Microbial Ecology

Analysis of Community Composition during Moderately Thermophilic Bioleaching of Pyrite, Arsenical Pyrite, and Chalcopyrite

M. Dopson and E.B. Lindström

Department of Molecular Biology, Umeå University, S-901 87, Umeå, Sweden

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Abstract

An analysis of the community composition of three previously undefined mixed cultures of moderately thermophilic bioleaching bacteria grown at 45°C on pyrite, arsenical pyrite, and chalcopyrite has been carried out. The bacterial species present were identified by comparative sequence analysis of the 16S rRNA gene isolated from the bioleaching vessels and analyzed by denaturing gradient gel electrophoresis, cloning, and sequencing. The mixed cultures leached all three minerals, as shown by the increase in iron released from the mineral concentrates. The species identified from the mixed cultures during bioleaching of pyrite, arsenical pyrite, and chalcopyrite were clones closely related to *Acidithiobacillus caldus* C-SH12, *Sulfobacillus thermosulfidooxidans* AT-1, "*Sulfobacillus montserratensis*" L15, and an uncultured thermal soil bacterium YNP. It was also found that the same mixed culture maintained for over a year on chalcopyrite mineral selected approximately the same consortia of bacteria as the original mixed culture grown on chalcopyrite.

Introduction

Acidophilic microorganisms play an important role in environmental and industrial systems, including the environmental problems of acid mine drainage (AMD), acid rock drainage (ARD), and the biotechnological process termed bioleaching. The microorganisms typically found in these environments are the chemolithotrophic and heterotrophic acidophiles (reviewed in [15]) where they enhance the dissolution of minerals.

Formerly, phylogenetic analysis of a mixed culture was restricted to plate-based assays. Plating is constrained by the selection of a limited number of species and led to the misconception that the microorganism populations at AMD, ARD, and bioleaching sites at ambient temperatures were dominated by a few species, such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* [4]. Previously, a mixed culture of moderately thermophilic microorganisms had been isolated from a coal spoil tip, Kingsbury, UK, where internal temperatures naturally reached ~45°C because of spontaneous and biologically mediated exergonic reactions [20]. The phylogeny of this environmental culture has not been extensively analyzed, although two species have been isolated from the Kingsbury coal spoils by plating: *Acidithiobacillus caldus* KU [16] and *Sulfobacillus thermosulfidooxidans* [9, 20].

The understanding of microorganisms present in acidophilic environments has been accelerated by the application of molecular phylogenetic techniques. These techniques have been applied to natural AMD sites [2, 3], coal-impacted ARD sites [5], biooxidation plants operating at mesophilic temperatures [8, 10, 11, 14, 26, 27], and acidic geothermal sites [6]. It has now been shown that "*Leptospirillium ferriphilum*" (formerly *Leptospirillium ferrooxidans* group II) [7] and *At. caldus* are widely distributed in bioleaching plants operating between 40 and 55°C [24, 27], as well as species from many other genera, including *Acidiphilium*, *Acidocella*, "*Ferromicrobium*", and *Ferroplasma* [23, 24, 31].

In this paper we identify the complete phylogeny of three mixed cultures derived from the Kingsbury coal spoil culture that have been used to catalyze the bioleaching of three minerals: pyrite, arsenical pyrite, and chalcopyrite. The species contained in the mixed populations were identified by extracting DNA from dena-

Correspondence to: M. Dopson; E-mail: mark.dopson@molbiol.umu.se

turing gradient gel electrophoresis (DGGE) bands, cloning, and sequencing of the 16S rRNA gene. It was also tested whether maintenance of one of the cultures on chalcopyrite for over a year affected the range of species in the mixed culture.

Methods

Mineral Concentrates and Growth Conditions. The mineral concentrates used in this study were provided by Boliden Mineral AB, Sweden, and their compositions are listed in Table 1. The concentrates were ground and passed through a sieve with pore size 75 μm. The mixed culture from the Kingsbury coal spoil tip was grown in shake flasks containing mineral salts medium [9] pH 2.5 and either 4% (wt/vol) pyrite, 1.5% arsenical pyrite, or 4% chalcopyrite at 45°C and sparged with 1% (vol/vol) CO_2 in air. The flasks were shaken at 200 rpm for up to 3 months, and then the enrichment cultures were frozen. These three frozen cultures were used to inoculate shake flasks [9] that were subsequently incubated for 24 to 48 h as above (until growth in the culture was observed under the microscope). A shake flask was also inoculated from a culture that had been grown on chalcopyrite, and then stored on the mineral residue for 15 months. From the flasks, 25 mL was inoculated into 1-L stirred-tank reactors (in triplicate) containing 4% (wt/vol) pyrite, arsenical pyrite, or chalcopyrite concentrates [9] and grown at 45°C with a stirring speed of 300 rpm and sparged with 300 mL CO_2 enriched air min^{-1}. Uninoculated stirred-tank reactors (in duplicate) were also incubated for 12 days to assess metal dissolution by chemical leaching. To ensure that the microorganism populations enriched in the stirred tank reactors were from the inoculum, rather than any microorganisms on the mineral, the concentrates were autoclaved prior to addition to the culture vessels. The vessels were sampled every 2 days. The total iron released from the mineral, i.e., dissolved iron and secondary minerals (Fe_{tot}), dissolved iron in the supernatant (Fe_{sup}), copper, and arsenic were analyzed by atomic absorption spectroscopy; dissolved ferrous iron was titrated with ceric sulfate; the redox potential was measured with a Ag/AgCl electrode; and the pH was recorded [9]. Also, 10-mL samples were taken for analysis of the microorganism population by DGGE. All analyses were carried in triplicate and mean ± SD ($n = 3$) presented, except for the uninoculated controls that were carried out in duplicate.

Preparation of Total DNA and PCR Amplification. Total DNA was extracted from the 10 mL sampled from the inoculated vessels by centrifuging at 10,000 g for 10 min to pellet all of the free-swimming and attached microorganisms. The pellet was then washed twice in 10 mM Tris containing 1 mM EDTA pH 8.5 (TE) before resuspending in 1 mL TE. A 100-μL aliquot was removed and incubated for 1 h at 37°C with 10 mg mL^{-1} lysozyme, followed by vortexing at full speed for 3 min with 300 mg glass beads (425 to 600 μm; Sigma, Stockholm, Sweden). The beads were prewashed overnight with 65% nitric acid and then rinsed with sterile ultrapure water (Millipore, Falkenberg, Sweden) until a constant pH was observed. Total DNA was isolated by use of the Wizard DNA Clean Up System (Promega, Sundbyberg, Sweden). DNA was quantified via the optical density at 260 nm on a Hitachi 150-20 spectrophotometer in a 1-cm cuvette.

Table 1. Mineral composition of the concentrates used in the study

	Pyrite	*Arsenical pyrite*	*Chalcopyrite*
FeS_2	94.0%[a]	72.0%	8.4%
FeS	ND[b]	ND	0.8%
FeAsS	ND	17.0%	ND
$CuFeS_2$	ND	ND	77.4%
ZnS	ND	ND	0.1%
SiO_2	6.0%	11.0%	13.2%

[a](wt/wt).
[b]None detected.

Control experiments were carried out to investigate which method of lysis of the microorganisms gave the best results. After washing with TE and treatment with lysozyme the cells were either freeze/thawed four times or heated for 15 min at 70°C up to three times in the presence of 1% sodium dodecyl sulfate. None of these methods increased the number of phylotypes identified or amount of DNA on the DGGE gel (data not shown). It was also tested to see if washing the mineral with 0.05% (vol/vol) Tween X-100 [13] increased the amount of DNA. This procedure should remove any microorganisms attached to the mineral that may not have been removed by lysozyme and bead beating. Again, no increase in the number of phylotypes or concentration of DNA was observed (data not shown). The moderately thermophilic cultures were also analyzed to see if they contained archaea by PCR amplification of the total DNA with the archaea specific primers: Arch21F [12] and 1492R [19]. No archaea-specific bands were observed in the mixed cultures (data not shown).

A portion of the bacterial 16S rRNA gene was amplified using the primers: GM5F with GC-clamp and 907R amplifying a 550-bp fragment containing sufficient sequence information for phylogenetic analysis [21]. PCR amplifications were performed using Ready-To-Go PCR Beads (Amersham Pharmacia, Uppsala, Sweden) on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Falkenberg, Sweden). The PCR program was 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 1 min at 72°C, and finally 72°C for 7 min.

Table 2. Clones containing 16S rDNA from species not selected in the leaching vessels[a]

Clone	*Accession number*	*Closest relative*	*Identity (%)*
MTU 2	AY099227	Feedlot manure bacterium B32 (AF317369)	98.6
MTU 3	AY099228	*Staphylococcus epidermis* SR1 (AF269314)	97.8
MTU 8	AY099233	*Arthrobacter* sp. MB6-20 (ASU85900)	97.9
MTU 30	AY099254	*Cyanobacterium* sp. TAF-A32 (AY038726)	95.1
MTU 31	AY099255	*Chryseobacterium scopthalmum* LMG 13028 (AJ271009)	97.2
MTU 32	AY099256	*Rhodococcus* sp. 67-BEN001 (AY044096)	97.2

[a]These clones are not included in the phylogenetic analysis. The closest relative (accession number in parentheses) and identity values are based on a BLAST search of the 16S rRNA gene sequence.

DGGE Analysis. PCR fragments (200 ng in each lane) were analyzed by DGGE (C.B.S. Scientific Company, Del Mar, U.S.A.) on a 5% (wt/vol) polyacrylamide gel in 20 mM Tris-acetate (pH 7.4), 10 mM acetate, 0.5 mM Na_2 EDTA with a denaturing gradient from 20 to 80% denaturant [100% denaturant corresponds to 7 M urea and 40% (vol/vol) formamide]. The gels (1 mm thick) were prepared using 30% (wt/vol) acrylamide stock solution (Severn Biotech Ltd., Kidderminster, U.K.) and allowed to polymerize for ≥1.5 h. Electrophoresis was run at 200 mV and 60°C for 12 h, after which the gels were stained with 10 mL of 0.003% (vol/vol) SYBR Green 1 nucleic acid stain (Molecular Probes, Leiden, Holland) for 40 min and scanned using a STORM PhosphorImager (Molecular Dynamics, Uppsala, Sweden). All three replicate cultures were analyzed by DGGE and the patterns compared. In the first instance, all bands from the pyrite enrichment culture were excised, cloned, and sequenced (see below). Subsequently, PCR products from the cloned 16S rDNA were then run alongside all three mixed cultures on DGGE gels to confirm the identity and purity of each phylotype. Any additional bands in the other enrichment cultures not present in the pyrite mixed culture were also cloned and sequenced, and the PCR products subsequently run on a DGGE gel to confirm that the correct phylotype had been cloned. This also ensured that identical bands were identified despite any inter-gel variability in migration of matching 16S rDNA bands. Bands presenting identical degrees of motility in the DGGE gels were assigned the same reference number (MTU 1 to 32).

Cloning and Sequencing of the PCR Fragments. A small portion of the bands of interest were punched out of the gel using a sterile 1-mL micropipette tip and suspended in elution buffer. DNA was isolated using the Qiaex II Gel Extraction Kit (Qiagen, Stockholm, Sweden), followed by PCR amplification (as above) and then cleaned using a Quantum Prep PCR Kleen Spin Column (BioRad, Sundbyberg, Sweden). The PCR product was cloned using the pGEM-T Easy Vector System (Promega) and transformed [17] into *Escherichia coli* strain DH5α (laboratory stock; [28]). The resulting transformants were plated onto Luria-Bertani (LB) plates containing 50 μg chloramphenicol mL^{-1}, 100 μm isopropylthio-β-D-galactoside, and 40 μg 5-bromo-4-chloro-3-indolyl-β-D-galactoside mL^{-1}. Colonies were grown in LB medium containing 50 μg chloramphenicol mL^{-1} and the plasmid isolated using the High Pure Plasmid Isolation Kit (Roche, Basel, Switzerland). The 550 bp PCR fragment cloned into the pGEM-T easy vector was PCR amplified in preparation for sequencing in both directions using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia) and oligonucleotides M13 universal (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACCATG-3′) (Amersham Pharmacia). The PCR product was sequenced using an ABI Prism 377 DNA Sequencer.

Phylogenetic Analysis. 16S rRNA gene sequences were compared with the GenBank database using BLAST [1]. Each clone was then tested to see if it was a chimera using the chimera check program at the Ribosome Database Project II site (http://rdp.cme.msu.edu/html/). Of all of the sequences only MTU 24 was found to be a chimera and has not been included in the phylogenetic trees. Those sequences found to be most closely related to species not normally associated with bioleaching were also not included in phylogenetic analysis (identities for these six clones were based on BLAST searches). The remaining sequences were aligned using ARB [29], which was then used to create a distance matrix using a Jukes and Cantor correction. Phylogenetic trees were created by DNA distance and neighbor joining, DNA parsimony, and maximum likelihood methods using ARB. Quoted sequence identities are based on a similarity matrix calculation based on 528 and 527 aligned 16S rDNA base pairs for the Gram-positive and -negative sequences, respectively.

Accession Numbers. The accession numbers for the cloned sequences are listed in Table 2 and Fig. 3.

Results

In order to identify the microorganisms present in the moderately thermophilic mixed cultures grown on different mineral concentrates they were pre-grown for 24 to 48 h to allow the bacteria to start to grow before in-

oculation into stirred-tank reaction vessels with 4% (wt/vol) pyrite, arsenical pyrite, or chalcopyrite at 45°C.

Chemical Leaching. During chemical leaching, neither the amount of metal released from pyrite nor the redox increased significantly over the time course of the experiment (changing from 30 ± 1 to 78 ± 60 mM (n = 2) and from 413 ± 4 to 488 ± 92 mV (n = 2) after 12 days, respectively). The Fe_{tot} values for chemical leaching of arsenical pyrite rose from 21 ± 1 to 29 ± 5 (n = 2) mM after 12 days, without a statistically significant change in the redox (378 ± 11 to 370 ± 28 mV (n = 2) after 12 days). Similarly low values for chemical leaching of chalcopyrite were found, with no significant changes in the redox or metal concentrations (from 11 ± 1 to 13 ± 5 mM and 6 ± 1 to 10 ± 4 mM (n = 2), respectively), and the redox did not significantly alter (353 ± 24 to 388 ± 21 mV (n = 2) after 12 days).

Bioleaching of Pyrite, Arsenical Pyrite, or Chalcopyrite. All of the tested mineral concentrates were leached by the moderately thermophilic mixed cultures (Fig. 1), with the amount of metal leached being significantly higher for all three mixed cultures than their uninoculated controls. As can be seen from the low ferrous iron values at the end of the experiments the microorganisms were oxidizing the ferrous iron released during chemical oxidation of the mineral, resulting in an increased redox at the end of the experiments. A larger amount of iron was released from the pyrite as compared to arsenical pyrite and chalcopyrite, due to the relative amounts of iron contained in each of the mineral concentrates (Table 1). The pH in the leaching vessels dropped below 1.6 for pyrite and arsenical pyrite after 4 and 8 days, respectively, resulting in most of the iron being in solution (data not shown). As the majority of the iron was in solution, the drop in pH was probably due to microbial or chemical oxidation of the reduced sulfur compounds. The pH of the chalcopyrite culture was not reduced below 1.6 until day 14, causing less iron to be in solution (data not shown). This was probably due to the recalcitrant nature of the chalcopyrite to bioleaching, therefore releasing less sulfur, which is oxidized via the main proton producing reactions.

Extractable DNA Present on the Autoclaved Mineral. Even though the mineral concentrates had been treated by potentially toxic flotation chemicals ([22, 25] and unpublished results) and autoclaved they were found to contain amplifiable DNA, probably from dead bacteria sorbed onto the surface of the mineral. The DGGE bands were extracted and sequenced to identify the DNA introduced into the moderately thermophilic mixed cultures from the minerals. The DGGE profiles of the 16S

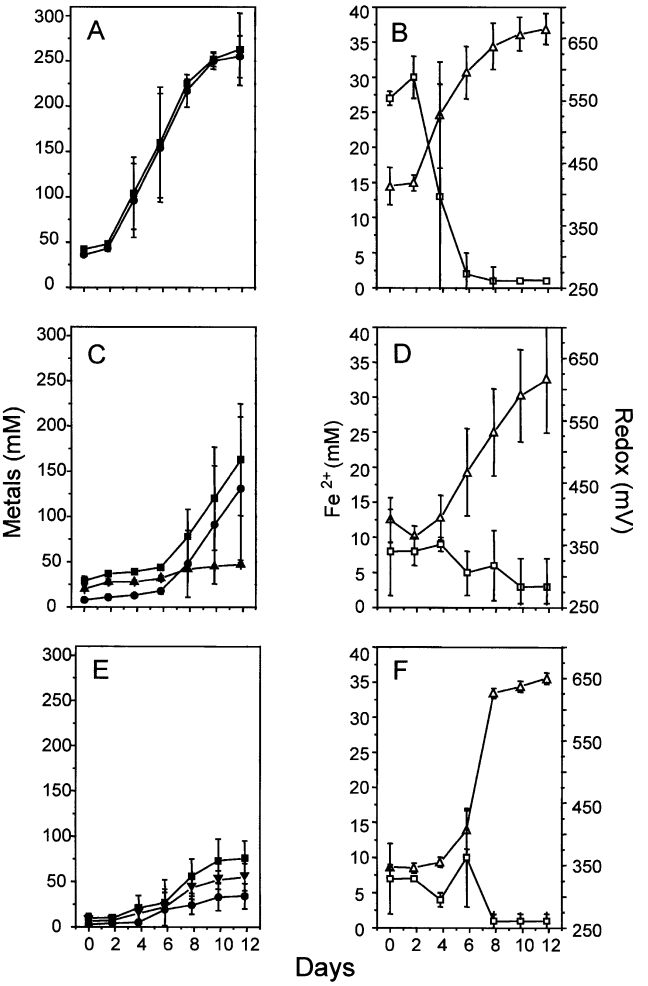

Figure 1. Pyrite (A and B), arsenical pyrite (C and D) and chalcopyrite (E and F) bioleaching by the moderately thermophilic mixed cultures. Symbols, ■: Fe_{tot}; ●: Fe_{sup}; ▲: As_{tot}; ▼: Cu_{tot}; △: redox; □: Fe^{2+}. The data points indicate mean ± SD (n = 3).

rDNA extracted from the three minerals are presented in Fig. 2, and the sequenced bands correspond to the species listed in Table 2 and Fig. 3. Not all of the species found on the autoclaved mineral would normally be found in bioleaching environments and these are listed in Table 2 with their closest relatives.

Other species present are more commonly associated with environments around 45°C, including MTU 1, 4, 6, and 7 that form a clade with an unpublished thermal soil bacterium YNP 10. There were also several clones (MTU 9, 10, and 17) most closely related to the moderately thermophilic sulfur oxidizer *At. caldus* strain C-SH12 [14]. A band corresponding to a group of *At. caldus* strain C-SH12 clones (MTU 11 to 15) had melting characteristics so close that their bands were indistinguishable under the conditions used. Finally, clones MTU 20 and 21 that were in a clade with "*Sulfobacillus montserratensis*" L15 [18] were identified. The phylogeny of

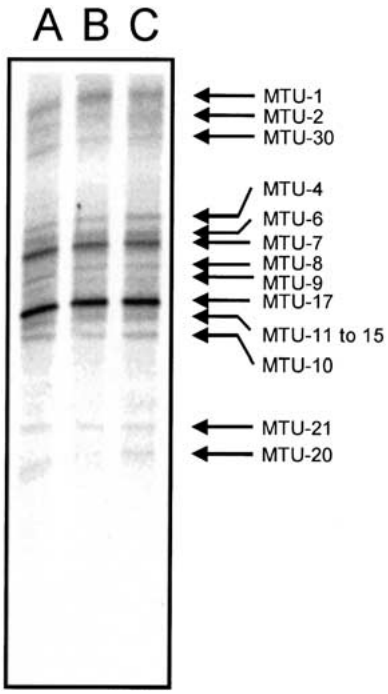

Figure 2. Negative image of the DGGE profile of the bacterial 16S rDNA amplified from pyrite (A), arsenical pyrite (B), and chalcopyrite (C) after autoclaving. All DGGE gels in Figs. 2, 4, and 5 have been cropped to show the same area of gel. The arrows indicate the clones isolated and sequenced from the total DNA.

the cultures and their relationship to other microorganisms implicated in bioleaching (based on comparative 16S rRNA gene sequences) are given in Fig. 3.

Phylogeny of the Frozen Cultures on Pyrite, Arsenical Pyrite, and Chalcopyrite. Although there were some variations in relative positions of identical 16S rDNA fragments between gels, the same phylotypes were identified from the three replicate bioleaching vessels carried out for each mineral concentrate, except in the case of chalcopyrite where one vessel contained three extra clones. The species present in the mixed culture grown on pyrite at day 0 (Fig. 4A) contained a number of DGGE bands that could have been introduced by the extractable DNA from the mineral, or they may also have been present in the mixed culture (MTU 1, 4, 17, 20, 21, and 30). There was also a band corresponding to at least one of the co-migrating *At. caldus* C-SH12 strains MTU 11 to 15. Last, MTU 27 was present that must have come from the mixed culture. MTU 27 is in a clade containing *Su. thermosulfidooxidans* strain AT-1 (99.3% identity) and the unpublished *Sulfobacillus* strain C-MT1 clone (Fig. 3A). After 2 days only the comigrating bands MTU 11 to 15, and MTU 17, 20, 21, and 27 were present. These clones remained until the end of the experiment (12 days), suggesting that several strains closely related to *At. caldus* C-SH12, "*Su. montserratensis*" L15, and *Su. thermosulfidooxidans* AT-1 are sufficient to leach pyrite efficiently. The amount of DNA in the mixed cultures increased from 110 ± 27 μg DNA mL^{-1} upon inoculation to a peak of 288 ± 84 μg DNA mL^{-1} after 8 days. From the relative strengths of the bands it appears that the cell numbers of *Su. thermosulfidooxidans* strain AT-1 increased over the time course of the leaching.

The species present at the start of the leaching of arsenical pyrite also contained, but were not limited by, DNA possibly carried over from the autoclaved mineral (Fig. 4B). The bacterial clones identified were MTU 3, MTU 1, MTU 4, the *At. caldus* C-SH12 co-localizing clones MTU 11 to 15, MTU 16 (99.8% identity with C-SH12), MTU 17, and MTU 27 (groups with the ferrous iron oxidizer *Su. thermosulfidooxidans* AT-1). Once again the amount of DNA in the leaching vessels increased, from 131 ± 55 μg DNA mL^{-1} to 256 ± 66 μg DNA mL^{-1} after 12 days. As with the mixed culture grown on pyrite, the 16S rRNA band from many of the phylotypes was no longer visible on the DGGE gels, and after 4 days only strains of *At. caldus* C-SH12 and *Su. thermosufidooxidans* AT-1 remained, although the relative concentrations of the *At. caldus* strains altered. These two phylotypes were sufficient to leach the arsenical pyrite, albeit slightly more slowly than for pyrite (Fig. 1A, B).

Many more bands were present at the start of the chalcopyrite culture (Fig. 4C). These included MTU 1, 3, 4, 6, 16, and 17. There were also three bands corresponding to clones that upon phylogenetic analysis grouped with *Su. thermosufidooxidans* AT-1: the co-localizing clones MTU 24 and 25, MTU 26 (weakly visible after 12 days, but clearer on replicate gels; data not shown), and MTU 29, and one clone (MTU 23) that is in the clade with "*Su. montserratensis*" L15. MTU 24 was found to be a chimera with bases 1 to 240 being most closely related to *Su. thermosulfidooxidans* and bases 241 to 587 being related to *At. caldus* strain GO-1, and has not been included in the phylogenetic analysis. The amount of DNA on chalcopyrite at the time of inoculum was 117 ± 21 μg DNA mL^{-1}, rising to 287 ± 67 μg DNA ml^{-1}. As with the other minerals only a few of the phylotypes remained at the end of the experiment, with MTU 1, 16, 17, 23, and 29 persisting in the culture, and MTU 27 increasing in number at the end of the bioleaching. Once again, the results suggested that phylotypes related to *At. caldus* C-SH12 and *Sulfobacillus* spp. increased in number and were sufficient to leach the chalcopyrite, although a higher number of clones persisted in the chalcopyrite culture. MTU 5, 18, and 28 were also present in other replicates of the chalcopyrite culture (all experiments carried out in triplicate; data not shown). MTU 5 has a 94.7% identity with the thermal soil bacterium YNP and was present at day 0 but not thereafter; MTU 18 is 94.5% identical to "*Su. montser-*

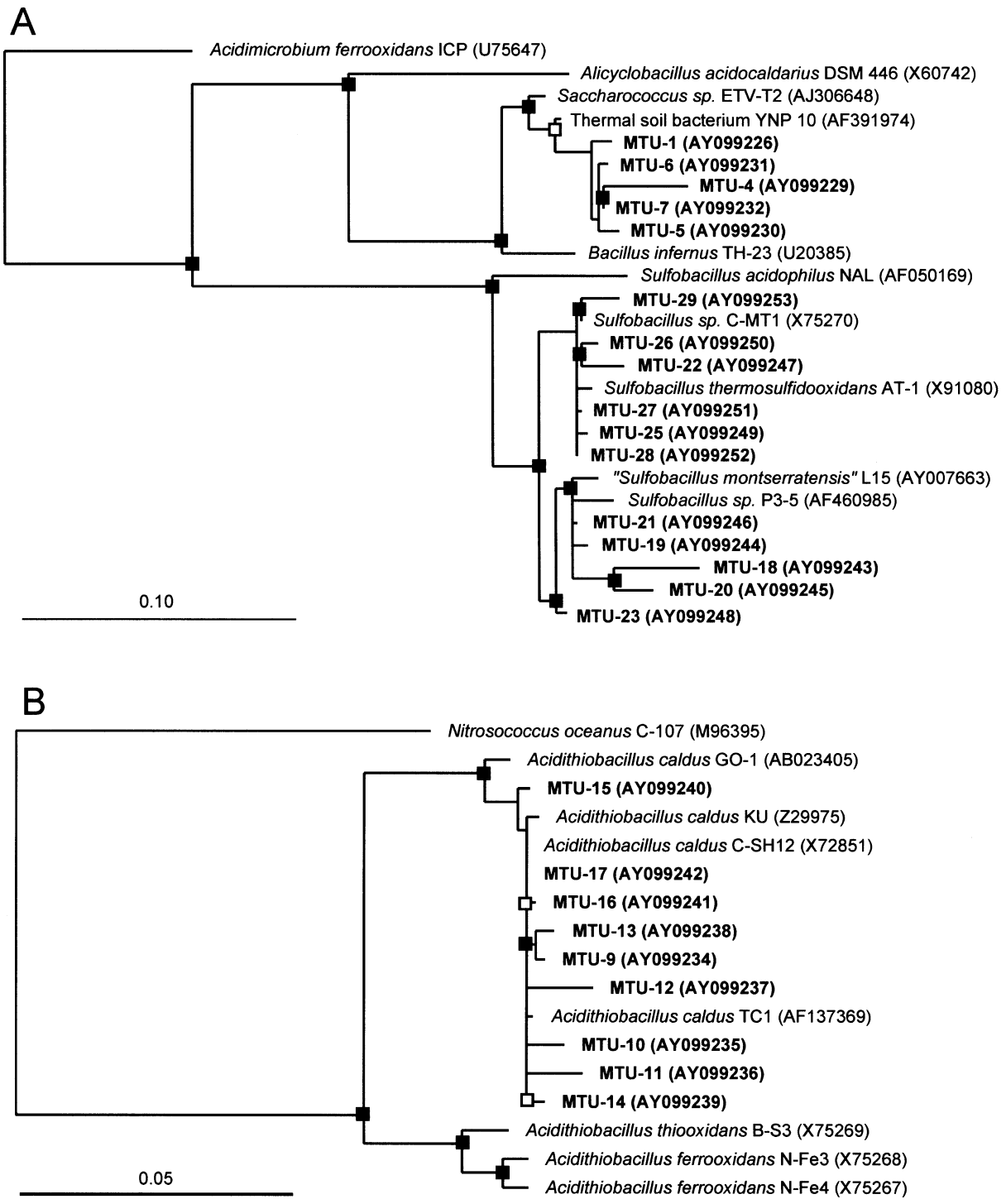

Figure 3. Maximum likelihood trees based on partial 16S rRNA gene sequences of the Gram-positive (A) and Gram-negative (B) clones isolated from the mixed cultures (in bold). The Gram-positive tree was rooted between the high and low GC Gram positives; the Gram-negative tree was rooted with *Thiobacillus thermosulfatus* and *Burkholderia cepacia* of the β-Proteobacteria. Phylogenetic analysis was carried out by the maximum likelihood, distance neighbor joining, and DNA parsimony methods and the nodes supported by all three trees (■) and two trees (□) have been marked. Accession numbers are given in parentheses. The scale bars correspond to 10 and 5% sequence similarity, respectively.

ratensis" strain L15 [18] and MTU 28 that is 99.5% similar to *Su. thermosulfidooxidans* AT-1 were present in the sample after 12 days.

Phylogeny of Chalcopyrite Maintained Culture. The original chalcopyrite leaching experiment and culture storage were not carried out aseptically;

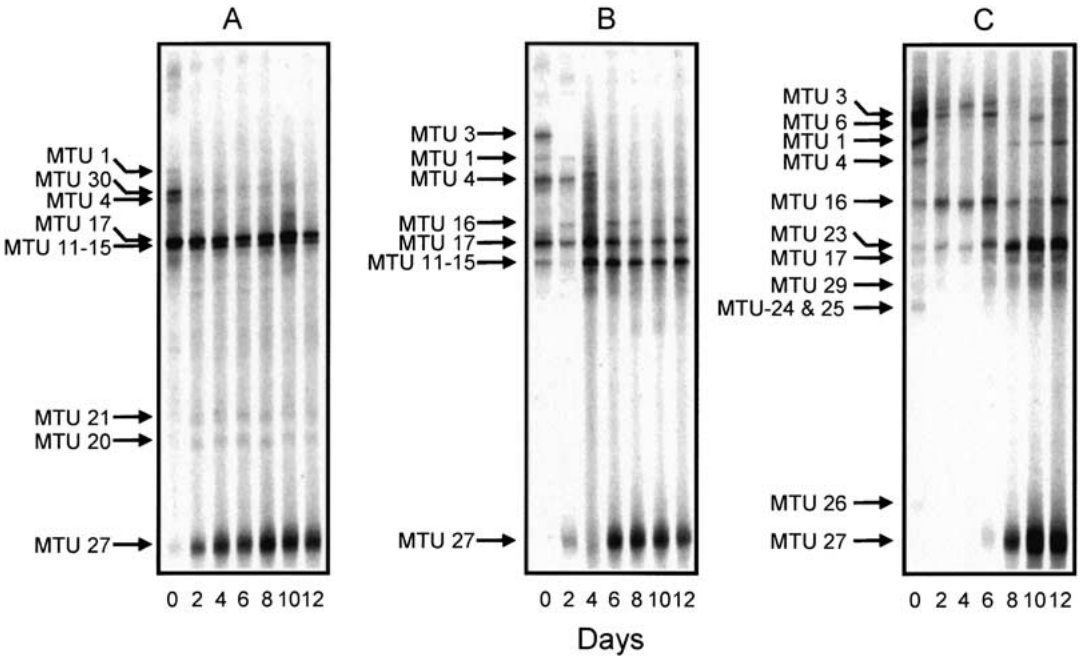

Figure 4. 16S rDNA PCR fragments separated by DGGE from samples taken during bioleaching of pyrite (A), arsenical pyrite (B), and chalcopyrite (C).

therefore, some species that are not acidophilic may have been introduced. The species present in the inoculum before re-inoculation were MTU 2, 6, 9, 11 to 15, 17, 27, 31, and MTU 32 (Fig. 5A). Bands pertaining to a number of species were no longer visible on the DGGE gels after only 24 h in shake flasks, whereas other species not visible in the mixed culture maintained on chalcopyrite mineral for 15 months are present. These include MTU 1, 30, 16, and 29 (Fig. 5B). Of the species not present in the inoculum, bands MTU 1 and 30 are possibly due to introduced DNA on the autoclaved mineral; the others must have been present in a low concentration in the chalcopyrite-maintained culture and increased in numbers under the conditions at the start of the leaching experiment. As the leaching progressed MTU 17 (100% identity with *At. caldus* C-SH12) and MTU 27 (99.3% identity with *Su. thermosulfidooxidans* AT-1) increased in cell numbers, with MTU 16, 19, 29, and 1 still being amplified in low amounts after 12 days. This suggests that the same species were selected in the stirred tank reactors irrespective of whether the inoculum was freshly grown from frozen cultures, or whether an inoculum maintained on mineral residue for over a year was used (Figs. 4C and 5B).

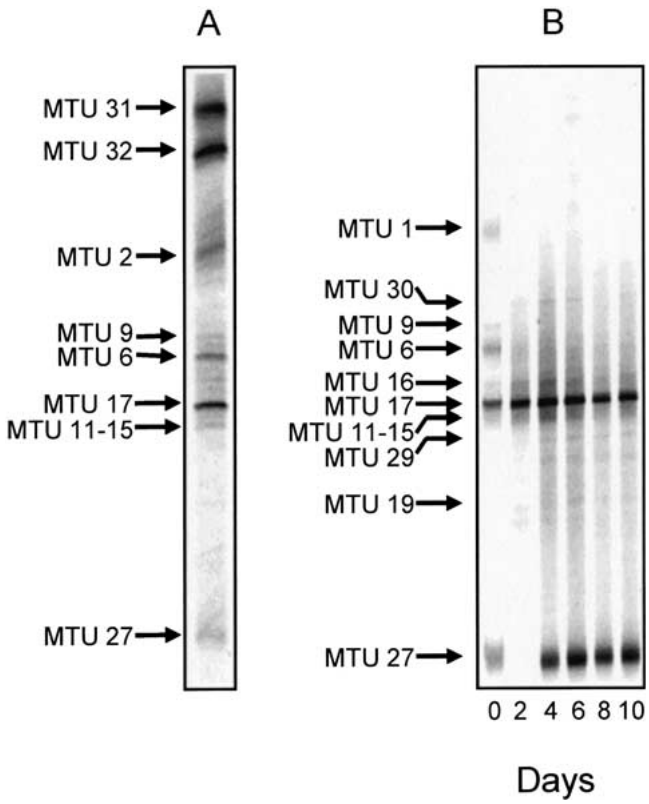

Figure 5. DGGE pattern of 16S rRNA gene fragments from microorganisms present in a mixed culture maintained on chalcopyrite for 15 months. The lanes show the inoculum culture prior to pre-growth overnight in fresh 1% (wt/vol) chalcopyrite (A) and during leaching of chalcopyrite for 10 days (B).

Discussion

From the original Kingsbury coal spoil culture, shake flasks containing pyrite, arsenical pyrite, and chalcopyrite were inoculated and the enrichment cultures frozen. These

original enrichment cultures were revived and the species diversity analyzed by DGGE, cloning, and sequencing.

The method used to extract total DNA from the samples is a critical step in sample preparation, as any microorganisms not lysed will introduce bias into the evaluation of the total species present in the mixed culture. However, the harsh techniques needed for lysis of Gram-positive cells could lead to fragmented DNA from Gram-negative species (a source of artifacts and chimeric sequences in the PCR reaction). The possible drawback of obtaining sheared DNA causing chimeras was encountered with the generation of one chimera (MTU 24). In control experiments the best method for extracting DNA was found to be treatment with lysozyme to weaken the cell wall and then bead beating, although the total number of bands was unchanged when the alternative methods were used. Although control experiments were carried out to test if any further phylotypes could be amplified, it is possible that some species were protected while growing in niches on the surface of the mineral, preventing lysis and DNA extraction from occurring.

The minerals used in the bioleaching experiments described here are flotation concentrates whereby an organic layer of promoter chemical is added to the mineral and then the concentrates stored. The flotation reagents may be toxic to the natural chemolithoautotrophic population of microorganisms present ([22, 25] and unpublished results). Of five species of moderately thermophilic acidophiles tested for sensitivity to flotation reagents, species from the genera *Leptospirillium* and *Ferroplasma* were found to be the most sensitive [25], and this could be why these species were not amplified from the mineral concentrate (Fig. 4, A–C). The treatment and storage for the minerals was the same, and this would contribute to the similarity of the DGGE banding pattern from the three minerals. Those clones corresponding to microorganisms not usually present in bioleaching environments were not found after the cultures had been grown for 48 h on 4% (wt/vol) pulp density. The disappearance of these bands combined with the lack of leaching by un-inoculated controls suggests these clones were most probably as a result of amplifiable DNA from dead microorganisms, subsequently degraded by DNases released by growing microorganisms or no longer amplified in the PCR reaction because of higher concentrations of DNA from other species.

Previous plating and molecular studies on the phylogeny of coal-impacted sites at Alvecote and Birch Coppice, UK (both in the geographic vicinity of Kingsbury), identified strains related to the genera *Leptospirillium*, *Sulfobacillus*, *Acidithiobacillus*, *Acidiphilium*, *Alicyclobacillus*, "*Ferromicrobium*," and a few other acidophilic heterotrophs (Paul Norris, personal communication). Another study by Brofft *et al.* [5] identified a wide range of bacterial and archaeal clones associated with a coal-impacted site.

Previous investigations into moderately thermophilic bioleaching enrichment cultures have identified species from the genera *Leptospirillium*, *Acidithiobacillus*, *Acidiphilium*, *Acidocella*, "*Ferromicrobium*," and *Ferroplasma* [23, 24, 31]. The species composition identified in the present study contained only a few of the species previously observed, although several phylotypes within the species classification were present. This suggests that the original enrichments on the three mineral concentrates strongly selected for a small consortium of phylotypes that out-competed the majority of the strains probably present in the original Kingsbury mixed culture. Further enrichment occurred during the bioleaching of the mineral concentrates, with several further phylotypes no longer being amplified after 12 days. Low species diversity in a bioleaching mixed culture has been previously reported, with strains from the genera *Leptospirillium*, *Acidiphilium*, *Sulfobacillus*, and *Acidithiobacillus* being identified from a commercial bioreactor [14]. Also, it was found that the predominant microorganisms in a separate commercial plant were also dominated by strains similar to *At. caldus*, *Leptospirillium*, *Sulfobacillus*, and *Ferroplasma* [24]. Therefore, although the original inoculum was likely to be highly complex, it appears that only a few strains are selected during bioleaching of mineral concentrates in either bench- or commercial-scale bioreactors. Storage of the chalcopyrite enrichment culture for over a year did not significantly alter the phylotypes present in the mixed culture. This suggests that the optimum microorganisms had been selected.

Although problems exist with quantifiable conclusions from PCR reactions, increases in band strength on DGGE gels suggest that the population is present and increasing in activity (reviewed in [30]). Therefore, it can be concluded that the two strains that increased in activity during bioleaching of all three minerals were most closely related to *At. caldus* C-SH12 and *Su. thermosulfidooxidans* AT-1. These two species (although a different strain of *At. caldus*) have both previously been isolated from the original mixed culture used in the present study and have successfully been used in defined co-culture for the bioleaching of arsenopyrite [9]. The *At. caldus* clones identified in the present study were different from the type strain (strain KU, previously identified from the Kingsbury coal spoil culture) possibly because of differential strain selection in the bioleaching environment compared to the high-tetrathionate, no-iron conditions used to isolate the type strain [16]. A further two phylotypes persisted during the bioleaching of the mineral concentrates, a phylotype related to "*Su. montserratensis*" L15, an iron and sulfur oxidizing acidophile isolated from

Montserrat, West Indies [18], and an uncultured thermal soil bacterium YNP.

In conclusion, under the conditions tested phylotypes similar to *At. caldus* C-SH12, "*Su. montserratensis*" L15 [18] and *Su. thermosulfidooxidans* AT-1 were selected for all three mineral concentrates, and in the case of chalcopyrite a phylotype similar to the uncultured thermal bacterium YNP also persisted. Species selection on chalcopyrite was unaffected after maintaining the mixed culture for over a year on the mineral. Although the microbial consortium present was highly simplified compared to the probable original culture, the selected strains effectively leached metal from pyrite, arsenical pyrite, and chalcopyrite mineral concentrates.

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