Extremophiles (2009) 13:39–48
DOI 10.1007/s00792-008-0195-1

ORIGINAL PAPER

Isolation and diversity analysis of arsenite-resistant bacteria in communities enriched from deep-sea sediments of the Southwest Indian Ocean Ridge

Shuangxi Chen · Zongze Shao

Received: 30 April 2008 / Accepted: 16 September 2008 / Published online: 8 October 2008

Abstract Microorganisms play an important role in the geobiocycling of arsenic element. However, little is known about the bacteria involved in this process in oceanic environments. In this report, arsenite-resistant bacteria were detected in deep-sea sediments on the Southwest Indian Ridge. From arsenite enriched cultures, 54 isolates were obtained, which showed varied tolerance to arsenite of 2–80 mM. Phylogenetic analysis based on 16S rRNA showed that they mainly belonged to Proteobacteria and Actinobacteria. Denaturing gradient gel electrophoresis revealed that *Microbacterium esteraromaticum* was the dominant member in the arsenite enriched communities, and this was reconfirmed by 16S rRNA gene library analyses. Thus, *M. esteraromaticum* showed highest resistant to arsenite among the detected bacteria. These results indicate that there are quite diverse bacteria of arsenite resistance inhabiting the deep sea sediment, which may play a role in the geobiocycling of arsenic element in marine environments.

Keywords Arsenite · Heavy metal tolerance · Deep sea · Sediment · *Microbacterium* · Biodiversity

Communicated by T. Matsunaga.

S. Chen · Z. Shao (✉)
Key Laboratory of Marine Biogenetic Resources,
Third Institute of Oceanography,
State Oceanic Administration,
Xiamen, Fujian 361005, China
e-mail: shaozz@163.com

Introduction

Arsenic is widely distributed in environments. Its pollution in both soil and groundwater has roused great concerns. Arsenic can cause serious health problems in humans, especially by its reduced inorganic form, arsenite, As(III). However, during the long history exposure to arsenic, microorganisms have evolved several kinds of mechanisms to reduce the harm effects of arsenite (Bhattacharjee and Rosen 2007).

By now, arsenic-resistant bacteria have mainly been isolated from arsenic-rich environments; while recent evidence has shown that arsenic-resistant bacteria can also be found in arsenic-free soils (Jackson et al. 2005). This finding implies a wide global interaction between bacteria and arsenic. Microbial metabolism was recently shown to be involved in mobilizing the subsurface arsenic and influence the speciation of arsenic in environments (Silver and Phung 2005; Oremland et al. 2004). Arsenate can be used as an electron acceptor by reduction to arsenite in the anaerobic sediment. Conversely, arsenite can be oxidized to arsenate by aerobic bacteria that are able to gain energy for their growth from the oxidation of arsenite. These studies emphasize the importance of microorganisms in element biogeocyling.

Since the first report of arsenite oxidizers and reducers in 1918, more than 30 strains representing at least nine genera have been reported to be able to oxidize arsenite (Stolz et al. 2006). By now, various arsenic-resistant bacteria have been found, including *Bacillus arsenoxydans, Pseudomonas arsenoxyda, Xanthomonas arsenoxydans, Achromobacter arsenoxydanss, Alcaligenes faecalis, Zoogloea* sp. ULPAs1, *Agrobacterium* sp. NT-26, *Hydrogenophaga* sp. (YED6-4, YED6-21 and YED1-18), *Thiomonas* sp., and so on. They were mainly isolated from arsenic-rich environments,

including soil (Quastel and Scholefield 1953; Osborne and Ehrlich 1976), cattle-dipping fluids (Green 1918; Turner 1949, 1954), mine water (Wakao et al. 1988; Leblanc et al. 1996), mines (Santini et al. 2000), acid mine drainage (Baker and Banfield 2003; Casiot et al. 2003, 2004; Morin et al. 2003; Duquesne et al. 2008), hot springs (Langner et al. 2001; Gihring et al. 2001; Salmassi et al. 2006; Hetzer et al. 2007), Mono Lake (Oremland et al. 2004; Hoeft et al. 2004, 2007) and Searles Lake (Oremland et al. 2005; Kulp et al. 2006).

Marine arsenic has many sources, such as land, subsurface and rock leaching. Arsenic in marine organisms and environment is relatively high (Lunde 1977; Neff 1997). For an instance, uncontaminated marine sediments contain from 5 to about 40 μg g^{-1} dry weight total arsenic (Neff 1997). In arsenic cycling of marine ecosystem, marine algae play a role by capture and delivery of this element from the surface seawater to deep sea environments (Dembitsky and Levitsky 2004). Hydrothermal activities on the ocean's inter-ridges bring about arsenic from the inner part of the Earth. Vent sites are thought to be where the autotrophic arsenite-oxidizers existed in the early stages of life on Earth (Lebrun et al. 2003), and potentially reflect a primitive microbial ecosystem on Mars or Europa (Oremland and Stolz 2003).

Marine arsenic-resistant bacteria have been found as *Serratia marinorubra* (Vidal and Vidal 1980), *Vibrio–Aeromonas* group bacteria (Hanaoka et al. 2004), *Pseudomonas fluorescens* (Prithivirajsingh et al. 2001), *Deferribacter desulfuricans* (Takai et al. 2003), and *Marinomonas communis* (Takeuchi et al. 2007). Most of them are reported arsenate-resistant, only a few show arsenite-resistance (Scudlark and Johnson 1982; Pepi et al. 2007; Takeuchi et al. 2007), such as marine bacterium *Marinomonas communis* which could resist both arsenate and arsenite, and capable of removing arsenic from culture medium (Takeuchi et al. 2007). According to our knowledge, no arsenic-resistant bacteria in deep sea environments have been investigated.

The aim of this study is to bioprospect the arsenite-resistant bacteria from deep sea sediment of two sites on the Southwest Indian Ridge, one of which is in the vicinity of an active hydrothermal vent. These bacteria, one side, provide clues to biocycling of arsenic in oceanic environments; the other side, of potential in bioremediation of arsenic pollution.

Materials and methods

Samples

Sediments from the Southwest Indian Ocean were collected during the cruise of DY105-17A of R/V "Da-Yang Yi Hao" in December 2005 at the site of IR-TVG2 (37.6866°S, 50.4873°E) with a depth of 1,420 m, and another site IR-TVG3 (31.0877°S, 59.1124°E) with a depth of 4,019 m.

Medium

PTA medium used for enrichment and bacterial isolate contained the following: 0.5 g l^{-1} yeast extract, 1.0 g l^{-1} tryptone, 2 g l^{-1} NH_4Cl, 2 g l^{-1} sodium lactate, 0.2 g l^{-1} sucrose and 0.2 g l^{-1} glucose. The medium was prepared with seawater, and the pH of the medium was adjusted to 7.4. 1.5% agar was added to make a solid medium. PTA medium was sterilized by autoclaving for 30 min at 115°C.

SLB medium used for determination of arsenite resistance contained 5 g l^{-1} yeast extract and 10 g l^{-1} tryptone and was prepared with seawater. This medium was sterilized by autoclaving for 20 min at 121°C.

Enrichment with arsenite

About 2 g sediment from each site was suspended in 100 ml PTA medium containing 2 mM arsenite and incubated at 28°C for about 3 days. Further enrichment was done by transferring 2 ml enrichment culture into the same medium and incubated under the same conditions, and repeated for two times. The culture from the final transfer was used for bacterial isolation and community composition analysis.

Detection of the predominant arsenite resistant bacteria

To detect the As-tolerance bacteria in the communities enriched with 2 mM arsenite at different incubation times, a 2 ml culture enriched as above was used to inoculate fresh PTA medium containing 2 or 20 mM $NaAsO_2$. The culture was incubated at 28°C, and sampled for denaturing gradient gel electrophoresis (DGGE) analysis every 4 h for 3 days. The OD_{600} was read by a Smart Spec™ plus spectrophotometer to monitor cell growth as a reference for bacterial composition analyses.

To detect the As-tolerance bacteria in the communities challenged with different arsenite concentrations, a 2 ml culture of the last round enrichment with 2 mM arsenite was used as an inoculant. Cells were harvested by centrifugation and washed twice with sterile sea water to remove arsenite; then resuspended and transferred to a fresh PTA medium with an arsenite concentration varying from 0 to 100 mM (the pH of the medium was adjusted to 7.4 with 2 M HCl) and incubated at 28°C for 5 days. The serial cultures were used for DGGE analyses. All tests except DGGE analyses were repeated in triplicate.

Bacterial isolation and determination of arsenite resistance

The enriched cultures of the two sites were diluted with sterile sea water and spread on PTA plates containing 2 mM $NaAsO_2$. Plates were incubated at 28°C for about 2 weeks. Colonies were streaked again for further purification, and the 16S rRNA sequence of about 800–1,500 bp (Weisburg et al. 1991) was analyzed using Blastn at http://www.ncbi.nlm.nih.gov/BLAST/, maintained by National Center of Biotechnology Information.

To evaluate the resistance of each isolate, all the isolates were inoculated into SLB medium containing 10 mM $NaAsO_2$ and incubated at 28°C for 3 days. Resistance was indicated by cell growth.

DNA preparation

Genomic DNA of all consortia and isolates was extracted by the modified method of SDS-CTAB, as previously described by Wilson (1999).

16S rRNA PCR for DGGE analysis

PCR amplification of the V3 variable region of the 16S rRNA fragment was performed prior to DGGE, as described by Muyzer et al. (1993). The sequences of the primers are as follows: DGGEr, 5′-ATTACCGCGGCTGCTGG-3′, and DGGEf, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′.

All PCRs were performed with a Mastercycler (Eppendorf, Hamburg, Germany). The PCR reaction mixture contained in a volume of 50 µl, 1.0 U of rTaq (TaKaRa), each primer at a concentration of 0.5 µM and 50 ng DNA template. After 6 min of an initial denaturation at 95°C, a touchdown thermal profile protocol was used, and the annealing temperature was decreased by 0.5°C per cycle from 65 to 55°C; then 20 additional cycles at 55°C were performed. Amplification was carried out with 1 min of denaturation at 95°C, 1 min of primer annealing and 1.0 min of primer extension at 72°C, followed by a final extension at 72°C for 10 min. PCRs were carried out fourfold using the consortium DNA, and then the mixtures were combined prior to DGGE analysis. PCR amplifications were checked on 1% (w/v) agarose gel before DGGE analysis.

Denaturing gradient gel electrophoresis

The purified PCR product was loaded onto an 8% (w/v) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gel with a gradient of 30 to 70% denaturant (100% denaturant contained 7 M urea and 40% (vv) formamide). The gels were electrophoresed using a D-Code instrument (Bio-Rad) in 1 × TAE buffer (40 mM Tris–acetate, 1 mM Na-EDTA; pH 8.0) at 30 V for 15 min and then 130 V for 4.5 h at 60°C.

After electrophoresis, the gel was stained with ethidium bromide (10 µg ml^{-1}) for 15 min, and images were captured with an Alpha-Imager Imaging System with AlphaEase FC image software 4.1.0 (Alpha Innotech).

Each band visible on the DGGE gels of the arsenite-resistant consortia was excised manually, and the DNA in the gel was extracted using the method described by Muyzer et al. (1993). With 5 µl DNA extracted as a template, PCR was carried out to generate more target DNA for cloning. PCR products in the reaction buffer were purified using the E.Z.N.A Cycle-Pure Kit (OMEGA Biotek, USA) and cloned into the pMD19-T Vector (TaKaRa). After confirmation with another DGGE gel, they were sequenced with M13+ sequencing primer by 3730 DNA Analyzer, ABI.

The 16S rRNA library analysis

The 16S rRNA clone library was constructed from enriched culture with 2 mM $NaAsO_2$ incubated for 3 days. Total DNA prepared in DGGE analysis was used as template for PCR, and two universal primers, 27F and 1492R (Delong 1992), were used to amplify the approximately 1.5 kb 16S rRNA genes. PCR product was purified and cloned into *Escherichia coli* DH5α. About 100 clones were randomly selected in each library and digested with restricted enzyme *Hae*III at 37°C overnight. The digested products were separated by electrophoresis on a 2.5% agarose gel. Unique clones identified from the RFLP analysis were selected and sequenced. Clones with the same sequence were grouped into the same operational taxonomic unit (OTU). Each OTU was counted and calculated for its percentage in the library.

Phylogenetic analysis

The determined sequences were manually aligned together with the reference 16S rRNA sequences available in the GenBank database and evolutionary distances were then computed with the DNAMAN software version 5.1. A rooted phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei 1987), after bootstrap analysis with 1,000 sample replications.

The neighbor-joining distance method based on p-distance was used to construct phylogenetic trees and the validity of the branches was ascertained with 1,000 bootstrap replicates.

PCR amplification of arsenite transporter genes

Two sets of degenerate primers targeting the *arsB* and *ACR3*(2), which were developed by Achour et al. (2007), were used to amplify arsenite transporter genes in the arsenite-resistant bacteria. The sequences of the primers are as follows: darsB1F/darsB1R (5′-GGTGTGGAACATCG TCTGGA AYGCNAC/5′-CAGGCCGTACACCACCAGR TACATNCC), and dacr5F/dacr4R (5′-TGATCTGGGT CATGATCTTCCCVATGMTGVT/5′-CGGCCACGGCC AGYTCRAARAARTT). The expected product size is about 750 bp. Two kinds of PCR products were purified, and directly sequenced without cloning with primers darsB1 (5′-GGTGTGGAACATCGTCTG) and ACR-1 (5′-TGATCTGGGTCATGATCT), respectively.

Nucleotide sequence accession numbers

The 16S rRNA sequences of the isolates have been deposited in the GenBank with accession no. from EU928727 to EU928780.

Results

Arsenite-resistant bacteria isolated from the sediments of Southwest Indian Ocean

Together, 103 isolates were selected according to colony morphology and further subjected to 16S rRNA analysis. Totally, 54 isolates of different sequence were obtained, among which, 25 isolates were obtained from IR-TVG2, and 29 isolates were obtained from IR-TVG3. The 54 isolates mainly belonged to Gamma-proteobacteria (25 isolates), followed by the Actinobacteria (14 isolates), Alpha-proteobacteria (11 isolates), and then the Cytophaga-Flavobacterium (CFB) group (4 isolates). A phylogenetic tree of the arsenite-resistant bacteria and the reference strains is shown in Fig. 1a, b, respectively.

Phylogenetically diverse bacteria were found within the group of Gamma-proteobacteria, the majority of which are *Halomonas*, *Pseudoalteromonas* and *Idiomarina*. In IR-TVG2, 15 isolates belonged to Gamma-proteobacteria, of which *Alcanivorax* and *Idiomarina* occupied the

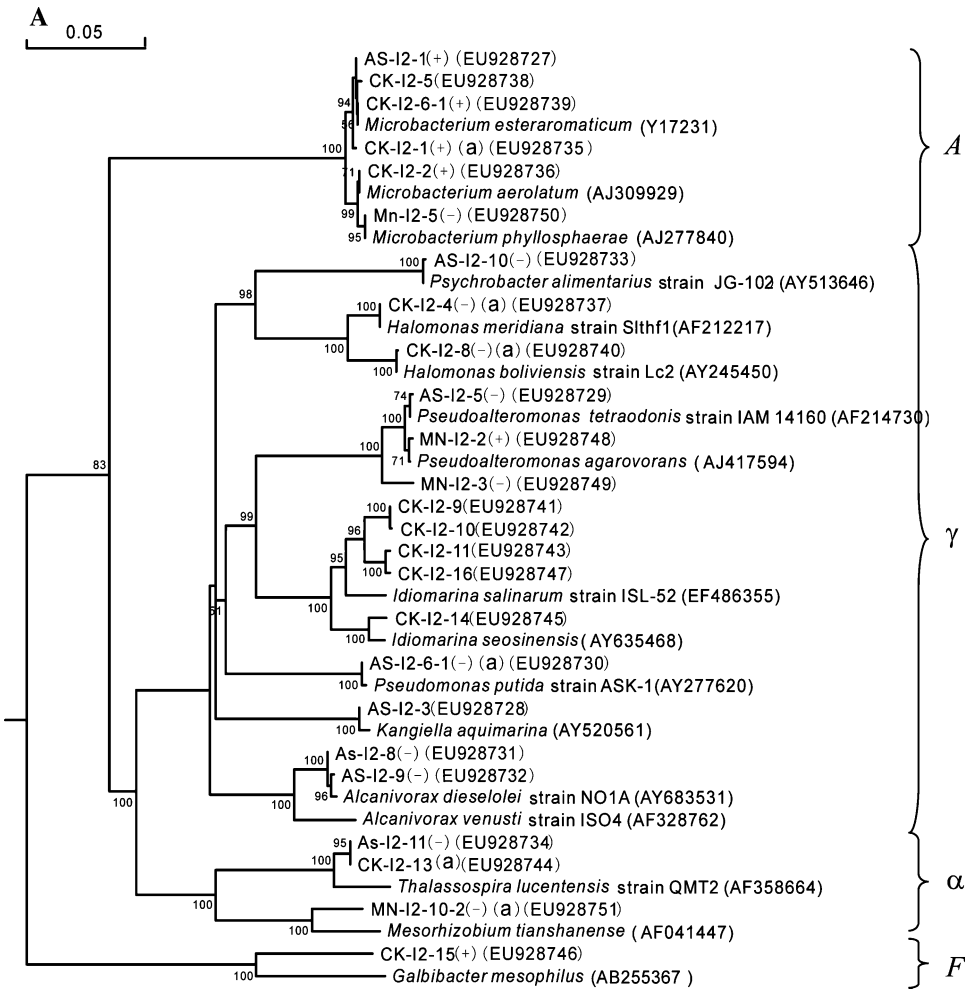
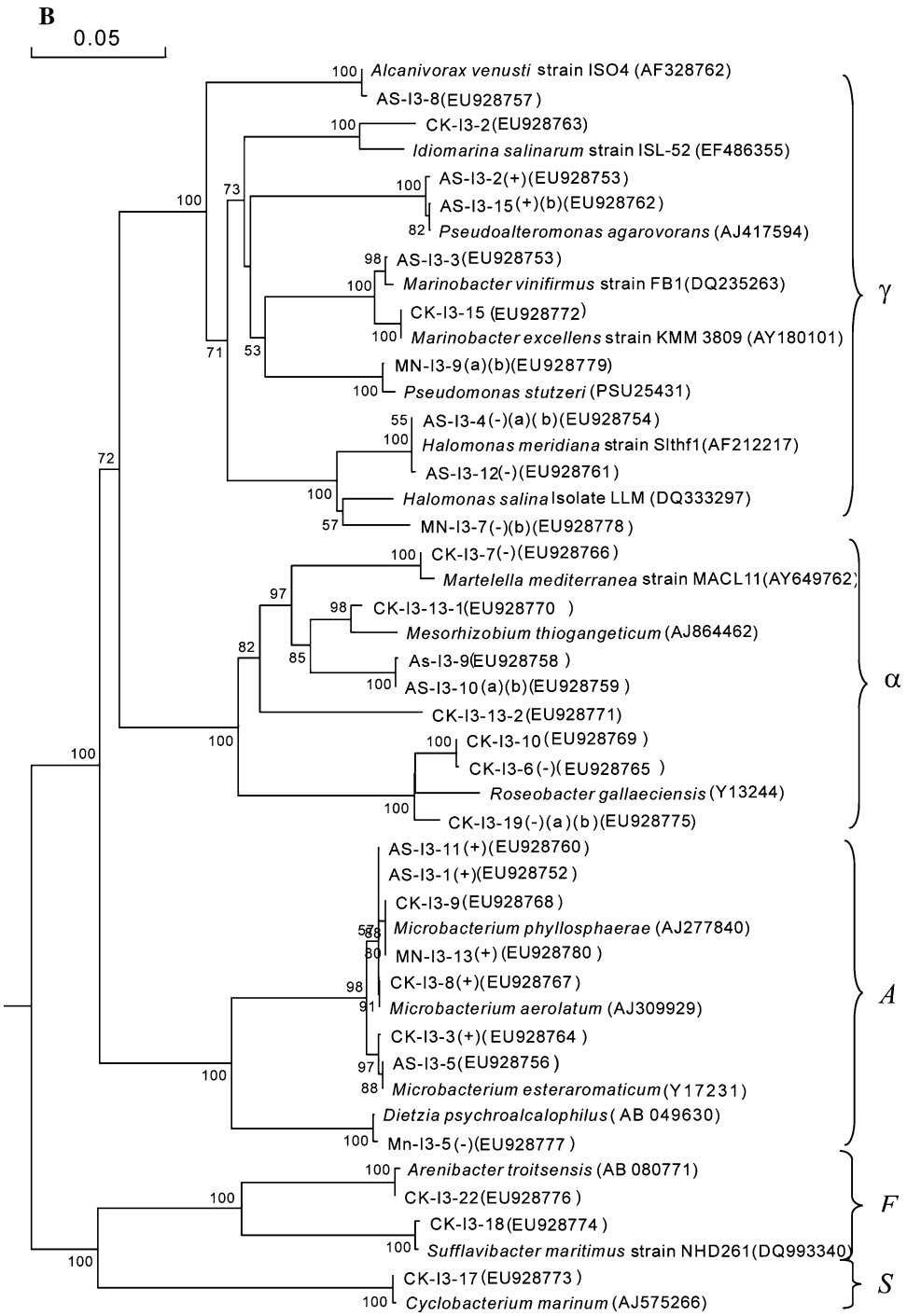


Fig. 1 Phylogenetic tree of partial 16S rRNA genes of the arsenite-resistant bacteria obtained from deep sediments of Southwest Indian Ocean. **a** Sampling site IR-TVG2; **b** sampling site IR-TVG3. The tree was constructed based on partial 16S rRNA sequences of the isolates and the reference strains. Bootstrap values (expressed as percentages of 1,000 replications) are shown at branch points. Bootstrap values over 50% were shown. Symbols: *plus*/*minus*, isolate can or cannot grow normally in SLB with 10 mM arsenite; **a** gene *ACR3*(1) was cloned; **b** gene arsB was cloned; *A* Actinobacteria, *α and γ* subgroups of Proteobacteria, *F* Flavobacterium, *S* Spirosomaceae

Fig. 1 continued



majority. Resistance tests showed that only two strains (Mn-I2-2, *Pseudoalteromonas tetraodonis*; AS-I2-6-1, *Pseudomonas putida*) could grow normally in the presence of 10 mM arsenite. While in the case of IR-TVG3, ten isolates belonged to Gamma-proteobacteria. *Idiomarina* was not as dominant as in IR-TVG2. Additionally, two strains of *Marinobacter* were only isolated in this site. Resistance tests showed both isolates of *Pseudoalteromonas* in IR-TVG3 could grow normally in the presence of 10 mM arsenite, while the others of this subgroup could only grow in 2 mM arsenite.

Although the high GC, Gram-positive group (Actinobacteria) is not as diverse as the Gamma-proteobacteria, which was mainly constituted of the species of *Microbacterium*. Plate screening revealed that *M. esteraromaticum* occupied the majority of culturable isolates in both IR-TVG2 and IR-TVG3. Moreover, most *Microbacterium* isolates could grow normally in presence of 10 mM arsenite.

Among the bacteria in the Alpha-proteobacteria and CFB group, most bacteria showed a low resistance, but there was a novel strain ck-I2-15 isolated from the site IR-TVG2 that could grow normally in 10 mM arsenite. Its closest phylogenetic relative is *Gillisia mitskevichiae*, sharing 92% sequence similarity.

PCR detected arsenite transporter genes

Six isolates of IR-TVG2 contained a gene related to *ACR3*; two isolates of IR-TVG3 contained a gene related to *arsB*; four isolates contained both related genes. The detected genes are most closely related to putative arsenical-resistance proteins with varied similarities. The positive detected isolates were indicated in Fig. 1a, b. Interestingly, the positively detected bacteria are not exclusively the isolates of high resistance. For an instance, isolates As-I2-1 and As-I3-5, which can tolerate 65 and 80 mM arsenite respectively, failed to be detected of both genes, whereas isolates As-I3-4, which can only tolerate 2 mM arsenite harbored both genes. Therefore, other mechanisms are possibly involved for high resistance, and thus no clear relationship between metalloid resistance levels and *arsB/ACR3* genotype, as observed by Ford et al. (2005) and Achour et al. (2007).

Bacterial community structures revealed by PCR-DGGE and 16S rRNA clone library

To examine the predominant bacteria in the arsenite-resistant communities, PCR-DGGE was used. The consortium was challenged with 2 mM $NaAsO_2$, in the community after 3 days incubation of the third enrichment. In the case of IR-TVG2, two bands were observed (not shown): Band 1 was closely related to *Alcanivorax dieselolei* NO1A (99%), and Band 2 was closely related to *M. esteraromaticum* DSM 8609 (100%). In the case of IR-TVG3, only one major band was detected (not shown), which was identified as *M. esteraromaticum* (100%). Its 16S rRNA sequence was identical to the one in IR-TVG2.

To reconfirm the results of DGGE analyses, the bacterial composition was further analyzed by the 16S rRNA clone library method. In site IR-TVG2, there were two OTUs: *A. dieselolei* (99%) and *M. esteraromaticum* (100%), which occupied 67.5 and 32.5% of the library, respectively. In the case of IR-TVG3, the OTUs were the same, but occupied 1.6 and 98.4%, respectively.

Dynamics of cell growth and the community structure during incubation

The dynamics of the community structure at different incubation times was examined; sampling was done every 4 h for 3 days. In addition, 2 and 20 mM initial arsenite concentrations were used in parallel for both sites.

Growth curves at OD_{600} of the culture community are showed in Fig. 2. From the curve of the enrichment of IR-TVG2 in both 2 and 20 mM initial arsenite concentrations, logarithmic growth ended at about 24 h; the culture reached the maximum biomass at about 48 h. In the enrichment of IR-TVG3, growth phases were not synchronized between the two treatments at different arsenite concentrations. In 2 mM arsenite, the OD_{600} reached a peak at 44 h, while in 20 mM arsenite, it took 60 h to reach a peak that was higher than that of IR-TVG2. In addition, a longer "lag phase" occurred in the 20 mM treatments in both cases. These results indicate that the 20 mM concentration inhibited growth to some degree in both communities while IR-TVG3 showed a better resistance.

The dynamics of the community structure were monitored with DGGE. The results of IR-TVG2 and IR-TVG3 communities are shown in Fig. 3a, b, respectively. In the case of IR-TVG2, only one dominant band occurred from inoculation to 24 h at 2 mM arsenite (Fig. 3a, lane 20 and 24); this band was derived from the isolate *M. esteraromaticum* As-I2-1. Interestingly, another dominant member appeared in the community at 28 h and onwards and shared similar brightness with Band 1. Band 2 corresponded to the isolate *A. dieselolei* As-I2-8. In 20 mM $NaAsO_2$, only the band identical to the isolate *M. esteraromaticum* As-I2-1 was retained in the community of IR-TVG2; that of *A. dieselolei* was lost (not shown). In the case of IR-TVG3, only one band corresponding to the isolate *M. esteraromaticum* As-I3-5 was observed in presence of 2 mM $NaAsO_2$ (Fig. 3b).

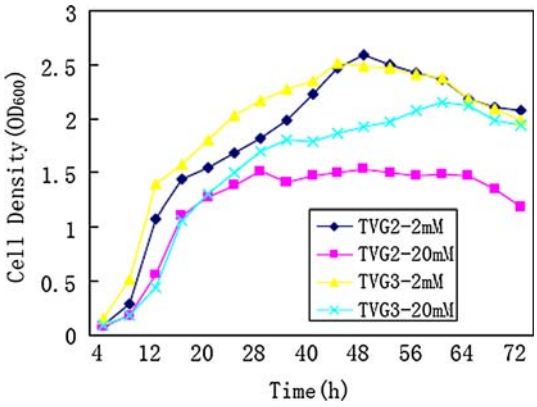


Fig. 2 Growth curve of community growth of two sites in 2 and 20 mM arsenite. Symbols: (*dark filled diamond*) community of IR-TVG2 with 2 mM arsenite; (*dark filled square*) community of IR-TVG2 with 20 mM arsenite; (*dark filled triangle*) community of IR-TVG3 with 2 mM arsenite; (*multiplication sign*) community of IR-TVG3 with 20 mM arsenite. In each treatment, three bottles were in triplicate and sampled in the time serials

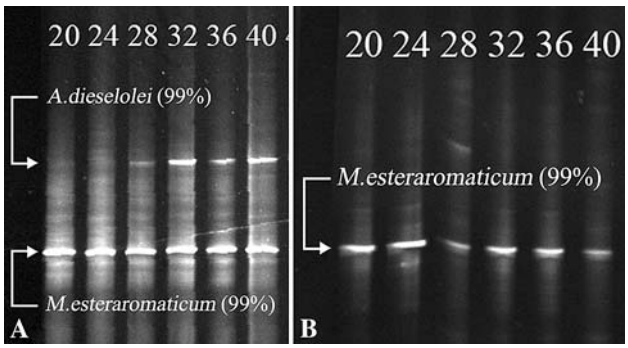


Fig. 3 The dynamic changes in DGGE patterns of bacterial community structure. **a** Sampling site IR-TVG2; **b** sampling site IR-TVG3. The community of IR-TVG2 and the community of IR-TVG3 were incubated in 2 mM arsenite. *Numbers* at the *top* of each *lane* refer to the sampling time (hours)

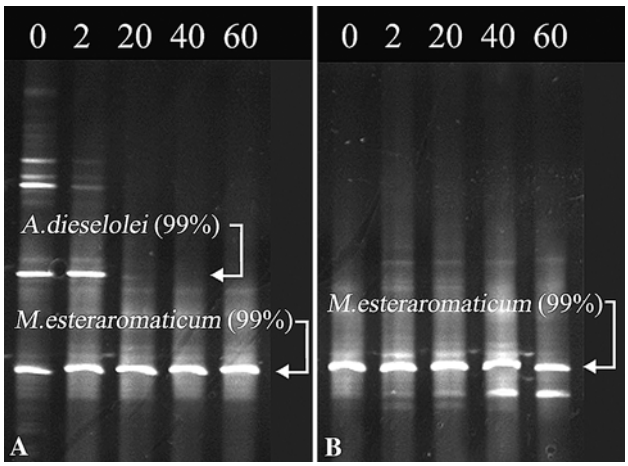


Fig. 4 Denaturing gradient gel electrophoresis profiles to show the dynamic changes of community structure in response to increased arsenite concentrations. **a** The community of IR-TVG2; **b** the community of IR-TVG3. In both **a** and **b**, the *lane numbers* are the concentration of $NaAsO_2$, from 0 to 60 mM. The communities were cultivated with varying arsenite concentrations for 5 days at 28°C

The dynamic changes in community structure in response to increasing arsenite concentration

As in the examination of the time-dependence, both PCR-DGGE and cell growth were used to evaluate the effect of arsenite concentration on the community structure. Serial dilutions of arsenite were made from 0 to 100 mM. IR-TVG2 could grow in arsenite up to 80 mM, while IR-TVG3 could grow but weakly in 100 mM arsenite. At 60 mM arsenite, the community of IR-TVG2 and IR-TVG3 reached 0.32 and 0.75 of OD_{600}, respectively. DGGE analyses were done with treatments ranging from 2 to 60 mM arsenite after incubation at 28°C, with a control containing no arsenite (Fig. 4).

In the case of the IR-TVG2 community, five bands occurred in the arsenite-absent control, including the above detected two bands; three of which became weak when arsenite was raised to 2 mM; two of which remained as major components, as those in Fig. 3a. The band representing *A. dieselolei* remained bright when arsenite concentration was set to 5 mM, but lost when arsenite concentration was raised to 10 mM (date not shown). When the arsenite concentration was raised higher than 10 mM, only one key band remained, it was corresponding to *M. esteraromaticum*.

IR-TVG3 community showed different results with IR-TVG2. No significant change was observed between the arsenite-absent control and 2 mM arsenite. In both treatments, *M. esteraromaticum* was found as the main band. Interestingly, however, with the increase of arsenite concentration, another band appeared in IR-TVG3 community, and turned out to be one of the two dominant bands above 20mM. Unfortunately, we failed to identify this band.

Therefore, *M. esteraromaticum* was the most important bacterium in both sites. It was the dominant member in the communities of different treatments and can resist high arsenite concentrations. Although two isolates of *M. esteraromaticum* in the two sites shared identical 16S rDNA sequence, they differed in arsenite resistance. The isolate As-I2-1 in IR-TVG2 can grow in presence of 65 mM arsenite, while As-I3-5 in TVG3 can resist arsenite 80 mM. Moreover, their Rep-PCR (Repetitive sequence-based PCR) patterns are not identical (not shown).

Discussion

In previous studies, we isolated bacteria and fungi from deep sea sediments of the Pacific Ocean with high resistance to manganese and lead, either by oxidation, intracellular accumulation or extracellular absorption (Tian and Shao 2006; Shao and Sun 2007; Sun and Shao 2007). Deep sea sediment probably is an ideal place to screen high metal resistance microbes. It is recognized that the arsenite-resistant bacteria are much less than those resisting arsenate in natural environment, because arsenite is more toxic than arsenate (Jackson et al. 2005). For example, *Pseudomonas stutzeri* sp. strain As-1, isolated from an electroplating industrial effluent, can tolerate up to 50 mM arsenate, but only 0.2 mM arsenite (Patel et al. 2007; Joshi et al. 2008). On the whole, however, microorganisms have developed various mechanisms of arsenic metabolism. Arsenite can be detoxified by efflux of cell, chaperone binding in cytoplasm and methylization (Bhattacharjee and Rosen 2007).

In this report, we present the first evidence of arsenite-resistant bacteria inhabiting in deep sea sediments of the

Southwest Indian Ridge. As a result, 54 bacterial strains of arsenite resistance were isolated from enriched cultures with 2 mM $NaAsO_2$. They were phylogenetically diverse, and belonged to five major bacterial clades, including Alpha-, Gamma-proteobacteria, Actinobacteria, Flavobacteria and Spingobacteria. Here, two groups should be intonated. One is Gamma-proteobacteria, which accounted for the majority of all the isolates, and of a quite broad biodiversity. Another is Actinobacteria, which was relatively less diverse but confirmed of high resistance, represented by species of *Microbacterium*.

Most Gamma-proteobacteria in this report were found of As-resistance for the first time as far as we know, such as *Halomonas*, *Pseudoalteromonas* and *Idiomarina*, which formed the largest clade in Fig. 1a, b. In our previous study, *Pseudoalteromonas tetraodonis* (100%) was proved to be the predominant bacterial species of arsenite resistance from another site on Southwest Indian Ocean Ridge at 49.6469°(E), 37.7719°(N) (Chen and Shao 2008). Although bacteria of *Pseudoalteromonas* are frequently isolated from deep sea samples as being resistant to many heavy metals like Hg, Se and Te (Iohara et al. 2004; Rathgeber et al. 2002), this is the first report to our knowledge of their resistance to arsenic.

Microbacterium bacteria have been isolated from various environments, including sludge, air and deep sea sediment, and can resist many metals, like Cr, U and Au (Humphries et al. 2005; Nedelkova et al. 2007; Inomata et al. 2007). *Microbacterium* was also detected in arsenic-resistant bacterial populations of an unsaturated soil (Macur et al. 2004), and can oxidize arsenite (Mokashi and Paknikar 2002). In this report, most isolates of *Microbacterium* could grow normally in presence of 10 mM arsenite. This tolerance is somewhat consistent with a previously reported result that an isolate of *Microbacterium* from forest soils can grow in the presence of 28 mM arsenite (Achour et al. 2007). Our results, together with these previous reports, indicate that *Microbacterium* bacteria are important arsenic-resistant bacteria in various environments. They may play a role in the geobiocycling of the arsenic element and have potential in bioremediation of arsenic-contaminated environments.

Bacteria of genus *Alcanivorax* are usually found as ubiquitous alkane degrading bacteria in marine environments (Head et al. 2006). Isolates from a hydrothermal vent field of East Pacific were also previously reported of resistance to mercury (Vetriani et al. 2005). In this study, *A. dieselolei* was found in the arsenite-resistant community of IR-TVG2; it grew slower than *M. esteraromaticum* (Fig. 3), and disappeared from the community when arsenite concentration was raised to 20 mM (Fig. 4a).

From the genome sequence annotation results of *Alcanivorax borkumensis* SK2 (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntab01), several arsenic resistance genes can be retrieved, encoding enzyme or proteins such as arsenite methyltransferase, arsenical resistance proteins (ArsH and ArsB), and the arsenical pump membrane protein ACR3. In our isolates of this genus, the gene *ArsB* and *ACR3* failed to be detected, while some other isolates were positively detected. Whether other resistance mechanisms exist in our *Alcanivorax* isolates would be an issue for further investigation.

Intracellular accumulation of arsenic was recently reported in *Pseudomonas stutzeri* sp. strain As-1 when exposed to arsenate (Patel et al. 2007; Joshi et al. 2008). Accumulation in cell has been observed in both prokaryotic and eukaryotic organisms. Even engineered bacteria of high accumulation can be developed to remove the metalloid (Singh et al. 2008). However, in our tested isolates (As-I2-1, As-I3-1, As-I3-5), there was no accumulation observed under transmission electron microscopes equipped with EDX (energy dispersive X-ray system) (not shown).

In summary, quite diverse bacteria with high arsenite resistance were discovered in deep sea sediments of the Indian Ocean, among which *M. esteraromaticum* showed the highest resistance. These bacteria probably participate in the arsenic cycling in marine system by mechanisms like arsenite methylization, bioaccumulation or passive absorption, arsenite oxidation, arsenate reduction and efflux. In addition, the effects of algae and other marine organisms, either in the aerobic upper layer of water column or the anaerobic subsurface, are incorporated into the big cycling of the element in marine environments.

Acknowledgments This work was financially supported by COMRA program (No. DYXM115-02-2-05) and National Infrastructure of Natural Resources for Science and Technology Program of China (No. 2005DKA21209), and National Basic Research Program of China (No.2004CB719601).

References

Achour AR, Bauda P, Billard P (2007) Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. Res Microbiol 158(2):128–137

Baker BJ, Banfield JF (2003) Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152

Bhattacharjee H, Rosen BP (2007) Arsenic metabolism in prokaryotic and eukaryotic microbes. In: Nies DH, Silver S (eds) Molecular microbiology of heavy metals. Springer, Berlin, pp 372–406

Casiot C, Morin G, Juillot F, Bruneel O, Personné JC, Leblanc M, Duquesne K, Bonnefoy V, Elbaz-Poulichet F (2003) Bacterial immobilization and oxidation of arsenic in acid mine drainage (Carnoulès creek, France). Water Res 37(12):2929–2936

Casiot C, Bruneel O, Personné JC, Leblanc M, Elbaz-Poulichet F (2004) Arsenic oxidation and bioaccumulation by the acidophilic protozoan, *Euglena mutabilis*, in acid mine drainage (Carnoulès, France). Sci Total Environ 320(2/3):259–267

Chen SX, Shao ZZ (2008) The diversity of arsenite-resistant bacteria in the enrichment of deep sea sediment of the Southwest Indian Ocean Ridge. Acta Microbiol Sin 48(10): (in press; in Chinese)

Delong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689

Dembitsky VM, Levitsky DO (2004) Arsenolipids. Prog Lipid Res 43:403–448

Duquesne K, Lieutaud A, Ratouchniak T, Muller D, Lett M-C, Bonnefoy V (2008) Arsenite oxidation by a chemoautotrophic moderately acidophilic *Thiomonas* sp.: from the strain isolation to the gene study. Environ Microbiol 10(1):228–237

Ford T, Jay J, Patel A, Kile M, Prommasith P, Galloway T, Sanger R, Smith K, Depledge M (2005) Use of ecotoxicological tools to evaluate the health of New Bedford Harbor sediments: a microbial biomarker approach. Environ Health Perspect 113:186–191

Gihring TM, Druschel GK, McCleskey RB, Hamers RJ, Banfield JF (2001) Rapid arsenite oxidation by *Thermus aquaticus* and *Thermus thermophilus*: field and laboratory investigations. Environ Sci Technol 35(19):3857–3862

Green HH (1918) Description of a bacterium that oxidizes arsenite to arsenate, and one which reduces arsenate to arsenite, isolated from a cattle-dipping tank. S Afr J Sci 14:465–467

Hanaoka K, Yamamoto H, Kawashima K, Tagawa S, Kaise T (2004) Ubiquity of arsenobetaine in marine animals and degradation of arsenobetaine by sedimentary micro-organisms. Appl Organomet Chem 2(4):371–376

Head IM, Jones DM, Röling WFM (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4:173–182

Hetzer A, Morgan HW, McDonald IR, Daughney CJ (2007) Microbial life in Champagne Pool, a geothermal spring in Waiotapu, New Zealand. Extremophiles 11(4):605–614

Hoeft SE, Kulp TR, Stolz JF, Hollibaugh JT, Oremland RS (2004) Dissimilatory arsenate reduction with sulfide as electron donor: experiments with mono lake water and isolation of strain MLMS-1, a chemoautotrophic arsenate respirer. Appl Environ Microbiol 70(5):2741–2747

Hoeft SE, Blum JS, Stolz JF, Tabita FR, Witte B, King GM, Santini JM, Oremland RS (2007) *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. Int J Syst Evol Microbiol 57:504–512

Humphries AC, Nott KP, Hall LD, Macaskie LE (2005) Reduction of Cr (VI) by immobilized cells of *Desulfovibrio vulgaris* NCIMB 8303 and *Microbacterium* sp. NCIMB 13776. Biotechnol Bioeng 90(5):589–596

Inomata T, Eguchi H, Matsumoto K, Funahashi Y, Ozawa T, Masuda H (2007) Adsorption of microorganisms onto an artificial siderophore-modified Au substrate. Biosens Bioelectron 23(5):751–755

Iohara K, Iiyama R, Nakamura K, Silver S, Sakai M, Takeshita M, Furukawa K (2004) The Mer operon of a mercury-resistant *Pseudoalteromonas haloplanktis* strain isolated from Minamata Bay, Japan. Appl Microbiol Biotechnol 56:736–741

Jackson CR, Dugas SL, Harrison KG (2005) Enumeration and characterization of arsenate-resistant bacteria in arsenic free soils. Soil Biol Biochem 37(12):2319–2322

Joshi DN, Patel JS, Flora SJS, Kalia K (2008) Arsenic accumulation by *Pseudomonas stutzeri* and its response to some thiol chelators. Environ Health Prev Med. doi:10.1007/s12199-008-0038-9

Kulp TR, Hoeft SE, Miller LG, Saltikov C, Murphy JN, Han S, Lanoil B, Oremland RS (2006) Dissimilatory arsenate and sulfate reduction in sediments of two hypersaline, arsenic-rich soda lakes: Mono and Searles Lakes, California. Appl Environ Microbiol 72(10):6514–6526

Langner HW, Jackson CR, McDermott TR, Inskeep WP (2001) Rapid oxidation of arsenite in a hot spring ecosystem, Yellowstone National Park. Environ Sci Technol 35(16):3302–3309

Leblanc M, Achard B, Othman DB, Luck JM, Bertrand-Sarfati J, Personne JC (1996) Accumulation of arsenic from acidic mine waters by ferruginous bacterial accretions (stromatolites). Appl Geochem 11(4):541–554

Lebrun E, Brugna M, Baymann F, Muller D, Lievremont D, Lett MC, Nitschke W (2003) Arsenite oxidase, an ancient bioenergetic enzyme. Mol Biol Evol 20(5):686–693

Lunde G (1977) Occurrence and transformation of arsenic in the marine environment. Environ Health Perspect 19:47–52

Macur RE, Jackson CR, Botero LM, McDermott TR, Inskeep WP (2004) Bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil. Environ Sci Technol 38(1):104–111

Mokashi SA, Paknikar KM (2002) Arsenic (III) oxidizing *Microbacterium lacticum* and its use in the treatment of arsenic contaminated groundwater. Lett Appl Microbiol 34(4):258–262

Morin G, Juillot F, Casiot C, Bruneel O, Personné JC, Elbaz-Poulichet F, Leblanc M, Ildefonse P, Calas G (2003) Bacterial formation of tooeleite and mixed arsenic(III) or arsenic(V)-iron(III) gels in the Carnoulès acid mine drainage, France. A XANES, XRD, and SEM study. Environ Sci Technol 37(9):1705–1712

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Neff JM (1997) Ecotoxicology of arsenic in the marine environment—review. Environ Toxicol Chem 16:917–927

Nedelkova M, Merroun ML, Rossberg A, Hennig C, Selenska-Pobell S (2007) Microbacterium isolates from the vicinity of a radioactive waste depository and their interactions with uranium. FEMS Microbiol Ecol 59(3):694–705

Oremland RS, Stolz JF (2003) The ecology of arsenic. Science 300:939–944

Oremland RS, Stolz JF, Hollibaugh JT (2004) The microbial arsenic cycle in Mono Lake, California. FEMS Microbiol Ecol 48:15–27

Oremland RS, Kulp TR, Blum JS, Hoeft SE, Basman S, Miller LG, Stolz JF (2005) A microbial arsenic cycle in a salt-saturated, extreme environment. Science 308:1305–1308

Osborne FH, Ehrlich HL (1976) Oxidation of arsenite by a soil isolate of *Alcaligenes*. J Appl Microbiol 41:295–305

Patel PC, Goulhen F, Botthman C, Gault AG, Charnock JM, Kalia K, Lloyd JR (2007) Arsenate detoxification in a *Pseudomonas* hypertolerant to arsenic. Arch Microbiol 187:171–183

Pepi M, Volterrani M, Renzi M, Marvasi M, Gasperini S, Franchi E, Focardi SE (2007) Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization. J Appl Microbiol 103(6):2299–2308

Prithivirajsingh S, Mishra SK, Mahadevan A (2001) Detection and analysis of chromosomal arsenic resistance in *Pseudomonas fluorescens* strain MSP3. Biochem Biophys Res Commun 280(5):1394–1401

Quastel JH, Scholefield PG (1953) Arsenite oxidation in soil. Soil Sci 75:279–285

Rathgeber C, Yurkova N, Stackebrandt E, Beatty JT, Yurkov V (2002) Isolation of tellurite- and selenite-resistant bacteria from hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean. Appl Environ Microbiol 68(9):4613–4622

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Salmassi TM, Walker JJ, Newman DK, Leadbetter JR, Pace NR, Hering JG (2006) Community and cultivation analysis of

arsenite oxidizing biofilms of Hot Creek. Environ Microbiol 8(1):50–59

Santini JM, Sly LI, Schnagl RD, Macy1 JM (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. Appl Environ Microbiol 66(1):92–97

Scudlark JR, Johnson DL (1982) Biological oxidation of arsenite in seawater. Estuar Coast Shelf Sci 14:693–706

Shao Z, Sun F (2007) Intracellular sequestration of manganese and phosphorus in a metal-resistant fungus *Cladosporium cladosporioides* from deep-sea sediment. Extremophiles 11(3):435–443

Silver S, Phung LT (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic Arsenic. Appl Environ Microbiol 71(2):599–608

Singh S, Mulchandani A, Chen W (2008) Highly selective and rapid arsenic removal by metabolically engineered *Escherichia coli* cells expressing *Fucus vesiculosus* metallothionein. Appl Environ Microbiol 74:2924–2927

Stolz JF, Basu P, Santini JM, Oremland RS (2006) Arsenic and selenium in microbial metabolism. Annu Rev Microbiol 60:107–130

Sun FQ, Shao ZZ (2007) Biosorption and bioaccumulation of lead by *Penicillium* sp. psf-2 isolated from the deep sea sediment of the Pacific Ocean. Extremophiles 11(6):853–858

Takai K, Kobayashi H, Nealson KH, Horikoshi K (2003) *Deferribacter desulfuricans* sp. nov., anovel sulfur-, nitrate- and arsenate-reducing thermophile isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol 53:839–846

Takeuchi M, Kawahata H, Gupta LP, Kita N, Morishita Y, Ono Y, Komai T (2007) Arsenic resistance and removal by marine and non-marine bacteria. J Biotechnol 127(3):434–442

Tian MJ, Shao ZZ (2006) Isolation and identification of manganese resistant bacteria from deep sea sediments. J Xiamen Univ (Nat Sci) 45(Suppl):272–276 (in Chinese)

Turner AW (1949) Bacterial oxidation of arsenite. Nature 164:76–77

Turner AW (1954) Bacterial oxidation of arsenite. I. Description of bacteria isolated from arsenical cattledipping fluids. Aust J Biol Sci 7:452–476

Vetriani C, Chew YS, Miller SM, Yagi J, Coombs J, Lutz RA, Barkay T (2005) Mercury adaptation among bacteria from a deep-sea hydrothermal vent. Appl Environ Microbiol 71(1):220–226

Vidal FV, Vidal VMV (1980) Arsenic metabolism in marine bacteria and yeast. Mar Biol 60:1–7

Wakao N, Koyatsu H, Komai Y, Shimokawara H, Sakurai Y, Shiota H (1988) Microbial oxidation of arsenite and occurrence of arsenite-oxidizing bacteria in acid mine water from a sulfur-pyrite mine. Geomicrobiol J 6:11–24

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Wilson K (1999) The extraction of genome DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Short protocols in molecular biology. Science Press, Beijing, pp 39–40