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MiniReview

Microbial communities in acid mine drainage

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Abstract

The dissolution of sulfide minerals such as pyrite (FeS_2), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and marcasite (FeS_2) yields hot, sulfuric acid-rich solutions that contain high concentrations of toxic metals. In locations where access of oxidants to sulfide mineral surfaces is increased by mining, the resulting acid mine drainage (AMD) may contaminate surrounding ecosystems. Communities of autotrophic and heterotrophic archaea and bacteria catalyze iron and sulfur oxidation, thus may ultimately determine the rate of release of metals and sulfur to the environment. AMD communities contain fewer prokaryotic lineages than many other environments. However, it is notable that at least two archaeal and eight bacterial divisions have representatives able to thrive under the extreme conditions typical of AMD. AMD communities are characterized by a very limited number of distinct species, probably due to the small number of metabolically beneficial reactions available. The metabolisms that underpin these communities include organoheterotrophy and autotrophic iron and sulfur oxidation. Other metabolic activity is based on anaerobic sulfur oxidation and ferric iron reduction. Evidence for physiological synergy in iron, sulfur, and carbon flow in these communities is reviewed. The microbial and geochemical simplicity of these systems makes them ideal targets for quantitative, genomic-based analyses of microbial ecology and evolution and community function.

Keywords: Acid mine drainage; Pyrite dissolution; Iron oxidation; Sulfide mineral; Sulfur oxidation; Geomicrobiology

1. Introduction

Acidic, metal-rich fluids are formed by chemical weathering of metal sulfide-rich rocks. These acid rock drainage (ARD) solutions are hot because metal sulfide oxidation reactions are highly exothermic. The predominant metal sulfide mineral in most rocks is pyrite (FeS_2). Pyrite-rich deposits are often mined for metals such as Au, Ag, Cu, Zn, and Pb, which are typically present as impurities in pyrite or occur in sulfide minerals such as chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and galena (PbS). Mining increases the surface area of sulfide ores exposed to air and water, thus, increases rates of acid generation. Regions where rocks have low buffering capacity generate highly acidic toxic solutions that are referred to as acid mine drainage (AMD).

Despite the extreme acidity, heat, and high concentrations of sulfate and toxic metals, a diverse range of microorganisms populate AMD environments. These organisms can form a chemoautotrophically-based biosphere in the subsurface, ultimately sustained by electron donors derived from sulfide minerals, CO_2, O_2, and N_2 derived from air, and phosphate liberated by water–rock interaction. Microbial activity increases the rate of AMD formation and may be responsible for the bulk of AMD generated [1].

Microbe–mineral interactions are of importance because AMD is a very widespread environmental problem. The organisms can be used in ore processing and are a source of novel biomolecules (especially enzymes) for industrial processes.

DNA-based studies of organisms populating mining environments have provided insights into the diversity of acidophilic, metal-tolerant species. Here, we review the importance of archaeal and bacterial lineages, and integrate microbiological, geochemical, mineralogical, and molecular information necessary for quantitative descriptions of the ecology of AMD. Eukaryotes (protists, fungi, and yeasts) are abundant and important in some parts of acid systems. However, due to the paucity of data on eukaryotes in AMD, our review focuses primarily on the

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prokaryotic components of these communities. We show that the prokaryotic richness of acidophilic communities is low compared to other extremophile and non-extremophile assemblages, yet the species are broadly distributed across the tree of life. Because of their biological and geochemical simplicity, AMD environments have potential as model systems for analysis of biogeochemical interactions and feedbacks and microbial community structure and function.

2. Dissolution of sulfide minerals

Many factors impact AMD generation. In part, rates of dissolution reactions are determined by fluid chemistry and flow, mineral and rock type, and temperature. The rate of supply of oxidant to the mineral surface influences the rate at which pyrite dissolves. The typical oxidants are oxygen and ferric iron. The concentration of oxygen in groundwater is very small compared to the large requirement for O_2 in the overall reaction:

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

Thus, the predominant source of oxygen in rapidly oxidizing systems is air. In fact, to create typical AMD, each packet of solution must be re-oxygenated hundreds to thousands of times along its flow path [2].

Geochemical studies have established that oxygen is a less effective sulfide oxidant than ferric iron. Thus, the dominant pathway for pyrite dissolution involves oxidation of ferrous iron by oxygen:

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O \quad (2)$$

followed by reduction of ferric iron by sulfide:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (3)$$

Note that the sum of reactions in Eqs. 2 and 3, required to describe the sustainable process, yields the reaction in Eq. 1. Ferrous iron oxidation by O_2 at low pH is slow, thus the rate of the reaction in Eq. 2 may limit the rate of AMD generation. However, iron-oxidizing prokaryotes catalyze ferrous iron oxidation, thus can determine the rate of pyrite dissolution [1,3]. The feedback between metabolic activity and mineral dissolution can drive the pH down to values <2, thus selecting for community members optimized for life in acid.

Physiological experiments have shown that AMD microorganisms can impact rates of sulfur oxidation during dissolution of pyrite, arsenopyrite, chalcopyrite, marcasite, and sphalerite [4,5]. Oxidation of sulfide ions to sulfate occurs via a series of intermediate sulfur-bearing compounds. Because protons are generated in the subset of reactions that add oxygen to the reduced sulfur species, microbial utilization of sulfide and intermediate sulfur compounds can significantly affect acidification, as well as pyrite dissolution rates.

There is debate about the details of the mechanisms by which microbe–sulfide mineral interactions occur. Pyrite oxidation has been proposed to proceed via indirect and direct pathways [1]. Increase in the rate of sulfide mineral dissolution due to oxidation of aqueous ferric iron is typically referred to as an 'indirect' mechanism. Oxidation of intermediate sulfur species can occur on the mineral surface or in solution. In contrast, 'direct' catalysis is used to describe the possibility of a direct enzymatic interaction with ions bound to the pyrite surface [6].

Enzymatic oxidation via a direct mechanism requires that the cells are either in close proximity or attached to the solid surface. Larsson et al. [7] showed that close proximity of the archaeon *Acidianus brierleyi* to pyrite was necessary for optimal growth and oxidation rates. Cell-sized pits, often observed on pyrite surface after reaction with *Acidithiobacillus ferrooxidans* [8], have contributed to speculation about a 'direct' enzymatic pathway.

Experiments were conducted by Edwards et al. [9] to determine whether cell attachment was necessary to generate cell-sized pits. High-resolution scanning electron microscopy (SEM) was used to characterize dissolution patterns on sulfides by *Ferroplasma acidarmanus* (at 37°C) and *Acidithiobacillus ferrooxidans* (at 25°C). Elliptical pits (1–2 μm) formed on pyrite surfaces in the presence of these species. However, they also formed in abiotic experiments with just Fe^{3+}, indicating that the presence of cells was not required for pit formation. Cell-sized pits were not observed on marcasite or arsenopyrite reacted with *A. ferrooxidans*, or on marcasite surfaces reacted with *F. acidarmanus*. However, rounded *F. acidarmanus* cells were found within round, deep pits (<0.5 μm) in arsenopyrite surfaces, clearly indicating enhanced dissolution in proximity to cells.

Fowler et al. [10] used constant ferric and ferrous ion concentrations in continuous flow reactors, with and without *Acidithiobacillus ferrooxidans*, to determine if that organism enhances leaching of pyrite by a mechanism other than increasing Fe^{3+} in the reactor. Dissolution rates with cells were faster, implying the presence of leaching enzyme(s) or a localized accumulation of Fe^{3+} at the cell surface.

Edwards et al. [11] conducted surface colonization experiments with pyrite in situ at Iron Mountain, CA, USA and in the laboratory using enrichments from the site. They found that bacteria tended to orient parallel pyrite surface steps in {110} and {100} orientations. Edwards et al. [5] also found that attachment of *Acidithiobacillus caldus* to pyrite, marcasite, and arsenopyrite was non-random. No cells attached to quartz inclusions within pyrite, indicating that attachment is mineral specific [12]. These observations suggest that cells are not simply using Fe^{2+} in solution, but exhibit chemotaxis, probably to gradients in $[Fe^{2+}]_{aq}$ outward from dissolving surfaces.

In addition to 'indirect' and 'direct' approaches to sulfide mineral dissolution, cells may make soluble organic

Table 1
List of prokaryotic diversity of acid mine drainage communities relative to other phylogenetically well-characterized environments

Environment	Number of putative divisions	Number of genera and novel lineages[a]	Reference
Non-extreme			
Arid southwestern US soil	7+	25	[18]
Wisconsin agricultural soil	17+	50	[19]
Grass pasture rhizospheres	22	145	[20]
Contaminated aquifer	25	56	[21]
Marine bacterioplanktonic community	9	37	[22]
Extreme			
High-temperature petroleum reservoirs	19	28	[23]
Mid-Atlantic Hydrothermal Vent	9+	19	[24]
Yellowstone N.P. hot spring	28	32	[25]
Antarctic sea ice and water	4	42	[26]
Deep subsurface paleosol	9+	42	[27]
Iron Mountain Mine, CA 1998	4	6	[16]
Total of all studied AMD sites	11	16	compiled here

'+' indicates that clones of unresolved phylogenetic positions were recovered.
[a]Novel lineages were counted subjectively by phylogeny provided in paper, each deeply branched cluster was counted.

molecules to shuttle electrons from insoluble elemental sulfur on pyrite surfaces to cell-associated enzymes [13]. It is also possible that oxygen and hydroxyl radical species are important in some sulfur transformations [2]. Alternatively, Sand et al. [6] proposed that cells attach to the mineral surface and accumulate Fe^{3+} in their outer membranes, causing local enhanced reactivity via a non-enzymatic direct mechanism.

Microorganisms accelerate iron oxidation rates at low pH by five orders of magnitude [14], thus can increase rates of pyrite dissolution by the reaction in Eq. 3. Numerous microbial pyrite dissolution rates have been reported. Typical experimental values range between 10^{-5} and 10^{-7} mol m^{-2} s^{-1} for microbial and ferric iron experiments compared to 10^{-6} to 10^{-9} mol m^{-2} s^{-1} for inorganic oxygen-mediated rates [11].

The degree to which microorganisms enhance sulfide mineral dissolution is determined by the number of iron-oxidizing cells present and the level of activity of the cells. These parameters are not incorporated in most reported microbial rates. Edwards et al. [11] determined the increase in release of iron from pyrite in two enrichments containing *Leptospirillum ferriphilum* to be 5×10^{-18} mol Fe $cell^{-1}$ s^{-1}, comparable to the *Thiobacillus ferrooxidans* iron oxidation rate of 6×10^{-18} mol Fe $cell^{-1}$ s^{-1} [15]. Using cell-normalized rates, it is possible to estimate the magnitude of the microbial impact in a natural environment if information about numbers of iron-oxidizing microorganisms is available. Using this approach, Edwards et al. [5] suggested that microbial activity accounts for about 75% of the AMD produced.

3. Diversity

It is essential to define the microbial population in order to analyze an ecological system. Currently, the primary method of determining which organisms comprise a microbial community involves determining the sequence of 16S ribosomal RNA genes (16S rRNA) from environmental samples. This approach provides information about species richness as well as the evolutionary relationships between lineages. Microbial species composition can then be correlated with environmental data to determine how communities are shaped by geochemical factors.

Any AMD system has many microbial niches. Variations in temperature, ionic strength, and pH yield communities characterized by different mixtures of organisms, but all habitats are restricted in membership to a few species (Table 1) [16,17]. This can likely be attributed to the limited number of energy-deriving reactions available in AMD environments. The number of substrates multiplied by the number of geochemically distinct habitats might be loosely related to the total number of AMD-adapted species. This would predict that the total number of AMD lineages would be small, given that AMD solution chemistry is strongly controlled by pyrite dissolution (see details below). Microbes from 11 putative divisions have been detected in AMD environments (Fig. 1).

The AMD system most intensively studied by culture-independent molecular methods is within the Richmond Mine at Iron Mountain in northern California. The methods used include 16S rDNA clone library analyses and cell imaging using multiple fluorescent probes designed to bind to the ribosomal RNA with varying levels of specificity. Pyrite-associated communities, subaqueous slime streamers, and subaerial biofilms in pH 0.5–1.4, 27–50°C solutions have been monitored and community responses to seasonal variations have been determined. Results confirm that a handful of prokaryotic taxa (often less than five groups distinct at the genera level) make up the communities in any specific microenvironment [17,28]. Low diversity has also been noted using cultivation-based approaches [29,30].

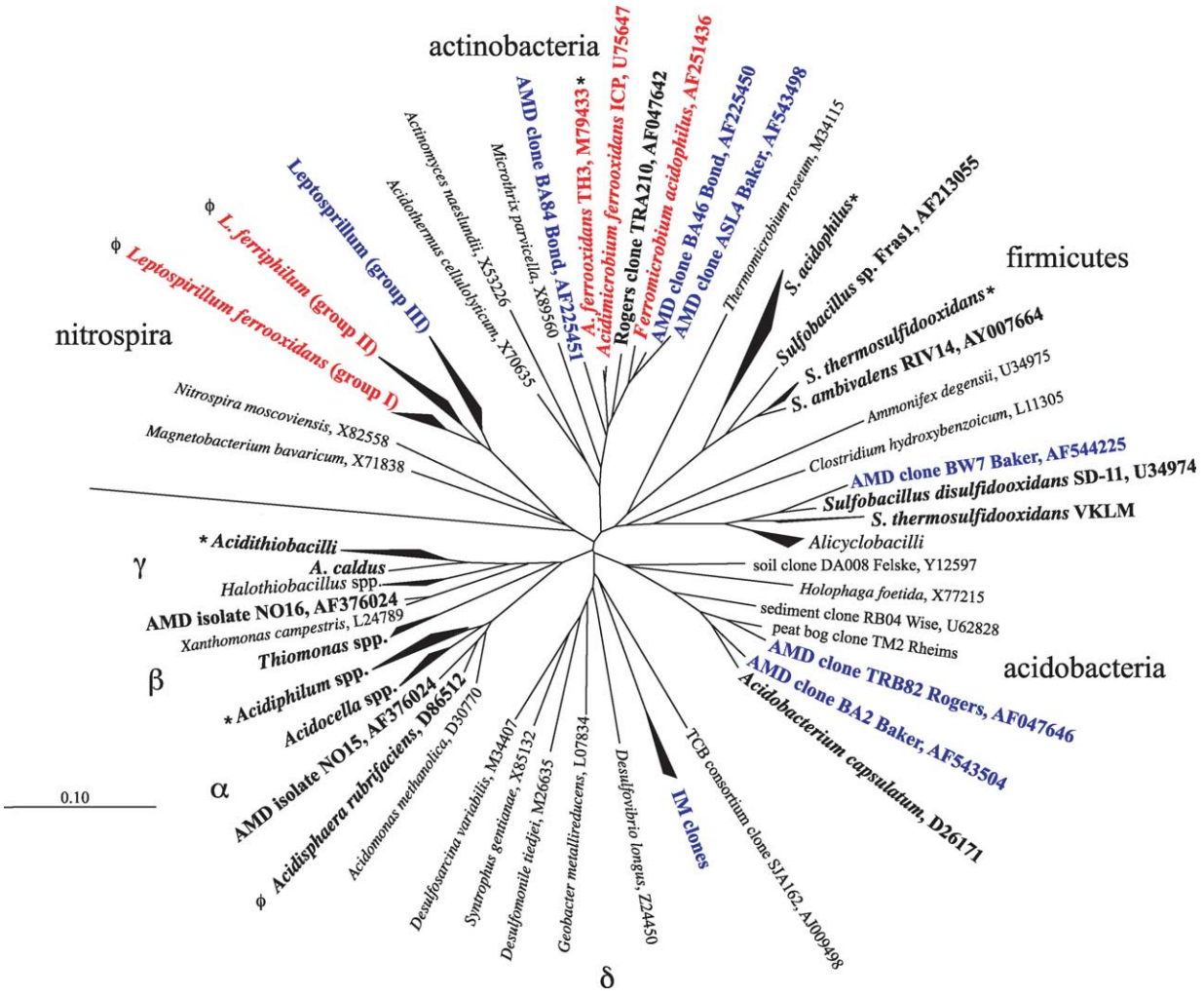

Fig. 1. Phylogeny of prokaryotic 16S rRNA genes from acid mine drainage and bioleaching sites (in bold) with reference lineages. * indicate lineages known to contain facultative anaerobes and ϕ appear to be obligate aerobes. The lineages in blue have no cultivated members to date. Lineages in red are not known to be able to utilize sulfur, only iron oxidizers. Putative divisions are shown near each of their branchings, the β-, γ-, δ-, α-proteobacteria are shown. Tree generated using neighbor joining method in ARB software package. Bar represents 0.1 changes per site or 10% difference in sequence, IM = Iron Mountain.

3.1. Bacteria

Among the bacterial lines of descent are divisions within the proteobacteria, nitrospira, firmicutes, and acidobacteria. The most extensively studied group (but possibly the least relevant under AMD-generating conditions, see below) are the γ-proteobacteria, specifically *Acidithiobacillus* spp. (formerly *T. ferrooxidans*, *Thiobacillus caldus* [31]) and *Thiobacillus* spp.

Two β-proteobacterial groups have been detected to date. Among this group are *Thiomonas* sp. (strains Ynys1 and Ynys3 [32]) and subsequently an isolate designated NO-16 from a Norwegian mine [30].

There are six species of heterotrophic α-proteobacteria of the genus *Acidiphilum* in pure culture. *Acidiphilum cryptum* (strain JF-5) was originally isolated from acidic (pH 3.0) iron-rich sediment from a lake associated with a coal mine in eastern Germany [33]. Another group of α-proteobacteria have been detected in clone libraries constructed from pH < 1.0 Iron Mountain AMD environments. However, these live in near neutral pH habitats inside acidophilic protists [34].

Recently, a novel group of δ-proteobacteria clones were recovered from Iron Mountain (Fig. 1) [16,17]. However, because this group has defined cultivation, their metabolic characteristics remain uncertain.

'*Leptospirillum ferrooxidans*'-group organisms are commonly detected in AMD and bioleaching systems. To date, *Leptospirillum* isolates and environmentally-derived clones cluster within one of three phylogenetically distinct groups (I, II, and III), as designated by Bond et al. [16]. Two species have been named: *L. ferrooxidans* [35] (group I) and *L. ferriphilum* (group II) [36]. To date, group III *Leptospirillum* has only been detected via clone library analysis of Iron Mountain microbial communities [16,17].

Three distinct groups of AMD organisms fall within the firmicutes division. These include an *Actinobacteria* group containing *Acidimicrobium ferrooxidans* and *Ferromi-*

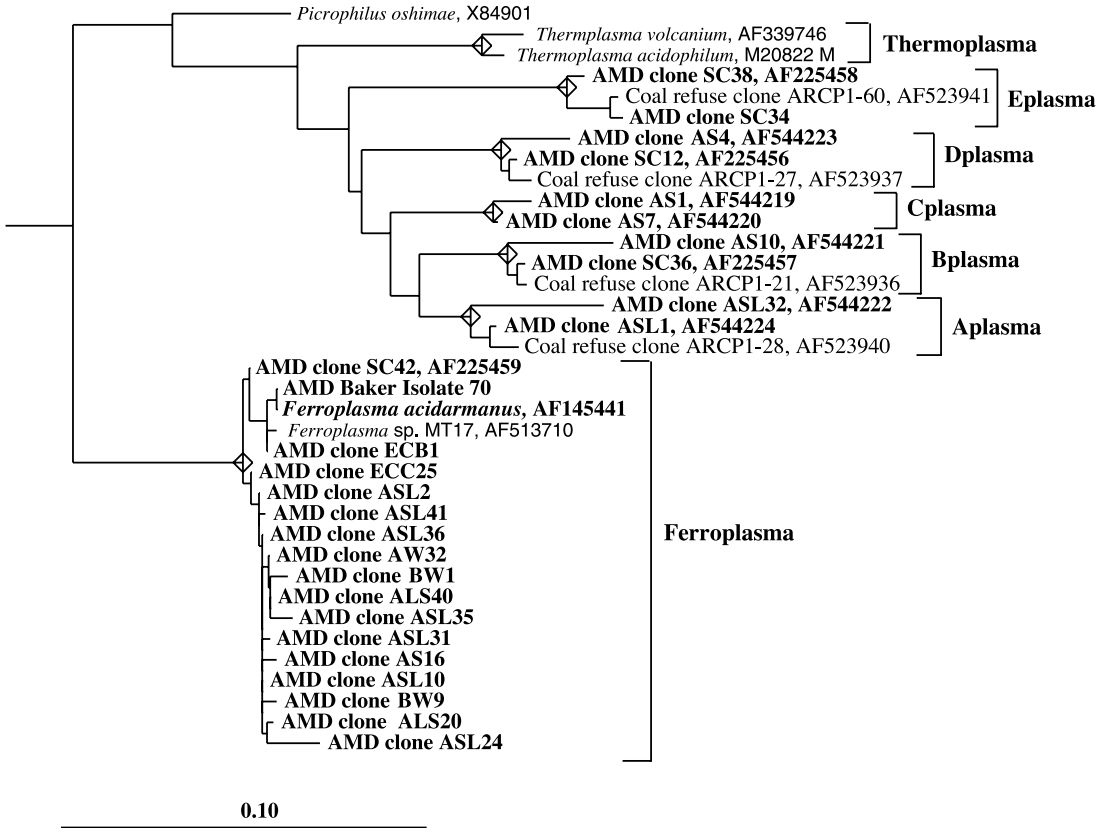

Fig. 2. Phylogeny of AMD-associated archaea within the *Thermoplasmatales*. Putatively proposed group names (the 'alphabet plasmas') shown on right and nodes for each corresponding group marked with diamonds. Clones from Iron Mountain site are shown in bold type. Tree generated via maximum likelihood method in ARB software package, outgroup not shown. Bar represents 0.1 changes per site, or 10%. Baker clones from [17].

crobium acidophilus (Fig. 1) and the polyphyletic (two lineages) low G+C Gram-positive *Sulfobacillus*. *Sulfobacillus thermosulfidooxidans* VKLM and *Sulfobacillus disulfidooxidans* SD-11 belong to the original *Sulfobacillus* spp. group, which is related to the *Alicyclobacillus* lineage (Fig. 1). Another large bacterial group named *Sulfobacillus*, represented by some *S. thermosulfidooxidans* and *Sulfobacillus acidophilus*, is distinct from the group represented by '*Sulfobacillus*' and separate from the *Alicyclobacillus* lineage. We suggest that in the future organisms within the *Alicyclobacillus* (including the type strain VKLM) be formally renamed to *Alicyclobacillus* based on phylogeny (Fig. 1).

The first isolate of the *Acidobacteria* division, *Acidobacterium capsulatum*, was recovered from an AMD site by Kishimoto et al. [37]. Closely related environmental clones have been reported from Iron Mountain (Fig. 1) [12,17].

3.2. Archaea

Archaeal lineages reported from AMD environments are restricted to the *Thermoplasmatales* and the *Sulfolobales*. A number of clones phylogenetically divergent from *Thermoplasma* spp. have been detected in clone libraries (Fig. 2) [16,17]. Further surveys and isolation efforts are needed to resolve the tentative groupings within this lineage, currently referred to as the 'alphabet plasmas' (Fig. 2).

One member of the *Sulfolobales*, *Metallosphaera prunae*, has been detected in AMD environments. Two other *Sulfolobales* genera, *Acidianus* and *Sulfolobus*, have only been found in geothermal acidic environments, thus are not considered further here.

3.3. Eucarya

There have been a few reports of eukaryotes in AMD environments [38–41]. Ciliates belonging to *Cinetochilium* genus and an amoeba related to *Vahlkampfia* sp. within the lineage *Heterolobosea*, and three flagellates (*Eutreptial* spp.) were isolated from an AMD site and shown to be able to graze on mineral-oxidizing acidophilic bacteria [42]. Lutz et al. [38] recovered clones related to *Vahlkampfia* sp. from Iron Mountain. At Iron Mountain fungal filaments provide structure to subaqueous biofilms termed 'slime streamers' (Figs. 3 and 4). Recently, Amaral Zettler et al. [43] reported a diverse community eukarya (including alga) present in the pH 2 Rio Tinto River, Spain. In this instance, eukaryotes comprised at least 60% of the total biomass. It remains to be seen if subsurface non-phototrophic protistan communities growing in more acidic environments (such as encountered at Iron Moun-

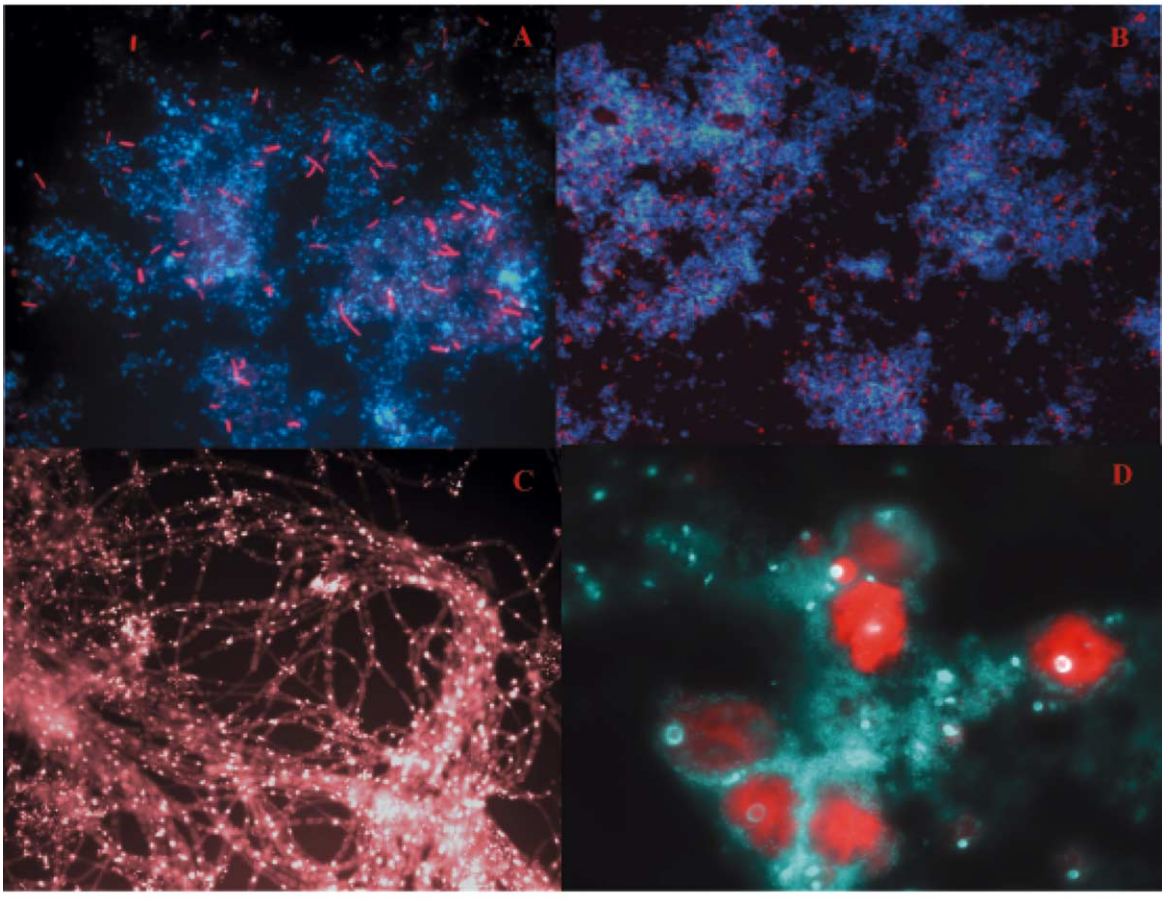

Fig. 3. Fluorescent in situ hybridization (FISH) biofilm samples from Iron Mountain Mine, CA. All images are the result of probes labeled with Cy3 colored with 4′,6-diamidino-2-phenylindole (DAPI) DNA stain for background cells. A is Sul228 specific for *Sulfobacillus* spp. [16], B Arc915 for archaea, C demonstrates archaea cells attached to fungal filaments, and D is eukarya-specific probe (Euk502). Note the nuclei of the protist are DAPI stained and many present in the sample are targeted by the Euk502 probe.

tain) are as diverse. Metabolically active protists are associated with Richmond Mine biofilms (pH 0.5–2.0; Fig. 3).

4. Evolution of acidophilic metal-tolerant organisms

It is widely believed that early Earth environments were relatively anoxic prior to the appearance of bacterial oxygenic photosynthesis. Given this, and the low abundance of sulfates in the early Earth record, it is likely that ARD environments were rare in the Archaean. Once the concentration of O_2 increased, new metabolic options, such as oxidation of iron and sulfur coupled to reduction of O_2, may have stimulated diversification within existing lineages, explaining the broad distribution of acidophiles over the tree of life (Figs. 1 and 2).

ARD and, more recently, AMD environments exist as isolated point sources that are exposed then removed via erosion within a few thousand to a few millions of years. Lateral gene transfer (LGT) is a mechanism by which some AMD survival genes could be introduced to create new acid- and metal-tolerant lineages. Recent analyses of LGT provide evidence for exchange of genes between extremely acidophilic trading partners [45]. However, there is no evidence to suggest that AMD organisms evolve from non-extremophiles when local acidic environments appear. In fact, organisms that colonize AMD environments possess habitat-specific genes (e.g., involved in metal resistance) [44] whose phylogeny matches the 16S rRNA gene phylogeny and modern inhabitants of AMD environments have very close relatives that are widely geographically distributed. Organisms are apparently introduced to newly created extremely acidophilic habitats from distant sites via as yet poorly understood dispersal mechanisms. The survival of these lineages over geologic timescales depends upon this.

5. Metabolic options and pathways

In regions of AMD systems exposed to sunlight, photosynthesis is an important source of energy and fixed carbon [46]. However, in subsurface ARD and AMD systems, inputs of externally-derived fixed carbon and

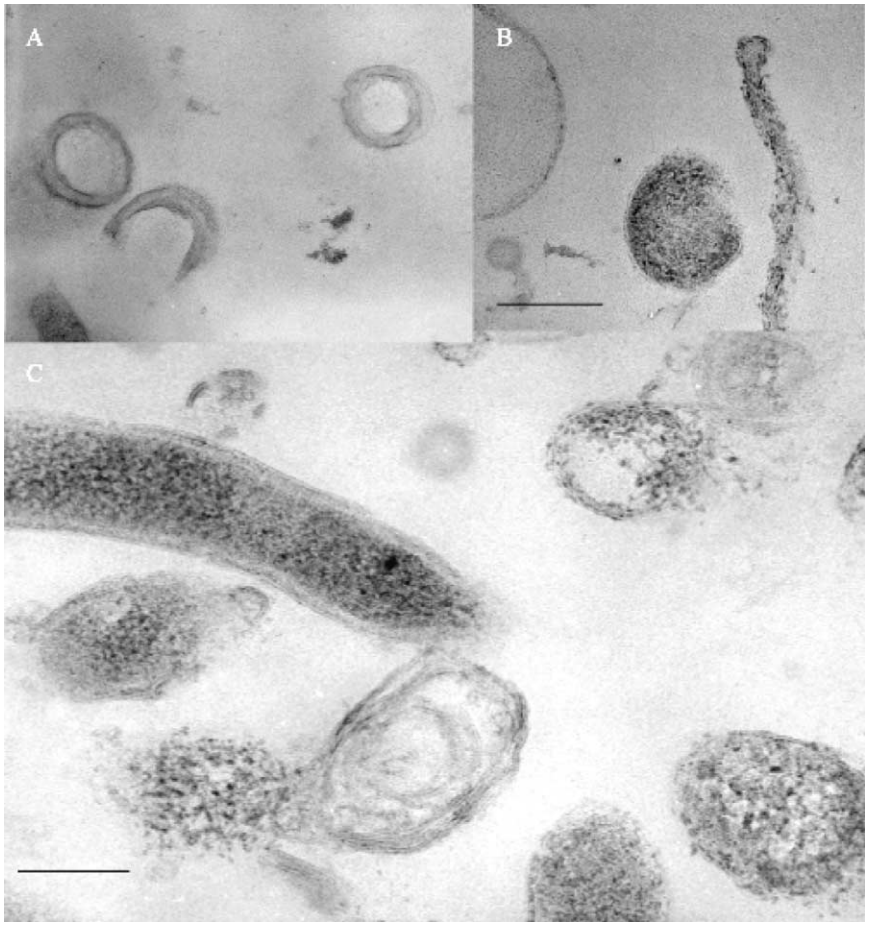

Fig. 4. High-resolution TEM images of Richmond Mine, CA environmental samples. A, C: A drift snottite. B: A slump subaerial slime. The size of the bar in B and C is 200 nm. Coccoid structures in A are less than 1 μm in diameter.

nitrogen are minimal. The primary metabolic groups detected in AMD-generating regions are lithoautotrophs that oxidize Fe^{2+} and S^{-} released by pyrite dissolution, organoheterotrophs that utilize carbon produced by lithoautotrophs, lithoheterotrophic Fe and S oxidizers, and anaerobes that couple oxidation of sulfur or organic carbon to Fe^{3+} reduction. A subset of these organisms must produce all fixed carbon and nitrogen required by the community.

In addition to S^{-}, intermediate sulfur and sulfoxy oxidation products can serve as electron donors. Electrons from these compounds are passed either to O_2 or Fe^{3+} (generated by oxidation of Fe^{2+} by O_2) because these are typically the only suitable electron acceptors available in significant concentrations.

Utilization of Fe^{3+}, and possibly sulfate, as electron acceptors is possible under microaerophilic and anoxic conditions. O_2 consumption by microbes and pyrite dissolution can induce some typically aerobic species such as *A. cryptum*, *Sulfobacillus*, and possibly *F. acidarmanus* and related 'alphabet plasmas' to grow anaerobically. To date, no obligate anaerobes have been cultivated from an AMD habitat. However, uncharacterized d-proteobacteria phylogenetically closely related to anaerobic species have been detected [16,17].

The acidophilic lineages documented to date have diverse evolutionary histories. If, as suggested above, most arose after the divergence of the three domains, the enzymes involved in the key transformations may be diverse. Our ability to test this hypothesis is limited due to the restricted amount of biochemical and genome sequence data currently available. Work of Blake and coworkers [47] suggests that the critical process of iron oxidation involves unrelated enzymes in most lineages examined to date.

5.1. Iron oxidation

The autotrophic, facultative anaerobic Fe oxidizer *Acidithiobacillus ferrooxidans* (formerly *T. ferrooxidans*) is readily cultivated and has been the focus of extensive genetic, genomic, and physiological studies [4,10,65]. Consequently, it is the only organism for which there are biochemical models of the electron transport chain for iron oxidation.

All characterized *L. ferrooxidans* and *L. ferriphilum* isolates are iron oxidizers [48,49,36,50,29]. Although no members of *Leptospirillum* group III (Fig. 1) have been isolated yet, it is likely that these are also iron oxidizers.

S. acidophilus, *S. thermosulfidooxidans*, and *Acidimicrobium ferrooxidans* strains TH3 and ICP are also iron oxidizers [51–53].

A few archaea associated with AMD have been shown to oxidize iron. *Ferroplasma acidiphilum* is an obligate autotroph able to oxidize ferrous iron as a sole energy source [54]. *F. acidarmanus* can oxidize ferrous iron and can grow heterotrophically [55]. A crenarchaeota, *Metallosphaera sedula*, can oxidize iron and sulfur at high temperatures (optimal growth at 74°C and pH 2.0). Thus, it is a candidate for high through-put industrial bioleaching [56].

5.2. Iron reduction

AMD solutions are iron rich because ferric and ferrous iron are very soluble at low pH (<2.5). In some cases, Fe^{3+} concentrations in AMD may exceed oxygen concentrations by several orders of magnitude [17]. Thus, Fe^{3+} may be widely used as an electron acceptor in microbial metabolism.

Johnson and McGinness [57] showed that the ability to reduce soluble Fe^{3+} is widespread among heterotrophic acidophiles. Moderately thermophilic species *S. thermosulfidooxidans* (strain TH1), *S. acidophilus* (strains ALV, THWX, and YTF1), and *Acidimicrobium ferrooxidans* (TH3) use ferric iron as the sole electron acceptor when grown heterotrophically under oxygen-limited conditions (with yeast extract and glycerol [57]). *Thiobacillus acidophilus* and a number of pure cultures of mesophilic heterotrophs are capable of mixotrophic Fe^{3+} reduction [58]. *A. cryptum* can couple Fe^{3+} reduction to oxidation of a variety of substrates including H_2 and glucose. For example, Kusel et al. [59] demonstrated that *A. cryptum* strain JF-5 couples oxidation of glucose to reduction of either O_2 or soluble Fe^{3+}.

Some microorganisms can utilize Fe^{3+} even if it is not in solution. *S. acidophilus* is capable of anaerobic dissolution of iron hydroxide, jarosite, and goethite [51]. Bridge and Johnson [57] demonstrated that *A. cryptum* strain SJH is capable of reductive dissolution of a wide range of ferric iron minerals (akageneite, goethite, jarosite, natrojarosite, magnetite, and amorphous ferric hydroxide). They reported that contact was necessary for dissolution to occur and that rates were faster at lower pH (2.0 vs. 2.8–3.0).

Anoxic conditions are not required for Fe^{3+} reduction by mixed cultures of acidophiles [58]. Recently, Kusel et al. [59] demonstrated that under oxic conditions JF-5 reduced soluble Fe^{3+} and schwertmannite (a Fe^{3+}-sulfate mineral) in sediment microcosms at pH 3.

In addition to coupling Fe^{3+} reduction to oxidation of organic carbon or hydrogen, some organisms can use Fe^{3+} to oxidize sulfur compounds. Bridge and Johnson [57] showed that *S. acidophilus* strains THWX, ALV, and TH1 use ferric iron to oxidize tetrathionate anaerobically.

5.3. Sulfur oxidation

Most members of AMD communities that oxidize sulfur also can fix CO_2. An exception is *A. cryptum*, which may utilize sulfur oxidation to some extent but is normally considered to be an organoheterotroph [60]. *T. acidophilus* can oxidize sulfur, iron, and organic carbon and is capable of autotrophic growth [61]. Autotrophic sulfur oxidation by pure cultures of *Acidithiobacillus ferrooxidans* [62], *Thiobacillus thiooxidans* [63], *Sulfobacillus* (more correctly *Alicyclobacillus*) *disulfidooxidans* SD-11 [64], *T. albertis* [65], *S. acidophilus* [66] has been demonstrated. *A. ferrooxidans* also can grow under anoxic conditions using ferric iron as the electron acceptor and S° the electron donor [62].

Several autotrophic sulfur oxidizers can also oxidize organic carbon and/or ferrous iron. For example, *S. disulfidooxidans* is capable of mixotrophic S oxidation [64], but does not oxidize iron. However, SB37, a recent *S. disulfidooxidans* isolate of close phylogenetic association, is able to oxidize Fe^{2+} (Baker, unpublished data). Fourteen strains of Gram-positive sulfide mineral-oxidizing bacteria, including *S. acidophilus* (one being ALV) and *S. thermosulfidooxidans* (including TH1), can grow autotrophically on ferrous iron and S°, mixotrophically in the presence of yeast extract, and heterotrophically on just yeast extract [66].

A variety of sulfur compounds with oxidation states intermediate between 2− (e.g., sulfide in sphalerite) and 6+ (sulfate) form during metal sulfide oxidation (e.g., polysulfide: S_n^{2-}, elemental sulfur: S°, thiosulfate: $S_2O_3^{2-}$). Some of these can be utilized by AMD microorganisms. Elemental sulfur and tetrathionate ($S_4O_6^{2-}$) are thought to be especially biologically important because they are relatively stable in low pH solutions. *T. acidophilus* grows autotrophically on trithionate ($S_3O_6^{2-}$), elemental sulfur, and tetrathionate [66,67]. As noted above, some strains of *S. acidophilus* couple autotrophic oxidation of tetrathionate to reduction of Fe^{3+} [51,66]. It is interesting to note that none of the strains described as *Thiobacilli* that were adapted to higher temperatures (~50°C) was capable of tetrathionate oxidation [66]. This is likely due to a decrease in bioavailability because of faster reaction rates.

5.4. Other metabolic requirements

Most subsurface AMD sites receive relatively minimal inputs of fixed carbon and nitrogen from external sources. The *Thiobacilli* (including *Acidithiobacillus ferrooxidans*) are probably the dominant group responsible for CO_2 fixation in lower temperature (<30°C), higher pH (>2) environments. In lower pH, higher temperature communities, autotrophic taxa include *Leptospirillum* spp., *Ferroplasma* spp., *Sulfobacillus* spp., *Ferromicrobium* spp., and *Acidimicrobium* spp. At high temperatures (~65–95°C) archaea such as *Metallosphaera* spp. may be the key sour-

ces of fixed carbon for the AMD biome. Although it is known that the *Acidithiobacillus ferrooxidans* pathway involves a bc_1 and an NADH-Q oxidoreductase complex functioning in reverse cycle for CO_2 fixation [68], little is known about the pathways used by other acidophilic autotrophs.

Fixation of N_2 in largely aerobic and microaerophilic AMD environments is potentially problematic due to inhibition of nitrogenase by O_2. *Acidithiobacillus ferrooxidans*, a moderate acidophile, may overcome this problem by using tetrathionate as an electron donor and ferric iron (rather than O_2) as an electron acceptor when fixing nitrogen [69]. The fixation of N_2 in very low pH environments is enigmatic, because no one has observed it directly. *L. ferriphilum* has been shown to possess *nifH* genes [70]. However, attempts to demonstrate active nitrogenase in pure cultures of *L. ferriphilum* were unsuccessful. Determination of which organisms are responsible for N_2 fixation in the full range of AMD environments is an important goal for further work.

6. Abundance, community structure, and physical/chemical regimes

Studies over the last decade indicate microbial communities in acidic environments are dominated by the *Thiobacilli*. It now appears this is an artifact of the culturing and sampling methods used, at least in some cases. Schrenk et al. [71] and Edwards et al. [72] used fluorescent in situ hybridization (FISH) probes to quantify the relative abundances of *Acidithiobacillus ferrooxidans* within the Richmond Mine at Iron Mountain (pH 0.3–0.7, 30–50°C). They reported that *A. ferrooxidans* were nearly undetectable. Subsequent studies demonstrated that *A. ferrooxidans* occur in relatively low numbers in pyrite-dominated bioreactor systems [73]. The failure of *A. ferrooxidans* to thrive in more extreme AMD environments is attributed to their mesophilic growth optima (26°C) and moderately acidophilic nature (pH 1.3–4.5). Druschel et al. [17] did not obtain any *A. ferrooxidans* clones in six libraries constructed from six pH < 1.0 AMD communities associated with the Richmond Mine. However, they demonstrated that an *A. ferrooxidans* strain was abundant in an oxidized, pH 1.4 pool.

Schrenk et al. [71] used FISH probes to show that *A. ferrooxidans* cells are abundant in environments with pH > 1.3 and temperatures < 30°C at the Richmond Mine. In this case, the higher pH, lower temperature environments occur outside the ore body, thus away from sites of active pyrite dissolution. However, pH 2–4, < 30°C environments are encountered in many AMD systems early in weathering, when rocks are less enriched in pyrite, or when the buffering capacity is higher.

Leptospirillum group isolates have been obtained from AMD environments characterized by a wide range of temperatures and pHs. They are as abundant, or more abundant, in bioleaching systems than *A. ferrooxidans* over much of the *A. ferrooxidans* growth range. Sand et al. [74] note that the growth rate of *A. ferrooxidans* only outcompetes that of *L. ferrooxidans* (group I) below 14°C. They also note that in their bioreactors high ratios of Fe^{3+} to Fe^{2+} appear to be less inhibitory to *Leptospirillum* than to *A. ferrooxidans*.

L. ferrooxidans DMZ2705 (group I) is reported to grow in the pH range of 1.3–4.0, with an optimal pH range of 1.6–2.0 (compared to 1.4–1.8 for *L. ferriphilum*) [36], and an upper temperature limit around 55°C [75,35]. Goebel and Stackerbrandt [29] isolated strain Lf30-A (group II, thus *L. ferriphilum*) from a continuous bioreactor (pH 1–2 and 35–40°C) and showed that the isolate was capable of growth at 28, 37, and 45°C. Coram and Rawlings [36] noted that *L. ferriphilum*-dominated commercial biooxidation tanks operated at 40°C, and that some strains were able to grow at 45°C (no *L. ferrooxidans* strains were capable of growth at 45°C). Golovacheva et al. [50] characterized an isolate from an iron-containing hydrothermal spring (pH 2.0, 45°C) and named it '*Leptospirillum thermoferrooxidans*' (also see [35]), based on greater G+C content and higher growth temperature (45–60°C) than previously described '*L. ferrooxidans*' strains. The status of isolates described as '*L. thermoferrooxidans*' is uncertain because these strains are not available for molecular characterization.

Oligonucleotide probe-based studies within the Richmond deposit indicate that *Leptospirillum* strains often dominate microbial communities growing at temperatures up to 50°C in solutions with pH values as low as 0.5 [16,17,71,72]. *Leptospirillum* (group III) were shown to comprise the majority of bacteria in subaerial slimes on the surface of a 'slump' of fine-grained pyrite ore [16] and, at times, to dominate subaqueous environments otherwise populated by *L. ferriphilum* [17]. *L. ferriphilum* and *Leptospirillum* group III primarily reside in lower pH microenvironments in the mine, while *L. ferrooxidans* occurs in higher pH environments (> 1) [17].

There are several other groups of bacteria whose abundance and distribution have been studied in AMD. *F. acidophilus* has a pH range of 1.3–4.8 and is adapted to temperatures from < 20 to 40°C [75]. Environmental clones closely related to *F. acidophilus* were recovered from the Richmond Mine AMD system (43°C) [16,17]. *Ferromicrobium* spp. were shown to be minor community members via probe-based studies [76]. *Acidimicrobium ferrooxidans* has been cultivated at temperatures between 34 and 57°C [53] and has been reported from a surprisingly diverse range of environments [77].

Sulfobacillus spp. have a broad range of physical growth regimes. Some isolates are capable of growth up to 65°C [78]. Recently, Yahya et al. [79] described *Sulfobacillus*-like strains (L-15 and Riv-14) isolated from geothermal sites. The strains are effective pyrite oxidizers at pH < 1, sug-

gesting that thermophilic species may be encountered in AMD systems. The FISH image in Fig. 3 shows *Sulfobacilli* rods within a Richmond Mine biofilm growing at pH ~0.7. *Sulfobacillus* clones have been recovered from pH 0.7–0.9, 35–43°C [16] and have been shown to comprise up to ~6–8% of microbial communities (in pH 0.8–1.4, 27–32°C environments [17]).

Six members of the genus *Acidiphilum* are adapted to temperatures ranging from 17 to 45°C and pH values from 1.5 to 6.0 [75]. Strain SJH has a pH optimum of ~3.0 [57]. One isolate was retrieved from a continuous bioreactor [29] and a clone was identified from the pH < 1.0 solutions at Richmond Mine [12], though later studies at this site have not detected this group [16,17]. Peccia et al. [80] used a 16S rRNA probe to determine the abundance of *Acidiphilum* in sediments from an active AMD site in Colorado and in laboratory reactors. They found that the *Acidiphilum* outnumbered *Acidithiobacillus ferrooxidans* in their mixed culture bioreactors and that *Acidiphilum* and *A. ferrooxidans* populations were approximately equal in the sediment samples. In general, *Acidiphilum* sp. tends to occupy lower temperature, higher pH microenvironments. Under anoxic conditions they may contribute to iron cycling by redissolving ferric iron-based minerals precipitated when the pH is increased by mixing with groundwater and in streams.

The metabolic role of the δ-proteobacteria has not been elucidated because no members have been isolated. As δ-proteobacteria are often anaerobic sulfate or metal reducers, Bond et al. [16] suggested that anaerobic microenvironments may exist within the slime samples from which the clones were recovered. Attempts to amplify the highly conserved dissimilatory sulfite reductase (Dsr) gene were unsuccessful, and therefore it has not been possible to confirm these bacteria as sulfate reducers (Baker and Banfield, unpublished data).

Acidobacteria spp. populate relatively moderate AMD environments (20–37°C and pH 3.0–6.0). Recent analyses suggest that this group is limited to the higher pH environments (~pH 1.4) at the Richmond Mine site [17].

Application of culture-independent approaches led to recognition that archaea are important and abundant in AMD environments. Iron-oxidizing archaea of the genus *Ferroplasma* have been isolated from a bioleaching plant [54] and the Richmond Mine [55]. *Ferroplasma* lack a cell wall and are bounded only by a cytoplasmic membrane. However, like other *Thermoplasma* and *Sulfolobus* spp., cells may possess an S-layer (Fig. 4) [81,82,76]. *F. acidarmanus*, isolated from the Richmond Mine, was shown to be the dominant organism in some habitats (see Fig. 3), comprising as much as 85% of the population in pH ~0.5, ~40°C, high ionic strength habitats [55]. *F. acidarmanus* has a lower pH growth range than *F. acidiphilum* (0–2.5 vs. 1.3–2.2).

Clones within the *Thermoplasmales* (e.g., *Thermoplasma acidiphilum* and *Thermoplasma volcanium*), but distinct from *Ferroplasma* spp., have been detected in clone libraries created from samples collected from many very acidic geothermal hot spring environments [83]. *T. acidophilum* and *T. volcanium* have moderately thermophilic (45–67°C) growth ranges [81,82] and are adapted to pH ranges typical of AMD. *T. acidophilum* has been isolated from a coal refuse pile [81].

The metabolic role of the 'alphabet plasmas' within the order '*Thermoplasmatales*' (Fig. 2) [16,17] is yet to be determined. Their environmental distribution indicates adaptation to high biomass, metal-rich, pH 0.5–1.4, 30–50°C habitats close to the air–biofilm interface [17]. Within the Richmond Mine system, all archaea are low abundance or absent from in pH 1.3–2.4 habitats [17,71]. *Metallosphaera* spp. (*Sulfolobales* order) can impact pyrite dissolution via catalysis of ferrous iron oxidation [84] and has been isolated from an acidic uranium mine [85] and a bioleaching reactor [56].

7. Synergistic interactions involving iron, sulfur, and carbon oxidizers

As indicated by the above discussion, community membership varies with pH and temperature, mineralogy, as well as with metal concentrations. However, in most environments (particularly in the subsurface) some species, or combination of species, within communities probably carry out iron oxidation, sulfur oxidation, organic carbon oxidation, carbon fixation, nitrogen fixation, extracellular polymeric slime production, as well as iron and sulfur reduction. It is also apparent from the above review that microbial communities in AMD systems tend to contain few distinct taxa (though these taxa may be remarkably phylogenetically diverse). Fig. 5 illustrates a simplified AMD system and provides examples of organisms that may control flow of iron, sulfur, nitrogen, carbon, and energy in different temperature regimes.

Interactions between members of microbial consortia are probably critical in optimization of AMD microbial community activity. For example, an important symbiosis exists between heterotrophic and certain autotrophic species: autotrophs may depend on coexisting heterotrophs to remove organic compounds that are toxic to them. Heterotrophic acidophiles are able to utilize organic materials produced by acidophilic autotrophs [86,56]. The culture filtrate from the autotroph *Acidithiobacillus ferrooxidans* contained sufficient organic matter to support heterotrophic growth of *S. thermosulfidooxidans* (strain TH1) [87]. Clark and Norris [53] conducted mixed culture experiments with *Acidimicrobium ferrooxidans* and both *S. acidophilus* and *S. thermosulfidooxidans*. They found iron oxidation by mixed cultures of *Sulfobacillus* spp. and *Acidithiobacillus ferrooxidans* was more extensive than in pure cultures containing any isolate. They attributed this greater leaching in mixed cultures to mixotrophic growth of the

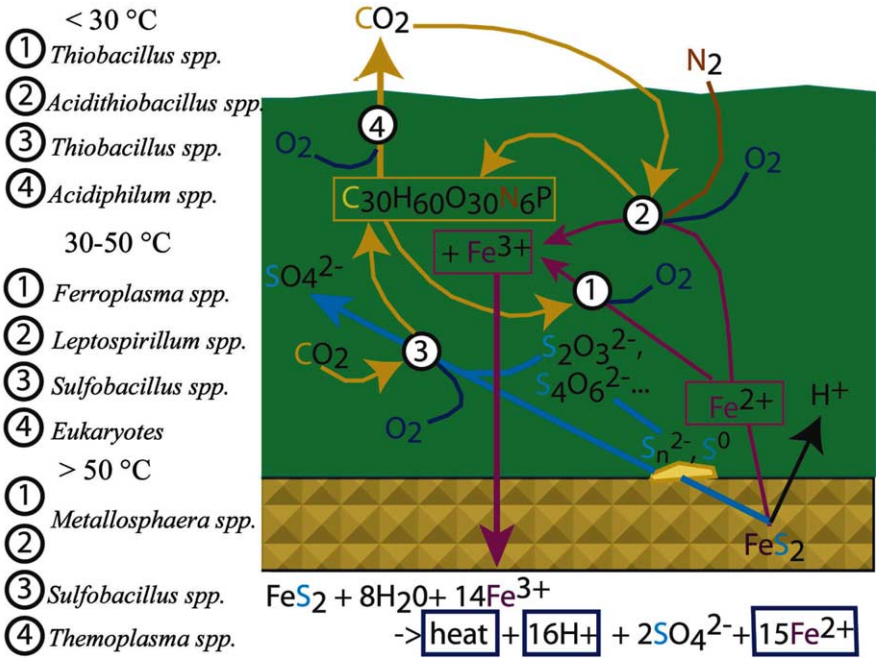

Fig. 5. Potential iron, sulfur, and carbon cycling based on known metabolic capabilities (1, 2, 3, and 4) associated with AMD members. Crystalline pyrite (Fe_2S) is in yellow at the bottom and green is representing AMD solution. Elemental sulfur is shown at the pyrite–water interface as a possible inhibitor of surface dissolution. The overall oxidation of pyrite is shown at the bottom, with Fe^{3+} indicated as the primary oxidant. Intermediate sulfur compounds are indicated as follows: $S_2O_3^{2-}$ being thiosulfate and $S_4O_6^{2-}$ is tetrathionate. $C_{30}H_{60}O_{30}N_6P$ indicates organic carbon compounds.

Sulfobacillus removing organic byproducts of *A. ferrooxidans*. This may explain the close correlation in populations of *A. ferrooxidans*/*T. thiooxidans* and *Acidiphilum* in environments studied by Peccia et al. [80].

Raman spectroscopy has been used to show that abundant S° forms on the surfaces of arsenopyrite [88] and galena [89] and pyrite [90] during oxidative dissolution. It has been hypothesized that microbial removal of elemental sulfur (S°) and other compounds is critical for continued access of the Fe^{3+} oxidant to pyrite surface sulfur-bearing groups. Dopson and Lindstrom [91] examined the effect of defined mixed cultures of the sulfur oxidizers *A. caldus* and *S. thermosulfidooxidans* (capable of Fe and S oxidation) on arsenopyrite dissolution. By following concentrations of released iron, tetrathionate, and sulfur in the cultures they found that *A. caldus* grows on surface sulfur compounds, resulting in an increase in the arsenopyrite dissolution rate. They also suggested that *A. caldus* produces surface-active agents to mobilize the extremely hydrophobic S°. Subsequently, Hu et al. [90] determined that elemental sulfur accumulations on galena significantly decrease dissolution rates. Because removal of S° from mineral surfaces is insignificant in abiotic controls, microbial oxidation of sulfur is likely to be a key determinant of acid generation rates (because all protons are released in conversion of sulfur to sulfate).

8. Outlook

Perhaps the most exciting aspect of AMD communities is that they provide the opportunity to study largely self-contained biomes (independent of sunlight and most other life). Culture-independent methods have provided a detailed understanding of the full diversity and phylogeny of organisms populating AMD systems. Progress in understanding the biochemistry of acidophiles has been accelerated due to availability of genome sequences for a few isolates (e.g., *F. acidarmanus* and *Acidithiobacillus ferrooxidans*). However, we know essentially nothing about the subset of organisms that have not been isolated.

An important approach for analysis of uncultivated organisms is the construction and sequencing of large insert genomic libraries from environmental DNA [92]. Cultivation-independent sampling of genomes of acidophiles will reveal enzymes potentially involved in the major metabolic pathways and provide insights into pH homeostasis and metal resistance mechanisms. The low species richness of AMD communities may permit us to largely reconstruct genomes of populations from whole community libraries. Related gene expression studies should enable development of AMD ecosystem models that resolve the contributions of individual species at the molecular (pathway) level, describe and quantify flows of materials and energy, and identify symbioses and competitive strategies. Thus, through integration of geochemical and biological information, a comprehensive model for AMD production may be feasible. Studies of AMD systems could have broader significance. The remarkable simplicity of these habitats may permit a more fundamental understanding of how microbial communities work than is possible through study of more complex ecosystems.

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