Direct 5S rRNA Assay for Monitoring Mixed-Culture Bioprocesses

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This study demonstrates the efficacy of a direct 5S rRNA assay for the characterization of mixed microbial populations by using as an example the bacteria associated with acidic mining environments. The direct 5S rRNA assay described herein represents a nonselective, direct molecular method for monitoring and characterizing the predominant, metabolically active members of a microbial population. The foundation of the assay is high-resolution denaturing gradient gel electrophoresis (DGGE), which is used to separate 5S rRNA species extracted from collected biomass. Separation is based on the unique migration behavior of each 5S rRNA species during electrophoresis in denaturing gradient gels. With mixtures of RNA extracted from laboratory cultures, the upper practical limit for detection in the current experimental system has been estimated to be greater than 15 different species. With this method, the resolution was demonstrated to be effective at least to the species level. The strength of this approach was demonstrated by the ability to discriminate between *Thiobacillus ferrooxidans* ATCC 19859 and *Thiobacillus thiooxidans* ATCC 8085, two very closely related species. Migration patterns for the 5S rRNA from members of the genus *Thiobacillus* were readily distinguishable from those of the genera *Acidiphilium* and *Leptospirillum*. In conclusion, the 5S rRNA assay represents a powerful method by which the structure of a microbial population within acidic environments can be assessed.

Bioprocesses which utilize pure cultures and sterile feeds predominate in the food, pharmaceutical, and specialty chemical industries. However, for large-scale bioprocessing in the mining industry, the same approaches are not feasible. For example, large-scale depyritization of coal is expected to use mixed populations of microorganisms for two reasons. First, mixed populations are more effective for coal desulfurization, and second, it would be very difficult and prohibitively expensive to sterilize the huge quantities of coal fed into large-scale bioprocesses. Not only do mixed populations of natural microbial floras behave as consortia, but also bacteria which have been added to enhance desulfurization may not be able to compete effectively and be maintained along with the population of indigenous microorganisms (22).

One requirement for mixed-culture bioprocessing is the development of appropriate methods for rapid analysis of consortium members. Conventional techniques are problematic for a number of reasons. Plating is a common method for enumerating bacteria. Unless bacteria are efficiently removed from the substrate, e.g., ore or coal particles, population determinations by plate-counting procedures are certain to be biased. Genera such as *Thiobacillus* and *Leptospirillum* are distinguished on the basis of colony appearance, deposition of iron, and incubation time to achieve visible colonies. The cultivation of *Thiobacillus* and *Leptospirillum* species requires 7 to 14 days, respectively, for colonies to be seen without magnification (16). The extended incubation times and the problems in distinguishing between *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* plate counts make this method inadequate for real-time processing control.

Many microscopic counting procedures that use fluorescent

dyes such as acridine orange to stain bacteria are straightforward but inadequate for at least two reasons: (i) the inability to discriminate between populations of morphologically similar bacteria and (ii) the inability to discriminate between metabolically active and dormant or dead microorganisms. While some fluorescent-dye staining procedures are able to assess viability, the issue of identification remains unaddressed. Microscopic enumeration with fluorescently labelled antibodies can be used to identify populations but is not designed to assess the metabolic activity or viability of the member populations. In many studies, oligonucleotide probes have been used for identification and for determination of relative abundances. However, for applications related to coal desulfurization or sulfidic ore oxidation, oligonucleotide probes based on 16S rRNA sequences may not be able to distinguish between *T. ferrooxidans* and *T. thiooxidans*.

The purpose of this study was to develop a general protocol and evaluate the direct 5S rRNA assay for its ability to discriminate among the bacteria that are integral to coal depyritization or the oxidation of sulfidic ores. The assay described herein has been evaluated for its ability to distinguish among genera, e.g., *Thiobacillus*, *Leptospirillum*, and *Acidiphilium*, as well as between closely related species, i.e., *T. ferrooxidans* and *T. thiooxidans*. The 5S rRNA assay was also applied to mixed cultures used for the biooxidation of refractory gold ores.

MATERIALS AND METHODS

Cultures and growth conditions. The bacteria used in this study are listed in Table 1. *T. ferrooxidans* and *Leptospirillum ferrooxidans* were cultivated with 50 mM FeSO₄ in a basal salts medium (pH 2) based on that of Johnson et al. (14), which contained (NH₄)₂SO₄, 1.25 g; MgSO₄ · 7H₂O, 0.50 g; K₂HPO₄, 0.25 g; and trace elements solution, 0.5 ml/liter. The *Acidiphilium* spp. were cultivated in basal salts medium (pH 3) to which glycerol, 1.8 g/liter, was added (14). The iron-oxidizing heterotroph SLC2 (11) was cultivated in basal salts medium (14) containing 50 mM FeSO₄ and glycerol at 1.8 g/liter. *T. thiooxidans* was cultivated at pH 3.5 in medium (36) that contained KH₂PO₄, 3.0 g; (NH₄)₂SO₄, 3.0 g; MgSO₄ · 7H₂O, 0.50 g; CaCl₂ · 2H₂O, 0.25 g; and K₂S₂O₈, 3.2 g in 1 liter of deionized water. The thermophilic iron-oxidizing enrichment culture was cultivated in medium containing (NH₄)₂SO₄, 0.4 g; MgSO₄ · 7H₂O, 0.25 g; K₂HPO₄,

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TABLE 1. Acidophilic microbial cultures used in this study

Culture	Original source	Reference(s)
<i>Acidiphilium angustum</i> ATCC 35903 (KLB)	Coal mine drainage, Pa.	39, 40
<i>Acidiphilium cryptum</i> ATCC 33463 (Lhet2)	Culture of <i>Thiobacillus ferrooxidans</i>	7
<i>Acidiphilium facilis</i> ATCC 35904 (PW2)	Abandoned deep mine, Pa.	39, 40
<i>Acidiphilium facilis</i> PW1	Abandoned deep mine, Pa.	39, 40
<i>Leptospirillum ferrooxidans</i> BU-1	Copper mine drainage, Bulgaria	6
<i>Leptospirillum ferrooxidans</i> MK	The original Markosyan isolate, Armenian mine	13
<i>Thiobacillus ferrooxidans</i> ATCC 23270	Coal mine effluent	6
<i>Thiobacillus ferrooxidans</i> ATCC 19859	Copper leachate, British Columbia, Canada	19
<i>Thiobacillus thiooxidans</i> ATCC 8085	Soil	37
Heterotroph NCIB 12826 (SJH)	Disused pyrite mine, North Wales	15
Thermophilic heterotroph (YTH13)	Hot spring, Yellowstone National Park, Wyo.	12
Thermophilic heterotroph (YTH15)	Hot spring, Yellowstone National Park, Wyo.	12
Iron-oxidizing heterotroph (SLC2)	Regolith sample	11

0.04 g; yeast extract, 0.2 g; and 100 mM FeSO_4 in 1 liter. Filter-sterilized stock solutions of $\text{K}_2\text{S}_2\text{O}_8$ and FeSO_4 were added to the culture media after autoclaving. FeSO_4 was prepared as a concentrated stock solution from $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, titrated against a KMnO_4 standard to determine the Fe^{2+} concentration, and then filter sterilized.

Cell suspensions of mixed microbial populations used for the leaching of sulfidic gold ores were provided by Gregory J. Olson, Little Bear Laboratories, Red Lodge, Mont., and shipped by overnight mail. One cell suspension was prepared from an enrichment culture maintained on pyrite, while the other cell suspension was prepared from a continuous stirred-tank reactor that had been inoculated 6 weeks previously with the same enrichment culture.

Extraction of RNA. The procedure for RNA extraction was based on the method of Peppel and Baglioni (30). For acidophilic heterotrophic bacteria, biomass was collected by centrifugation and approximately 0.1 ml (2 loopfuls) of collected biomass was transferred to 0.5 ml of lysis solution (2% sodium dodecyl sulfate [SDS], 200 mM Tris-HCl [pH 7.5], 1 mM EDTA) at room temperature for 5 to 10 min. After lysis, 150 μl of 5 M potassium acetate solution was added to each microcentrifuge tube and the contents were mixed. Following chilling on ice or at -20°C for 10 to 15 min, the solutions were clarified by centrifugation at maximum speed in a microcentrifuge for 5 min at 5°C . The supernatants were removed, placed in clean microcentrifuge tubes, and extracted with 0.3 ml of chloroform-isoamyl alcohol (24:1). The aqueous phase was transferred to a new tube, and nucleic acids were precipitated by the addition of 0.6 ml of ice-cold isopropanol. The material was held at -20°C for a minimum of 20 min. RNA was resuspended in diethylpyrocatechol-treated deionized water, and the amount of nucleic acid was estimated by using DNA Quik Strips (Laboratory Research Products, Kodak, New Haven, Conn.).

For cultures such as *T. ferrooxidans*, *T. thiooxidans*, *L. ferrooxidans*, and SLC2, which were cultivated in 2- to 10-liter volumes, biomass was collected by filtration onto HT Tuffryn (Gelman Sciences, Ann Arbor, Mich.) filter (pore size, 0.2 μm) cartridges. The filters were rinsed with 200 to 300 ml of salts solution (pH 2) or 0.1 or 0.2 N H_2SO_4 . Biomass was lysed on the filter with 10 to 15 ml of lysis solution. The filter units were rolled intermittently for 5 to 10 min at room temperature. Lysed material was withdrawn from the inlet port of the filter units and split between two 50-ml centrifuge tubes. The rest of the RNA preparation procedure was continued as described above with proportionally higher volumes of reagents. Nucleic acids were precipitated from solution with isopropanol or ethanol overnight at -20°C .

The thermophilic iron-oxidizing bacterium required treatment with lysozyme prior to lysis with SDS. This culture was collected by filtration and rinsed as described above. Cells were removed from the filter with 20 ml of a solution containing 250 mM Tris-HCl and 1.25 mM EDTA and were incubated at 45°C with 1 mg of lysozyme per ml for 20 min. The cells were lysed by addition of 2% (final concentration) SDS to the incubation buffer, and the rRNA was prepared as described above.

DGGE. A modification of the denaturing gradient gel electrophoresis (DGGE) method of Myers et al. (24, 25) was used to separate the 5S rRNA species. DGGE was performed with a dual-capacity DGGE system (model DGGE-2000; C.B.S. Scientific Co., Inc., Del Mar, Calif.). Denaturing gradient gels (14.5 by 20 cm) containing urea and acrylamide gradients that were perpendicular to the direction of electrophoretic separation were formed by casting gels with the apparatus on its side. A gradient between 7 M urea–10% acrylamide (37.5:1; acrylamide/bisacrylamide) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 1 mM EDTA, 20 mM acetate) and 15% acrylamide (37.5:1) in TAE was formed over half of the gel, with the remaining half of the gel was formed from 15% acrylamide (37.5:1) in TAE. RNA preparations (10 to 100 μg of nucleic acid for each bacterium) were distributed within the single well that ran along the entire top edge of the gel. Electrophoresis was performed at 65°C for 5 h at a constant voltage of 250 V (model 3000xi power supply; Bio-Rad, Hercules, Calif.). The gels were stained with SYBR Green II RNA gel stain (Molecular

Probes, Eugene, Oreg.) in TAE, visualized with a UV transilluminator (model T1202; Sigma Chemical Co., St. Louis, Mo.), and photographed with a Polaroid MP-4 land camera (model 44-01; Polaroid Corp., Cambridge, Mass.) with a SYBR Green gel photographic filter (Molecular Probes, Inc.).

RESULTS

DGGE analysis of iron-oxidizing autotrophs. The 5S rRNA assay was evaluated with known cultures of microorganisms whose origins were in acidic mining environments (Table 1), particularly microorganisms for which 5S rRNA or 16S rRNA sequence information was available. Among the acidophilic cultures used for this study are the autotrophic bacteria of the genera *Thiobacillus* and *Leptospirillum*, heterotrophic bacteria of the genus *Acidiphilium*, and iron-oxidizing heterotrophic

FIG. 1. Negative image of a SYBR II-stained gel obtained for *T. ferrooxidans* ATCC 23270. Within the left half of the gel, about one-quarter of the way down from the top, the double-banded pattern for the 5S rRNA species of the *Thiobacillus* microorganism is visible. The dark bands in the upper region of the gel have been assigned to the higher-molecular-weight RNAs, i.e., 16S and 23 rRNAs, while the bands clustered within the lower portion of the gel have been assigned to the tRNA species.

FIG. 2. Negative image of a SYBR II-stained gel obtained for *T. ferrooxidans* ATCC 23270 and *L. ferrooxidans* MK. Within the left half of the gel, the single band assigned to the 5S rRNA of *L. ferrooxidans* migrated to a position above that of the double-banded pattern for the 5S rRNA of *T. ferrooxidans*.

FIG. 3. Negative image of a SYBR II-stained gel obtained for *T. ferrooxidans* ATCC 23270 and *T. thiooxidans* ATCC 8085. Within the left half of the gel, the double-banded pattern assigned to the 5S rRNAs of *T. thiooxidans* migrated to a position above that of the double-banded pattern assigned to *T. ferrooxidans*.

bacteria. Members of the genera *Thiobacillus* and *Leptospirillum* are autotrophic microorganisms which mediate the oxidation of iron and sulfur and are required for ore and coal bioprocessing. The double-banded pattern for *T. ferrooxidans* ATCC 23270 (Fig. 1) was readily distinguished from the band obtained for *Leptospirillum ferrooxidans* MK (Fig. 2). With the separation of 5S rRNAs isolated from *T. ferrooxidans* and *T. thiooxidans* (Fig. 3 and 4), we have demonstrated that DGGE, which has the capability to resolve fragments differing by a single base in as many as 450 bp (26), is more than sufficient to resolve the 5S rRNAs from two very closely related bacteria (5, 17, 18).

DGGE analysis of acidophilic heterotrophs. Heterotrophic bacteria are thought to affect the overall oxidation of pyritic ore in several ways. Heterotrophs indirectly enhance the oxidation process by scavenging organic acids that inhibit *Thiobacillus* and *Leptospirillum* species (11). Heterotrophs may also affect bioleaching rates by their ability, in some cases, to reduce Fe^{3+} or oxidize Fe^{2+} (11). The 5S rRNA assay was capable of resolving the 5S rRNAs from some members of the genus *Acidiphilium*. For example, the 5S rRNAs from *Acidiphilium cryptum* Lhet2 (Fig. 5) and *Acidiphilium angustum* KLB (Fig. 6) were distinguished from that of *Acidiphilium facialis* PW2. The 5S rRNA of *A. angustum* KLB migrated above the 5S rRNA of *L. ferrooxidans* BU-1 (Fig. 7). The 5S rRNA of an iron-oxidizing heterotrophic microorganism, designated SLC2 (Fig. 8), migrated in a pattern that is strikingly different from the patterns obtained for the genera *Thiobacillus*, *Leptospirillum*, and *Acidiphilium*. The migration pattern for SLC2 was similar to those obtained for two thermophilic isolates, YTH13 and YTH15 (Fig. 9), obtained from an acidic hot spring in Yellowstone National Park, Wyo. (10, 12), and a thermophilic enrichment culture from a heap leach operation (Fig. 10) (see below).

DGGE analysis of mixed cultures. The 5S rRNA assay was applied to three uncharacterized, presumably mixed cultures that were being maintained for the purpose of oxidizing refractory gold ores in industrial leaching operations. A gram-

FIG. 4. Negative image of a SYBR II-stained gel obtained for *T. ferrooxidans* ATCC 19859 and *T. thiooxidans* ATCC 8085. Within the left half of the gel, the double-banded pattern assigned to the 5S rRNAs of *T. thiooxidans* migrated to a position above the single band assigned to *T. ferrooxidans* ATCC 19859.

FIG. 5. Negative image of a SYBR II-stained gel obtained for *A. cryptum* Lhet2 and *A. facilis* PW2. The double-banded pattern assigned to the 5S rRNA species of *A. cryptum* Lhet2 migrated to a position above the double-banded pattern for the 5S rRNAs of *A. facilis* PW2.

FIG. 6. Negative image of a SYBR II-stained gel obtained for *A. angustum* KLB and *A. facilis* PW2. The double-banded pattern assigned to the 5S rRNA species of *A. angustum* KLB migrated to a position above the double-banded pattern for the 5S rRNAs of *A. facilis* PW2.

positive, thermophilic, iron-oxidizing culture, provided by J. A. Brierley, Newmont Metallurgical Services, Salt Lake City, Utah, was derived from a heap-leaching operation in which the temperatures within the piles exceeded, through self-heating, the mesophilic range of *Thiobacillus*, *Leptospirillum*, and *Acidiphilium* species. The thermophilic culture was enriched for and maintained by incubation at 45°C in a basal salts solution (pH 1.8) containing yeast extract and FeSO₄. Sterile techniques were used to establish and maintain the enrichment culture, but no attempt was made to isolate and establish a pure culture. The 5S rRNA profile for the thermophilic enrichment culture (Fig. 10) showed two closely spaced 5S rRNA bands in the lower region of the gel, just above the region for tRNA. The location within the gel was similar to that for 5S rRNA of the iron-oxidizing heterotroph SCL2 (Fig. 8) and the thermophilic isolates YTH13 and YTH15 (Fig. 9).

Cell suspensions provided by G. J. Olson, Little Bear Laboratories, Inc., were derived from mixed cultures maintained on gold-bearing pyritic ores. A batch culture of iron-oxidizing bacteria was maintained in low-ionic-strength basal salts medium (pH 1.8) with pulverized ores or ore concentrates. The batch culture was originally inoculated with ore and acidic mine waters from Montana, Chile, and Pennsylvania. The culture was incubated with shaking at 25°C in 1-liter flasks and periodically freshened with medium and ore. The basal salts medium and ores were not autoclaved; therefore, microorganisms indigenous to the ores were introduced each time the mixed cultures were replenished. This batch culture was used to inoculate a continuous stirred-tank reactor, which operated for approximately 6 weeks before the cells were harvested for this study. The pyrite-arsenopyrite ores used to maintain cultures contained 6 to 8% iron or approximately 15% pyrite. The ore concentrates had been enriched for the gold-bearing sulfide fraction by flotation technology and contained 35 to 40% iron or approximately 80% pyrite.

Both the batch (Fig. 11) and continuous mixed (Fig. 12) cultures had relatively simple 5S rRNA profiles consisting of three major bands. The two bands in the upper region of the gels were suggestive of the iron-oxidizing autotroph genera, *Thiobacillus* and *Leptospirillum*. Indeed, microscopic examination of the mesophilic enrichment cultures revealed a "leptospirillum-like" cell morphology for approximately 50% of the cells (28). The band in the lower region of the gel was similar in placement to the 5S rRNA bands for the iron-oxidizing heterotroph, SLC2 (Fig. 8) and the thermophilic cultures derived from the acidic hot spring in Yellowstone National Park (Fig. 9) and from the industrial heap leach operation (Fig. 10). In the three cases evaluated, the 5S rRNA profiles of the acidic enrichment cultures were relatively simple, suggesting that mixed cultures used for some leaching operations are dominated by a few species. The culture complexity observed was well within the resolution capacity of this assay, which has been estimated to be between 15 and 20 species.

DISCUSSION

DGGE uses gradients of denaturant, typically urea and formamide, in polyacrylamide gels. It has been possible to detect single point mutations in GC-clamped fragments ranging up to several hundred base pairs in length (24). In aqueous solution, 5S rRNAs are highly folded (21, 31), and while the impact of sequence differences on folding is subtle, the differences in the free energies of secondary structures are not (27). Through the exploitation of DGGE, subtle sequence-related differences in the Gibbs free energy of the folded structures of the various 5S rRNAs translate to differential loss of secondary structure in a gradient of denaturant (20). This provides the means for resolving the individual 5S rRNAs from mixtures by differenti-

FIG. 7. Negative image of SYBR II-stained gel obtained for *A. angustum* KLB and *L. ferrooxidans* BU-1. The double-banded pattern assigned to the 5S rRNA species of *A. angustum* KLB migrated above the band assigned to *L. ferrooxidans* BU-1.

FIG. 8. Negative image of a SYBR II-stained gel obtained for an iron-oxidizing heterotrophic bacterium (SLC2), *L. ferrooxidans* BU-1, and *T. ferrooxidans* ATCC 19859. The band assigned to strain SLC2 was a horizontal band that migrated to a position approximately halfway down the gel.

ating among similar 5S rRNAs on the basis of the denaturant concentration required for complete unfolding.

In this study, gradients of urea and acrylamide were formed perpendicular to the direction of the electrical field. As viewed in Fig. 1 to 12, gels were formed such that the urea concentration decreased and the acrylamide concentration increased from the right edge to the middle of the gel. The left half of the gel contained no denaturant and an acrylamide concentration of 15%. Within the gel, particularly the portion of the gel where the denaturant concentration was high (right side), obvious groupings of 5S rRNA and the class 1 and class 2 tRNAs were observed (Fig. 1 to 12). These groupings were similar to the patterns observed for small rRNA species separated by denaturing gel electrophoresis (1, 8).

As a laboratory tool, the 5S rRNA assay may well provide an effective first estimate of the relatedness of microbial isolates, particularly uncharacterized environmental isolates, prior to the investment of the time and resources required for rRNA sequence analyses. The 5S rRNAs of closely related microorganisms (17, 18), such as those in Fig. 4, were observed to migrate more closely together than did the more distantly related microorganisms (17, 18), such as those in Fig. 8. The acidophilic heterotrophic bacterium designated SJH had been classified, on the basis of its ability to reduce ferric iron, with *Acidiphilium organovorum* (15). The comigration of the double-banded pattern for the 5S rRNA of isolate SJH with the 5S rRNA of *A. cryptum* Lhet2 (data not shown) is consistent with the recent sequence analysis of the 16S rRNA of strain SJH that indicated that this microorganism was closely related to *A. cryptum* Lhet2 (32). Comigration of 5S rRNA bands was observed for isolates YTH13 and YTH15 (Fig. 10), which were isolated from an acidic hot spring in Yellowstone National Park, Wyo. (12). This observation was consistent with the 16S

rRNA sequence data, which indicated that YTH13 and YTH15 were closely related (32). Comigration was also observed for *Acidiphilium facilis* PW1 and PW2 (data not shown), which were isolated from an abandoned deep mine in Penn-

FIG. 9. Negative image of a SYBR II-stained gel obtained for two thermophilic cultures (YTH13 and YTH15) obtained from an acidic hot spring in Yellowstone National Park, Wyo. The 5S rRNAs of these isolates migrated in a horizontal band that overlapped with the tRNAs in the left-hand portion of the gels.

FIG. 10. Negative image of a SYBR II-stained gel obtained for a thermophilic enrichment culture derived from an industrial heap-leaching operation. The bands attributed to the 5S rRNAs migrated to a position just above the visible tRNA bands.

FIG. 11. Negative image of a SYBR II-stained gel for a cell suspension obtained from a batch enrichment culture that had been maintained on pyrite ores. The bands were suggestive of *Leptospirillum* and *Thiobacillus* species and the gram-positive or gram-positive-like microorganisms.

sylvia (39) and identified by classical determinative methods (40).

DGGE analysis of 5S rRNA species was determined to be an effective approach for evaluating the population of microorganisms associated with the oxidation of sulfidic ores or iron pyrite. This assay can be completed within a time frame that is appropriate for real-time ore or coal bioprocessing. Depending on the number of samples, the extraction procedure requires approximately 1 to 2 h to complete followed by approximately 6 to 8 h for the electrophoresis component of the procedure, which includes setup, electrophoresis for 4 to 5 h, staining, and photography. While detection limits were not determined, in a formal sense, a working knowledge of the biomass required for the assay was acquired. Because of the large well size, between 10 and 100 μg of nucleic acid was added per culture tested. One gel requires RNA from approximately 1×10^{10} to 3×10^{10} cells. Because this assay was intended for large-scale bioprocessing, biomass limitation was not an issue. In situations in which biomass may be limited, it would be possible to increase the sensitivity of the assay by using radioactive or fluorescent end-labelling procedures (1, 9).

One of the observations made during the course of this study was the presence of double bands in some of the 5S rRNA profiles, e.g., *T. ferrooxidans* ATCC 23270, *T. thiooxidans* ATCC 8085, and *A. angustum* KLB. Double bands could be interpreted as an indication of mixed cultures. However, double bands were observed for two cultures grown from single colonies: *A. angustum* KLB and *A. facilis* PW2. In two other cases, *T. thiooxidans* ATCC 8085 and *T. ferrooxidans* ATCC 23270, the cultures exhibited single-colony morphology. A single-colony morphology, while not definitive evidence, is consistent with a pure culture. Multiple 5S rRNA bands may also be due to the presence of multiple 5S rRNA genes, multiple 5S rRNA species, or terminal-length heterogeneities of 5S rRNA

in bacterial cells (3, 4, 18, 29, 33). Digweed et al. (3) used denaturing gels (12% acrylamide, 7 M urea) to isolate multiple 5S rRNA from *Escherichia coli* A19. In that study, one of the 5S rRNA species isolated differed from the published sequence for this microorganism by only one base. The concentrations of acrylamide and urea that were used by Digweed et al. (3) were comparable to those used in this study. Thus, our observation of double bands suggested that the 5S rRNA assay was extremely sensitive and had the capacity to separate closely related 5S rRNA species or those with terminal length heterogeneities. This observation illustrated one of the difficulties that may be encountered when using 5S rRNA for microbial ecology studies (29). Yet, in the three examples of undefined, potentially mixed cultures, only one, the thermophilic culture, had bands that were suggestive of a doublet belonging to a single bacterial species. The sensitivity of the 5S rRNA assay and the ability to discriminate between closely related rRNA species may make this assay an excellent tool with which to examine 5S rRNA gene expression and precursor processing.

DGGE analysis of 5S rRNA has the potential of providing an estimate of the relative contribution of a species to a community. Relative contribution of a species to a community may be estimated by using the relative intensity of the fluorescently stained or end-labelled 5S rRNA bands. The relative contribution may be the result of the contribution of a few cells that are rapidly growing or a larger number of less active cells. Höfle (9) used low-molecular-weight RNA analysis to monitor microbial population dynamics in a mesocosm. Scans of autoradiograms of the RNA profiles were used to assess changes in the relative amounts of 5S rRNA and tRNA in response to perturbations (9). Similarly, densitometry scans of low-molecular-weight rRNA profiles were also used to compare free-living and particle-associated bacterial populations in estuarine environments (1). In any study in which quantitative assessments

FIG. 12. Negative image of a SYBR II-stained gel for a cell suspension obtained from a continuous stirred-tank reactor operating with pyritic ores. The bands were suggestive of *Leptospirillum* and *Thiobacillus* species and the gram-positive or gram-positive-like microorganisms.

are sought, biases may be introduced by the procedures used. For the 5S rRNA assay, bias may be introduced by methods used for collection of biomass, lysis of cells, and visualization of the nucleic acids. In our example, to minimize the bias introduced by biomass collection, the pore size must be small enough to retain even the smallest bacteria. Unless rigorous methods to lyse bacteria are used, results will be biased toward the more easily lysed members of the microbial population (38). Thus, for acidic mining environments, the lysis procedure must be suitable for both gram-negative and the somewhat more difficult to lyse gram-positive microorganisms. Finally, bias may be introduced by differences in the efficiencies of end-labelling procedures (1) or the type of fluorescent stain used. For SYBR Green II stain, the fluorescent quantum yield varies according to the base composition of the homopolymer (33). This variation is also dependent on whether the nucleic acid is DNA or RNA and whether it is in the single- or double-stranded form. Considering this, bias determinations will have to be made with purified 5S rRNA of target microorganisms, such as *Thiobacillus*, *Leptospirillum*, and *Acidiphilium* species.

The 5S rRNA assay has the potential of finding the identity of community members. Direct extraction and sequence analysis of the electrophoretically separated 5S rRNA species was used to identify an Antarctic endolithic bacterium (2), bacteria in the mud obtained from the bottom of a copper leaching pond (18), bacteria from a thermal spring in Yellowstone National Park (35), and the dominant bacteria within a freshwater mesocosm (9). Muyzer et al. (23) used DGGE to estimate genetic diversity in biofilms from PCR-amplified fragments of 16S rRNA. In that example, several members of the undefined microbial population were identified by hybridization analysis with oligonucleotide probes.

In summary, the DGGE 5S rRNA assay has the resolution required to separate the 5S rRNA of bacteria indigenous to

acidic mining environments or integral to ore bioprocesses. When fully developed, the assay has the potential of providing an estimate of the relative abundance or activity as well as the identity of the members of mixed microbial populations. Coupled with other methods of community characterization, the 5S rRNA assay may well provide a new perspective on population trends within a natural ecosystem, a heap leach operation, or a continuous stirred-tank reactor.

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