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PCR-Mediated Detection of Acidophilic, Bioleaching-Associated Bacteria

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The detection of acidophilic microorganisms from mining environments by culture methods is time consuming and unreliable. Several PCR approaches were developed to amplify small-subunit rRNA sequences from the DNA of six bacterial phylotypes associated with acidic mining environments, permitting the detection of the target DNA at concentrations as low as 10 fg.

A variety of chemolithotrophic and heterotrophic microorganisms are responsible for the solubilization of metals from sulfide minerals in acidic environments. Although *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, in the presence of heterotrophic *Acidiphilium* bacteria, were commonly regarded as the principal biological catalysts, recent analysis showed the important role played by other acidophilic bacteria, such as *Thiobacillus caldus* (11), "*Leptospirillum ferrooxidans*" (31), and *Sulfobacillus* (10, 26). To study the ecological relationships of these microorganisms and the population dynamics during the bioleaching processes, specific methods for their identification and enumeration are required. Although numerous (and often laborious) plating methods have been described (17, 18, 29), none of them can circumvent the problems linked to the long wait for the colony to develop and/or the inability of some bacteria to grow on solid media. Immunological assays were developed to overcome these problems, and they are sensitive enough to detect 10^3 to 10^4 cells in suspension (2, 3). However, multiple serotypes can be present in different isolates of a single species (12). Molecular methods based on the detection of genomic diversity, such as G+C content, DNA-DNA hybridization, and rRNA analysis, have been used to obtain a phylogenetic survey of the sulfur- and iron-oxidizing bacteria (15, 19). Detection and differentiation among bacteria found in acidic mining environments were recently assessed by PCR-based techniques, such as randomly amplified polymorphic DNA (27) and analysis of the 16S-23S intergenic spacer region (ISR) (30). Denaturing gradient gel electrophoresis analysis of 5S rRNA has been applied to some important bacteria associated with bioleaching; however, besides problems linked to a complicated experimental procedure and low sensitivity, distinct species could have similar migration patterns and double-band migration patterns were observed with pure cultures (33). A PCR-mediated method has been applied for the detection of *Thiobacillus cuprinus* with 23S ribosomal DNA (rDNA) and 16S-23S ISR-targeted primers (24), but high mutability in some parts of the 23S rRNA gene and in the ISR could preclude the detection of these microorganisms in environmental samples. Finally, clone-specific oligonucleotide probes have been successfully used to screen low-diversity 16S rRNA clone libraries generated from commercial and natural bioleaching sites (8, 9). The objective of the present study was to develop and apply a PCR-based method with primers derived from 16S rRNA sequences to sensitively and specifically detect six groups of important microorganisms involved in the commercial bioleaching of mineral ores.

Bacterial strains and DNA extraction. Bacteria used in this study and their sources are listed in Table 1. They were cultivated in a mineral salt medium which contained 0.6 mM K_2HPO_4, 1.0 mM $MgSO_4$, 15.1 mM $(NH_4)_2SO_4$, and 1.3 mM KCl. *T. ferrooxidans* was cultivated at pH 2.0 and 28°C with 50 mM $FeSO_4$. The "*L. ferrooxidans*" strains were cultivated at pH 1.6 and 28°C with 50 mM $FeSO_4$. *Sulfobacillus thermosulfidooxidans* was cultivated at pH 2.0 and 48°C with 0.025% yeast extract, 50 mM $FeSO_4$, and 0.5 mM $K_2S_4O_6$. *Acidiphilium cryptum* was cultivated at pH 3.0 and 28°C with 0.03% yeast extract and 0.1% glucose. The *T. thiooxidans* strains were cultivated at pH 3.5 and 28°C with 0.5% sterile powdered sulfur. *T. caldus* was cultivated at pH 2.5 and 45°C with 0.5% sterile powdered sulfur. Ore-containing experiments were performed in mineral salt medium at pH 2.5 with 5% ore (50- to 200-μm particle size). All cultures but "*L. ferrooxidans*" were cultivated in gyratory shakers at 200 rpm. Bacterial growth was determined by direct counting under a phase-contrast microscope in a Thoma counting chamber (depth, 0.02 mm; Weber, Teddington, England). Nucleic acids from liquid samples were extracted within Sterivex-GS filter units, as described by Somerville and collaborators (32). They were precipitated overnight at −20°C with 2.5 M ammonium acetate and 2.5 volumes of 100% ethanol. DNA pellets were washed with 70% ethanol, dried, and redissolved in 500 μl of 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0). The concentration of the DNA was determined spectrophotometrically, and its quality was observed on a 1% (wt/vol) agarose gel. This DNA extraction method was used successfully with the pure acidophilic cultures and with liquid samples from bioleaching environments. DNA extraction from ore samples followed the same protocol as for the liquid samples, including elements for direct extraction of DNA from soil (34) and ore (30). Ore samples (10 g) were washed twice with an equal volume of 0.04 N sulfuric acid and twice in 20% sucrose–50 mM EDTA–50 mM Tris hydrochloride (pH 7.6). The washed ore was resuspended in 2.5 ml of the same buffer containing 15 mg of lysozyme/ml and incubated at 37°C for 1 h, and then 0.1% sodium dodecyl sulfate was added. Three cycles of freezing at −20°C and thawing at 70°C were conducted. Then, 0.2 ml of a proteinase K solution (10 mg/ml in double-distilled H_2O) was added and the mixture was incubated at 50°C for 1 h. After these lysis steps, the ore was centrifuged at 3,000 × *g* for 10 min and the supernatant

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TABLE 1. Acidophilic microbial cultures used in this study

Species	Strain	Accession no.	Original source	Reference
A. cryptum	Lhet2	DSM 2389Ta	Coal mine water, United States	14
"L. ferrooxidans"	BU1	DSM 2391	Copper mine drainage, Bulgaria	15
	L15	DSM 2705^{T}	Copper deposit, Armenia	22
S. thermosulfidooxidans	AT-1	DSM 9393^{T}	Ore deposit, Russia	10
T. caldus	KU	DSM 8584^{T}	Coal spoil enrichment, United Kingdom	12
T. ferrooxidans	N-Fe4	DSM 9465	Mine shaft water, Australia	7
T. thiooxidans	Type	ATCC 19377^{T}	Sulfur-producing lake, Libya	15
	B-S2	DSM 9463	Bioleaching reactor, Australia	7

[a] T, type strain.

was purified and concentrated as described for the liquid samples.

Selection of primers. Primers based on 16S rRNA sequences were designed to be specific for bacterial groups and clones described in bioleaching environments: *Acidiphilium*, *T. thiooxidans*, *T. ferrooxidans*, *T. caldus*, "*L. ferrooxidans*," and *S. thermosulfidooxidans*. The 16S rRNA sequence data was obtained from the EMBL and GenBank databases. Four distinct types of analysis were performed due to the phylogenetic diversity of the sulfur- and iron-oxidizing bacteria (19). Phylogenetic analysis of the bioleaching microorganisms was restricted to the comparison of highly to moderately conserved nucleotide positions that were unambiguously alignable in all sequences, corresponding to 1,238 nucleotides for *Acidiphilium*, 837 for *Leptospirillum*, 777 for *Sulfobacillus*, and 717 for *Thiobacillus*. Phylogenetic placements were conducted through distance matrix analysis and parsimony analysis with the Phylogeny Inference Package (PHYLIP) version 3.5c (5). The PCR primers were designed based on the 16S rRNA sequence groups (phylotypes) derived from the phylogenetic analysis (Fig. 1 and Table 2). Primers were considered specific to a phylotype group if they had two or more mismatches with the targeted region of the 16S rRNA sequences in the databases (from bioleaching microorganisms or not) tested with the programs CHECKPROBE 2.0 (21) and BLAST (1).

Reactivities of the primer sets. Small-subunit rRNA genes were amplified by two-stage heminested or nested PCR (Table 2). The final choice of the PCR protocol depended on the available primers and the optimal results. Conditions for both amplification reactions were as previously described (4). A 1-μl volume of the completed first-round reaction mixture was added to the second reaction mixture as the target DNA. The products of the PCRs were detected by gel electrophoresis of 5 μl of PCR reaction mixture on a 1% (wt/vol) agarose gel, stained with ethidium bromide and viewed under UV light. Cross-hybridization and nonspecific-amplification products with the other strains used in this study were carefully checked. Identities of some of the PCR products generated were confirmed by determining their nucleotide sequence by automated sequencing by using methods previously described (4). Optimal conditions were determined to be those producing a strong amplicon, without nonspecific bands and without cross-hybridization with DNA from the other tested species. The combination of reagents and conditions employed in PCR which resulted in maximum specific amplification of the target sequences was considered to be optimal with 1.5 mM $MgCl_2$ and a hybridization temperature of 60°C. We determined each primer pair's sensitivity by 10-fold serial dilution of the target DNA. After the first round of PCR, 16S rDNA was detected from a quantity of template DNA comprised of between 10 and 100 pg for all the microorganisms tested (Table 2). A nested PCR increased the sensitivity by 2 to 3 $\log_{10}$, and a heminested PCR increased it by 4 $\log_{10}$. However, a heminested protocol was not applicable to all the bacteria tested due to the few positions available for the design of specific primers. In repeated experiments, extraction of DNA from pure cultures by the filter method yielded a recovery of ~20 ng of DNA per 10^6 cells for the mixotrophs and heterotrophs and of ~2 ng of DNA per 10^6 cells for the autotrophs (data obtained from at least two DNA extractions per strain), which corresponds to the yields obtained by Somerville and collaborators (32). Hence, these PCR protocols permit the detection of less than 50 cells in pure culture and represent the lowest possible detection level among all the detection methods described for these bioleaching-associated bacteria (2, 3, 16, 24, 30). Besides its sensitivity, a great advantage of this method over the immunoassay method is that it can be used for cells in suspension as well as with ore-attached bacteria.

Bacterial population in bioleaching experiments. The above-described PCR procedures were applied to analyze the microbial population during the silver-catalyzed bioleaching of a chalcopyrite ore (28). General features of the columns operated at MIM Holding Hydrometallurgy Research Laboratory (Brisbane, Australia) are as follows: 35 kg of ore crushed to 1/4 in. was agglomerated and loaded in polycarbonate columns (200 by 14 cm). Columns were inoculated with 20 liters of a mixed culture. The culture serving as the inoculum was obtained from a batch reactor kept at 37°C and contained ~10^9 cells per ml. Its bacterial diversity has been assessed previously to contain only the six types of microorganisms discussed in this study (6, 7). The solution in the columns was recirculated with a peristaltic pump. After DNA extraction by the filter method (50 ml filtered), microorganisms present in the culture were detected with a single PCR from 150 ng of DNA, corresponding to 1 μl (0.2% vol/vol) of the initial DNA solution (Fig. 2A). *T. ferrooxidans* and *T. thiooxidans* were not detected, even after a nested PCR. Although their presence was detected in enrichments from an initial batch culture, they were shown to grow poorly at 37°C and to disappear when the mixed culture was placed in a continuous bioreactor under the same physicochemical conditions (6, 7). Addition of these microorganisms at different concentrations before extraction of the bulk DNA produced a PCR amplification with their specific primers (data not shown). Moreover, in pure culture, these microorganisms gave good DNA recovery rates and high PCR sensitivity identical to *T. caldus*, detected in the batch samples. "*L. ferrooxidans*" is known to tolerate far more ferric iron than *T. ferrooxidans* (24) and to outcompete *T. ferrooxidans* at temperatures above 30°C (13). Hence, it seems probable that the *Thiobacillus* species have been progressively lost due to the better growth rate of "*Leptospirillum*" and the moderately thermophilic *Sulfobacillus* and *T. caldus* bacteria under the selected conditions. The bacterial population attached to the ore was analyzed after passage of the batch culture through

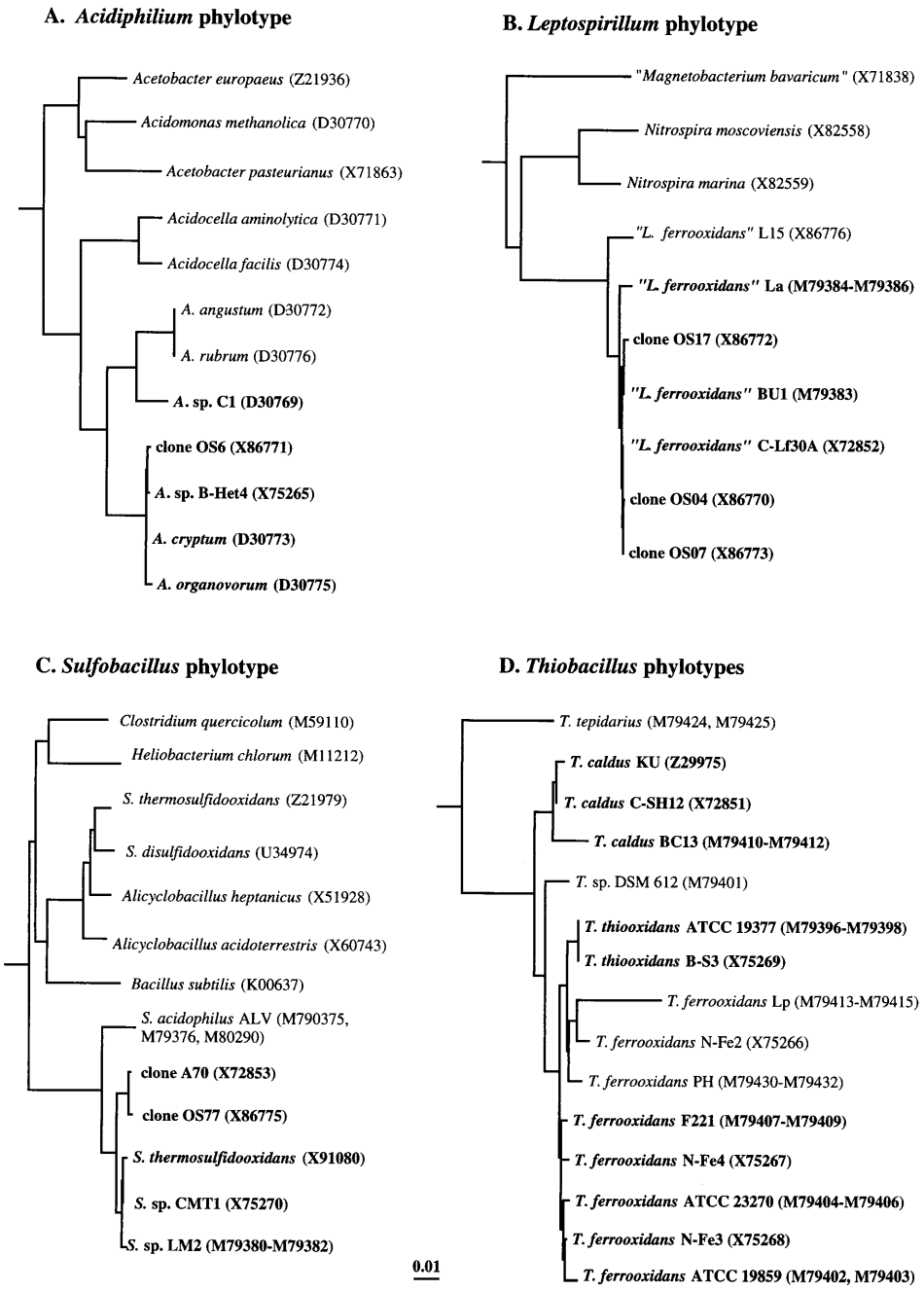

FIG. 1. Phylogenetic relationships of the four groups of bioleaching microorganisms to members of related taxa. The scale bar represents 0.01 fixed mutation per nucleotide position. The strains in boldface have 16S rRNA sequences targeted by the specific primers.

the column and recirculation for a period of 30 days (Fig. 2B). The number of cells in the effluent was lower than in the batch inoculum (stabilized at ~10^7 cells per ml), and only "*L. ferrooxidans*" could be detected with a single PCR, producing a faint band on an agarose gel (with 10 μl [2% vol/vol] of DNA solution from the effluent and the ore), due to the lower cell concentration (and hence lower DNA concentration). A nested protocol was necessary to detect the other microorganisms associated with the ore and the effluent, which were the same as in the batch culture. It is proposed that the role of the bacteria in silver-catalyzed bioleaching of chalcopyrite is to regenerate the ferric iron as a primary leaching agent through the oxidation of the ferrous iron produced (28). "*L. ferrooxidans*" seems to be the principal species responsible for iron oxidation in the column environment studied (ore and effluent). Phase-contrast microscopy of effluent samples showed that spiral-shaped bacteria were the dominant population. This observation agrees with some findings of other researchers on the important role of this poorly defined taxonomic group of acidophilic, spiral-shaped, iron-oxidizing bacteria (8, 13, 23, 25).

Bacterial leaching is an alternative for the treatment of low-

TABLE 2. 16S rRNA gene-targeted oligonucleotides used for PCR detection of bioleaching microorganisms

Group specificity	Probe[a]	Nucleotide sequence 5′–3′	First PCR			Second PCR		
			Primer set	Amplicon (bp)	Sensitivity (pg)	Primer set	Amplicon (bp)	Sensitivity (fg)
Acidiphilium	ACIDO594F	ACA GTC AGG CGT GAA ATT CCT G	√			√		
	ACIDO1150R[b]	AGA GTG CCC ACC CAA ACA T		921	100	√	557	100
	EUB1492R[c]	TAC GGY TAC CTT GTT ACG ACT T	√					
"Leptospirillum"	EUB27F[c]	GAG TTT GAT CCT GGC TCA G	√					
	LEPTO176F[b]	CGA ATA GTA TCC GGT TCC G		642	10	√	520	10
	LEPTO679R	AAA TTC CGC TTC CCT CTC C	√			√		
Sulfobacillus	EUB27F	GAG TTT GAT CCT GGC TCA G	√					
	SULFO170F	CAA TCC CGC ATA CGT TCC		1,091	100	√	429	10
	SULFO606R	AAA CCG CTA CGT ATC GCA C				√		
	SULFO1137R	GAA CCG CTG GCA ACA CAC	√					
T. caldus	CALD460F	ATC CGA ATA CGG TCT GCT A	√			√		
	CALD1475R	TAT ACC GTG GTC GTC GCC		1,054	10	√	1,016	100
	EUB1492R	TAC GGY TAC CTT GTT ACG ACT T	√					
T. thiooxidans	THIO458F	GGG TGC TAA TAW CGC CTG CTG	√			√		
	THIO1473R	TAC CGT GGT CAT CGC CCT		1,057	10	√	1,017	100
	EUB1492R	TAC GGY TAC CTT GTT ACG ACT T	√					
T. ferrooxidans	FERRO458F	GGG TTC TAA TAC AAT CTG CT	√			√		
	FERRO1473R	TAC CGT GGT AAC CGC CCT		1,057	100	√	1,017	100
	EUB1492R	TAC GGY TAC CTT GTT ACG ACT T	√					

[a] Number corresponds to the position (*Escherichia coli* numbering) of the first nucleotide of the primer (5′–3′). F, forward primer; R, reverse primer.
[b] Primer designed by Goebel and Stackebrandt (9).
[c] Primer designed by Lane (20).

grade copper sulfide ores which are economically unattractive under conventional treatment methods. The efficient manipulation and design of such complex operations can be better approached with the aid of this new method, which provides a useful tool for understanding the biological parameters of the bioleaching.

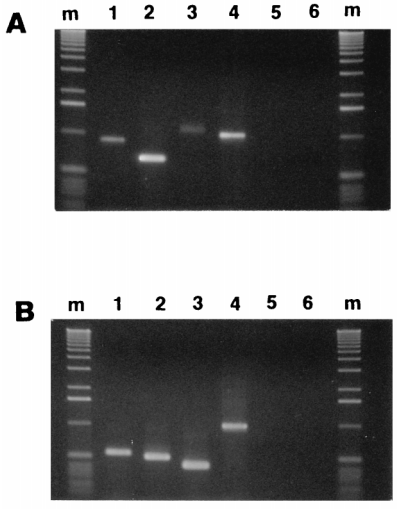

FIG. 2. PCR amplifications with specific primers. (A) DNA from the batch reactor with a single PCR. (B) DNA from 30 days leached ore with nested or heminested PCR. Lanes m, 1-kb marker (Gibco BRL); lanes 1, *Acidiphilium* primers; lanes 2, "*Leptospirillum*" primers; lanes 3, *S. thermosulfidooxidans* primers; lanes 4, *T. caldus* primers; lanes 5, *T. ferrooxidans* primers; lanes 6, *T. thiooxidans* primers.

Nucleotide sequence accession numbers. In order to analyze the 5′ end of *T. ferrooxidans* DSM 9465 and *T. thiooxidans* ATCC 19377, 16S rDNA sequences were obtained during this study (EMBL accession no. Y11595 and Y11596).

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