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Bacterial Community in Copper Sulfide Ores Inoculated and Leached with Solution from a Commercial-Scale Copper Leaching Plant

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Most copper bioleaching plants operate with a high concentration of sulfate salts caused by the continuous addition of sulfuric acid and the recycling of the leaching solution. Since the bacteria involved in bioleaching have been generally isolated at low sulfate concentrations, the bacterial population in ores leached with the high-sulfate solution (1.25 M) employed in a copper production plant was investigated. The complexity of the original population was assessed by the length pattern of the spacer regions between the 16S and 23S rRNA genes, observed after PCR amplification of the DNA extracted from the leached ore. Six main spacers were distinguished by electrophoretic migration, but they could be further resolved into eight spacers by nucleotide sequence homology. The degree of homology was inferred from the electrophoretic migration of the heteroduplexes formed after hybridization. One of the spacers was indistinguishable from that found in *Thiobacillus thiooxidans*, four could be related to *Thiobacillus ferrooxidans*, and three could be related to *Leptospirillum ferrooxidans*. Only five of the spacers in the original sample could be recovered after culturing in media containing different inorganic energy source. Altogether, the results indicate that the bacteria in the leached ore formed a community composed of at least three species: a fairly homogeneous population of *T. thiooxidans* strains and two heterogeneous populations of *T. ferrooxidans* and *L. ferrooxidans* strains.

Copper bioleaching consists of the acid leaching of copper from the copper sulfides after oxidation enhanced by acidophilic autotrophic bacteria (22). Culture studies reveal the presence of a number of bacterial species, including the commonly found *Thiobacillus ferrooxidans* and other autotrophs, such as *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* (8, 9, 11). Heterotrophs belonging to the genus *Acidiphilium* and iron-oxidizing heterotrophic acidophiles have also been observed, and a role for these bacteria in bioleaching has been proposed (15, 16, 27). However, the culture media utilized for isolation and subsequent studies are generally quite different from the leaching solution employed in commercial-scale bioleaching operations, specially in salt concentration, and the bacteria finally observed are those selected under conditions quite different from those prevailing during leaching. In situ predominance of non-*T. ferrooxidans* bacteria in leaching industrial operations (2) and in desulfuration of coal (20) has been suggested by studies with fluorescent antibody techniques.

Most bioleaching operations involve recovery of copper from a leaching solution that is subsequently returned after replenishment of the sulfuric acid that reacted with salts in the ore (22). This recycling results in a continuous enrichment of non-copper sulfate salts until an equilibrium is reached by the replenishment of missing solution with fresh water (4). Solution is lost during operation by disposal of wet ores after the copper has been recovered and occasionally by seepage. In some commercial-scale leaching operations, sulfate ions may reach concentrations higher than 120 g per liter (19). The bacteria growing at this salt concentration (1.25 M or higher) must actively counterbalance their internal osmotic pressure or water activity and could be considered halotolerant, although this term is usually applied to NaCl tolerance (5, 17, 26). Since the bacteria prevalent in the high sulfate salt concentration of these leaching solutions might differ from those cultivated in low-salt media, a study was made of the bacterial population composition in ores leached with the solution employed in the Lo Aguirre copper production plant in Santiago, Chile (4, 19). Direct molecular analysis of DNA was employed in this study, since conventional identification methods that require culturing generally fail to detect many of the bacteria originally present in the system (1). The DNA complexity was analyzed by the length patterns of the spacer regions between the 16S and 23S rRNA genes observed after PCR amplification (14, 21). Comparison of the lengths of the amplification products with those from the main species isolated from bioleaching systems permitted a preliminary correlation to particular bacterial species. This correlation was validated by hybridization with spacer regions from culture collection species. Denaturation and subsequent renaturation of a mixture of different spacers generates, in addition to homoduplexes, heteroduplexes between related sequences with reduced electrophoretic mobility in a polyacrylamide gel (13). The presence of single-stranded regions in the heteroduplex, due to sequence divergence, results in a decrease in the electrophoretic mobility that in turn can be interpreted in terms of genetic differences between the DNAs forming the complex. Since the reduction in mobility can be related to the degree of sequence divergence between annealed strands, this analysis permits the estimation of genetic relationships (6, 12). We present the results of the analysis of the spacer regions in the bacteria present in leached chalcocite and covellite ores and in those selected upon culturing with different energy sources.

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MATERIALS AND METHODS

Bioleaching and sampling. Ore containing 1.36% copper, mainly as oxide (33%), chalcocite (61%), and covellite (4.5%), crushed to less than 1/4 in. was

agglomerated and leached daily as described previously (21), with 600 ml of the solution employed in the Lo Aguirre copper production plant in Santiago, Chile (19). The pH of this solution was 1.6, and the solution contained 120 g of sulfate (mainly as Al, Mg, Mn, and Fe salts) and 4.5 g of iron per liter with 2.7 g as ferrous iron per liter.

DNA preparation. DNA was obtained from the ores by direct lysis of attached cells, after 120 days of leaching when 97% of the copper had been extracted. Ten grams of agglomerated ore withdrawn from the columns was treated as described previously (21), except the ore was washed and resuspended in 0.1 M Tris–0.01 M EDTA (pH 8.5). The amount of DNA was estimated visually by comparison of the stained bands with appropriate standards after electrophoresis in 1% agarose. DNA was obtained by lysis with sodium dodecyl sulfate (21) from cultures of the following strains: *T. ferrooxidans* DSM 583, "*L. ferrooxidans*" DSM 2391, and *T. thiooxidans* ATCC 19377.

PCR amplification. A fraction of the recovered DNA was treated with RNase A and proteinase K (21) and subsequently extracted once with phenol-chloroform and dialyzed against TE (0.01 M Tris, 0.001 M EDTA [pH 7.5]). The final concentration of DNA was about 0.5 ng/μl; after dilution to 1/50 in TE, 0.15 ng was used for amplification, for which both primers and conditions have been described previously (21). Electrophoresis was performed with 7% polyacrylamide gels (16 cm long by 18 cm wide by 0.15 cm thick) with Tris-borate buffer (24) at 200 V, and the DNA was visualized by staining of the gel with silver nitrate (7).

Growth in media. Cultures in media containing different energy sources were grown by inoculation of 5 ml of the same solution employed for leaching the ore (sterilized by filtration) with 0.5 g of leached ore. Subsequent cultures were made by transfer of 0.1 ml of the culture to 5 ml of the same medium. A final culture of 100 ml was used for DNA extraction. The irrigation solution, without further addition, was employed for cultivation in ferrous iron. MS9b medium (21) containing 1.5% $FeSO_4 \cdot 7H_2O$ was used for the cultivation of cells under low-sulfate conditions. For cultivation with other energy sources, the ferrous iron in the irrigating solution was oxidized by titration with hydrogen peroxide and supplemented with different energy sources: 0.5% elemental sublimed sulfur, 0.5% of a 90% chalcopyrite mineral obtained by flotation, or 0.2% yeast extract.

Hybridization. Denaturation and renaturation of the amplification product were performed by the procedure described for human immunodeficiency virus type 1 (HIV-1) heteroduplex formation of amplification products (6). Amplification products were diluted to an approximate concentration of 1 ng of each amplified spacer in 9 μl of PCR amplification buffer (without *Taq* polymerase) plus 1 μl of 10× annealing buffer (6). Denaturation and renaturation were performed in a thermocycler (Perkin-Elmer Gene PCR system 2400) in which the reaction mixture was heated to 96°C for 2.5 min, heated to 72°C for 2.5 min, and then cooled to 4°C. For denaturation, the step at 72°C was omitted. Radioactive labeling was performed by PCR amplification as described above, except that concentration of deoxynucleoside triphosphate was 30 μM, and the mixture additionally contained 0.6 μCi of [α-^{32}]dATP per μl. One microliter of a 1/10 to 1/30 dilution of this product was employed for hybridization by denaturation and annealing as described above.

RESULTS

Analysis of 16S to 23S rRNA intergenic spacers in bacteria grown in ore leached at high sulfate concentrations. Agglomerated chalcocite-covellite ore was leached with a solution employed in a copper production plant containing, among other ions, 120 g of sulfate per liter. DNA was extracted from the leached ore, and the spacer regions between the 16S and 23S rRNA were amplified by PCR and analyzed by gel electrophoresis. A complex pattern was observed, composed of six main bands with sizes of approximately 550, 530, 515, 510, 480, and 460 bp plus several additional faint bands (Fig. 1). The sizes of the main bands were within the range previously observed in spacer regions of *T. ferrooxidans*, *T. thiooxidans*, or *L. ferrooxidans* strains. (The apparent length of the spacer regions of *L. ferrooxidans* calculated with more appropriate markers is approximately 460 bp and not 500 bp as previously published [21].)

Nucleotide sequence homology between the amplified spacer regions was analyzed by the electrophoretic migration of the heteroduplexes formed after melting and renaturation. The observed heteroduplexes were compared with those formed between amplified spacer regions of culture collection strains of *T. ferrooxidans*, *L. ferrooxidans*, and *T. thiooxidans* (Fig. 2). The heteroduplexes *L. ferrooxidans-T. ferrooxidans*, and *L. ferrooxidans-T. thiooxidans* remained at the top of the gel (lanes Tf/Lf and Lf/Tt), while the *T. ferrooxidans-T. thiooxidans* heteroduplex migrated behind the single-strand bands (lane Tt/Tf). These migrated for about one-third of the gel and were usually resolved into the two complementary strands. The different heteroduplexes, single strands, and renatured homoduplexes formed after denaturation and renaturation of a mixture of the amplified spacers of these three strains could be resolved after electrophoresis (lane Tt/Lf/Tf). Denaturation and renaturation of the amplification product of the DNA extracted from the leached ore rendered different putative heteroduplexes (lane O). Some had a migration rate similar to or equal to that of the heteroduplexes formed between the

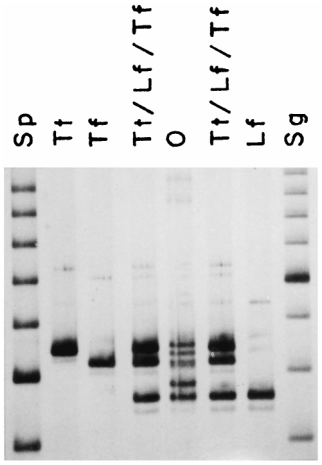

FIG. 1. PCR amplification products of the spacer regions between the 16S and 23S RNA genes of DNA from leached ore. Lanes: Sp, Promega 100-bp DNA ladder; Tt, *T. thiooxidans* ATCC 19377; Tf, *T. ferrooxidans* DSM 583; O, leached ore; Lf, *L. ferrooxidans* DSM 2391; Tt/Lf/Tf, mixture of amplified spacers from the corresponding strains; Sg, Gibco 100-bp DNA ladder.

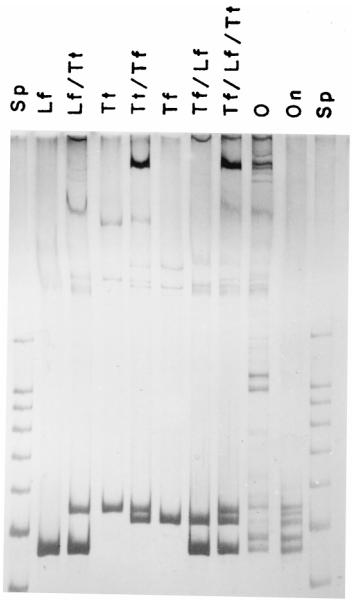

FIG. 2. Homo- and heteroduplex formation after denaturation and renaturation of amplified 16S to 23S rDNA spacers. Results from acrylamide gel electrophoresis upon denaturation and subsequent renaturation of the amplification products, single or mixed, are shown. Sp, Tt, Tf, Lf, and O DNAs, as defined in the legend to Fig. 1; On, spacers from leached ore without denaturation and renaturation.

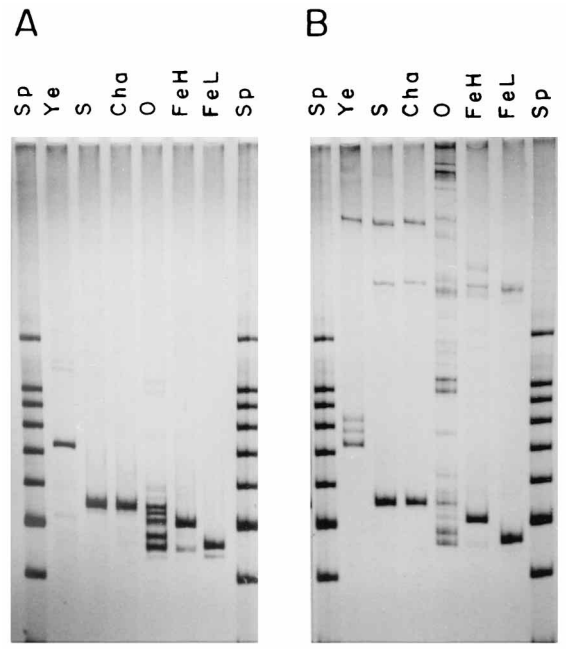

FIG. 3. (A) PCR amplification products of the spacer regions between the 16S and 23S RNA genes of DNA from leached ore and from cultures with different energy sources. (B) Homo- and heteroduplex formation after denaturation and renaturation of the amplified spacers. Amplification products of DNA from cultures with the following energy sources were used: Ye, yeast extract; S, elemental sulfur; Cha, chalcopyrite; FeH, ferrous iron in leaching high-salt solution; FeL, ferrous iron in MS9b medium low-salt solution; O, DNA from leached ore. Sp, Promega 100-bp ladder.

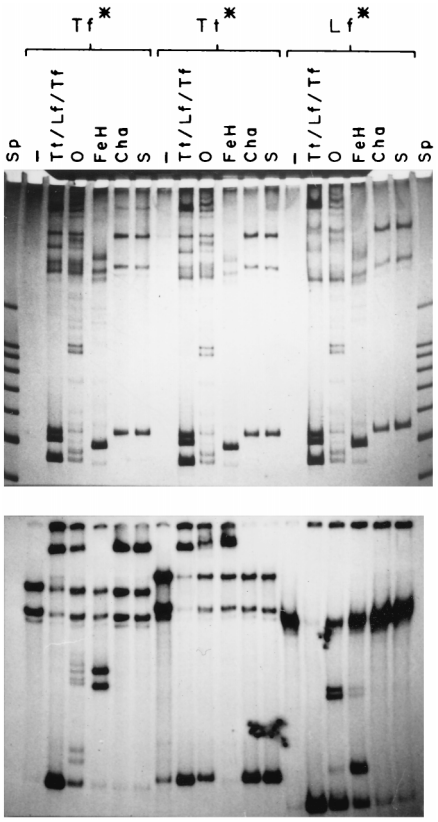

FIG. 4. Hybridization of radioactively labeled spacers from *T. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans* with the amplification product of DNA from leached ore and cultures. The three different labeled spacers indicated on top of the figure were denatured and subsequently renatured alone (−), with the amplification product of DNA extracted from the leached ore (O), or with the product from the bacteria selected with the following energy sources: S, elemental sulfur; Cha, chalcopyrite; FeH, ferrous iron in leaching high-salt solution. Sp, Tt, Tf, and Lf are defined in the legend to Fig. 1. The top panel shows a gel stained with silver nitrate. The bottom panel shows results from autoradiography of the stained gel.

culture collection species, while others migrated significantly faster. The formation of these latter heteroduplexes suggested the presence of spacers with greater sequence homology than that found between those of *T. ferrooxidans* and *T. thiooxidans.*

Bacterial population obtained with different energy sources and high sulfate concentrations. The bacteria in the leached ore were cultured in the plant leaching solution supplemented with different energy sources: ferrous iron, elemental sulfur, chalcopyrite, or yeast extract. After four or more subcultures, the composition of the selected bacterial population was analyzed by both the size of the amplified spacers (Fig. 3A) and the formation of heteroduplexes (Fig. 3B), as described above. Culture in high-salt ferrous iron selected for bacteria with spacers approximately 530 and 480 bp in length, similar to those observed in *T. ferrooxidans* and *L. ferrooxidans* strains, respectively (Fig. 3A, lane FeH). Heteroduplexes were formed upon denaturation and renaturation of these spacers, but they were difficult to distinguish by staining (see below). Culture in ferrous iron but with MS9b medium (low in sulfate concentration) selected for bacteria with a spacer similar to those found in *L. ferrooxidans* (Fig. 3A, lane FeL). Abundant and rapid growth occurred upon cultivation in yeast extract (data not shown), but the bacteria selected had a main spacer quite different in size from any of those observed in the original leached ore (Fig. 3A, lane Ye). This spacer was apparently heterogeneous in sequence, since upon denaturation and annealing, it formed two products with slower migration, probably corresponding to heteroduplexes (Fig. 3B, lane Ye). The bacteria selected in both elemental sulfur and chalcopyrite contained a spacer of the size observed in *T. thiooxidans* (Fig. 3A, lanes S and Cha, respectively). These did not form heteroduplexes, suggesting that a single strain had been selected in both cultures. Altogether, only three of the six spacers distinguished by size in the leached ore could be recovered from the cultures in the plant leaching solution, despite the different energy sources employed.

Sequence homology of the spacers observed in leached ore and in cultures to those of collection strains. The spacers amplified from the DNAs extracted from leached ore and cultures were hybridized with radioactively labeled amplified spacers from the *T. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans* strains. Samples of labeled and unlabeled spacers were mixed, denatured, and renatured, and the formation of hybrids was subsequently analyzed by gel electrophoresis and autoradiography (Fig. 4). A low concentration of radioactive spacer was used to avoid self-reannealing; thus, radioactive bands observed under this condition represent only the single-stranded DNAs (Fig. 4, lanes −, lower panel). The radioactive amplified spacer of *T. ferrooxidans* yielded at least eight radioactive bands when reannealed with the amplification product from leached ore DNA (Fig. 4, lane O, lower panel): one at the position of the homoduplex, another close to the *T. ferrooxidans-T. thiooxidans* heteroduplex, and six between these two bands. These last six bands probably correspond to heteroduplexes with higher homology than the *T. ferrooxidans-T. thiooxidans* heteroduplex. The hybridization with the spacers am-

plified from the bacteria selected in ferrous iron gave only two of the radioactive bands observed with the product of leached ore DNA. With the amplification product of cultures in either elemental sulfur or chalcopyrite, the *T. ferrooxidans* amplified spacer gave a single band at the position of the *T. ferrooxidans-T. thiooxidans* heteroduplex. Hybridizations of the labeled amplified spacer from *T. thiooxidans* with the amplification product of DNA extracted from leached ore yielded radioactive hybrids which migrated either like the homoduplex or like the *T. ferrooxidans-T. thiooxidans* heteroduplex. With the product of the bacteria grown in ferrous iron, the hybrids formed had migration rates similar to those of the *T. ferrooxidans-T. thiooxidans* heteroduplex. The hybrid formed with the product of bacteria grown in either elemental sulfur or chalcopyrite migrated like the homoduplex. Hybridization of the labeled amplified spacer from *L. ferrooxidans* with the spacers of leached ore DNA yielded a radioactive hybrid that migrated like the homoduplex along with additional radioactive bands that probably corresponded to heteroduplexes. Hybridization with the amplified spacers from the bacteria selected in ferrous iron medium yielded the same products observed with leached ore DNA, although in very different proportions. The heteroduplex between labeled *L. ferrooxidans* and *T. thiooxidans*, expected with the cultures in elemental sulfur and chalcopyrite, probably corresponds to the radioactivity remaining very close to the top of the gel.

DISCUSSION

The observation of six 16S to 23S ribosomal DNA (rDNA) spacers of different sizes after PCR amplification of the DNA extracted from the leached ore suggested the presence of at least six different bacterial strains in the leached ore. Analysis of the heteroduplexes formed between amplified spacers permitted the study of the degree of sequence homology among them as well as the homology with the spacers of culture collection strains of the species *T. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans*. Heteroduplexes between amplified 16S to 23S rDNA spacers have been described previously (12, 13). The two different spacer classes of *Escherichia coli* form a heteroduplex of decreased electrophoretic mobility that is separated into two bands. Each band probably corresponds to the hybrid formed by the plus and minus complementary strands of each spacer class. According to the sequences reported for the spacers of *T. ferrooxidans* and *T. thiooxidans* (23, 25), they would form a heteroduplex with a similar structure, containing a double-stranded section of about 270 and 130 bases at the 16S and 23S ends, respectively, and a single-stranded loop of 100 bases. The correlation between electrophoretic mobility and sequence homology of the heteroduplexes has been shown for amplification products of different HIV-1 types or subtypes (6). Assuming that this relationship holds for the heteroduplexes formed by the 16S to 23S genes coding for rRNA spacers, the formation of radioactive hybrids with mobility equal to that of the homoduplex indicated the presence of spacers practically homologous to the radioactively labeled spacers of the culture collection strains. On the other hand, the formation of heteroduplexes migrating between the homoduplexes and the *T. ferrooxidans-T. thiooxidans* heteroduplex indicated the presence of spacers with higher homology than those between these two closely related species (8, 10, 18). Although the phylogenetic distance reflected by differences in nucleotide sequence of the spacers is not well defined (3, 12), spacers with higher homology than that between *T. ferrooxidans-T. thiooxidans* spacers were assumed to be derived from different strains of the same species. Accordingly, our results indicate that the bacteria present in bioleached ore form a community composed of a fairly homogeneous population of *T. thiooxidans* and populations of *T. ferrooxidans* and *L. ferrooxidans*, comprised of at least four and three different strains, respectively. The number of different strains was estimated under the assumption that most of the heteroduplexes were resolved into two bands after gel electrophoresis. Our results indicate that the same species described for low-salt leaching (21) are found under high-salt leaching conditions. The differences found in the bacterial communities under both conditions can be attributed to strain variants within the same species. Although the type of analysis used here and in the previous work (21) would have detected other bacterial species even if not culturable, all of the bacteria detected appear to be closely related to those already described by methods involving cultivation. This observation differs from those commonly obtained by analyses of DNA extracted from samples of different environments which have revealed a much larger microbial diversity than that observed by methods involving culturing (1). Moreover, the diversity observed for each of the three bacterial species found in the leached ore was similar to that previously observed when independent isolates of these species were compared; while isolates of *T. ferrooxidans* and *L. ferrooxidans* show large genotypic differences, *T. thiooxidans* isolates form a fairly homogeneous group (9, 10, 21).

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