



## Detection of *Acidithiobacillus ferrooxidans* in acid mine drainage environments using fluorescent in situ hybridization (FISH)

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### Abstract

An important microorganism of acid mine drainage (AMD) and bioleaching environments is *Acidithiobacillus ferrooxidans* which oxidizes ferrous iron and generates ferric iron, an oxidant. Most investigations to understand microbial aspects of sulfide mineral dissolution have focused on understanding physiological, metabolic, and genetic characteristics of *A. ferrooxidans*. In this study, a 16S rRNA oligonucleotide probe designated S-S-T.ferr-0584-a-A-18, and labeled at the 5'-end with indocarbocyanine dye (CY3), was used in a fluorescent in situ hybridization (FISH) procedure on pure cultures of nine isolates of *A. ferrooxidans*. These isolates were recovered from acid mine drainage and mining environments. The probe was also used to detect cells of *A. ferrooxidans*, recovered from AMD samples, growing on FeTSB and FeSo solid media in a FISH procedure. In addition, the presence of cells of *A. ferrooxidans* in an environmental water sample from an AMD site in Copper Cliff, Ontario, Canada was analyzed using the FISH technique. Probe specificity was first confirmed with *A. ferrooxidans* ATCC 19859 (positive control) and *Acidithiobacillus thiooxidans* ATCC 19377, *Acidiphilium acidophilum* ATCC 27807, and *Lactobacillus plantarum* ATCC 8014 (negative controls). Positive and negative control cells were also used to determine optimal stringency conditions for hybridizations with the probe. Cells of the nine isolates of *A. ferrooxidans* stained positive, although the fluorescent signal varied in intensity from isolate to isolate. Colonies of *A. ferrooxidans* from the environmental water sample of the AMD site were recovered only on FeTSB solid medium after 22 days of incubation. The probe was able to detect cells of *A. ferrooxidans* in a FISH procedure. However, no cells of *A. ferrooxidans* were detected in the AMD water sample without cultivation. Thus, probe S-S-T.ferr-0584-a-A-18 hybridized effectively with cells of *A. ferrooxidans* recovered from pure cultures but failed to directly detect cells of *A. ferrooxidans* in the AMD site.

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## 1. Introduction

Mining activities result in the formation of a severe environmental problem known as acid mine drainage (AMD). AMD is an aqueous pollutant formed as a result of chemical and biological oxidation of sulfide minerals. It is produced in tailings ponds and waste rock dumps and is characterized by a low pH and high concentration of sulfate and dissolved metals (Stichbury et al., 1995). Annually, nearly 500,000,000 t of waste rock are deposited in tailings impoundments covering roughly 15,000 ha of Canadian wilderness (Davis et al., 1995). Of all Canadian reactive tailings, the Copper Cliff Tailings Area is the largest, covering approximately 2226 ha and containing more than 10% of all tailings in Canada (Puro et al., 1995). The chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* is one of the major microorganisms in AMD as it accelerates the rate at which some of the key chemical reactions proceed, thereby increasing the rate of acid generation. *A. ferrooxidans* is also the most important microorganism in microbiological mining.

*A. ferrooxidans* is a Gram-negative, non-spore forming rod-shaped acidophilic bacterium (Leduc and Ferroni, 1994). It is an obligate chemolithoautotrophic bacterium that uses elemental sulfur and/or reduced sulfur compounds as well as ferrous iron as a source of energy and carbon dioxide as a source of carbon. Various strains of the species *A. ferrooxidans* have been isolated from natural (rocks, ores and mine waters) and technological (ore concentrations and pulps of the gold and non-ferrous industries) sources. The habitats of *A. ferrooxidans* strains are geographically extremely diverse and vary in their physico-chemical conditions (presence of particular sulfide minerals and their ratio, pH, temperature and the content of toxic compounds in the liquid phase). This might explain the polymorphism of *A. ferrooxidans* strains, in terms of both their physiological properties and genotypic characteristics (Karavaiko et al., 2003).

Nucleic acid-based, genotypic methods promise a faster and more reliable identification than cultivation-based methods. The in situ identification of fixed whole bacterial cells using fluorescently labeled, ribosomal RNA-targeted oligonucleotides originally described by DeLong et al. (1989) has found numerous applications in microbiology (Amann and Kühn, 1998; Amann et al., 1995). Certain attributes of

the rRNAs favor their use as molecular chronometers because the sequences that code for rRNA are among the most highly conserved and enable definitive identification of target microorganisms (Amann et al., 1995). The rRNAs can be viewed as composed of structural domains within which sequence variation increases with respect to increasing phylogenetic distance. Regions that vary sufficiently slowly allow inference of relationships between members of the three domains (Bacteria, Eukarya, and Archaea), whereas the most variable regions provide for discrimination between organisms of approximate genus and species rank differences (Woese, 1987). Regional differences in sequence conservation have provided the basis for designing nucleic acid probes varying in specificity; group- and species-specific oligonucleotide probes have been used for direct assessment of environmental diversity.

The purpose of this study was to use an oligonucleotide probe to detect cells of pure cultures of nine isolates of *A. ferrooxidans* recovered from different AMD and mining environments in a fluorescent in situ hybridization (FISH) procedure. The probe was also used as a confirmation test to detect cells of colonies of *A. ferrooxidans* recovered from AMD samples growing on solid media. Finally, the probe was used to detect the presence of cells of *A. ferrooxidans* in acid leaching environments at Copper Cliff, Ontario, Canada using a FISH procedure.

## 2. Materials and methods

### 2.1. Growth of control cells and isolates of *A. ferrooxidans* in liquid culture media

Type cultures of control cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cultures of *A. ferrooxidans* ATCC 19859 (positive control) and nine isolates of *A. ferrooxidans* recovered from mining and acidic environments (Table 1; Leduc and Ferroni, 1993) were grown in TK liquid medium (Tuovinen and Kelly, 1973) and incubated on a gyratory shaker (200 rpm) at room temperature (23 °C). Three negative controls were used in this study (Table 1). *A. thiooxidans* ATCC 19377 was grown in Thiobacillus medium (ATCC medium # 125 Broth) and incubated at room temper-

Table 1  
Control cells and isolates of *A. ferrooxidans* recovered from acidic and mining environments used in FISH procedure

Organisms	Source	Phylogenetic grouping (subdivision)	Metabolic characteristics
<i>Acidithiobacillus ferrooxidans</i>		Proteobacteria ( $\gamma$ )	Sulfur and iron oxidizer Autotrophic
Positive control	ATCC 19859		
Isolates D2, D6, and D7	Uranium mine Denison Mines, Elliot Lake, Ontario		
Isolates N2, W1, and A1	Gifts from Dr. Marvin Silver Dalhousie University, Halifax, Nova Scotia		
Isolate F1	Indigenous to tailings areas from Falconbridge Mines, Falconbridge, Ontario		
Isolate R1	Collected from Rio Algom Mines, Elliot Lake, Ontario		
Isolate S2	ATCC 33020		
<i>Acidithiobacillus thiooxidans</i>		Proteobacteria ( $\gamma$ )	Sulfur oxidizer Autotrophic
Negative control	ATCC 19377		
<i>Acidiphilium acidophilum</i>		Proteobacteria ( $\alpha$ )	Sulfur oxidizer Heterotrophic
Negative control	ATCC 27807		
<i>Lactobacillus plantarum</i>		Gram-positive <i>Bacillus</i> , <i>Lactobacillus</i> , <i>Streptococcus</i>	Heterotrophic
Negative control	ATCC 8014		

ature (23 °C). Cultivation of *A. acidophilum* ATCC 27807 was done in tetrathionate liquid medium (O.H. Tuovinen, Ohio State University) and incubated on a gyratory shaker (200 rpm) at room temperature (23 °C). Growth of *Lactobacillus plantarum* ATCC 8014 was done in Lactobacilli MRS broth (Difco 0881-17-5) and incubated on a gyratory shaker (200 rpm) at 37 °C. For each microorganism, a shake-flask containing 100 mL of the appropriate fresh culture was inoculated with 3.0 mL of stationary phase cells and incubated at the appropriate temperature.

## 2.2. Environmental samples from an AMD site

Samples were obtained from an AMD environment from an INCO tailings pond located in Copper Cliff, Ontario, Canada. A surface water sample was collected in a sterile 1-L polypropylene bottle in May and June of 2003. Values for pH and temperature were taken on site using an Accumet portable AP5 pH meter. Each sample was kept on ice and transported to the laboratory where it was processed within 6 h.

## 2.3. Recovery of isolates from environmental samples on solid media

The environmental water sample was serially diluted using sterile basal salts solution (Tuovinen

and Kelly, 1973). Prior to inoculation, 0.5% Tween 80, a surfactant, was added to the dilutions to facilitate the spreading of the inoculum onto the solid medium. A 0.2-mL aliquot of each dilution was then plated. The isolation of acidophilic iron-oxidizing bacteria, as well as acidophilic heterotrophic bacteria, was achieved using the spread plate technique on FeTSB solid medium described by Johnson (1995). The isolation of acidophilic iron-oxidizing bacteria, acidophilic sulfur-oxidizing bacteria, and acidophilic heterotrophic bacteria was done using the spread plate technique on FeSo solid medium described by Johnson (1995). Positive and negative controls were streaked for isolation of colonies onto a suitable solid medium. All plates were incubated at room temperature (23 °C) for 15 and 22 days with the exception of the plates of *L. plantarum* ATCC 8014 grown on Lactobacilli MRS agar (Difco 0882-17-0), which were incubated at 37 °C for 48 h. The procedure was done in duplicate.

## 2.4. Preparation and fixation of cells used for FISH

Bacterial cells of pure cultures of positive and negative controls as well as cells of isolates of *A. ferrooxidans* grown in TK liquid medium were harvested at log phase as determined by measuring the rate of ferrous sulfate oxidation as a function of

time. As a result of the ferrous sulfate oxidation, the ferric iron concentrations produced were estimated by measuring absorbance at 300 nm using a Beckman DU-65 spectrophotometer. Cells were concentrated by centrifuging a volume of 100 mL of each pure culture at  $31,000\times g$  for 15 min at 10 °C. The supernatant was discarded and pellets were collected. Each pellet was washed in 30 mL of 10% sterile filtered oxalic acid (w/v) to eliminate the precipitate of ferric hydroxide generated during the growth of *A. ferrooxidans* in TK liquid medium. Cells were washed by sequential resuspension in 30 mL of sterile filtered phosphate-buffered saline (PBS, pH 7.4) and centrifugation at  $31,000\times g$  for 15 min at 10 °C. Cells were resuspended in 10 mL of sterile PBS and fixed in 1:3 (v/v) dilutions of 4% paraformaldehyde (w/v) on ice at 4 °C for 3 h. As for *L. plantarum* ATCC 8014, cells were fixed with 4% paraformaldehyde (w/v) and then with 70% ethanol (1:1, v/v). The fixative was removed by sequential centrifugation and resuspension. Prior to hybridization, cells were stored at -20 °C in sterile 10 mL PBS/96% ethanol solution (1:1, v/v).

Colonies of positive and negative control cells as well as target colonies recovered from the environmental samples on FeTSB and FeSo plates were picked twice, after a period of 15 and 22 days of incubation, and then suspended in one mL aliquots of sterile PBS. Cells were resuspended by vortexing vigorously for up to 90 s. Several colonies of each organism were needed to reach an optical density of 0.2 at a wavelength of 650 nm. Cells were fixed, washed and stored as previously described.

The environmental water samples taken were prefiltered through a sterile Whatman No.1 paper to remove large eukaryotic microorganisms, as well as particles and debris. A volume of 200 mL of the filtered environmental water was centrifuged at  $25,900\times g$  for 15 min at 4 °C. The supernatant was discarded and the cells were resuspended in 2-mL aliquot of sterile PBS and then fixed in 1:3 (v/v) dilutions of 4% paraformaldehyde (w/v) on ice at 4 °C for 3 h. The fixative was removed by centrifugation at  $31,000\times g$  for 15 min at 4 °C. Cells were washed twice in 6 mL sterile PBS by resuspension and centrifugation at  $31,000\times g$  for 15 min at 4 °C. The cells were resuspended in 2-mL aliquot of sterile PBS/96% ethanol solution (1:1, vol/vol) and stored at -20 °C prior to hybridization.

## 2.5. Oligonucleotide probe

An oligonucleotide probe specific for *A. ferrooxidans*, designed by Schrenk et al. (1998) and designated as S-S-T.ferr-0584-a-A-18 by Alm et al. (1996), was used in our studies. The probe sequence was 5'-CAGACCTAACGTACCGCC-3'. It was labeled at the 5'-end with indocarbocyanine dye (CY3) and it targets a region of the 16S rRNA. The probe was manufactured by Sigma-Aldrich Canada (Oakville, Ontario). Based on a comparative analysis of recently available aligned 16S rDNA sequences by BLAST of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) as well as the assistance of the Probe Match program of the RDP-II (Cole et al., 2003), specificity of the probe was examined theoretically with target and nontarget microorganisms used in this study.

## 2.6. Fluorescent in situ hybridization (FISH)

Prior to hybridization, the fixed cells were mixed by vortexing and by repeated trituration through a 10- $\mu$ L pipette tip. Ten microliters aliquots of each sample were spotted to each of three wells on a CLEARCELL™ slide ER-203B-2 (Erie Scientific, Portsmouth, NH). The samples on the slides were dried at room temperature, then dehydrated and permeabilized by passing through a 50%, 80% and 96% ethanol series (3 min each). Samples were air dried again at room temperature. For each slide, a master mix resuspended in 1.7-mL Eppendorf centrifuge tubes was made by adding 100  $\mu$ L of hybridization buffer, 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.1% SDS (w/v), containing 100 ng of labeled probe, in the presence of 20% deionized formamide (v/v). The mixture was gently mixed. A 25- $\mu$ L aliquot of master mix containing the probe was applied to each well on a slide. A cover glass (22 $\times$ 50 mm, FISHERfinest™) was placed on top of each slide. Slides were incubated for 90 min at 46 °C in a model C24 New Brunswick incubator shaker, but without shaking.

After hybridization, washes were done in diffuse light to avoid the bleaching of fluorescence. Cover glasses were removed from slides by dipping them in a Coplin staining jar filled with prewarmed wash buffer. Then, slides were immediately placed in a

wash buffer and incubated at 48 °C for 20 min without shaking. The wash buffer was composed of 20 mM Tris/HCl (pH 7.4), 0.1% SDS (w/v), 5 mM EDTA, and 45 mM NaCl. The formamide was omitted. The NaCl concentration was calculated to achieve the same stringency during washing and during hybridization using the formula of Lathe (Lathe, 1985). Slides were rinsed in sterile distilled water to remove salts and SDS, and air-dried in the dark at room temperature. The slides of the environmental water sample were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), final concentration 1 mg/mL, for 10 min, and then rinsed in sterile distilled water before being dried in the dark at room temperature. Each slide was mounted with the anti-fading agent Vectashield™ (Vector Laboratories, Burlingame, CA), covered with a cover glass, and observed after storage at –20 °C. Each treatment was replicated once within an experiment, and the experiment was repeated once.

Prior to use as control cells in hybridization experiments, fixed, positive and negative controls as well as target cells were examined for autofluorescence. A volume of 25 µL of each suspension was dropped to each of three wells on a CLEARCELL™ slide and subjected to the in situ hybridization protocol described, without the addition of probe. Fixed, positive and negative control cells were used to determine probe specificity with target and nontarget species, and optimal stringency conditions for hybridization with the oligonucleotide probe. A series of hybridizations conducted on positive and negative control cells at formamide concentrations ranging from 0 to 40%, at 5% increments, were performed.

### 2.7. Microscopy and image acquisition

Images of colonies recovered from the environmental water samples and grown on solid media were taken using a USB microscope (Scalar). Images were stored as 24-bit true color in Microsoft Photo Editor software version 3.0. Specimens were examined with a Nikon Labophot Phase Contrast microscope equipped for epifluorescence with an HBO 100 W/2 mercury lamp (OSRAM). The Nikon Episcopic-fluorescence EFD-3 attached to the Phase Contrast microscope was fitted with Chroma Technology 51011 DAPI/CY3™ filter set (Brattleboro,

Vermont), for detection of DAPI and CY3. Having been stored at –20 °C, each slide was brought to room temperature for 5 min before being examined. A drop of non-fluorescent immersion oil Citifluor AF87 (Marivac, Montreal, Quebec) was placed on the cover glass, before viewing with the 100× objective. Each microscopic field was first viewed with phase contrast before switching to the DAPI/CY3™ filter set. Images were captured with a Nikon coolpix 995 digital camera attached to an MDC-A relay lens (Nikon, Tokyo, Japan), in the dark. Exposure time was 1 s for phase contrast and 15 s for CY3. Exposure time for viewing cells hybridized with the probe and counterstained with DAPI using the 51011 DAPI/CY3™ filter set was 6 s. Twenty to twenty-five microscope fields were taken per slide, randomly. The images were processed by use of ACDSee software version 6.0 (ACD Systems, Saanichton, British Columbia). Microscope, camera, and image capture software settings were constant throughout the acquisition of images collected for all hybridizations.

## 3. Results

### 3.1. Growth of isolates of *A. ferrooxidans* in liquid media

Measurements of the amount of ferrous sulfate oxidation as a function of time were used to monitor the growth of cultures of *A. ferrooxidans* in TK liquid medium. Thus, this procedure indicated when the culture was in the log phase of growth. The cultures of *A. ferrooxidans* yielded a low number of cells because *A. ferrooxidans* must oxidize large amounts of ferrous iron in order to produce even a very small amount of cell biomass. Therefore, cells were concentrated by centrifugation and washed with 10% oxalic acid to eliminate ferric hydroxide precipitate formed during growth of *A. ferrooxidans*. Although the washing step with oxalic acid was performed prior to the fixation step, it did not show any effect on FISH results. All cells were examined microscopically for shape and size using a Nikon phase contrast microscope with a green interference filter. The cells of *A. ferrooxidans* were rod shaped and appeared as singles and pairs.

### 3.2. Probe specificity

The purpose of designing species-specific probes is to find a variable region within the 16S rRNA gene that is different even in closely related species (DuTeau et al., 1998). The sequence of probe S-S-T.ferr-0584-a-A-18 perfectly matches the sequences of *A. ferrooxidans* ATCC 19859 (positive control) and *A. ferrooxidans* ATCC 33020 (isolate S2 in this study). Oligonucleotide probes can distinguish between complementary and nearly complementary sequences on the basis of single mismatches if high stringency hybridization conditions are established (Amann et al., 1990). *A. thiooxidans* ATCC 19377, a closely related species to *A. ferrooxidans* and used as a negative control in this study, had two internal mismatches with the sequence of the probe. For *Acidiphilium acidophilum* ATCC 27807, a heterotrophic acidophile, the probe sequence exhibited several mismatches. The number of mismatches with the probe sequence exhibited by *L. plantarum* ATCC 8014, a more distantly related species to acidophilic microorganisms, was unknown. Nucleotide sequences of 16S rDNA for *L. plantarum* ATCC 8014 were not found on the GenBank database. The results indicate that in the FISH conditions used in this investigation, two mismatches were sufficient to discriminate positive and negative target cells (Table 2).

### 3.3. Optimization of hybridization conditions

The effective use of FISH requires that target labeled cells be distinguished from autofluorescence cells and from cells fluorescing due to nonspecific binding. Since whole cell hybridization requires

minimization of thermic stress to maintain cell integrity, the stringency was adjusted by gradually increasing (in 5% intervals) the formamide in the hybridization buffer (Manz et al., 1992). The appropriate percent formamide in the hybridization buffer was determined microscopically by assessing the fluorescent signal of positive control cells. Twenty percent formamide was found to give the optimal stringency, since no signals were detected with the nontarget organisms. All hybridizations were done at a fixed temperature of 46 °C because this temperature offered a compromise between specificity (sufficient to clearly distinguish one mismatch or more) and signal intensity (Amann et al., 1990). The concentration of NaCl in the wash buffer was calculated by the formula of Lathe to achieve the same stringency by eliminating most of the nonspecific binding of the probe with nontarget species.

### 3.4. FISH of isolates of *A. ferrooxidans*

Control cells grown in liquid media and cells of pure cultures of isolates of *A. ferrooxidans* grown in TK liquid medium, processed without the hybridization step and examined for autofluorescence, were uniformly negative. In the stringent conditions, the probe hybridized effectively with positive control cells. All cells stained positive and the FISH signal was strong. Cells of *Acidithiobacillus thiooxidans* ATCC 19377 did not show hybridization with the probe. Cells of *A. acidophilum* ATCC 27807 and *L. plantarum* ATCC 8014, hybridized with the probe, were not detectable when viewed with the epifluorescence microscope.

All cells of the nine isolates of *A. ferrooxidans* were found to hybridize with probe S-S-T.ferr-0584-a-

Table 2

16S rDNA nucleotide sequences of target and nontarget species with sequence of probe (S-S-T.ferr-0584-a-A-18)

Target and nontarget organisms	Probe sequence and target sequences 3'-CCGCCATGCAATCCAGAC-5'
<i>Acidithiobacillus ferrooxidans</i> ATCC 19859, AF362022 <sup>a</sup>	5'-GGCGGTACGTTAGGTCTG-3'
<i>Acidithiobacillus ferrooxidans</i> ATCC 33020, AJ278719 <sup>a</sup>	5'-.....-3'
<i>Acidithiobacillus thiooxidans</i> ATCC 19377, Y11596 <sup>a</sup>	5'-.....G..A.....-3'
<i>Acidithiobacillus thiooxidans</i> ATCC 19377, AJ459803 <sup>a</sup>	5'-.....G..A.....-3'
<i>Acidiphilium acidophilum</i> ATCC 27807, M79399 <sup>a</sup>	5'-.....CGACC..G..T.....AT-3'
<i>Lactobacillus plantarum</i> ATCC 8014	Unknown

<sup>a</sup> GenBank accession number from the National Center for Biotechnology Information (NCBI).

A-18, showing that their rRNA was accessible to hybridization. However, the fluorescent signal varied in intensity from isolate to isolate. Fig. 1 shows the whole cell hybridizations of the nine isolates of *A. ferrooxidans* with probe S-S-T.ferr-0584-a-A-18.

Cells of isolates D6 showed the strongest fluorescent signals, whereas cells of isolate F1 showed a weak but detectable fluorescent signal. It should be noted that the hybridization intensities differed within the same field.

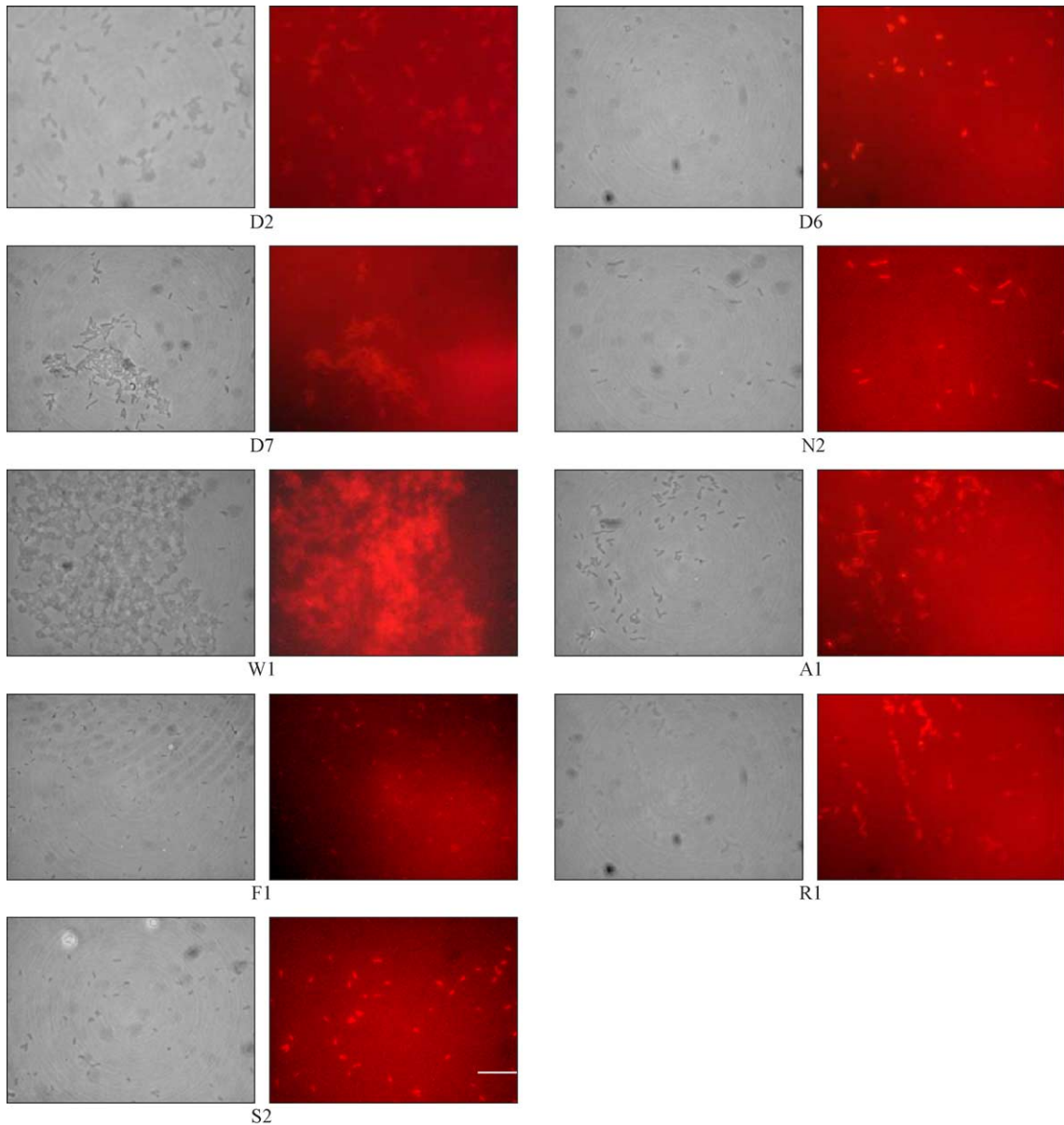


Fig. 1. In situ hybridization of cells of isolates of *A. ferrooxidans* from acidic and mining environments with CY3 labeled probe S-S-T.ferr-0584-a-A-18. The identical microscopic field was viewed, phase contrast (left) in gray scale and epifluorescence (right). The bar for the S2 isolate micrograph is 10  $\mu$ m and applies to all panels.

### 3.5. The recovery of isolates from the environmental water samples on solid media

The temperature of the water sample taken on May 2003 was 12 °C with a pH of 3.0, whereas the temperature of the water sample taken on June 2003 was 16 °C with a pH of 3.0. Iron-oxidizing acidophiles and acidophilic heterotrophs were recovered on FeTSB medium using the spread plate technique. Generally, the heterotrophs were more numerous than the iron-oxidizers, on FeTSB plates. Iron-oxidizing colonies were distinguished by their ferric iron staining, which appeared in the center of developing colonies and became more pronounced with prolonged incubation forming an orange/brown colour. Representative colonies of the major iron-oxidizing colony type, designated as C1 in this study, were used in a FISH protocol to detect the presence of *A. ferrooxidans* (Fig. 2). Acidophilic heterotrophs exhibited different types of colonies. Two major types, typical of heterotrophic acidophiles grown on FeTSB solid medium, designated as C2 and C3 in this study (Fig. 2), were picked for use in a FISH protocol. Iron-oxidizing colonies as well as sulfur-oxidizing colonies were not recovered on FeSo plates. Acidophilic heterotrophs grew on FeSo plates. Representative colonies of the major colony type recovered on FeSo plates, designated as C4 in this study, were used in a FISH protocol (Fig. 2).

### 3.6. FISH of cells of colonies recovered from the environmental water sample on solid media

Positive and negative control cells grown on solid media were examined for autofluorescence and were uniformly negative. Cells of iron-oxidizing colonies (C1) were negative for autofluorescence. However, a number of cells of heterotrophic acidophiles colonies (C2 and C3) showed autofluorescence. Cells were dark blue (violet) when viewed with the filter set 51011 DAPI/CY3™ (data not shown). Cells of the major colony type recovered from the environmental water sample on FeSo solid medium (C4), exhibited no intrinsic autofluorescence.

Cells of colonies originating from a pure culture of *A. ferrooxidans* ATCC 19859 (positive control) streaked on FeTSB solid medium as well as cells of colonies of *A. ferrooxidans* recovered after 15 days of incubation on FeTSB solid medium did not reach the optical density of 0.2 as determined for use in FISH. Colonies were tiny, covered by the iron precipitate, and did yield a few cells to be brought to the examined microscopic field. Usually 20 days of incubation are needed for growth of *A. ferrooxidans* on FeTSB solid medium (Johnson, 1995; Johnson et al., 1987). The probe strongly hybridized with positive control cells grown on FeTSB solid medium after 22 days of incubation as well as positive control cells grown on FeSo solid medium after 15 and 22 days of incubation (Fig. 3). The FISH signal for

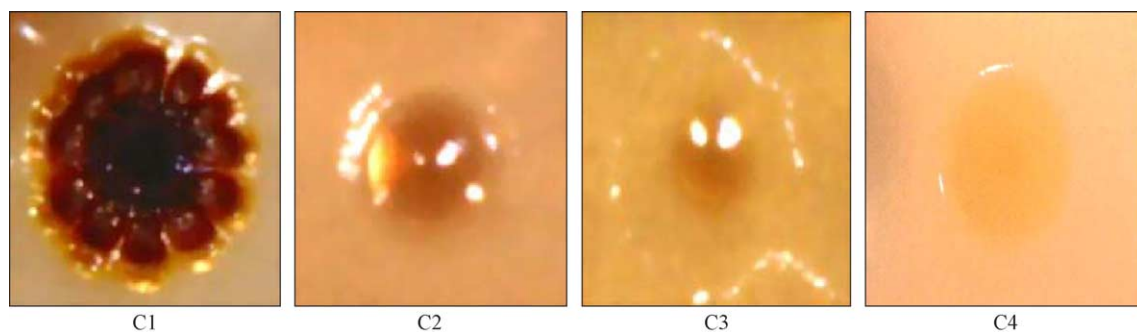


Fig. 2. Colonies recovered from an AMD water sample at Copper Cliff, Ontario and grown on solid media after 15 and 22 days of incubation. C1, C2, and C3 were grown on FeTSB solid medium. C1 represents an iron-oxidizing colony. Colonies are orange/brown colour and flower shaped. C2 shows a colony of an heterotrophic acidophile, such colonies are pink and fade at edges. C3 shows another type of heterotrophic acidophile colonies which are beige with a white contour. Major type of colony (C4) was grown on FeSo solid medium. Colonies are large, creamy white and circular shaped. Cells of the four colonies were picked, after 15 and 22 days of incubation, to be used in a FISH procedure.

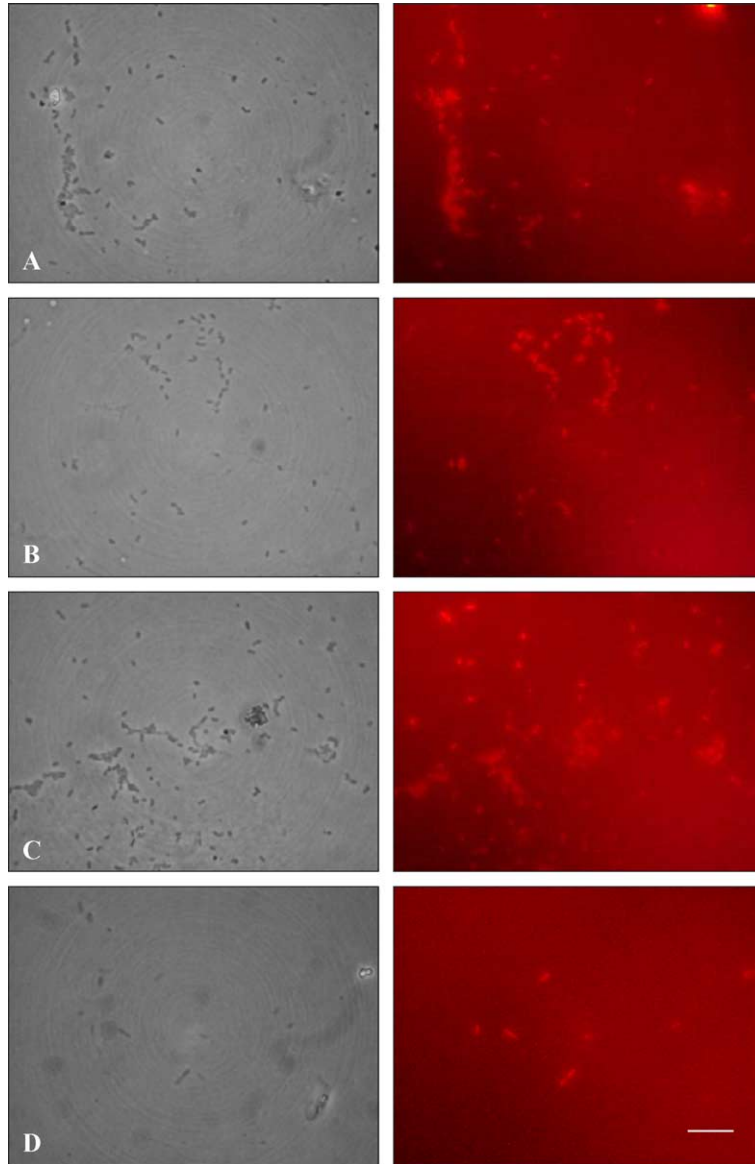


Fig. 3. In situ hybridization of cells of colonies of *A. ferrooxidans* ATCC 19859 with CY3 probe S-S-T.ferr-0584-a-A-18: recovered after 22 days of incubation on FeTSB solid medium (image A), recovered after 15 days of incubation on FeSo solid medium (image B), and recovered after 22 days of incubation on FeSo solid medium (image C). Image D represents FISH of cells of colonies (C1) recovered after 22 days from FeTSB solid medium with CY3 labeled probe S-S-T.ferr-0584-a-A-18. The identical microscopic field was viewed, phase contrast (left) and epifluorescence (right). The bar in (D) is 10  $\mu\text{m}$  and applies to all panels.

positive control cells grown on solid media and taken after 22 days of incubation was diffuse. For negative control cells, grown on solid media, FISH signals were negative and cells were not detectable when viewed with the epifluorescence microscope. Some

images showed a weak FISH signal but similar to the background fluorescence (data not shown).

Cells of colonies were attached to particles resulting from the organic materials present in the solid media. The lowest number of cells of iron-oxidizers

colonies (C1), grown on FeTSB solid medium and recovered after 15 days of incubation, prevented a reliable use of the cells in a FISH procedure. Few cells of iron-oxidizers colonies (C1), recovered after 22 days of incubation on FeTSB medium, stained positive with the probe (Fig. 3). Cells of heterotrophic acidophile colonies (C2 and C3) did not hybridize with the probe after 15 and 22 days of incubation on solid media. Although cells of C2 and C3 colonies showed autofluorescence, cells did not hybridize with the probe and the FISH signal was not detectable when viewed with the epifluorescence microscope. Cells of C4 colonies, recovered after 15 and 22 days of incubation on FeSo solid medium, were not detected with probe S-S-T.ferr-0584-a-A-18.

### 3.7. Detection of *A. ferrooxidans* in AMD water sample

The designed probe S-S-T.ferr-0584-a-A-18 was used to detect *A. ferrooxidans* among cells from the AMD site. The number of cells recovered from the environmental water sample, after concentration by centrifugation, was low. Although the water sample was filtered through a Whatman filter paper, many cells were attached to particles from sediments on slides. To be distinct from the particles exhibiting an orange fluorescent color, cells were counterstained

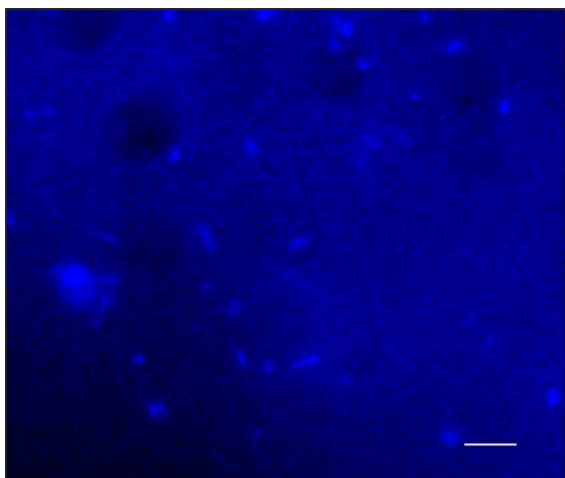


Fig. 4. In situ hybridization of cells of the environmental water sample from the AMD site at Copper Cliff, Ontario, Canada with CY3 labeled probe S-S-T.ferr-0584-a-A-18 and counterstained with DAPI. No FISH signal was detected. The bar is 10  $\mu\text{m}$ .

after hybridization with the probe with DAPI, a dye targeting only the DNA in cells. Cells in the water sample that hybridized with the probe were completely absent and cells stained with DAPI did not show a FISH signal (Fig. 4).

## 4. Discussion

An important step in developing in situ hybridization protocols is to ensure that probes have access to the rRNA (Hahn et al., 1993; Amann et al., 1992). The results of the whole cell hybridization experiments clearly showed that probe S-S-T.ferr-0584-a-A-18 entered the cells of the nine isolates of *A. ferrooxidans* and hybridized with the ribosomes (Fig. 1). The variation in the intensity of hybridization signals of cells of the nine isolates could be explained by a low number of ribosomes, since the signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content (Poulsen et al., 1993). Cells in stationary or death phase would have a lower cellular rRNA content. The variation in fluorescence levels among isolates of *A. ferrooxidans* could also be explained by differences in target region availability in isolates for the probe. The presence of an intramolecular binding site in the target region of the 16S rRNA could interfere with the probe binding which would give lower FISH signals. For in situ hybridization with rRNA-targeted oligonucleotides, the target molecules which are integral parts of the ribosome, remain in the cell. rRNA–protein and intramolecular rRNA–rRNA interactions may influence the accessibility of the target sites (Amann et al., 1995; Langendijk et al., 1995). The results demonstrated the ability of probe S-S-T.ferr-0584-a-A-18 to detect pure cultures of isolates of *A. ferrooxidans* in FISH protocols. The probe was highly specific under the FISH conditions used in this study since nonspecific binding of the probe did not occur with negative controls, especially with *A. thiooxidans* ATCC 19377 that exhibited only two mismatches with the probe.

FeTSB is a dual purpose medium, which has been used to grow both *A. ferrooxidans* and acidophilic heterotrophic bacteria as it contains ferrous iron and organic substrates in the tryptone soya broth (TSB) component (Johnson, 1995). Colonies of iron-oxidizing acidophiles recovered from the environmental

water sample on both sampling occasions suggested the presence of *A. ferrooxidans* in the AMD site, the only thiobacillus to oxidize ferrous iron. Colonies were orange/brown in colour. The numerically predominant colonies, designated as C1, were flower shaped and were used in a FISH protocol as a confirmation test to detect cells of *A. ferrooxidans*. Cells examined by phase contrast microscopy were typical of *A. ferrooxidans*.

Heterotrophs are important members of the microflora of AMD. Acidic drainage typically contains low concentrations of dissolved carbon in the water (Wichlacz and Unz, 1981). The carbon source is, therefore, from the secretions of other cells or is introduced by vegetation from outside the site. Dugan (1975) has proposed organic depot products of iron-oxidizing thiobacilli, excretions of algae, and coal leachates as possible nutrient sources for heterotrophic bacteria. This would explain the high numbers of heterotrophic acidophile colonies recovered on FeTSB solid medium. Cells of pink colonies (C2) and beige colonies (C3) were typical of *Acidiphilium* spp. Cells of C2 and C3 exhibited autofluorescence. This was probably due to the pigmentation of colonies that is characteristic of many acidophilic heterotrophs.

No universal solid medium exists that allows for the recovery of the microbial populations inhabiting AMD environments. FeSo solid medium is a suitable medium for the AMD site studied since it supports the growth of *Acidithiobacillus thiooxidans* as well as iron-oxidizing acidophiles (*A. ferrooxidans* and *Leptospirillum ferrooxidans*) and many heterotrophic bacteria. Iron-oxidizing bacteria from the environmental water sample were not recovered on FeSo solid medium. Microscopic observations revealed the absence of both *A. ferrooxidans* and *L. ferrooxidans*. No curved motile rods typical of *L. ferrooxidans* or short motile rods in singles, typical of *A. ferrooxidans*, were observed.

Most strains of *A. ferrooxidans* can utilize both ferrous iron and tetrathionate in FeSo; isolates grow initially as rust-colored, jarosite-encrusted colonies of varying sizes. Then, colonies change gradually to gelatinous cream-colored colonies within 7–14 days of incubation (Johnson, 1995). This sequence was observed when pure cultures of *A. ferrooxidans* ATCC 19859 (positive control) were streaked on FeSo but it did not occur with colonies recovered from the environmental water sample.

Sulfur-oxidizing colonies were also not recovered on FeSo solid medium. Isolated colonies of a pure culture of *A. thiooxidans* ATCC 19377 did grow on FeSo solid medium. Colonies were creamy white and very tiny, characteristics of *A. thiooxidans* colonies on solid medium. However, colonies of the autotrophic *A. thiooxidans* from the environmental water sample were not recovered. This was confirmed by microscopic examination since no straight, single or paired motile rods were observed from the major type colony (C4) growing on FeSo solid medium and picked to be used in the FISH protocol. The major type colony (C4) was large, creamy white, and circular shaped; these are characteristic of heterotrophic colonies grown on FeSo plates.

The probe hybridized effectively with *A. ferrooxidans* ATCC 19859 (positive control) grown on FeSo plates after 15 days of incubation (Fig. 3). However, the FISH signal for positive control cells grown on both solid media (FeTSB and FeSo) and taken after 22 days of incubation was slightly diffuse within the same field (Fig. 3). The variation in signal intensities was probably due to a lower number of ribosomes present in the older cells (Alfreider et al., 1996).

Of 25 images taken, four microscopic fields' images showed hybridization of the probe with cells of *A. ferrooxidans* recovered from colonies (C1) grown on FeTSB solid medium after 22 days of incubation (Fig. 3). The detection of few cells of *A. ferrooxidans* was probably due to target region availability, cell permeability, or ribosome content of the cells. A problem that could compromise the FISH protocol was the clustering of cells in a single colony and the attachment of cells to particles resulting from the organic materials of the media used. The probe did not hybridize with cells of heterotrophic acidophile colonies C2, C3, and C4 recovered on both media (data not shown). The orange fluorescent signal viewed was from the media particles and was unrelated to hybridization.

One aim in the use of fluorescent probes is the direct identification of microorganisms in complex ecosystems. For the AMD site used in this investigation, colonies of *A. ferrooxidans* were recovered on FeTSB solid medium after 22 days of incubation and cells of *A. ferrooxidans* stained with the probe. However, cells of *A. ferrooxidans* recovered from the environmental water sample were not detected with

the probe. This suggested that the bacteria at this site were in a state of dormancy. The low metabolism of these cells would result in low ribosome numbers and restrict the use of rRNA-targeted probes (Alfreider et al., 1996).

In summary, the effectiveness of a 16S rRNA fluorescent oligonucleotide probe specific to *A. ferrooxidans* was examined in a FISH procedure. The probe was specific to *A. ferrooxidans*. There was variation in the FISH signals among isolates probably because of the inaccessibility of some target regions to the probe due to intramolecular binding and differing contents of cellular rRNA. The probe hybridized with cells of *A. ferrooxidans* grown on FeTSB solid medium after 22 days of incubation but not with cells of heterotrophic acidophile colonies recovered from the AMD site. The probe confirmed the presence of *A. ferrooxidans* at the AMD site. The last objective of the study focused on the detection of cells of *A. ferrooxidans* at the AMD site, without cultivation, using the fluorescent oligonucleotide probe. However, cells of *A. ferrooxidans* were not detected. In general, probe S-S-T.ferr-0584-a-A-18 worked effectively in nutrient-rich environments with rapidly growing cultures of *A. ferrooxidans* but not with uncultured cells. This problem can be addressed by the use of fluorescently labeled rRNA-targeted polynucleotide probes since they yield higher signal intensities than oligonucleotide probes (Ludwig et al., 1994; Trebesius et al., 1994) and may thus represent a better means of detecting cells with a low ribosome content. Such probes are RNA transcripts from PCR amplicons of 16S and 23S rRNA genes from either environmental DNA or fosmid clones (DeLong et al., 1999).

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## References

- Alfreider, A., Pernthaler, J., Amann, R., Sattler, B., Glöckner, F.-O., Wille, A., Psenner, R., 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. *Appl. Environ. Microbiol.* 62, 2138–2144.
- Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., Raskin, L., 1996. The oligonucleotide probe database. *Appl. Environ. Microbiol.* 62, 3557–3559.
- Amann, R., Kühl, M., 1998. In situ methods for assessment of microorganisms and their activities. *Curr. Opin. Microbiol.* 1, 352–358.
- Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172, 762–770.
- Amann, R., Stromely, J., Devereux, R., Key, R., Stahl, D.A., 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* 58, 614–623.
- Amann, R., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Appl. Environ. Microbiol.* 59, 143–169.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., Tiedje, J.M., 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* 1, 442–443.
- Davis, B.S., Fortin, D., Beveridge, T.J., 1995. Acidophilic bacteria, acid mine drainage and Kidd Creek Mine Tailings. *Sudbury '95 Mining and the Environment*, Canmet, Sudbury, vol. 1, pp. 69–78.
- DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* 243, 1360–1363.
- DeLong, E.F., Taylor, L.T., Marsh, T.L., Preston, C.M., 1999. Visualisation and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. *Appl. Environ. Microbiol.* 65, 5554–5563.
- Dugan, P.R., 1975. Bacterial ecology of strip mine areas and its relationship to the production of acidic mine drainage. *Ohio J. Sci.* 75, 266–279.
- DuTeau, N.M., Rogers, J.D., Bartholomay, C.T., Reardon, K.F., 1998. Species-specific oligonucleotides for enumeration of *Pseudomonas putida* F1, *Burkholderia* sp. Strain JS150, and *Bacillus subtilis* ATCC 7003 in Biodegradation experiments. *Appl. Environ. Microbiol.* 64, 4994–4999.
- Hahn, D., Amann, R.I., Zeyer, J., 1993. Whole-cell hybridization of *Frankia* strains with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 59, 1709–1716.
- Johnson, D.B., 1995. Selective solid media for isolating and enumerating acidophilic bacteria. *J. Microbiol. Methods* 23, 205–218.
- Johnson, D.B., Macivar, J.H.M., Rolfe, S., 1987. A new solid medium for the isolation and enumeration of *Thiobacillus ferrooxidans* and acidophilic bacteria. *J. Microbiol. Methods* 7, 9–18.
- Karavaiko, G.I., Turova, T.P., Kondrat'eva, T.F., Lysenko, A.M., Kolganova, T.V., Ageeva, S.N., Muntyan, L.N., Pivovarova,

- T.A., 2003. Phylogenetic heterogeneity of the species *Acidithiobacillus ferrooxidans*. Int. J. Syst. Evol. Microbiol. 53, 113–119.
- Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H.F., Welling, G.W., 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. 61, 3069–3075.
- Lathe, R., 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. J. Mol. Biol. 183, 1–12.
- Leduc, L.G., Ferroni, G.D., 1993. The need for *Thiobacillus ferrooxidans* strain selection in applications of bioleaching. In: Gould, W.D., Lortie, L., Rodrigue, D. (Eds.), Proceedings of the Tenth Annual Meeting of BIOMINET, pp. 25–42 (CANMET Special Publication SP94-1, Mississauga, Canada).
- Leduc, L.G., Ferroni, G.D., 1994. The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. FEMS Microbiol. Rev. 14, 103–120.
- Ludwig, W., Dorn, S., Springer, N., Kirchhof, G., Schleifer, K.-H., 1994. PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl. Environ. Microbiol. 60, 3234–3244.
- Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H., 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.
- Poulsen, L.K., Ballard, G., Stahl, D.A., 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. 59, 1354–1360.
- Puro, M.J., Kipkie, W.B., Knapp, R.A., McDonald, T.J., Stuparyk, R.A., 1995. Inco's Copper Cliff tailings area. Sudbury'95 Mining and the Environment, Canmet, Sudbury, Ontario, vol. 1, pp. 181–191.
- Schrenk, M.O., Edwards, K.J., Goodman, R.M., Hamers, R.J., Banfield, J.F., 1998. Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: implications for generation of acid mine drainage. Science 279, 1519–1522.
- Stichbury, M., Bechard, G., Lortie, L., Gould, W.D., 1995. Use of inhibitors to prevent acid mine drainage. Sudbury'95 Mining and the Environment, Canmet, Sudbury, Ontario, vol. 2, pp. 613–622.
- Trebesius, K., Amann, R., Ludwig, W., Mühlegger, K., Schleifer, K.-H., 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. 60, 3228–3235.
- Tuovinen, O.H., Kelly, D.P., 1973. Studies on the growth of *Thiobacillus ferrooxidans*: I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison with <sup>14</sup>CO<sub>2</sub>-fixation and iron oxidation as measures of growth. Arch. Microbiol. 88, 285–298.
- Wichlacz, P.L., Unz, R.F., 1981. Acidophilic, heterotrophic bacteria of acidic mine waters. Appl. Environ. Microbiol. 41, 1251–1261.
- Woese, C.R., 1987. Bacterial evolution. Microbiol. Rev. 51, 221–271.