

Genetic and physiological diversity of phylogenetically and geographically distinct groups of *Arthrobacter* isolated from lead–zinc mine tailings

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Abstract

The phylogenetic positions of 60 bacterial strains isolated from three tailing piles were determined by analyzing their partial 16S rRNA gene sequences. These strains were divided into three phylogenetically distinct groups of *Arthrobacter* and likely represented several non-described species. The physiological diversities of these phylogenetically and geographically distinct populations were evaluated based on their resistance to five heavy metals (Zn, Pb, Cd, Cu and Co) and four antibiotics (Kan, Rif, Str and Amp), and differences in utilization of 49 carbon sources. Genetic differentiations were demonstrated with randomly amplified polymorphic DNA (RAPD) analysis. These biological parameters were significantly different among three phylogenetically distinct groups. Notably, detectable differences were also observed among three geographically distinct subdivisions with similar taxonomic position. These results indicate that mine tailings are an ideal site for investigating the differentiation of natural bacterial populations in response to extreme metal contamination. Additionally, these environments appear to harbor many previously not characterized bacterial species, which are potentially important candidates for application in bioremediation due to their remarkable resistance to multiple metals.

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Keywords: Lead–zinc tailings; *Arthrobacter*; Phylogenetic analysis; Physiological and genetic diversity

1. Introduction

Ecotoxicological effects of heavy metals on soil microbes have been recently widely investigated at the ecosystem level using culture-independent methods including BIOLOG microplate techniques [1–3], DNA-based approaches [4–6], and phospholipid fatty acid analyses (PLFA) [7,8]. The majority of these studies are concerned with the microbiological differences at the community level between non-polluted and contami-

nated sites. Little is known about the phylogenetic position and physiology of microbial populations that have adapted appropriate physiological and genetic characteristics to allow them to survive in highly toxic environments.

In the areas of evolutionary biology and conservation biology, it is routine to investigate the physiological and genetic variation in natural animal or plant populations [9]. Although different geographical locations appear to harbor physiologically and genetically distinct bacterial populations, this has not been extensively investigated in naturally-occurring bacterial populations. Until recent years there have been a handful of such studies

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performed on several pathogenic microbial populations [10,11], and one non-culturable bacterium [12]. These studies indicate that there are molecular variations between populations from different geographic locations. Another study demonstrates the effects of geographic location and host taxonomic group on the genetic differentiation for *Escherichia coli* [13]. However, no work has been carried out to examine the effects of heavy metals on microbial populations.

Tailings are one of the large-volume wastes produced in the metal industry, and contain a high variety of metals including Pb, Zn, Cu, Cd and Ni [14–16]. Tailings piles have loose texture and low water capacity. They are frequently devoid of vegetation, or have only sparsely growing shrubs and/or grasses [15]. Tailing could thus be viewed as an extreme heavy-metal-contaminated environment that has adverse impacts on biological activity. Nonetheless, only few investigations have demonstrated that there are diverse lithotrophic and chemoorganotrophic (COT) bacteria in sulfidic and uranium tailings [17–21].

In previous experiments we investigated the chemoorganotrophic bacteria of three lead–zinc tailings, abandoned nearly 10, 20 and 100 years ago, respectively (Zhang et al., unpublished results). It was observed that all three lead–zinc tailings had one kind of numerically dominant bacterial colony type growing on rich medium. In some cases, the colony morphology was uniform and appeared to be a pure culture. We presumed that: (1) these colonies belonged to a group of phylogenetically close bacteria, dominating in the presence of a strong selective pressure of high metal concentrations; (2) these colonies from three tailings must differentiate in their physiological and genetic characteristics due to their adaptation to the different metal concentrations of tailings.

We conducted further studies to elaborate the phylogenetic positions of these chemoorganotrophic bacteria. In the experiments presented herein we tested the hypothesis that different tailing piles select genetically and physiologically distinct bacterial subpopulations with similar taxonomic position. The results of this study provide information on the characteristics of chemoorganotrophic bacteria able to withstand extreme metal-contaminated environments, with potential application in bioremediation of heavy metal-contaminated environments in future.

2. Materials and methods

2.1. Site description and sampling

The county of Huize, Yunnan province, China, has several hundred years of producing the lead and zinc. There are some ancient tailing piles that have been abandoned and are untouched since 1920s. Around in 1980s and 1990s, sporadic smelting activities of peasants produced some tailings again. Therefore three tailing piles A, B and C, with ages of approximately 10, 20 and 100 years, respectively, were located in a relic site: pile A located at 26°35'78"N lat., 103°39'28"E long. with an altitude of 2180 m, B located at 26°35'79"N lat., 103°39'28"E long. with an altitude of 2200 m, and C located at 26°35'75"N lat., 103°39'37"E long. with an altitude of 2250 m.

Samples were collected at about 5–10 and 25–30 cm from the top of the tailings pile surface and were immediately brought back to the laboratory in sealed sterilized plastic bags. Samples were screened using 2 mm mesh screens and immediately analyzed chemically and biologically. Their chemical characteristics were reported previously [22]. In this report only soluble Pb, Zn, Cd and pH were shown in Table 1. Biological analyses were carried out as described below.

2.2. Enumeration and isolation of strains

Solid TY medium (1000 ml distilled water containing 3 g of beef extract, 5 g of peptone, 3 g of tryptone and 15 g of agar, pH 7.2–7.4) was used to culture bacteria. A sample (10 g) of each sieved tailing was put into a 250 ml conical flask containing 90 ml washing solution [4] with glass beads and shaken for 20 min. Then the supernatant (or diluent of the supernatant) was spread on the solid plates, three plates for each dilution. After being cultured for 9 days at 30 °C, colonies were observed and counted. The data were expressed as colony forming units (CFU) per gram dry weight of each sample.

On the plates with well-separated colonies, total colonies and one numerically dominant type of creamy and non-diffusible (CND) colony were enumerated. To obtain enough CND colonies, optimal dilutions (i.e., 10^{-3} and 10^{-4}) were spread on ten plates. Then 60 CND

Table 1
Chemical characteristics of the tailing piles^a

Tailing piles ^b	Soluble Pb (mg kg ⁻¹)	Soluble Zn (mg kg ⁻¹)	Soluble Cd (mg kg ⁻¹)	pH
A	35.0	27.9	17.5	7.2
B	151.6	27.8	18.0	6.8
C	295.5	26.8	5.8	5.7

^a The means of the layers of 5–10 cm below surface and 25–30 cm below surface.

^b Letter A, B and C represent 10-year, 20-year and 100-year tailings, respectively.

colonies were chosen randomly and re-streaked on the TY solid plates to obtain purified isolates.

2.3. Analysis of 16S rRNA gene

To determine the phylogenetic position of above CND colonies, their chromosomal DNA was extracted using Bacteria Genomic DNA Isolation Mini Kit (WATSON). Forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer (5'-AAG GAG GTG ATC CAG CCG CA-3') [23] were used to amplify an approximately 1500 bp fragment of 16S rRNA gene. Chromosomal DNA (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 MgCl₂), 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, and 2 min extension at 72 °C; and a final extension step of 10 min at 72 °C. The resulting PCR products were examined by horizontal electrophoresis on a 1% agarose gel. Fragments of approximately 1500 bp were cut out and extracted using the Gel Extraction Mini Kit (WATSON). Purified PCR products were sequenced with an ABI PRISM 377-96 sequencer using BigDye as terminator. The forward primer was used as the sequencing primer. Sequences were subjected to an NCBI (National Center for Biotechnology Information) BLAST search to determine which microorganisms were most closely related to these strains.

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) [24]. *Streptomyces ambofaciens* was included as an outgroup. Nucleotide positions where there were gaps were excluded from subsequent phylogenetic analysis. Neighbor-joining (NJ) analysis [25] was also performed using CLUSTAL X. The neighbor-joining tree was constructed from the distance matrix calculated with the algorithm of Kimura's two-parameter model [26]. Bootstrap confidence values were obtained with 1000 resamplings.

2.4. Physiological analysis

The utilization of 49 carbon sources was tested in liquid mineral medium ((NH₄)₂SO₄ 2.0 g, NaH₂PO₄ · H₂O 0.5 g, K₂HPO₄ 0.5 g, MgSO₄ · 7H₂O 0.2 g, CaCl₂ 0.1 g, 1000 ml of distilled water) supplemented with 0.5% of each carbon source. Briefly, 1 ml of 24-h old culture was centrifuged and the cell pellet was flooded

twice and re-suspended with 1 ml of 0.85% (w/v) NaCl. Cell suspension (0.01 ml) was transferred into 10 ml of above fresh liquid medium. After aerobic growth for 72 h at 30 °C, an increase in turbidity greater than that observed in the carbon-free broth inoculated as a control was considered to represent growth on the offered carbon source.

To determine the resistance of heavy metals and antibiotics, aqueous heavy metal stocks of 1 mol l⁻¹ of ZnSO₄, Pb(NO₃)₂, CdCl₂, CuSO₄ and CoCl₂, and antibiotics stocks of 1 g l⁻¹ of streptomycin, rifampicin, kanamycin, and ampicillin were sterilized (heavy metals were autoclaved and antibiotics were filter-sterilized) and added to liquid TY medium with final heavy metal concentrations ranging from 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 16 to 32 mmol l⁻¹ or antibiotics concentrations ranging from 0.5, 1, 2, 4, 6, 8, 16, 32, 64 to 128 mg l⁻¹. After growth for three days (for detecting antibiotics resistance) or 15 days (for detecting heavy metal resistance) at 30 °C, the minimal concentration for inhibition (MIC) of cell growth was recorded.

2.5. RAPD analysis

Firstly we screened 100 oligonucleotide primers (size 10 bp) for the ability to produce a good band pattern with five *Arthrobacter* strains. Then five 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'; 5'-GTG ATC GCA G-3') were selected and used to obtain RAPD profiles of 60 strains. 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 MgCl₂), 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: 2 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, and 2 min extension at 72 °C; and a final extension step of 5 min at 72 °C. The RAPD products were electrophoresed at 80 V for approximately 3 h on a 1.5% agarose gel. Then the gels were stained with ethidium bromide and scanned with Gene Snap of SynGene. Images were calibrated and data analysis was performed using Gene Tools of SynGene, a match tolerance equivalent to 2.0% of the molecular weight of each band was used. Very faint bands were excluded from the analysis and the presence or absence of major bands was recorded in a binary matrix. The patterns generated with each of the primers were combined for each isolate, and the genetic distance was calculated as described by Nei [27] using Popgene program (ver. 1.32). Then

neighbor-joining tree was constructed from distance matrices using MEGA2 program (ver. 2.1).

3. Results

3.1. The total number of culturable bacteria and isolation frequency of *Arthrobacter* spp.

In TY medium, the population size of the culturable bacteria was similar either between three tailing piles or the layers of 5–10 cm below surface and 25–30 cm below surface. The samples collected at 25–30 cm depth from each of the three tailings were also similar, with approximately 6.7×10^5 CFU g^{-1} dry tailings. However, in samples collected at 5–10 cm depth, it was about two-fold higher in tailings B and C than tailing A (Table 2).

Among these culturable bacteria, creamy and non-diffusionable (CND) colonies were numerically dominant. Because each of the 60 randomly selected colonies with this morphology was related to *Arthrobacter* strains (see analyses below), the isolation frequencies of *Arthrobacter* strains were roughly estimated as the frequencies of CND colonies. The average percentages of *Arthrobacter* isolated from samples of both depths at each tailings pile were 55.5%, 61.0% and 34.0% for A, B and C, respectively, without significant difference. However, the layer of 5–10 cm yielded about 88.6% *Arthrobacter* spp., which is approximately three times higher than that of the deeper layer (25–30 cm) sample, which had an average of 34.7%.

3.2. Phylogenetic analysis

The partial 16S rRNA gene sequence (from 474 to 708 bp) was determined for a total of 60 isolates from

three tailings. These strains were placed into three phylogenetically distinct groups of the genus *Arthrobacter* (Fig. 1). The largest group, group 1 (G1), included 48 strains and was mostly closely related to *A. keyseri* and *A. ureafaciens*. Sequence similarity ranged from 95.8% to 100% and no nucleotide substitution occurred among most of our sequences (excluding *A. keyseri* and *A. ureafaciens*, 48 sequence similarities ranged from 99.1% to 100%), indicating that these isolates were phylogenetically very close. In this group, 20 strains were from tailing A, representing 87.0% of the all isolates (23). Seventeen strains were from tailing B and 11 from tailing C, which is about 81% (17/21) and 68.8% (11/16), respectively. The distribution was different among three tailings ($\chi^2 = 17.83, P < 0.001$). In total, there were 21 *Arthrobacter* strains from the 5–10 cm layer (about 44% of 60 isolates) and 27 from the 25–30 cm layer (56%). Statistically there was no significant difference between the *Arthrobacter* frequency of the two layers ($\chi^2 = 0.75, P = 0.39$).

Group 2 (G2) included nine strains, two from tailing A, four from B and three from C. Sequence similarity ranged from 95.8% to 100%. This group was most closely related to *A. chlorophenicus*. Spatially, seven out of nine *Arthrobacter* strains originated from the 5–10 cm layer. Group 3 (G3) included only three strains with sequence similarity ranging from 99.0% to 100%. They showed a close relationship with two species, *A. aurescens* and *A. ilicis*.

3.3. Physiological diversities

Among 49 carbon sources tested, five carbon sources including sucrose, glucose, dextrin, maltose and glycerol could be utilized by all strains, while 15 carbon sources including D-arabinose, glycogen, aesculin, thymidine,

Table 2
Isolation frequencies of *Arthrobacter*-like strains

Samples ^a	Culturable bacteria ($\times 10^5$ CFU g^{-1} of tailings) ^b	Depth-integrate average	CND ^c colonies			Non-CND ^c	Total observed colonies	<i>Arthrobacter</i> colonies (%)	Depth-integrate average
			Total	Sequenced	<i>Arthrobacter</i>				
A1	6.0 \pm 1.0	6.8	255	9	9	34	289	88.2	55.5
A2	7.5 \pm 1.5		228	14	14	354	582	39.2	
B1	16.3 \pm 0.3	11.6	207	12	12	25	232	89.2	61.0
B2	6.8 \pm 1.3		60	9	9	146	206	29.1	
C1	12.0 \pm 0.5	9.0	22	9	9	3	25	88.0	34.0
C2	5.9 \pm 1.5		26	7	7	90	116	22.4	
Total			798	60	60	652	1450		
Statistical significance ^c	NS ^d	NS						*** ^d	NS

^a A, B and C represent 10-year, 20-year and 100-year tailings, respectively. Number 1 and 2 represent the layers of 5–10 cm below surface and 25–30 cm below surface, respectively.

^b The mean \pm 1 SE of two measurements.

^c Significance levels were determined by one-way ANOVA. NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^d Between the layers of 5–10 cm below surface and 25–30 cm below surface.

^e CND, creamy and non-diffusionable colonies.

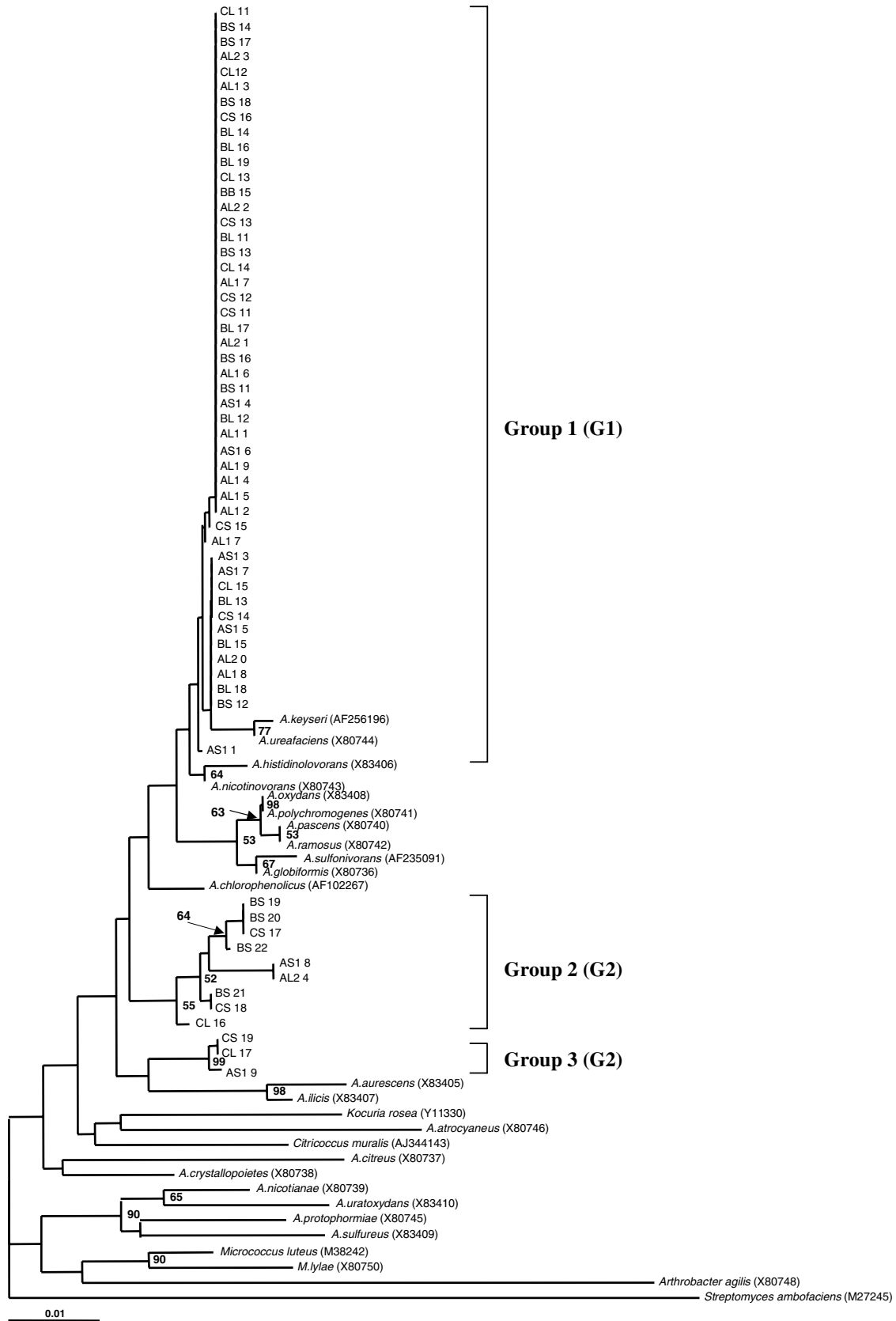


Fig. 1. Phylogenetic tree constructed from the 16S rRNA gene sequences of 60 strains and its closest relatives. *Streptomyces ambofaciens* was used as outgroup. The numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped tree; only values that are 50% or greater are shown. The GenBank Accession No. (in parentheses) is indicated for each previously described strain, and our 60 tailing strains are registered in Genbank as from AY371202 to AY371261.

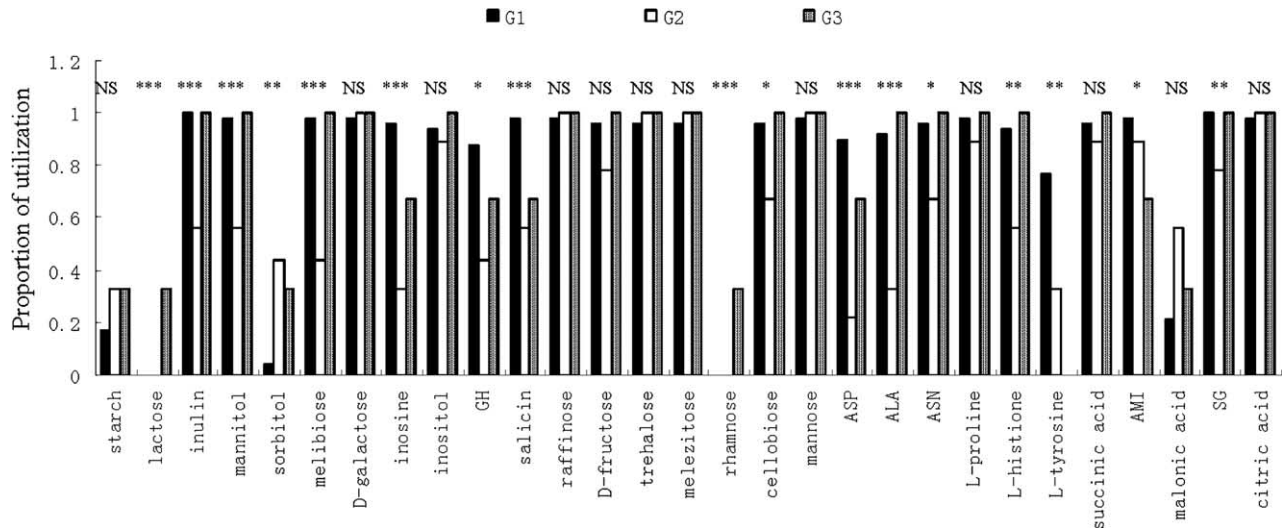


Fig. 2. Variation in utilization of 29 carbon sources among three phylogenetically distinct groups. GH (glucosamine hydrochloride), ASP (L-aspartic acid), ALA (DL- α -alanine), ASN (L-asparagine), AMI (γ -aminobutanoic acid), SG (sodium gluconate). Significance levels were determined by χ^2 test. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. G1, G2 and G3 represent three phylogenetically distinct groups of *Arthrobacters* (see Fig. 1).

urea, L-leucine, L-serine, hydroxy-L-proline, itaconic acid, sebacic acid, formic acid, acetate, propionic acid, phenylalanine and tween 80, could not be utilized by any of the strains. Statistical significance of the utilization of the other 29 carbon sources was investigated performing a χ^2 test. This established that usage of 17 carbon sources differed significantly between the three phylogenetically distinct groups (Fig. 2). The differences within G1 and G2 strains, further subdivided geographically according to three tailings, were also analyzed. The five carbon sources starch, inositol, trehalose, DL- α -alanine and malonic acid were utilized in a statistically different manner among three subdivisions of G1, but without differences among three subdivisions of G2 (data not shown). Geographical diversity of G3 was excluded from statistical analysis because the strain number was too small.

The three phylogenetically distinct groups were significantly different in their resistance to Zn ($\chi^2 = 8.774, P = 0.012$). Average minimal concentrations for inhibition ranged from 8.67 mmol l⁻¹ for G3 to 25.71 mmol l⁻¹ for G1. However, no difference was observed in resistance to Pb, Cu, Co and Cd; average MICs were 7.20, 9.63, 3.71 and 1.54 mmol l⁻¹, respectively (Fig. 3(a)). Resistance to Kan, Rif, and Amp was also significantly different between the three phylogenetically distinct groups G1, G2 and G3 ($\chi^2 = 13.409, P = 0.001$ for Kan; $\chi^2 = 25.508, P < 0.001$ for Rif; $\chi^2 = 9.271, P = 0.012$ for Amp) (Fig. 3(a)). Resistance to Str was similar between G1, G2 and G3, with an average MIC of 3.2 $\mu\text{g ml}^{-1}$. Geographically, G1 strains only differed from one another in resistance to Cd and Kan ($\chi^2 = 20.012, P < 0.001$ for Cd;

$\chi^2 = 7.135, P = 0.028$ for Kan) (Fig. 3(b)), but no difference was observed among G2 strains (data not shown).

3.4. RAPD analysis

RAPD analysis was successfully performed on 55 strains. Nei genetic distance was 0.0736 between G1 and G2, 0.0481 between G1 and G3, and 0.0225 between G2 and G3. Excluding the G3 populations, the differentiation between geographically-related subdivisions was analyzed. Relative to subdivision A, there was a smaller genetic distance between subdivision B and C, in either G1 or G2. Moreover, the divergence within G1 is smaller than those of G2 (Fig. 4).

4. Discussion

In three lead–zinc mining tailings, which contained extremely high soluble heavy metals (Table 1), numerically dominant colonies appeared to be members of the genus *Arthrobacter* (Table 2). In heavy-metal-contaminated soils the dominant culturable bacteria are frequently gram-negative bacteria (i.e., *Ralstonia*), which harbor a megaplasmid containing a gene cluster for heavy metal-resistance [28–30]. Such a large number of *Arthrobacter* isolates from tailings suggests the potential of gram-positive bacteria to resist heavy metals. It is possible that this resistance is also related to a megaplasmid-mediated resistance mechanism. *Arthrobacters* are considered to be ubiquitous and predominant members of culturable soil microorganisms [31,32].

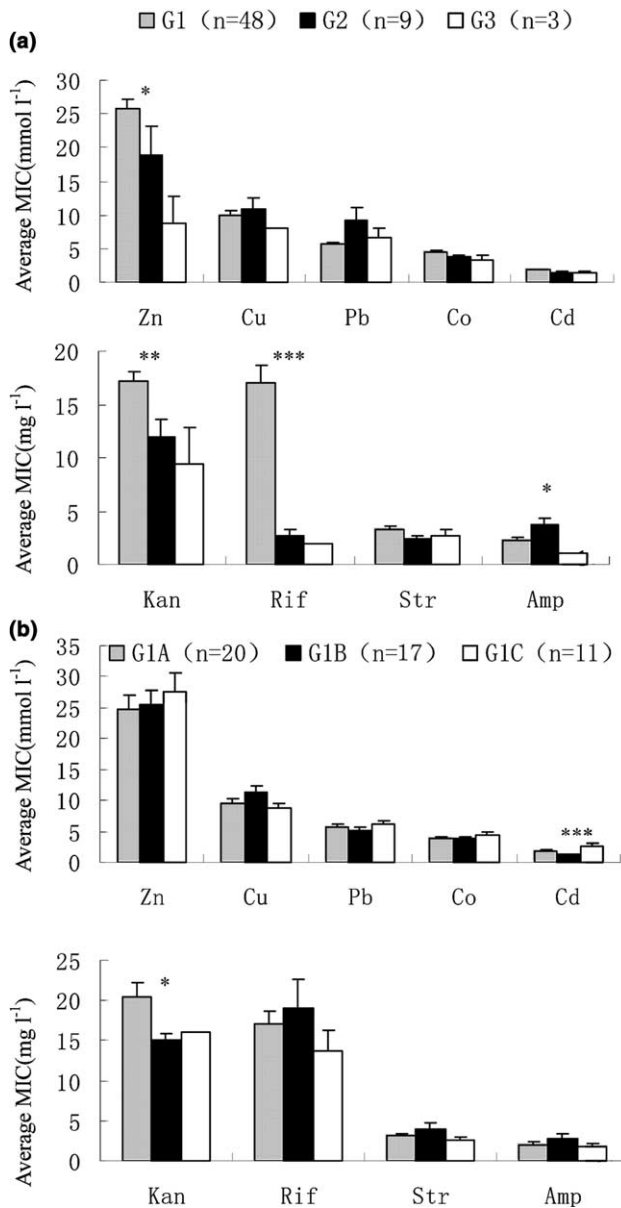


Fig. 3. Variation in MIC values of heavy metals and antibiotics between three phylogenetically distinct groups G1, G2 and G3 (a) and three geographically distinct subdivisions G1A, G1B and G1C from G1 strains (b). *n* represents the number of strains tested. Notes of significance levels see Fig. 2. Error bar represents +SE.

Some studies have reported that 20% of isolated bacteria in sediments were *Arthrobacter* spp. [33,34]. In the present study, *Arthrobacter* isolates from the three mine tailings constituted 30% of the population from layers of 25–30 cm depth, and 90% from 5–10 cm depth (Table 2), indicating that arthrobacters are ubiquitous in these mine tailings. However, these frequencies may be over-estimates due to the bias of using only one culture medium in the isolation protocol. Future investigations are needed to elaborate the abundance of culturable and non-culturable species by extracting DNA directly from tailings and analyzing their fingerprinting.

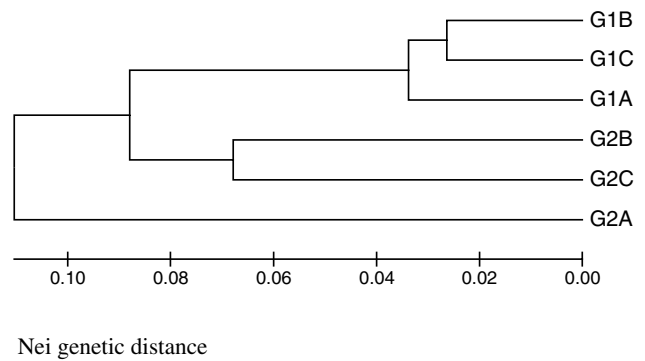


Fig. 4. Nei distance calculated using RAPD data of three geographical subdivisions G1A, G1B and G1C from G1 strains and three subdivisions G2A, G2B and G2C from G2 strains.

Arthrobacter spp. can utilize a wide variety of organic components and play an important role in transformation of organic matter in natural environment [35]. However, their ecological role in these lead–zinc tailings is not clear. Moreover, although numerically dominant colonies appeared to be *Arthrobacter*-like bacteria (Table 2), sequence analysis of 16S rRNA gene showed that the 60 strains included at least three species (Fig. 1). Does it imply that, relative to other genera, at the genus level *Arthrobacter* has an advantage in surviving in mine tailings? In contaminated soils, most studies have been performed to investigate microbiological parameters at a community level, and thus cannot establish whether or not dominant bacteria involve only a few common genera. Biological approaches to remediation of metal-contaminated mine tailings are desirable, for reasons including low cost and ease of applications, for example, recently, phytoremediation of mine tailings has been evaluated using the transgenic Indian mustard plants [36]. Therefore as important background information for bioremediation it is necessary to extensively investigate the taxonomic position of microorganisms surviving in mine tailings.

Recently, environmental effects that modify the physiological and genetic characters of microorganisms were described, either in natural microbial populations [10–13] or in laboratory-evolved subpopulations from one ancestor [37–39]. The free-living *Arthrobacter* strains isolated in this study were physiologically and genetically diverse (Figs. 2–4). There were also detectable differences among three geographically distinct subpopulations of G1 in their utilization of five carbon sources and resistance to Cd and Kan (Figs. 2 and 3). Similarly, the Nei genetic distance calculated with RAPD data also showed the differentiation among arthrobacters from the three geographically distinct mine tailings (Fig. 4). A smaller genetic distance between subdivision B and C, in either G1 or G2, implies that there are actually environmental effects shaping genetic structure of phylogenetically distinct groups of ar-

throbacters. However, a smaller divergence within G1 than those of G2 indicates the latter is relatively sensitive to environmental modifications.

Metal contamination is an extreme environment to which microorganisms can respond quickly [4,40], making metal-contaminated sites ideal for testing the differentiation of populations under extreme selective pressure. However, because the genetic background of microbial populations in mine tailings is unknown, it is difficult to exactly judge the role of environmental factors in the modification of natural populations therein. In this study, we cannot categorically conclude whether or not the characteristics of microbial populations are directly related to the selective pressure of heavy metal toxicity. Nonetheless, the different contaminant concentration, notably soluble Pb changes in tailings from three different ages, does imply the possibility of environmental modification on the physiological and genetic characters of *Arthrobacter* living in three tailings due to the selection pressure of high metal concentration (Table 1). Further investigation is required to separate environmental factors for this differentiation through laboratory evolutionary experiments.

Overall, one type of numerically dominant colony investigated in this study may include more than three novel *Arthrobacter* species. Although their exact taxonomies need additional analyses such as DNA–DNA reassociation experiments and further biochemical data, this finding suggests that tailing piles could be used as a new source of novel heavy-metal-tolerant microorganisms. The remarkable resistance of these unique species to multiple heavy metals might be applicable in future bioremediation.

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