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Bacterial community succession during the enrichment of chemolithoautotrophic arsenite oxidizing bacteria at high arsenic concentrations

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Abstract

To generate cost-effective technologies for the removal of arsenic from water, we developed an enrichment culture of chemolithoautotrophic arsenite oxidizing bacteria (CAOs) that could effectively oxidize widely ranging concentrations of As(III) to As(V). In addition, we attempted to elucidate the enrichment process and characterize the microbial composition of the enrichment culture. A CAOs enrichment culture capable of stably oxidizing As(III) to As(V) was successfully constructed through repeated batch cultivation for more than 700 days, during which time the initial As(III) concentrations were increased in a stepwise manner from 1 to 10–12 mmol/L. As(III) oxidation activity of the enrichment culture gradually improved, and 10–12 mmol/L As(III) was almost completely oxidized within four days. Terminal restriction fragment length polymorphism analysis showed that the dominant bacteria in the enrichment culture varied drastically during the enrichment process depending on the As(III) concentration. Isolation and characterization of bacteria in the enrichment culture revealed that the presence of multiple CAOs with various As(III) oxidation abilities enabled the culture to adapt to a wide range of As(III) concentrations. The CAOs enrichment culture constructed here may be useful for pretreatment of water from which arsenic is being removed.

Key words: arsenic; bacterial community; chemolithoautotrophic arsenite oxidizing bacteria; enrichment; terminal-restriction fragment length polymorphism

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Introduction

Arsenic is a toxic element that occurs in soil and water naturally and as a result of anthropogenic activities. Background concentrations of arsenic in groundwater are generally less than 10 μg/L, and sometimes substantially lower. However, the concentration of arsenic can be much higher than the background values, with levels higher than 100,000 μg/L being present in some locations (Nordstrom, 2002). The worst areas of arsenic contamination in the world are in Bangladesh and West Bengal, India (Roychowdhury et al., 2005; Jahan et al., 2006; Wang and Zhao, 2009). Ahamed et al. (2006) reported that many well water samples from Eruani Village in Bangladesh had arsenic concentrations greater than 1000 μg/L. Chatterjee et al. (1995) found that groundwater in the vicinity of a chemical plant in Calcutta, India contained arsenic levels of 58 mg/L. Arsenic has acute and chronic toxicities and carcinogenic properties; therefore, long-term exposure to arsenic via drinking water has the potential to cause cancer of the skin, lungs, urinary bladder and kidney, as well as other skin diseases (Karim, 1999; Nordstrom, 2002; Ng et al., 2003). Because of these health problems, the World Health Organization set an arsenic safety level of 10 μg/L as the drinking water guideline in 1993. Since then, development of effective processes for the removal of arsenic from drinking water sources has been an important issue.

In aquatic systems, arsenic is primarily found in inorganic forms such as arsenate (As(V)) and arsenite (As(III)). Among these forms of arsenic, As(III) is more toxic than As(V) (Mondal et al., 2006). Several surveys have revealed that As(III) is often predominant under natural conditions. For example, in groundwater, As(III) was found to account for 74% and 98% of the total arsenic contamination in India and Bangladesh, respectively (Rasul et al., 2002; Postma et al., 2007; Nguyen et al., 2009; Routh and Hjelmquist, 2011). In water with pH values typically found in the environment (i.e., pH 3 to 9), As(V) exists primarily as negatively charged $H_2AsO_4^-$ or $HAsO_4^{2-}$, which makes it easy to interact with solid surfaces. By contrast, As(III) is primarily present as zero-charged H_3AsO_3 at these pH values; thus, it is more mobile and less likely to be adsorbed onto solid surfaces than As(V) (Wang and Zhao, 2009). As a result, pre-oxidation

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of As(III) to As(V) is needed to enhance the removal of arsenic from water by conventional treatment methods such as precipitation, adsorption, ion exchange and membrane filtration (Ghurye and Clifford, 2001; Battaglia-Brunet et al., 2002; Lièvremont et al., 2003; Casiot et al., 2006; Halttunen et al., 2007). Chemical oxidants such as chlorine, permanganate and ozone have been found to be effective for As(III) oxidation (Ghurye and Clifford, 2001, 2004; Dodd et al., 2006). However, because these oxidants are not specific to As(III), and may also oxidize other reducing substances present in the water, excess amounts are required to ensure sufficient As(III) oxidation, which results in high remediation costs (Ghurye and Clifford, 2001, 2004). Moreover, unspecific reactions with coexisting reducing substances can generate harmful byproducts such as trihalomethanes (Gallard and Von Gunten, 2002). Consequently, chemical oxidation reactions are unfavorable in practical applications. Biological oxidation of As(III) has been recognized as an attractive alternative due to its specificity for As(III), which enables high efficiency and cost effectiveness in addition to being environmentally friendly (Mondal et al., 2006; Jahan et al., 2006; Shrestha et al., 2008; Lièvremont et al., 2009; Wang and Zhao, 2009).

As(III) oxidizing bacteria are classified into heterotrophic and chemolithoautotrophic groups (HAOs and CAOs, respectively). HAOs oxidize As(III) for detoxification, while CAOs utilize As(III) as an electron donor for energy acquisition. CAOs are more beneficial as remediating agents because their use can prevent increasing remediation costs and the occurrence of secondary contamination associated with the need to add external organic carbon for HAOs. Several CAOs have been isolated from contaminated aquatic environments, mine residues, and drainage and employed for As(III) remediation studies (Weeger et al., 1999; Santini et al., 2000, 2002; Battaglia-Brunet et al., 2002, 2005; Duquesne et al., 2007, Michel et al., 2007). However, practical use of pure bacterial cultures is not realistic because they generally require well defined feedstocks and prior sterilization of water, which results in high remediation costs. Mixed cultures of CAOs (enrichment) are preferable during practical water remediation because they have several advantages over pure cultures. For example, although pure CAOs cultures may exhibit reduced As(III) oxidizing activity if the environmental conditions are not preferable to their growth, the As(III) oxidizing activity of mixed cultures remains stable regardless of minor changes in the environmental/operational conditions (e.g. temperature, pH and nutrient and As(III) concentrations). Despite this, only a few studies have attempted As(III) oxidation via CAOs enrichment cultures for arsenic remediation to date (Rhine et al., 2006; Elizabeth et al., 2008). Furthermore, no studies have been conducted to monitor the behavior of CAOs during the enrichment process and characterize the obtained enrichment cultures in detail.

The objectives of this study were: (1) to obtain an enrichment culture of CAOs that is useful for the pretreatment step during arsenic removal from water, and (2) to elucidate the enrichment process and characterize the microbial composition of the enrichment culture. As described above, the arsenic concentrations in the environment vary markedly and reach higher than 100,000 μg/L in some cases. Thus, in an effort for effective arsenic bioremediation in such highly contaminated environment, we applied high concentrations (from 1 mmol/L to 10–12 mmol/L) of As(III) with a stepwise increase manner to effectively enrich CAOs from soil samples that had the potential to contain a variety of bacteria including non-As(III) oxidizing bacteria. This enrichment procedure would be useful to obtain an enrichment of As(III) oxidizing bacteria that can exhibit a high As(III) oxidation potential irrespective of the arsenic contamination level. The dynamics of the bacterial community structure in the enrichment culture were monitored by terminal-restriction fragment length polymorphism (T-RFLP) analysis targeting eubacterial 16S rRNA genes throughout the enrichment period. Furthermore, bacteria in the enrichment culture were isolated and examined for their As(III) oxidizing ability.

1 Materials and methods

1.1 Inoculum source

A soil sample collected from the subsurface zone (a depth of a few centimeters) of an arsenic contaminated area located near a mine in Japan was used as the inoculum for construction of the enrichment culture of CAOs. Analysis of arsenic species after shaking extraction of the soil sample in a tenfold volume of water revealed that it contained around 0.13 mmol/L of soluble arsenic.

1.2 Culture media

A slightly modified version of the enrichment medium described by Battaglia-Brunet et al. (2002) was used as a basal salt medium (BSM) for enrichment of CAOs and As(III) oxidation studies for isolated CAOs. The medium consisted of K_2HPO_4, 0.25 g/L; KH_2PO_4, 0.25 g/L; NaCl, 0.25 g/L; $(NH_4)_2SO_4$, 0.1 g/L; $MgSO_4$, 0.05 g/L; $CaCl_2$, 0.1 g/L; trace element solution (Battaglia-Brunet et al., 2002), 1 mL/L; and vitamin solution, 10 mL/L (Battaglia-Brunet et al., 2002). In addition, $NaHCO_3$ (0.5 g/L) was added as the carbon source, and the pH of the BSM was adjusted to 6.0 with H_2SO_4. Aliquots of As(III) stock solution were added to BSM to give appropriate As(III) concentrations. The 500 mmol/L As(III) stock solution was prepared by dissolving $NaAsO_2$ in ultra pure water, and then sterilizing the solution by filtration through a Dismic-25 cellulose acetate filter (pore size 0.22 μm, Advantec, Japan).

CAOs have been reported to grow both autotrophically and heterotrophically (Garcia-Dominguez et al., 2008; Duquesne et al., 2007; Battaglia-Brunet et al., 2005; Santini et al., 2000). Thus, to enable rapid and better growth of CAOs, tryptic soy broth (TSB; Becton-Dickinson, USA) was used for bacterial counts of the enrichment culture, bacterial isolation from the enrichment culture, and routine cultivation of the isolated bacteria. For isolation of bacteria

in the enrichment culture and cultivation of the isolates, aliquots of As(III) stock solution were added to TSB to give a final As(III) concentration of 1 mmol/L. To prepare solid media, 1.8% (*W*/*V*) agar was added to TSB.

1.3 Enrichment of CAOs

The enrichment of CAOs was initiated by adding approximately 2 g (wet) of the soil sample to 300 mL Erlenmeyer flasks containing 100 mL of BSM spiked with 1 mmol/L As(III). The flasks were then shaken aerobically at 28°C on a rotary shaker at 120 r/min. Aliquots (1 mL) of the culture were periodically sampled to confirm the oxidation of As(III) to As(V). After the initially added As(III) was completely oxidized to As(V), 1% (*V*/*V*) of the culture was repeatedly transferred to fresh medium and cultured further. The As(III) concentration in the medium was increased in a stepwise manner as follows: cycle 1–4, 1 mmol/L; cycle 5–7, 2 mmol/L; cycle 8, 2.6 mmol/L; cycle 9–13, 5 mmol/L; after cycle 14, 10–12 mmol/L.

In addition to periodic measurement of the arsenic concentration, the bacterial growth in the enrichment culture was monitored at certain cycles. Samples were also collected at the end of certain cycles to monitor the bacterial community structure by T-RFLP analysis. The effects of the addition of organic carbon on As(III) oxidation by the CAOs enrichment culture were evaluated by adding 0.05% (*W*/*V*) yeast extract in addition to regular subculturing. The control experiment was not prepared in our study as the abiotic oxidation of As(III) by dissolved oxygen or air is very slow, which may take several days or even months (Battaglia-Brunet et al., 2005; Rhine et al., 2006).

1.4 Isolation of CAOs

Aliquots (100 μL) of the enrichment culture after cycle 32 and 46 were plated onto TSB agar containing 1 mmol/L As(III) and then incubated at 28°C. Morphologically distinct colonies were selected and purified by streaking onto the same agar medium, after which they were incubated in BSM containing 1 mmol/L As(III) and again plated onto TSB agar containing 1 mmol/L As(III). Isolated strains were subjected to As(III) oxidation studies to evaluate their As(III) oxidizing ability as well as to T-RFLP analysis to determine their presence and dominance in the enrichment culture.

1.5 As(III) oxidation studies of isolated CAOs

As(III) oxidation studies were conducted using the growing cells and whole cells. All cultivations were carried out on a rotary shaker (120 r/min) at 28°C. Prior to the As(III) oxidation studies, isolated strains were grown to the late logarithmic phase in TSB supplemented with 1 mmol/L As(III). Next, 1 mL aliquots of the culture were transferred to 50 mL glass vials containing 20 mL of fresh medium and grown again to the late logarithmic phase. The cells were then harvested by centrifugation (15,000 ×*g*, 4°C, 10 min) and washed three times with 5 mg/L sodium tripolyphosphate solution. For cell growth assays, the washed cells were inoculated into 50 mL glass vials containing 20 mL BSM supplemented with 1, 5, or 10 mmol/L As(III) to a final cell density of approximately 0.02 (based on the optical density at a wavelength of 600 nm (OD_{600})). For whole cell assays, As(III) was added to BSM at 0.1, 0.5, 1, 5, or 10 mmol/L, and the bacterial cells were then inoculated at an OD_{600} of 0.2. During As(III) oxidation studies, 1 mL of the culture was collected at appropriate intervals to measure the concentrations of As(III) and As(V). The As(III) oxidation kinetic parameters of six CAO strains were determined from Lineweaver-Burk plots of the data.

1.6 Analytical methods

Bacterial growth was monitored based on changes in the OD_{600} using a UV1200 spectrophotometer (Shimadzu, Japan). The dry cell weight was calculated from the linear relationship between the OD_{600} and the dry cell weight, which was determined prior to the experiments.

The bacterial community structure of the enrichment culture was monitored by T-RFLP analysis. DNA templates were prepared by the proteinase K method, as previously described (Sei et al., 2000). T-RFLP analysis was carried out as previously described using *Hha*I (Matsuda et al., 2010). To determine the sizes of the terminal restriction fragments (T-RFs) of the isolated strains, their colonies on TSB agar were directly subjected to PCR amplification of the 16S rRNA genes. The resultant PCR products were subsequently subjected to T-RFLP analysis as described above.

To determine the arsenic concentrations, aqueous samples taken from the culture were centrifuged (20,000 ×*g*, 4°C, 10 min) and then filtered through a Dismic-25 cellulose acetate filter (pore size, 0.45 μm, Advantec, Japan), after which the filtrates were stored at 4°C until analysis. As(V) and the total arsenic concentrations were determined by ion chromatography (HIC-20A Super System, Shimadzu, Japan) using an HIC-SA3(G) guard column (Shimadzu, Japan), a HIC-SA3 analytical column (Shimadzu, Japan), and a CDD-10Asp electric conductivity detector (Shimadzu, Japan). $NaHCO_3$ at 5 or 6 mmol/L was applied as the mobile phase at a flow rate of 1.0 mL/min. Prior to measurement of the total arsenic concentration, H_2O_2 was added to the samples at a final concentration of 3% (*V*/*V*) to completely oxidize the remaining As(III) to As(V). The As(III) concentration was calculated based on differences between the total arsenic and As(V) concentrations.

2 Results

2.1 Construction of the enrichment culture of CAOs

We attempted to enrich CAOs from an arsenic contaminated soil sample by repeated subculturing without the addition of any external organic carbon sources (Fig. 1).

During cycle 1 and 2, the arsenic concentration was not measured, and subculturing was carried out after 10 days for each cycle. From cycle 3, the As(III) and As(V) concentrations were measured to evaluate the As(III) oxidation, and subculturing was conducted after almost

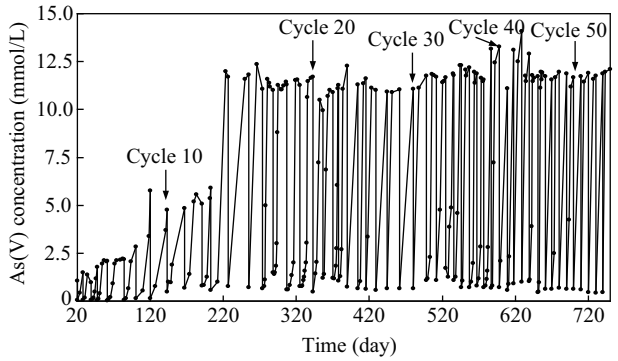

Fig. 1 Chronological profile of As(III) oxidation by the enrichment culture. The As(V) concentration was monitored from cycle 3.

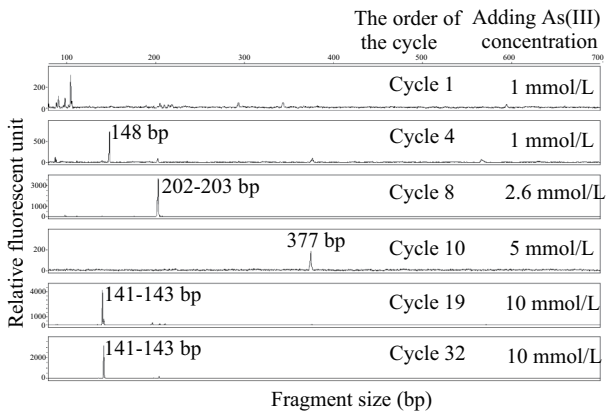

Fig. 2 Typical T-RFLP profiles of the enrichment culture during different enrichment cycles.

complete As(III) oxidation. During cycle 3 and 4, 1 mmol/L of As(III) was completely oxidized to the equivalent concentration of As(V) within eight days. Thereafter, the culture was sequentially subcultured with As(III) concentrations that were increased in a stepwise fashion. During enrichment, As(III) added at each subculturing cycle was almost completely oxidized to As(V), even when the As(III) concentration increased. Furthermore, the As(III) oxidizing ability of the enrichment culture gradually improved. For example, although the enrichment culture required 14 days to oxidize approximately 2 mmol/L As(III) during cycle 7, 5 mmol/L As(III) was oxidized within 12 days during cycle 13, and 10 and 12 mmol/L As(III) were oxidized within 8 and 6 days during cycles 23 and 46, respectively. Finally, after more than 60 cycles (> 700 days), the enrichment culture stably oxidized approximately 12 mmol/L As(III) within 4 days.

Successful As(III) oxidation was achieved during the long-term enrichment process without supplementation of any organic carbon sources, indicating that autotrophic bacterial growth utilizing As(III) as the energy source was occurring in the enrichment culture. This was further supported by a clear positive correlation between As(III) oxidation and bacterial growth in the culture (data not shown). These results confirmed that CAOs, which utilize As(III) as the energy source for their growth, were the main constituents of the enrichment culture. Moreover, the addition of 0.05% (*W*/*V*) yeast extract led to a significant acceleration of the As(III) oxidation and enhanced the bacterial growth in the enrichment culture (data not shown).

2.2 Change in bacterial community during the enrichment process

Variations in the bacterial community during the enrichment process were analyzed by T-RFLP (Fig. 2). A few dominant and minor T-RFs were detected at the beginning of enrichment (cycle 1). However, the number of T-RFs decreased and specific T-RFs became dominant during the enrichment process. Furthermore, the dominant T-RFs varied depending on the As(III) concentration in the culture. During cycle 4, when 1 mmol/L As(III) was added, a T-RF of 148 bp dominated. After increasing the initial As(III) concentrations to 2.6 and 5 mmol/L, T-RFs of 202–203 bp and 377 bp, respectively, became dominant. After the initial As(III) concentration was increased to 10–12 mmol/L, the enrichment culture had a stable bacterial community in which a T-RF of 141–143 bp always dominated and the minor T-RFs fluctuated temporally.

2.3 Isolation of CAOs from the enrichment culture

After cycle 32 and 46, during which approximately 10 mmol/L As(III) was stably oxidized and the microbial community composition determined by T-RFLP analysis was unchanged, the bacterial members present in the enrichment culture were isolated. Overall, ten morphologically unique bacterial colonies were isolated and designated as strains A, B1, B2, C, D, E1, E2, F, G and H. Preliminary screening of their As(III) oxidizing abilities with 1 mmol/L As(III) indicated that seven strains (B1, B2, C, D, E1, E2 and F) were CAOs, while strains A, G and H did not exhibit significant As(III) oxidizing abilities and were considered to be coexisting bacteria with As(III) tolerance (data not shown). Strain F lost its As(III) oxidizing ability during continuous subculturing.

T-RFLP analysis of ten isolated strains revealed that each isolate represented the following sizes of T-RF: strain A, 201–203 bp; strains B1, B2 and C, 141–143 bp; strains D, E1 and E2, 568–569 bp; strain F, 338–340 bp; strains G and H, 342–346 bp (Fig. 3). The T-RF of 201–203 bp represented by strain A corresponded to the dominant T-RF during cycle 8, while the T-RF for strains B1, B2 and C was identical to the dominant T-RF during cycle 19 (Figs. 2 and 3). However, T-RFs represented by the other isolates were not dominant in the enrichment culture.

2.4 As(III) oxidation ability with growing cells of isolated strains

Six strains (B1, B2, C, D, E1 and E2) that were confirmed as CAOs and stably exhibited As(III) oxidizing ability were further evaluated for their As(III) oxidizing ability using 1, 5 and 10 mmol/L As(III). Although all six strains were able to oxidize 1 to 10 mmol/L As(III), their oxidizing characteristics differed (Fig. 4). Specifically, strains B1, B2, E1 and E2 showed high As(III) oxidizing ability in the presence of all As(III) concentrations examined, and could oxidize 5 and 10 mmol/L As(III) completely within 72 and 120 hr, respectively. Strains B1, B2 and E1 also oxidized 1 mmol/L As(III) completely within 12 hr,

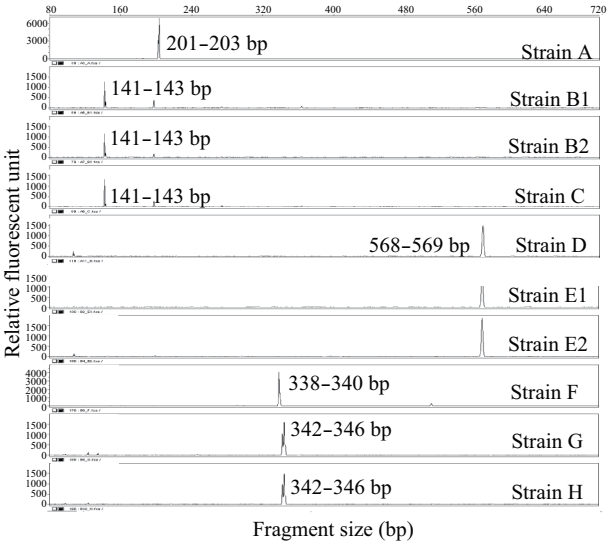

Fig. 3 T-RFs of ten isolated strains.

while strain E2 required 24 hr. Strain C showed similar As(III) oxidizing ability at 1 mmol/L As(III) to that of strains B1, B2, E1 and E2. In addition, strain C had the highest ability among the six strains at 5 mmol/L As(III), being able to complete oxidize it within 48 hr. However, when As(III) was added at 10 mmol/L, this strain oxidized only approximately 2 mmol/L within 120 hr. The As(III) oxidizing ability of strain D was lowest among the six strains investigated, requiring 24 and 120 hr for complete oxidation of 1 and 5 mmol/L of As(III), respectively. However, the As(III) oxidizing ability of strain D at 10 mmol/L was slightly higher than that of strain C.

2.5 As(III) oxidation ability with whole cells of isolated strains

The effects of As(III) concentration (0.1 to 10 mmol/L) on As(III) oxidation by the six CAO strains were also examined by whole cell assays. The results are summarized in Fig. 5. The specific As(III) oxidation rates of strains B1, B2, D, E1 and E2 increased with increasing As(III) concentration from 0.1 to 10 mmol/L, and reached similar maximum values between 0.22 and 0.28 mmol As(III)/(mg cell·hr) at 10 mmol/L. In contrast, the specific As(III) oxidation rate of strain C reached a maximum of 0.20 mmol As(III)/(mg cell·hr) at 5 mmol/L, and declined to 0.12 mmol As(III)/(mg cell·hr) at 10 mmol/L.

The experimental data were further analyzed to determine the oxidation and substrate inhibition kinetics of As(III) oxidation. Oxidation of up to 10 mmol/L of As(III) by strains B1, B2, D, E1 and E2 and up to 5 mmol/L As(III) by strain C can be represented by the Monod equation:

$$V = V_{\max} \times S/(K_m + S) \quad (1)$$

where, V (mmol As(III)/(mg cell·hr)) and $V_{\max}$ (mmol As(III)/(mg cell·hr)) are the actual and maximum specific oxidation rate, respectively, S (mmol/L) is the initial As(III) concentration, and K_m (mmol/L) represents the half saturation constant. The data for the six CAO strains fit the equation well, with correlation coefficients (r^2) ranging from 0.96 to 0.99. The As(III) oxidation kinetic parameters ($V_{\max}$ and K_m) determined for the six CAO strains are shown in Table 1.

Table 1 Kinetic parameters for As(III) oxidation by six isolated CAO strains determined from the Lineweaver-Burk plot based on the experimental data obtained from whole cell assays

Strain	$V_{\max}$ (mmol As(III)/(mg cell·hr))	K_m (mmol/L)
B1	0.28	0.61
B2	0.28	0.73
C	0.22	0.51
D	0.24	0.66
E1	0.25	0.68
E2	0.26	0.60

Although all six CAO strains had similar $V_{\max}$ values, those of strains B1 and B2 were the highest (0.28 mmol As(III)/(mg cell·hr)). The K_m values of the six CAO strains ranged from 0.51 to 0.73 mmol/L. Strain C had the lowest K_m value of 0.51 mmol/L, indicating that it has the highest affinity for As(III) among the six CAO strains.

3 Discussion

In this study, repeated batch cultivation for more than 700 days (more than 60 cycles of subculturing) using a contaminated soil sample as the inoculum resulted in successful construction of an enrichment culture capable

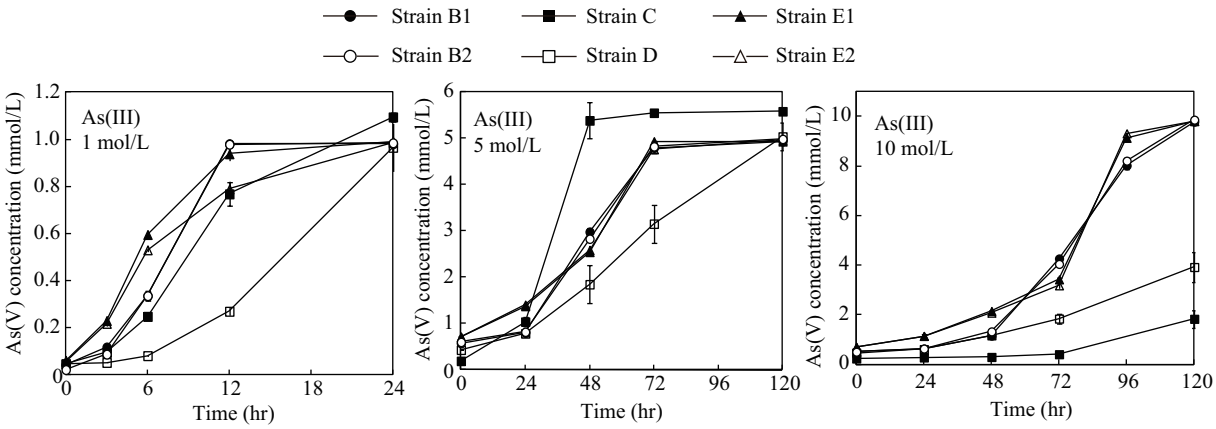

Fig. 4 Oxidation of 1, 5 and 10 mmol/L As(III) by six isolated CAO strains. Error bars indicate the standard deviation obtained from three independent experiments.

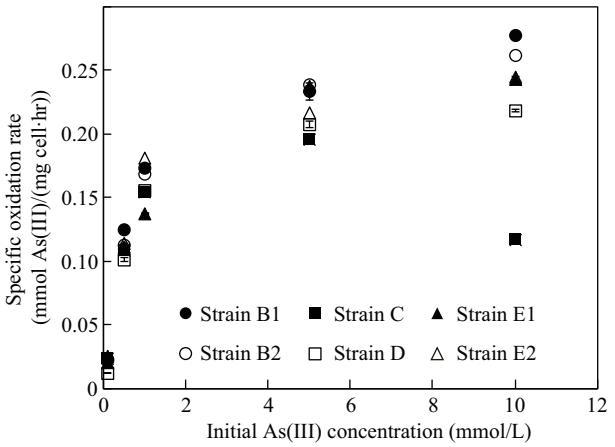

Fig. 5 Effects of the initial As(III) concentration on the specific As(III) oxidation rate of six isolated CAO strains. Error bars indicate the standard deviation obtained from three independent experiments.

of stably oxidizing 1 to 12 mmol/L As(III) to As(V) within four days. Achievement of continuous As(III) oxidation for a long period without supplementation of organic carbon and energy sources, and the correlation between As(III) oxidation and bacterial growth in the enrichment culture confirmed that As(III) oxidation in the enrichment culture was mainly a result of the activity of CAOs. As previously described, it was assumed that CAOs in the enrichment culture gained energy for cell growth from the As(III) oxidation as follows: $2H_3AsO_3 + O_2 \longrightarrow H_2AsO_4^- + HAsO_4^{2-} + 3H^+$, $\Delta G^0 = -256$ kJ/Rx (Santini et al., 2000). Enhancement of As(III) oxidation and bacterial growth in the enrichment culture by supplementation of the yeast extract indicated that the CAOs present in the enrichment culture are not obligatory autotrophic bacteria, but mixotrophic bacteria that are able to utilize both inorganic and organic carbon sources for their growth. These findings are in agreement with those of previous studies (Garcia-Dominguez et al., 2008; Duquesne et al., 2007; Battaglia-Brunet et al., 2005; Santini el al., 2000).

As(III) oxidation activity of the enrichment culture gradually improved as the number of enrichment cycles increased. This indicates that the continuous subculturing with a stepwise increase of As(III) concentration applied in this study would be effective for successful enrichment of CAOs with a high As(III) oxidizing ability from environmental samples that contain divergent bacteria.

T-RFLP analysis of the bacterial community in the enrichment culture showed that it varied drastically during the enrichment process. Although the soil sample used here contained various types of bacteria (as indicated by the number of T-RFs), the diversity of the bacterial community decreased greatly after initiation of the enrichment process with 1 mmol/L As(III). This reduction of bacterial diversity was likely due to the selection of specific bacteria, possibly CAOs, by the strong toxicity of As(III) and limited carbon and energy sources. Interestingly, the main T-RFs, which represented the predominant bacteria, varied with increasing As(III) concentration from 1 mmol/L to 10–12 mmol/L, and the bacterial community composition became stable with a dominant T-RF of 141–143 bp at 10–12 mmol/L As(III). These results indicated that distinct CAOs that were well-adapted to a given As(III) concentration became dominant at each As(III) concentration. Accordingly, the As(III) concentration was a significant selective pressure for CAOs in the enrichment culture, but CAOs other than the dominant one persisted at various As(III) concentrations as minor populations in the enrichment culture. Thus, some of the CAOs originally present in the soil sample survived even when the selective pressure was unfavorable for their growth, and became dominant when preferable conditions were provided with the enrichment procedure applied here (i.e., the stepwise increase of selective pressure in repeated batch cultivation).

The aforementioned assumptions were supported by the isolation of a variety of bacterial strains from the established enrichment culture. Isolated strains actually included those showing a T-RF of 141–143 bp (strains B1, B2 and C), which was the dominant T-RF in the enrichment culture at 10–12 mmol/L As(III), and a T-RF of 201–203 bp (strain A), which was dominant at 2.6 mmol/L (Figs. 2 and 3). Furthermore, six of the ten isolated strains (B1, B2, C, D, E1 and E2) showed stable As(III) oxidation ability under autotrophic conditions. These results verified that the stable enrichment culture obtained in this study was composed of dominant and non-dominant CAOs, as well as other various bacteria.

Six CAO strains had As(III) oxidation characteristics that varied depending on the As(III) concentration. When plotted against the initial As(III) concentration, their specific As(III) oxidation rates followed saturation kinetics, suggesting that As(III) oxidation by these strains was catalyzed by the activity of arsenite oxidase. The V_{max} and K_m values of the six CAO strains ranged from 0.22–0.28 mmol As(III)/(mg cell·hr) and 0.51–0.73 mmol/L, respectively. Kinetic parameters that can be compared with our data were available for four previously isolated CAO strains (Garcia-Dominguez et al., 2008; Dastidar and Wang, 2009). The V_{max} values of our strains were much higher than those of other strains (0.01–0.04 mmol As(III)/(mg cell·hr)), while the K_m values for our strains were slightly higher than or nearly equal to those of the other strains (0.14–0.44 mmol/L). These findings suggest that the CAOs present in our enrichment culture are very efficient As(III) oxidizers.

Although strains B1, B2 and C exhibited the same T-RF, which was identical to the dominant T-RF in the enrichment culture adapted to 10–12 mmol/L As(III) (Figs. 2 and 3), their As(III) oxidation activities differed. Strains B1 and B2 were capable of completely oxidizing 10 mmol/L As(III) within five days in the growing cell assays, and no significant inhibition at concentrations of As(III) up to 10 mmol/L was observed in the whole cell assays. In contrast, although strain C could oxidize 1 and 5 mmol/L As(III) rapidly and completely, at 10 mmol/L As(III) its oxidation did not proceed efficiently. Therefore, it was assumed that strains B1 and B2 played a primary role in the oxidation of 10 mmol/L As(III) in the stabilized enrichment culture, while other CAOs grew slightly via oxidation of As(III) after B1 and B2 oxidized it to some

extent. Consequently, a variety of CAOs with distinct As(III) oxidation characteristics appeared to coexist in the enrichment culture. Coexistence of multiple bacterial species in CAOs enrichment culture was also reported in a study conducted by Battaglia-Brunet et al. (2002).

Three isolated strains, A, G and H, did not exhibit significant As(III) oxidizing ability under autotrophic conditions. Those isolates might simply be co-existing bacteria with high As(III) tolerance that are capable of growth by utilizing inorganic carbon in the medium or organic carbon derived from dead CAOs. However, T-RFLP analysis indicated that strain A was dominant in the enrichment culture at 2.6 mmol/L As(III). This suggests that strain A contributed to As(III) oxidation in the enrichment culture. One possible explanation for this finding is that strain A can only oxidize As(III) in mixed cultures via certain interactions with other bacteria; nevertheless, further study of strain A is warranted. Moreover, strain F could not oxidize As(III) during continuous subculturing. This may have been due to the lack of some trace (but vital) nutrients produced by other bacteria in the enrichment culture, or the deprivation of plasmids in which the As(III) oxidation genes were located.

In this study, an enrichment culture of CAOs for As(III) oxidation composed of various bacteria was established and maintained. The results of this study demonstrated that specific CAOs that are well-adapted to various As(III) concentrations can be selectively enriched by providing $NaHCO_3$ and As(III) as the sole carbon and energy sources, respectively. In contrast, other CAOs that do not play a major role in oxidizing As(III) at certain As(III) concentrations can survive without complete disappearance in the enrichment culture. This might have occurred as a result of the stepwise increase of the As(III) concentration during repeated batch cultivation. Because CAOs co-existing in the resultant enrichment culture had various As(III) oxidation characteristics, some specific CAOs that exerted higher As(III) oxidation and growth under given environmental conditions could become dominant over other CAOs. Thus, the CAOs enrichment culture demonstrated the high potential of efficient As(III) oxidation activity under various levels of arsenic contaminated water. Considering the application for the pretreatment step in the remediation of arsenic-contaminated water, these characteristics of the enrichment culture are highly desirable since As(III) concentrations are continuously changing in the batch cultivation system. After As(III) oxidation, the obtained As(V) can be effectively removed from water by adsorption and/or co-precipitation process with high capacity adsorbents such as iron hydroxides and activated alumina. Overall, the CAOs enrichment culture developed here has higher flexibility and stability than pure CAO cultures for the preliminary As(III) oxidation step during the removal of arsenic from contaminated water. Further studies are under way to gain a more detailed understanding of the physiological and phylogenetic properties and As(III) oxidizing ability in actual environmental conditions of isolated strains.

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