World J Microbiol Biotechnol (2008) 24:1607–1611
DOI 10.1007/s11274-007-9624-z

SHORT COMMUNICATION

Isolation and characterization of selenite- and selenate-tolerant microorganisms from selenium-contaminated sites

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Received: 15 August 2007 / Accepted: 19 November 2007 / Published online: 28 November 2007

Abstract Eight bacterial strains designated ARI 1-8 were isolated from soil and water samples collected from selenium-contaminated sites in India using the enrichment culture technique. They exhibited very high MIC values ranging from 300 to 750 mM for different forms of selenium (selenite and selenate). On the basis of various biochemical tests, fatty acid methyl ester profile and 16S rRNA gene sequencing, these isolates were identified belonging to the classes β-Proteobacteria and Bacilli. These microorganisms could be further used for bioremediation of contaminated sites.

Keywords Bacterial strains · Fatty acid profile · MICs · Phylogenetic analysis · Selenate · Selenite · Tolerance

Introduction

Human activities have been contaminating the environment with toxic heavy metals and metalloids over the past 200 years and, consequently, have resulted in severe disturbance of ecological balance in most ecosystems (Kozdroj and van Elsas 2001). Selenium (Se), a metalloid, occupies a unique position as regard to continuing conflicting aspects of its toxicological and nutritional significance. Agricultural drainage, industrial effluents and sewage sludges are major sources of various toxic forms (selenate, Se^{+6}; selenite, Se^{+4}, selenide, Se^{-2}) of selenium (Stadtman 1990; Stolz et al. 2006). However, quite a few microorganisms are reported to tolerate or reduce these toxic selenium species into non-toxic forms and, thus, bioremediation has been considered as an effective means of cleaning up of selenium-contaminated water and sediments (Garbisu et al. 1995; Haq et al. 1999; Frankenberger and Arshad 2001; Klonowska et al. 2005).

Since the contaminated sites are potential sources of competent microorganisms, it is of substantial significance to explore the naturally adapted microbial population present in such habitats. In this regard, culture-dependent isolation methods have been proved to be successful (Haq et al. 1999; Ellis et al. 2003). Further, phylogenetic and phenotypic characterization of the isolates would identify environmentally relevant microorganisms and this taxonomic information, in turn, would help in understanding the genetic systems that regulate diverse metabolic enzymes involved in metal tolerance and/or reduction (de Souza et al. 2001; Kozdroj and van Elsas 2001). Only a few attempts have been made to metabolically engineer some of the members of genera *Geobacter* and *Deinococcus* for in situ bioremediation of heavy metal-contaminated environment (Wang et al. 2000; Brim et al. 2006). In view of the above, the main objective of the present study was the isolation and polyphasic taxonomic characterization of microorganisms that were tolerant to higher concentrations of selenium. Furthermore, phylogenetic affiliation of these isolates with other known selenium-tolerant and/or -reducing microorganisms was determined, to evaluate their novelty in terms of bioremediation.

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Materials and methods

Sample collection, elemental analysis and isolation of metalloid-tolerant bacterial strains

Selenium-contaminated soil and water samples were collected from sites around the industrial area of Nawanshahr in Punjab, a polluted creek of Thane in Maharashtra and Bhavanpadu dam in Andhra Pradesh. These samples were subjected to elemental analysis using flame atomic absorption spectrophotometry (Unicam 929, UK). The pH values of soil slurry (10% w/v in distilled water) and water samples were measured using a digital pH meter. Bacterial strains were isolated using enrichment culture technique on enrichment and growth medium [EG medium; composition (l^{-1}): 0.03 g NH_4Cl, 0.01 g NaCl, 0.01 g $MgSO_4$, 0.15 g yeast extract and 0.5 g peptone, pH 7.5 (Mokashi and Paknikar 2002)]. Filter sterile 0.2 mM sodium salts of selenite ($\sim$34 mg Na_2SeO_3 l^{-1}) or selenate ($\sim$16 mg Na_2SeO_4 l^{-1}) were added to the EG medium after autoclaving.

Determination of minimum inhibitory concentration (MIC)

In order to determine MICs, the strains were grown in EG medium supplemented with sodium selenite or sodium selenate at increasing concentrations. The concentrations used were in increments of 10 mM over the range of 0–100 mM and, thereafter, in increments of 50 mM up to the final concentration of 1 M. The pH of the EG medium was adjusted to 7.5 throughout the experiments. Growth of the microorganisms was measured by c.f.u. counting after 48 h of incubation at 30°C.

Characterization of the bacterial isolates

Morphological characterization such as colony and cell morphology, Gram-reaction, motility etc. was performed as described by Ghosh et al. (2006). Physiological and biochemical tests were carried out according to the standard protocols described in *Bergey's Manual of Systematic Bacteriology* (Krieg et al. 1984). Fatty acid methyl esters (FAMEs) were obtained from cells grown on tryptic soy agar (TSA; HiMedia, India) for 24 h at 30°C followed by saponification, methylation and extraction as described by Pandey et al. (2002). The methyl ester mixtures were separated by gas chromatography and analysed by Sherlock Microbial Identification Systems software (MIDI, USA).

Phylogenetic analysis

Genomic DNA was isolated from the bacterial isolates using a genomic DNA isolation kit (Qiagen, GmbH, Germany). The concentration and purity of DNA was measured spectrophotometrically at 260 nm. PCR amplification, cloning of 16S rRNA gene amplicon and sequencing were performed as described previously (Ghosh et al. 2006). The sequences were determined following the dideoxy chain-termination method using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and analysed by ABI 310 Genetic Analyzer (Applied Biosystems, USA). Sequences of closely related taxa of the bacterial isolates and other known selenite/selenate-tolerant and/or -reducing microorganisms were retrieved from the GenBank database using BLASTN (Altschul et al. 1997). These sequences were aligned using the CLUSTAL X program (Thompson et al. 1997) and a neighbor-joining phylogenetic tree was constructed using TREECON program (Van de Peer and De Wachter 1994) as illustrated by Ghosh et al. (2006).

Results and discussion

The present study was aimed to characterize selenium-tolerant bacterial strains isolated from contaminated sites. Selenium concentrations of the collected samples were found to be 2.25–3.00 mg kg^{-1} soil and 0.08–1.10 mg l^{-1} water which are comparable with the selenium concentration (3.50 mg kg^{-1}) determined for samples from Kesterson reservoir (Burton et al. 1987). The pH values of all the soil samples were alkaline (8.0–9.2) and that of the water samples ranged from 6.0 to 7.4. A total of 38 morphologically different colonies were isolated from these samples and, among them, eight bacterial strains (designated ARI 1-8) were found to grow on selenite- or selenate-supplemented EG medium after enrichment.

MIC is the minimum concentration of a metal or metalloid in a given culture medium at optimum condition below which bacterial growth is not inhibited. It is interesting to note that all the strains ARI 1-8 could tolerate very high concentrations of different forms of selenium. The MIC values exhibited for selenate were 650–750 mM (52–60 g l^{-1}), and that for selenite ranged from 300 to 600 mM (51–102 g l^{-1}) (Table 1). The determined MIC values were in agreement with the fact that selenite is more toxic to microorganisms than selenate (Stadtman 1990). de Souza et al. (2001) reported similar MIC values for sodium selenite (>200 mM) and sodium selenate (>200 mM) in the case of bacterial strains isolated from selenium-contaminated hypersaline evaporation ponds. Although the

MICs determined with the traditional media approach could not be related to the actual metal concentrations in the habitat from which bacteria were isolated, this technique is still considered as a valid approach to evaluate the impact of heavy metals on microbial activity in polluted habitats. The toxicity of heavy metals and metalloids toward soil bacteria has been proposed as an indicator of potential toxicity of the elements to other forms of life (Stolz et al. 2006).

Since there is an increasing awareness regarding the exploitation of natural microbial population in the abatement of pollution, polyphasic characterization of the adapted bacterial strains would be of interest in terms of bioremediation of selenium-contaminated environment. Keeping this in mind, culture condition-dependent (based on phenotypic properties and FAMEs profile) and culture condition-independent (based on genetic characters) approaches were followed for their identification. Table 2 shows the presence of both Gram-negative and Gram-positive bacteria belonging to the genera *Pseudomonas*, *Bacillus* and *Enterobacter* based on the results obtained from phenotypic characterization. Furthermore, FAMEs analysis revealed that $C_{16:\ 0}$ was the major fatty acid present in strains ARI 1 (36%), ARI 2 (33%), ARI 4 (26%) and ARI 7 (34%), whereas ISO $C_{15:\ 0}$ was the predominant one in strains ARI 3 (56%), ARI 5 (28%), ARI 6 (28%) and ARI 8 (26%). The microorganisms were identified based on the comparison of total fatty acid profile of individual isolates with that reported in literature as well as considering the MIDI similarity indices obtained from TSA library match (Table 2).

Partial 16S rRNA gene-sequencing of strains ARI 1–8 was also performed for further confirmation. The sequences obtained were deposited to GenBank with accession numbers AY684781–AY684787 and DQ102370; BLAST homology search of partial 16S rRNA gene sequences indicated 98–99% sequence similarities with their closest matches (Table 2). The evolutionary distance dendrogram constructed based on the 16S rRNA gene sequences revealed that strains ARI 1–8 along with other selenite/selenate-reducing and/or -tolerant microorganisms belonged to the domain *Bacteria* and fell into seven lineages of this domain (Fig. 1). The most dominant group of isolates was placed in the beta subdivision of Proteobacteria which included *Achromobacter xylosoxidans* ARI 1, *Delftia tsuruhatensis* ARI 5 and *D. tsuruhatensis* ARI 7. An earlier report demonstrated that a betaproteobacterium *Thauera selenatis* could respire selenium anaerobically (Rech and Macy 1992); however, strains isolated in this study were strict aerobes. The second major phylogenetic group belonged to the class Bacilli and consisted of *Bacillus fusiformis* ARI 3, *Bacillus* sp. ARI 6 and *Bacillus sphaericus* ARI 8. Members of the genus *Bacillus* are in general well known for conferring resistance to various other metals and metalloids (Nies 1999; Stolz et al. 2006). In another study Garbisu et al. (1995) showed that a strain of *Bacillus subtilis* was able to tolerate higher concentration of selenate and convert selenite into elemental selenium. *Pseudomonas* sp. ARI 2 and *Enterobacter cowanii* ARI 4 were found to cluster with other members of the γ-Proteobacteria. Resistance of strains of *Enterobacter cloacae* toward cadmium, chromium and selenium is well documented and they are being considered as potential candidates for the treatment of contaminated agricultural drainage water (Losi and Frankenberger 1997; Haq et al. 1999). Among other members of the γ-Proteobacteria, a strain of *Pseudomonas stutzeri* has been shown to reduce both selenate and selenite (Lortie et al. 1992). Phylogenetic analysis in the present study revealed that most of the selenium-tolerant microorganisms were common soil bacteria belonging to the class of β-Proteobacteria and Bacilli. Importantly, the identified strains encompass most of the genera hitherto reported to be involved in selenium tolerance/reduction, however, the association of members of the genus *Delftia* with selenium tolerance has been shown for the first time.

Table 1 Determination of MICs of strains ARI 1–8 on selenite and selenate

Strain	MICs (mM)[a]	
	Selenite	Selenate
ARI 1	300	700
ARI 2	600	750
ARI 3	450	650
ARI 4	550	700
ARI 5	600	700
ARI 6	500	700
ARI 7	600	750
ARI 8	600	700

[a] Final concentration of the compound in EG medium

Conclusions

A total of eight different bacterial strains, ARI 1–8 isolated from the selenium-contaminated sites showed tolerance toward high concentrations of selenite and selenate. These microorganisms were characterized following polyphasic identification approach. Phylogenetic analysis revealed that most of the selenium-tolerant microorganisms were common soil bacteria belonging to the classes of β-Proteobacteria and Bacilli and thus may offer an insight into the tolerant bacterial population present in selenium-laden sites of India. Furthermore, in future studies, these

Table 2 Identification of the strains ARI 1-8 using polyphasic taxonomy

Strain	Phenotypic identification	FAMEs analysis (similarity index)	16S rRNA gene sequence (% identity)	Group	Strain match
ARI 1	*Achromobacter* sp.	0.815	99.7	*β-Proteobacteria*	*Achromobacter xylosoxidans*
ARI 2	*Pseudomonas* sp.	0.357	99.2	*γ-Proteobacteria*	*Pseudomonas* sp.
ARI 3	*Bacillus* sp.	0.632	99.8	*Bacilli*	*Bacillus fusiformis*
ARI 4	*Enterobacter* sp.	0.862	98.8	*γ-Proteobacteria*	*Enterobacter cowanii*
ARI 5	*Delftia tsuruhatensis*	0.775	99.7	*β-Proteobacteria*	*Delftia tsuruhatensis*
ARI 6	*Bacillus* sp.	0.143	99.3	*Bacilli*	*Bacillus* sp.
ARI 7	*Delftia tsuruhatensis*	0.903	99.9	*β-Proteobacteria*	*Delftia tsuruhatensis*
ARI 8	*Bacillus sphaericus*	0.687	99.8	*Bacilli*	*Bacillus sphaericus*

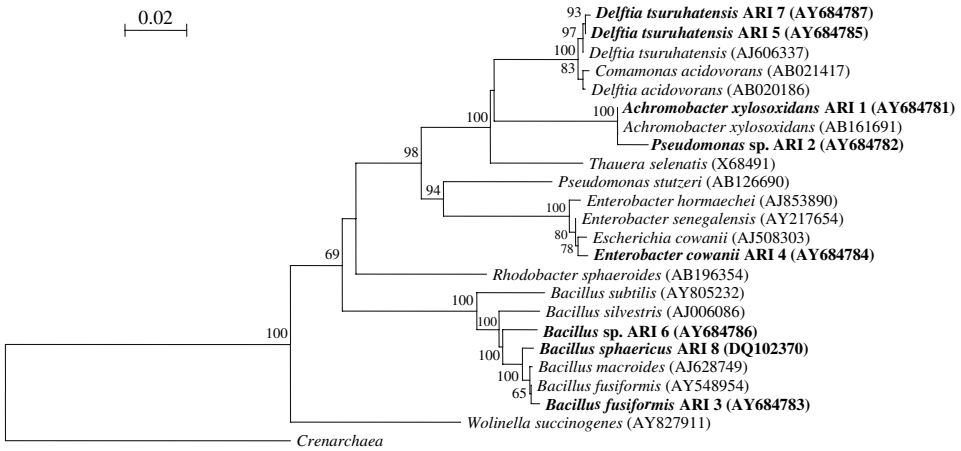

Fig. 1 16S rRNA gene sequence-based dendrogram showing the phylogenetic position of the selenium-tolerant bacterial strains ARI 1–8 and their relationship with other selenium-tolerant/resistant microorganisms. The tree was constructed using the neighbour-joining method (Saitou and Nei 1987) and rooted using *Crenarchaea* as outgroup. GenBank accession numbers are given in parentheses. Numbers at node indicate bootstrap values of >50% (for 1,000 iterations). Bar, 0.02 substitution per nucleotide

bacterial strains could be tested individually or in consortia for their capability to reduce selenite or selenate, so that they can be further genetically manipulated and exploited for in situ bioremediation of selenium-containing wastewaters and soil under Indian climatic conditions.

Acknowledgements We express gratitude to Mr. Mayilraj for his help in carrying out the FAMEs analysis. We thank Mr. Dhan Prakash and Ms. Meenu Khurana for their excellent technical assistance. We are also thankful to Mr. Janmejay Pandey and Mr. Narinder K. Sharma for reading the manuscript. This work was supported, in parts, by CSIR and DBT, Government of India. A.G. acknowledges research fellowship awarded by CSIR. This is IMTECH communication number 014/2007.

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