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Structure and Diversity of Arsenic-Resistant Bacteria in an Old Tin Mine Area of Thailand

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The microbial community structure in Thailand soils contaminated with low and high levels of arsenic was determined by denaturing gradient gel electrophoresis. Band pattern analysis indicated that the bacterial community was not significantly different in the two soils. Phylogenetic analysis obtained by excising and sequencing six bands indicated that the soils were dominated by *Arthrobacter koreensis* and *β-Proteobacteria*. Two hundred and sixty-two bacterial isolates were obtained from arsenic-contaminated soils. The majority of the As-resistant isolates were Gram-negative bacteria. MIC studies indicated that all of the tested bacteria had greater resistance to arsenate than arsenite. Some strains were capable of growing in medium containing up to 1,500 mg/l arsenite and arsenate. Correlations analysis of resistance patterns of arsenite resistance indicated that the isolated bacteria could be categorized into 13 groups, with a maximum similarity value of 100%. All strains were also evaluated for resistance to eight antibiotics. The antibiotic resistance patterns divided the strains into 100 unique groups, indicating that the strains were very diverse. Isolates from each antibiotic resistance group were characterized in more detail by using the repetitive extragenic palindromic–PCR (rep-PCR) DNA fingerprinting technique with ERIC primers. The PCR products were analyzed by agarose gel electrophoresis. The genetic relatedness of 100 bacterial fingerprints, determined by using the Pearson product-moment similarity coefficient, showed that the isolates could be divided into four clusters, with similarity values ranging from 5–99%. Although many isolates were genetically diverse, others were clonal in nature. Additionally, the arsenic-resistant isolates were examined for the presence of arsenic resistance (*ars*) genes by using PCR, and 30% of the isolates were found to carry an arsenate reductase encoded by the *arsC* gene.

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Contamination of drinking water supplies with the inorganic soluble forms of arsenite and arsenate has often been reported, and arsenic has been identified as a major risk for human health in northeast India, Bangladesh, northwest United States, and other parts of the world [42]. Arsenic contamination in Thailand was first recognized in 1987 when people living in the Ronphibun District of Nakhon Si Thammarat reported health problems due to arsenic contamination of drinking water. Over 1,000 people, particularly those living in and close to Ronphibun town, have been diagnosed with arsenic-related skin disorders [58]. Fordyce *et al.* [22] and William *et al.* [59] reported that about 15,000 people drank water with arsenic contamination of more than 50 mg/l concentration. The affected area lies within the Southeast Asian tin belt. Primary tin–tungsten–arsenic mineralization and alluvial placer tin deposits have been mined in the district for over 100 years, although mining activities have now ceased. Legacies of mining operations include waste from arsenopyrite-rich piles, ore-dressing plants, and dissemination from small-scale panning by villagers.

Arsenic is considered to be a semimetal with metallic and nonmetallic properties. Arsenic is toxic to not only bacteria, but also other domains of life. Arsenic present in diverse environments is released either by natural weathering of rocks or by anthropogenic processes (*e.g.*, by mining industries and agricultural practices). In the environment, arsenic is present in the pentavalent As(V) (arsenate) and trivalent As(III) (arsenite) forms [15]. Arsenite is more toxic than arsenate and has been shown to inhibit several dehydrogenases [19]. The biogeochemical cycle of As strongly depends on microbial transformation, which affects the

mobility and the distribution of arsenic species in the environment [43, 55]. Whereas arsenite (AsO_2^- or AsO_3^{3-}) has the ability to bind to sulfhydryl groups of proteins and dithiols such as glutaredoxin, arsenate (AsO_4^{3-}) acts as a structural analog of phosphate and inhibits oxidative phosphorylation by producing unstable arsenylated derivatives [5, 7, 16].

Microbial As(III) oxidation and As(V) reduction activities are cellular strategies for either detoxification or for generating energy, and microorganisms can use arsenic compounds as electron donors, or electron acceptors, or possess one or more arsenic detoxification mechanisms [2, 11, 29, 35, 40, 46, 47]. These mechanisms include (i) minimizing the uptake of arsenate through the phosphate uptake system [11], (ii) peroxidation reactions with membrane lipids [1], and (iii) using microbial arsenic detoxification pathways involving the *ars* operon [49]. The genetics, regulation, and function of detoxification-based As(V) reduction have been reported by Silver and Phung [50], and genes encoding respiratory As(V) reductases have been cloned and characterized. Similarly, genes encoding As(III) oxidase have also been cloned and characterized [37, 47], and this enzyme has been extensively characterized [5], including the elucidation of the crystal structure [20]. The genetic basis for the regulation of As(III) oxidation, however, remains unknown.

Operons encoding analogous arsenic resistance genes (*ars*) have been found on the chromosome and on transmissible plasmids in a wide variety of both Gram-positive and Gram-negative microorganisms. These operons generally consist of either the *arsRBC* or *arsRDABC* genes that have been organized into a single transcriptional unit [49]. The three-gene system, encoding the arsenic transcriptional repressor (*arsR*), arsenite permease (*arsB*), and arsenate reductase (*arsC*), are present on the chromosome of *Escherichia coli*, *Pseudomonas aeruginosa* [10], and other enterobacteria [17]. The *arsRDABC* operon encodes for an arsenite-inducible repressor (*arsR*), a negative regulatory protein (*arsD*), an ATPase, a membrane-located arsenite efflux pump (*arsA* and *arsB*, respectively), and an arsenate reductase (*arsC*). This operon was initially discovered on *E. coli* plasmids R773 and R46 [13] and subsequently on plasmid pKW301 from *Acidiphilum multivorum* [54]. Moreover, some of the arsenic-resistant genes have been described in different bacteria, such as *Bacillus subtilis* [48], *Acidithiobacillus ferrooxidans* [8, 9], and a *Synechocystis* sp. [32]. The *ars*C gene occurs in the *ars* operons of many bacteria as well as in some archaeal genomes [50]. ArsC (in the absence of other ars operon gene products) reduces arsenate to arsenite, which is exported by the ArsB protein [29]. ArsC functions as an intracellular substrate-binding protein [31, 51, 56] analogous to the periplasmic substrate-binding proteins for ATP-dependent membrane uptake systems [29]. ArsC would then make arsenate accessible to the ArsA/ArsB membrane complex, functioning as an arsenate efflux ATPase. ArsB plus ArsA alone was thought to export arsenite and antimonite [13, 31, 29, 44]. Recently, several studies have focused on the detection of *ars* genes in environmental samples, the arsenic-transforming capacities of bacterial isolates [4, 23, 34, 45], or used these genes as potential molecular biomarkers to detect arsenic contamination [21, 53].

The objectives of this study were to better understand the microbial community structure of arsenic-contaminated soil in old tin mine areas located in Tambon Ong-pra, Amphoe Dan-Chang, in Suphan-Buri Province in Thailand, and to characterize arsenic-resistant bacteria isolated from these soils using a variety of genotypic and phenotypic methodologies.

Materials and Methods

Sampling Site

Soil samples (0–10 cm depth) were collected along the Makham creek near old tin mines located in Tambon Ong-pra and Amphoe Dan-Chang, in Suphan-Buri Province, Thailand. The field-moist soil samples were stored at 4°C until use for later study.

Soil Characteristics

Soil pH was determined using a 1:1 ratio of soil to deionized water. Texture classification was measured using the pipette method [25], and organic matter content was determined by wet oxidation and titration using the Walkley and Black method [39]. Total arsenic was analyzed as described by Stewart and Bettany [52], and other heavy metals in the soils were determined as described by Amacher [3].

Denaturing Gradient Gel Electrophoresis Analysis

DNA was extracted from soil sampled by using PowerSoil DNA kits (MOBio Laboratory, Inc., CA, U.S.A.). Soil DNA extracts were amplified by PCR, using primers for bacterial groups; PRBA338F (5'-ACT-CCT-ACG-GGA-GGC-AGC-AG-'3), with a GC clamp needed to stabilize products for DGGE analysis, and PRUN518R (5'-ATT-ACC-GCG-GCT-GCT-GG-'3) [14]. The PCR technique was used to amplify the approximately 250-bp fragment of bacterial 16S rDNA gene as previously described [6]. The DGGE was performed using a polyacrylamide gel [13% (w/v) of 37.5:1 acrylamide/bisacrylamide in 1× TAE buffer] with a urea formamide denaturing gradient of 40–70%. Electrophoresis was performed at 60°C with a constant voltage of 90 V for 13 h. [6, 60]. Following electrophoresis, gels were stained with ethidium bromide, images were visualized on a UV transilluminator, and band profiles were quantified and interpreted by using the BioNumerics v3.5 software (Applied Maths, Sint-Martens-Latem, Belgium). Several dominant DGGE bands were excised from gels by using a sterile knife, placed in 25 µl of PCR water (RNase- and DNase-free water), incubated at 40–50°C for 1 h, and stored at 4°C. DNA from excised bands was sequenced at the Biomedical Genomics Center at the University of Minnesota, St. Paul, MN, U.S.A.

Comparison of DGGE Fingerprints from Contaminated Soil Sites

Comparisons of individual DGGE fingerprints were made using the BioNumerics v3.5 software. DGGE gel profiles were normalized in BioNumerics to eliminate lane-to-lane variations. Jaccard similarity coefficients (band-base analysis) were determined by comparing the portion of common bands present within the total number of bands between samples [18].

Isolation of Arsenic-Resistant Bacteria

Arsenic-resistant bacteria were isolated from eight contaminated soils as follows: soil samples were mixed and extracted with 0.85% NaCl and bacteria were grown on TGE medium, containing (per liter) tryptone 5 g, D-glucose 1 g, and meat extract 3 g. The medium was supplemented with 10, 50, 100, 250, or (500 mg/l arsenite $NaAsO_2$). The plates were incubated aerobically at 30°C for 24 h. Bacteria growing on plates were re-streaked to obtain pure cultures. All arsenic-resistant bacteria were stored in 25% glycerol and kept at -80°C until used for later study.

Identification and Characterization of Arsenic Resistant Bacteria

Gram staining. One drop of culture medium was placed onto a glass microscope slide and Gram stains was performed under bright field illumination using a Zeiss microscope [33].

Determination of Minimum Inhibitory Concentrations (MICs)

The MIC is defined as the lowest concentration of an agent that completely inhibited growth of bacteria. Isolates were streaked onto LB agar plates containing 10 mg/l arsenite (As^{+3}, as $NaAsO_2$) and single colonies were grown in LB broth and incubated at 30°C for 24 h. The MICs of arsenic and antibiotic were determined by replica plating onto LB agar medium containing arsenite (10, 50, 100, 250, 500, 1,000, or 1,500 mg/l of $NaAsO_2$), arsenate (10, 40, 100, 200, 500, 1,000, or 1,500 mg/l of $NaHAsO_2$), or antibiotics. The eight antibiotics tested were chloramphenicol, ampicillin, spectinomycin (at 1, 5, or 10 μg/ml), streptomycin (at 5 or 10 μg/ml), tetracycline (at 0.5, 1, or 2 μg/ml), kanamycin (at 1 or 5 μg/ml), naladixic acid (at 1, 2.5, or 5 μg/ml), and rifamycin (at 1, 12, or 15 μg/ml). MIC values were entered into the BioNumerics v3.5 software as binary data and the resulting matrices were analyzed by using simple matching analysis with binary coefficients. Dendrograms were produced to show the relationship of bacterial strains based on arsenic resistance.

Rep–PCR DNA Fingerprinting

All unique isolates from MIC examination were further characterized by DNA fingerprint analysis. Isolates were chosen based on profiles of antibiotic resistance. DNA fingerprints were obtained by using rep–PCR DNA fingerprinting as follows: single colonies were suspended in 0.05 M NaOH, boiled at 95°C for 15 min, and centrifuged at 200 rpm for 10 min. The rep–PCR fingerprints were generated using the ERIC primers: ERIC1R (5'-ATG TAA-GCT-CCT-GGG-GAT-TCA-C-3') and ERIC2 (5'-AAG–TAA-GTG-ACT-GGG-GTG AGC-G-3') [36]. PCR was performed with an MJ Research PTC 100 (MJ Research, Waltham, MA, U.S.A.) thermocycler, using the conditions described by Johnson *et al.* [30]. Fingerprint data were normalized and analyzed using the Bionumerics v3.5 software. DNA fingerprint similarities were calculated by using Pearson's product-moment correlation coefficient.

Arsenic-resistant gene determination. One hundred isolates were examined for the presence of the arsenate reductase gene encoded by *arsC* by using the PCR technique. Primers for the amplification of the *arsC* gene were 5'-GTA-ATA-CGC-TGG-AGA-TGA-TCC-G-3' and 5'-TTT-TCC-TGC-TTC-ATC-AAC-GAC-3' as described by Saltikov and Olson [45]. PCR reaction mixtures contained approximately 1 μl of DNA template, 2.5 μl of 10× PCR buffer, 1 μl of 50 mM $MgCl_2$, 0.5 μl of *arsC* primers (10 μM each), 0.5 μl of 100 μM deoxynucleoside triphosphates (dNTPs), and 0.1 μl of 5 U *Taq* polymerase in a 25-μl final volume. PCR was performed using a TC512 thermal cycler (Techne, U.K.) using the conditions described by Chang *et al.* [12]. PCR products were analyzed on 1% agarose gels, stained with ethidium bromide in 1× TAE buffer, and photographed. For direct sequencing, PCR products were purified with a Gel/PCR DNA fragments extraction kit (Geneaid Biotech, Taiwan) according to the manufacturer's directions. DNA sequencing was performed by Macrogen (Seoul, Korea).

RESULTS AND DISCUSSION

Soil Characteristics

Soil samples contaminated with arsenic 40 years ago were collected from an old tin mining area. This site is located in Amphoe Dan-Chang in Suphan-Buri Province, Thailand. The physicochemical properties of the soils are summarized in Table 1. All soils were characterized generally as loams,

Table 1. Characteristics of the soil samples taken from Makham creek, Tambon Ong-pra, Amphoe Dan-Chang, Suphan Buri province, Thailand.

Soil	Texture	pH	OM (%)	Heavy Metal Concentration (mg/kg soil)						
				As	Cu	Cr	Mn	Pb	Zn	Cd
Site 1	loam	7.8	0.81	389	26	26	138	9	29	0.61
Site 2	silty clay loam	6.5	2.50	711	54	111	302	17	97	1.44
Site 3	clay loam	6	2.63	124	12	32	279	9	50	0.88
Site 4	sandy clay loam	5.8	2.14	160	12	34	167	2	28	0.61
Site 5	clay loam	7.4	2.22	315	15	38	142	8	31	1.02
Site 6	loam	8.5	0.23	467	38	71	467	60	101	1.58
Site 7	sandy loam	7.4	1.48	356	13	34	0	43	32	0.78
Site 8	loam	7.9	2.90	381	16	29	112	66	68	0.95

with soil pH ranging from 5.8 to 8.5 and the percentage of organic matter ranging between 0.23% and 2.9%. The concentration of total arsenic in the soils varied between 124 and 711 mg/kg soil. The highest concentration was found at site 2. Other heavy metal concentrations were also generally high at site 2. Results in Table 1 show that high arsenic concentration generally related with high concentrations of other heavy metals, except Pb. Elevated concentrations of arsenic were found both in the soils nearest and farthest away from the smelter. There was no significant correlation between soil texture, heavy metal content, and soil organic matter.

Soil Microbial Community Analysis by DGGE

The bacterial community structures in the eight soil samples obtained near the mine and the one control soil were assessed by analyzing the DGGE banding patterns of 16S rDNAs amplified by PCR. There was a greater number of bands present in the DGGE profile of 16S rDNA from site 7 compared with the number of bands from site 5 and site 1 (data not shown). Based on the band intensity, the most dominant bacteria were seen in soils from site 1 and site 5. The relative abundance of species within the microbial community, based on band number, is shown in Fig. 1. The number of bands resolved by DGGE analysis ranged from 26 to 52, with soil from site 7 having the greatest species diversity. In contrast, DGGE analysis of 16S rDNA from site 6 only showed 26 bands, which was lower than the number found in the control soil. This difference may, in part, result from DNA extraction problems in site 6, which resulted in the presence of light bands. It should be noted, however, that it has been estimated that only 1% of the sample diversity is observed as bands in a DGGE fingerprint [26], despite the fact that soils are composed of complex

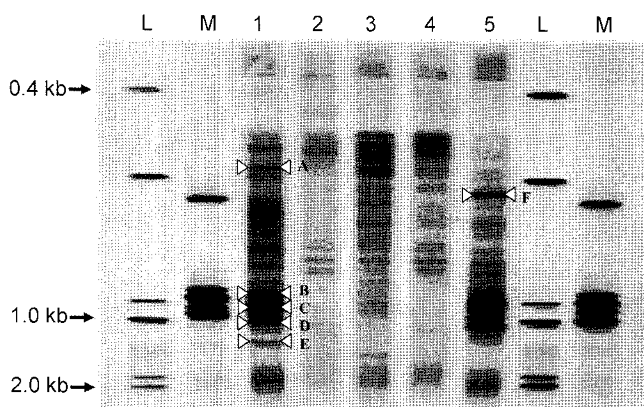

Fig. 1. DGGE analysis of PCR-amplified 16S rRNA gene fragments.

Bands marked were excised and sequenced. 1, site 1; 2, site 2; 3, site 3; 4, site 4; 5, site 5; L, 1 kb plus DNA ladder; M, a mixture of PCR-amplified 16S rRNA gene fragment of five individual bacteria, *Pseudomonas putida*, *Acinetobacter* ADP1, *E. coli* DH5α, *Comamonas testosteroni*, and environmental isolate BW7.

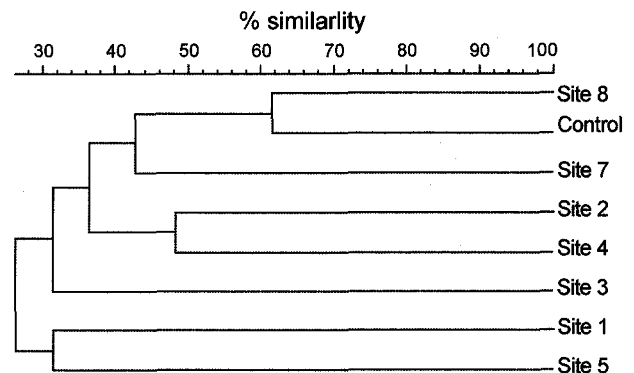

Fig. 2. Comparison of DGGE banding profiles of 16S rRNA gene from contaminated soil using UPGMA cluster analysis.

microbial communities with population richness exceeding several hundred phylotypes [24, 57]. Thus, although 20 to 40 bands in a DGGE profile can be clearly resolved in a gel, it may not be possible to detect the entire diversity in most soil samples [38].

Dendrogram analysis of DGGE banding profiles of 16S rDNA (Fig. 2) indicated that the microbial communities were quite different in the soils impacted by As. Overall, the banding patterns ranged from 25 to 60% similarity. The 16S rDNA banding patterns could be separated into two major clusters at the 30% similarity level, which were not related with the distance from the mine. Moreover, results of this study indicated that the microbial communities detected were related not only to arsenic concentration, but also likely correlated with pH, organic matter content, and the presence of other heavy metals.

The six dominant bands present in the DGGE profiles of 16S rDNA from site 1 and site 5 were excised (Fig. 1) and examined for the presence of single microbial species by re-running DGGE analysis prior to DNA sequencing. DGGE analysis indicated that all the excised bands contained a single 16S rDNA molecule. The DNAs in the bands were subsequently sequenced using the same primers that were used for PCR. Sequence analysis indicated that two of the bands comprised cultured members of *Arthrobacter koreensis* (band A) and a *β-Proteobacteria* (band B), with sequence similarities of 97% and 100%, respectively. In contrast, 16S rDNAs in the remaining four bands were only distantly related (at the 94 to 100% similarity levels) to known 16S rDNA genes present in members of yet undescribed bacterial divisions. Bands C and F had sequence similarities with unknown bacterial clones FLSED16 and LNR A2-16, respectively, and bands D and E sequences did not belong to other previously reported species.

Isolation and Characterization of Arsenic-Resistant Bacteria

The 262 bacterial strains, isolated from soils with different arsenic concentrations (Table 1), showed significant variation

in their resistance to both arsenite and arsenate (data not shown). Most of the bacterial cells were either cocci or rod shaped, and the majority of the isolates were Gram-negative bacteria. However, several Gram-positive strains were also detected. The MIC analysis of the 262 unique arsenite- and arsenate-resistant isolates was determined. Over 60% of the isolates from sites 1, 2, and 3 were resistant to arsenite at concentrations up to 1,000 mg/l. More than 50% of the isolates from sites 6 and 7 were resistant to arsenite at concentrations up to 500 mg/l, whereas isolates from site 4 were resistant to arsenite at concentrations of 10–250 mg/l. In contrast, MICs analyses of isolates from site 8 showed that only 25% were able to grow with arsenite at concentrations exceeding 250 mg/l, and no isolates were resistant to arsenite at concentrations exceeding 500 mg/l. While cultures were originally isolated based on their resistance to arsenite, we were also interested to determine if they also grew in the presence of arsenate. MIC analysis of arsenate showed that more than 60% of the isolates from sites 1, 2, and 6 were resistant to arsenate at concentrations up to 1,500 mg/l, whereas more than 40% of the isolates from sites 3, 4, and 7 were resistant to arsenate at concentrations of 1,000 mg/l, 500 mg/l, and 200 mg/l, respectively. Highly resistant strains capable of growth on arsenate up to 1,500 mg/l were isolated from all of the sites, except site 8. From the MICs analysis, arsenite and arsenate resistance decreases with increasing arsenite and arsenate concentrations.

For MICs of eight antibiotics, bacteria were resistant to eight antibiotics in different concentrations (data not shown). Most bacteria showed resistant to chloramphenicol and kanamycin at 5 μg/ml, to spectinomycin, streptomycin, and ampicillin up to 10 μg/ml, and to rifamycin at 15 μg/ml.

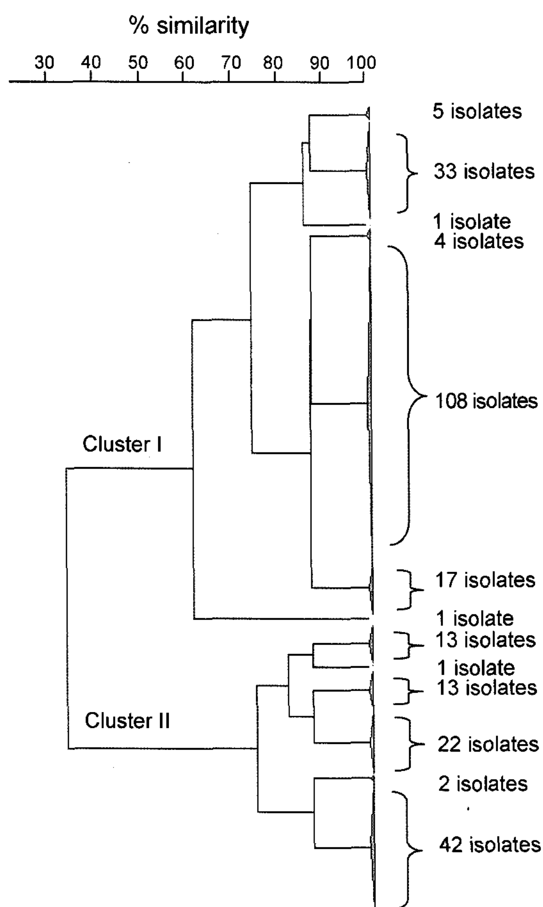

Fig. 3. Dendrogram analysis of 262 arsenite-resistant isolates showing the relatedness of arsenite-resistant bacteria from old tin mine in different soils.

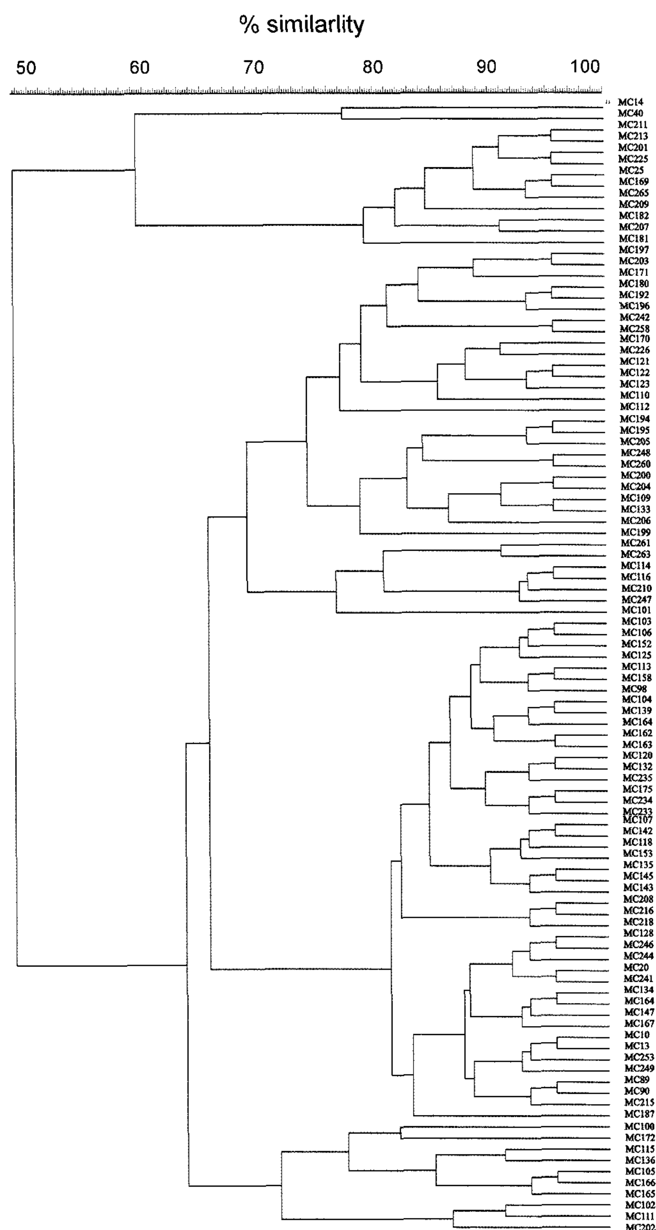

Fig. 4. Dendrogram analysis showing the relatedness of arsenic-resistant isolates by antibiotic- resistant data.

Moreover, they were resistant to tetracycline and naladixic acid at 2 µg/ml and 2.5 µg/ml, respectively.

To determine the relatedness of bacteria, a dendrogram based on MICs data was constructed using the BioNumerics program. Although most of the bacteria had close similarity values based on arsenite resistance, they could be arranged into 13 unique groups as shown in Fig. 3. Although the arsenite-resistant ability of isolated bacteria could be determined with a minimal inhibitory concentration on different arsenic concentrations, antibiotic resistant may be useful for grouping of these isolates. A dendrogram was constructed based on antibiotic resistantce data (Fig. 4). They could be divided into 4 clusters, with a similarity range of 60–96%.

Rep–PCR DNA Fingerprinting

The rep–PCR DNA fingerprinting technique was conducted to differentiate arsenic-resistant isolates. Complex fingerprint patterns were obtained for the 100 isolates studied. Isolates were chosen based on profiles of resistance to arsenite and antibiotics. The rep–PCR DNA fingerprint generated with ERIC primers indicated that arsenic-resistant bacteria from arsenic-contaminated soil were genotypically diverse (Figs. 5 and 6). Approximately 90% of isolates produced high-quality DNA fingerprints when primer ERIC was used. However, individual lanes generally contained 10 to 20 PCR product bands.

Dendrogram analysis showed that the isolates could be divided into four clusters, with a similarity ranging from 5 to 99%. Although many isolate were genetically diverse, others were highly related, and isolates with 94% or greater similarity can be considered to be of the same strain. Our results indicated that the rep–PCR DNA fingerprinting technique done by using ERIC primers is a useful and effective tool for rapidly determining diversity among bacteria isolated from arsenic-impacted soils.

Detection of Arsenic-Resistant Gene Homolog in Arsenic Resistant Isolates

The gene common to all *ars* operons is *arsC*, encoding for the enzyme that reduces less toxic arsenate [As(V)] to more toxic arsenite [As(III)] [27–29]. ArsC is a cytoplasmic arsenate reductase that is found widely in microbes. Resistance to arsenate is conferred by the reduction of arsenate to arsenite by the *arsC* gene product; the resulting arsenite is extruded by the transport system [27, 41]. Therefore, in this study, 100 isolates were examined using PCR technique for the presence of the *arsC* gene. Results of *arsC* detection showed that only 30% of isolated bacteria contained this gene. A representative agarose gel of PCR products from arsenic-resistant isolates, using primers for *arsC*, is shown in Fig. 7. The results suggested that the *arsC* gene may be diverse in these isolates. It is also possible that these isolates have other arsenic-resistant

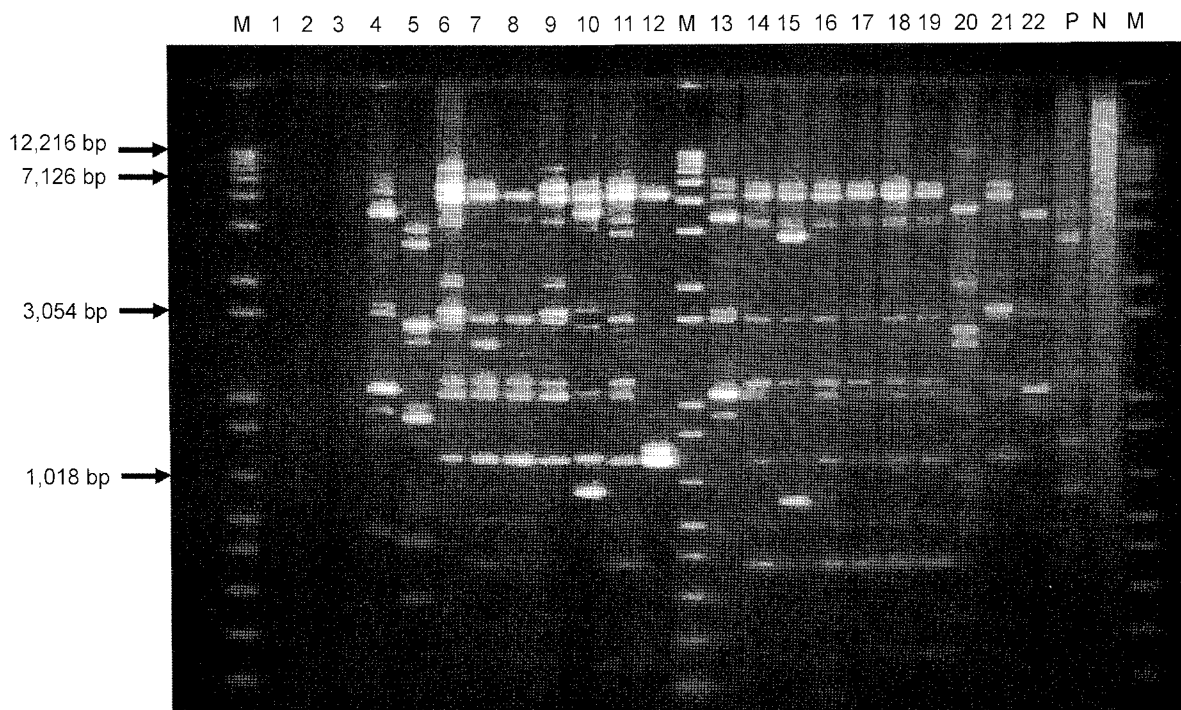

Fig. 5. Rep–PCR DNA fingerprint patterns of arsenic-resistant isolates obtained from arsenic-contaminated soil.
PCR DNA fingerprint patterns generated with primer ERIC. Lanes: M, molecular mass markers (100-kb ladder); P, positive control (*E. coli* 294); 1–22, isolates number MC204, MC205, MC206, MC207, MC208, MC209, MC210, MC211, MC213, MC215, MC216, MC218, MC225, MC226, MC233, MC234, MC235, MC241, MC242, MC244, MC247; N, negative control.

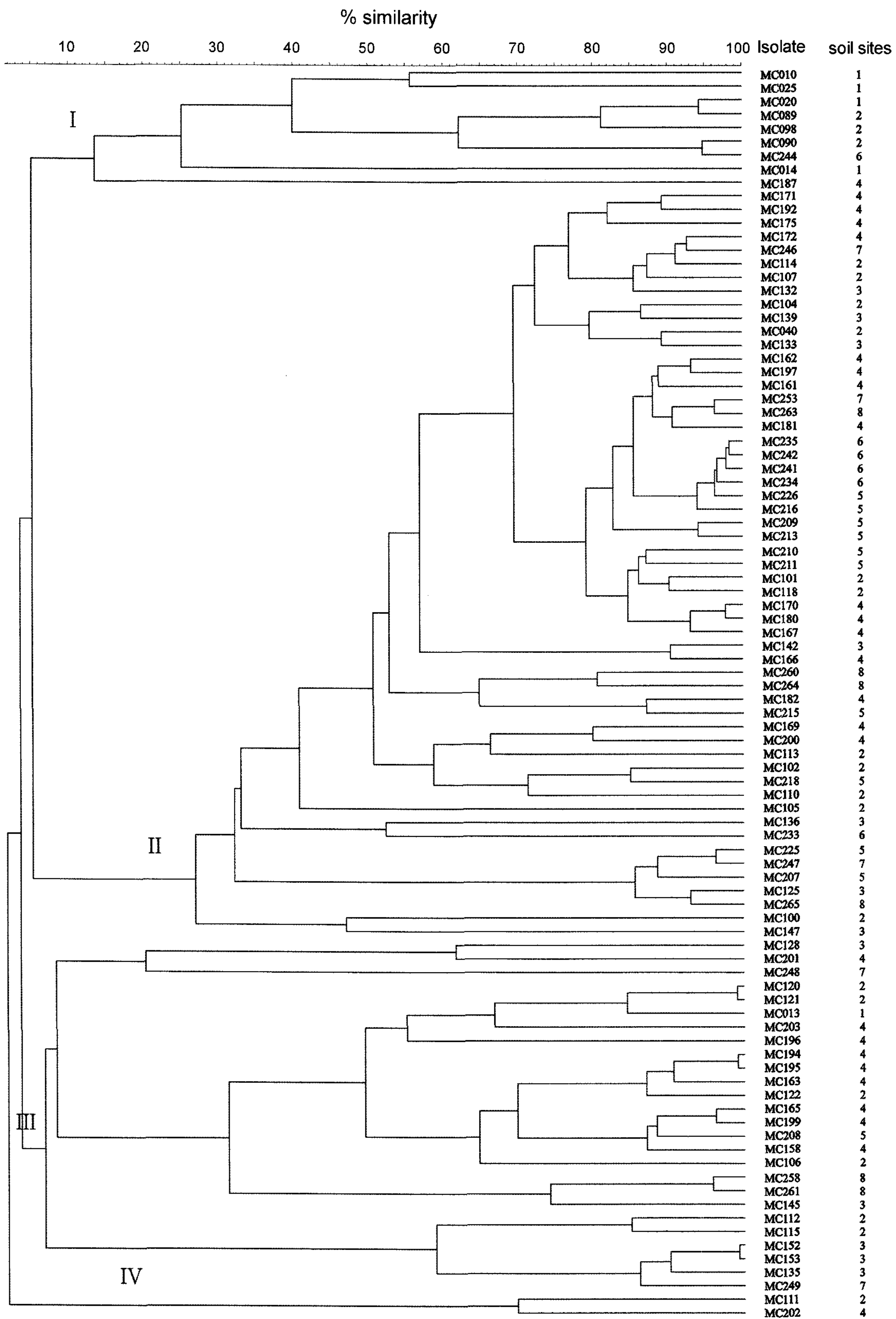

Fig. 6. Dendrogram showing the relatedness of arsenic-resistant strains isolated from 8 arsenic-impacted areas by a PCR DNA fingerprint analysis performed with primer ERIC.

Relationships were determined by using Jaccard similarity coefficients and the neighbor-joining clustering method.

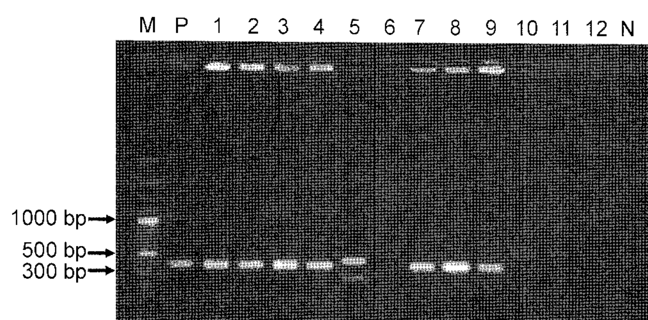

Fig. 7. Representative of PCR products from arsenic-resistant isolates, using an *arsC* primer.
Lanes: M, molecular mass markers (GeneRuler 100-bp ladder); P, positive control (*E. coli* W3110); 1, MC10; 2, MC14; 3, MC20; 4, MC25; 5, MC89; 6, MC90; 7, MC98; 8, MC100; 9, MC101; 10, MC102; 11, MC103; 12, MC103; N, negative control.

mechanisms rather than containing *arsC*. Taken together, these results suggest that the environmental isolates examined in this study have diverse arsenic resistance genes and mechanisms to cope with relatively large concentrations of this metal in soils.

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