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The bacterial community structure during bioleaching of a low-grade nickel sulphide ore in stirred-tank reactors at different combinations of temperature and pH

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ABSTRACT

The focus of this study is to characterize the bacterial community structure present during stirred-tank bioleaching of a low-grade nickel sulphide ore at different temperatures (5 to 45 °C) and pH levels (3 and 5). This is a continuation of previous work, which was designed to assess the technical feasibility of applying elevated-pH bioleaching to this same ore from Manitoba, Canada. A combination of classical microbiological and molecular biological techniques has been used to identify and enumerate the members of the bacterial consortia over the course of the five-week experiments. DGGE analysis revealed the presence of at least 16 distinct 16S rRNA gene sequences, 14 of which are closely related to existing GenBank sequences. Two sequences were detected that are not closely related to existing GenBank sequences and may possibly be from novel species. Thirteen sequences are related to gene sequences of genera that have previously been detected in bioleaching environments (*Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus*, *Acidiphilium*, *Ferrimicrobium*, and *Acidimicrobium*). Members from the genus *Acidithiobacillus* were dominant at all temperatures except 45 °C, at which *Sulfobacillus* spp. were dominant. Many of the acidithiobacilli are most closely related to strains of *Acidithiobacillus ferrooxidans*, and different strains were dominant under different experimental conditions, indicating considerable phenotypic heterogeneity within the species.

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1. Introduction

The widespread application of molecular biological techniques has led to significant advances in the understanding of the bacterial species present in acidic mining-related environments and bioleaching operations in the last decade. Several studies have applied molecular techniques to examine the bacteria community structure of acidic mining-related environments such as acid mine drainage (AMD) sites (Bond et al., 2000; Hallberg and Johnson, 2003; Xiao et al., 2009), mine water sites (He et al., 2007; Xiao et al., 2009), mine tailings sites (Mendez et al., 2008), and acidified metal-laden waterways (Gonzalez-Toril et al., 2003). Molecular techniques have also been used to identify and enumerate the prevalent bacterial species in a number of bioleaching environments, although those applications are usually targeted to specific microorganisms of interest (i.e. sulphur- and iron-oxidizers) and reveal less information on the total bacterial community structure. These studies include bench-scale stirred-tank bioleaching experiments (Xingyu et al., 2009, 2010), commercial stirred-tank bioleaching operations (Coram and Rawlings, 2002), column bioleach-

ing experiments (Coram-Uliana et al., 2006; Halinen et al., 2009), and heap bioleaching operations (Coram-Uliana et al., 2006; Demergasso et al., 2005; Qin et al., 2009).

This study is a continuation of previous work, which was designed to assess the technical feasibility of applying elevated-pH bioleaching to a low-grade metamorphosed ultramafic-dominated nickel sulphide ore from Manitoba, Canada. Cameron et al. (2009a,b) reported on the stirred-tank bioleaching of this ore at 30 °C (pH 2 to 6), and at 5 to 45 °C (pH 3 to 5) respectively. The ore is characterized by low nickel content and a high fraction of acid-soluble magnesium silicate gangue minerals. Attempts to bioleach this ore at conventional pH levels (~2) resulted in an unacceptable amount of solubilized magnesium and excess sulphuric acid consumption. It was concluded that operating at elevated pH (>3) resulted in a substantial increase in the nickel to magnesium ratio in the leachate, and also resulted in a substantial reduction in the consumption of sulphuric acid.

In the previous work, the effect of the presence of bacteria and the effect of temperature and pH on the extraction of metals, the consumption of sulphuric acid, and the resulting solid state alterations were extensively studied. The focus of the present study is to characterize the microbial consortia involved in the bioleaching of the ore at different combinations of temperature (5 to 45 °C) and pH (3 to 5). A combination of classical microbiological and molecular biological techniques was used

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to identify and enumerate the members in the consortia over the course of the five-week experiments.

The application of molecular biological techniques to bioleaching systems is not novel; however, there is currently no other study that comprehensively examines the microbial community structure under such controlled conditions and over such a wide range of temperature and pH. The effect bacteria have in the bioleaching environment has been well-documented; however, the effect of changing physico-chemical parameters on the bacterial community structure is poorly understood. This study furthers the understanding of the micro-organisms present during the bioleaching of sulphide ores and the potential impacts of temperature and pH on the bacterial community structure.

2. Materials and methods

Cameron et al. (2009a) reported on the mineralogical and chemical characterization of the ore used in this study. The ore is characterized by a high content of magnesium silicate minerals (~64% lizardite) and a low content of sulphide minerals (<3%). Pentlandite was determined to be the primary nickel-bearing phase. Chemical analysis revealed the ore to contain 0.31% Ni, 21% Mg, 0.016% Cu, 7.2% Fe, and 0.65% total-S.

2.1. The original microbial consortium

The original microbial culture used in this study was derived from water and soil samples collected from mining-related locations in Sudbury, Ontario in October 2006. A total of 16 samples were taken from locations with a range of acidity (pH 3.2 to 6.5) and oxidation-reduction potential (125 to 438 mV vs. Ag/AgCl). Iron- and sulphur-oxidizing microorganisms were enriched from each environmental sample using media designated mTK and Att respectively (Table 1).

All enrichment cultures were combined, and the resulting mixed culture was maintained on a finely-ground low-grade nickel sulphide ore from the Sudbury region by serial sub-culturing in mTK media at pH 2.2. Initial molecular biological characterization revealed that the original mixed culture contained microorganisms closely related to *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and *Acidiphilium* spp. (Dinardo and Mohapatra, 2008). No magnesium supplement or energy sources were added during growth on sulphide ores, unless otherwise noted. Magnesium was omitted because the ore supplied enough dissolved magnesium, and iron was omitted in order to encourage the bacteria to attach to the ore and utilize the sulphide minerals as an energy source.

A maintenance culture was acclimated to the ore used in this study for several months by serial sub-culturing at 30 °C and pH 3 before being used as inoculum for new maintenance cultures at 5, 15, and 45 °C. The maintenance cultures at the four different temperatures were acclimated to the ore used in this study for more than one year by serial sub-culturing prior to the commencement of the stirred-tank

experiments. The introduction of new species by contamination from equipment cannot be ruled out, as sterile practices were not used during the stirred-tank reactor experiments. Furthermore, it is likely that the ore contained bacteria, as iron- and sulphur-oxidizing bacteria are ubiquitous in nature. The ore also contained some organic material, which most likely included heterotrophic micro-organisms. The ore was received as drill cores packed in burlap sacks, and several of the sacks contained nuts and/or seeds that had been deposited by animals during storage.

2.2. Bioleaching experiments and bacterial enumerations

Six stirred-tank experiments were conducted for this study at different combinations of temperature and pH: 5 °C (pH 3 and 5), 15 °C (pH 3), 30 °C (pH 3 and 5), and 45 °C (pH 3). The materials and methods were the same as in the previously reported stirred-tank reactor experiments (Cameron et al., 2009a,b), unless otherwise noted.

The inocula for each experiment was developed by combining 5 g of ore, 100 mL of McCready media (adapted from McCready et al., 1986), and 8 mL of 10% H₂SO₄ (vol/vol) in a 250 mL Erlenmeyer flask. After 48 h in an orbital shaker at 30 °C, the pH was adjusted to 3 and the flask was brought to the temperature of the experiment in which it was to be used. The shake flask was then inoculated with 5 mL of slurry from a well-shaken maintenance culture, which had been grown at the appropriate temperature, and the resulting inoculum was maintained in an orbital shaker for eleven days at pH 3. The inoculum was then combined with 145 g of ore in the reaction vessel and made up to a total volume of 1.5 L with McCready media.

The nature of these experiments required the periodic removal of large samples. Leachate samples (10 mL after 5 min settling) were taken for metal analysis on days 0, 2, 7, 14, 21, 28 and 35. Three 125 mL slurry samples were taken while mixing for molecular biological analysis on days 7, 21, and 35 (the samples taken on day 21 were not analyzed). Slurry samples (10 mL each) were taken while stirring on days 2, 7, 14, 21, and 35 for bacterial enumerations. In order to maintain the original pulp density of 10% (mass/vol), evaporative losses were made up with deionised water prior to sampling and the volume of leachate taken for metal analysis was replaced with an equal volume of fresh media. The reactors were operated with a new volume after the removal of the large slurry samples; 1375 and 1250 mL after days 7 and 21 respectively. The dilution resulting from the removal of the slurry samples that were drawn for enumeration purposes was ignored, as it represents less than a 3% dilution error.

Bacterial determinations were accomplished with a five-tube most-probable-number (MPN) method (Cochran, 1950). The media used for the enumeration tests are listed in Table 1. Tubes from the experiments conducted at 30 and 45 °C were incubated in the dark for 28 days at their respective temperatures, whereas the tubes from the experiments conducted at 5 and 15 °C were incubated in the dark for 42 days in a temperature-controlled room at approximately 17 °C.

Table 1

Composition of the growth media used during enrichment, culture maintenance, and bioleaching experiments.

	Modified TK (mTK)	McCready	ATCC Medium 23 (Att)	Thermophilic media (thermo)
Nutrients (g/L)	0.5 (NH ₄) ₂ SO ₄ 0.5 K ₂ HPO ₄ 0.5 MgSO ₄ ·7H ₂ O	0.0661 (NH ₄) ₂ SO ₄ 0.0174 K ₂ HPO ₄ 0.123 MgSO ₄ ·7H ₂ O	0.1 NH ₄ Cl 3.0 KH ₂ PO ₄ 0.2 MgCl ₂ ·6H ₂ O 0.1 CaCl ₂	1.3 (NH ₄) ₂ SO ₄ 0.05 CaCl ₂ ·2H ₂ O 0.28 K ₂ HPO ₄ 0.25 MgSO ₄ ·7H ₂ O 0.1 Yeast extract
Energy source (g/L)	33.4 FeSO ₄ ·7H ₂ O	33.4 FeSO ₄ ·7H ₂ O	5.0 Na ₂ S ₂ O ₃ ·5H ₂ O or 0.5 Elemental sulphur	33.4 FeSO ₄ ·7H ₂ O, 5.0 Na ₂ S ₂ O ₃ ·5H ₂ O, or 0.5 Elemental sulphur
Initial pH	2.1	2.1	4.2	2.1 (Fe ²⁺) 4.2 (Na ₂ S ₂ O ₃ ·5H ₂ O or S ₈)
Reference	Modified from Tuovinen and Kelly (1973)	McCready et al. (1986)	Modified from Gherna et al. (1989)	Modified from Salo-Zieman et al. (2006)

Sterile techniques were used to ensure no false positive tests due to contamination. A positive test for sulphur-oxidizing bacteria was indicated by a drop in pH (0.5 units below the average value of the control tubes). At temperatures other than 45 °C, a positive test for iron-oxidizing bacteria was indicated by the formation of an orange precipitate. The rate of abiotic ferrous ion oxidation (and subsequent precipitation) at 45 °C was fast enough to make it difficult to visually differentiate between positive and negative tubes. For this reason, an increase in oxidation reduction potential (ORP) of +50 mV relative to the average of the control tubes was used to identify positive tubes at 45 °C. There is no precedence in the technical literature to identify positive tubes in the context of an MPN test based on ORP; however, the average of the control tubes in this study was observed to be very consistent within an individual rack of tubes (± 1 mV). Throughout the enumeration tests, not a single positive control tube was detected.

The bacterial consortium at each temperature was tested for the ability to oxidize ferrous ion and elemental sulphur. A test consisted of combining 1 mL of slurry from a well-shaken maintenance flask with 100 mL of filtered media (+0.5 g precipitated elemental sulphur when appropriate) in a 250 mL Erlenmeyer flask, which was then incubated in a temperature-controlled orbital shaker at 150 rpm. The flasks at 15 to 45 °C were incubated for one month, whereas the flasks at 5 °C were incubated for two months. A positive test for elemental sulphur-oxidizing bacteria was a drop in pH of 0.5 units below the sterile control flask.

An effort was made to ensure that experiments conducted at different pH levels reflect only the changes that can be attributed to differences in pH. For this reason, all six experiments were commenced and terminated on the same day and experiments conducted at the same temperature were inoculated with inocula prepared identically. All inocula were maintained at pH 3 for the eleven-day incubation period prior to the commencement of the stirred-tank experiments, making it as likely as possible that each experiment at a given temperature was inoculated with the same bacterial community structure (both species and cell densities); therefore, there were only four initial conditions (T_0 at 5, 15, 30, and 45 °C) for the six experiments conducted at distinct temperature and pH conditions.

Estimates of the bacterial community structure and of the cell densities on day 0 were obtained by enumeration and molecular biological analysis of inocula prepared according to the same protocol described above. The reported numbers for the cell densities are an arithmetic average of duplicates after applying the appropriate dilution factor of approximately 100 mL in 1.5 L. It is recognized that it would have been preferable to determine the bacterial densities and community structure on day 0 from the same set of bioleaching tests; however, it was not logistically possible. Furthermore, it was thought that the 1:15 dilution on day 0 would have potentially caused detection problems during molecular biological analysis, particularly at 5 and 45 °C, at which the total community DNA was determined to be quite low.

2.3. Molecular biological techniques

2.3.1. DNA extraction

Prior to total community DNA extraction, the samples were vortexed for about 1 min to re-suspend the settled ore material. One hundred milliliter of the sample was centrifuged at 5600 rpm ($4000\times g$) for 10 min at 4 °C. The pellet was washed with 30 mL of PBS (pH 7.4) and vortexed at maximum speed for about 1 min. The sample was centrifuged again at 5600 rpm ($4000\times g$) for 10 min at 4 °C. The DNA extraction method made use of the MoBio PowerMax Soil DNA Extraction Kit and was performed following the manufacturer's instructions. The DNA on the white filter membrane was eluted with 3 mL of Solution C6 from the MoBio Soil Kit and incubated at room temperature for 10 min before the final centrifugation. The total community DNA was quantified using a NanoDrop instrument. For

the samples with low DNA concentration, the total community DNA was further concentrated by ethanol precipitation. The DNA pellet was re-dissolved in 50 μ L of TE (pH 8.0) before PCR amplification.

2.3.2. PCR amplification of the 16S rRNA gene

For PCR amplification of the 16S rRNA gene, the bacteria-specific forward primer F1 (5'-GAGTTTGATCTGGCTCAG-3') and reverse primer 519R (5'-GTATTACCGGGCTGCTGG-3') were used. These primers, complementary to conserved regions of the 16S rRNA gene, were used to amplify a 491-bp fragment corresponding to positions 27 to 519 of the *Escherichia coli* sequence and covered the variable regions V1 to V3. The bacteria-forward primer used for DGGE possessed a GC-clamp (5'-GGCGGGCGGGGGCACGGGGGGCGGGCGGGCGGGCGGGGG-3') at the 5' end. This GC-clamp stabilizes the melting behaviour of the amplified fragments (Sheffield et al., 1989). Each 50 μ L PCR mixture contained 1 μ L of the template DNA (~ 1 ng/ μ L), 25 pmol of each oligonucleotide primer, 200 μ M of each dNTP, 1 mM MgCl₂ and 2.5 units of Taq polymerase (Amersham Biosciences, Piscataway, NJ, USA) in 10 $\times$ Taq polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂). Briefly, after an initial temperature of 96 °C for 5 min and thermocycling at 94 °C for 1 min, the annealing temperature was set to 65 °C for 1 min and decreased by 1 °C every cycle for 10 cycles, and 3 min elongation time at 72 °C. Additional cycles (15 to 20) were performed with annealing temperatures of 55 °C. PCR products were loaded onto a 1% agarose gel with SYBR Safe (Molecular Probes, Eugene, OR, USA), using a 100-bp ladder (MBI Fermentas, Amherst, NY, USA) to determine the presence, size and quantity of the PCR products.

2.3.3. Denaturing gradient gel electrophoresis

The 16S rRNA gene PCR products from 3 to 8 PCR reactions were combined for each sample and concentrated by ethanol precipitation for DGGE analysis. About 500 ng of the 16S rRNA PCR product from each sample was applied to a lane, and was analyzed on an 8% polyacrylamide gel containing a gradient of 30–70% denaturant (7 M urea and 40% deionized formamide were considered to be 100% denaturant). DGGE was performed with a DCode Universal Mutation Detection System (Bio-Rad). Electrophoresis was run at a constant voltage of 80 V for 16 h at 60 °C in 1 $\times$ TAE running buffer. The gels were then stained with VistaGreen (Amersham Biosciences, Piscataway, NJ, USA). The gels were imaged with the FluorImager System Model 595 (Molecular Dynamics, Sunnyvale, CA, USA). The gel images were analyzed using GelCompar II v4.6 (Applied Maths, Sint-Martens-Latem, Belgium) to generate dendrogram profiles. The genotypes were visually detected based on the presence or absence of bands in each lane. A band was defined as "present" if its peak intensity was at least 2% of the most-intense band in the sample. After conversion and normalization of gels, the degree of similarity of DNA pattern profiles were computed using the Dice similarity coefficient (Dice, 1945), and dendrogram patterns were clustered by the Unweighted Pair Group Method using Arithmetic average (UPGMA) groupings with a similarity coefficient (S_{AB}) matrix.

2.3.4. Sequencing and phylogenetic analyses

Individual bands of the DGGE gels were excised and eluted with 25 μ L of dH₂O for 2 to 3 days at 4 °C before being re-amplified with the same set of primers without the GC-clamp. One microliter of DNA was re-amplified with the appropriate primers without the GC-clamp as follows: an initial denaturation of 5 min at 96 °C; followed by 30 cycles of 94 °C for 1 min; 65 °C for 30 s; and 72 °C for 1 min. PCR products for sequencing were purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Baie d'Urfe, QC). Sequencing was performed at the Université Laval Plate-forme d'analyses biomoléculaires using a model ABI Prism 3130XL (Applied Biosystems, Foster City, CA, USA) with their respective primers. Raw sequence data were assembled in BioEdit v7.0 (Hall, 1999). The sequences were manually aligned by comparing forward and reverse sequences. The occurrence of chimeric

sequences was determined manually with the CHECK_CHIMERA function from the Ribosomal Database Project-II (<http://35.8.164.52/cgiis/chimera.cgi?su=SSU>, Cole et al., 2003) and Bellerophon (<http://foo.maths.uq.edu.au/~huber/bellerophon.pl>, Huber et al., 2004). Close relatives of the final selection of different sequences (phylotypes) were tentatively identified by NCBI BLASTN search (<http://ncbi.nlm.nih.gov/blast/>). Sequences were aligned by the MacVector 9.0 software package (Accelrys) with both closely-related representatives from NCBI BLASTN and as well as novel complete and partial sequences obtained from GenBank. Additional manual alignment was done when necessary. Phylogenetic relationships were constructed with evolutionary distances (Jukes–Cantor distances) and the neighbor-joining method using the MacVector software package. The bootstrap analyses for the phylogenetic trees were calculated by running 1000 replicates for the neighbor-joining data.

The 16S rRNA gene sequences obtained in this study (BL-1 to BL-16) have been deposited in the GenBank database under accession numbers HM124428 to HM124443 respectively.

3. Results and discussion

3.1. Effect of pH on nickel extraction from pentlandite

Nickel extraction from the low-grade ore as a function of time at the different combinations of temperature and pH is shown in Fig. 1. The extraction of nickel at 5, 15, and 30 °C is similar to previously reported results (Cameron et al., 2009a,b); however, the initial rate of nickel extraction at 45 °C in the current study appears to be slightly lower compared to the previously reported study, perhaps suggesting lower biological activity. The extraction of the other metals, acid consumption, and ORP measurements are comparable (data not shown).

3.2. Substrate utilization tests

Each bacterial consortium was tested for the ability to oxidize ferrous ion and elemental sulphur using media listed in Table 2. If the culture was able to oxidize a substrate in the absence of yeast extract, then the culture was not tested with yeast extract. Cultures at 5, 15, and 30 °C exhibited the ability to oxidize both substrates autotrophically, whereas the culture at 45 °C required the presence of yeast extract to oxidize both substrates.

3.3. Monitoring of the microbial consortium

3.3.1. DGGE analysis

Murray et al. (1996) determined there is a relationship between DGGE band intensity and the relative abundance of the corresponding

Fig. 1. Nickel extraction from the low-grade nickel sulphide ore as a function of time at different combinations of temperature and pH.

Table 2
Substrate utilization tests.

Test #	Substrate (media)	5 °C	15 °C	30 °C	45 °C
1	Ferrous ion (mTK)	Growth	Growth	Growth	No growth
2	Ferrous ion (thermo + FeSO ₄ ·7H ₂ O)	N/A	N/A	N/A	Growth
3	Elemental sulphur (Att + S ₈)	Growth	Growth	Growth	No growth
4	Elemental sulphur (thermo + S ₈)	N/A	N/A	N/A	Growth

phylotypes during DGGE analysis when using a mixture of DNA templates. More recent work (Fromin et al., 2002) has suggested that the relative band intensity from DGGE could be used to estimate the relative density of the corresponding sequence type within the sample, strongly implying that band intensity has a diagnostic value in estimating the relative abundance of an organism in a sample.

The DGGE patterns for the four inocula (T_0 at 5, 15, 30, and 45 °C) and the DGGE patterns for the six experiments at days 0, 7, and 35 (T_0 , T_7 , and T_{35} respectively) are given in Figs. 2 and 3 respectively. Fig. 3 reveals generally low similarity values (S_{AB}) between the T_0 samples and both the T_7 and T_{35} samples at the same temperature, indicating large changes in the bacterial community structure. There are additional potential reasons for these apparent differences besides pH and the progression of time, including: the pulp density in the shake flask inocula was 5% (mass/vol), whereas the pulp density in the stirred-tank reactor experiments was 10% (mass/vol); and the Reynolds number in the stirred-tank reactor experiments was substantially higher compared to the Reynolds number in the shake flask during incubation of the inocula. For this reason, it is more appropriate to compare the T_0 samples separately from the T_7 and T_{35} samples, and to rely on the comparison between the T_7 and T_{35} samples to evaluate shifts in the bacterial community structure on a time and pH basis.

In general, temperature affected the bacterial community structure more than did pH. The T_0 samples at 5, 15, and 30 °C exhibited a great degree of similarity, whereas T_0 at 45 °C was substantially different. This suggests that the dominant bacteria at 5, 15, and 30 °C were mostly the same species, whereas the dominant species at 45 °C were unique. Upon comparison of the T_0 samples in the temperature range of 5 to 30 °C, it can be concluded that the sequences BL-5 and -8 decrease in relative abundance with decreasing temperature, whereas sequences BL-2, -3, -11, and -13 increase in relative abundance with decreasing temperature (bands labelled in Fig. 3), suggesting a strong response to temperature. Surprisingly, the samples at 5 °C are more closely clustered by time rather than pH, suggesting that at 5 °C, time has more impact on the microbial community structure than does the pH in this pH range. All 30 °C samples were clustered closely together, which suggests that the bacterial community structure at that temperature was relatively stable throughout the 35-day experiments. Sequences BL-10 and BL-4 were enriched only in the 5 °C samples.

3.3.2. Sequencing and phylogenetic analysis

The phylogenetic relationship of the 16 bacterial 16S rRNA gene sequences obtained from the bioleaching samples is given in Fig. 4. All sequences, except BL-12 and BL-16 are closely related (>97% identity) to sequences of previously-sequenced bacteria. All sequences, except BL-1, -10, and -14 are related to sequences of microorganisms that have previously been detected in bioleaching environments, belonging to the genera *Acidithiobacillus* (Halinen et al., 2009; Xingyu et al., 2009, 2010), *Leptospirillum* (Coram and Rawlings, 2002; Halinen et al., 2009; Xingyu et al., 2009, 2010), *Sulfobacillus* (Halinen et al., 2009;

Fig. 2. DGGE analysis of the T_0 bioleaching samples using the 16S rRNA gene primer set F1–519R.

Xingyu et al., 2009, 2010), *Acidiphilium* (Coram-Uliana et al., 2006), *Ferrimicrobium* (Halinen et al., 2009), and *Acidimicrobium* (Cleaver et al., 2007).

Two sequences were detected that are not closely related to existing GenBank sequences, and may possibly be from novel species: sequence BL-12, which is most closely related (93% identity) to the gene from an uncultured *Acidithiobacillus* sp. (EF612415) from a lead-zinc mine tailings site in Arizona (Mendez et al., 2008); and sequence BL-16 that is most closely related (90% identity) to the gene from *Sulfobacillus acidophilus* strain YTF-1 (AY007665), which is a microorganism that was isolated from an acidic geothermal site in Yellowstone National Park (Johnson et al., 2001).

Most of the sequences that were detected are closely related to iron- and sulphur-oxidizing microorganisms that are commonly found in bioleaching environments. Members from the genus *Acidithiobacillus* were dominant at all temperatures except 45 °C, at which *Sulfobacillus* spp. were dominant. Sequences BL-6 and -7 both

belong to the genus *Leptospirillum*, which virtually disappeared from all samples after T_0 . There is no conclusive explanation for this observation, but it is possible these microorganisms were sensitive to either shear or metal concentration; however, this is inconsistent with observations that *Leptospirillum* spp. often dominate in commercial stirred-tank bioleaching operations (Rawlings et al., 1999), which are typically characterized by high pulp densities and high shear.

The genus *Acidithiobacillus* are a group generally considered to be mesophilic, obligately acidophilic, autotrophic aerobes that oxidize numerous reduced-sulphur compounds, with some species having the ability to oxidize ferrous ion and/or hydrogen (Kelly and Wood, 2000). Their presence in all samples is expected, as all the inocula were grown at pH 3; however, one might expect to see the presence of some species that are widely considered to be moderate acidophiles in the experiments at pH 5, which are conditions that more closely resemble moderately-acidic AMD rather than a typical bioleaching operation. The dominance of *Acidithiobacillus* spp. at pH 5 may seem somewhat

Fig. 3. DGGE analysis of all the bioleaching samples using the 16S rRNA gene primer set F1–519R. Bands excised for sequencing are identified with numbers (1–16).

Fig. 4. Phylogenetic relationship of the 16 bacterial 16S rRNA gene sequences obtained from the bioleaching samples. The bands were labelled bioleaching (BL) with their band numbers, as shown in Fig. 3. The tree was inferred by neighbor-joining analysis of sequence from each band. *Aquifex pyrophilus* was used as the outgroup. Numbers on the nodes are the bootstrap values based on 1000 replicates. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

inconsistent with the perceived preferred habitat of *Acidithiobacillus* spp.; however recent work by Ni et al. (2008) suggests that the preferred habitat of certain *Acidithiobacillus* spp. might be more diverse than originally thought. Ni et al. (2008) compared the growth characteristics of five unidentified sulphur-oxidizing *Acidithiobacillus* spp. isolated from different mining-related environments in China with the growth characteristics of two *Acidithiobacillus* type strains (ATCC 19377 and DSM 8584). They observed a wide range of optimum growth conditions (T_{opt} of 28 to 45 °C; and pH_{opt} of 2 to 5). Coincidentally, the pH_{opt} of the two type strains were towards the lower end of the range. There is clear evidence of this phenotypic heterogeneity in the current study. Sequences BL-2, -3, -4, -5, and -11 are all most closely related to strains of *A. ferrooxidans*; however, they each show a different response to temperature.

The dominant iron- and sulphur-oxidizing species at 45 °C belong to the genus *Sulfobacillus*, which is comprised of moderately thermophilic spore-forming Gram-positive rods that are strictly aerobic mixotrophs capable of growth on ferrous ion and some reduced-sulphur compounds (Karavaiko et al., 2006). Sulfobacilli are capable of either autotrophic or heterotrophic growth for a limited number of transfers; whereas they are capable of faster and extended growth in the presence of yeast extract (Karavaiko et al., 2006).

Sulfobacillus acidophilus strain YTF-1 (AY007665) is the fastest growing known iron-oxidizing microorganism (Johnson et al., 2001), and is the species most closely related to sequence BL-16. Sequence BL-11 is closely related (99% identity) to the gene sequence from *Sulfobacillus thermosulfidooxidans* (EU491199). Sulfobacilli exhibit a number of characteristics that make them of interest for use in bioleaching; Watling et al. (2008) compared some characteristics of the four known *Sulfobacillus* spp., reviewed their occurrence in acidic and mining-related environments, and concluded that their versatility and resilience makes them valuable contributors in the bioleaching environment.

Heterotrophic microorganisms are often detected in bioleaching and acidic mining-related environments, and were detected in all samples other than at 45 °C in this study. Sequence BL-13 from the genus *Acidiphilium* was the dominant heterotroph in many of the sample at $T \leq 30$ °C. The genus *Acidiphilium* is composed of acidophilic, mostly obligately heterotrophic species (Johnson and Bridge, 2002) and *Acidiphilium* spp. are widely distributed throughout acidic mining-related environments. Sequence BL-10 (related to *Propionibacterium acnes*) was the dominant heterotroph in most of the samples at 5 °C. There was no detectable band for any obligately heterotrophic species at 45 °C; however, *Sulfobacillus* spp. are widely

considered to be mixotrophic microorganisms (Karavaiko et al., 2006).

Sequences BL-1, -10, and -14 are not typically found in bioleaching environments. Sequence BL-10 shows 99% homology to the gene from *Propionibacterium acnes*, which is an organism that is generally considered to be an anaerobic heterotroph and is known for causing the skin condition known as acne; however, it has been found in AMD (Hallberg and Johnson, 2003). Sequence BL-1 shows 99% homology to the gene from *Ilyobacter tartaricus*, which is a strict anaerobe capable of fermenting tartrate and has been found in numerous anoxic environments (Schink, 1984). Sequences BL-14 shows 99% homology to the gene from a bacterium of the phylum Gemmatimonadetes, which is a recently-discovered phylum, in which the first cultured species was *Gemmatimonas aurantiaca*; a rod-shaped Gram-negative aerobe capable of heterotrophic growth on a limited range of substrates in a minimal nutrient environment at mesophilic temperatures and near-neutral pH (Zhang et al., 2003).

It is unclear the role (if any) played by these microorganisms in the bioleaching process, nor how anaerobes were able to survive in a highly-aerated environment. It is possible these species were introduced by contamination during bioleaching (i.e. electrodes or glassware) or were present on the original ore sample. As previously mentioned, the ore was found to contain some organic material deposited by animals; however, it seems unlikely that the microorganisms were present on the original ore in sufficient numbers to be detected in DGGE analysis, which has a detection limit of approximately 1%.

3.3.3. Bacterial density measurements

Bacterial density curves were constructed by enumerating both iron- and sulphur-oxidizing bacteria using the MPN method six times over the course of the five-week experiments. Slurry samples were taken from the reactors while stirring, so the bacterial density determinations

represent a combination of both planktonic and sessile bacteria. The first tube in each row was shaken vigorously in an attempt to dislodge sessile bacteria from the ore particles; however, no surfactant was used for fear it would negatively impact reproduction (Torma et al., 1976). The bacterial growth curves for the experiments conducted at 45, 30, 15, and 5 °C are given in Fig. 5a–d respectively.

The MPN method estimates the population density of a particular group of microorganisms based on their ability to use a specific substrate. The MPN method does not differentiate between species like the molecular-based enumeration techniques; however, the MPN method offers the advantage of only enumerating live bacteria that are healthy enough to be capable of reproduction.

Thiosulphate has been used for the purpose of enumerating sulphur-oxidizing bacteria in this study; however, thiosulphate disproportionates in mildly acid media to produce primarily elemental sulphur and sulphite. According to Xu and Schoonen (1995), the initial reaction proceeds rapidly according to Eq. (1); however, the reaction does not go to completion and achieves a steady-state, which results in a solution that contains varying amounts of elemental sulphur, sulphite, and small quantities of other reduced-sulphur compounds.

Oxidation of any of these products would produce a drop in pH, which is the indicator for a positive tube in the MPN tests. Since it is not possible to conclude which reduced-sulphur compound is being oxidized, the results of the sulphur-oxidizing enumeration tests in this study have been interpreted as determining the total number of microorganisms capable of oxidizing a range of reduced-sulphur compounds, and have been consistently referred to as “sulphur-oxidizing bacteria” in this report.

Fig. 5. a. Density of iron- (Fe) and sulphur-oxidizing (S) bacteria at pH 3 as a function of time at 45 °C. b. Density of iron- (Fe) and sulphur-oxidizing (S) bacteria at pH 3 and 5 as a function of time at 30 °C. c. Density of iron- (Fe) and sulphur-oxidizing (S) bacteria at pH 3 as a function of time at 15 °C. d. Density of iron- (Fe) and sulphur-oxidizing (S) bacteria at pH 3 and 5 as a function of time at 5 °C.

Each enumeration experiment in this study was run concurrently with 10 un-inoculated control tubes. The initial pH of the media was set at 4.2; however, after incubation, the average pH of the un-inoculated tubes was always higher than 4.2, which is consistent with the consumption of protons during disproportionation (Eq. (1)). A tube was identified as positive for sulphur-oxidizing bacteria if the pH was found to be 0.5 units lower than the average of the control tubes. Generally speaking, positive tubes were determined to be $\text{pH} < 2.5$, making the identification of positive tubes unambiguous.

The results at 45 °C are unusual and generally characterized by low cell concentrations (Fig. 5a). The concentration of iron-oxidizing bacteria exhibited a bimodal growth curve. There was no detectable presence of sulphur-oxidizing bacteria in the first 14 days, after which the concentration of sulphur-oxidizers increased and appeared to track the concentration of iron-oxidizers over the last two enumeration periods. There is currently no conclusive explanation for these observations.

The experiments at 15 and 30 °C (Fig. 5c and b respectively) exhibited similar trends, which are characteristic of bacterial growth in batch culture: a robust increase in cell concentrations in the first few days (log phase), followed by a period of constant cell concentrations (stationary phase), and then a decline in cell concentrations in some cases (death phase). It is not unexpected for cell concentrations to have declined as the substrate was exhausted (particularly at higher temperatures and pH 5), considering the ore contains less than 3% total sulphide mineral and the majority of the iron reports to either iron-containing oxides or iron-rich silicates, both of which were shown to be stable in this system at pH 5 (Cameron et al., 2009a).

In the 30 °C experiments, iron-oxidizers at pH 3 and sulphur-oxidizers at both pH 3 and 5 multiplied quickly in the first few days, whereas the concentration of the iron-oxidizing bacteria at pH 5 exhibited less robust growth. Poor growth of the iron-oxidizing bacteria at pH 5 is somewhat expected considering the total dissolved iron determinations at 30 °C pH 5 were consistently in the range of 1 to 2 ppm. Similar cell densities and trends were observed in two different sets of growth experiments with the same ore: 35-day growth experiments at 30 °C pH 3 and 5 (unpublished data), and 10-day growth experiments at 30 °C pH 3 and 5 (Cameron et al., 2009a).

At 5 °C (Fig. 5a), the iron-oxidizing bacteria consistently outnumbered the sulphur-oxidizing bacteria by approximately 2 to 3 orders of magnitude, and time appeared to be the most important factor affecting the bacterial densities. The observation that time appeared to be the most important factor at 5 °C is supported by the DGGE similarity analysis, which clustered the 5 °C bands most closely by time rather than pH (Fig. 3). It is surprising that a difference of two orders of magnitude in proton activity (i.e. pH 3 to 5) appeared to have little effect on the rate of growth at 5 °C.

4. Conclusions

This report offers a characterization of the microbial consortia involved in the stirred-tank bioleaching of a low-grade nickel sulphide ore. The experiments were conducted over a range of temperatures (5 to 45 °C) and pH (3 and 5) conditions. A combination of classical microbiological and molecular biological techniques was used to identify and enumerate the microorganisms. There was agreement between the results obtained by the classical enumeration tests and the molecular biological techniques.

DGGE analysis revealed the presence of at least 16 distinct 16S rRNA gene sequences, 14 of which are closely related to existing GenBank sequences (>97%). The other two sequences are not closely related to existing GenBank sequences, and may possibly be from novel species; they are most closely related to sequences from an *Acidithiobacillus* sp. and a strain of *Sulfobacillus acidophilus*. Thirteen sequences are related to gene sequences of genera that have previously been detected in

bioleaching environments (*Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus*, *Acidiphilium*, *Ferrimicrobium*, and *Acidimicrobium*), and one sequence that is most closely related to a gene sequence of a microorganism that has been previously detected in AMD (*Propionibacterium acnes*). At 5 °C, two sequences were detected that are not typical of either bioleaching or acidic mining-related environments, and are closely related to the species *Ilyobacter tartaricus* and to the phylum Gemmatimonadetes. The 16S rRNA gene sequences obtained in this study (BL-1 to BL-16) have been deposited in the GenBank database under accession numbers HM124428 to HM124443 respectively.

Substrate utilization tests determined that the mixed cultures at 5, 15, and 30 °C were able to oxidize elemental sulphur and ferrous iron in minimal nutrient media, whereas the culture at 45 °C required yeast extract in order to oxidize both substrates. Considering the low sulphide content of this ore, the growth curves generally show robust growth of both iron- and sulphur-oxidizing bacteria at all temperatures except 45 °C. A difference of two orders of magnitude in proton activity (i.e. pH 3 to 5) had an insignificant effect on the rate of bacterial growth at 5 °C.

DGGE analysis revealed large changes in the bacterial community structure as a function of temperature, pH, and time. This is a strong indication that the original microbial consortium that was enriched at 30 °C was quite diverse and robust. Members from the genus *Acidithiobacillus* were dominant at all temperatures except 45 °C, at which *Sulfobacillus* spp. were dominant. Many of the acidithiobacilli were most closely related to strains of *A. ferrooxidans*, and different strains were dominant under different experimental conditions, suggesting considerable phenotypic heterogeneity within the species.

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