# 12 Techniques for Detecting and Identifying Acidophilic Mineral-Oxidizing Microorganisms

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## 12.1 Biodiversity of Acidophilic Microorganisms That Have Direct and Secondary Roles in Mineral Dissolution

The majority of microorganisms that have the potential to accelerate the dissolution of minerals in acidic milieu are prokaryotic, though some species of fungi can also solubilize minerals, chiefly as a result of their production of metal-chelating organic acids such as citric and oxalic (Ehrlich 2002). The minerals that have been most widely studied in this context are sulfides, which may be found within igneous, and (nonoxidized) sedimentary and metamorphic rocks. Sulfide minerals may be categorized into those that are "acid-soluble" and others that are "acid-insoluble" (Rohwerder et al. 2003). The former (which includes chalcopyrite, $CuFeS_2$, and sphalerite, ZnS) are solubilized by protons (or soluble ferric iron), while the latter (which includes pyrite, $FeS_2$, and molybdenite, $MoS_2$) are attacked by chemical oxidants, of which ferric iron is the primary reagent in biomining operations and in most environmental situations. Since ferric iron is highly insoluble above approximately pH 2.5, it follows that it is only effective at oxidizing sulfides in acidic liquors. Two classes of acidophilic microorganisms are therefore important primary agents in accelerating the dissolution of sulfide minerals at low pH: (1) those that generate (sulfuric) acid by oxidizing sulfur, sulfide and reduced inorganic sulfur compounds (e.g., thiosulfate and tetrathionate) – the "sulfur-oxidizing prokaryotes," – and (2) those that oxidize iron(II) (ferrous iron) to iron(III) (ferric iron) – the "iron-oxidizing prokaryotes."

In addition to those acidophiles that have direct roles in accelerating mineral dissolution in acidic environments, there is a second group that can have a major impact in the overall process owing to their positive (or negative) interactions with the primary mineral sulfide-oxidizing prokaryotes. These "secondary" microorganisms are mostly heterotrophic, and include eukaryotes (some protozoa, microalgae and fungi) as well as bacteria and archaea. Some of these microorganisms may also contribute directly to mineral oxidation. For example, many *Acidiphilium* spp. can oxidize reduced forms of sulfur, but since characterized species (with the exception of *Acidiphilium acidophilum*) require organic carbon, these acidophiles only have important

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roles in mineral oxidation when they are members of consortia that include autotrophic prokaryotes.

The biodiversity of mineral-oxidizing acidophiles was described in Chap. 10; however, it is important to note, when considering the various approaches that are available for their detection and identification, that these microorganisms can have very different physiological characteristics. For example, amongst iron- and sulfur-oxidizing acidophiles, there are some, such as *Acidithiobacillus* spp. and *Leptospirillum* spp., that are autotrophic and fix inorganic carbon ($CO_2$), and others (obligate heterotrophs) that have an absolute requirement for organic carbon. An added complexity is another group can use either organic or inorganic carbon as a carbon source. These prokaryotes have sometimes been described as "mixotrophs," though this term has also been used to describe prokaryotes that use organic carbon as a carbon source and inorganic electron donors. A second, frequently used means of differentiating acidophiles is on the basis of their response to temperature. Although there are no fixed rules for delineating temperature cutoffs, mesophilic acidophiles are usually considered as microorganisms that grow optimally between 20 and 40˚C, while thermo-acidophiles grow optimally at higher temperatures – moderate thermophiles at approximately 40–60˚C and extreme thermophiles at above 60˚C. It is important to note that these are temperature optima, and that some mesophilic acidophiles (such as some *Leptospirillum* spp.), will grow at above 40˚C and some moderate thermophiles (such as *Acidicaldus organivorans*) at above 60˚C. Thermal characteristics need to be considered when targeting specific mineral-oxidizing prokaryotes, by using suitable incubation temperatures. Another important physicochemical variable is pH. Acidophiles vary greatly in the degree to which they tolerate acidity. Some archaea, such as "*Ferroplasma acidarmanus*" (an iron-oxidizer) and *Picrophilus* spp. (moderately thermophilic heterotrophs) can grow at pH 0, while most mineral-oxidizing bacteria do not grow below pH 1. The terms "extreme acidophiles" and "moderate acidophiles" have been used to describe those microorganisms that grow optimally at pH<3 or pH 3–5, respectively. Recently, it has been shown that some novel species of iron-oxidizers (*Thiomonas* spp. and *Frateuria*-like γ-proteobacteria) are moderately acidophilic. Although it is unlikely that such bacteria are important in commercial heap-leaching and stirred-tank biomining operations, there is increasing evidence for their widespread distribution in mine drainage waters (Hallberg and Johnson 2003).

## 12.2 General Techniques for Detecting and Quantifying Microbial Life in Mineral-Oxidizing Environments

### 12.2.1 Microscopy-Based Approaches

Mineral-oxidizing microorganisms may be observed using light and electron microscopes. However, a significant drawback to most microscopic techniques, with the notable exceptions of those involving the use of specific

oligonucleotide probes or specific antigen/antibody systems described in Sect. 12.5, is that they do not differentiate or identify different species of acidophilic prokaryotes. Phase contrast microscopy allows the visualization of live microorganisms, and acidophiles that occur as highly motile cells with distinctive morphologies (such as vibrioid and spirilla *Leptospirillum* spp.) are readily recognized. This is also the case with sporulating bacteria, such as *Sulfobacillus* spp.; endospores appear as bright, phase-dense objects within cells, though these can sometimes be confused with other cellular inclusions, such as sulfur granules. Counting chambers (such as Thoma cells) can be used to enumerate bacteria, but these require a minimum of more than $10^6$ cells per milliliter to obtain meaningful counts. For more accurate and sensitive enumeration, microorganisms present in liquid samples may be concentrated by filtering through membrane filters (pore size 0.1–0.2 μm), and may be stained with a general dye. There are a number of alternative stains that may be used, but 4′,6-diamidino-2-phenylindole (DAPI) and SYBR Green (both of which bind to DNA, and also RNA in the case of SYBR Green ) are particularly effective at detecting living (though not necessarily metabolically active) cells, and have greater sensitivity than other fluorescent dyes such as acridine orange.

One of the major problems in using microscopy to assess microbial populations in mineral-leaching environments is to differentiate living organisms from particulate matter. Liquid samples are usually much more easily processed than solid materials, though large amounts of suspended colloidal materials can be problematic. Ferric iron compounds are often amongst the most frequently encountered materials that interfere with microscopic detection of microorganisms in mineral-leaching environments. Oxalic acid may be used in a pretreatment to remove amorphous and poorly crystalline ferric iron minerals, such as schwertmannite and ferrihydrite (Ramsay et al. 1988), though these acids can also destroy the bacteria being examined. When dealing with solid materials, such as sulfidic minerals, the major problem often encountered in detecting and enumerating acidophiles is dislodging them from mineral surfaces. Many bacteria have a great propensity to attach to surfaces, and this is certainly the case for mineral-oxidizing acidophiles, many of which appear to attach selectively to certain minerals (e.g., sulfides, rather than to gangue minerals) and locations on the minerals (e.g., fracture planes, in preference to smooth surfaces; Tributsch and Rojas-Chapana 2004). Detaching bacteria and archaea from solid surfaces is often difficult, particularly those that synthesize copious amounts of exopolymeric materials, which increases the strength of their attachment (Harneit et al. 2005).

### 12.2.2 Biomass Measurements

There are a number of different techniques available for measuring total microbial biomass in environmental samples and most of these may be used, in theory, in acidic mineral-leaching situations. Modifications of standard

protocols are often necessary owing to the high concentrations of protons, metals and other solutes that are characteristic of mineral leachates. However, a major drawback of many of these techniques is, again, their inability to differentiate between different types of microorganisms present in a particular system.

One compound that is ubiquitous in all living microorganisms is adenosine triphosphate (ATP), which is often considered to be the "energy currency" of life. Concentrations of ATP may be readily measured in laboratory and environmental samples. The most widely used method involves the use of an enzyme (luciferase, which is usually sourced from the tails of fireflies) and a long-chain aldehyde substrate (luciferin). In the presence of ATP, luciferase oxidizes luciferin, releasing photons which may be detected in a luminometer. This is a highly sensitive technique, readily detecting femtomolar concentrations of ATP, and commercial reagents and equipment for this purpose are widely available. A "typical" acidophilic mineral-oxidizing bacterium contains about $10^{-21}$ mol ATP, though this depends both on the size and on the metabolic activity of the organism. However, because it is an enzymatic assay, careful handling of mineral leachates and similar test materials is required to ensure that their elevated concentrations of dissolved metals and protons do not inhibit measurements of ATP.

Other cellular components of microorganisms may also be quantified to determine the total microbial biomass present in an environmental (or industrial) sample (White et al. 1997), though not all of these have been used in the context of biomining. These include proteins (Ramsay et al. 1988; Karan et al. 1996), muramic acid (for Gram-positive bacteria) and other cell wall components, and lipids (White et al. 1997). Most polar lipids in microorganisms are phospholipids, and may be determined as lipid phosphates or phospholipid ester-linked fatty acids (PFLA). PFLA patterns may be used to indicate spatial and temporal variations in microbial community compositions (White et al. 1997).

### 12.2.3 Measurements of Activity

Measurements of microbial activity are important in mineral-leaching environments. Because of the importance of redox transformations of iron and sulfur in these situations, measurements of rates of oxidation and reduction of these two elements are particularly meaningful. The oxidation of iron is readily assessed by monitoring changes is ferrous iron concentrations (e.g., using the "ferrozine" assay; Lovley and Phillips 1987), while sulfur oxidation can be determined by measuring changes in sulfate concentrations (Kolmert et al. 2000). Alternatively, an oxygen electrode (e.g., a Clarke electrode) can be used to determine rates of oxygen consumption associated with these reactions. Rates of ferric iron or sulfate reduction can also be determined by measuring concentrations of ferrous iron or sulfate, though in such cases test

materials need to be incubated under zero or limiting oxygen concentrations. By coupling measurements of redox transformation of iron or sulfur with those of biomass (e.g., protein concentrations) it is possible to determine specific rates of transformations of these elements. Activities of heterotrophic acidophiles (apart from those involved in iron and sulfate reduction) may be assessed by monitoring changes in an organic substrate/electron donor, or by measuring rates of oxygen consumption, which is more appropriate when a complex organic substrate, such as yeast extract, is used.

The heat generated through the biologically catalyzed oxidation of pyrite and other sulfidic minerals may be used to quantify the activity of these acidophiles (Rohwerder et al. 1998). Since the amount of heat output in a typical sample is very small, a specialized instrument (a microcalorimeter) that can accurately record microwatt outputs of thermal energy is required. With this technique, a representative sample of solid material (typically 2–20 g) is placed into a glass container, leaving sufficient air space (50–70%) so that the reaction does not become oxygen-limited. The glass vessel is securely sealed and placed in the microcalorimeter. Following an equilibration period (typically 30 min) at a nominated temperature (generally the average temperature of the site from where the sample was taken), heat output is measured over a 2–4-h period. If the dominant sulfide mineral present is pyrite, its rate of oxidation (as milligrams per kilogram per hour) in the sample can be evaluated by using the $\Delta H$ value (1,546 kJ $mol^{-1}$ pyrite) that has been calculated for the complete oxidation of this mineral to ferric iron and sulfate (Schippers and Bosecker 2005).

## 12.3 Cultivation-Dependent Approaches

Cultivation-based techniques, as the term implies, have the prerequisite that the microorganism(s) in question can be grown under defined conditions in the laboratory. While there is the possibility, or even probability, that mineral-leaching environments contain microorganisms that may not be cultivated *in vitro*, currently most known acidophiles can be cultivated in synthetic liquid and also on solid media.

### 12.3.1 Enrichment Media

Liquid media for enriching populations of mineral-leaching and other acidophilic prokaryotes may be variously formulated to encourage the growth of target microorganisms. Also important is the temperature, pH and oxygen status under which enrichment cultures are incubated, in order to select for specific groups of acidophiles (thermophiles, anaerobes, etc.). Even so, care needs to be taken when interpreting data from enrichment cultures. For

example, the widespread use of ferrous sulfate liquid media to enrich for iron-oxidizing chemolithotrophs invariably favors the growth of *Acidithiobacillus ferrooxidans* rather than *Leptospirillum* spp., as the former has a faster growth rate on ferrous iron. On the other hand, with use of suitably formulated enrichment cultures together with selective solid media, it is possible to both select for and isolate most characterized mineral-oxidizing and other acidophilic microorganisms (Table 12.1). Alternatively, enrichment cultures may be diluted to extinction in order to obtain the dominant microorganism present; the highest dilution displaying positive growth is assumed to be a pure culture of the most numerous organism in the original enrichment culture.

### 12.3.2 Most Probable Number Counts

Acidophilic microorganisms can be enumerated using a statistical approach whereby samples are diluted and inoculated into a series of tubes containing growth media. Following incubation, the tubes are examined and scored for positive or negative growth, and the results are compared with standard tables to determine the most probable number (MPN) of microorganisms present (Schippers and Bosecker 2005). By varying the growth medium and

**Table 12.1.** Possible routes for isolating target mesophilic and moderately thermophilic acidophilic mineral-oxidizing prokaryotes, based on initial growth in liquid enrichment cultures followed by isolation on solid media

| Target acidophile | Liquid medium for enrichment (pH; temperature, ˚C) | Streak to plate[a] |
|---|---|---|
| *Acidithiobacillus ferrooxidans* | Ferrous sulfate (2.0; 30) | Fe<u>o</u> |
| *At. thiooxidans* | Elemental S (2.5; 30) | FeS<u>o</u> |
| *At. caldus* | Elemental S (2.5; 45) | FeS<u>o</u> |
| *Leptospirillum* spp.[b] | Pyrite (1.5; 37) | iFe<u>o</u> |
| "*Ferrimicrobium acidiphilum*" | Ferrous sulfate/yeast extract (2.0; 30) | Fe<u>o</u> |
| *Sulfobacillus thermosulfidooxidans* | Ferrous sulfate/yeast extract (1.8; 45) | FeS<u>o</u> |
| *Sb. acidophilus* | Ferrous sulfate/yeast extract (1.8; 45) | FeS<u>o</u> |
| "*Sb. montserratensis*" | Ferrous sulfate/yeast extract (1.8; 30) | FeS<u>o</u> |
| *Acidimicrobium ferrooxidans* | Ferrous sulfate/yeast extract (1.8; 45) | FeS<u>o</u> |
| *Ferroplasma* spp. | Ferrous sulfate/yeast extract (1.5; 37) | FeS<u>o</u> |
| *Thiomonas* spp. | Ferrous sulfate/thiosulfate/ yeast extract (4.0, 30) | FeT<u>o</u> |

[a]See Table 12.2
[b]Protracted incubation (10–20 days) is recommended in order to obtain greater numbers of *Leptospirillum* spp. than *At. ferrooxidans* in liquid pyrite-containing medium.

incubation conditions, it is possible to enumerate different physiological groups of acidophiles. Again, care is required to correctly interpret the data obtained. For example, 9K liquid medium (Silverman and Lundgren 1959) has frequently been used to determine MPN counts of iron-oxidizing acidophiles, yet neither heterotrophic iron-oxidizers such as *Ferroplasma* and "*Ferrimicrobium*," nor *Thiomonas*-like moderate acidophiles grow in 9K medium, owing to the absence of organic carbon and inappropriate pH, respectively. Therefore, MPN counts using 9K or other highly acidic, organic substrate-free liquid media are likely to underestimate total numbers of iron-oxidizing prokaryotes present in a sample.

### 12.3.3 Cultivation on Solid Media and on Membrane Filters

Solid media are used routinely in microbiology to cultivate neutrophilic microorganisms. In contrast, growth of acidophiles on solid media, in particular iron-oxidizers and sulfur-oxidizers, was, for many years, reported to be nonreproducible or, in the case of *Leptospirillum* spp., nonexistent. A number of different media formulations have been published, mostly designed to improve the plating efficiency of *At. ferrooxidans* (described in Johnson 1995). A radically different approach, referred to as the "overlay technique," has been used to facilitate the growth of the entire range of known moderately thermophilic and mesophilic acidophiles (Johnson and McGinness 1991; Johnson 1995; Hallberg and Johnson 2003; Johnson et al. 2005). With this, toxic organic materials, invariably present in agar-based gelling agents and also produced during plate incubation as a result of on-going acid hydrolysis of the polysaccharide, are removed by a heterotrophic acidophile which is incorporated into the lower layer of a two-layered gel. A number of different overlay plate formulations have been used to select for different groups of mineral-oxidizing and other acidophiles (Table 12.2). In most cases, the heterotroph used in the gel underlayer is an *Acidiphilium cryptum* like bacterium (strain SJH), though an "*Acidocella aromatica*" like isolate (strain PFBC) is superior for plating heterotrophic acidophiles, as this bacterium metabolizes the major toxins (organic acids) present in the solid media, but is unable to utilize materials such as yeast extract and glucose, which serve as carbon/energy sources for heterotrophs present in the inoculum.

There are a number of inherent advantages in using the overlay technique for detecting and enumerating mineral-oxidizing and other acidophilic bacteria, though a major detraction is the time required (approximately 3–20 days) for microorganisms to develop on solid media. Firstly, direct plating of samples eliminates the problems of bias (and potential elimination of poorly competitive strains) associated with enrichment cultures, allowing a more accurate picture of indigenous biodiversity of samples to be assessed. Secondly, colonies of acidophilic microorganisms generally display very different morphological characteristics, which allows them to be differentiated

**Table 12.2.** Overlay solid media for isolating and cultivating mineral-oxidizing and other acidophilic microorganisms

| Medium code | Energy sources | pH | Target isolates |
|---|---|---|---|
| ***Acidiphilium* SJH in the gel underlayer** | | | |
| iFeo | Ferrous iron | ~2.5 | Fastidious iron-oxidizers |
| Feo | Ferrous iron/TSB | ~2.5 | Iron-oxidizers (also some heterotrophs) |
| FeSo | Ferrous iron/tetrathionate/TSB | ~2.5 | Iron-oxidizers and sulfur-oxidizers (also some heterotrophs) |
| FeTo | Ferrous iron/thiosulfate/TSB | ~4.0 | Moderately acidophilic iron-oxidizers, sulfur-oxidizers and heterotrophs |
| ***Acidocella* PFBC in the gel underlayer** | | | |
| YE3o | Yeast extract | ~3.0 | Extremely acidophilic heterotrophs |
| YE4o | Yeast extract | ~4.0 | Moderately acidophilic heterotrophs |

All of the media are gelled with agarose (e.g., Sigma type I) at a final concentration of 0.5% (w/v). In most cases, three separate solutions are prepared: (1) basal salts/tryptone soya broth (*TSB*) (or yeast extract)/(tetrathionate or thiosulfate), acidified to pH 2.5 (pH 6.5 in the case of FeTo medium) and heat-sterilized; (2) ferrous sulfate (a 1 M acidified (pH 2.0) and filter-sterilized stock solution); (3) a concentrated (2%) heat-sterilized agarose solution. On cooling to about 45˚C, the three solutions are combined to give the desired final concentrations. In the case of ferrous iron, these are 25 mM (iFeo, Feo and FeSo media), 5 mM (FeTo medium) and 500 μM (yeast extract overlay media). The final concentration of tetrathionate in FeSo medium is 2.5 mM, and that of thiosulfate (in FeTo medium) is 10 mM. TSB is added to a final concentration of 0.025% (w/v) in Feo, FeSo and FeTo media, and yeast extract is added to a final concentration of 0.02%. Combined media are inoculated with either *Acidiphilium* SJH or *Acidocella* PFBC (grown in corresponding liquid media) and poured as thin gels in sterile Petri plates. Once these have solidified, a top layer of sterile medium is added.

and aids their preliminary identification (Johnson et al. 2005). For example, colonies of iron-oxidizing isolates are readily identified from their deposition of ferric compounds, causing them to be encrusted in rust-like materials. The sizes and detailed morphologies of colonies of iron-oxidizers differ between species and often strains, though a single strain will usually produce uniform colonies on any particular medium formulation. Confirmation of the identities of plate isolates is most accurately made by using a biomolecular approach (generally by analysis of their 16S ribosomal RNA, rRNA, genes; Johnson et al. 2005).

A significant limitation of agar(ose)-gelled solid media is their use at elevated temperatures (above approximately 50˚C), so they are generally limited to cultivating mesophilic and moderately thermophilic acidophiles. Gelrite is an alternative polysaccharidic gelling agent produced from bacteria, in contrast to agar, which is derived from algae. Solid media produced using Gelrite are far more thermostable than agar gels, their strength and stability being determined by the concentrations of divalent cations present. Lindström and Sehlin (1989) used a double-layered Gelrite-gelled solid medium to grow colonies of the thermo-acidophile *Sulfolobus acidocaldarius* strain BC65

(now known to be *Sulfolobus metallicus*). The inoculated plates were incubated at 65˚C, and small colonies were evident after about 4 days of incubation. The efficiency of the plating technique was essentially 100%.

A different approach that circumvents the problems of the toxicity and thermostability of gelling agents is to grow acidophiles on membrane filters. Tuovinen and Kelly (1973) pioneered this work with *At. ferrooxidans*, filtering liquid cultures through membrane filters (which varied in their effectiveness) that were then placed onto agar-gelled solid media, as avoiding direct contact of the cells with agar was considered to be very important. Later, de Bruyn et al. (1990) eliminated the need for a gelled medium altogether, floating inoculated polycarbonate membranes on low-pH (1.6) ferrous sulfate liquid medium. Colonies of a fastidious *At. ferrooxidans* like acidophile were evident after about 5 days of incubation at 30˚C.

## 12.4 Polymerase Chain Reaction (PCR)-Based Microbial Identification and Community Analysis

Advances in molecular biological tools have transformed the field of microbial ecology, allowing for the analysis of microbial communities without prior cultivation and revealing a remarkable diversity of microorganisms (Hugenholtz et al. 1998), including acidophiles. The most commonly used approaches in molecular microbial ecology focus on the small subunit of the rRNA, SSU rRNA, or its gene, and these have been described in detail elsewhere (Amann et al. 1995; Head et al. 1998). In general, the biodiversity of microorganisms in a particular ecological niche can be assessed by extraction of nucleic acid from that environment followed by amplification of the SSU rRNA gene by the polymerase chain reaction (PCR) using universal or strain-specific primers. The resulting PCR products are then subjected to a variety of analyses to assess microbial identification, community diversity and composition and, with some techniques, quantitative analysis of specific microorganisms within a community. While these molecular approaches are useful for understanding microbial communities without the reliance on cultivation techniques, they are not always capable of providing information concerning the physiology of unknown microorganisms. Therefore, the combined use of biomolecular tools and cultivation techniques is recommended (Fig. 12.1), as this approach not only provides information on the diversity of microorganisms in samples being analyzed, but also results in the isolation of indigenous microflora that can be then subjected to further studies in the laboratory.

The focus of most molecular ecological studies, the SSU rRNA, is common to all organisms, though the molecule is somewhat smaller (16S) in prokaryotes than in eukaryotes (18S). It is a highly conserved molecule (e.g., it shares a high degree of sequence identity among all organisms) that is made up of regions of near identity interspersed with regions of high sequence variability.

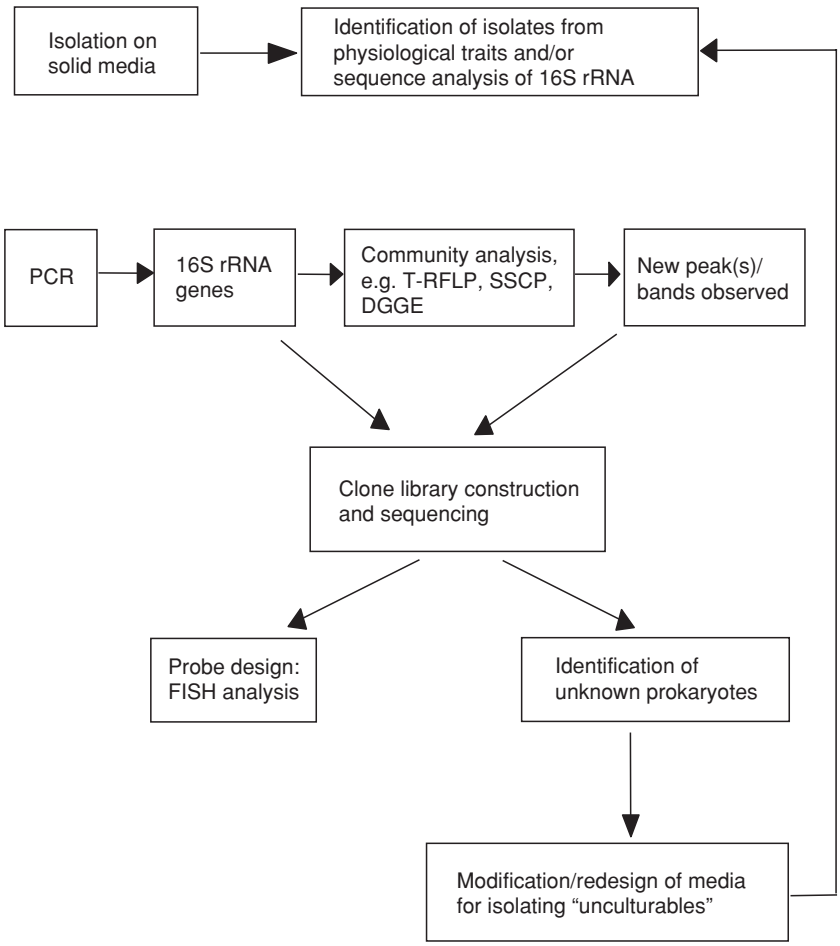


**Fig. 12.1.** Scheme for analysis of acidophilic communities using the dual approach of cultivation and molecular microbial ecology. Enrichment cultures may be used ahead of plate isolation or polymerase chain reaction (*PCR*) based analyses, though the resulting diversity of microorganisms is likely to be greatly diminished, compared with that for non-enriched samples. *rRNA* ribosomal RNA, *T-RFLP* terminal restriction fragment length polymorphism, *SSCP* single-stranded DNA conformation polymorphism, *DGGE* denaturing gradient gel electrophoresis, *FISH* fluorescent *in situ* hybridization

The regions of the 16S rRNA that are nearly identical in all organisms are targets for PCR primers, to facilitate the amplification (formation of multiple copies) of the gene from all microorganisms – the so-called universal primers. Universal primer pairs are available to amplify nearly the entire 16S rRNA gene from bacteria and archaea, as well as internal portions of the gene (Table 12.3). In contrast, the regions of high variability can serve as targets for species-specific PCR primers.

**Table 12.3.** Target sites, sequences and specificity of oligonucleotides used for amplification of 16S ribosomal RNA (*rRNA*) genes from bacteria and archaea, including those used to amplify variable regions of the gene for community analysis by denaturing gradient gel electrophoresis or single-stranded DNA conformation polymorphism (*SSCP*)

| Primer[a] | Target site[b] | Sequence (5′ to 3′)[c] | Specificity | Reference |
|---|---|---|---|---|
| 27F | 8–27 | AGAGTTTGATCMTGGCTCAG | Bacteria | Lane (1991) |
| 1387R | 1404–1387 | GGGCGGWGTGTACAAGGC | Bacteria | Marchesi et al. (1998) |
| 20F | 3–20 | TCCGGTTGATCCYGCCRG | Archaea | Orphan et al. (2000) |
| 1392R | 1407–1392 | GACGGGCGGTGTGTRC | Universal | Lane (1991) |
| 1492R | 1513–1492 | TACGGYTACCTTGTTACGACTT | Universal | Lane (1991) |
| 357F-GC[d] | 341–357 | CCTACGGGAGGCAGCAG | Bacteria | Lane (1991) |
| 907R | 926–907 | CCGTCAATTCMTTTGAGTTT | Universal | Lane (1991) |
| ARC363F-GC | 344–363 | ACGGGGYGCAGCAGGCGCGA | Archaea | Raskin et al. (1994) |
| ARC915R | 934–915 | GTGCTCCCCCGCCAATTCCT | Archaea | Stahl and Amann (1991) |
| w49[e] (F) | 331–350 | ACGGTCCAGACTCCTACGGG | Bacteria | Zumstein et al. (2000) |
| w34[e] (R) | 533–515 | TTACCGCGGCTGCTGGCAC | Universal | Zumstein et al. (2000) |
| w36[f] (F) | 333–348 | TCCAGGCCCTACGGGG | Archaea | Leclerc et al. (2001) |

[a]Numbers corresponds to the position of the 16S rRNA to which the 3′ end of the primer anneals. *F* and *R* are forward and reverse primers, respectively.
[b]*Escherichia coli* numbering of Brosius et al. (1981)
[c]*M* is A or C, *Y* is C or T, *R* is A or G, *W* is A or T
[d]GC is a 40 nucleotide GC-rich sequence attached to the 5′ end of the primer for use with DGGE, and has the sequence 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′ (Muyzer et al. 1996).
[e]w49 is the forward primer and w34 is the reverse primer for amplification of the V3 variable region of bacterial 16S rRNA genes for SSCP analysis.
[f]w36 is the forward primer used in conjunction with the w34 primer to amplify the V3 region of archaeal 16S rRNA genes for SSCP analysis.

### 12.4.1 Rapid Identification and Detection of Specific Acidophiles in Communities

In the simplest application of molecular biology to identify microorganisms, these primers are used to amplify the 16S rRNA gene from an isolate for sequence analysis. Following amplification, the gene can be cloned prior to sequencing; however, given that the Taq polymerase used to replicate the DNA during PCR is prone to making errors (i.e., inserts incorrect nucleotides), it is often better to sequence using the PCR product as the template. A cloned

gene will contain any misincorporated nucleotides that have been inserted into that molecule and may affect sequence comparison, while the PCR product will contain a mixture of faithfully replicated genes in addition to the few mistakes. Sequencing with the latter as a template will most likely assure that the correct sequence of the gene is obtained. With use of this gene sequence, a phylogenetic comparison (Sect. 12.4.4) with those of known microorganisms can yield a presumptive identification of that isolate.

Biomolecular approaches other than cloning and sequencing have also been used for the rapid analyses of mineral-leaching populations. These include restriction enzyme mapping of the 16S rRNA genes amplified from bioleachate liquors (Rawlings 1995; Rawlings et al. 1999; Johnson et al. 2005), often referred to as amplified ribosomal DNA restriction enzyme analysis (ARDRA or ARDREA). For use of this technique, however, 16S rRNA gene sequence information is required. Another genetic marker that can be amplified with universal PCR primers and used to rapidly study populations of acidophiles is the spacer region between the 16S and 23S rRNA genes which can be distinguished on the basis of its size in different prokaryotes (Pizarro et al. 1996; Bergamo et al. 2004). While these regions can vary in size even within a species, partial sequencing of the 16S rRNA gene adjacent to a spacer region can be used to confirm which organism is represented by a particular amplified spacer region. Species-specific primers, on the other hand, can be used to determine the presence of microorganisms of interest in any given environment. Such an approach has been used to detect iron- and sulfur-oxidizing acidophiles in laboratory-scale bioreactors (De Wulf-Durand et al. 1997).

### 12.4.2 Techniques for Microbial Community Analysis

In contrast to the amplification of a 16S rRNA gene from an individual microorganism, community analysis based on 16S rRNA genes requires a means to separate genes that have different sequences, as these represent distinct microbial species. This can be accomplished by constructing a 16S rRNA gene library in a host bacterium, usually *Escherichia coli*. These libraries are constructed by cloning the individual PCR products into plasmids and transforming *E. coli* with these plasmids. Each colony of *E. coli* arises from a single cell that has been transformed with one plasmid (i.e., has an individual cloned 16S rRNA gene). Genes that are contained within these colonies are then screened to determine their uniqueness, and to determine the number of each unique gene cloned. Screening is typically done by restriction enzyme fragment length polymorphism (RFLP) following PCR amplification using PCR primers that target the plasmid (to avoid amplifying the *E. coli* gene). By plotting the number of unique clones identified against the total number of clones screened, important information on the comprehensiveness of the clone library (in terms of accounting for the entire microbial community under investigation) can be obtained. Once unique genes ("clones") have been

identified by RFLP analysis, they can be sequenced to determine the phylogenetic relationship of the microorganism represented by that gene.

While gene libraries are powerful tools for microbial community analysis, their construction and analysis is a time consuming and expensive process and thus they are not usually used to compare spatial or temporal variation of microbial communities. Rapid methods for such comparisons exist, and are often referred to as gene "fingerprinting." Two of these methods are based on analysis of a portion of the gene amplified by PCR using primers that target internal regions of the RNA gene, while the third can be applied to the nearly completely amplified genes using the universal primers. In the first of the techniques (Muyzer and Smalla 1998), portions of the 16S rRNA gene containing the variable regions are amplified using archaeal or bacterial primers with a "GC clamp" (Table 12.3), and the resulting products are separated by denaturing gradient gel electrophoresis (DGGE). The denaturant typically used is a mixture of urea and formamide, or else a temperature gradient is used (often referred to as TGGE); both serve to separate (melt) the DNA into single strands that are prevented from completely denaturing by the GC clamp. The double stranded genes migrate through the gel up to the point at which they are partially denatured, which is sequence-dependent. In this way, genes with different sequences will separate from each other. The resulting bands can be quantified by densitometry to give a measure of the relative abundance of individual microorganisms within a population. Further work, however, is required to assign bands to specific microorganisms, such as band extraction and gene sequencing, or to perform DGGE analysis on clones from a clone library made in parallel with the DGGE analysis. Care must be taken with the latter approach, however, as two different sets of primers are used for the respective PCR, which may yield different results in terms of relative abundances of microorganisms.

Another fingerprinting method, single-stranded DNA conformation polymorphism (SSCP), is also based on the amplification of a small, variable region of the 16S rRNA gene, and also relies on sequence-specific secondary structure for the separation of the resulting PCR products (Orita et al. 1989). In this case, however, the small product is denatured and allowed to renature during electrophoresis, which affects the mobility of the products through the gel. The renaturation occurs in a sequence-dependent manner, thus allowing the separation of unique genes within a community. In SSCP, the PCR products are labeled with a fluorescent molecule during PCR (using a primer synthesized with a fluorochrome attached to the 5′ end), and are standardized (e.g., given a "size") by comparison with known size standards labeled with a different fluorochrome. This allows for comparison of SSCP products with those of known acidophiles, or with products from clones, to identify microorganisms within a community. In addition, the relative abundance of the products can be determined by measuring the fluorescence intensity of each product relative to the total fluorescence of all products. As with DGGE/TGGE, though, comparison of relative abundances of microorganisms

determined by clone libraries and SSCP may differ owing to the different primer sets used.

A third fingerprinting method commonly employed for microbial ecological studies is terminal RFLP (T-RFLP; Marsh 1999). Here, nearly complete 16S rRNA genes in a sample are amplified using a fluorescently labeled primer to yield a mixture of labeled 16S rRNA genes. These amplification products are digested with restriction enzymes to produce labeled terminal restriction enzyme fragments (T-RFs), which are then denatured, and the single-stranded T-RFs are separated by electrophoresis under denaturing conditions (e.g., in the presence of urea). Comparison of the migration time of the T-RFs to internal standards, labeled with a different fluorochrome, allows accurate sizing of the fragments to within ±1 nucleotide. Ideally, each T-RF represents a single microorganism, though in practice microorganisms of different species often share one T-RF. Therefore, digestion with up to three different restriction enzymes is usually necessary to accurately identify a microorganism on the basis of T-RF size. As in SSCP, the relative abundance of microorganisms represented by a T-RF can be determined by measuring the fluorescence of each T-RF relative to the sum of the fluorescence. An advantage of T-RFLP over the other fingerprinting methods is that computer analysis of gene sequences can be carried out to determine the theoretical T-RFs of known microorganisms for comparison with T-RFs obtained from environmental samples. In this way, identification of microorganisms in a sample can be determined rapidly. Alternatively, a database of experimentally derived T-RFs from known microorganisms can be created to allow for environmental microorganism identification. Lastly, by using the same primers as those typically used to make gene clone libraries, comparative information on microorganism abundance can be obtained, as well as identification of unknown T-RFs.

### 12.4.3 PCR Amplification from Community RNA for Identification of Active Microorganisms

Each of the previous approaches is based on amplification of the 16S rRNA gene. While this is a rapid and generally reliable approach, the detection of DNA is only an indication of the presence of the microorganism containing that gene. To get information on the active microorganisms of a sample, RNA-based analyses should be performed. Two such approaches that do not involve PCR are described later in this chapter (Sect. 12.5.2). To detect RNA by PCR methods, and thus employ the community analysis techniques described before, a viral enzyme is employed to transcribe RNA into DNA (referred to as copy DNA or cDNA) in a process known as reverse transcription (or transcriptase) PCR (RT-PCR). Following conversion of RNA isolated from a sample into cDNA, PCR amplification is carried out as normal. This product can then be subjected to further analysis by any of the previously

described techniques to investigate the active microbial community. RT-PCR can also be employed on genes other than the 16S rRNA genes, but much greater care needs to be taken as messenger RNA is notoriously unstable relative to rRNA.

### 12.4.4 Phylogenetic Analysis of Amplified Genes for Microbial Identification

A final key step to identifying mineral-oxidizing microorganisms is phylogenetic (relationships based on molecular similarity) analysis of the 16S rRNA gene (or any other gene) sequences obtained by any of the methods already described. A new gene sequence can be compared with those from classified microorganisms, isolates obtained in other studies, or other sequences obtained by molecular methods, to provide information on the identity of the microorganism in question. Gene sequences can be found in a host of databases, the largest of which include GenBank (http://www.ncbi.nlm.nih.gov/), EMBL (http://www.embl.de/) and the DNA Databank of Japan (http://www.ddbj.nig.ac.jp/). The Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) is a specialist database containing only rRNA sequences and a variety of tools for sequence analysis. A new gene sequence can be compared with any of these databases, often using the basic local alignment search tool (BLAST) at GenBank, to find those genes that are most identical. The information gained from such a search can be quite informative, but caution is required when analyzing such results. There is no firm rule that relates the degree of 16S rRNA gene sequence identity to taxonomical relationships (those based on physiological and phenotypic traits) but, as a rule of thumb, gene sequences that share greater than 95% identity are from two microorganisms that probably belong to the same genus, while identities above 98% indicate a high probability that the two microorganisms belong to the same species (Stackebrandt and Goebel 1994). Currently, the only accepted definition that two microorganisms belong to the same species is if they share greater that 70% similarity of their entire genomes. Also, it is important to remember when determining phylogenetic relationships that caution should be used when attempting to assign phenotypic traits to microorganisms discovered only by molecular means. A good example of this is the well-known acidophiles *At. ferrooxidans* and *At. thiooxidans*, which both share 16S rRNA gene sequence identity of approximately 97%, and yet the latter is an obligately aerobic sulfur-oxidizing microorganism, while the former is also capable of oxidizing iron and is a facultative anaerobe.

More detailed phylogenetic analysis requires specialized computer software. Once related microorganisms have been identified by homology searches, the gene sequences of all the microorganisms are aligned. Several alignment programs exist, but the commonly used ones are ClustalX (Thompson et al. 1997) and ARB (http://www.arb-home.de/). ClustalX runs

on Windows, while the ARB package operates on UNIX or Linux, and both are available at no cost on the Internet. These gene sequence alignments are then used for the construction of phylogenetic trees, again using a variety of software packages available on the Internet; ARB also includes tools for the production of trees. To assure that the phylogenetic relationships are accurate, different algorithms should be used for tree production, giving a final consensus tree. In addition, statistical analysis of the topology of the tree (the phylogenetic relationships) can be performed by bootstrap analysis (Felsenstein 1985). Trees that are thus formed can easily be viewed with the freely available Treeview (Page 1996). Great care must be taken when performing true phylogenetic analysis, especially if microorganisms that are very distantly related to any others are discovered, but this process can be rapid when one simply wants to make a tree showing the relationship of newly discovered microorganisms to known microorganisms.

### 12.4.5 Other Genes Useful for Microbial Identification and Community Analysis

While most of the molecular microbial ecology methods discussed here focus on the 16S rRNA gene, it is by no means the only one that is useful in this context. Other genes that are highly conserved can also be used in the previously described methods, including a range of functional genes that encode for growth and maintenance functions in bacteria and archaea (so-called housekeeping genes) as well as for enzymes responsible for different modes of growth. While there is a range of universal primers to amplify genes encoding for functional enzymes, those that may be more relevant to acidophiles include the genes encoding for sulfate reduction (in the context of mine effluents), rusticyanin and those that target the large subunit of type I and type II ribulose-1,5-bisphosphate carboxylase/oxygenase (*cbbL* or *cbbM*), enzymes involved in the fixation of carbon dioxide. Primers have been designed to target two different enzymes in the sulfate reduction pathway, and include the adenosine 5′-phosphosulfate reductase subunit A gene (*apsA*) (Friedrich 2002) and the dissimilatory sulfite reductase subunits A and B genes (*dsrAB*) (Wagner et al. 1998). These genes have been amplified from nearly all classified sulfate-reducing prokaryotes, including the archaeon *Archaeoglobus profundus*, and an extensive database of these gene sequences exists for comparative analysis and potential microbial identification. Primers have also been designed to amplify the genes encoding two different isoforms of the blue-copper protein rustacyanin from strains of *At. ferrooxidans* (Sasaki et al. 2003). The latter primer pair is more restricted in nature than those used for sulfate-reducing prokaryote analysis, but nevertheless it provides a means to target a specific species of mineral-oxidizing bacteria, since the only acidophile known to contain rusticyanin is *At. ferrooxidans*.

## 12.5 PCR-Independent Molecular Detection and Identification of Acidophiles

Although the approaches described in Sect. 12.4 are useful in assessing environmental biodiversity, they are at best only semiquantitative. They all rely on the use of PCR to amplify target genes, a process that has been shown to be subject to various biases (von Wintzingerode et al. 1997). In addition, although PCR-based techniques yield useful information concerning population sizes and diversity, they are often time-consuming and require specialist skills and equipment, and more rapid and simpler approaches for enumerating and identifying microorganisms are more useful in an industrial situation, such as biomining.

### 12.5.1 Immunological Detection and Identification of Acidophiles

Immunoassay is a PCR-independent approach, providing rapid, yet accurate enumeration of specific microorganisms. In the immunoassay, cells are immobilized on nitrocellulose membranes and are detected by antibodies that recognize target microorganisms. Antibodies have been produced that target different acidophiles, including iron- and sulfur-oxidizing bacteria and the archaeon *Sulfolobus metallicus* (Apel et al. 1976; Arredondo and Jerez 1989; Jerez and Arredondo 1991; Amaro et al. 1994). However, the existence of multiple serotypes among Gram-negative acidophiles (Koppe and Harms 1994; Hallberg and Lindström 1996) limits the use of this approach. Lipopolysaccharides (LPS), a major constituent of the outer membrane of Gram-negative bacteria, are potent antigens, and differences in the LPS among bacteria of the same species are recognized by different antibodies (a method of determining serotypes). To use immunoassays effectively, a thorough knowledge of microorganisms present in a leaching operation is needed, as is a judicious choice of antibodies that target cellular components other than the variable LPS molecules.

As with all enumeration techniques, immunoassays are far less effective for detecting microorganisms attached to solids. Aside from using methods to detach the microorganisms, such as vigorous shaking in dilute detergent solutions, an innovative modification of immunoassays has been described to enumerate attached acidophiles. Enzyme-linked immunofiltration assay (ELIFA) makes use of specially adapted 96-well plates that have filters in the wells (Dziurla et al. 1998). These filters have small enough pores to retain both planktonic microorganisms and those attached to mineral particles. Following a washing step, the bacteria can be detected with antibodies that have been cross-linked to an enzyme, which produces a colored product or fluorescence. The amount of product is directly related to the total number of bacteria contained in the well, and the solids do not interfere with detection of the product. Providing care is taken, ELIFA is reported to be able to allow

detection of all microorganisms on the mineral particles that are applied to the wells.

### 12.5.2 Detection and Enumeration of Acidophiles by RNA-Targeting Methods

A different approach to characterizing populations of acidophiles (a variant of DGGE; Stoner et al. 1996) makes use of the differential migration patterns of 5S rRNA in acrylamide gels run with a gradient of urea. In this method, RNA is purified directly from microbial populations and separated by DGGE. The 5S rRNA from each different species migrates in a unique manner in these gels, and therefore the presence of a specific band in an electrophoresis gel can be used to confirm the presence of a particular microorganism in a mixed population. Since rRNA is the target molecule, this method gives information on the presence of active cells in the population, as only active cells contain appreciable amounts of RNA. Quantification of the microorganisms present can be performed by measuring the relative intensities of each band, but this is difficult to relate directly to cell number as each microorganism may have a different number of rRNA molecules, which will also vary depending on the growth rate of that microorganism. A drawback to this method is that the composition of the culture examined needs to be well known, or else a large number of pure cultures of acidophiles must be tested in this manner in order to assign 5S rRNA bands to specific microorganisms.

Fluorescent *in situ* hybridization (FISH) is a cultivation-independent technique that can yield highly accurate counts of different groups or species of acidophiles in a particular sample (Amann et al. 1990b). As the target molecule in FISH is typically 16S rRNA, this technique gives quantitative data on the active microorganisms in a population. FISH is a multistep technique that starts with the fixation of samples of acidophiles using ethanol or formaldehyde, preserving the structure of the microbial population at the moment of sampling (the *in situ* population). Next a gene probe (an oligonucleotide coupled to a fluorescent dye) is applied to the fixed sample, which binds to complementary nucleotide sequences in the 16S rRNA molecule (not the gene) present in the target microorganism(s). After washing out unbound probes from the sample, a fluorescence microscope is used to observe and count the number of target cells present in the original mixed population, although other methods of counting, such as flow cytometry, can also be used (Amann et al. 1990a). Given the nature of the 16S rRNA molecule, probes can be designed to target regions of the RNA that are shared amongst many microorganisms (e.g., those that target *Bacteria* or *Archaea*), those that are shared by subgroups of microorganisms such as the α-, β- and γ-*Proteobacteria*, etc., or species-specific regions.

Probe design to target specific microorganisms requires knowledge of the 16S rRNA sequence, which is usually obtained from gene sequence analysis

(Sect. 12.4). Once a short region of 18–20 nucleotides unique to the targeted acidophile has been found, an oligonucleotide complementary to this region can be synthesized (there are a number of companies that offer this service). It has been shown that some regions of the 16S rRNA molecule are more accessible to probes than others (Fuchs et al. 1998), though by using "helper oligonucleotides" (unlabeled oligos that bind to the RNA on either side of the probe) this problem can be overcome (Fuchs et al. 2000). When novel species of acidophiles are discovered and targeted by FISH probes, great care has to be taken to ensure the stringency of new probes. It is important to ensure that they detect target cells, but also that the oligonucleotide sequence of the probe and the hybridization conditions are such that all other microorganisms that may be present do not also retain the fluorescent probe. This entails a considerable amount of careful practical work, though this is aided by the fact that extremely acidic environments tend to contain few if any active neutrophilic microorganisms, reducing the likelihood that new probes will detect nontarget microorganisms.

A number of species-specific oligonucleotide probes that target more familiar and recently discovered acidophiles have been described (Table 12.4). These probes have been used to study bacteria and archaea found in mine water and acid streamer growths (Bond et al. 2000b; Bond and Banfield 2001; Hallberg et al. 2006). In other studies, FISH has been used to assess the planktonic microbial community of the Rio Tinto (Gonzalez-Toril et al. 2003), and in laboratory-scale bioreactors with pyrite as a growth substrate or sediment samples from a mine tailings pile (Peccia et al. 2000). A problem with the use of FISH for some environmental samples, especially solids from heaps, is that the low activity of the microorganisms results in low fluorescence against a high background of nonspecific fluorescence. This has been overcome by the adaptation of catalyzed reporter deposition FISH (CARD-FISH), where an enzyme that enhances the fluorescence signal is conjugated to the probe (Pernthaler et al. 2002). CARD-FISH has been successfully applied to a heap-leaching operation (Demergasso et al. 2005).

## 12.6 Future Perspectives on Molecular Techniques for Detection and Identification of Acidophiles

Although a number of molecular techniques for detecting and identifying mineral-oxidizing and other acidophiles have been described in this chapter, rapid advances continue to be made in tools used for molecular microbial ecology. One important advance is the use of oligonucleotide arrays to detect microbes (Zhou 2003). Here, the probes to detect microorganisms are immobilized on a solid surface, which can hold many thousands of such probes, and are then hybridized with labeled RNA extracted from the community. Microarray technology, therefore, allows for the potential rapid detection and

**Table 12.4.** Oligonucleotide probes that target the 16S rRNA molecule of commonly encountered mineral-oxidizing acidophiles

| Probe Name | Target Organism | Sequence (5′ to 3′) | Reference |
|---|---|---|---|
| TH1187 | *Thermoplasmales* | GTACTGACCTGCCGTCGAC | Bond and Banfield (2001) |
| FER656 | *Ferroplasma* | CGTTTAACCTCACCCGATC | Edwards et al. (2000b) |
| LF581 | *Leptospirillum* groups I and II[a] | CGGCCTTTCACCAAAGAC | Schrenk et al. (1998) |
| LF1252 | *Leptospirillum* group III[a] | TTACGGGCTCGCCTCCGT | Bond and Banfield (2001) |
| LF655 | *Leptospirillum* groups I, II and III[a] | CGCTTCCCTCTCCCAGCCT | Bond and Banfield (2001) |
| ACM732 | *Acidimicrobium* and relatives[b] | GTACCGGCCCAGATCGCTG | Bond and Banfield (2001) |
| ACM995 | *Am. ferrooxidans* | CTCTGCGGCTTTTCCCTCCATG | Paul Norris, University of Warwick, UK |
| SUL228 | *Sb. thermosulfidooxidans*[c] | TAATGGGCCGCGAGCTCCC | Bond and Banfield (2001) |
| ACD840 | *Acidiphilium* spp. | CGACACTGAAGTGCTAAGC | Bond and Banfield (2001) |
| Acdp821 | *Acidiphilium* spp. | AGCACCCCAACATCCAGCACACAT | Peccia et al. (2000) |
| Thio820 | *Acidithiobacillus* spp.[d] | ACCAAACATCTAGTATTCATCG | Peccia et al. (2000) |
| TF539 | *At. ferrooxidans* | CAGACCTAACGTACCGCC | Bond and Banfield (2001) |
| ATT0223 | *At. thiooxidans* | AGACGTAGGCTCCTCTTC | Hallberg et al. (2006) |
| THC642 | *At. caldus* | CATACTCCAGTCAGCCCGT | Edwards et al. (2000a) |

[a]The *Leptospirillum* grouping is based on phylogenetic analysis of environmental clones from the Iron Mountain site (Bond et al. 2000a).
[b]This probe targets *Am. ferrooxidans* as well as related clones from Iron Mountain and "*Fm. acidiphilum*," but not the environmental clones IMBA84 and TRA2-10 from Iron Mountain.
[c]This probe also targets *Sb. acidophilus*, but with a one-base mismatch in the target region, and thus it may not be specific for *Sb. thermosulfidooxidans* under certain hybridization conditions.
[d]*At. thiooxidans*, *At. ferrooxidans* and *Acidithiobacillus* sp. DSM612 (previously known as *Thiobacillus thiooxidans* DSM612) are the targets for this probe, but *At. caldus* is not a target.

identification of a great many more microorganisms in a community than has previously been possible. Given that the number of known acidophiles is vastly limited compared with the number of environments, such as active sewage sludge, it is possible that a carefully designed array could be made to detect all known acidophiles. A powerful extension of microarrays is the coupling of arrays with the labeling of macromolecules such as DNA or RNA with isotopes of a substrate (e.g., $^{13}CO_2$). Separation of the macromolecules that incorporated the isotope from those that remained unlabeled, followed by hybridization to a microarray allows for the identification of those microorganisms in a community that are metabolically active with that substrate (Adamczyk et al. 2003).

Other advances include the recent sequencing of the dominant genomes in an abandoned pyrite mine, which has allowed a hypothetical reconstruction of the community interactions (Tyson et al. 2004). This work identified microorganisms that were important to the oxidation of pyrite, leading to the formation of acid mine drainage, and showed that a key microbe in the process was a novel, nitrogen-fixing *Leptospirillum* spp. (Tyson et al. 2005). Such a genome-based approach to studying microbial communities is further enhanced by a recently described approach to study gene expression in acidophiles (Parro and Moreno-Paz 2003). The application of these new techniques will further advance our understanding of the roles of acidophilic microorganisms in mineral processing operations and the wider environment.

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