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Arsenite Oxidation Using Biogenic Manganese Oxides Produced by a Deep-Sea Manganese-Oxidizing Bacterium, *Marinobacter* sp. MnI7-9

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Marinobacter sp. MnI7-9, a deep-sea manganese [Mn(II)]-oxidizing bacterium isolated from the Indian Ocean, showed a high resistance to Mn(II) and other metals or metalloids and high Mn(II) oxidation/removal abilities. This strain was able to grow well when the Mn(II) concentration reached up to 10 mM, and at that concentration, 76.4% of the added Mn(II) was oxidized and 23.4% of the Mn(II) was adsorbed by the generated biogenic Mn oxides (total 99.9% Mn removal). Scanning electron microscope observation and X-ray diffraction analysis showed that the biogenic Mn oxides were in stick shapes, adhered to the cell surface, and contained two typical crystal structures of γ-MnOOH and δ-MnO_2. In addition, the biogenic Mn oxides generated by strain MnI7-9 showed abilities to oxidize the highly toxic As(III) to the less toxic As(V), in both co-culture and after-collection systems. In the co-culture system containing 10 mM Mn(II) and 55 μM As(III), the maximum percentage of As(III) oxidation was 83.5%. In the after-collection system using the generated biogenic Mn oxides, 90% of the As(III) was oxidized into As(V), and the concentration of As(III) decreased from 55.02 to 5.55 μM. This study demonstrates the effective bioremediation by a deep-sea Mn(II)-oxidizing bacterium for the treatment of As-containing water and increases the knowledge of deep-sea bacterial Mn(II) oxidation mechanisms. Supplemental materials are available for this article. Go to the publisher's online edition of *Geomicrobiology Journal* to view the supplemental file.

Keywords: Arsenic oxidation, biogenic Mn oxides, deep-sea *Marinobacter*, manganese oxidation, manganese-oxidizing bacterium

Introduction

Manganese(II) can be oxidized to Mn oxides, mainly Mn(IV), via biotic and abiotic processes. Such Mn oxides are ubiquitous in deep-sea, soil and sediment (Jeffery 1999) and have high sorption capacities for many metal cations, such as Pb(II) (Nelson et al. 1999; Villalobos et al. 2005), Zn(II) (Tani et al. 2004a; Toner et al. 2006), Cd(II) (Meng et al. 2009), Ni(II), Co(II) (Tani et al. 2004a) and Cu(II) (Jin et al. 2009). The Mn oxides are also recognized as important oxidants that can oxidize As(III) to As(V) (Tani et al. 2004b), Cr(III) to Cr(VI) (He et al. 2010; Murray and Tebo 2007), Co(II) to Co(III) (Lee and Tebo 1994), U(IV) to U(VI) (Chinni et al. 2008), and Ce(III) to Ce(IV) (Ohnuki et al. 2008). They play important roles in the cycling of mineral nutrients and heavy metals in the environment.

Manganese (II) oxidation in natural aquatic systems often proceeds at a very slow rate in the absence of Mn(II)-oxidizing microorganisms, which ubiquitously occur in fresh water, marine water and soil (He et al. 2010; Villalobos et al. 2003; Wang et al. 2009). Mn(II)-oxidizing microorganisms can accelerate the rate of Mn(II) oxidization compared to the abiotic process (Hastings and Emerson 1986). Therefore, the majority of natural, environmental Mn oxides are derived from microbial Mn(II)-oxidation processes and can therefore be considered biogenic Mn oxides (Kim et al. 2003; Tebo et al. 2004). Studies have demonstrated that biogenic Mn oxides were highly reactive minerals that had much higher sorption and oxidation capacities for a wide variety of metal ions than abiotic Mn oxides (Meng et al. 2009; Murray and Tebo 2007). For example, the biogenic Mn oxides produced by *Leptothrix discophora*

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SS-1 had a higher Pb(II) sorption capacity, with a Pb/Mn molar ratio of up to 0.5, due to the higher surface areas (224 m^2/g) than those of abiotic Mn oxides (< 4.7 m^2/g) (Nelson et al. 1999); the biogenic Mn oxides generated by *Brachybacterium* sp. strain Mn32 exhibited a 2–3-fold greater capacity to adsorb Zn(II) or Ni(II) than the commercially or chemically synthesized Mn oxides, which contained crystal structures of birnessite (δ-MnO_2) and manganite (γ-MnOOH) (Wang et al. 2009). The effectiveness of biogenic Mn oxides was mainly dependent upon their large surface area (Nelson et al. 1999), high binding energy per unit area (Miyata et al. 2007) and fast growing ability, which ensured that the adsorbed substance was incorporated into their crystal structures (Webb et al. 2006).

Arsenic (As) is a very toxic metalloid pollutant in the environment, and As(III) is more toxic than As(V). Furthermore, As(V) is much easier to be removed from solution than As(III). Thus, pre-oxidation of As(III) to As(V) is generally required in arsenic-containing wastewater treatment technologies. Tani et al. (2004b) reported that biogenic Mn oxides generated by a fungus strain *Acremonium* sp. KR21-2 were able to oxidize As(III) to As(V) in a co-culture system. The researchers also studied the interaction of inorganic As with the biogenic Mn oxides. They found that As(III) began to be oxidized to As(V) after the formation of Mn oxides, and the As(III) oxidation rate decreased with increasing dissolved Mn(II) (Tani et al. 2004b).

Katsoyiannis et al. (2004) found that low concentrations of As (0.47 and 0.56 μM for As(III) and As(V), respectively) in simulated groundwater were removed through a fixed-bed upflow filtration system containing unpurified soil colonized by Fe(II)/Mn(II)-oxidizing bacteria. They later reported that biogenic Fe oxides could partially oxidize As(III) to As(V) and that the oxidized form was removed from the groundwater through adsorption (Zouboulis and Katsoyiannis 2005). To our knowledge, the successful use of purified Mn(II)-oxidizing bacteria for As removal has not yet been reported.

Manganese is a nutrient element in the marine system and participates in the photosynthetic process. Microbial Mn(II) oxidation and the precipitation of Mn in the deep sea are important oceanic biogeochemical processes (Zouni et al. 2001). To date, the knowledge of Mn(II) oxidation mechanisms by deep-sea microbial species is still very limited. This study was designed (1) to identify and detect the Mn(II) oxidation/removal abilities of a deep-sea bacterial strain, MnI7-9, and (2) to examine the oxidation and adsorption of As(III) by strain MnI7-9 in co-culture and in after-collection systems with the produced biogenic Mn oxides. The results of this study explain the possible deep-sea bacterial Mn(II)-oxidation mechanisms and are valuable for the application of bacterial biogenic Mn oxides to the bioremediation of arsenic-polluted water.

Materials and Methods

Identification of Deep-Sea Mn(II)-Oxidizing Bacterial Strain MnI7-9

A Mn(II)-resistant bacterial strain MnI7-9 was kindly provided by Dr. Zongze Shao (The Third Institute of Oceanography, Xiamen, China). The strain was isolated from sediment of the outer part of a hydrothermal vent chimney in the deep-sea of middle Indian Ocean (25.32ºS, 70.04ºE; 2,474 m deep), in 2009. The deep-sea sediment samples were collected using sediment collecting tubes and isolated immediately in the microbiology lab on the ship using liquid A medium plates [(L^{-1}), 10 g sodium acetate, 0.5 g yeast extract (Oxoid, Basingstoke, UK), 0.5 g peptone (Oxoid), 150 g agar, 0.126 g $MnCl_2$, 13.31 g NaCl, 0.563 g KCl, 9.99 g $MgSO_4 \cdot 7H_2O$, 0.833 g $CaCl_2$ and 20 mmol N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.2] and incubated at 28°C for 7 d according to Tian and Shao (2006).

A nearly full-length 16S rRNA gene sequence was amplified using PCR with the universal 16S rRNA gene primers Uni-27F and Uni-1492R as previously described (Wilson et al. 1990). The DNA sequencing was performed at the Beijing Genomics Institute (Beijing, China). The sequences were initially compared with the sequences in the GenBank database using blastN (http://www.ncbi.nlm.nih.gov) and subsequently aligned with closely related strains using the CLUSTAL W algorithm with default parameters. A phylogenetic analysis was carried out using the MEGA 4.0 program (Tamura et al. 2007). The distances and clustering were determined using the neighbor-joining method, with bootstrap analyses based on 1000 replications (Saitou and Nei 1987). The cellular fatty acid composition was analyzed using gas chromatography following the instruction of the Sherlock Microbial Identification System method (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10).

Metal Resistance Levels of Strain MnI7-9

The minimum inhibitory concentration (MIC), defined as the lowest metal/metalloid concentration that completely inhibited the growth of the strain, was determined using liquid A medium amended with different concentrations of $MnCl_2$ (0–60 mM), $NaAsO_2$ (0–20 mM), Na_3AsO_4 (0–20 mM), K_2CrO_4 (0–5 mM) and $ZnCl_2$ (0–6 mM). After one week of incubation at 28°C and shaking at 150 rpm, the cell growth was measured at OD_{600} using a spectrophotometer (Perkin-Elmer, Waltham, MA, USA) as previously described (Wang et al. 2009).

Mn(II) Oxidation and Adsorption by Strain MnI7-9

Manganese(II) was removed by strain MnI7-9 via two methods: oxidation and adsorption. The Mn(II) oxidation ability of strain MnI7-9 was qualitatively determined using the LBB method (Krumbein and Altmann 1973) and quantified using an atomic absorption spectrometry (AAS; 986A, Beijing Puxi General Instrument Co., Beijing, China).

To study whether the concentration of Mn(II) affected the Mn-removal capability of strain MnI7-9, 0.1 mL of the overnight culture was inoculated into each of triplicate aliquots of 100 mL liquid A media with different $MnCl_2$ concentrations ($c_{Mn\ (0)}$) (0–14 mM). After 10 days' cultivation at 28°C and shaking at 150 rpm, 10 mL of the liquid culture was taken and centrifuged at 12,000 rpm for 10 min.

The supernatant was filtered through a 0.22-μm filter, and the dissolved Mn(II) concentration ($c_{Mn(D)}$) was measured using the AAS. The brown precipitates (Mn oxide particles and the bacterial mass) were washed 3 times with deionized water,

mixed with 10 mL of 20-mM $CuSO_4$ solution and kept at 28°C for 12 h with 150 rpm shaking.

This operation was completed to release the adsorbed Mn(II) from the biogenic Mn oxides ($c_{Mn(A)}$) (Tani et al. 2004b). The solution was then separated by centrifuging and filtering, and the Mn(II) concentration in the filtrate ($c_{Mn(A)}$) was measured using an AAS as described previously.

To measure the amount of solid Mn oxides produced by strain MnI7-9, the remaining precipitates were washed 3 times with deionized water and mixed with 10 mL of 20-mM hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$). The mixture was kept at 28°C for 12 h with 150 rpm shaking, separated by centrifuging at 12,000 rpm for 10 min, and filtered through a 0.22-μm filter.

The Mn(II) concentration ($c_{Mn\,(OX)}$) of the filtrate was measured using an AAS as described previously. In the control experiments, heat-killed cells of strain MnI7-9 were incubated with the same amount of $MnCl_2$ in the liquid A medium.

The percentage of Mn(II) removed from the solution by strain MnI7-9 (*Re*%) on day 10 was calculated as follows:

$$\mathrm{Re}\% = \frac{c_{Mn(0)} - c_{Mn(D)}}{c_{Mn(0)}} \times 100$$

The percentage of Mn(II) oxidation (Ox%) was calculated as follows:

$$Ox\% = \frac{c_{Mn(OX)}}{c_{Mn(0)}} \times 100$$

The percentage of Mn(II) adsorption (Ad%) was calculated as follows:

$$Ad\% = \frac{c_{Mn(A)}}{c_{Mn(0)}} \times 100$$

where $c_{Mn\,(0)}$ was the initiative $MnCl_2$ concentrations in the liquid A media, $c_{Mn(D)}$ was the dissolved Mn(II) concentration in the supernatant, $c_{Mn\,(A)}$ was the adsorbed Mn(II) by the biogenic Mn oxides, $c_{Mn\,(OX)}$ was the Mn(II) concentration oxidized by strain MnI7-9, *Re*% was the percentage of Mn(II) removed from the solution by strain MnI7-9, Ox% was the percentage of Mn(II) oxidized by MnI7-9, and Ad% was the percentage of Mn(II) adsorbed by the biogenic Mn oxides.

Characterization of the Morphology and Crystal Structures of Biogenic Mn Oxides

Scanning electron microscopy (SEM) was used to observe the morphology of the biogenic Mn oxides and the cells. First, strain MnI7-9 was grown in liquid A medium for 7 days, with and without the addition of 10 mM $MnCl_2$, and centrifuged at 10,000 rpm for 5 min. The precipitant was transferred onto a small cover slip that was fixed with a double layer of glue and examined using a SEM (JSM-6390/LV, JEOL, Japan) with 10 or 20 kV accelerating voltage. To determine whether Mn was present on the cell surface, an energy-dispersive spectroscopy (EDS) (JSM-6390/LV, NTC, Japan) analysis was performed using the same cultures as were used for the SEM analysis, except that the washed precipitant was dried in a vacuum-freeze dryer.

X-ray diffraction (XRD) was used to analyze the crystal characteristics of the biogenic Mn oxides using an X-ray diffraction meter (Y-2000, Dandong Aolong Radial Instrumental, China). Ten milliliters of a 7-day-old strain MnI7-9 culture (in liquid A medium containing 10 mM $MnCl_2$) was centrifuged at 10,000 rpm for 5 min. The precipitate was air-dried, placed into a sample groove, covered with a quartz slice and analyzed using CuKα radiation ($\lambda = 0.1543$ nm) at 20 mA and 30 kV over the scanning range 5–90° (2θ), with an interval of 0.08° (Wang et al. 2009).

As(III) Oxidation and Adsorption by Biogenic Mn Oxides in the Co-culture System

First, 2 mL of a strain MnI7-9 culture with an OD_{600} of approximately 0.5 was inoculated into 200 mL of liquid A medium containing 10 mM $MnCl_2$ and incubated at 28°C was shaken at 150 rpm for 3 days. After this time, the growth of strain MnI7-9 reached stationary phase, and the biogenic Mn oxides was produced. Then $NaAsO_2$ was added to the culture, with a final As(III) concentration of 55 μM, and the culture was incubated for another 6 days. Two milliliters of the culture was taken each day: 1 mL of the suspension was used to analyze the Mn(II) concentration using the method as described above, and the other 1 mL was used to analyze the As(III)/As(V) concentrations.

The As(III)/As(V) concentrations were detected using a combination of high-performance liquid chromatography with hydride-generation atomic fluorescence spectroscopy (HPLC-HG-AFS). The operating conditions of the HPLC-HG-AFS are listed in Supplementary material Table S1. These procedures were defined as "co-culture systems." To examine if As(III) was oxidized or adsorbed by the bacterial cells, strain MnI7-9 was cultured in 200 mL of liquid A medium containing 55 μM As(III) and without an addition of $MnCl_2$.

As(III) Oxidation by the Biogenic Mn Oxides in the After-Collection System

To test the As(III) oxidation and As(III) removal capability of the biogenic Mn(II) oxides produced by strain MnI7-9, strain MnI7-9 was cultured in A medium containing 10 mM $MnCl_2$, for 10 days at 28°C and was shaken at 150 rpm. The biogenic Mn(II) oxides were collected by centrifuging and repeatedly washing with deionized water. The biogenic Mn(II) oxides were vacuum-dried at -56° C for 24 h, ground in an agate mortar and passed through a 0.16-mm sieve. A 0.1 mmol aliquot of the biogenic Mn oxides was added into 100 mL of 55 μM $NaAsO_2$ solution (pH 6.78, not buffered) and kept at 28°C for 100 h with 150 rpm shaking. One milliliter of the solution was taken away at an appropriate time, separated by centrifugation and analyzed for the As(III)/As(V) concentrations as described earlier. This procedure was defined as the "after-collection system."

Results

Phenotypic Identification and Fatty Acid Analysis of Strain MnI7-9

Strain MnI7-9 is a Gram-negative, rod-shaped, halotolerant Mn(II)-oxidizing bacterium. Colonies of this strain are white, round, and translucent on A medium plates. The colonies became clearly brown-colored after they grew for 4 days on A medium plates containing Mn(II), and they changed rapidly to bright blue when LBB was added to the plates (data not shown). These color changes indicated that biogenic Mn oxides containing high-valence Mn(III/IV) had been generated by strain MnI7-9.

Strain MnI7-9 exhibited high 16S rRNA gene sequence similarities to *Marinobacter flavimaris* SW 145^T (99.02%), *M. adhaerens typus* strain HP15 (99%), *Marinobacter gudaonensis* SL014B61A^T (97.56%) and *Marinobacter sediminum* R65^T (97.29%). A phylogenetic analysis showed that strain MnI7-9 was grouped in the same cluster as these *Marinobacter* species (Figure 1). The predominant cellular fatty acids (>5%) of strain MnI7-9 were $C_{18:1}\omega9c$ (28.8%), $C_{16:1}\omega7c$ (13.3%), C16:1ω7*c*/15 iso 2-OH (11.2%), C16:0 (10.4%), C17:0 10-methyl (7.2%), and C16:1ω9*c* (5.3%). The fatty acids of strain MnI7-9 were similar to the major fatty acid types of other *Marinobacter* species (Supplementary material, Table S2). The morphological, phylogenetic and fatty acid analyses all identified strain MnI7-9 as a *Marinobacter* member. Thus, strain MnI7-9 was named as *Marinobacter* sp. MnI7-9.

Growth Curves and Metal/Metalloid Resistance Levels of Strain MnI7-9

Strain MnI7-9 approached stationary phase after cultivation for 3 days in liquid A medium. The effect of different concentrations of Mn(II) on the growth of strain MnI7-9 is shown in Figure 2. The growth (OD_{600}) did not obviously change when the $MnCl_2$ concentrations were lower than 10 mM, and

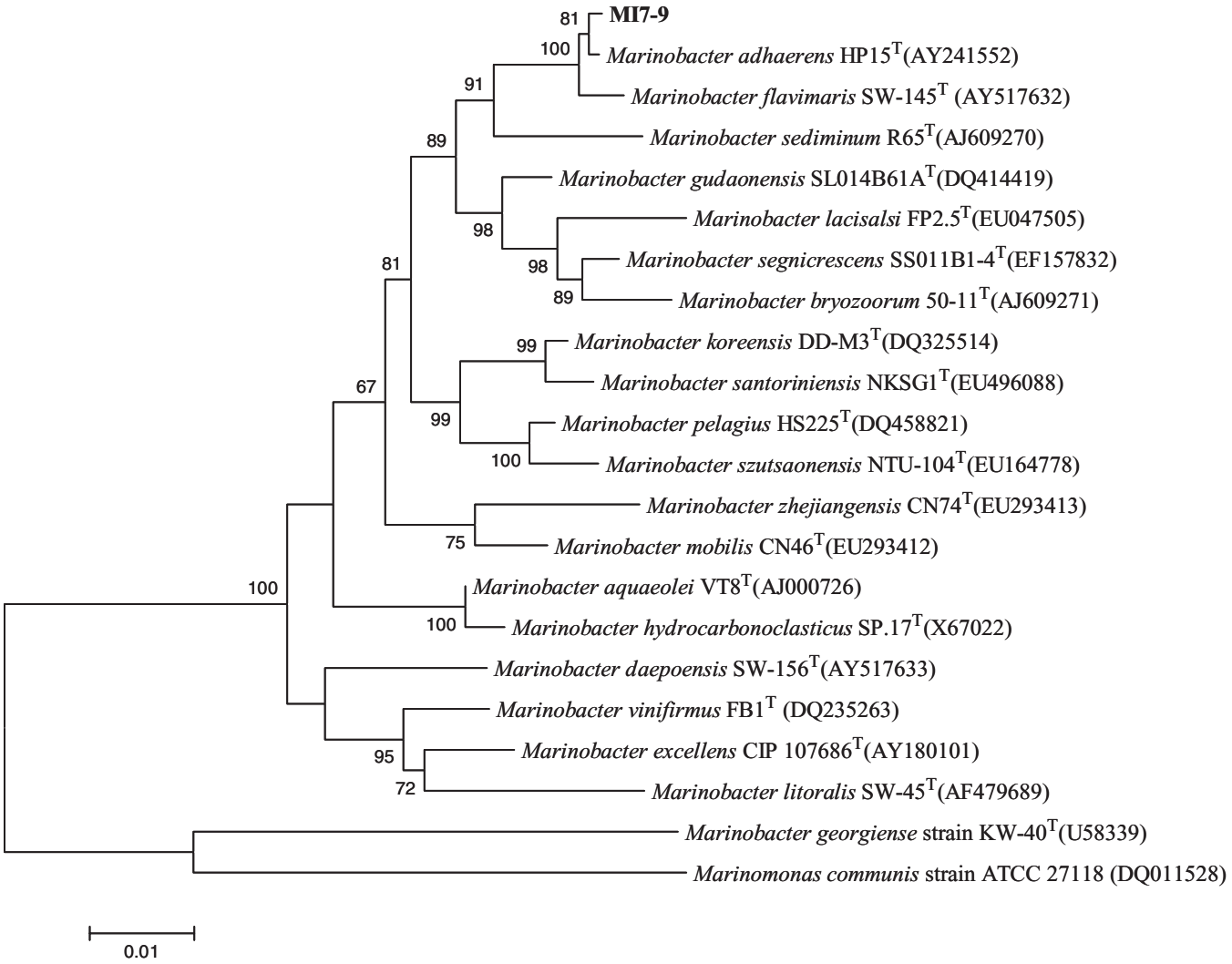


Fig. 1. A neighbor-joining phylogenetic dendrogram based on the nearly complete 16S rRNA gene sequences, showing the phylogenetic position of the strain MnI7-9 and the type strains of other *Marinobacter*, *Marinobacterium* or *Marinomonas* species. The numbers near the branches are the bootstrap probability values (%) with 1000 replicates. The scale bar represents a 2% difference in nucleotide sequences. The numbers in parentheses represent the accession numbers of the sequences in the GenBank database.

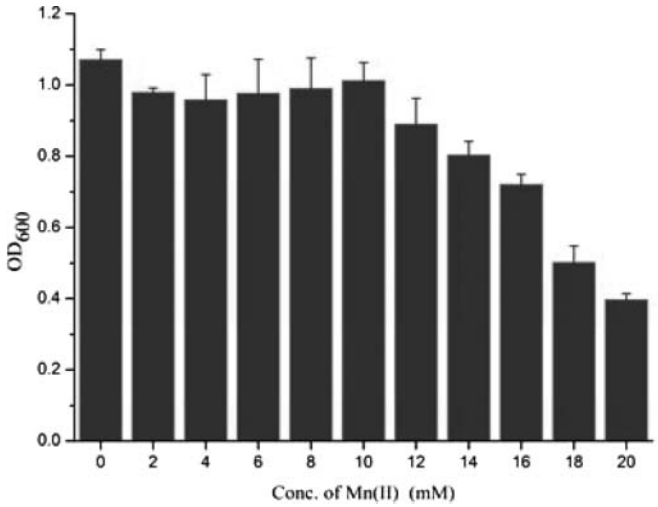


Fig. 2. The growth of strain MnI7-9 (OD_{600}) after being cultured for 5 days in liquid A medium with different concentrations of Mn(II).

the growth (OD_{600}) was inhibited by the increasing Mn(II) amounts when the $MnCl_2$ concentrations were higher than 10 mM (Figure 2). Strain MnI7-9 showed high resistances to some metal/metalloids. The MICs for Mn(II), As(III), As(V), Cr(VI) and Zn(II) were 45, 5.0, 17.0, 0.5 and 0.4 mM, respectively.

Mn(II) Oxidation and Removal by Strain MnI7-9

The percentage of Mn(II) removal by strain MnI7-9 was calculated as the total percentage of Mn(II) oxidation plus Mn(II) adsorption as described in the methods section. The percentages of Mn(II) oxidation and removal by strain MnI7-9 in different Mn(II) concentrations on the 10th day were calculated (Figure 3A). The Mn(II) removal ability of strain MnI7-9 remained stable under different Mn(II) concentrations (0–14 mM), and fractions of Mn(II) that were removed (*Re*%) were greater than 95%. The results demonstrated the potential of using strain MnI7-9 in the treatment of highly Mn(II)-polluted wastewater. The percentage of Mn(II) oxidation (*Ox*%) increased when the Mn(II) concentrations ranged from 2 to 10 mM, and the percentage of Mn(II) oxidation declined along with the increased Mn(II) concentrations (Figure 3A).

The liquid A medium with the optimal Mn(II) concentration (10 mM) was used for a detailed study of the Mn(II) oxidation and adsorption by strain MnI7-9 over different culturing time periods. As shown in Figure 3B, the Mn(II) concentration in the suspension began to decrease rapidly on the 2nd day, and the Mn(II) was almost completely removed after a 7-day incubation, during which the Mn(II) concentration had declined from 10 ± 0.30 to 0.37 ± 0.02 mM. Brown biogenic Mn oxides appeared on the 3rd day and kept increasing in abundance throughout the incubation period. The amount of Mn(II) adsorbed on the biogenic Mn oxides increased, reached a peak (59.6%) on the 4th day, and then decreased. Until the system reached equilibrium on the 9th day, 7.64 ± 0.08 mM of 10 mM Mn(II) was converted into Mn oxides by strain MnI7-9 (76.4%), 2.34 ± 0.01 mM Mn(II) was adsorbed

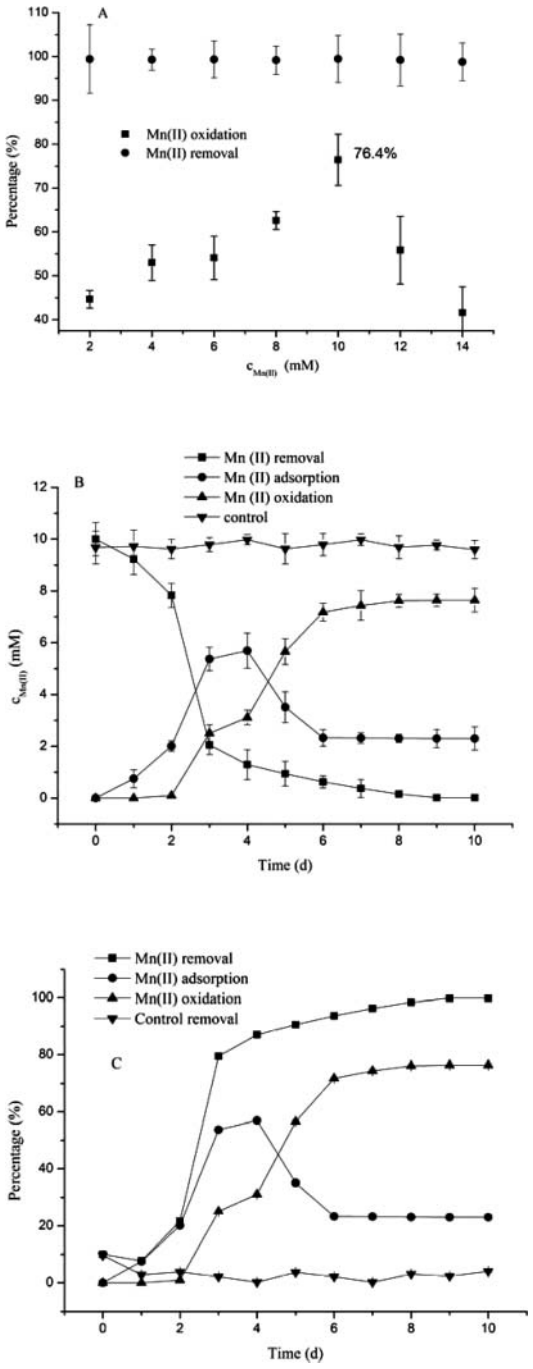


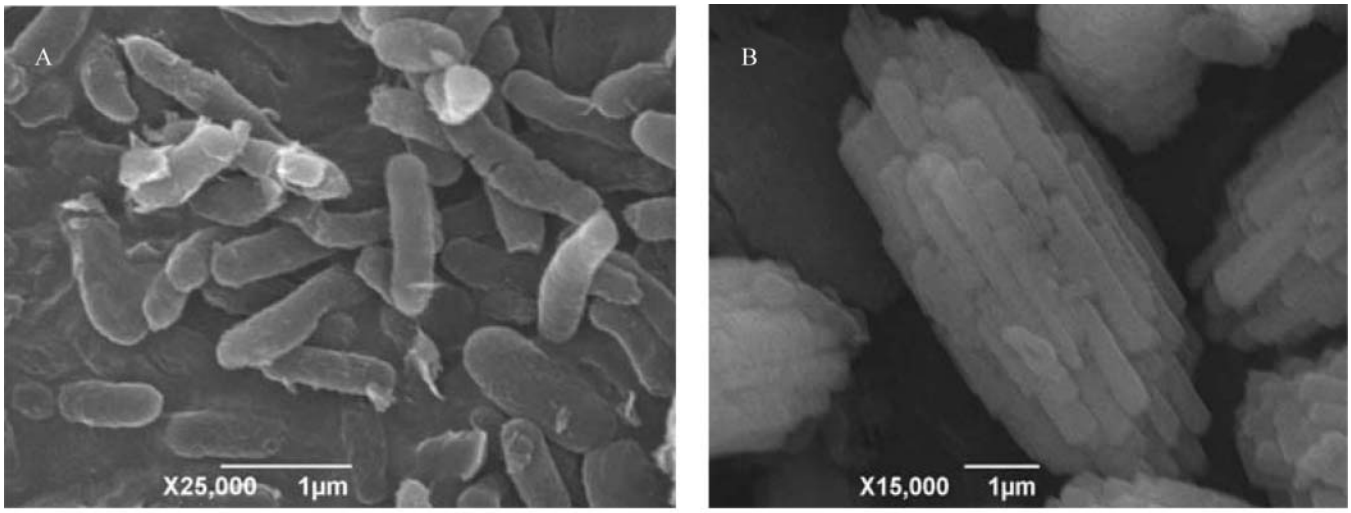
Fig. 3. Mn(II) oxidation and removal by Mn32 in liquid A medium. A, The percentages of Mn(II) oxidation (■) and Mn(II) removal (●) by strain MnI7-9 under different concentrations of Mn(II) in liquid A medium. B, Time course of Mn(II) oxidation and removal by strain MnI7-9 in liquid A medium. ■, concentration of Mn(II) remaining in the supernatant; ●, concentration of Mn(II) adsorbed onto the biogenic Mn oxides; ▲, concentration of the generated solid biogenic Mn oxides; ▼, concentration of Mn(II) in control experiments using dead cells of MnI7-9 with the addition of 10 mM $MnCl_2$. C, The percentages of Mn(II) oxidation and removal by strain MnI7-9 in liquid A medium. ■, Mn(II) removal; ●, Mn(II) adsorption; ▲, Mn(II) oxidation; ▼, control removal. Error bars represent the variation of the results from three replicate experiments.

by the biogenic Mn oxides that coated the cells inside (23.4%), and only 0.01 ± 0.01 mM soluble Mn(II) was left in the suspension. The removal of Mn(II) (*Re*%) reached the maximum value of 99.9% on the 9th d and remained stable on the 10th day (Figures 3B and 3C). In the control experiments, dead cells of strain MnI7-9 were incubated with $MnCl_2$ in liquid A medium, and no obvious oxidation or adsorption of Mn(II) was detected, indicating that there was no Mn(II) physically adsorbed onto the MnI7-9 cells (Figures 3B and 3C).

Morphologies and XRD Patterns of the Biogenic Mn Oxides Produced by Strain MnI7-9

The morphologies of strain MnI7-9 and biogenic Mn oxides observed using SEM are shown in Figure 4. The cells of strain MnI7-9 were 0.5–0.8 μm in diameter and 1.5–2.0 μm in length (Figure 4A). The biogenic Mn oxides were stick-shaped and adhered to the surface of strain MnI7-9 cells; the combination of the biogenic Mn oxides and the cells were regular spindle-like particles (Figure 4B). An energy-dispersive spectroscopy analysis revealed the presence of Mn on the cell surfaces of strain MnI7-9 (data not shown).

There were obvious crystalline peaks of δ-MnO_2 and γ-MnOOH on the CuKα XRD patterns of the biogenic Mn oxides produced by strain MnI7-9. The CuKα XRD patterns of the biogenic Mn oxides showed the following features: (1) four typical peaks of birnessite at 36.3°, 37.9°, 43.0°, 53.1° (Figure 4C; peaks a, b, c and d) with D values of 0.2379 nm, 0.2338 nm, 0.2105 nm and 0.1726 nm, respectively, which were designated as δ-MnO_2 peaks; (2) three narrow



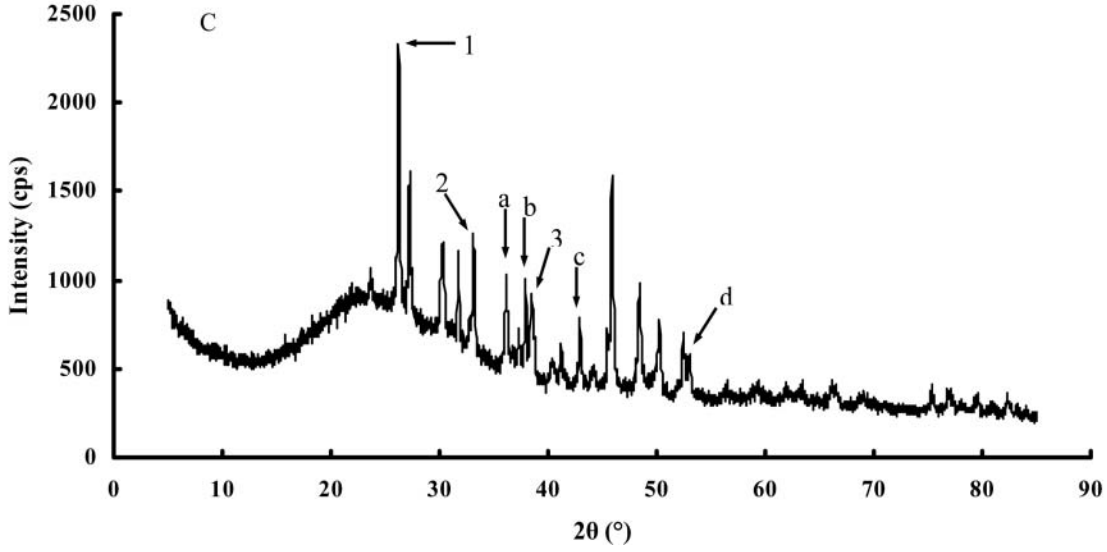


Fig. 4. A, SEM images of MnI7-9 cells cultivated in liquid A medium for 7 days without an addition of $MnCl_2$. B, SEM images of strain MnI7-9 incubated for 7 d and coated with the biogenic Mn oxides. C, XRD patterns of the biogenic Mn oxides generated by strain MnI7-9. Peaks a, b, c and d are typical of birnessite (δ-MnO_2); peaks 1, 2 and 3 are typical of manganite (γ-MnOOH).

diffraction peaks at 26.3°, 33.3° and 38.5° (Figure 4C; peaks 1, 2 and 3) with D values of 0.3393 nm, 0.2696 nm and 0.2338 nm, respectively, which were typical peaks of γ-MnOOH.

As(III) Oxidation and Adsorption in a Co-culture Experiment

To detect the As(III) oxidation ability of the biogenic Mn oxide, As(III) was added after treating MnI7-9 cells with 10 mM $MnCl_2$ for 3 days, when biogenic Mn oxides were generated. During the entire incubation period, no As(V) was detected in the biogenic system. It is possible that As(III) was first oxidized to As(V) and then adsorbed to the brown solids containing a mixture of Mn oxide and the bacteria cells. When strain MnI7-9 was cultured in liquid A medium without Mn(II), the As(III) concentration in suspension did not decrease (Figure 5), indicating that the MnI7-9 cells did not adsorb As(III) and that the As(III) removal by the biogenic Mn oxide was responsible for the decrease of As(III) concentration in the co-culture system. When strain MnI7-9 was grown in liquid A medium with 55 μM As(III), the As(III) concentration decreased from 54 $\pm$ 1.62 μM on the 3rd day to 9.07 $\pm$ 0.28 μM on the 6th day, then increased slightly and stabilized at approximately 16.87 μM on the 7th day. The maximum percentage of As(III) removal was 83.5% on the 6th day (Figure 5). The As(III) removal occurred along with the growth (OD_{600}) of strain MnI7-9 (Figure 5).

As(III) Oxidation Using the Biogenic Mn Oxides in the After-Collection System

In the experiment processes of after-collection systems, the As(III) concentration in the solution decreased quickly within the first 20 h, stabilized between 20–40 h and then continued to decline to 5.55 $\pm$ 1.53 μM As(III) (Figure 6A). The As(V) concentration in the solution appeared at the 20th h and then continuously increased to 49.98 $\pm$ 1.90 μM. Approximately

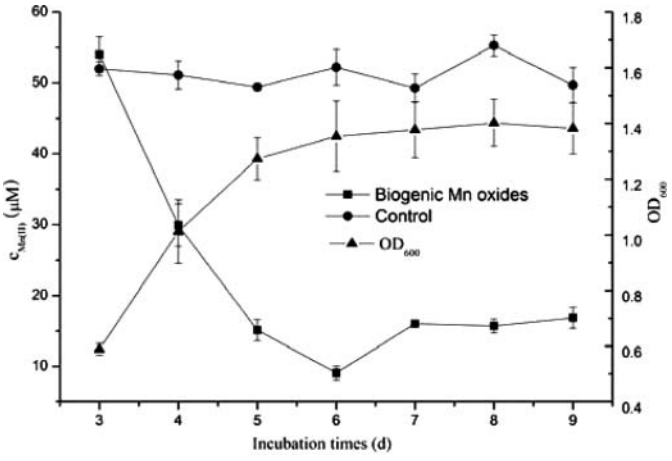


Fig. 5. As(III) oxidation and adsorption in the co-culture system containing 10 mM Mn(II), and added 55 μM As(III) after 3 days of incubation. ■, As(III) concentrations in the system containing the biogenic Mn oxides; ▲, growth curve of strain MnI7-9 (OD_{600}); ●, As(III) concentrations in the system containing strain MnI7-9 without an addition of Mn(II). Error bars represent the variation of the results from three replicate experiments

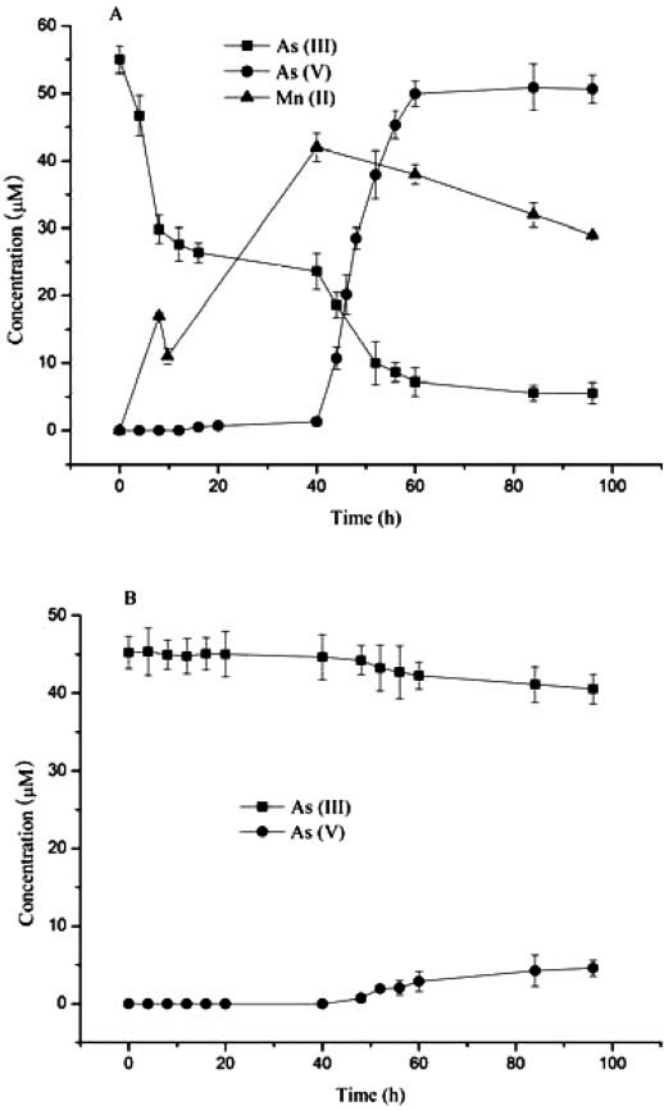


Fig. 6. Time course of As(III) oxidation using the biogenic Mn oxides generated by strain MnI7-9. A, 0.1 mmol biogenic Mn oxides added in 55 μM $NaAsO_2$ solution; B, control experiments, 55 μM $NaAsO_2$ solution without an addition of the biogenic Mn oxides; ■, As(III) concentration; ●, As(V) concentration; ▲, Mn(II) concentration.

90% of the As(III) had been oxidized into As(V) at 96 h. The Mn(II) concentration in the supernatant had increased from 0 to 42 $\pm$ 2.1 μM at 40 h, then decreased to 29 $\pm$ 0.2 μM by 96 h (Figure 6A). This result indicated that Mn(III/IV) reduction occurred along with the oxidation of As(III).

During the incubation period in the control experiments without the addition of the biogenic Mn oxides, the As(III) concentrations changed only slightly (from 40.52 $\pm$ 1.90 to 45.36 $\pm$ 3.12 μM), and only a small amount of As(V) (from 0.70 $\pm$ 0.37 to 4.56 $\pm$ 1.02 μM) was detected (Figure 6B). These results indicated that the As(III) oxidation was mainly achieved by the biogenic Mn oxides and was not a natural oxidation process. Furthermore, the pH of the solution changed

from 6.78 ± 0.42 (at 0 h) to 7.28 ± 0.01, 8.75 ± 0.06, 8.79 ± 0.05 and 8.82 ± 0.13, at 8, 40, 60 and 96 h, respectively.

Discussion

This study provides the first evidence of the capability of a pure Mn(II)-oxidizing bacterial strain to oxidize As(III) in both co-culture and after-collection systems. *Marinobacter* sp. MnI7-9, isolated from the deep sea in the Indian Ocean, showed a high resistance to multiple metal/metalloids and was able to efficiently remove Mn(II) from solution by oxidizing soluble Mn(II) to insoluble biogenic Mn oxides and through the adsorption of Mn(II) to the generated biogenic Mn oxides. A particular characteristic of this deep-sea Mn(II)-oxidizing bacterium is its high Mn oxidation capability. This strain could grow and efficiently oxidize up to 10 mM Mn(II), which is much higher than the efficiently oxidized Mn(II) concentration (0.3 mM) by our previously reported deep-sea Mn(II)-oxidizing *Brachybacterium* sp. Mn32 (Wang et al. 2009). The optimal Mn oxidation concentrations of other bacterial or fungal strains all appeared to be lower than that of *Marinobacter* sp. MnI7-9 [e.g., the Mn(II)-oxidizing fungus KR21-2 with an optimal Mn concentration of 1.1 mM (Tani et al. 2004b) or the marine *Bacillus* sp. SG-1 with an optimal Mn concentration of 0.2 mM (Francis and Tebo 2002)].

It appeared that strain MnI7-9 oxidized Mn(II) to Mn(III) and Mn(IV), because the biogenic Mn oxides contained poorly crystallized structures of birnessite (δ-MnO_2) and manganite (γ-MnOOH) oxides. These two types of structures are similar to those produced by the deep-sea *Brachybacterium* sp. Mn32 (Wang et al. 2009) and the marine *Bacillus* sp. SG-1 (Webb et al. 2005). Such Mn oxide structures were reported to have a large surface area and have strong adsorption ability for some metal ions (Murray and Tebo 2007; Wang et al. 2009). To date, it is still not clear whether there is a significant difference among the structures of biogenic Mn oxides generated from marine, freshwater and soil bacteria or fungi. Interestingly, in our research, most of the biogenic Mn oxides generated from soil Mn(II)-oxidizing *Bacillus* species did not have crystallized structures and could not adsorb metals efficiently (Liu et al. 2009; Wang et al. in preparation).

Another soil *Bacillus* strain, WH4, also produced biogenic Mn oxides with no obvious crystalline peaks on their XRD patterns, but this strain could still efficiently adsorb Cd (Meng et al. 2009). A freshwater bacterium, *Pseudomonas putida* MnB1, had a poorly crystalline structure of δ-MnO_2, but no γ-MnOOH peaks were detected using an XRD analysis (Villalobos et al. 2003). In addition, *Marinobacter* sp. MnI7-9, *Brachybacterium* sp. Mn32 (Wang et al. 2009) and *Bacillus* sp. SG-1 (Webb et al. 2005), isolated from a deep-sea hydrothermal vent in the Indian Ocean, a deep-sea Mn-Co nodule in the Pacific Ocean and the surface sediment of San Diego Bay, respectively, showed similar crystal structures and even morphologies (for strains Mn32 and MnI7-9) of the biogenic Mn oxides that each strain produced.

These results indicate that such structures may be typical and dominate in marine environments regardless of the differences among bacterial species. Mn(III) is a strong oxidant that is required in the photosystem II pathway to produce oxygen and also important for the oxidation of sulfur and nitrogen compounds (Zouni et al. 2001). An understanding of the mechanisms of Mn(II) oxidation and transformation by deep-sea microorganisms is very important for the study of oceanic biogeochemical processes.

The contamination of drinking or waste water by inorganic As is an important environmental problem worldwide. Pre-oxidation of As(III) is generally required for treatment because As(III) is less effectively removed than As(V) by most As-treatment technologies. The biogenic Mn oxides are redox-active and can potentially react with As and change its redox state. In this study, in the co-culture system, As(III) was efficiently removed in conjunction with the formation of the insoluble biogenic Mn oxides, and no As(V) was generated in the supernatant during the incubation times. It is possible that the produced As(V) was efficiently adsorbed by the biogenic Mn oxides mixed with strain MnI7-9 cells. Tani et al. (2004b) studied the interaction of inorganic As with the biogenic Mn oxide produced by an Mn(II)-oxidizing fungus, strain KR21–2.

During the cultivation of strain KR21-2 with 14.7 μM As(III) and 1.1 mM Mn(II), As(III) started to decline at 40 h, almost all of the As(III) was removed in 60 h, and approximately 25% of the As(V) from the oxidation of As(III) was adsorbed by the biogenic Mn oxides. Obviously, As removal (55 μM) was significantly higher in our co-culture experiments than in the experiments of Tani et al. (2004b). This difference may have resulted because the As(III) was first oxidized to As(V), and then almost all of the As(V) was adsorbed by the generated Mn oxides. The unpurified soil Fe(II)/Mn(II)-oxidizing bacteria could remove 0.47 to 0.56 μM As(III), a concentration that was also lower than that removed by strain MnI7-9 (Katsoyiannis et al. 2004).

In the after-collection system, approximately 90% of the As(III) was oxidized into As(V) using the biogenic Mn oxides produced by strain MnI7-9, and most of the As that could be detected in the solution was the As(V) type at 100 h [50.66 ± 2.10 μM for As(V) and 5.55 ± 1.53 μM for As(III)]. The oxidation of As(III) by the biogenic Mn oxides is a complex process, including As(III) oxidation, Mn(III/IV) reduction and adsorption of As(III/V) and Mn(II). Theoretically, the Mn(II) from the reduction of Mn(III/IV) and the As(V) from the oxidation of As(III) were almost equal according to oxidation-reduction reaction theory.

Moreover, the Mn(II) adsorbed by the biogenic Mn oxides would release into the solution as the Mn oxides were reduced. Thus, the Mn(II) concentration in the solution might be increased. However, in our study, the Mn(II) concentration (29.0 ± 0.20 μM) in the solution was much lower than the As(V) concentration (50.66 ± 2.10 μM) after reaction for 60 h (Figure 6A), indicating that the biogenic Mn oxides tended to bind Mn(II) rather than As(V). As the after-collection experiments proceeded, Mn(III/IV) was reduced to Mn(II) which may occupy more adsorption sites of the generated biogenic Mn oxides. As a result, almost all of the As(V) generated from the As(III) oxidation in the system existed in the solution.

In the after collection system, the As(III) was oxidized to As(V), and the pH of the aqueous solutions changed from neutral to an alkaline condition which may be caused by the reduction of Mn(III/IV). This result is different from that of the co-culture system. In that case, the medium was buffered at pH 7.2 and the pH values did not change obviously during the entire As(III) removal period (data not shown). In such buffered medium, Mn(II) oxidation should be enhanced enzymatically. Moreover, as the As(III) was added at the 3rd d when the Mn oxides already generated, we believe that the biogenic Mn oxides were responsible for the decrease of As(III) concentration instead of an enzymatic reaction, such as the arsenite oxidase (Cai et al. 2009). This can also be approved that no As(III) oxidation phenotype was observed when the As(III) was co-cultured with strain MnI7-9 without the addition of $MnCl_2$ (data not shown). Recently, we analyzed the genome sequence of strain MnI7-9 and revealed interesting genes associated with Mn(II) oxidation, but not with As(III) oxidation (Wang et al. forthcoming 2012), and we are in the process of investigating the deep-sea bacterial Mn(II) oxidation mechanisms.

In summary, the deep-sea Mn(II)-oxidizing bacterium *Marinobacter* sp. MnI7-9 was highly resistant to Mn(II), As(III) and As(V) and had powerful abilities for oxidizing/removing Mn(II) from the surrounding media. Strain MnI7-9 removed Mn(II) by two steps: 1) the soluble Mn(II) was oxidized to insoluble biogenic Mn oxides with structures of δ-MnO_2 and γ-MnOOH; 2) the biogenic Mn oxides further adsorbed more Mn(II) from the culture. Such structured deep-sea biogenic Mn oxides should be very useful for the cycling of the toxic element and nutrient composition of seawater. In addition, the biogenic Mn oxides could oxidize As(III) in both co-culture and after-collection systems, suggesting a great potential for the use of biogenic Mn oxides in the treatment of arsenic-polluted water.

Abbreviations

MIC, minimum inhibitory concentration; SEM, Scanning electron microscopy; EDS, energy-dispersive spectroscopy; XRD, X-ray diffraction; HPLC-HG-AFS, high-performance liquid chromatography with hydride-generation atomic fluorescence spectroscopy.

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