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# Bacterial Populations in Samples of Bioleached Copper Ore as Revealed by Analysis of DNA Obtained before and after Cultivation

JOSÉ PIZARRO,[1] EUGENIA JEDLICKI,[2] OMAR ORELLANA,[2] JAIME ROMERO,[1] AND ROMILIO T. ESPEJO[1,3]*

*Department of Microbiology[1] and Department of Biochemistry,[2] Faculty of Medicine, University of Chile, and Division of Research and Development, Pudahuel Mining Society,[3] Santiago, Chile*

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**The composition of bacterial populations in copper bioleaching systems was investigated by analysis of DNA obtained either directly from ores or leaching solutions or after laboratory cultures. This analysis consisted of the characterization of the spacer regions between the 16 and 23S genes in the bacterial rRNA genetic loci after PCR amplification. The sizes of the spacer regions, amplified from DNAs obtained from samples, were compared with the sizes of those obtained from cultures of the main bacterial species isolated from bioleaching systems. This allowed a preliminary assessment of the bacterial species present in the samples. Identification of the bacteria was achieved by partial sequencing of the 16S rRNA genes adjacent to the spacer regions. The spacer regions observed in DNA from columns leached at different iron concentrations indicated the presence of a mixture of different bacteria. The spacer region corresponding to *Thiobacillus ferrooxidans* was the main product observed at high ferrous iron concentration. At low ferrous iron concentration, spacer regions of different lengths, corresponding to *Thiobacillus thiooxidans* and "*Leptospirillum ferrooxidans*" were observed. However, *T. ferrooxidans* appeared to predominate after culture of these samples in medium containing ferrous iron as energy source. Although some of these strains contained singular spacer regions, they belonged within previously described groups of *T. ferrooxidans* according to the nucleotide sequence of the neighbor 16S rRNA. These results illustrate the bacterial diversity in bioleaching systems and the selective pressure generated by different growth conditions.**

Copper bioleaching is increasingly being used because of its economical and environmental advantages. It consists of the acid leaching of copper from the copper sulfides after oxidation, enhanced by acidophilic autotrophic bacteria (11, 23). Agglomeration of mineral ores has been incorporated into an industrial copper bioleaching process increasingly applied in Chile since 1984. This process essentially consists of irrigation of nonflooded heaps of agglomerated crushed ore with diluted sulfuric acid solutions at ambient temperature, permitting a high bacterial growth (2) that allows studies of complex bacterial populations expected in bioleaching systems (29). Culture studies reveal the presence of a small number of bacterial species including the commonly found *Thiobacillus ferrooxidans*, other autotrophs, such as *Thiobacillus thiooxidans* and "*Leptospirillum ferrooxidans*," and frequently also heterotrophs belonging to the genus *Acidiphilium* (7, 10, 14, 27) (the genus *Leptospirillum* did not appear on the Approved List of Bacterial Names and has not been validated). In situ predominance of non-*T. ferrooxidans* bacteria in leaching industrial operations (1) and in desulfuration of coal (19) has been suggested from studies using fluorescent antibody techniques. Also the presence of an unidentified 5S RNA, different from that of *T. ferrooxidans*, has been previously reported in an industrial leaching pond (17). These observations and the evolutionary widespread capacity for sulfur and/or iron oxidation observed among the cultured bacteria (16) suggest the participation of potentially diverse bacterial species in bioleaching.

Direct molecular analysis of DNA has greatly enhanced the ability to assess the diversity of microorganisms growing in an ecosystem (31), and analyses of rRNA genes have confirmed the view that conventional identification methods requiring culturing miss many of the bacteria originally present in the system (6, 18, 28, 32). The complexity of the DNA obtained from samples derived from bioleached agglomerated copper sulfide ore was analyzed by PCR of the spacer regions between the 16 and 23S rRNA genes (12) to improve the characterization of the bacterial population participating in the bioleaching of copper. Comparison of the size of the amplification products by gel electrophoresis with those from the main species isolated from bioleaching systems showed relationships to particular bacterial species. Here we describe the analysis of the spacer regions and 16S rDNAs observed in the bacterial population present after leaching chalcosite/covellite ores and the selection observed upon culturing of the bacteria in the leaching solution in medium containing ferrous iron as energy source.

## MATERIALS AND METHODS

**Bioleaching and sampling.** Ore containing 1.36% copper as oxide (33%), chalcosite (61%), and covellite (4.5%) crushed to 1/4 in. (6.35 mm) was agglomerated (22). Approximately 2,000 g, loaded in glass columns (8 by 20 cm) was leached daily with 600 ml of the MS9b solution, containing ferrous iron at different concentrations. MS9b contains (grams per liter) $(NH_4)_2SO_4$ (0.1), $K_2HPO_4 \cdot 2H_2O$ (0.04), and $MgSO_4 \cdot 7H_2O$ (0.25). Before leaching, these columns were inoculated with 100 ml of effluent obtained from columns containing the same mineral leached with a solution containing 1 g of ferrous iron per liter. Copper coming off the column was determined by atomic spectroscopy. Samples were obtained from both agglomerated ore and effluent solution when copper recovery was about 90%. The sample from the heap was a solution coming off the bottom of heaps 2 to 4 months old, from the Lo Aguirre industrial plant of the Pudahuel Mining Society. These heaps were being leached with the solution

* Corresponding author. Phone: 56-2-7370081, ext. 5342, or 56-2-6019071. Fax: 56-2-7774216. Electronic mail address: respejo@med.uchile.cl.

resulting from the equilibrium in the plant process (raffinate), which includes solvent extraction usually called SX (23).

**DNA preparation.** DNA was obtained from the ores by direct lysis of attached cells. Ten grams of agglomerated ore withdrawn from the columns was washed twice with 10 ml of 0.04 N sulfuric acid, leaving behind the larger particles by decantation. These particles made this procedure more laborious. As was determined, they contained DNA with an amplification pattern identical to that of the smaller particles. Though the smaller ore particles recovered represent only 1/10 to 1/20 of the total weight of the original sample, they contain approximately 70% of the total DNA (results not shown). This may be due to the fact that they represent a similar proportion of the total ore surface in the sample because of their small size. Ore suspension was centrifuged at 2,000 × *g* per 10 min, washed a second time with acid, and twice with TE (0.01 M Tris, 0.001 M EDTA [pH 8.5]). Then, the washed ore was suspended in 1 ml of TE–0.15 M NaCl–1% sodium dodecyl sulfate (SDS), heated at 70°C for 15 min, and centrifuged at 3,000 × *g* for 5 min. Although lysis with SDS has proved effective for thiobacilli and leptospirilli (16), this procedure was repeated three more times to avoid missing the microorganisms less sensitive to this lysis treatment. The four supernatants were then mixed for subsequent analysis; the amount of DNA obtained in the fourth lysis treatment was 1/5 to 1/10 of that recovered in the first. DNA from bacteria, present in effluent leaching solutions or batch cultures, was obtained by direct lysis after concentration and subsequent washing with 0.04 N sulfuric acid and TE, as described above, except that centrifugation was at 10,000 × *g* for 20 min. Lysis was performed (though only once) as described above for bacteria attached to the ore. The amount of DNA was estimated visually by comparison of the stained bands with appropriate standards after electrophoresis in 1% agarose.

**PCR amplification.** A fraction of the recovered DNA was treated with ribonuclease A (100 μg/ml for 2 h at 37°C) and proteinase K (50 μg/ml for 1 h at 37°C). DNA concentration in the lysates ranged from 10 up to 400 ng/μl. The enzyme-treated lysate was used for amplification without further purification after dilution at least 1/100 in TE for PCR (0.01 M Tris, 0.001 M EDTA [pH 7.5]) and heating in boiling water for 5 min. The spacer regions between the 16 and 23S genes were amplified by PCR using 1.5 ng of DNA and the primers (G1 and L1) under conditions described by Jensen et al. (12), except that 25 cycles at 15 s at 94°C, 7 min at 55°C, and 30 s at 72°C were performed and the one additional cycle was run at 72°C for 10 min. PCR amplification for the region containing the spacer and most of the 16S rDNA was performed by using the thermal cycling described by DeLong (3) and primers Eubac27F (3) and L1 (12). Electrophoresis was performed on 7% polyacrylamide gels (16 by 18 by 0.15 cm) with Tris-borate buffer (26), and the DNA was visualized by staining the gel with silver nitrate (5).

**Cloning and sequencing.** Amplified DNA was treated with the Klenow fragment of the DNA polymerase and phosphorylated with T4 polynucleotide kinase, extracted with phenol-chloroform, purified by Glassmax (Gibco), and ligated with pUC19 (Gibco) previously digested with *Sma*I (26). This DNA was used to transform *Escherichia coli* DH5 α as described elsewhere (20). Clones containing inserts were identified by digestion of the plasmid with *Eco*RI followed by agarose gel electrophoresis. The spacer region in each recombinant plasmid was identified by PCR amplification with primers G1 and L1 as described above. Denatured, double-stranded plasmid templates were sequenced by using primer 357f (13) with Sequenase 2.0 (United States Biochemicals) according to the manufacturer's recommendations. Direct sequencing of the amplification product was performed with the dsDNA cycle sequencing system (Gibco-BRL), after purification by Glassmax, according to the manufacturer's recommendations.

**Growth in media.** Cultures in synthetic media containing ferrous iron were grown by inoculation of 100 ml of MS9b–3% $FeSO_4 \cdot 7H_2O$ with either 1 ml of effluent leaching solution or 0.5 g of agglomerated ore, at 28°C in an orbit Environ shaker. Growth in elemental sulfur was performed in the same salt solution containing 0.5% sublimed sulfur instead of ferrous iron.

**Reference strains.** DNA was obtained from cultures of the following strains or isolates. *T. ferrooxidans* ATCC 19859 and Torma and isolates Chi 002, Chi 85, Michilla, and Teniente, obtained in different geographical regions of Chile, were originally provided by M. Rodriguez (Catholic University of Chile) to O. Orellana. Isolate Lo Aguirre was obtained from a leaching solution of the Lo Aguirre plant. These isolates had already been purified by plating in agarose-gelled medium. "*L. ferrooxidans*" DSM 2705, DSM 2391, and ATCC 49881 and *T. thiooxidans* ATCC 19377 were provided by C. Jerez (University of Chile). The other ATCC, DSM, and IFO strains were obtained directly from the respective culture collections.

**Sequence analysis.** Sequences were compared with those in the EMBL database. Positions which consistently yielded ambiguous nucleotide assignments or band compression anomalies were excluded from the analysis. The sequences have been deposited in the EMBL under accession numbers X91222 to X91230.

## RESULTS

**Spacer regions between the 16 and 23S rRNA genes in strains isolated from bioleaching operations.** The capability to distinguish bacterial species related to bioleaching by the size of these spacers according to the method described by Jensen et al. (12) was initially explored. Spacers of particular strains of *T. ferrooxidans*, *T. thiooxidans*, and "*L. ferrooxidans*" showed appreciable length differences (Fig. 1A). The three strains of "*L. ferrooxidans*" examined showed similar short spacers. Three strains of *T. thiooxidans* with high DNA homology by DNA-DNA hybridization (10) contained indistinguishable spacer regions, about 50 bp larger than those in the "*L. ferrooxidans*" strains (Fig. 1B). Strain B-S2 (DSM 9463), isolated by Goebel and Stackebrant (7), contained a slightly longer spacer (Fig. 1C). This strain clusters with the other bona fide strains of *T. thiooxidans* by sequence similarity of the 16S rRNA (7), though it has not been classified by DNA-DNA hybridization. Strain DSM 612, originally classified within this species, though with a low percentage of DNA-DNA hybridization with a reference *T. thiooxidans* strain (10), contained a much shorter spacer region (Fig. 1D). This strain has been lately designated a *Thiobacillus* sp. and is probably a strain of *Thiobacillus albertis* (4, 10). Seven isolates of *T. ferrooxidans*, including strain Torma (25) and six other isolates obtained from different geographical regions in Chile, showed an indistinguishable spacer region by gel electrophoresis (Fig. 2A). Considering, however, the low DNA homology among some strains of this species (9), spacer length polymorphism across strains seemed more likely than the observed uniformity. This could be attributed to the unintentional selection of strains from a particular group of DNA homology (10). When strains previously classified within different groups by DNA homology (10) were included in further comparisons, considerable length polymorphism across group lines was observed (Fig. 2B). Table 1 shows the estimated spacer region lengths for the different strains examined.

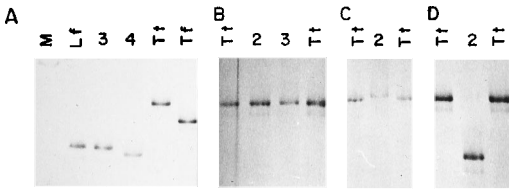


FIG. 1. PCR amplification products of the spacer regions between the 16 and 23S RNA genes of DNAs from different strains. (A) Lanes: M, 603-bp ϕX174 *Hae*III fragment; Lf, "*L. ferrooxidans*" DSM 2705; 3, "*L. ferrooxidans*" DSM 2391; 4, "*L. ferrooxidans*" ATCC 49881; Tt, *T. thiooxidans* ATCC 19377; Tf, *T. ferrooxidans* ATCC 19859. (B, C, and D) Lanes Tt, ATCC 19377; lanes 2, DSM 594 (B), DSM 9463 (C), or *Thiobacillus* sp. strain DSM 612 (D); lane 3, DSM 622.

**16-23S intergenic spacer in bacteria grown throughout leaching.** The complexity of the bacterial population was stud-

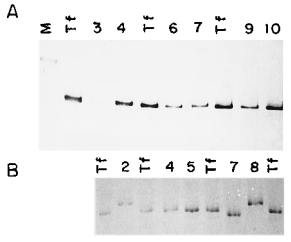


FIG. 2. PCR amplification products of the spacer regions between the 16 and 23S RNA genes of DNAs from different strains of *T. ferrooxidans*. (A) Lanes M and Tf, 603-bp ϕX174 *Hae*III fragment and strain ATCC 19859, respectively; lanes 3, Chi 002; lane 4, Chi 85; lane 6, Michilla; lane 7, Teniente; lane 9, Torma; lane 10, Lo Aguirre. (B) Lanes: 2, IFO 14245 (Lp); 4, DSM 583; 5, DSM 9464 (N-Fe3); 7, DSM 9465 (N-Fe4); 8, IFO 14262 (Ph).

TABLE 1. Estimated lengths of the 16-23S intergenic spacer region in strains belonging to different species and groups associated with bioleaching

| Strain(s)[a] | Group[b] | Supplier[c] | Approx length[d] |
|---|---|---|---|
| *T. ferrooxidans* | | | |
| ATCC 19859 | 3a | ATCC | 530 |
| DSM 583 | 3a | DSM | 530 |
| DSM (NFe3) | NG | DSM | 530 |
| IFO 14245 (Lp) | 2 | IFO | 545 |
| IFO 14262 (Ph) | 4 | IFO | 545 |
| DSM 9465 (NFe4) | NG | DSM | 520 |
| Torma | NG | O. Orellana | 530 |
| Six Chilean isolates | NG | O. Orellana | 530 |
| *T. thiooxidans* | | | |
| ATCC 19377 | 1 | ATCC | 550 |
| DSM 504 | 1 | DSM | 550 |
| DSM 622 | 1 | DSM | 550 |
| DSM 9463 (BS2) | NG | DSM | 555 |
| DSM 612 | 2 | DSM | 480 |
| "*L. ferrooxidans*" | | | |
| ATCC 49881 (LfP3A) | | C. Jerez | 495 |
| DSM 2705 | | C. Jerez | 505 |
| DSM 2391 (BU-1) | | C. Jerez | 505 |

[a] Alternate designations are in parentheses.
[b] On the basis of DNA-DNA hybridization (10).
[c] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; IFO, Institute for Fermentation, Osaka.
[d] In nucleotides.

ied in samples from columns with agglomerated chalcosite/covellite ore, irrigated with solutions containing either 5, 1, or 0 g of ferrous iron per liter. Ferrous iron concentration did not change either the rate or the total recovery of copper by more than 5% among these columns (results not shown). The spacer regions of the DNAs obtained from both the agglomerated ore and effluent leaching solution from each of these columns were amplified. Gel electrophoresis analysis of the products revealed a complex band pattern. This pattern was different for each ferrous iron concentration. The band expected after amplification of DNA from *T. ferrooxidans* strains commonly isolated in Chile was observed significantly only in the agglomerated ore leached with 5 g of ferrous iron per liter (Fig. 3A, lane $O^5$) and in the effluent solutions from the columns leached with 5 and 1 g of ferrous iron per liter (lanes $S^5$ and $S^1$). An additional band corresponding to an amplification product approximately 10 bp smaller was also observed in all these samples. The band corresponding to *T. ferrooxidans* was very light or absent in the amplification products of DNAs obtained from both the agglomerated ore and the effluent solution of the column leached without added ferrous iron (Fig. 3B, lanes O° and S°) and from the agglomerated ore leached with 1 g of ferrous iron per liter (Fig. 3A, lane $O^1$). The two main amplification products observed in the columns leached without ferrous iron had electrophoretic mobilities similar to those of the products obtained from DNAs of strains of *T. thiooxidans* and "*L. ferrooxidans*" (lanes Tt and Lf). To make sure that the apparent absence of *T. ferrooxidans* was not due to a failure in recovery, *T. ferrooxidans* was added to the washed ore before extraction, as a control. Amplification of the extracted DNA showed an efficient recovery (Fig. 3B, lane O°/Tf). A light band, smaller than the main band obtained for each strain after amplification, was frequently observed. It also appeared after amplification of DNA from culture collection strains. This

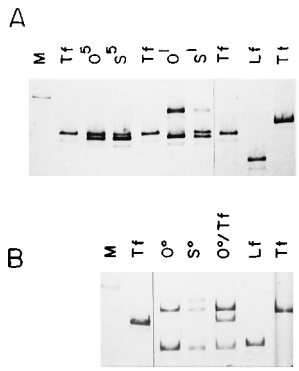


FIG. 3. PCR amplification products of the spacer regions between the 16 and 23S RNA genes of DNA obtained from samples of leached ores (lanes O) and effluent solutions (lanes S). (A) Samples from columns leached with solutions containing 5 or 1 g of ferrous iron per liter (indicated by a superscript). Lanes Tf, Tt, and Lf, amplification products of DNAs from *T. ferrooxidans* ATCC 19859, *T. thiooxidans* ATCC 19377, and "*L. ferrooxidans*" DSM 2705, respectively; lane M, 603-bp ϕX174 *Hae*III fragment. (B) Samples from columns leached without ferrous iron. Lane O°/Tf, amplification products of DNA recovered after addition of $5 \times 10^7$ bacteria of *T. ferrooxidans* (determined by epifluorescent microscopy) per gram of ore. M, Tf, Tt, and Lf are defined above.

seems to be an artifact of amplification because it was not consistently observed.

**Identification of bacteria grown when leaching occurred without ferrous iron.** The bacteria found when *T. ferrooxidans* was not detected after amplification were identified by sequencing portions of the 16S rRNA genes adjacent to the spacer regions. To obtain the 16S rRNA region, the original DNA was amplified by using primers complementary to conserved sequences near the 5′ ends of the 16S rRNA and the 23S rRNA. Amplification with these primers allowed us to obtain the 16S rDNA linked with its corresponding spacer region, for convenient identification (see below). Amplification of the spacer regions in these products produced a pattern similar or equal to that observed after amplification of the spacers in the original DNA (Fig. 4, lanes 1 and 5, respectively). The amplification products containing the 16S rRNA gene were subsequently cloned into pUC19. Nine clones were analyzed; amplification of the spacer regions in each clone showed that five contained the spacer similar to that of *T. thiooxidans* while three others contained one similar to that in "*L. ferrooxidans*" (Fig. 4). These eight clones were sequenced in the region corresponding to approximately nucleotides 400 to 700 of the *E. coli* 16S rRNA. The amplification of the ninth clone generated a product approximately 200 bp long (not shown), which was not sequenced, since it was observed only once in the numerous amplification reactions of the original DNA.

16S rRNA sequences obtained from the five clones with longer spacers were identical. Comparison with the reported sequences of the 16S rRNA in strains found in bioleaching conditions showed the highest similarity with strain *T. thiooxi-*

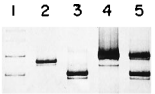


FIG. 4. PCR amplification products of the spacer regions between the 16 and 23S RNA genes. Lane 1, product of the amplification to obtain the 16S rRNA gene and spacer region together; lane 2, *T. ferrooxidans* ATCC 19859 DNA; lane 3, recombinant plasmid pss 14; lane 4, recombinant plasmid pls 24; lane 5, DNA obtained from ore leached without ferrous iron.

TABLE 2. Similarity matrix based on 16S rRNA and 16S rDNA sequences

| Strain or clone | % Similarity with[a]: | | | |
|---|---|---|---|---|
| | pls[b] | pss[c] | | BU-1[d] |
| | | 21, 14 | 38 | |
| *T. thiooxidans* B-S3[e] | 98.5 | 69.6 | 70.3 | 71.1 |
| pls[b] | | 69.4 | 70.4 | 70.1 |
| pss[c] | | | | |
| 21, 14 | | | 94.6 | 94.6 |
| 38 | | | | 100 |

[a] The data are uncorrected percentages based on a comparison of 194 nucleotides from position 429 through 630 (*E. coli* numbering). Some positions which consistently yielded ambiguous nucleotide assignments or band compression anomalies were excluded from the analysis.
[b] pls, recombinant plasmids with a large spacer region (clones 23, 24, 45, and 47).
[c] pss, recombinant plasmids with a small spacer region.
[d] "*L. ferrooxidans*." The data are from reference 16.
[e] The data are from reference 7.

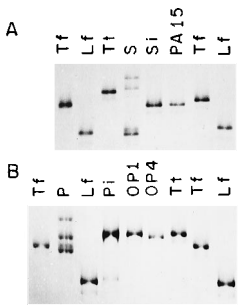


FIG. 5. PCR amplification products of DNA from bacteria in the leaching solutions and after growth in ferrous iron medium. Tf, Lf, and Tt are defined in the legend to Fig. 1. (A) Selection from the leaching solution of the experimental column. Lanes: S, leaching solution; Si, culture in ferrous iron inoculated with leaching solution; PA15, isolate obtained after the culture of leaching solution was plated. (B) Selection from leaching solution from an industrial plant. Lanes: P, leaching solution; Pi, culture in ferrous iron inoculated with leaching solution; OP1 and OP4, strains from culture of leaching solutions isolated by dilution.

*dans* B-S3 (Table 2). The sequences of the three clones enclosing the shorter spacer region (similar in length to spacers found among "*L. ferrooxidans*" strains) showed some diversity. Two clones (clones 21 and 14) had identical sequences, while the third contained a sequence identical to that reported by Lane et al. (16) for "*L. ferrooxidans*" BU-1 (DSM 2391) (Table 2).

Base changes between 16S rRNAs of strains of *T. thiooxidans* and *T. ferrooxidans* species are concentrated mainly in 10 bases within the region from positions 443 to 480. The differences within this stretch allowed Goebel and Stackebrandt (7) to distinguish seven groups among strains of these two species. In the clones containing the longer spacers, this sequence stretch was almost identical to that reported for the group of strain *T. thiooxidans* BS-3. (Table 3). Identification of the strains with the longer spacers as *T. thiooxidans* was confirmed phenotypically because the bacteria with this spacer grew on elemental sulfur but not on ferrous iron.

**Selection upon growth in culture media with different energy sources.** The bacterial population composition changed drastically after cultivation of the effluent leaching solutions in ferrous iron medium. Figure 5A shows the spacer region pattern after amplification of DNAs obtained from the effluent solution of the column leached without ferrous iron (lane S) and from a culture of the bacteria in this solution in medium containing ferrous iron as energy source (lane Si). The bacteria that grew from the effluent solution and produced the short spacer region (about 525 bp) were isolated by plating in agarose-gelled medium. This strain was designated PA15.

A similar analysis was performed with DNA obtained from a leaching solution coming off a heap at a copper production plant. The heap consisted of similar ore, leached with the solution resulting in the process employed in the industrial plant (raffinate), which contains high sulfate and iron concentrations (about 150 and 5 g/liter, respectively). Amplification of this DNA produced a spacer pattern with many bands (Fig. 5B). The bacteria in this solution were cultured in MS9b-ferrous iron medium, and the amplified DNA produced instead two main spacer regions. These were significantly larger than that in *T. ferrooxidans* (approximately 555 and 550 bp). Bacteria with each spacer were isolated by serial dilution to extinction in liquid medium, because they did not form colonies in agarose-gelled medium. These strains were designated OP1 (555-bp spacer) and OP4 (550-bp spacer).

**Characterization of the bacteria selected in medium containing ferrous iron.** Further characterization of the isolated bacteria by both restriction enzyme fragment length pattern of the spacer region and nucleotide sequence of the neighbor 16S rRNA were performed. Restriction fragment length polymorphisms of the amplification products of these strains were similar to those obtained for *T. ferrooxidans* ATCC 19859 (Fig. 6). The restriction sites for *Alu*I and *Cfo*I were conserved in PA15, but strains OP1 and OP4 contained an additional site for *Cfo*I. The region corresponding to nucleotides 370 to 625 (approximately) of the *E. coli* 16S rRNAs was sequenced in the 16S rDNA next to the spacer region, so as to have a better estimation of relatedness. On this occasion, sequencing of the 16S rDNA stretch was performed directly with the amplification product of the spacer region and adjacent 16S rRNA. Similarity between PA15 and OP4 was 98.4%. Comparison

TABLE 3. Comparison of 16S rDNA positions 443 through 480 (*E. coli* numbering) with those in previously described *T. ferrooxidans* and *T. thiooxidans* groups

| Organism(s) | Sequence (5′ to 3′)[a] |
|---|---|
| *T. thiooxidans* BS3 and ATCC 19377[b] | GG**A**GGACGAAAAGG**T**GG**G**T**G**C**T**AATA**ACGC**CTGCT**G**TT |
| pls[c] | GG**A**GGACGAAAAGG**T**GG**G**T**G**C**T**AATA**TCGC**CTGCT**G**TT |
| *T. ferrooxidans* N-Fe4, PH, and F221[b] | GG**A**GGACGAAAAGG**C**GG**G**TTC**T**AATA**CAAT**CTGCT**G**TT |
| Isolate PA15 | GG**A**GGACGAAAAGG**C**GG**G**TTC**T**AATA**CAAT**CTGCT**G**TT |
| *T. ferrooxidans* N-Fe2 and Lp[b] | GG**G**GGACGAAAAGG**C**GG**G**TCC**T**AATA**CGAT**CTGCT**G**TT |
| Isolate OP4 | GG**G**GGACGAAAAGG**C**GG**G**TCC**T**AATA**CGAT**CTGCT**G**TT |

[a] Potentially discriminatory positions are boldfaced.
[b] The data are from reference 7.
[c] pls, clones containing a large spacer region.

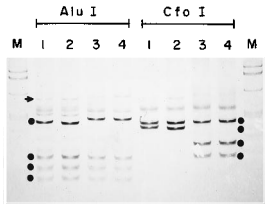


FIG. 6. Restriction fragment length pattern of the 16-23S intergenic amplification product from isolated strains and *T. ferrooxidans*. Lanes: 1, *T. ferrooxidans* ATCC 19859; 2, PA15; 3, OP1, 4, OP4. Dots, fragment products; arrow, remaining unrestricted amplification product. Lane M, ϕX174 *Hae*III restriction fragments.

with the respective available sequences for *T. ferrooxidans* (7, 16) showed similarities between 97.4 and 100%. In each comparison, differences were concentrated in the region between nucleotides 443 and 480, as previously observed for *T. thiooxidans* and *T. ferrooxidans* strains (7). The sequences of this region in PA15 and OP4 were identical to that reported for two different groups of *T. ferrooxidans* strains (Table 3).

The isolated strains PA15, OP1, and OP4 were straight rods able to oxidize elemental sulfur besides ferrous iron and indistinguishable from *T. ferrooxidans* ATCC 19859 in size and shape. OP1 and OP4 were unable to form colonies in ferrous medium gelled with agarose; PA15 formed small colonies with low efficiency.

## DISCUSSION

It is important to identify and characterize lithotrophic bacteria involved in bioleaching processes properly, since some properties in a few popular laboratory strains are extrapolated for the whole group and considered for process design. To be able to observe the genetic differences so as to characterize the bacterial population, the different amplification products of the DNA obtained from leached ores were analyzed. Though the relative abundance of each amplification product cannot be directly related to the amount of particular strains in the ore (because of potential differences in lysing or amplification efficiency among strains), meaningful shifts in population composition can be observed in changes in the amplification product patterns. The spacer regions of *T. ferrooxidans* strains were prevalent at high ferrous iron concentration, while at low concentration of this element spacer regions of *T. thiooxidans* and "*L. ferrooxidans*" prevailed. The determination of portions of the 16S rRNA sequences allowed the identification of the bacteria. When an exact discrimination of *T. ferrooxidans* and *T. thiooxidans* was not possible, conclusive identification of *T. thiooxidans* strains was done according to their abilities to grow on elemental sulfur but not on ferrous iron. It should be noted that the bioleaching potentials of both *T. thiooxidans* and "*L. ferrooxidans*" are high (90% of the copper was recovered) when the concentration of ferrous iron is low.

Our results indicate that *T. ferrooxidans* strains, undetectable by amplification, probably because of the small amounts of their genomic DNAs (<0.015 ng), can become prevalent strains after growth in medium containing ferrous iron. Its selection from a sample in which the main spacer regions were those from *T. thiooxidans* and "*L. ferrooxidans*" demonstrate the high selective pressure of culturing. It also suggests that "*L. ferrooxidans*" strains could be more common in bioleaching conditions than previously estimated by cultural analysis. The lack of growth of the observed "*L. ferrooxidans*" strain in the culture medium is difficult to explain. A low $K_s$ value for ferrous iron along with an inhibition of growth at high ferrous iron concentrations, as that employed for culturing, would explain both its prevalence at low ferrous iron concentration and its failure to prevail after culturing. Norris et al. (21) have reported a $K_s$ value for ferrous iron of *Leptospirillum* spp. well below that of *T. ferrooxidans*.

The characterization of the spacer region in different *T. ferrooxidans* strains including those isolated in this work reveal the large heterogeneity of this region within the species. Two rRNA operons (rrnT1 and rrnT2) are present in the genome of *T. ferrooxidans* Torma (24). These operons have an identical 16-23S intergenic spacer, containing $tRNA^{Ile}$- and $tRNA^{Ala}$-like sequences, with a total size of 530 bp. The spacer in this strain has 97% homology with that in strain A4, isolated in a Chilean mine (25, 30). However, strains of *T. ferrooxidans* are genotypically more diverse than is customary for a single species. At least five homology groups have been identified by DNA-DNA hybridization (9, 10), and polymorphism of the spacer regions within strains assigned to this species is likely, as has been reported for strains of a few other species (12, 15). We isolated strains OP1 and OP4, which have larger spacer regions than any of those observed among the examined *T. ferrooxidans* strains from the culture collection. This difference in genotype could account for distinct phenotypical properties not described till now because plating would not have detected these strains and their inability to form colonies would have discouraged further studies. Whether the observed polymorphism in *T. ferrooxidans* strains reflects intrinsic variabilities within this species or is a consequence of a definition that does not correspond to a cohesive taxon remains to be determined.

The results obtained encourage us to urge specifically aimed studies to define bacterial populations at each particular bioleaching plant. Conditions prevailing in production plants are very different from our experimental conditions (temperature, dissolved salts, etc.), and this may select an alternative bacterial population. For example, the amplification product patterns of the Lo Aguirre plant isolates were highly complex, suggesting that these studies should further include comparison with amplification products and 16S rRNA sequences from species such as *Acidiphillum* spp., *Sulfobacillus* spp., *T. cuprinus*, etc., also found in bioleaching systems at ambient temperature.

The method originally proposed by Jensen et al. (12) for the identification of bacteria seems suitable to assess bacterial population complexity in the bioleaching system. However, identification of the strains requires further procedures including sequencing of the 16S rDNA adjacent to the observed spacers, as described in this article.

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