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SHORT COMMUNICATION

Phylogenetic and phenotypic analyses of arsenic-reducing bacteria isolated from an old tin mine area in Thailand

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Abstract An agar plate screening assay was used to determine whether 100 arsenic-resistant bacterial isolates, previously obtained from arsenic-contaminated soils, had the ability to transform arsenite and arsenate. Ninety-five percent of the isolates were capable of reducing arsenate on agar plates. The isolates also grew in the presence of high concentrations of arsenite, but none of the bacterial isolates oxidized arsenite to arsenate under the growth conditions tested. About 14 % (13 of 95) of the tested isolates transformed high levels of arsenate (33–70 μM) when tested using the molybdenum blue method. Partial sequence analysis of 16S rDNA genes indicated that the isolates belonged to two broad taxonomic groups: Firmicutes and Proteobacteria. Ten isolates were assigned to four species in the genus *Bacillus*, and three isolates belonged to two species in the genera *Enterobacter* and *Ochrobactrum*. Taken together these results indicate that phylogenetically diverse bacteria isolated from arsenic-contaminated soils in an old tin mine area in Thailand have the ability to transform arsenate to arsenite.

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Introduction

Arsenic is mainly of geochemical origin and is one of the 20 most abundant elements in the Earth's crust. Arsenic has been characterized as an "essential toxin" because it is both required in trace amounts for growth and metabolism, but it is toxic at high concentrations (Stolz et al. 2002). The toxicity of arsenic depends on its oxidation state and chemical form. Inorganic arsenic compounds are more hazardous than organic forms. The soluble forms of inorganic arsenic include trivalent arsenite (AsO_2^- or AsO_3^{3-}) and pentavalent arsenate (AsO_4^{3-}), and these are the most common arsenic species found in the environment. The toxicity of arsenate in large part stems from its ability to act as a structural analog of phosphate and it inhibits oxidative phosphorylation by producing unstable arsenylated derivatives (Anderson et al. 1992; Tamaki and Frankenberger 1992). In contrast, since arsenite binds to sulfhydryl groups of proteins and dithiols such as glutaredoxin, it disrupts proteins and interferes with enzyme function (Anderson et al. 1992).

The biogeochemical cycling of arsenic in nature strongly depends on microbial transformation, which affects its mobility and the distribution of arsenic species in the environment (Quinn and McMullan 1995; Tamaki and Frankenberger 1992). Arsenic solubility under oxidizing conditions is low because arsenate is the predominant species and strongly adsorbs onto rock surfaces or forms minerals with iron, manganese, and aluminum. Arsenate is generally found to be the major species in

arsenic-contaminated soils (Garcia-Manyes et al. 2002; Bissen and Frimmet 2000).

The bacterial oxidation of arsenite to arsenate represents a potential partial detoxification mechanism because it generates the less toxic and less mobile form of arsenic, this has broad applications in bioremediation (Simeonova et al. 2004). In contrast, arsenic can lead to the mobilization of arsenite under reducing or anaerobic conditions, such as in groundwater (Mok and Wai 1994). The transformation of arsenate to arsenite by arsenate-reducing bacteria, however, results in the direct release of arsenite, which poses serious environmental concerns due to the higher toxicity and mobility of arsenite than arsenate.

The objectives of the present study were to investigate the transformation potential of the novel arsenic-transforming bacterial isolates for future applications in the bioremediation of As-contaminated soils in Thailand.

Materials and methods

Screening for arsenic-transforming ability

One hundred bacterial isolates having unique antibiotic resistance patterns, obtained from a previous study (Jareonmit et al. 2010), were used for screening arsenic transformation ability. The arsenic transformation capacity of the bacteria was tested by cultivation of strains in chemically defined medium (CDM) that was incubated in the dark at 30 °C for 48–72 h. The CDM (Weeger et al. 1999) was prepared by mixing the three following solutions: Solution A (100 ml): 0.0812 M of $MgSO_47H_2O$, 0.187 M of NH_4Cl, 0.07 M of Na_2SO_4, 0.574 mM of K_2HPO_4, 4.57 mM of $CaCl_22H_2O$, 0.446 M Na lactate; Solution B (2.5 ml): 4.8 mM $FeSO_47H_2O$; and Solution C (10 ml): 0.95 M $NaHCO_3$ in 887.5 ml water. The final pH of the medium was 7.2. Solution A was further sterilized by autoclaving (121 °C, 20 min) and solutions B and C were sterilized by filtration through a 0.45 μm pore size filter. Arsenic transformation screening was carried out using the silver nitrate screening method for the detection of arsenite-oxidizing and arsenate-reducing bacteria (Krumova et al. 2008). Each bacterial isolate was grown in LB liquid medium supplemented with 10 mg $NaAsO_2$/l and incubated at 30 °C for 24 h. The cell suspension was centrifuged at 4,750×*g* for 10 min and adjusted to an optical density (OD_{600nm}) of 0.6. A 20 μl aliquot of cells was transferred to 130 μl of CDM medium and further incubated at 30 °C for 48 h at 150 rev/min. Sterile filter paper, 0.5 cm (diam), was soaked with cell culture and placed on CDM agar containing 2 mM $NaAsO_2$ or 20 mM $NaHAsO_2$. The CDM agar plate was incubated in the dark at 30 °C for 48 h and then flooded with a solution of 0.1 M $AgNO_3$ to verify the As-transforming ability of the isolates. A brownish precipitate revealed the presence of arsenate in the medium (arsenite-oxidizing bacteria), while the presence of arsenite was detected by a yellow precipitate (arsenate-reducing bacteria) (Lett et al. 2001).

The As-transforming ability of the isolates was confirmed by using a microplate screening assay (Simeonova et al. 2004). Aliquots (5 ml) of 2-day cultures of CDM-grown cells (without arsenic) were centrifuged at 4,750×*g* for 10 min, and cell pellets were washed twice with sterile deionized water and suspended in 0.5 ml deionized water. Twenty μl aliquots of the cell suspensions were added to round-bottomed 96-well plates containing 80 μl 0.1 M Tris–HCl buffer (pH 7.4) supplemented with $NaAsO_2$ or $NaHAsO_2$ to final concentrations of 1.33 and 2.66 mM, respectively. The inoculated microtiter plates were incubated for 48 h and 100 μl 0.1 M $AgNO_3$ was added to each well. The arsenite-oxidizing reaction was indicated by a change of the medium to a bright yellow color to brownish color, while the change of a brownish color to a bright yellow color indicated an arsenate-reducing reaction.

Arsenic transformation determination using the molybdenum blue method

An arsenic speciation assay, using the molybdenum blue method, was used to determine the fate of the arsenate following reaction with the isolated bacteria. This method is based on the reaction between arsenate ions and the molybdenum blue reagent (6 g ammonium molybdate, 10.8 g ascorbic acid, 0.136 g potassium antimony tartrate and 67.3 ml sulfuric acid per liter of mixed reagent). This resulted in the production of a molybdenum-blue color complex which was detected spectrophotometerically (Niggemyer et al. 2001). The speciation and amounts of arsenite were measured using a modification of the spectrophotometric method described by Johnson and Pilson (1972). All isolates were grown in LB medium, pH to 7.2, not supplemented with $NaAsO_2$ or $NaHAsO_2$. Cell suspensions were centrifuged at 4,750×*g* for 10 min, cell pellets were washed twice with PIPES buffer (20 mM, pH 7.0), and washed cell were suspended in PIPES buffer supplemented with 1 mM $NaHAsO_2$ to an $OD_{600} = 0.6$. Cells were incubated at 30 °C for 24 h, with shaking at 150 rev/min. One ml aliquots of each sample was centrifuged at 4,750×*g* for 10 min and pipetted into duplicate 1.5 ml tubes containing oxidized or unoxidized samples. Since the assay was only suitable for arsenate and arsenite concentrations up to 100 μM, samples were diluted with PIPES buffer, pH 7.0, prior to assay. Oxidized samples were prepared by oxidizing 300 μl samples with 100 μl of KIO_3 solution (5 mM KIO_3 in 50 mM HCl). Non-oxidized samples were prepared by acidifying 300 μl samples with

100 µl of 25 mM HCl. All treatments were incubated at 25 °C for 10 min and 600 µl of the molybdenum-containing reaction mixture (Johnson 1971) was added to each treatment. Samples were immediately incubated at 78 °C for 10 min, followed by 5 min incubation on ice. Absorbance was measured at 865 nm and a standard curve was prepared for arsenate concentrations from 0 to 100 µM. Arsenite concentrations were determined by subtracting the absorbance values of oxidized samples from those of unoxidized samples. To avoid phosphorus interference problems, new 1.5 ml tubes were used and all glassware was cleaned with 0.1 M HCl before use.

Bacterial identification using 16S rDNA sequence analysis

A single-colony was picked from each isolate by using a sterile loop, and the colony was suspended in 0.05 M NaOH, boiled at 95 °C for 15 min, and centrifuged at 200 rev/min for 10 min. PCR reaction mixtures contained approximately 1 µl of DNA template, 5 µl of 10× PCR buffer, 3 µl of 25 mM $MgCl_2$, 0.5 µl of 16S rDNA primers (100 µM each), 0.4 µl of 100 µM deoxynucleoside triphosphates (dNTPs), and 0.25 µl of 5 U Taq polymerase (Fermentas, Canada) in a final volume of 50 µl. PCR was performed with a TC512 thermal cycler (Techne, UK) using the conditions described by Sajjaphan et al. (2010). The forward amplification primer was 341F (5′-CCT ACG GGA GGC AGC AG-3′), and the reverse primer was 928R (5′-CCC CGT CAA TTC CTT TGA-3′). PCR products were analysed on 0.8 % agarose gels, stained with ethidium bromide in 1× TAE buffer, and photographed. For direct sequencing, PCR products were prepared and DNA sequencing was performed by Macrogen (Seoul, Korea).

All 16S rDNA sequence data files were uploaded to the RDPII server (http://rdp.cme.msu.edu/) and automatically aligned to the RDP database using the program defaults of the internal secondary structure based aligner. The aligned sequences were used as input files for the Tree-building subroutine of RDP and the program was run using default settings. The outgroup was manually selected. A distance matrix was generated using the Jukes-Cantor corrected distance model. Alignment inserts were ignored and the minimum comparable position was 200. The tree was created using Weighbor, with an alphabet size 4 and length size 1,000. The final tree was created after performing 100 bootstrapping replication with 100 iterations to build a majority consensus tree. Trees were exported as a Newick file to Tree Fig software (http://tree.bio.ed.ac.uk/software/) for manual annotation. The 16S rDNA sequencing files were also compared with sequences in the NCBI database using the BLASTN algorithm. The 16S rDNA gene sequences (560 nucleotides) were aligned with closely related sequences retrieved from the RDP database. Phylogenetic analysis was performed using the neighbor-joining method. Trees were generated as described above.

Nucleotide sequence accession numbers

The 16S rDNA gene sequences of 13 of the tested strains were deposited in GenBank under accession numbers JN208225 through JN208237.

Results and discussion

Screening for arsenic-transforming ability

Arsenic transformation capacity of 100 unique isolates from a previous study (Jareonmit et al. 2010) was screened using the agar plate assay. Before testing arsenic transformation on agar plates, suitable growth conditions of these bacteria was tested on CDM-supplemented with As(III) or As(V). The As(III) and As(V) were added to CDM at a final concentration of 2 and 20 mM, respectively. Plates were incubated at 30 °C for 48 h, and transformation ability was evaluated by using the silver nitrate screening method. The interaction of silver nitrate with arsenite generates a bright yellow precipitate while a brownish precipitate forms with arsenate (Simeonova et al. 2004). Ninety-five percent of the tested isolates were capable of transforming arsenate to arsenite based on the formation of bright yellow precipitate surrounding the colonies (Fig. 1a). However, none of the bacteria produced a red- brownish precipitate, suggesting that no arsenate was generated (Fig. 1b). This indicated that 95 isolates had reducing activity while none of the colonies displayed arsenic-oxidizing activity under the condition of the assay.

The microplate screening method was used to confirm that the 95 isolates were capable of reductive arsenic transformations. After the addition of silver nitrate, the microplate screening assays indicated that the tested strains reduced arsenate to arsenite. Unfortunately, this study could not determine the proportion of arsenite and arsenate from the microplate analyses because of the rapid color reaction and the lack of color scale standard. However, our results indicated that the agar plate screening assay done using silver nitrate was a useful and effective tool for rapidly determining arsenic transformation of bacterial isolates on a large scale. Moreover, application of this method in petri dishes was successful only when it was tested using the CDM medium (Simeonova et al. 2004).

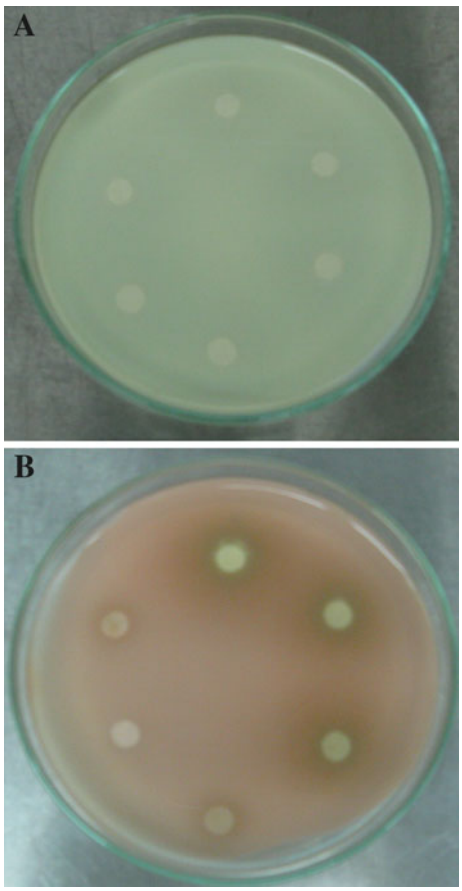

Fig. 1 Arsenic resistance among isolated bacteria by using the silver nitrate screening method in CDM agar plates supplemented with 2 mM sodium arsenite (**a**) and 20 mM of sodium arsenate (**b**). Plates were incubated in dark at 30 °C for 2 days

Arsenic transformation determined by using the molybdenum blue method

The arsenic reduction potential of the 95 arsenic-resistant bacteria was also assessed by using the molybdenum blue method. About 75 % (71 of 95) of the isolated bacteria reduced arsenate to arsenite. This implied that these isolates possess the enzymatic machinery necessary for arsenate reduction. However, these bacteria had low reduction effectiveness when cultivated in 1 mM of arsenate and incubated at 30 °C for 24 h. Nevertheless, 13 isolates reduced more than 10 % of the As(V) (1 mM) within 24 h (Table 1). Under these conditions, only about 14 % (13 of 95) of the isolates were capable of reducing high concentrations of arsenate ranging from 33 to 70 μM. Of these isolates, MC196, MC197, and MC204 showed high arsenate reduction effectiveness at 26.5, 32.2 and 38.1 %, respectively. However, it is possible that growth conditions affect arsenate reduction through the inhibition of arsenate reductase activity. Srivastava et al. (2009) reported that *Pseudomonas* sp. strain DRBS1 was efficient in arsenate reduction, with more than 97 % of As reduced at higher initial arsenate concentrations (60, 80, and 100 mM) and more than 90 % of arsenate reduced at initial concentrations of 40 and 20 mM. An *Aeromonas* sp. and *Exiguobacterium* sp. strain were able to transform arsenate (50 mM) to arsenite with alkalinization of the growth medium during exponential growth, but they could not transform arsenite (Anderson and Cook 2004).

Table 1 Effectiveness of arsenic reduction by arsenate reducing bacteria

Isolated	Arsenite concentration (μM)	Effectiveness of arsenate reduction (%)[a]
MC010	38.32	15.1
MC013	39.09	14.8
MC120	56.94	19.7
MC123	36.64	14.1
MC169	37.12	11.2
MC194	33.16	10.0
MC196	54.44	26.5
MC197	66.22	32.2
MC202	36.30	14.1
MC203	39.67	19.5
MC204	69.59	38.1
MC205	35.30	13.9
MC265	38.92	12.2

[a] Effectiveness of arsenate reduction was calculated from arsenate concentration remaining in the buffer. Bacteria were incubated in the presence of 1 mM arsenate concentrations incubated in PIPES buffer (pH 7.0) at 30 °C

Bacterial identification by using 16S rDNA sequence analyses

The 16S rDNA of 13 arsenic-resistant isolates was amplified using the primers 341F and 928R, resulting in a characteristic single band of about 560 bp. Results indicated that the 13 arsenic-reducing isolates could be separated into three primary groups based on partial 16S rDNA sequence analysis (data not shown). Ten isolates belonged to cluster I with similarities ranging from 57 to 99 %. Cluster II contained one isolate (MC197) and cluster III contained two isolates, MC010 and MC204, showing 91 % similarity. Moreover, all isolated bacteria were identified using phylogenetic analysis of their nearest bacterial relatives. The isolates were assigned to six bacterial species belonging to three genera. All identified bacterial isolates were associated with Firmicutes and Proteobacteria (Fig. 2). Most isolates belonged to the genus *Bacillus* (sequence similarity 83 %), except for MC010, MC197, and MC204. In contrast, 16S rDNA sequence analysis

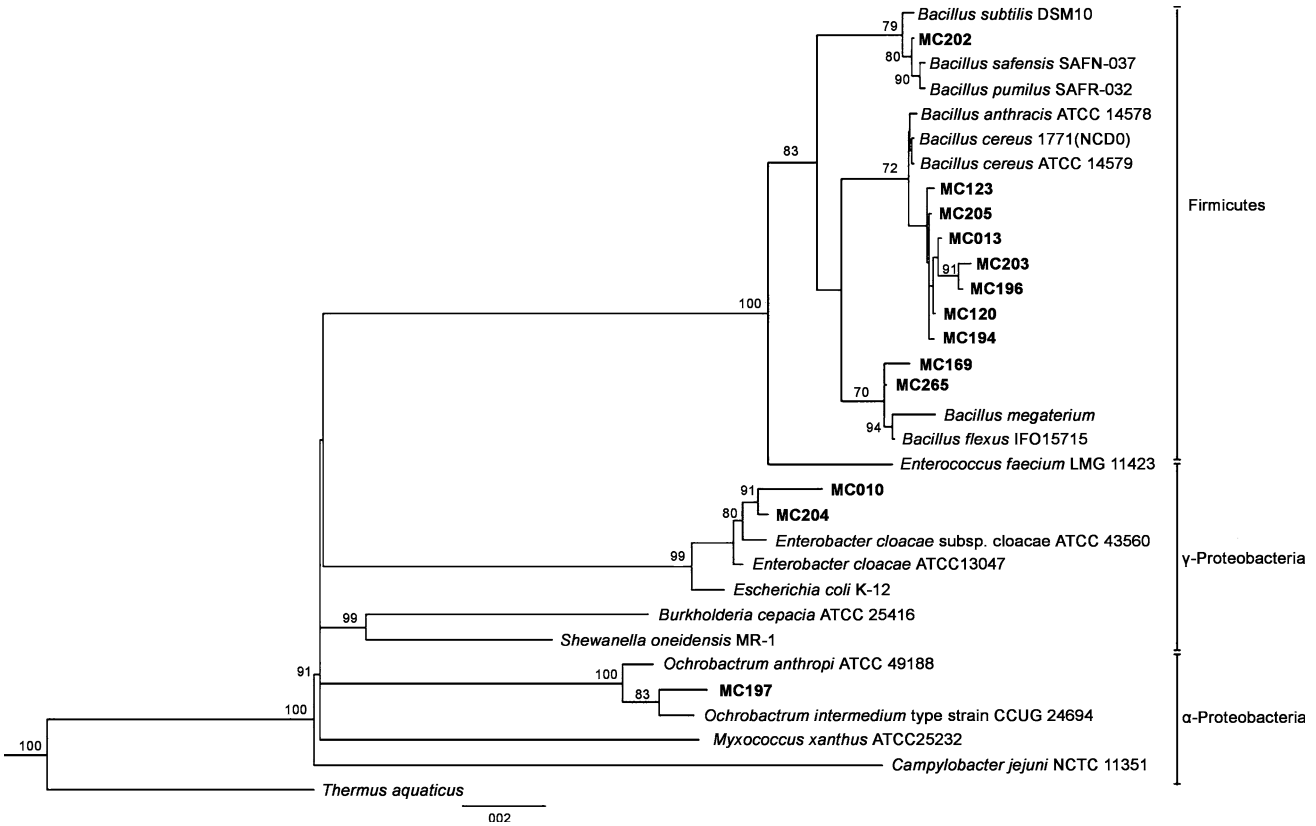

Fig. 2 Phylogenetic tree of 13 of arsenate-reducing bacteria and related taxa based on a combined sequence analysis of partial 16S rDNA genes. The *scale bar* represents 0.02 substitutions per 100 nucleotides within the16S rDNA sequence, at least 100 iterations were used for bootstrap analysis

showed that MC010 and MC204 were most closely related to *Enterobacter cloacae*, with 80 % sequence identity, and isolate MC197 belonged to the genus *Ochrobactrum*, with 99 % sequence identity. However, the genus *Bacillus*, and the γ-Proteobacteria have evolved strong resistance mechanisms supported by arsenate reduction (Anderson and Cook 2004; Matlakowska et al. 2008; Salam et al. 2009). While members of genera *Exiguobacterium*, *Aeromonas*, *Bacillus*, *Pseudomonas*, *Escherichia*, *Acinetobacter*, and γ-Proteobacteria, Actinobacteria, and Flavobacteria have been previously reported to evolve strong resistance mechanisms supported by arsenate reduction, none of these bacteria can utilize arsenite or arsenate in respiratory processes (Anderson and Cook 2004; Matlakowska et al. 2008; Salam et al. 2009).

The present work indicates the possibility of isolating a relatively large number phylogenetically different arsenate-reducing bacteria from Thai soils. It also showed that bacteria capable of arsenate reduction might be used as candidates for arsenic remediation in arsenic-contaminated sites. This will have great use in Thailand and other counties as excessive dumping and use of arsenic has contaminated many soils and has created health concerns.

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