *arsB* gene was found to encode a membrane transport regulatory protein within the arsenite-oxidizing bacterial strains. The partial sequence exhibited a 93% homology with the same gene from *Pseudomonas aeruginosa* strain ATCC 15522 and an 89% homology with the plasmid-borne gene in *Salmonella typhimurium* (AP0005147). Each of the four strains showed a clear PCR product, yet this also varied from strain to strain, *i.e.*, *arsR* was found in 20% of the responding strains; *arsD*, - 40%; *arsA* - 20%; *arsB* - 100%; *arsAB* - 0%; *arsC* - 0%; *arsAB* - 0%; *arsH* - 20%; *arrA* - 20%; *arrB* - 0%; *aoxA* - 0%; *aoxB* - 0%; *aoxC* - 0%; *aoxD* - 0%; *aroA* - 0%, and *aroB* - 0% (Table 2). This was clearly shown in the results for *Pseudomonas putida*, *Pseudomonas aeruginosa*, and *E. coli*, which varied greatly as regards the *ars* genes present and their arsenate-reducing or arsenite-oxidizing capabilities.

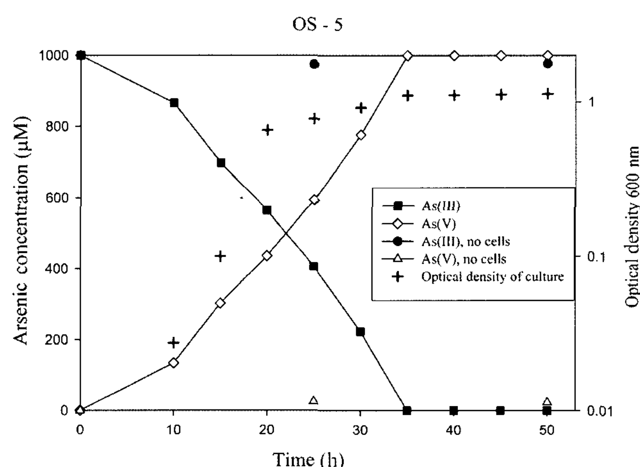


**Fig. 4.** Arsenite oxidation, arsenate reduction, and culture density during aerobic culturing of OS-5 (AY952321).

The experiment was performed independently in triplicate in batch modes with a working volume of 60 ml at 30°C. Each data point represents the average value for the readings from each experiment.

All the genotype results were performed independently and are listed in Table 2.

**Arsenic Redox Assay of Ars$^r$ Indigenous Bacteria**

OS-5 was cultivated under aerobic conditions, while monitoring the redox speciation of arsenic (Fig. 4). After 22.5 h, more than half of the arsenite was oxidized to As(V). The concentration of sodium arsenite (III) slowly decreased during the first 10 h of incubation, and then decreased more rapidly until it disappeared after 25 h. The initial low conversion rate of arsenite (V) was likely due to a lag phase in microbial growth. Therefore, the results demonstrated that strain OS-5 could oxidize arsenite to arsenate in less than 35 h in batch tests. Furthermore, strain OS-19 was also shown to be capable of completely oxidizing arsenite to arsenate (Table 2). However, the time required for the oxidization of 1,000 μM As(III) varied according to the strain, from 35 h [strains OS-19 (AY866406) and OS-5 (AY952321)] to more than 75 h [strains RW-28 (DQ112333) and OS-80 (ABB76686)].

## DISCUSSION

The arsenic concentrations in the tailings and sediments from the Myoung-bong and Duck-um areas were significantly high and likely to have an impact on the areas surrounding the mine dumps. The metal concentrations in tailings and sediments are influenced by various characteristics at each site, such as the soil composition, pH, Eh, the presence of mobile microorganisms, and redox conditions. From the two mines in the present study, a total of four new indigenous *ars* bacteria were isolated based on their abilities to grow in the presence of arsenic. Each was characterized to lead the oxidation of As(III) to As(V) or the reduction of As(V) to As(III). Therefore, these results suggest that indigenous bacteria and pH conditions can influence arsenic speciation in natural settings, and this phenomenon is characterized by mobility, since the bacteria lead the oxidation or reduction. Arsenite-oxidizing bacteria inhabiting natural systems require high-level arsenic detoxification systems that are not yet understood. Thus, the activity of the indigenous bacteria from the abandoned mines was investigated on the basis of their 16S rRNA growth under facultative anaerobic bacteria conditions (Table 2). Specifically, strain OS-19, which utilized a high concentration of arsenite(III) (up to 6 mM under aerobic conditions, lived 180 days; up to 3 mM under anaerobic conditions, lived 7 days), was isolated from soil obtained from the Duck-um mine and found to have a doubling time of 25 h. This may also explain why more chemolithoautotrophic arsenite-oxidizing bacteria were found in the abandoned mine. The other arsenite-oxidizing organisms isolated from the arsenic-contaminated mines included species with a low G+C content, strain OS-5, at 44%; strain OS-19, at 50%; and RW-28, at 52%.

The best-characterized arsenic resistance was encoded by the *arsR* and *arsD* operons of strain RW-28 and strain OS-80 found in the Duck-um mine. This leader gene was observed under weakly acidic conditions (pond 1, pH 4.0; pond 2, pH 5.0) (Fig. 1). For bacteria existing in weakly acidic conditions, this *ars* system can be active with a variety of arsenic species in As-contaminated mines. PCR detection and *ars* gene probes are often used to characterize bacterial strains and establish a general genetic model [4]. The present investigation into the diversity of the chromosomal *ars* gene population using a PCR found that this technique was also useful for screening *ars* gene sequences in indigenous bacteria, as a variety of genes involved in *ars* resistance were identified using gene homology, including the *arsR*, -*B*, and -*H* genes. According to Diorio [4], the *E. coli* chromosomal *ars* operon showed strong homology with the genes present in the *E. coli* plasmid R773, as well as the chromosomal *ars* operon found in *P. aeruginosa.* Cai *et al.* [2] stated that the chromosomal *ars* operon homolog in *Pseudomonas aeruginosa* was not found in other strains of *Pseudomonas*. Similarly, Salitkov *et al.* [25, 26] studied chromosomes based on the *ars* system of arsenic-resistant bacteria using the primers for *E. coli* pUM3; and the results showed that no *ars* genes were found in *Pseudomonas* sp. In this study, although four of the strains were classified as *Pseudomonas putida*, it was observed that the *ars* operon was not always the same, even though the strains were categorized within the same genera. Therefore, this type of diversity should be considered when *ars* systems are examined using PCR-based techniques. The studies of Macur *et al.* [14] and Cai *et al.* [2] found that the *Pseudomonas aeruginosa* chromosomal *ars* operon responsible for detoxification contained three potential ORFs encoding proteins with a significant sequence similarity to those encoded by the *arsR*, *arsB*, and *arsC* genes of *E. coli* chromosomal *ars* operons. In the current study, the presence of an efflux pump system, encoded by the *arsB* sequences, was found in all 4 isolates. Therefore, the purported requirement of ArsB for arsenic resistance would seem to be incorrect, as none of the isolates showed any PCR products. Since this *ars* genotype is correct, the *arsB* pumping plays a very important ecological role in arsenic toxicity and mobility in mine soil, as it facilitates the modern biogeochemistry of arsenite-oxidizing bacteria in abandoned mines. The present results revealed a wide diversity between the amino acid sequences for the *arsB* gene. In ArsB (strain OS-5, ABB83931; OS-19, ABB04282; OS-80, ABB76686; and RW-28, ABB88574), there were four putative enzymes, His131, Arg135, Arg161, and Trp164, each located in a different region of the partial sequence. Therefore, this information indicates that indigenous arsenite-oxidizing bacteria may provide an understanding

of the molecular geomicrobiology that can be applied to the bioremediation of arsenic-contaminated soil. When investigating the arsenite-oxidizing bacterial strain OS-5 using biochemical testing, it showed different substrate utilization patterns, yet 30 similar physiological characterizations. The bacterial strain also grew on solid media with various substrates, including mannose, melibose, p-n-p-β-galactose, proline nitroanilide, urea, and several other types of medium.

Various researchers have already reported that amplification of the *ars* genes and unknown chromosomal *ars* areas during a PCR is influenced by many factors, such as the type of primer used, conformational variations in the extracted DNA, thermal cyclic conditions, and the composition of the buffers or agents. Most previous studies have dealt with the problems involved in the PCR amplification of the *ars* genes for cloning the anticipated redox band into another plasmid-encoded *ars* operon. However, in the present study, the chromosomal DNA was amplified differently in terms of size, as shown in Fig. 2. For example, a 745-bp segment was produced from OS-5, and a 914-bp segment created from *Pseudomonas aeruginosa*. Although Rosen [23] and Silver and Phung [32] maintained that the plasmid-encoded *arsB* gene product is similar to the chromosome-encoded *arsB* gene product (R773, 100%; R46, 92%; *E. coli*, 90%; pI258, 56%; pSX267, 56%), this study found a low degree of similarity between the *ars*B gene product sizes (bp). In addition, four bacterial strains capable of oxidizing arsenic were isolated, where the first, OS-5, was identified as a *Pseudomonas* sp. and found to be capable of completely oxidizing 1,000 μM arsenite in 35 h. The maximal activity was achieved after only 20 h. The three other strains were also capable of completely oxidizing 1,000 μM arsenite, yet generally required a longer time to trigger or complete the process.

In summary, this paper described the first investigation of arsenic redox and the presence of *arsR*, *arsD*, *arsA*, *arsB*, *arsAB*, *arsC*, *arsH*, *arrA*, *arrB*, *aoxA*, *aoxB*, *aoxC*, *aoxD*, *aroA*, and *aroB* in indigenous arsenic-resistant bacteria, resulting in the suggestion that indigenous bacteria can influence arsenic speciation in natural settings, owing to oxidation or reduction. Therefore, this could be applied to enhance the bioremediation of arsenic-contaminated sites. However, further studies are required to understand the roles of the chromosomal *ars* genes in bacterial respiration in such environments.

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