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Bacterial *aox* genotype from arsenic contaminated mine to adjacent coastal sediment: Evidences for potential biogeochemical arsenic oxidation

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ABSTRACT

The potential biogeochemical redox activity of arsenic was investigated by examining bacterial arsenic (As) redox genes such as *aox*, *ars*, and *arr* in arsenic-contaminated abandoned mine area and adjacent coastal sediments. Consistent with aerobic sediment and water samples from the mine through coastal areas, bacterial genes involing arsenic(V) (arsenate, AsO_4^{3-}) reduction such as *ars*C and *arr*A were identified only in a few samples, wheres bacterial *aox*B gene encoding arsenite oxidase which is a central role in arsenic(III) (AsO_2^-) oxidation of *aox* operon. This study suggests that evaluation of arsenite-oxidizing bacteria including *aox* genotype may lead to a better understanding of molecular geomicrobiology in arsenic biogeochemistry, which can be applied to the bioremediation of arsenic contaminated mines along the coast of Gwangyang Bay. In this study, high concentrations of arsenic were observed in the mines and Gwangyang Bay and it was speculated that As(III)-oxidizing bacteria isolated from those highly arsenic-contaminated areas contributed the biogeochemical cycling of arsenic by transforming arsenic species and resulting in change of mobility, though further in situ biogeochemical and/or microbial ecological investigations are needed for confirming the phenomena in natural environment. *Acinetobacter junni* and *Marinobacter* sp. which were isolated in the contaminated area contained the *aox* genes and were able to oxidize As(III) to As(V), which is a more soluble form in oxic aqueous environments and apt to migrate from the mine to the coast. This might suggest a potential of a significant redox role of *aox* genes of arsenic-oxidizing bacteria in biogeochemical cycle of arsenic.

1. Introduction

The biogeochemical cycle of arsenic has been brought by the evolution of arsenic-redox gene systems in various enviornment in response to arsenic released either by natural or anthropogenic influences [1–4]. Biogeochemical activity is involved in a surprising range of metals and metalloids' transformations including arsenic, where Ars (arsenic resistance system), Arr (respiratory arsenate reduction system), and Aox (respiratory arsenite oxidation system) resulting in arsenic species change are included [1,5,6]. In terms of arsenic chemistry, the bacterial Aox system oxidizes arsenite (As(III)) to arsenate (As(V)), while Ars and Arr systems contribute to As(V) reduction to As(III), nonetheless for the different purposes [7,8]. The reason why microorganisms utilize toxic inorganic arsenic, arsenate and arsenite is unclear. However, microbial oxidation of As(III) to As(V) has long been recognized, especially with aerobic isolates from arsenic-impacted environments. In the energy source-limiting conditions, As(III) can serve energy to certain microorganisms for their respiration such as Aox (respiratory arsenite oxidation) system [8]. Or certain microorganisms can oxidize As(III) to As(V) merely to detoxify, without gaining energy from the process [9]. In contrast, reduction of As(V) to more toxic As(III) is also known as detoxification, since bacteria pump out the reduced As(III) species from the cytoplasm using Ars (arsenic resistance system) [8]. In terminal electron acceptor-limiting conditions, certain bacteria can transfer electrons to As(V) for their anaerobic respiration using Arr (respiratory arsenate reduction) system [10].

As(III) oxidation genes (*aox*AB) were found in *Ochrobactrum tritici* SCII24, *Agrobacterium tumefaciens*, and β-proteobacterial strain ULPAs1 [11–13]. Cai et al. described three gene clusters involed in arsenic redox transformation, which mediate respiratory arsenic oxidation by *aox*A–*aox*B–*aox*C and arsenic detoxification through reduction by *ars*R–*ars*D–*ars*C from *Achromobacter* sp. SY8 and *Pseudomonas* sp. TS44, respectively [14]. The *ars*C gene from plasmide pI258 of *Staphylococcus aureus* was shown to play a role in bacterial arsenic resistance through reduction of As(V) to As(III) with help of efflux pump of ArsAB [15,16]. In addition, it was found that the reduced toxic As(III) was transported out of cells through Ars-ATPase involved in efflux pump of arsenic resistance system (ArsA, ArsB, or ArsAB) [1,5,17]. In addition, dissimilatory arsenate-

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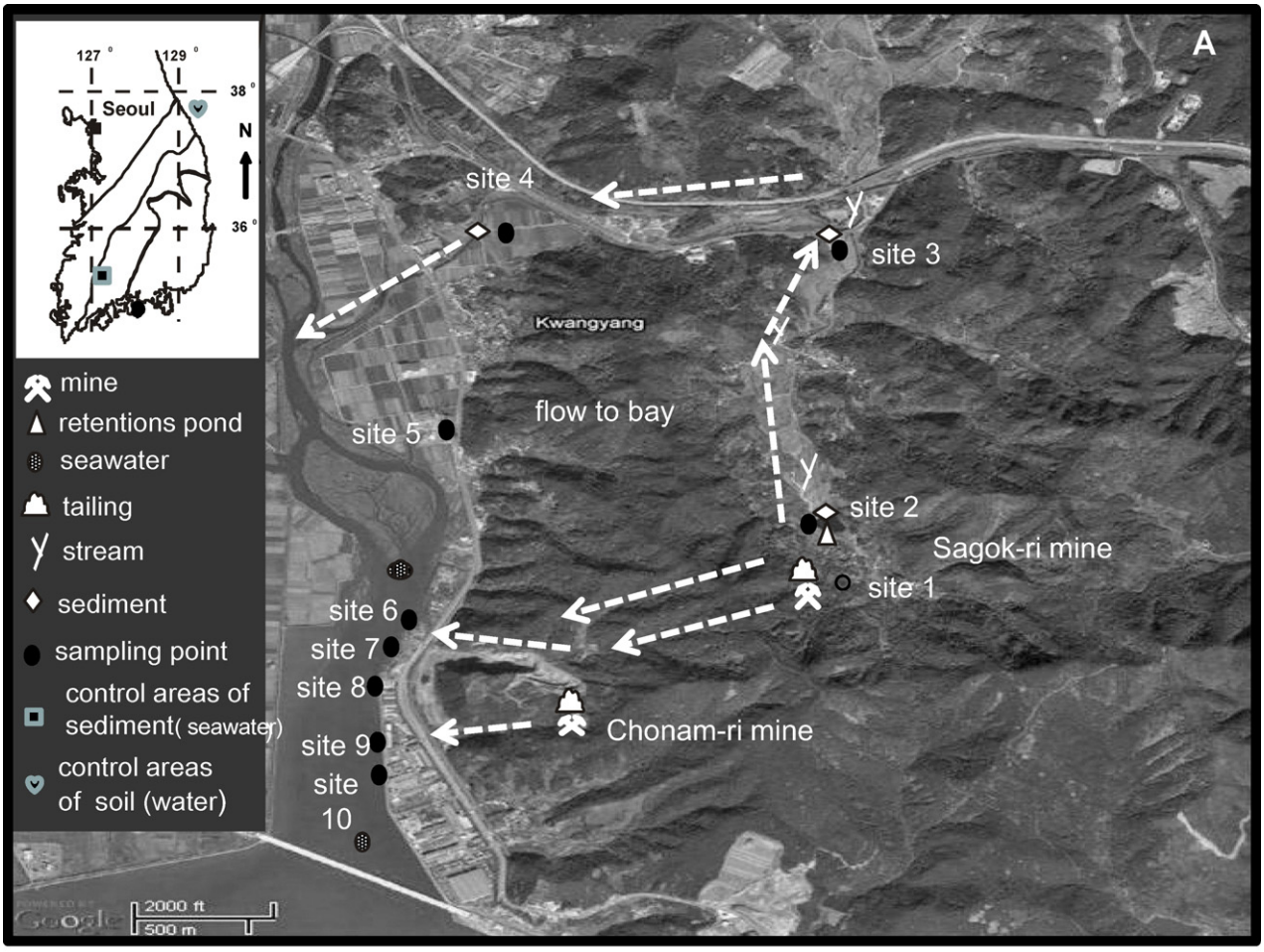

B

Measurements In sample point	Control	site 1	site 2	site 3	site 4	site 5	site 6	site 7	site 8	site 9	site 10
pH	8.0	2.6	6.6	7.1	7.4	7.6	7.6	7.6	6.8	7.8	7.6
Temp. (°C)	20.0	19.5	26.8	21.3	22.0	24.0	22.0	24.9	26.6	21.2	21.0
Eh (mV)	- 70.0	-26.6	- 26.9	- 72.9	- 70.7	- 74.2	- 70.7	- 57.9	- 60.2	- 60.2	- 60.6
Salimity	30.2 [a]					31.7 [a]	33.5 [a]	33.0 [a]	33.5 [a]	34.5 [a]	34.5 [a]

Fig. 1. (A) Sampling sites in Chonam-ri and Sagok-ri creeks of the Gwangyang Au–Ag mine area, the Republic of Korea. (B) Sampling point for. In roral districts (A), arsenic contrations were measured in seawater, tailing, and sediment from mine surface (squares, triangles, and dots). These site ananysis include pH, temperature, Eh, salinity (dot markers).

respiring bacteria (DARB) which were characterized by a specific *arr* gene cluster (*arr*A, *arr*B, *arr*C, and *arr*D) reduce arsenate to arsenite and may play a significant role in arsenic mobilization or immobilization [15]. Those results suggest that arsenic species are subject to change in response to bacterial As(III) oxidation and/or As(V) reduction. Therefore looking insight into molecular geomicrobiology on arsenic biogeochemistry may lead to a better understanding of ecological role of such bacteria.

However, roles of the genetic mechanisms by which mobile As chemistry is influenced in remote environments but closely linked with contamination sources are poorly understood. Lièvremont et al. [6] proposed bioprocesses for treatment of arsenic-contaminated water based on bacterial metabolism and biogeochemical cycle of arsenic. Effort to correlate microbial genetic systems to biogeochemical transformations of contaminants may provide a better understanding of biogeochemical behaviors of the contaminants, arsenic in this research, through the ecosystems remote but geographically connected to contamination sources [18,19]. In this study, arsenic-redox related genotypes were targeted to assess evidence of biogeochemical transformations of arsenic, especially bacterial arsenite oxidation by an isolated arsenic-oxidizing baterium from the As-contaminated mine sediment, which is located adjacent to and supposed to influence on the coastal sediment of Gwangyang Bay, South Korea. We hypothesized that detecting microbial arsenic-redox genes along with arsenic species through two remoted but geographically connected environments would provide some evidences of potential migration and biogeochemical transformations of the contaminant, arsenic in this study, through both ecosystems.

2. Materials and methods

2.1. Sampling and trace metals analysis

Seawater, tailing, and sediment samples were collected from the Gwangyang Au–Ag mine (latitude 34°53′ N, longitude 127°47′ E),

Gwangyang Bay, Republic of Korea in August 2008 (Fig. 1). The temperature and pH of the water samples were measured in the field using an Orion electrode (Orion model 290A portable meter fitted with an Orion model 9107 electrode). All seawater samples were transported to the laboratory on ice and then stored at 4 °C until analyzed. Samples of seawater were separated into two portions, diluted and then filtered through 0.45 μm filter (Whatman), after nitric acid was added to give a pH of approximately 2 [20,21]. One of the portions was then used to determine the concentration trace metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) by ICP-MS (Agilent 7100) [7,22]. The other portion of seawater was used for speciation of arsenic. To accomplish this, the sample was allowed to pass through a silica-based anion-exchange cartridge (LC-SAX SPE Tube, Supelco) that retained the As(V), but allowed the As(III) to pass through [23]. The total As and As(III) concentration were then determined by Graphite-AAS (Perkin Elmer 5100) and the concentration of As(V) was determined by subtraction of the As(III) from the total As. Each soil sample was composed of approximately 2–3 subsamples taken at 30 cm depths from each site. To determine the total concentration of arsenic and trace metals in the tailings and sediments, the soil samples (0.25 g) were mixed with aqua regain [1 ml of HNO3 (65%, Merck) and 3 ml of HCl (37%, J.T. Baker)], heated at 70 °C with shaking for 1 h, and then diluted with 6 ml of distilled water [24]. The extracted solution was then filtered through a 0.45-μm micropore filter. The concentration of As and trace metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) in each sample was determined by Graphite-AAS (Perkin Elmer 5100) and ICP-MS (Agilent 7100), respectively. All analytical measurements were performed in duplicate.

2.2. Isolation and pure culture condition of arsenic-resistant bacteria

For the isolation of arsenic-resistant microorganisms, each seawater (1 ml), tailing (1 g), sediment (1 g) and water (1 ml) was added to marine broth (agar) (2216 Difco TM) [25] containing sodium arsenite ($NaAs(III)O_2$) or sodium arsenate ($Na_2HAs(V)O_4$) (Aldrich, St. Louis, MO, USA). In order to isolate arsenic-resistant bacteria, the subsurface soils from 20 to 30 cm deep were used. After several transfers, isolated colonies were assessed for the amounts of As(III) and As(V) present, and a single isolate was selected. The As(III) and As(V) were used at concentrations up to 66.7 mM and 66.7 mM, respectively, by using the 7th pure cultures (As(III): NaAsO2; 0, 5, 10, 15, 20, 25, 30 and 66.7 mM; As(V): $Na_2AsO_4{\cdot}7H_2O$; 0, 5, 10, 15, 20, 26, 40, 50, and 66.7 mM). All of the As(III)- or As(V)-resistant isolates were tested for As(III)-oxidizing or As(V)-reducing, respectively. All the arsenite-oxidizing and arsenate-reducing microorganisms were cultured at 30 °C in marine broth (agar) (2216 Difco TM) with 1 mM D(+)-glucose and ethanol as the carbon sources. After an aerobic test with each pure culture, Petri dishes containing the media were placed in an anaerobic chamber (Coy Laboratory, MI, USA) at 30 °C (gas mixture; 5% CO_2, 10% H_2, and 85% N_2). The isolated colonies were again assessed for their tolerance levels of As(III) and As(V), and a single isolate was selected. Arsenic resistance experiments were carried out using the same arsenic concentrations as that of the aerobic tests. For anaerobic liquid cultures, the precultured cells in anaerobic marine broth (agar) (2216 Difco TM) media were used, which was preincubated in the glove box for at least 3 days. The arsenic resistant bacterium grew in marine broth (agar) (2216 Difco TM) plus (1 mM) D(+)-glucose.

2.3. Genomic DNA isolation

Bacterial genomic DNAs were prepared from the bacterial cultures using the standard methods [25], and were placed in a 1 ml microcentrifuge tube with the appropriate individual colony. Bacterial suspensions were composed of 10^8 CFU/ml of each isolate. Each culture was incubated overnight at 30 °C with intermittent shaking. Culture was 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^{-1}), which was reacted 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. This solution was then manually mixed for 3 min and centrifuged at 14,240 × *g* for 15 min, and the supernatant was removed. This process was repeated 3 times. The supernatant was then mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Tubes were shaken slowly and the genomic DNAs were gently removed and then 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 measured using a spectrophotometer set at 260 nm and 280 nm. All of DNA had an absorbance ratio (A 260 nm/A 280 nm) ranging from 1.7 to 2.0.

2.4. PCR amplification of the 16S rRNA gene and arsenic-resistance genes

Bacterial genomic DNA was isolated from the bacterial cultures by the standard methods [25] and placed in a 1 ml microcentrifuge tube for PCR amplification. In order to amplify the arsenic-redox genes, the oligonucleotide primer was designed using the published sequence. The primers used to amplify the *aox* genes were designed specifically to amplify *aox*A, *aox*B, *aox*C, *aox*D, *aox*S, and *aox*R, (Table 1) based on the GenBank database GIST-*aox*A – AF509588, AB020482, AM502288, AY791695, DQ151549, and Z48565; GIST-*aox*B – DQ151549, CU207211, AF509588, AM502288, and AB020482; GIST-*aox*C – AB020482, and AF509588; GIST-*aox*D – AF509588; GIST-*aox*S – DQ151549, and CU207211; and GIST-*aox*R – DQ151549, CU207211, and CAL60694. The primer sequences for PCR of the arsenic resistance genes were shown in Table S1. The primer sequences included targeted regions for the specific genes [21, Table S1]. The *ars* gene primers were designed to obtain a Tm of approximately 55–62 °C, and specifically obtained by aligning the *ars* gene sequences from GenBank using BLAST. PCR amplification was performed with a final volume of 50 μl containing 0.5 μg genomic DNA and 10 pmol (0.2 μM) of the primers at an initial heating at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, followed by an additional extension at 72 °C for 7 min. PCR was conducted in a Mastercycler Gradient (Eppendorf, Germany), and the PCR products were analyzed in 0.7–1.5% agarose gels. All procedures for pure culture of arsenic-resistant bacteria, PCR amplificatiopn of 16S rRNA gene, and sequencing were performed according to well-described protocals [5,25]. Sequencing for the 16S rRNA genes was conduced using an automated DNA sequencer (Model 3100; ABI PRISM Genetic Analyzer System). The 16S rRNA gene sequences were compared with the NCBI database using the BLAST algorithm integrated with the Vector NTI Suite v5.5.1 (InforMax, USA). Database sequences with fewer than 1500 nucleotides were excluded from the phylogenetic analysis, and the almost complete 16S rRNA gene sequence (<1486 nucleotides) of the isolated bacterium was aligned with closely-related sequences retrieved from GenBank using CLUSTAL_X [9]. Phylogenetic analysis was performed using the neighbor-joining method [26].

2.5. Arsenic oxidation and reduction assays

To test the ability of the strains to oxidize arsenite and/or reduce arsenate, the isolates were inoculated in sea salts medium (Sigma, USA) with either 1 mM of arsenite (As(III)) or 1 mM of arsenate (As(V)). For the batch tests, marine broth (agar) (2216 Difco TM)

Table 1
Gwangyang As–Ag miens and Bay areas in the soil, sediment, water, seawater in the concentration of arsenic species were analyzed.

Sample	Arsenic measurements in sample points (mean ± S.D.)[a]		
	As(III) (mg kg^{-1})	As(V) by difference (mg kg^{-1})	As (total) (mg kg^{-1})
Gwangyang Au–Ag mine soil			
Control[b]	nd	nd	nd
Station 1	3.4	469.6	473.0
Station 2	ND	ND	49.6
Station 3	ND	ND	79.8
Station 4	21.2	460.8	482.0
Gwangyang Bay coastal sediment			
Control[c]	nd	nd	nd
Station 5	51.4	148.6	200.0
Station 6	99.8	255.2	355.0
Station 7	78.3	135.7	214.0
Station 8	nd	ND	42.1
Station 9	nd	ND	6.89
Station 10	nd	ND	ND
	As(III) (μg l^{-1})	As(V) by difference (μg l^{-1})	As (total) (μg l^{-1})
Gwangyang Au–Ag mine water			
Control[d]	nd	nd	nd
Station 1	9.7	16.9	26.4
Station 2	12.1	17.6	29.7
Station 3	11.1	15.0	26.1
Station 4	4.2	25.9	30.1
Gwangyang Bay coastal seawater			
Control[e]	nd	nd	nd
Station 5	7.1	26.1	1.1
Station 6	7.9	20.0	33.2
Station 7	7.8	9.4	27.9
Station 8	8.0	7.6	17.2
Station 9	nd	ND	ND
Station 10	3.2	6.9	10.1

nd: not determined; ND: below detection limits (1 μg l^{-1}).
Error (±) is equal to the range (for $n = 3$) or relative standard deviation (for =3).
The third replicate analyses were performed for each sample.
[a] Average ± standard deviation (S.D.).
[b] Control areas of soil.
[c] Control areas of sediment.
[d] Control areas of water.
[e] Control areas of seawater.

media supplemented with 1 mM of arsenite was inoculated with each of the strains (10^7 CFU) and incubated aerobically at 22 °C for 10 days with shaking (170 rpm), after which the concentrations of As(III) in the culture media were determined. All experiments were conducted in triplicate using 60-ml Erlenmeyer flasks. The controls consisted of sterile media (i.e., un-inoculated) with 1 mM arsenite and were incubated under the same condition. Aliquots of 2 ml were collected from the incubation flasks periodically throughout the experimental period to measure the cell density and determine the arsenic speciation and concentration. These samples were centrifuged at 14,240 × g for 10 min, decanted, and stored at 4 °C prior to arsenic analysis [24]. To monitor the oxidation of As(III) to As(V) during the incubation period, the As(III) concentration was determined as described above. All analytical measurements were performed in duplicate.

3. Results

3.1. Characteristics of arsenic contaminated sites

Quantitative analyses revealed the characteristics of tailing, sediment, and water collected at an abandoned mine, and sediment collected from the coastal Gwangyang Bay. The results revealed that high concentrations of heavy metals were present at all sampling locations (Fig. 1 and Table S2). In addition, As from Sagok-ri and Chonami-ri mine was spected to have migrated to Gwangyang Bay. High concentrations of total As were observed at site 1 (473.0 mg kg^{-1} in tailing as 3.4 mg kg^{-1} As(III) and 469.6 mg kg^{-1} As(V); 26.4 μg l^{-1} in water as 9.7 μg l^{-1} As(III) and 16.7 μg l^{-1} As(V)) and site 4 (482.0 mg kg^{-1} in sediment as 21.2 mg kg^{-1} As(III) and 460.8 mg kg^{-1} As(V); 30.1 μg l^{-1} in water as 4.2 μg l^{-1} As(III) and 25.9 μg l^{-1} As(V)) in Sagok-ri mine (Table 1). High concentrrations of total As were also observed at site 6 (355.0 mg kg^{-1} in sediment; 33.2 μg l^{-1} in seawater) and site 7 (214.0 mg kg^{-1} in sediment; 27.9 μg l^{-1} in seawater) from Gwangyang Bay. The migration of metal contaminants from the abanoned mine to Gangyang Bay was speculated by showing correlations of heavy metal profiles between two remote but geographically connected areas. As shown in Table S2, a high concentration of heavy metals was found in the mine (7894.8 mg kg^{-1} Fe and 139.0 mg kg^{-1} Pb in tailings from site 1; 18.0 μg l^{-1} Cd, 2583.0 μg l^{-1} Cr, 200.0 μg l^{-1} Pb, and 457.3 μg l^{-1} Zn in water from site 1) and coastal areas of the bay (44.2 mg kg^{-1} Cr, 55.0 mg kg^{-1} Ni, and 410.0 mg kg^{-1} Zn in sediment from site 6; 332.8.0 μg l^{-1} Zn in seawater from site 5; 1231.8 μg l^{-1} Mn in seawater from site 6). Water temperature of the sample site ranged from 19.5 to 26.6 °C and pH of the water ranged from 2.6 to 7.1 and from 6.8 to 7.8 in the mine and the Bay areas, respectively (Fig. 1B). The heavy metals and As in the mine tended to migrate to the adjacent environment, coastal bay. The two abandoned mines had been used since early 20th century, and since then the mine tailings have contaminated nearby ecosystems including rice-fields, orchards, etc. They were sources of heavy metals, especially arsenic, found in the nearby bay area and coastal seawater [25], and contaminated ecosystem of tidal flat. This research

Table 2
There identification, *ars* genotype, doubling time, residual concentration of As (uM) and sodium arsenite tolerance (mM) of arsenite-oxidizing bacterial that were exposed to arsenic under facultative anaerobic conditions. Each strain was analyzed for the presence of the *ars*R, *ars*D, *ars*A, *ars*B, *ars*C, *ars*H, *arr*A, *arr*B, *aro*A, *aro*B, *aox*A, *aox*B, *aox*C, *aox*D, *aox*S, and *aox*R gene within its genomic DNA using PCR, as described in Experimental section.

Sample	Isolate	Isolate accession no.[a]	16S rRNA similarly (%) to known bacteria	*ars* genotype[b]	Doubling time (h)	As(III) tolerance (mM); day[c]	Initiation time (days)[d]	Residual concentration of arsenic (uM)
Alcaligenes sp. KCTC[e] 338; ATCC[f] 3178				*ars*H[g]				
Gwangyang Au–Ag mine soil								
Site 1	SeaH-As1s[h]	FJ607345	99/*Citrococcus* sp.	*aox*B$^+$	36	15; 3	3	700
Site 2	SeaH-As2s[h]	FJ607347	99/*Bacillus* sp.	*aox*C$^+$	24	20; 14	4	750
Site 3	SeaH-As3s[h]	FJ607349	98/*Stenotrophomonas* sp.	*aox*C$^+$	72	15; 10	3	750
Site 4	SeaH-As4s[h]	FJ607351	98/*Pseudomonas putida*	*aox*B$^+$	24	15; 14	2	700
Gwangyang Bay coastal sediment								
Site 5	SeaH-As5s[h]	FJ607353	99/*Bacillus* sp.	*aox*R$^+$	72	45; 7	4	750
Site 6	SeaH-As6s[h]	FJ607355	98/*Acinetobacter junii*	*ars*B$^+$ *aox*B$^+$	24	45; 14	1	0
Site 7	SeaH-As7s[h]	FJ607357	98/*Bacillus* sp.	*aox*C$^+$	36	45; 10	3	800
Site 8	SeaH-As8s[h]	FJ607359	98/*Halomonas* sp.	*aox*B$^+$	36	45; 7	5	800
Site 9	SeaH-As9s[h]	FJ607361	99/*Bacillus* sp.	*aox*B$^+$ *aro*B$^+$	36	35; 14	4	650
Site 10	SeaH-As10s[h]	FJ607363	98/*Micrococcus* sp.	*ars*H[h] *aox*B$^+$ *aox*C$^+$	24	45; 10	5	850
Gwangyang Au–Ag mine water								
Site 1	SeaH-As1w[h]	FJ607346	99/*Bacillus licheniformis*	*aox*B$^+$ *aox*R$^+$	72	15; 3	3	700
Site 2	SeaH-As2w[h]	FJ607348	99/*Acinetobacter* sp.	nd	72	25; 5	3	600
Site 3	SeaH-As3w[h]	FJ607350	98/*Stenotrophomonas* sp.	*ars*D$^+$	36	25; 10	2	550
Site 4	SeaH-As4w[h]	FJ607352	98/*Pseudomonas monteilii*	*ars*H[h] *arr*A$^+$	24	25; 14	3	750
Gwangyang Bay coastal seawater								
Site 5	SeaH-As5w[h]	FJ607354	99/*Bacillus* sp.	*ars*H[h]	36	45; 14	4	800
Site 6	SeaH-As6w[h]	FJ607356	99/*Marinobacter* sp.	*aox*C$^+$	72	45; 14	1	0
Site 7	SeaH-As7w[h]	FJ607358	99/*Pseudomonas* sp.	*ars*B$^+$	36	45; 7	3	850
Site 8	SeaH-As8w[h]	FJ607360	98/*Halomonas* sp.	*ars*D$^+$	24	15; 10	4	850
Site 9	SeaH-As9w[h]	FJ607362	99/*Bacillus* sp.	*ars*B$^+$ *aox*B$^+$	34	35; 10	2	600
Site 10	SeaH-As10w[h]	FJ607364	97/*Bacillus* sp.	*aox*B$^+$ *aox*C$^+$ *aox*R$^+$	36	45; 5	3	600

[a] NCBI http://www.ncbi.nlm.nih.gov/ (National Center for Biotechnology Information).
[b] *ars* resistance relationship analyzed for the *ars* genotype: + positive PCR product generated.
[c] Arsenic concentration mM test (As(III); $NaAsO_2$ [Sigma])/survival (days).
[d] The initial concentration of arsenite was 1000 μM in sea salts (Sigma S9883) of MSB medium.
[e] Control strain: Korean Collection for Type Cultures.
[f] Control strain: American Type Culture Collection.
[g] Unknown chromosomal *ars* gene.
[h] The initiation time refers to the beginning time of arsenite-oxidiation by the newly isolated bacteria during cultivation.

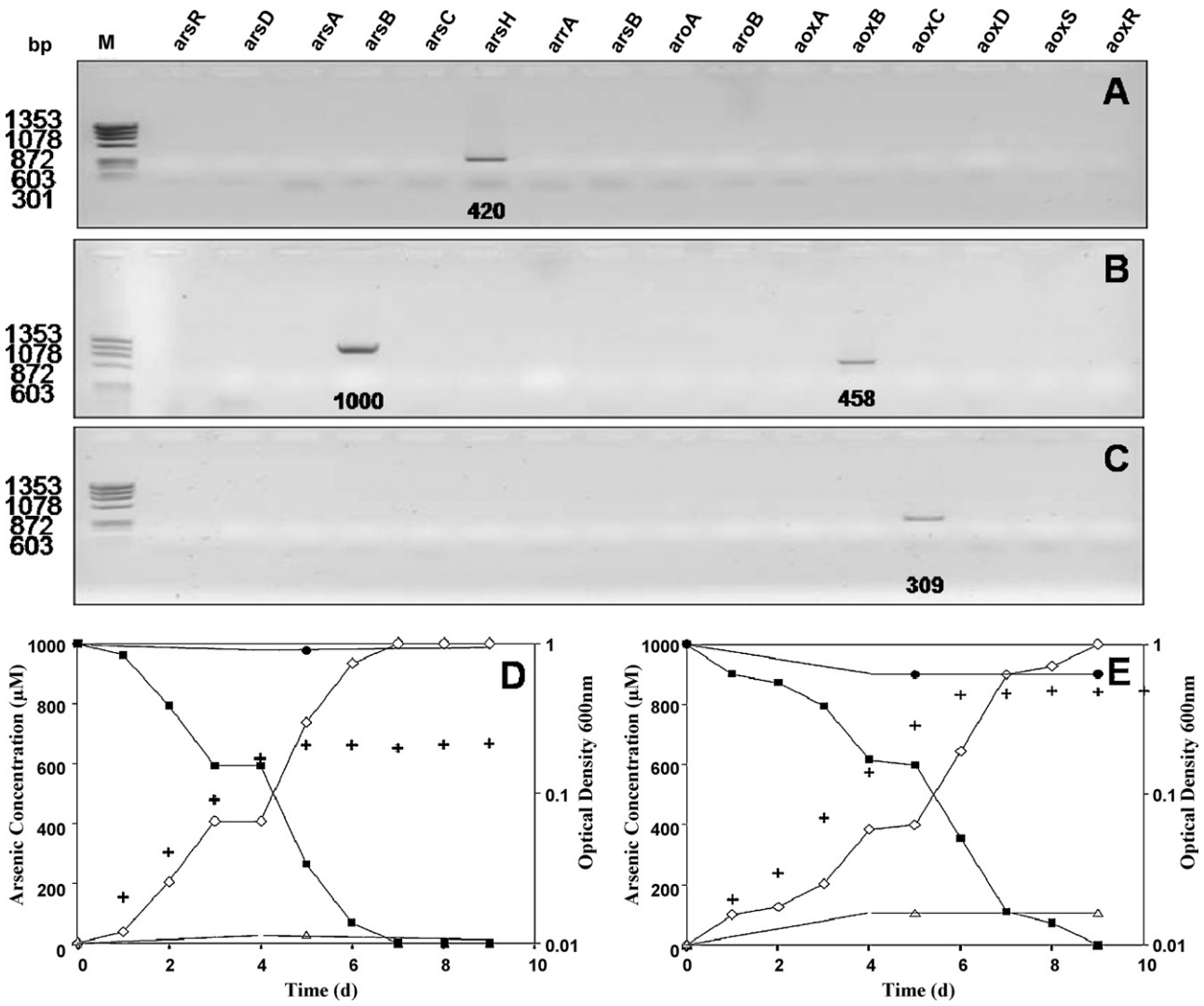

Fig. 2. Agarose gels showing PCR products amplified from genomes of several arsenite-resistant bacterial strains. Lanes M: Lambda DNA/HindIII size mark (Promega, USA). The strains shown are *Alcaligenes* sp. KCTC 338; ATCC 3178) (A), *A. junii* SeaH-As6s (FJ607355) (B) and *Marinobacteria* sp. SeaH-As6w (FJ607356) (C). Time course variation in culture turbidity (OD_{600}) and arsenite/arsenate concentration in the culture between *A. junii* SeaH-As6s (FJ607355) (D) and *Marinobacteria* sp. SeaH-As6w (FJ607356) (E) in sea salts (S9883, Sigma) medium containing arsenite (1 mM). The experiment was performed independently in triplicate in batch modes using a working volume of 60 ml at 25 °C. Each data point represents the average value of the readings from each experiment. (■, concentration of arsenite; ◊, concentration of arsenate; ●, concentration of arsenite without cell inoculation; △, concentration of arsenate without cell inoculation; +, culture turbidity (OD_{600}).

investigated microorganisms resistant to As, the highly contaminated pollutant in the environments, and their potential on As redox transformation by arsenic-redox genotypes, which in turn can disperse or precipitate the contaminant, As, through the studied environments from the mine to bay areas.

3.2. Characterization of arsenic-redox genes from the isolated Bacteria

Using the waters and sediments sampled from aerobic mine and coastal environments, cultures for both aerobic As(III)-oxidation and anaerobic As(V)-reduction were enriched, from which aerobic As(III)-oxidizers and anaerobic As(V)-reducers were isolated. Although anaerobic A(V)-reduction was observed by the isolates from aerobic As(III)-enrichments as well as anaerobic As(V)-enrichments, genes related with As(V)-reduction were not identified unexpectedly (data not shown). Aerobic As(III)-oxidation, however, was observed by most of aerobic isolates from As(III)-enrichments. Thereafter, this study only focused on aerobic isolates showing As(III)-oxidation, since the water and sediment samples were from aerobic condition. From 20 isolated bacterial strains from arsenic-enrichments using the mine and bay sediment and waters, genes were screened by PCR using a range of marker primers for *aox*, *aro*, *ars*, and *arr* genes. Table 2 and Fig. 2 show that the arsenic-oxidizing bacteria were found to have the *ars* genes as well as *aox* genes. Among the identified genes, *aox*B gene was the most abundant than other genes (Table 2), which is an arsenite oxidase subunit II and idem with *aro*A and *aso*A. We detected *ars* genes such as *ars*B, *ars*D, and *ars*H, but excluded for evidence of potential biogeochemical roles in arsenic redox at least in the mine-bay environmental system, because the identified ars genes are not essential in arsenic detoxification process of *ars* operon, through reduced As(III) generated by As(V) reduction system is pumped out of cell cytoplasm via efflux pump encoded by *ars*AB. Interetingly, *aro*B gene was identified in the isolate, SeaH-As9s (16Sr RNA gene accession numner: FJ607361) from site 9 sediment of the bay (Table 2). The *aro*B gene is idem with *aox*A, arsenite oxidase subunit I, and so the isolate contained both *aox*A and *aox*B, complete set of arsenite oxidase. Also, the gene responsible for arsenate respiratory reduction, *arr*A was identified in the isolate, Sea-As4w (FJ607352). The genes related with As(V) reduction such as *arr*A and *ars*C, however, were not identified much,

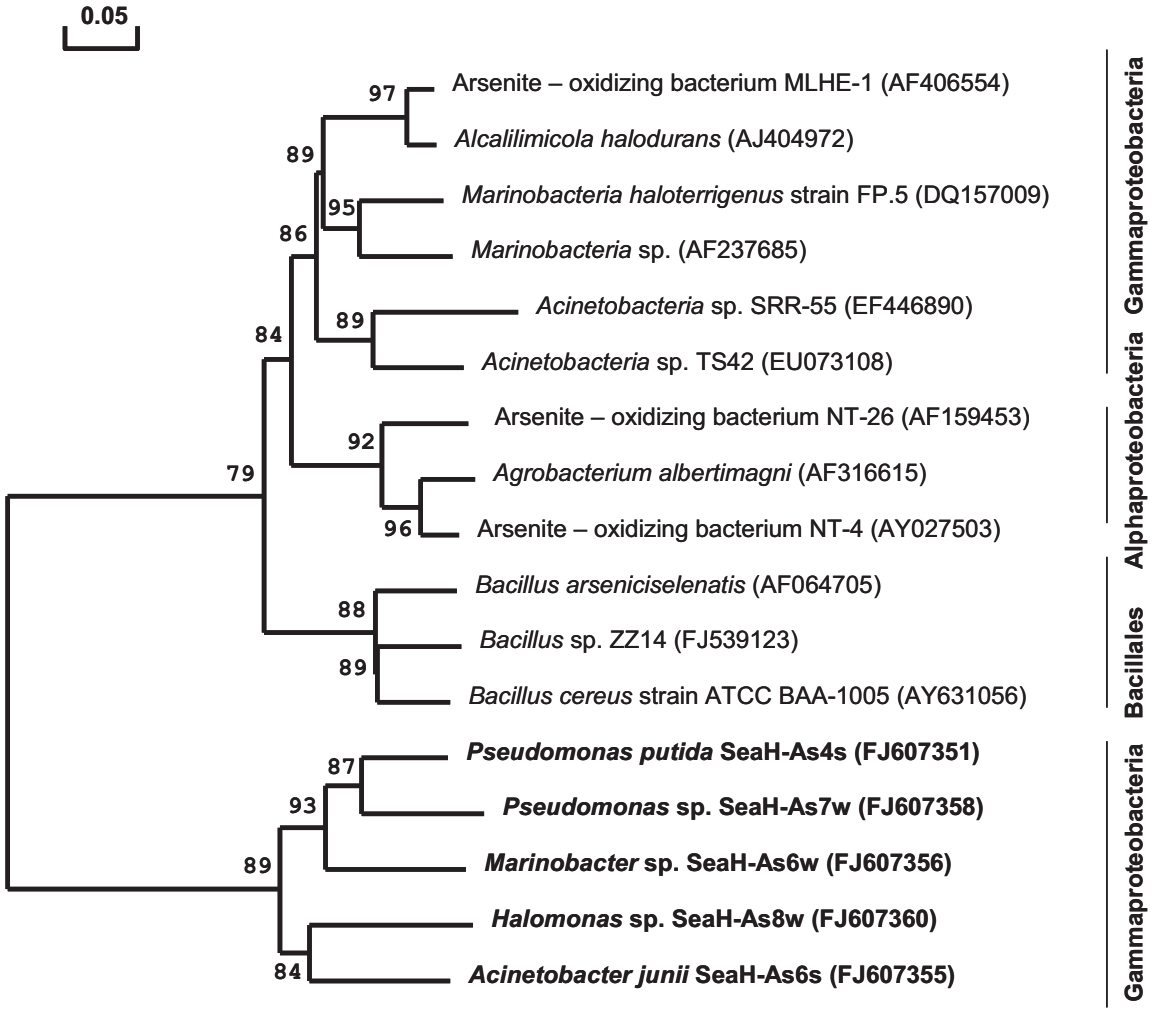

Fig. 3. Phylogenetic tree based on 16S rDNA sequence showing the position of arsenite-bacterial isolate *A. junii* SeaH-As6s (FJ607355), *Marinobacteria* sp. SeaH-As6w (FJ607356) and gammaprotebacteria. The tree was constructed from a matrix of pair-wise genetic distances using the neighbor-joining tree method. The phylogenetic data was obtained by aligning the different arsenic-resistant bacteria 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.

probably because the samples were rather aerobic and incubation and enrichment were in aerobic condition. The gel-electrophoresis patterns of arsenic-redox genes from two strains, *Acinetobacter junii* SeaH-As6s and *Marinobacteria* sp. SeaH-As6w are presented in Fig. 2 with a reference strain of *Alcaligenes* sp. (KCTC 338; ATCC 3178) which has been reported for arsenite-oxidation: the isolate SeaH-As6s (FJ607355) from the coastal sediment of Gwangyang Bay contained *aox*B (458 bp) (Fig. 2B), which is arsenite oxidase, while the isolate SeaH-As6w and reference strain *Alcaligenes* sp. were lack of any arsenic-redox gene, such as *aox*A, *aox*B, *ars*C, *arr*A, etc. (Fig. 2A and C). Phylogenetic analysis of the partial 16S rRNA gene sequence obtained from arsenite-oxidizing bacteria revealed that the isolated strains SeaH-As6s and SeaH-As6w were the close relatives to the Gammaproteobacteria *A. junii* (FJ607355) and *Marinobacteria* sp. (FJ607356), respectively (Fig. 3). From the experimental results, we speculated that the arsenite-oxidizing reaction could play a biogeochemical role in the studied As-ecosystem of mine-bay system.

3.3. Evaluation of arsenite oxidation by the isolated bacteria

Using those aerobic isolates with arsenic-redox genes, the isolated bacteria were characterized for their As(III)-oxidizing abilities. As shown in Table 2, two isolated arsenite-oxidizing bacteria *Acinetobacter junni* strain SeaH-As6s (FJ607355) and Marinobacter sp. SeaH-6w (FJ607356) were resistant to relatively high concentrations of As(III) (up to 45 mM). The doubling time of *A. junii* (FJ607355) strain SeaH-As6s and Marinobacteria sp. strain SeaH-As6w under the experimental condition was 24 h and 72 h, respectively. Batch cultures in seawater revealed that strains SeaH-As6s and SeaH-As6w oxidized 1 mM As(III) to As(V) almost completely in 4 and 9 days, respectively (Fig. 2D and E). The generated aqueous As(V) concentrations reached almost 1 mM in both the bacterial cultures (Fig. 2D and E), indicating nearly 100% As(V) in aqueous phase, with no precipitation in the experimental condition. For all the isolates, the lag time required for the isolates to oxidize 1 mM of sodium arsenite varied by the strains as follows: 2 days for SeaH-As4s, SeaH-As3w, and SeaH-As9w; 3 days for SeaH-As1s, SeaH-As3s, SeaH-As7s, SeaH-As1w, SeaH-As2w, SeaH-As4w, SeaH-As7w, and SeaH-As10w; 4 days for SeaH-As2s, SeaH-As5s, SeaH-As9s, SeaH-As5w, and SeaH-As8w; and more than 5 days for SeaH-As8s and SeaH-As-10s (Table 2). We isolated arsenic-resistant bacteria both in the aerobic and anaerobic conditions using As(III) and As(V), respectively. In the aerobic cultures, the microorganisms were tolerant to As(III) up to 45 mM (Table 2), while the isolates, in the anaerobic cultures, showed As(V) tolerance up to 66.7 mM. Reduction of As(V) was not observed in the anaerobic experiments using all the anaerobic As(V)-tolerant isolates. Whereas the aerobic As(III)-tolerant isolates which showed activities of

As(III)-oxidation were *A. junii* SeaH-As6s (FJ607355) and *Marinomacteria* sp. SeaH-As6w (FJ607355). The environmental samples where the two As(III)-oxidizing bacteria were isolated were analyzed to contain relatively high concentrations of heavy metals as well as arsenic: 8.4 mg kg^{-1} Cd, 44.2 mg kg^{-1} Cr, 154.0 mg kg^{-1} Cu, 1943 mg kg^{-1} Fe, 828.0 mg kg^{-1} Mn, 55.0 mg kg^{-1} Ni, 74.0 mg kg^{-1} Pb, 410.0 mg kg^{-1} Zn from the sample where *A. junii* SeaH-As6s was isolated; 1.23 μg l^{-1} Cd, 62.4 μg l^{-1} Fe, 773.1 μg l^{-1} Mn, 31.4 μg l^{-1} Ni, 1.4 μg l^{-1} Pb, 195.4 μg l^{-1} Zn from the sample where *Marinomacteria* sp. SeaH-As6w was isolated. Although the microorganisms were isolated from the highly contaminated environments, the correlation between the microbial metabolism and relatively high concentrations of heavy metals remains uncertain. However, it was speculated that the As(III)-oxidizers were evolved to cope with the stresses from the heavy metals by utilizing them for their metabolism, e.g., As(III) as electron donor in this study.

4. Discussion

The mobilization of arsenic from an abandoned mine to the coastal areas of Gwangyang Bay was speculated to be induced by biogeochemical activity as well as hydrological migration. The arsenic-related biogeochemical activities can lead to species change, mobility, bioavilability of arsenic, and therefore adverse effects on human by environmental exposure. It is important to ensure that drinking water in coastal areas has not been contaminated [1–3,5,6,18,27]. Chang et al. [1,5] and Silver et al. [8] studied the bacterial *ars* genes by which arsenic species change occurs, and Edmonds and Francesconi [18] focused on the biogeochemical activity of the *ars* genes involved in redox pathways and trace metal cycles in the marine environment. In addition, a study of the arsenic cycle conducted by Oremland et al. [38] revealed that bacteria that contained the *ars* genotype could not grow in salt-saturated environments, but could be used to change the As species from As(V) to As(III) in marine environments [28]. It is also possible that the use of various sources can affect the redox activity of the *ars* and *aox* genotype. Among the diverse bacterial arsenic-redox systems, we focused to *aox* genes (respiratory arsenite oxidation system), because the investigation was performed on oxic environments of the mine areas and adjacent coastal seawater and sediments.

Anthropogenic point sources contribute to arsenic or heavy metals found in the environment. Jung et al. [27] examined Chonam-ri and Sagok-ri creek in the abandoned Gwangyang Au–Ag mine area and found that transport and sediment-water partitioning of trace metals had occurred (Cr: 0.66 mg kg^{-1}, Co: 19.4 mg kg^{-1}, Fe: 3656 mg kg^{-1}, Pb: 82.5 mg kg^{-1}, Cu: 631 mg kg^{-1}, Ni: 26.3 mg kg^{-1}, Zn: 1265 mg kg^{-1}, and Cd: 9.40 mg kg^{-1}) (Fig. 1, Table S2). However, they did not measure consentration of As. The present study, to our knowledge, is the first to report the concentration of arsenic in the mine area (SeaH-As4s: 482.0 total mg kg^{-1} (21.2 III mg kg^{-1}; 460.8 V mg kg^{-1}); (SeaH-As6w: 33.2 total 7.9 III μg l^{-1}; 26.1 V μg l^{-1}) indicating arsenic contamination, as well as to demonstrate that the arsenic species changed in response to arsenic redox genotypes from the mine area. Arsenic is a toxic contaminant regulated in seawater in many countries. Recent amendment of the maximum contaminant level (MCL) of arsenic from 50 to 10 μg l^{-1} in drinking water regulation of the United States has influenced the technology used for follow-up/monitoring and toxicity assessment of marine environments [18,29].

We showed that arsenic-resistant bacteria were isolated from the contaminated mine area and remote sea water and the isolates were able to oxidize As(III) to As(V) with *aox* genes. We speculate that As is mobilized and/or transported from the arsenic-contaminated mine through to bay area via the species change by microbial redox activities as well as geochemical and hydrological factors. Additionally, the results of this study suggest that arsenic could migrate as a result of biogeochemical cycle of arsenic including bacteria with *aox*, *ars*, *arr*, etc. Fig. 2 shows a potential transformation pathways by arsenic-redox related genes by which, we propose As is mobilized and the species change in the arsenic-contaminated mine/bay. We suggest that important microbial genes in the biogeochemcial activities in arsenic-contaminated surfacial and shallow depth environments could be *aox* rather than *ars*, *arr*, etc. due to arsenic oxidation occurring in such oxic environments as contaminants are usually exposed. The bacterial arsenic-oxidizing activity extends from cytoplasm across the outer membrane to arsenic-contaminated environment. It is not known how the energy sources and/or substrates affect the *aox* genotype systems. However, the *aox* genotype could have significant ecological roles, such as effect on arsenic species change and mobility in oxic groundwater, and allow arsenic-oxidizing bacteria to be involved in biogeochemical cycle of arsenic from an environment (the mine area in this study) through another environment (the bay area in this study). Especially, *aox* genotype activities that were determined from the abandoned mine site and from Gwangyang Bay site could be an important clue for the change and migration of arsenic species through the ecologically connected but remote environments.

Takeuchi et al. [30] evaluated arsenic resistance and removal by marine and non-marine bacteria. They found that seawater enriched with arsenate had a higher concentration of Marinomonas community. Until now, *ars* genotype activity and molecular biogeochemistry have been rather widely studied to reduce ecological damage to levels below those specified in the regulations and to evaluate effects of the genotype on arsenic contaminated areas [1–6,31]. *A. junii* SeaH-As6s (FJ607355) and *Marinobacter* sp. SeaH-As6w (FJ607356) which were isolated in the water and sediment of Gwanyang Bay, respectively, were found to oxidize As(III) to As(V). This could mean that indigenous bacteria play a role in biogeochemical cycle of As in the contaminated areas of this study. There have been studies correlating arsenic-oxidation in the environments and the isolated arsenic-oxidizing bacteria. Handley et al. [32,33] evaluated *Marinobacter santoriniensis* sp. nov., which was an arsenite-oxidizing bacterium isolated from hydrothermal sediment. In addition, arsenite oxidation and anaerobic nitrate-dependent oxidation of Fe(III) were facilitated by the presence of an organic carbon source [34]. The present study suggests the oxidizing activity of the *aox*B genotype isolated from the As-contaminated mine and bay area. Arsenic contamination of tailing, water, and sediment from a mine and seawater from the adjacent coast could cause drinking water contamination or damage to marine ecological systems [2,3,18,27,35]. Oremland et al. [3,4] reported that the full biogeochemical arsenic cycle was operative in the sediment of a salt-saturated extreme environment. Similarly, the ecology of arsenic metabolizing bacteria could be a potential impact on As-species and mobilization of arsenic in nature. The results from previous studies conducted biological arsenite oxidation [20], arsenite oxidation by *Alcaligenes faecalis* [34,36], and heterotrophic characteristics of microbes in the demethylation in seawater [10] have revealed that the oxidation rate of arsenite was very slow [29]. In this study, it was evidenced that the mobilization of arsenic from abandoned mines to coastal areas of the bay could potential that induced by biogeochemical activities. In addition to further in situ investigation to probe actual occurrence of biogeochemical transformation and transport of arsenic in the studied environmental system, further studies are needed to evaluate the mechanism by which arsenic is mobilized from anthropogenic sources to coastal regions via bacteria with the arsenic-redox genotypes such as *ars*, *arr*, *aro*, *aox*, and *aso* gene systems [37] and to determine if human damage is persued by monitoring arsenic and heavy metals [35] in the ecosystem [3,4,25,28,38]. Although mobi-

lization of high concentrations of arsenic does not immediately cause ecological damage, it may affect the arsenic-redox genotype activities such as microbial As(III) oxidation and As(V) reduction among the indigenous prokaryotes, where horizontal gene transfer might be one of the mechanisms [39]. Bacteria containing the arsenic-redox genes could alter the As species, however, the mechanism by which those changes occur are not yet completely understood. Taken together, the results of this study indicate that it may be possible to probe arsenic-redox gene for the arsenic contamination in the ecologically and geographically connected but remote areas. Bacteria with the arsenic-redox genes are also proposed as clues for biogeochemical cycle of arsenic through different environmental systems, where arsenic migrated from the source area to another area such as the mine areas to the coastal areas in this study.

5. Conclusions

From the sediment samples of the arsenic-contaminated abandoned mine and adjacent coastal seawater, As(III)-resistant bacteria were isolated. The strains were found to have As(III)-oxidizing potential, which can play an important role in the marine-biogeochemical cycling activity. It is speculated that the organisms would be involved in arsenic detoxification of coastal seawater in the Gwangyang Bay by converting toxic As(III) to less toxic As(V). The microorganisms include *Acinetobacter* sp., *Bacillus* sp., *Citrociccus* sp., *Halomonas* sp., *Marinobacteria* sp., *Pseudomonas* sp., and *Stenotrophomonas* sp. However, further studies are required to understand the roles of *aox* genotype and other arsenic-redox genotypes in in situ environmental conditions. The arsenic biogeochemical cyclic activity was proposed by arsenic-transforming bacteria which carry *aox*, *ars*, *arr*, etc., and specifically in this study As(III)-oxidizing bacteria of *aox*-genotype. We suggest that the microbial As(III)-oxidation system could be applied to enhance the arsenic detoxification of coast seawater of Gwangyang Bay, as it facilitates the biogeochemical transformation of As(III) to As(V) in the marine environments. In addition, ecologically the microbial arsenic redox transformation including As(II)-oxidation in this study can play an important role in biogeochemical cycle and migration of arsenic in the As-contaminated Sagok-ri and Chonami mine areas and adjacent coast seawater of Gwangyang Bay ecosystem.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.07.055.

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