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EXPERIMENTAL ARTICLES

# Compositions of Microbial Communities in Sulfide Nickel Ore Waste Piles

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**Abstract**—Bacterial communities of moderately acidic waste piles of sulfide nickel ore and of the nickel-leaching enrichments obtained from them are analyzed. The structure of bacterial communities was determined by molecular biological techniques. The PCR profiles of bacterial communities were obtained with the primers to a variable 433 bp site of the eubacterial 16S rRNA gene. The differences in community compositions were determined by comparison of their DGGE profiles. Sequencing of the DNA fragments was then carried out and the results were compared with the GenBank gene sequences. Analysis of the 16S rRNA gene sequences revealed few bacterial genera in the moderately acidic waste piles of sulfide nickel ore, with predomination of *Acidithiobacillus* sp. and *Leptospirillum* sp. A number of the bacteria revealed belonged to the species never obtained in pure culture. Molecular biological analysis showed the presence of the same groups of bacteria in enriched cultures obtained by inoculating the liquid medium containing ground ore with the waste pile samples (5 : 1). The geochemical activity of these bacteria was confirmed by their capacity for leaching nickel from the sulfide ore in enriched cultures, resulting in nickel solubilization. Thus, new information was obtained concerning the structure composition of the bacterial communities of sulfide ore waste piles: the dominant forms were determined, their leaching activity was confirmed, and the activity of thiobacilli from the waste, which have not been isolated in pure cultures, was confirmed in liquid medium in the presence of ore.

*Keywords*: sulfide nickel ore, bacterial community, thiobacilli, *Acidithiobacillus, Leptospirillum*, molecular biological analysis, 16S rRNA, DGGE.

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The microbiological characteristics of ore waste piles have been investigated in a number of research projects for screening and isolation of microbial cultures for industrial bioleaching. These works, however, have been focused on isolation of the most geochemically active strains, rather than characterization of the community structure. For example, extremely acidophilic bacteria *Acidithiobacillus ferrooxidans* (previously, *Thiobacillus ferrooxidans* [1]) and *Leptospirillum ferrooxidans* are typical inhabitants of sulfide ores and may be also used as industrial microorganisms for nickel bioleaching [2, 3]. The presence of these microorganisms was shown for microbial communities of acidic mine waters [4].

We investigated the composition of bacterial communities of the samples of sulfide nickel ore provided by CANMET, Canada. The neutral or moderately acidic reaction of the samples and some results of preliminary experiments indicating a possibility of nickel leaching by moderate acidophiles [5] suggested predominance of neutrophilic lithotrophic microorganisms resembling, for example, *Alicyclobacillus ferrooxidans* [6]. The present work was therefore aimed at investigation of the occurrence and diversity of bacteria in ore waste piles with neutral pH.

Our investigation of bacterial diversity was supplemented by simulation experiments on nickel bioleaching from the waste piles by the aboriginal microbial community. Although bioleaching is usually associated with such extremely acidophilic bacteria as *A. ferrooxidans* and *L. ferrooxidans*, biogeocenoses with sulfide oxidation support a broad spectrum of chemolithotrophic and heterotrophic bacteria [7]. Earlier works did not answer the question of the microorganism predominating in the neutral zones, so that knowledge of the diversity and leaching activity of moderate acidophiles is insignificant.

Molecular biological investigation of microbial communities in the samples of similar ores and their waste revealed that most of the bacteria detected in

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these environments have not been isolated in pure culture, although they were phylogenetically close to the known species. For example, 16S rRNA cloning demonstrated the presence of new organisms belonging to the following groups or genera: *Leptospirillum*, *Thermoplasmales, Sulfobacillus, Acidimicrobium*, and *Acidiphilum*, which are known to prefer acidic environments [8, 9]. It was therefore important to carry out molecular genetic investigation of the structure of bacterial communities of neutral or moderately acidic waste piles and compare the results with previously published data on the structure of microbial communities in acidic ores. The next stage was comparison of the natural microbiota of the waste piles with the diversity of bacteria in laboratory enrichments capable of bioleaching of sulfide nickel ore aimed at determination of the active components.

Thus, the goal of the present work was to investigate the composition of the bacterial communities of neutral or moderately acidic waste piles of sulfide nickel ore, determine the dominant forms, and establish the possible activity of these bacteria (including uncultured forms) in the processes of leaching.

## MATERIALS AND METHODS

**Characterization of the ore.** The samples of nickel ore and its waste piles (samples A, B, and C) were provided by CANMET (Canada Centre for Mineral and Energy Technology). Nickel content in the ore did not exceed 1%. According to CANMET, the content of industrial metals was Ni, 0.8%; Cu, 0.2%; Fe, 13.9%; and Mg, 2.7%. The major sulfide minerals in the ore were pyrrhotite (33%), pentlandite (3%), and chalcopyrite (0.7%). Thus, the samples were a poor sulfide iron–nickel ore with overall sulfide content sufficient for growth of autotrophic sulfur-oxidizing bacteria. The content of reduced iron and, therefore, the possibility for growth of iron-oxidizing bacteria were not determined.

Magnesium hornblende $Ca_2(Mg, Fe)_4Al(Si_7Al)O_{22}(OH, F)_2$ (13%) and plagioclase $(Na, Ca)AlSi_3)_8$ (22%) were the predominant silicates. The nickel content in pentlandite and pyrrhotite was 36.3 and 0.7%, respectively.

Initially, the pH of unoxidized waste pit samples was neutral (pH ~7), while, after prolonged storage (for months), the upper 1- to 3-mm layer oxidized, with pH decreasing to 5.

**Molecular biological analysis of bacterial diversity.** Bacterial diversity in the original samples (A0, B0, and C0) and in the enrichments was carried out by separating the PCR fragments by denaturing gradient gel electrophoresis (DGGE). The fingerprints of microbial populations were obtained by amplification of 16S rRNA gene fragments with the primers U968GC and L1401 [11]. The sequences were compared to the GenBank sequences. The procedures are described in detail below.

Microbial cells in the ground samples of ore waste (5 g) were lysed. For direct DNA extraction, a MoBio Ultra Clean Soil DNA Kit (MoBio Laboratories, Canada), was used. Extracted DNA did not require additional purification. To obtain the optimal concentrations of the PCR amplification products, the solutions were diluted 20- to 50-fold. The primers U968GC (5'-CGC CCG GGG GGC GCC CCG GGC GGG GCG GGG GCA CGG GGG CAA CGC GAA GAA CCT TAC-3') and L1401 (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') were used. The U958GC sequence contained a 40-nucleotide GC clamp for improved DGGE separation of the fragments.

For PCR, AmpliTaq DNA polymerase and the Stoffel Kit buffer system (Applied Biosystems) were used. The reaction mixtures contained the following (in a ratio of 2.5 : 3.75 : 2 : 0.5 : 0.5 :0.5 : 0.5 : 0.5 : 0.5 : 0.5) : 10 Stoffel Buffer, 25 mM $MgCl_2$, 50° acetamide, 1.25 mM dATP, 1.25 mM dCTP, 1.25 mM dGTP, 1.25 mM dTTP, R-1401, F-968-GC, and 5 U AmpliTaq DNA polymerase. The samples were incubated in an Eppendorf Thermocycler (Netheler-Hinz, Germany) after addition of 1-μl DNA preparation (dilutions of the DNA preparation were from 1: 1 to 1 : 50). The programmed cycle was 5 min at 94°C. Amplification was carried out as follows: 35 cycles of 1 min at 94°C, 1 min at 53°C, 2 min at 72°C, and additionally 10 min at 72°C. Electrophoresis of the aliquots (5 μl) was carried out in 1% agarose gel; ethidium bromide staining was used. PCR products were separated by denaturing gradient gel electrophoresis (DGGE) in polyacrylamide gel with a gradient (30–80%) of denaturing factors (urea and formamide) at 65 V for 20 h in 1× TAE buffer at 60°C. The gel was stained with SYBR Green or silver nitrate.

The silver-stained DGGE bands were excised with a scalpel, transferred into 1.5-ml Eppendorf tubes with 0.5 ml of the decolorizing solution (6 mM $K_3Fe(CN_6)$, and incubated for 10 min at 37°C. The decolorizing solution was then removed and the gel bands washed twice with deionized water (MilliQ) for 5 min at 37°C. The DNA fragments were isolated from the gel according to the accepted procedure [10]. DNA was then reamplified and treated by DGGE in order to confirm its electrophoretic motility in the gel. Amplification of 16S rRNA fragments from the bands and cloning of the PCR products were carried out as described in [11]. The cloning was carried out using the pGEM-T Easy Vector System I (Promega) according to the manufacturer's recommendations. The cloned DNA fragments were sequenced with the standard M13 primers.

The PCR fragments contained in the major excised gel bands were reamplified. This band usually contained one homogeneous amplicon. Silver was

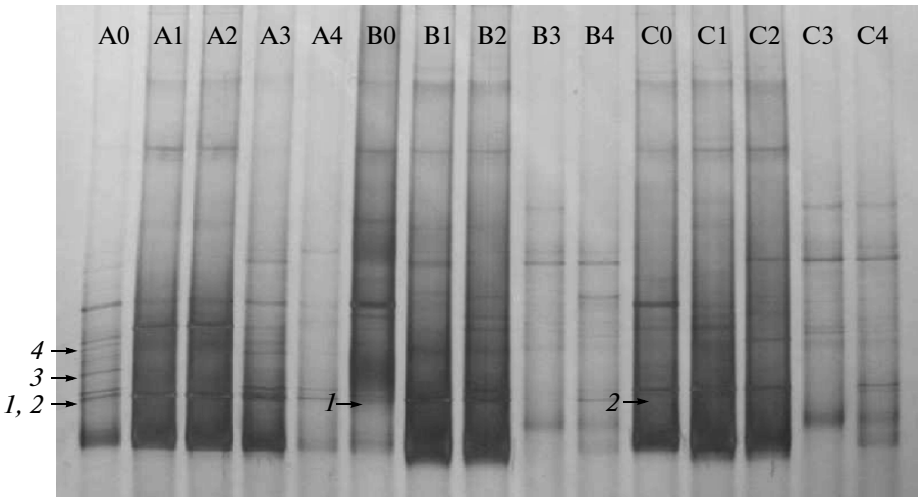


**Fig. 1.** Denaturing gradient gel with the PCR fragments of bacterial DNA from the samples of sulfide nickel ore waste. A, B, and C designate the waste pile samples; A0, B0, and C0 designate the fragments for the original microflora. During the leaching experiments, the enrichments inoculated with the samples were incubated under the following conditions: pH 2, 28°C (A1); pH4, 28°C (A2); pH 2, 45°C (A3); pH 4, 45°C (A4); pH 2, 28°C (B1); pH4, 28°C (B2); pH 2, 45°C (B3); pH 4, 45°C (B4); pH 2, 28°C (C1); pH4, 28°C (C2); pH 2, 45°C (C3); and pH 4, 45°C (C4). The fragments marked by arrows (*1, 2, 3*, and *4*) were taken for the sequencing.

removed from the gel by thorough washing with $K_3Fe(CN_6)$ in order to achieve uninhibited reamplification.

The sequences were analyzed using the BLAST and Similarity Matrix (RDP) software packages in order to determine their closest relatives in the available databases. The sequences were aligned using the CLUSTAL software package. Phylogenetic trees were constructed using TREECON [13], and the distance matrix [14] was constructed by analysis of 1000 trees. The 16S rDNA trees were constructed using sequences of 24 clones from enrichment cultures, 23 clones from waste piles, and 29 reference GenBank sequences.

**Model laboratory experiments on ore leaching by the waste pile bacterial community.** Weighed portions of ground sterilized sulfide nickel ore were placed into cotton-stoppered conical flasks and supplemented with synthetic medium containing the following (g/l): $K_2HPO_4 \cdot 2H_2O$, 2.0; $KH_2PO_4$, 0.4; $Na_2S_2O_3 \cdot 5H_2O$, 1.0; $NH_4Cl$, 0.4. The flasks were inoculated with the A, B, and C waste samples. This medium with neutral-to-alkaline pH is used to cultivate neutrophiles.

The solid/liquid phase ratio was 1 : 5 (40 g : 200 ml).

Nickel content in the medium was tentatively determined by the turbidimetric analytic procedure based on the qualitative reaction between nickel and dimethylglyoxime. Since glycerol was added to the reaction mixture in order to stabilize the nickel dimethylglyoximate suspension, both qualitative (color reaction) and quantitative turbidimetric analysis were possible. Adsorption of the suspension at 550 nm, i.e., in the adsorption maximum of the dimethylglyoximate suspension in visible light, was proportional to the nickel concentration in the standard solutions. The method detected nickel in solutions at concentrations from 16 to 250 mg/l, corresponding to the linear part of the calibration curve. Analyses were carried out on a Shimadzu UV-160A spectrophotometer.

Precise analysis of nickel content in the solution was carried out by ion chromatography on an 844 UV/VIS Compact IC computerized ion chromatograph for cation detection (Metrosep 6.1010.300 column, diode matrix UV/VIS detector for three wavelengths, IC Net software package). Solutions of $Ni^{2+}$ and $Co^{2+}$ in 20 mM ascorbic acid (0.5, 1.0, 2.0, 5.0, and 10.0 mg/l) were used as the standards. Prior to analysis, the sample was centrifuged to remove solid particles.

## RESULTS AND DISCUSSION

**Bacterial diversity in the Waste piles.** Bacterial diversity was determined in the original waste samples (designated as A0, B0, and C0 in Fig. 1) and in the nickel-leaching enrichments inoculated with these samples. Depending on the ambient pH and temperature, the samples were designated as A1, A2, etc (see caption to Fig. 1). DGGE analysis revealed differences between microbial communities of the waste piles (A0, B0, and C0). However, subsequent sequencing of the DNA fragments (lanes *1, 2, 3*, and *4* in Fig. 1) and comparison with the GenBank 16S rRNA gene sequences (using the BLAST and CLUSTAL software packages) demonstrated the presence of *Acidithiobacillus* in all samples. Microbiota of the A waste pile had the highest content of the members of this genus. DGGE analysis showed changes in the

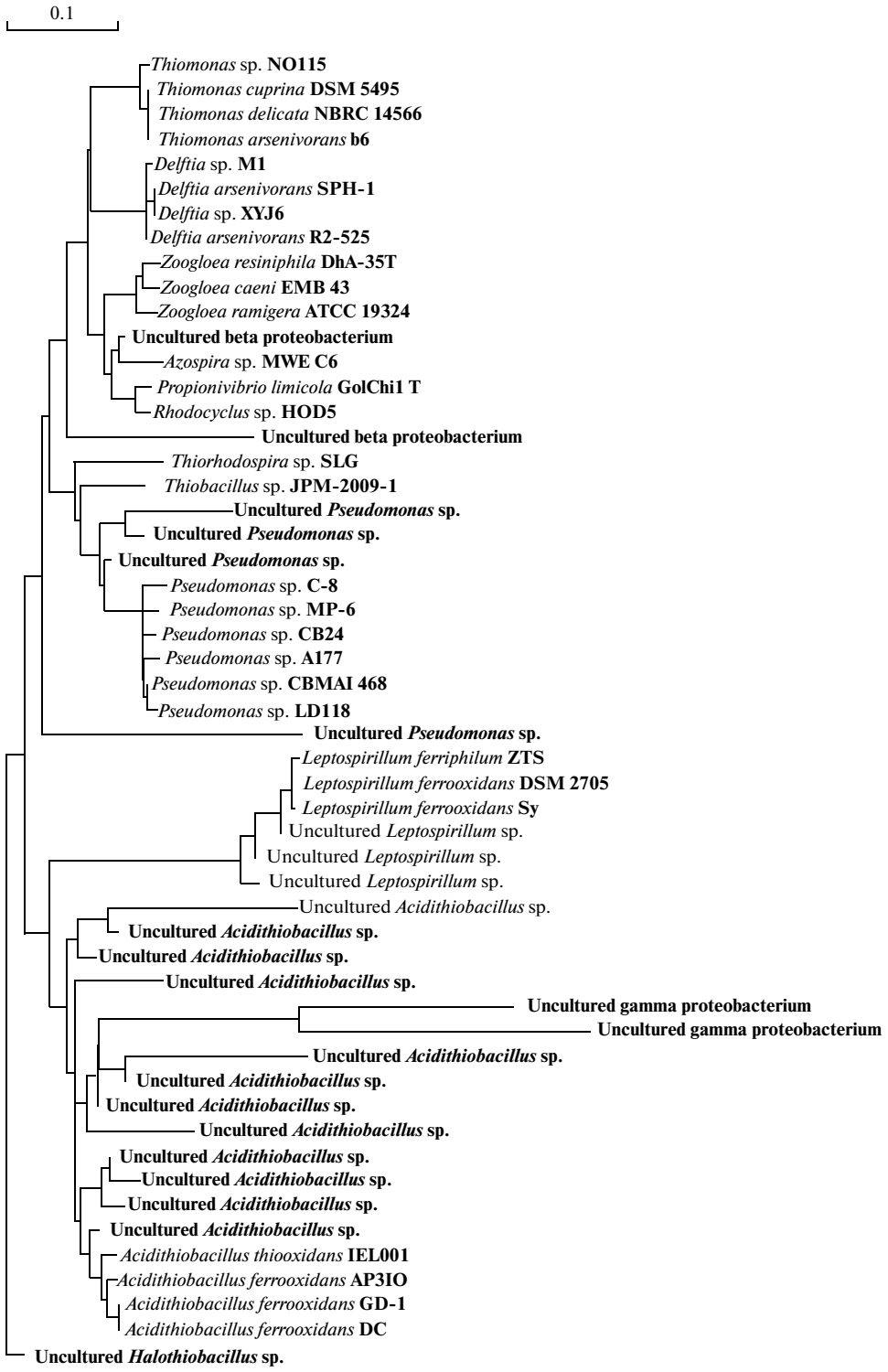


**Fig. 2.** Phylogenetic analysis of the partial 16S rDNA sequences from the ore waste pile samples, including the clones of the major DGGE bands (marked by boldface) and the relevant reference sequences.

microbial community depending on pH and temperature. Experimental variants at different temperature and pH values revealed that *Acidithiobacillus* predominated at 45°C, pH 2 and 4.

The differences in the initial composition of the microbiota of the samples were relatively small, so that the results may be presented as a generalized tree (Fig. 2). According to the determined similarity level

Leaching of nickel sulfide ore by the waste pile aboriginal microbial community

| Temperature, °C | pH | Final Ni concentration in solution, mg/l |
|---|---|---|
| 28 | 2 | 50.445 |
| 45 | 2 | 75.473 |
| 28 | 4 | 52.247 |
| 45 | 4 | 31.489 |

(~97%) of the 39 discovered sequences with those from GenBank database, they were identified as members of the genera *Acidithiobacillus, Leptospirillum, Halothiobacillus*, and *Pseudomonas*. The genus *Acidithiobacillus* was the most widespread. In general, the domination in the bacterial diversity of the ore waste piles (in descending order) may be presented as *Acidithiobacillus* > *Leptospirillum* > *Halothiobacillus* > *Thiomonas* > *Pseudomonas* > *Delftia*.

**Nickel leaching from sulfide ore and bacterial diversity in laboratory experiments.** Direct analyses not involving leaching experiments are sufficient for investigation of bacterial diversity in ore waste piles. However, determination of the major species in the mineral medium with ore after inoculation with the waste samples with a known biota composition was also of interest.

Analysis of the biota composition revealed the presence of acidophiles in neutral (pH 7) ore waste piles. Cultivation under different conditions is a selective factor stimulating growth of the groups for which these conditions are more favorable. To stimulate the possible different groups of acidophiles within the microbiota, leaching of the sterile ore was carried out at different temperatures (10, 28, and 45°C) and at initial pH 2 or 4. For acidification of the originally neutral or alkaline medium, sterile sulfuric acid was added after the autoclaving. In the variant with pH 2, chemical transformation of thiosulfate was expected. According to Volynskii [15], under acidic conditions, it occurs according to the following equations:

$$S_2O_3^{2-} + H^+ \longrightarrow HS_2O_3^-,$$

$$HS_2O_3^- \longrightarrow HSO_3^- + S^0,$$

$$HSO_3^- + S_2O_3^{2-} \longrightarrow HS_2O_3^- + SO_3^{2-} \text{ и т.д.}$$

Thus, both at pH 4 and at pH 2, ore mineral sulfides (supplemented with thiosulfate in the first case) could act as the basic energy substrates for bacteria.

Laboratory experiments on leaching of sterile sulfide iron–nickel ore were also carried out in order to confirm the viability of the leaching bacteria revealed in the waste piles by molecular biological techniques. The experiments showed that these bacteria were viable. Inoculation with the waste material resulted in nickel leaching. The final concentrations of the leached nickel varied depending on the inoculum source, pH, and temperature. The highest nickel output of about 75% was achieved in the variant inoculated with sample C and incubated at pH 2 and 45°C (table). Thus, the leaching bacteria belonged to various groups. Their selection and predominance (the majority for molecular biological analysis) in the individual experiments depended on pH and temperature.

Identification of the communities in enrichment cultures was carried out as described above and revealed that they were similar to the original structure of the waste pile microbiota. Similarly to the original communities of the waste pile samples, the differences between the experimental variants were small, so that the results may be presented as a single generalized tree (Fig. 3). Interestingly, enrichment cultures in the leaching experiments also contained significant numbers of pseudomonads. The prevalence of this group among nonautotrophic bacteria is in agreement with publications concerning the capacity of pseudomonads for oligocarbophilic growth. For example, both the ability of *Pseudomonas* to grow in mineral solutions at a concentration of organic substrates of about several μg/l [16] and their predominance among oligocarbophilic organisms in natural mineral water [17] have been previously reported. In our case, however, it should be mentioned that the pseudomonads exhibited a high adaptive potential to the heavy metals present in the ore and its wastes. Apart from adaptation, transfer of the plasmids responsible for this resistance from thiobacilli to the pseudomonads is also possible [18].

Bacteria of the microbial community of the moderately acidic nickel ore waste piles were identified. Identification was carried out for the dominant organisms for which major bands of the amplified 16S rDNA were obtained in the DGGE gels. Thus, they were more numerous than the minor microorganisms by at least an order of magnitude.

These results are compatible with the data on the composition of the enrichment cultures that developed in liquid mineral medium inoculated with waste pile material. Interestingly, comparative analysis makes it possible to determine which bacteria were inactive, with their DNA present in the samples (these ones were not retrieved as the major components) and which remained or became the major ones. The results indicated (Figs. 2 and 3) a basic change in the structure of the microbiota with the prevalence of the com-

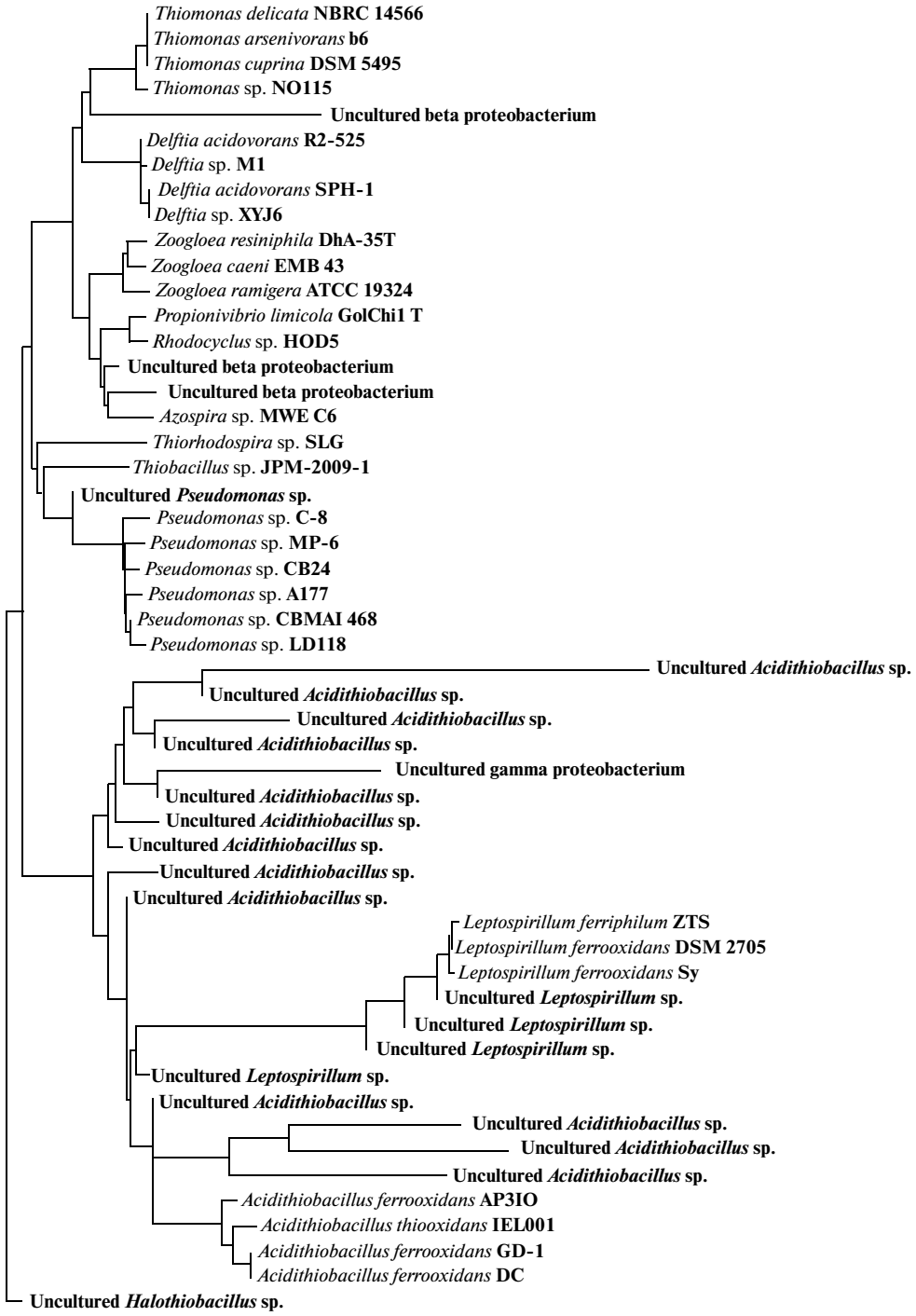


**Fig. 3.** Phylogenetic analysis of the partial 16S rDNA sequences from enriched cultures, including the clones of the major DGGE bands (marked by boldface) and the relevant reference sequences.

mon tendency: the presence of bacteria of the genera *Acidithiobacillus* and *Leptospirillum*, which are capable of leaching. In all the variants, most bacterial clones belonged to the species unknown in pure cultures but belonging to the genera known as inhabitants of acidic mine waters. These results complement our knowledge of the microbial communities of ore wastes of acidic and moderately acidic environments. In particular, these data show that the zone of occurrence of active leaching microorganisms is far broader than acidic biogeocenoses.

Investigation of microbial diversity in ore wastes is of interest both for general microbiology and for the search for potentially efficient industrial leaching

strains. Moreover, comparative analysis of the preferred cultivation conditions, amount of leached metal, and the most pronounced DGGE bands makes it possible to carry out screening and search for active cultures.

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