

## In situ assessment of active *Thiobacillus* species in corroding concrete sewers using fluorescent RNA probes

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### Abstract

Culture-dependent studies have implicated sulfur-oxidizing bacteria as the causative agents of concrete corrosion in sanitary sewers. *Thiobacillus* species are often considered the major representative of the acid-producing bacteria in these environments, and members of the genus *Acidiphilium* have been implicated to support their growth. Active populations of selected *Thiobacillus*, *Leptospirillum*, and *Acidiphilium* species were compared to total bacterial populations growing on the surfaces of corroding concrete using three oligonucleotide probes that have been confirmed to recognize unique sequences of 16S rRNA in the following acidophilic bacteria: *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* (probe: Thio820), *Leptospirillum ferrooxidans* (Probe: Lept581) and members of the genus *Acidiphilium* (probe: Acdp821). With these genetic probes, fluorescent in situ hybridizations (FISH) were used to identify and enumerate selected bacteria in homogenized biofilm samples taken from the corroding crowns of concrete sewer collection systems operating in Houston, Texas, USA. Direct epifluorescent microscopy demonstrated the ability of FISH to identify significant numbers of active acidophilic bacteria among concrete particles, products of concrete corrosion (e.g. CaSO<sub>4</sub>), and other mineral debris. As judged by FISH analyses with the species-specific probe Thio820, and a domain-level probe that recognizes all Bacteria (Eub338), *T. ferrooxidans* and *T. thiooxidans* comprised between 12% and 42% of the total active Bacteria present in corroding concrete samples. Although both *Acidiphilium* and *Leptospirillum* have also been postulated to have ecological significance in acidic sulfur-oxidizing environments, neither genera was detected using genus-specific probes (Lept581 and Acdp821). © 2002 Elsevier Science Ltd. All rights reserved.

### 1. Introduction

The production of sulfuric acid by certain acidophilic bacteria may cause significant structural damage to sub-surface infrastructure by promoting the acidic deterioration of concrete sewer pipes (Edwards et al., 1997; Morton et al., 1991; Parker, 1951). Members of the genus *Thiobacillus*, particularly *Thiobacillus thiooxidans*, have often been cultured from biofilms growing in corroding concrete sewers, and are therefore commonly used as models to describe how the metabolism of sulfur-oxidizing bacteria can catalyze acid production in sewer pipes. Other acidophilic bacteria have also been associated with corroding concrete sewer crowns and other acidophilic environments, including *Leptospirillum ferrooxidans* and members of the genus *Acidiphilium* (Goebel and

Stackebrandt, 1994; Islander et al., 1991; Wulf-Durand et al., 1997). While some investigators have agreed that *Thiobacillus* is a dominant genus in acid mine drainage environments (Evangelou and Zhang, 1995; Harrison, 1984) recent genetic-based studies have suggested that *Thiobacillus* species may play a different and perhaps lesser role in maintaining acidic environments than previously proposed (Peccia et al., 2000).

In acidic environments, members of the genus *Acidiphilium* may have significant interactions with sulfur- and iron-oxidizing bacteria, and, specifically with regard to the microbial ecology catalyzing the crown corrosion of concrete sewers, a mutualistic relationship between *Thiobacillus* species and members of the heterotrophic *Acidiphilium* genus has been suggested (Islander et al., 1991). Pure culture studies on *T. thiooxidans* have shown these microorganisms excrete pyruvic and oxalacetic acids that can be self-inhibitory at levels between  $2 \times 10^{-5}$  and  $7 \times 10^{-5}$  M (Borichewski, 1967), and it has therefore

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been suggested that the growth of *T. thiooxidans* in the environment may require a relationship with an acidophilic heterotroph.

Culture-based techniques (Harrison, 1984; Islander et al., 1991) have been useful in identifying responses of selected *Thiobacillus* species to environmental pH and sulfide levels in the headspace of sanitary sewers; however, given the many limitations and biases introduced by quantitative culture-based techniques (Amann et al., 1995), extrapolating these responses to determine microbial community relationships in situ, should be considered tentative. Molecular techniques have proven useful in more accurately describing the microbial ecology of acidic environments. Signature fatty acid analysis (Kerger et al., 1987) has identified neutrophilic and acidophilic *Thiobacillus* species in corroding concrete sewers. Genetic amplification methods (Polymerase chain reaction (PCR)) and 16S rDNA bacterial sequence analysis have been used to identify chemolithoautotrophic bacteria in natural systems containing high sulfide concentrations (Angert et al., 1998). However, PCR is not yet reliably quantitative in many environmental matrices, and relative abundance cannot be determined by this method alone. Monoclonal antibodies against *Thiobacillus ferrooxidans* (Baker and Mills, 1982) have been developed and can be used quantitatively, but they are difficult to use for broader population description, as they are limited to a single serotype. The use of 16S rRNA fluorescent in situ hybridizations (FISH) targeting *T. ferrooxidans* and *L. ferrooxidans* cells “nested” with Bacterial domain FISH cell counts suggest the presence and significance of other active species in mine-associated wastes and provided an important step towards the quantitative population analysis in these acidic environments (Schrenk et al., 1998; Edwards et al., 1999). These studies concluded that *T. ferrooxidans* occur mainly in peripheral slime-based communities (pH > 1.2) and not in the site of substrate acid formation (pH 0.3–0.7). This previously reported genetic probe targeting members of the genus *Thiobacillus* (Schrenk et al., 1998) is specific for the species *T. ferrooxidans* only. The presence of *T. thiooxidans* and *Acidiphilium* species in acidic environments as determined by PCR and DNA analysis coupled with results from population description studies with *T. ferrooxidans* and *L. ferrooxidans*, suggest a need to examine different species that promote sulfuric acid production in sulfide-rich environments.

We previously reported the development and testing of two synthetic oligonucleotide probes capable of identifying selected bacteria in acidophilic environments; one circumscribing *T. thiooxidans* and *T. ferrooxidans*, and the other circumscribing the members to the genus *Acidiphilium* (Peccia et al., 2000). Using a widely accepted direct microscopy technique (FISH), we report here the quantitative use of multiple, fluorescently labeled genetic probes to

characterize active acidophilic bacteria growing on the crowns of operating sewage collection systems among concrete particles, concrete corrosion products (e.g. CaSO<sub>4</sub>), and other mineral debris.

## 2. Materials and methods

### 2.1. Organisms, culture techniques, and nucleic acid hybridizations

Pure cultures of microorganisms used in this study are listed in Table 1. Cultures were obtained from the American Type Culture Collection (ATCC, Rockville, MD), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All organisms were cultured according to ATCC and DSM recommendations. For positive hybridization controls (FISH), liquid pure cultures were harvested in mid-log phase.

### 2.2. Environmental samples

Samples of corroding concrete and its associated biofilm were taken during the summer, 1999 from the surfaces of operating sanitary sewers in Houston, Texas, USA. Samples were taken by scraping several centimeters of pipe/corrosion product surfaces with a clean metal spatula. Immediately following their collection, samples were aseptically transferred to sterile 50 ml Oakridge centrifuge tubes containing a fresh 4% paraformaldehyde-based fixative solution in a 1:3 volumetric dilution to prepare for fluorescent in situ hybridizations. Samples were transported on ice to the laboratory, and within 48 h, were washed 3× in a 50 mM phosphate-buffered saline (PBS) (150 mM NaCl) solution (pH 7.2) using sequential centrifugation and resuspension (5 min @ 10,000 × g) according to previously described methods (Amann et al., 1990).

### 2.3. Oligonucleotide probes

The following oligonucleotide probes were used in this study because they have a demonstrated specificity to active whole bacterial cells in acidic environmental samples (Probe notation is in accordance with Ribosomal Database

Table 1  
Pure cultures used to confirm probe integrity, hybridization specificity, and recovery of known additions

Organism	Phylogenetic grouping phylum (subdivision)
<i>Acidiphilium organovorum</i> (ATCC 43141)	Proteobacteria (α)
<i>Thiobacillus thiooxidans</i> (DSM 612)	Proteobacteria (γ)
<i>Thiobacillus ferrooxidans</i> (ATCC 23270)	Proteobacteria (γ)
<i>Leptospirillum ferrooxidans</i> (ATCC 29047)	Proteobacteria (γ)

Project convention (RDP) (Maidak et al., 1999): (i) Acdp-0821-a-A-24 (Acdp821) designed to circumscribe all RDP catalogued members of the genus *Acidiphilium*; (ii) Thio-0820-a-A-22 (Thio820) designed to circumscribe all RDP catalogued *T. thiooxidans* and *T. ferrooxidans* species except the more distantly related mixotrophic *T. ferrooxidans* str. m-1 DSM 2392 (Peccia et al., 2000), (iii) Eub-0338-a-A-18 (Eub338) designed to circumscribe all catalogued members of the domain *Bacteria* (Amann et al., 1990), and (iv) Lept-0581-a-A-20 (LEPT581) designed to circumscribe all species of *L. ferrooxidans*. Fluorochrome-conjugated oligonucleotides used in FISH analysis were obtained from Genosys Biotech (Woodlands, TX) and HPLC or cartridge purified, depending on the recommendation of the fluorescent dye manufacturer. FISH analysis probes were 5' end labeled with CY3 (Eub338), Oregon Green 538 (Acdp820 and LEPT581), or Texas Red (Thio821).

#### 2.4. Fish

Probe-targeted cells from sewer pipe samples and pure cultures were observed with an epifluorescent microscope using the following procedures: Cells were fixed on ice in 1:3 (*v/v*) dilutions of 4% paraformaldehyde for 1 h. The fixative was removed by sequential centrifugation and re-suspension; cells were washed 3 × in 0.1% Tergitol type NP-40 (Sigma Chemical) and re-suspended in sterile filtered PBS. FISH stained cells were observed on membrane filter surfaces using the modifications to a previously reported hybridization procedure used for microbial visualization on glass slides (Heidelberg et al., 1993): hybridization and incubation was performed in 1.7 ml microcentrifuge tubes. Ten microliter of fixed cell solution was added to pre-warmed hybridization buffer (0.1% sodium dodecyl sulfate (SDS), 0.9 M NaCl, 100 mM Tris pH 7.2) containing 200 ng of fluorochrome-conjugated oligonucleotide probe. Cells were allowed to hybridize overnight slightly below the predetermined disassociation temperatures ( $T_d$ ) (empirical  $T_d$  values were determined at the temperature where 50% of the probe was eluted from rRNA obtained from pure-cultures that contain rRNA completely homologous to each probe). Fifty microliter of this solution was then removed, added to 450  $\mu$ l of fresh hybridization buffer (pre-warmed to  $T_d$ ), and incubated for 1 h at  $T_d$ . Cells were then counter-stained with 10  $\mu$ g/ml final concentration 4',6-damidino-2-phenylindole (DAPI, Sigma Chemical) and filtered onto 0.22  $\mu$ m black polycarbonate filters (Osmonics Inc., Livermore, CA). The filters were washed with hybridization solution at  $T_d$  to remove any unbound probe. Filters were mounted on microscope slides for observation with low fluorescence immersion oil. Bacteria retained on membrane filter surfaces were observed using a Nikon Eclipse E400 series epi-fluorescent microscope (Nikon Corp., Tokyo, Japan). Excitation and emission

filter sets were chosen in accordance with fluorescent dye manufactures' recommendations. The quantities of DAPI and FISH stained bacteria in the samples were determined according to a widely accepted protocol (Hobbie et al., 1977) using a total of three filters per sample where a minimum of 10 fields was counted per filter. A coefficient of variance < 30% was chosen as the criteria for an acceptable uniform distribution of FISH-stained bacteria on the filters' surfaces. Images were captured by a 24-bit cooled color digital camera (Spot Camera, Diagnostic Instruments Inc., Sterling Heights, MI) and recorded using Adobe Photoshop (v 5.0) software (Adobe Systems, San Jose, CA).

#### 2.5. Whole cell hybridization and specificity confirmation (control)

Probe integrity and specificity was confirmed using FISH techniques in parallel with all observations. A fixed pure culture of mid-log growth target species *Acidiphilium organovorum* (50  $\mu$ l) was mixed with cultures (50  $\mu$ l each) of fixed non-target species. To confirm identical probe recovery, the same amount of *A. organovorum* (ATCC 43141) was added to 150  $\mu$ l of sterile deionized water and both treatments were hybridized with Acdp821 probe according to the methods described above. Similar confirmation experiments were performed with target species *T. thiooxidans* (DSM 612) and the Thio820 probe, as well as with *L. ferrooxidans* species (ATCC 29047) using probe Lept581. Three

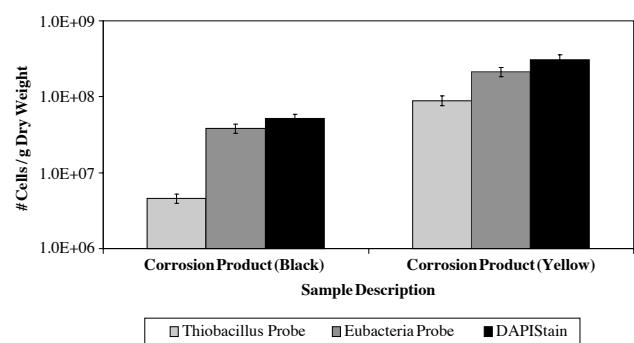


Fig. 1. Averages of FISH- and DAPI-stained cell counts in homogenized corrosion product samples taken from the surface of an active sewage collection system in Houston, Texas, USA. Bars represent # cells/g dry corrosion product circumscribed by Thio820 probe □, Eub338 probe ■, and DAPI-stained cells ■. Site A corresponds to a black surface biofilm and its associated corrosion product sample taken near a concrete manhole appurtenance; and Site B corresponds to a yellow surface biofilm and its associated corrosion product sample taken from the top of (crown) a concrete pipe near Site A. Error bars represent one standard deviation ( $n = 3$ ), which in all cases was < 14% of the average count.

microscope slides were prepared for each treatment and cells were counted according to the methods described above.

### 3. Results and discussion

#### 3.1. Whole cell FISH stringency, and recovery of known additions

To verify the recovery of oligonucleotide probes in this environmental matrix, known quantities of pure culture, log-growth target cells (Table 1) were added to dilutions of mixed cultures containing non-target microorganisms from environmental samples. FISH based counts from environmental samples “spiked” with lab-grown target cells were compared to direct DAPI-based counts of sterile water containing identical dilutions of pure culture cells. Regardless of the probe and pure culture used, results of paired *t*-tests showed that, with 95% confidence, there was no statistical difference between the increases in the amounts of cells added to environmental samples as judged by FISH, and those measured in corresponding dilutions of distilled water as judged by DAPI. This result was the same for the Thio820, Acdp821, and Lept581 probes when tested either individually or in combination. In all cases, coefficients of variance for all counts were < 25%; thus, probe specificity in FISH application was sufficient to distinguish between *Acidiphilium* species, *Thiobacillus* species and *Leptospirillum* species in this environmental matrix.

#### 3.2. Implementation of probes—environmental samples

Environmental samples collected from concrete sewer pipes (Houston, TX), were subject to DAPI staining and whole cell hybridization with the following four oligonucleotide probes: Thio820, Acdp821, LEPT581, and Eub338. Results of whole cell hybridizations are presented in Fig. 1 for two locations; Site A corresponds to a black surface biofilm and its associated corrosion product sample taken near a concrete manhole appurtenance; and Site B corresponds to yellow surface biofilm and its associated corrosion product sample taken from the top of (crown) a concrete pipe near the Site A manhole appurtenance. These sites were characterized by history of collection systems operation at less than capacity, and elevated hydrogen sulfide concentrations. At the respective sites, direct microscopic cell counts of active *Thiobacillus* species accounted for  $\sim 6.5 \times 10^6$  cells/g dry corrosion product (site A), and  $5.9 \times 10^7$  cells/g dry corrosion product (site B). While *Acidiphilium* species were present, their average cell concentration was below the statistical threshold for the method detection limit in all samples observed. No *Leptospirillum* species were detected. The ability to microscopically discriminate and count FISH-hybridized species in an environ-

mental matrix containing high concentrations of concrete particles, various solid phase corrosion products, and other debris is presented in Fig. 2 (for Site A). Total counts of cells hybridizing with the Eub338 probe were higher than the sum of those hybridizing to all probes in both samples. At Site A, 12% of the cells hybridizing with the Eub338 probe were accounted for by those also hybridizing with the Thio820 probe, while 42% of the cells hybridizing with the Eub338 probe at Site B could be accounted by hybridization with the Thio820 probe. As determined by the non-specific DNA intercalating agent DAPI, total counts of intact cells containing DNA were significantly higher than counts associated with the hybridization of Eub338 or Thio820 in both samples. At Site A, only 9% of DAPI positive cells were accounted for by those hybridizing with the Thio820 probe, while 13% of DAPI positive cells at Site B could be accounted by hybridization with the Thio820 probe.

#### 3.3. Summary

The successful in situ environmental applications of oligonucleotide probes, specific for sulfur-oxidizing and other acidophilic bacteria, are significant steps toward quantitative descriptions of the microbial ecology that supports sustained biogenic sulfuric acid production on the surfaces of concrete pipes in sewage collection systems. As judged by oligonucleotide probes, which reflect significant ribosomal RNA content and thus viability, active bacteria were quantified on pipe surfaces and in corrosion products that contain high concentrations of solid-phase precipitates. The oligonucleotide probes used did not circumscribe all the active bacterial species that are significant to the ecosystem associated with concrete sewer pipe corrosion. Additionally, these probing results indicate that active acidophilic populations are maintained within biofilms on both pipe surfaces and manhole appurtenances that are well removed from the raw wastewater flow.

### 4. Uncited References

Espejo and Romero, 1997; Hiraishi et al., 1998; Holdeman Moore and Moore, 1984; Pizarro et al., 1996; Vasquez and Espejo, 1997

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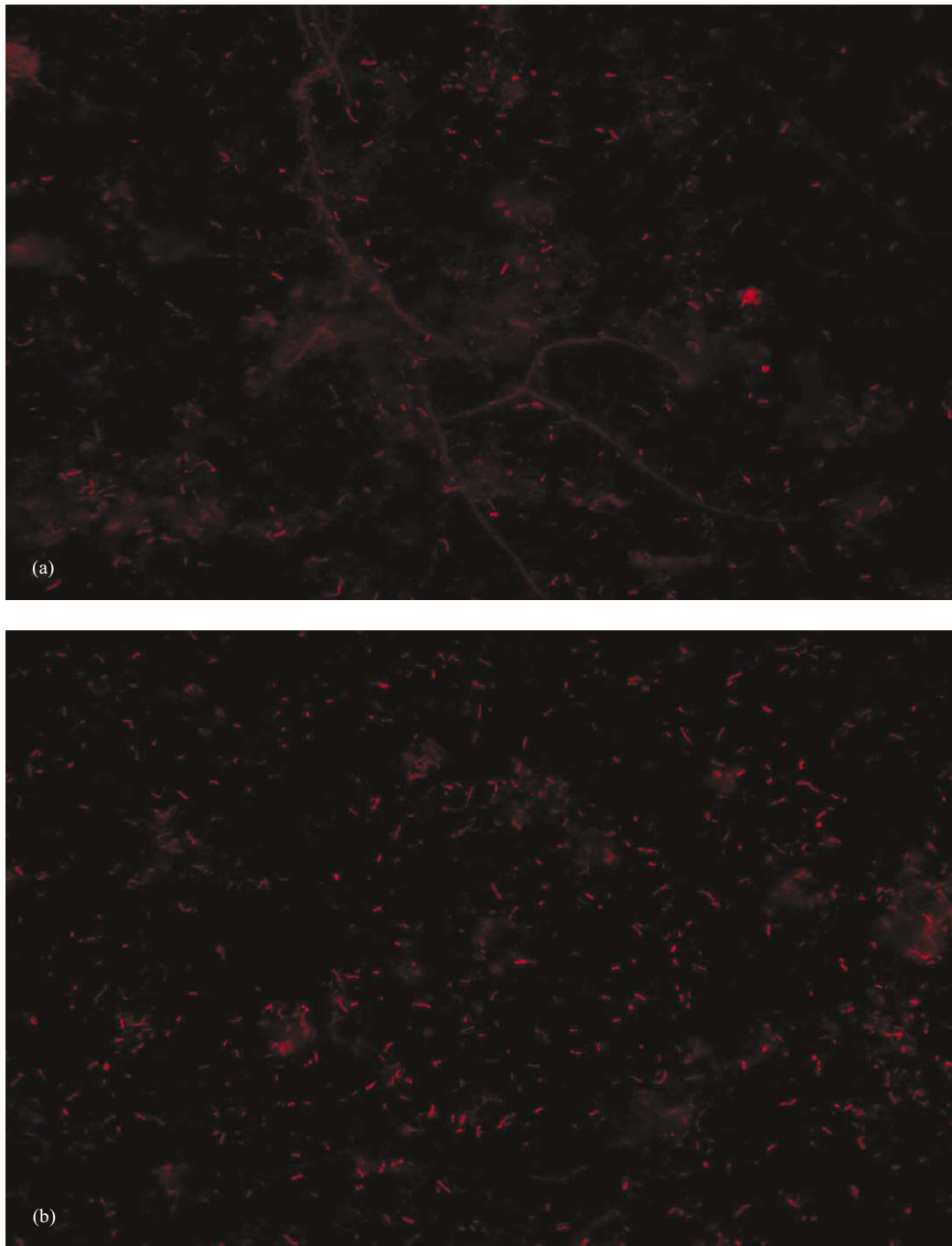


Fig. 2. Epifluorescent micrograph (1000 $\times$ ) of 16S rRNA in situ hybridization for: (a) Texas Red-labeled probe Thio820 specific for *T. thiooxidans* and *T. ferrooxidans* and (b) CY3-labeled probe Eub338. Environmental samples are from scraping the inside surface of a manhole appurtenance—Site A. Images were captured by Spot 24-bit digital camera and produced with Adobe Photoshop 5.0 for Windows.

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