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RESEARCH ARTICLE

Genetic differentiation of *Arthrobacter* population from heavy metal-contaminated environment

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Abstract Six samples containing extremely high concentration of Pb, Zn, and Cd were obtained from the layers of 5−10 cm and 25−30 cm three tailing piles, with ages of about 10, 20 and more than 80 years, respectively. Then, 48 bacterial strains were obtained from these samples, and subsequently their phylogenetic positions were determined by analysis on the partial sequence of 16S rRNA gene (fragment length ranging from 474 to 708 bp). These isolates were members of the *Arthrobacter* genus, phylogenetically close to *A. keyseri* and *A. ureafaciens*, with sequence ranging from 99.1% to 100%. Furthermore, genetic variation between subpopulations from different samples was revealed by analysis on their randomly amplified polymorphic DNA profile. Nei genetic distance showed that the greatest differentiation occurred between subpopulation A and C. Notably, either genetic distance between subpopulations from the layers of 5−10 cm and 25−30 cm of each tailing pile or between same layers of different tailing pile increased with the history of tailings. Moreover, correlation analysis showed that soluble Pb has a significantly negative relationship with Nei' gene diversity of subpopulation. It was assumed that soluble Pb may be responsible for the reduced genetic diversity of the *Arthrobacter* population. Our data provided evidence that genetic differentiation of microbial populations was consistent with the changes of environmental factors, particularly heavy metals.

Keywords lead-zinc mine tailings, *Arthrobacter*, phylogenetic analysis, randomly amplified polymorphic DNA, genetic structure

1 Introduction

Recently, determining the effect of a long-term, chronic exposure of a chemical contaminant on the genetic diversity of natural populations (Bickham et al., 2000) has become an emerging field. As an important anthropic contaminant, heavy-metal is relatively more resistant to biodegradation than organic pollutants, thus very persistent in the environment. Therefore, its effects on genetic structure of organisms should be given more attention to. Related works have been carried out to investigate their effects on plant populations (Bergmann and Hosius, 1996; Duan et al., 2000). To soil microorganisms, most works have been limited in investigating changes in total microbial biomass, metabolic activity, and community structure (Insam et al., 1996; Kandeler et al., 1996; Pennanen et al., 1996; Roane and Kellogg, 1996; Knight et al., 1997; Kuperman and Carreiro, 1997; Giller et al., 1998; Barajas et al., 1999; Fritze et al., 2000), but little is known about changes in genetic diversity of stressed microbial populations.

It is a challenge to determine the genetic variation of free-living microbial populations. Time-consuming isolation and identification of microbes make it difficult to obtain phylogenetically close populations from different geographical locations. Previously, we reported the chemoorganotropic bacteria of three lead–zinc tailings, abandoned nearly 10, 20, and 80 years ago, respectively (Zhang et al., 2003; 2004a; 2004b), and observed that all three lead–zinc tailings had one kind of numerically dominant bacterial colony with creamy and nondiffusionable (CND) morphology. These colonies possibly belong to one narrow taxonomic group (i.e., one species) thus it will surpass the difficulty faced in most normal soil environments.

Additionally, soluble metal concentration of mine tailings usually increased with the history of being abandoned (Zhang et al., 2003). This would exert stronger selective pressure to microorganisms in older tailings. Therefore, it is expected that these geographically distinct bacteria with closely phylogenetic position tend to differentiate in genetic structure due to their adaptation to the different metal concentrations. In the present study, phylogenetic positions of these CND bacteria were determined based on their partial 16S rRNA gene sequences and their genetic differentiation represented

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by randomly amplified polymorphic DNA (RAPD) patterns in different tailings was characterized.

2 Materials and methods

2.1 Sampling

Six samples, designated as A1, A2, B1, B2, C1, and C2, were obtained from 5−10 cm and 25−30 cm from the top of three tailing piles on the same day in September 2001. These samples were characterized in detail chemically and physically in a previous report (Zhang et al., 2003, 2004a).

2.2 Phylogenetic analysis

A total of sixty CND strains were isolated from the samples, and stored on TY solid medium (Zhang et al., 2004b). Their chromosomal DNA was extracted using the Bacteria Genomic DNA Isolation Mini Kit (WATSON), then an approximately 1500-bp fragment of 16S rRNA gene was amplified using a forward primer (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Edwards et al., 1989). Chromosomal DNA of 20 to 100 ng was amplified in 50 μL of the following reaction mixture: 5 μL of 10 × PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$), 4 μL of dNTP mixture (2.5 mM of each dNTP) (TaKaRa), 1.25 U *Taq* polymerase (TaKaRa), 1 μL of each primer (final concentration 0.4 μM). PCR amplification was done in a DNA thermal cycler (GeneAmp PCR system) as follows: 4 min at 94°C to denature DNA, 32 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, 2 min extension at 72°C, and a final extension step of 10 min at 72°C.

The resulting PCR products underwent electrophoresis and then were purified using the Gel Extraction Mini Kit (WATSON). The purified PCR products were sequenced with an ABI PRISM 377−96 sequencer, using BigDye as terminator.

According to the BLAST, the 16S rRNA gene sequence was aligned with the 16S rRNA gene of other *Arthrobacter* species and closely related microorganisms of other genera by using the program CLUSTAL X (ver. 1.8). The *Streptomyces ambofaciens* was included as an outgroup. Nucleotide positions that had gaps were excluded from subsequent phylogenetic analysis. Neighbor-joining (NJ) analysis was also performed by using CLUSTAL X. The NJ tree was constructed from the distance matrix calculated by the algorithm of Kimura's two-parameter model (Kimura, 1980). Bootstrap confidence values were obtained with 1000 re-samplings.

2.3 Randomly amplified polymorphic DNA (RADP) analysis

To determine the genetic variation, RAPD profile was produced by integration of RAPD patterns amplified with five random 10-mer primer 5′-CAG CGA CAA G-3′, 5′-GTG ACA GGC T-3′, 5′-AAT CGG GCT G-3′, 5′-GAA ACG GGT G-3′, and 5′-GTG ATC GCA G-3′. Each 20 μL RAPD reaction mixture contained the following reagents: 0.5 μL of chromosomal DNA, 2 μL of 10 × PCR buffer (100 mM Tris–HCl, Ph 8.3; 500 mM KCl; 15 mM $MgCl_2$), 1 μL of dNTP mixture (2.5 mM of each dNTP) (TaKaRa), 0.2 U *Taq* polymerase (TaKaRa), 2 μL of primer (final concentration 0.4 μM), and 14.3 μL of distilled water. PCR amplification was done in a DNA thermal cycler (GeneAmp PCR system) as follows: two cycles of 4 min at 94°C, 2 min at 35°C, and 2 min at 72°C, then 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 35°C, 2 min extension at 72°C, and a final extension step of 5 min at 72°C. Images were calibrated and data analysis was performed by using Gene Tools of SynGene, with a match tolerance equivalent to 2.0% of the molecular weight of each band. The presence or absence of major bands was recorded in a binary matrix. The genetic distance and gene diversity were calculated as described by Nei (1978), using Popgene program (ver. 1.32).

2.4 Statistical analysis

Relation analysis was performed by using the SPSS program (SPSS Inc. USA, Release 10.0, 1999). $P < 0.05$ was the criteria of significant difference.

Table 1 GenBank accession numbers and length of 16S rRNA gene sequence of 48 *Arthrobacter* strains

Strains	Accession No.	Length/bp	Strains	Accession No.	Length/bp	Strains	Accession No.	Length/bp	Strains	Accession No.	Length/bp
AS11	AY371216	553	AL16	AY371207	483	BS15	AY371238	567	BL19	AY371233	474
AS12	AY371217	548	AL17	AY371208	553	BS16	AY371239	478	CS11	AY371253	587
AS13	AY371218	632	AL18	AY371209	534	BS17	AY371240	505	CS12	AY371254	587
AS14	AY371219	492	AL19	AY371210	483	BS18	AY371241	536	CS13	AY371255	535
AS15	AY371220	540	AL20	AY371211	534	BL11	AY371225	531	CS14	AY371256	600
AS16	AY371221	676	AL21	AY371212	599	BL12	AY37126	553	CS15	AY371257	553
AS17	AY371222	543	AL22	AY371213	536	BL13	AY37127	553	CS16	AY371258	615
AL11	AY371202	587	AL23	AY371214	524	BL14	AY37128	529	CL11	AY371246	586
AL12	AY371203	553	BS11	AY371234	483	BL15	AY37129	495	CL12	AY371247	535
AL13	AY371204	538	BS12	AY371235	493	BL16	AY371230	553	CL13	AY371248	520
AL14	AY371205	542	BS13	AY371236	562	BL17	AY371231	582	CL14	AY371249	553
AL15	AY371206	600	BS14	AY371237	561	BL18	AY371232	563	CL15	AY371250	552

3 Results

3.1 Phylogenetic position of strains

In TY medium, CND colonies were numerically dominant. All the 60 randomly selected colonies with this morphology belonged to *Arthrobacter* according to the analysis on their partial 16S rRNA gene sequences (fragment lengths ranging from 474 to 708 bp). These strains were placed into three phylogenetically distinct groups of *Arthrobacter* genus (data not shown). The largest group included 48 strains (Table 1) and was phylogenetically close to *A. keyseri* and *A. ureafaciens* (Fig. 1). Excluding two known species, 48 sequence similarities ranged from 99.1% to 100%. Additionally, these strains showed high similarity in cell morphology and utilization of 48 carbon sources (data not shown). These data implied that they had very close phylogenetical position at species level. Because these individuals were approximately distributed in three tailing piles, forming three geographically distinct subpopulations A, B, and C, or six geographically distinct subpopulations A1, A2, B1, B2, C1, and C2 if further subdivided according to the layers of 5−10 cm and 25−30 cm of each tailing pile, consequently we made further analysis on their genetic variation.

3.2 Genetic differentiation of *Arthrobacter* populations

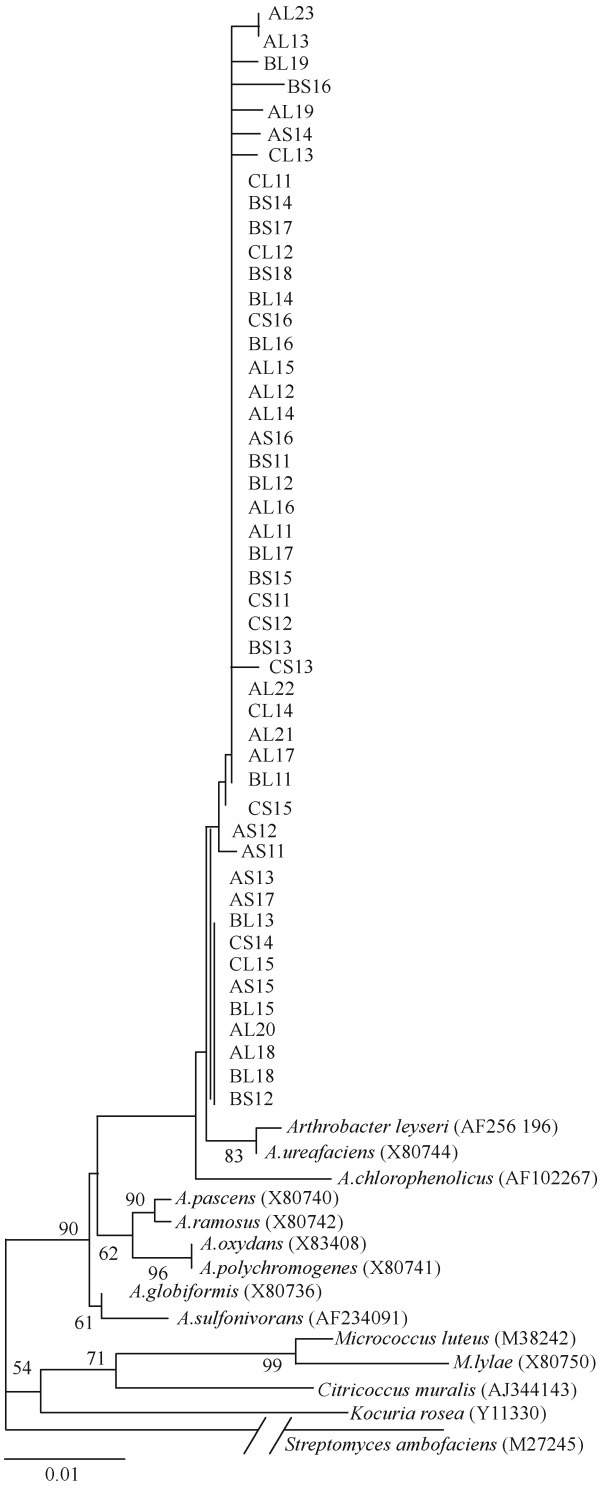
One hundred 10-mer primers were screened for positive signal of PCR amplification. Among 19 primers with positive amplification, 5 primers, 5′-CAG CGA CAA G-3′, 5′-GTG ACA GGC T-3′, 5′-AAT CGG GCT G-3′, 5′-GAA ACG GGT G-3′, and 5′-GTG ATC GCA G-3′, were used to amplify 48 strains.

Randomly amplified polymorphic DNA (RAPD) analysis was successfully performed on 44 strains. On the average, each strain produced 11 DNA bands, however, no significant difference in band number was found among the three populations (Table 2). The patterns generated with each of the primers were combined for each isolate, and the presence or absence of major bands was recorded in a binary matrix. A total of 40 gene loci were determined and 39 loci were polymorphic loci. Nei genetic distance was 0.35 among 44 strains. Genetic differentiation was detectable between subpopulations in different tailing piles. For example, distance was 0.043 between subpopulation A and B, 0.058 between A and C, and 0.034 between B and C. The greatest differentiation occurred between subpopulation A and C. Notably, genetic distance between subpopulations from the

Table 2 The number of RAPD bands produced by amplification of 5 primers

Populations	A ($n = 20$)	B ($n = 16$)	C ($n = 8$)
Number of bands ± SE	12.35 ± 0.57	11.88 ± 0.66	10.88 ± 1.16
χ^2 test		$\chi^2 = 1.778$, $P = 0.411$	

n: the number of strains detected



Streptomyces ambofaciens was used as an outgroup; The numbers at the nodes indicated the percentages of occurrence in 1000 bootstraped tree; Only values that were 50% or greater were shown. For each previously described strain, the GenBank accession number was indicated in parentheses and for 48 tailing strains, described in Table 1

Fig. 1 Phylogenetic tree constructed from the 16S rDNA sequences of 48 strains isolated from tailings and its closest relatives

Table 3 Nei distance of geographically distinct subpopulations of *Arthrobacter*

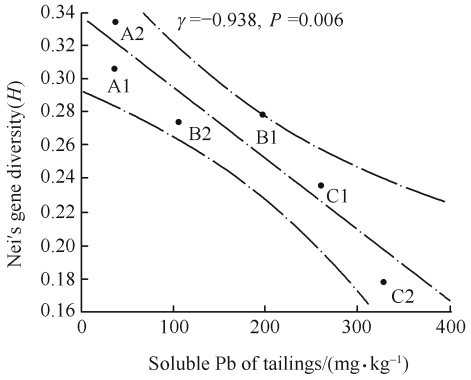
Subpopulation	A1 (n = 7)	A2 (n = 13)	B1 (n = 8)	B2 (n = 8)	C1 (n = 5)	C2 (n = 3)
A1						
A2	0.063					
B1	0.089	0.075				
B2	0.119	0.050	0.072			
C1	0.121	0.078	0.056	0.067		
C2	0.091	0.098	0.068	0.114	0.085	

Data in box emphasized the difference between the layers of 5–10 cm below surface and 25–30 cm below surface in each tailing pile

layer of 5−10 cm and 25−30 cm of each tailing pile increased with the history of tailings (Table 3). For example, distance was 0.063 between subpopulation A1 and A2. However, this number increased to 0.072, 0.085 between subpopulation B1 and B2, and between C1 and C2, respectively. Similarly, distance between different piles in the same depth also increased with history. For example, distance was 0.089 between A1 and B1, but 0.121 between A1 and C1. At deeper layer, distance was 0.050 between A2 and B2, but 0.098 between A2 and C2.

3.3 Correlation analysis

To rule out which chemical factor might be responsible for genetic differentiation of the *Arthrobacter* population, correlation analysis between environmental factors and Nei' gene diversity (H) of six subpopulations was carried out. Among nine environmental factors, involving total and soluble metals (Pb, Zn, and Cd), pH and organic biomass, only soluble Pb showed a significantly negative relationship with Nei' gene diversity of the subpopulation (Fig. 2).



Dash lines: 95% of confidence internal for means

Fig. 2 Correlation between Nei's gene diversity of *Arthrobacter* subpopulation and soluble Pb of tailings

4 Discussion

In the field, because many environmental factors involving water, oxygen, and pH, etc., can affect microbial growth at macro-, meso-, and microscale level (Holden and Firestone, 1997), different geographical locations appear to accommodate genetically and physiologically distinct microbial populations with phylogenetically close relationship. However, most previous reports have focused on the genetic differentiation of several pathogens (Eldar et al., 1999; Gray et al., 1999; Souza et al., 1999; Grayson et al., 2000), and little is known on the genetic variation of stressed microbial population under chemical pollutants. A major limitation was time-consuming isolation and identification of microbes with phylogenetically close microbes. In this study, high concentrations of metals selected dominant microbes with close phylogenetics (Fig. 1) and allowed us to carry out such investigations.

Nei genetic distance represented by RADP patterns showed that either genetic distance between subpopulations from the layer of 5−10 cm and 25−30 cm of each tailing pile or between same layers of different tailing pile increased with the history of tailings (Table 3). Because spatial chemical heterogeneity of tailings, caused by long-term natural processes, such as flooding of rainfall and plant colonization, was also significant (Zhang et al., 2004a), which implied that there were actually environmental effects on shaping genetic structure of phylogenetically distinct groups. Particularly, correlation analysis showed that soluble Pb was significantly negatively related with Nei' gene diversity of subpopulation (Fig. 2). It was assumed that increased soluble Pb in tailings may be responsible for the reduced genetic diversity of the *Arthrobacter* population.

However, due to the complexity of environmental factors in nature, together with unclear original genetic background and selection experience of these *Arthrobacters* in tailings, it is presently very difficult to rule out the affirmatory factor responsible for their evolution under heavy-metal stress. In the future, more research works are needed to evaluate the effects of chemical pollutants on genetic structure of soil microbes.

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