Acidophile Microbiology in Space and Time

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Abstract

The study of extreme acidophiles, broadly defined as microorganisms that grow optimally at pH values below 3, was initiated by the discovery by Waksman and Joffe in the early 1900s of a bacterium that was able to live in the dilute sulfuric acid it generated by oxidizing elemental sulfur. The number of known acidophiles remained relatively small until the second half of the 20th century, but since then has greatly expanded to include representatives of living organisms from within all three domains of life on earth, and notably within many of the major divisions and phyla of Bacteria and Archaea. Environments that are naturally acidic are found throughout the world, and others that are man-made (principally from mining metals and coal) are also widely distributed. These continue to be sites for isolating new species, (and sometimes new genera) which thrive in acidic liquor solutions that contain concentrations of metals and metalloids that are lethal to most life forms. The development and application of molecular techniques and, more recently, next generation sequencing technologies has, as with other areas of biology, revolutionized the study of acidophile microbiology. Not only have these studies provided greater understanding of the diversity of organisms present in extreme acidic environments and aided in the discovery of largely overlooked taxa (such as the ultra-small uncultivated archaea), but have also helped uncover some of the unique adaptations of life forms that live in extremely acidic environments. Thanks to the relatively low biological complexity of these ecosystems, systems-level spatio-temporal studies of model communities have been achieved, laying the foundations for 'multi-omic' exploration of other ecosystems.

This article introduces the subject of acidophile microbiology, tracing its origins to the current *status quo*, and provides the reader with general information which provides a backdrop to the more specific topics described in Quatrini and Johnson (2016).

Acidophilic microorganisms defined

Acidophiles are broadly defined as life forms that grow preferentially in natural or man-made environments where the pH is well below 7. Together with other categories of 'extremophiles', they have greatly expanded our knowledge of the diversity of life, our understanding on how microorganisms can adapt to seemingly hostile situations, and provided scenarios for the possibility that life forms similar to those that colonize inhospitable niches on Earth, our own planet, may be found on planets and satellites within and outside of our solar system.

While there is no formal definition of what constitutes an acidophile, one proposed by Johnson (2007) has gained general acceptance. That delineated 'extreme acidophiles' as those that have pH optima at 3 or below, and 'moderate acidophiles' as those with pH optima of 3–5, though some species are capable of growing at pH < 3. Acid-tolerant species have pH optima above 5, though they can grow at pH values lower than this. The most acidophilic of all known life forms (the euryarchaeote *Picrophilus*, which currently contains two validated species) has a pH optimum of 0.7 and can grow at pH 0. Given that pH is a logarithmic scale, this means that *Picrophilus* spp. can grow in liquors where the hydronium ion (H_3O^+) concentration is 50–100 times greater than that tolerated even by many

extreme acidophiles. The term 'hyper-acidophiles' (analogous to 'hyper-thermophiles' which was introduced when prokaryotes that had temperature optima > 80°C were discovered, some of which grew well above 100°C) is a more appropriate way to describe species that grow optimally at pH < 1, though such prokaryotes appear at present to be very rare.

The same general rule applies with pH as other environmental parameters (temperature, salinity, pressure, etc.) which is that, as the stress factor (in this case, acidity) increases in intensity, so the number of species that can tolerate or grow in such environments decreases. Extreme acidophiles are exclusively microbial. Macroscopic life forms are sometimes found in low-pH environments, such as the angiosperms *Erica* spp. that grow in acidic soils and *Juncus bulbosus* which can colonize the fringes of acidic pit lakes. However, cell membranes of higher plants are susceptible to damage at pH < 4, and the roots of wetland plants such as *Juncus* spp. are generally found in the sediments of acidic ponds and lakes where the pH is significantly greater than that of the water body itself. While it is the case that most extremely acidophilic eukaryotes are unicellular, some multicellular life forms, including rotifers and some crustaceans, can thrive in some low-pH environments. Acidophiles are also widely distributed in the 'tree of life', occurring in all three domains of life, Bacteria, *Archaea* and *Eukarya*, and within multiple phyla in the same domain (Fig. 1.1). See also Quatrini and Johnson (2016).

The number of species of extremely acidophilic microorganisms that have been isolated and characterized has increased almost exponentially from just two in the mid-20th century to > 50 in the early 21st century. It is now clear that, as a group, acidophiles are highly heterogeneous from physiological and metabolic perspectives, and can utilize different sources of energy (solar, organic and inorganic chemicals), electron acceptors (oxygen, ferric iron and sulfur) and carbon sources (organic, inorganic, or both) although some metabolic functions seem to be rare (e.g. fermentation) or absent (e.g. dissimilatory nitrate reduction), and chemolithotrophy unusually commonplace amongst acidophilic microorganisms (Johnson and Aguilera, 2015).

The evolution and now routine application of molecular techniques for studying biological systems has resulted in many uncultivated species of microorganisms being detected in extremely low pH, as in other environments. Improved and probably novel cultivation-based approaches and techniques are currently required to catch up and keep pace with what cultivation-free studies are revealing about the biodiversity of extreme environments, including those that are extremely acidic.

Nature, genesis, and global distribution of extremely acidic environments

Extremely acid environments can vary greatly in scale, nature and origin. Gastric acid produced in the lining of the human stomach has a pH of ~1.5 to 3.5 due to the production of hydrochloric acid. In contrast, in both man-made and natural extremely acidic environments, sulfuric acid is usually the mineral acid responsible for the low-pH conditions. In situations where low pH is due to the presence of organic acids, such as humic acids/colloids (as in acid peats) these tend to be moderate (>3) rather than extreme. Notable exceptions are the pH of lemon and lime fruits, which have pH between 2 and 3 due to their elevated concentrations of undissociated citric acid (a tricarboxylic acid with pK_a values of 3.1, 4.8 and 6.4).

Many biological processes, for example fermentation, generate acidity due to the production of small molecular weight aliphatic acids (such as lactic and propionic acids) as metabolic waste products. Acetogenic bacteria have long been used to generate malt and wine vinegars, which have pH values of ~2.5. However, biological processes that generate mineral (inorganic) acids have, at least in theory, greater potential for generating extremely low-pH environments due to the generally lower pK_a values of the acids involved. Nitrification is a two-stage oxidative process that generates nitrous (pK_a 3.4) and nitric (pK_a −1.6) acids. However, the vast majority of prokaryotes that catalyse these dissimilatory reactions (mostly chemolithotrophs) are acid sensitive and only thrive in environments that are self-buffered, such as marine waters and non-acidic soils. In contrast, the dissimilatory oxidation of elemental and reduced forms of sulfur is carried out by prokaryotes that have widely different pH ranges and optima for growth, and includes a number of species of extremely acidophilic bacteria and archaea.

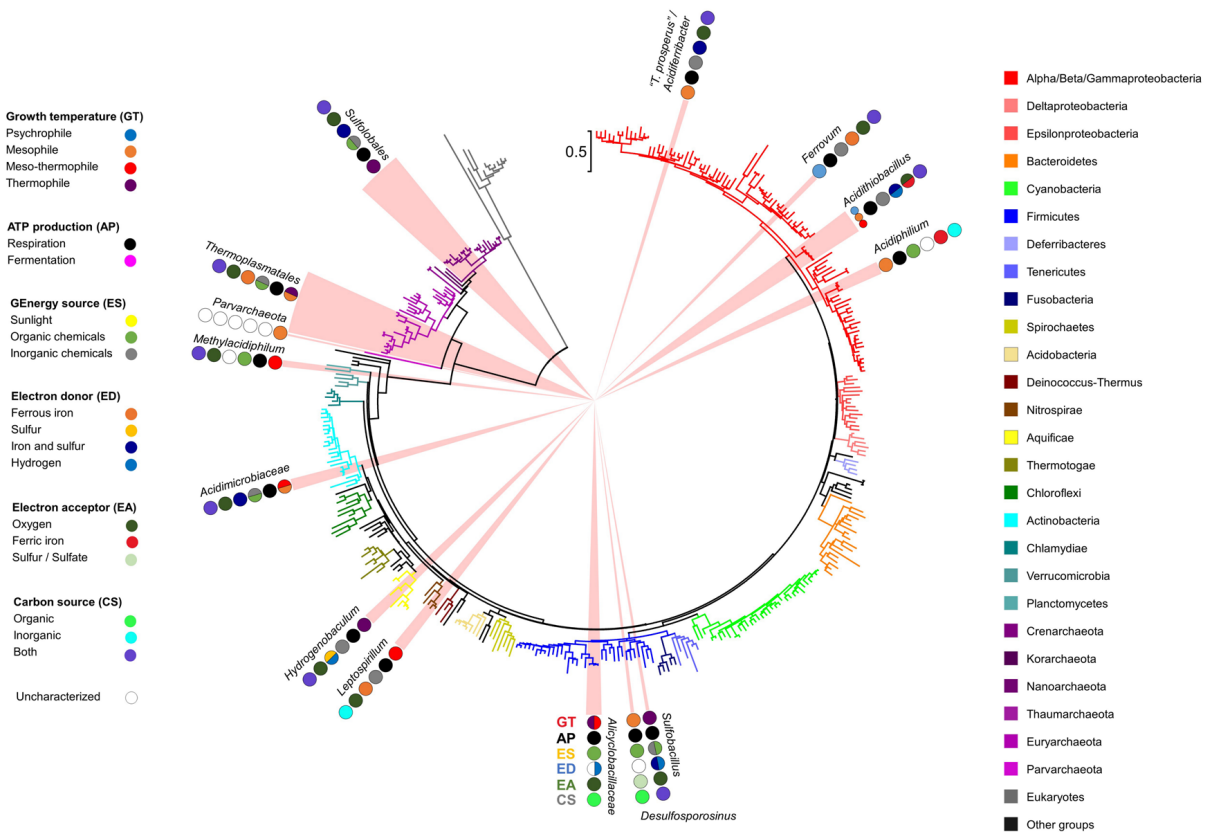

Figure 1.1 Phylogenetic tree of bacterial and archaeal phyla based on the 16S small subunit rRNA gene sequence, constructed as described in Quatrini and Johnson (2016). taxonomic groups are defined by the clade colour index at the right side of the figure and the presence of acidophiles in each clade is indicated in pink. Metabolic properties of each group of acidophiles are defined by the coloured circles outside the tree (coded as in the left side index). The figure was prepared by Juan Pablo Cárdenas (Fundación Ciencia and Vida and Universidad Andres Bello, Santiago, Chile).

Sulfur is one of the more abundant elements in the lithosphere, occurring at ~0.05% on a weight basis. It is also a complex element, and can exist in up to nine different oxidation states ranging from −2 (e.g. in hydrogen sulfide; H_2S) to +6 (e.g. in the sulfate anion; SO_4^{2-}; Steudel, 2000). Catenation (the ability of sulfur atoms to bond to each other to form chains of practically unlimited lengths) is another characteristic of this element. In polythionates (sulfur oxyanions) the sulfur atoms are present in different oxidation states, e.g. in tetrathionate, where the two central sulfur atoms (which are covalently bonded to each other) have oxidation states of zero, while the two terminal sulfur atoms (which are covalently bonded to oxygen as well as to the central sulfur atoms) have oxidation states of +5. Redox transformations of sulfur are common in nature, though mostly this is directed at assimilating the element (e.g. into amino acids, or sulfate esters) and dissimilatory processes (sulfur species being used directly or indirectly as electron donors or acceptors) are confined to some species of prokaryotic microorganisms. The terminal products of sulfur oxidation are the sulfate or bisulfate (HSO_4^-) anions. In situations where dissimilatory sulfate production exceeds the ability of the local environment to counter-balance the net acidity generated by this reaction, pH will decline, and species of prokaryotic microorganisms mediating the reaction will also change from neutrophiles to moderate extremophiles, and ultimately to extreme acidophiles. Sulfuric acid has two dissociation constants, shown in Equation 1.1:

$$H_2SO_4 \underset{pK_a=-2.8}{\longleftrightarrow} HSO_4^- + H^+ \underset{pK_a=+1.92}{\longleftrightarrow} SO_4^{2-} + 2H^+ \quad (1.1)$$

While in most acidic environments associated with sulfur oxidation sulfate is the dominant form, in others (such as water bodies found within the Richmond Mine at Iron Mountain, California) bisulfate is more abundant. In acidic sulfate-rich waters, the sulfate/bisulfate couple can therefore act as an important pH buffer.

Three factors are important in determining whether an extremely acidic environment may be formed as a consequence of dissimilatory sulfur oxidation: (i) reduced forms of sulfur (including elemental sulfur) need to be present in relatively large abundance; (ii) conditions need to be conducive to the (microbial) oxidation of the reduced sulfur, which usually (though not necessarily) requires the presence of oxygen, and (iii) the acidity generated needs to exceed the neutralizing capacity of the niche in which it is occurring. Geothermal sites often fulfil all three criteria. Crystals of prismatic sulfur can form in the vents of volcanoes from the condensation of sulfur dioxide and hydrogen sulfide gases (Equation 1.2):

$$SO_2 + 2H_2S \rightarrow 3S^0 + 2H_2O \quad (1.2)$$

Solfatara (which derives from the Italian for 'sulfur place') are volcanic and geothermal areas where sulfur gases occur in association with water vapour. Temperatures in solfatara water bodies can approach water boiling point (85° to > 100°C, depending on altitude and the presence of solutes) but tend to cool rapidly as waters flow out from the immediate heat source, giving the opportunity for colonization of the sulfur-rich waters by microorganisms with widely differing temperature ranges. Oxidation of sulfur by archaea and bacteria then generates sulfuric acid (Equation 1.3):

$$2S^0 + 3O_2 + 2H_2O \rightarrow 3H^+ + HSO_4^- + SO_4^{2-} \quad (1.3)$$

Acidic hot springs are common features within solfatara fields, and thixotropic acidic mud pools form from the destruction of silicates and other minerals by the combined action of extreme acidity and high temperatures. Solfatara can be found around the margins of active and some dormant volcanoes and where the Earth's crust is relatively thin. Examples include Yellowstone National Park (USA), Whakarewarewa (New Zealand), Krisuvik (Iceland), the Kamchatka peninsula (Russia), Sao Michel (Azores), Volcano, Naples and Ischia (all Italy), Djibouti (Africa), the Purico complex (Chile) and some Caribbean islands, such as Montserrat and St. Lucia. Submarine volcanic areas and hydrothermal systems, such as the Mid-Atlantic ridge, the East Pacific Rise, the Guaymas Basin, and active seamounts (e.g. around Tahiti), also discharge large amounts of reduced sulfur from magmatic sources. In contrast to land-based solfatara fields, the acidity generated by sulfur oxidation from submarine discharges is neutralized by the buffering capacity of sea water, though localized

areas of (extreme) acidity which act as niches for acidophilic microorganisms can exist close to the venting ducts.

The other major source of reduced sulfur that can act as the point of origin of extremely acidic environments are where sulfide minerals are concentrated, e.g. in metal ore bodies. These may occur as igneous (as in the Iberian Pyrite Belt), sedimentary (e.g. the extensive kupferschiefer deposits in central Europe) or metamorphic (e.g. polymetallic black schist ores in Finland) deposits. Gossans, intensively weathered, oxidized rock strata that are often highly coloured (orange/brown) due to surface layers of ferric iron-rich deposits, are geological features found in many parts of the world. These overlie unweathered rock containing pyrite and other sulfide minerals, and are the primary source of the iron seen at the land surface. Water in the vicinity of, and draining out of, the gossans can be acidic due to the microbially catalysed oxidative dissolution of the sulfidic minerals. Gossans and associated acid rock drainage waters have been reported in northern Greenland (Langdahl and Ingvorsen, 1997) and in Antarctica (Dold *et al.*, 2013). Acid production in such situations is often limited by the physical nature of the unweathered rock, restricting its exposure to both oxygen and water, both of which are necessary for the potentially acid-generating minerals to be degraded. However, when unweathered sulfidic rock strata are mined as ore bodies, the processes involved (excavation, comminution and disposal of reactive mineral wastes) provide conditions that are highly conducive for the activities of lithotrophic ('rock eating') acid-generating acidophiles, and consequently the mining of many metals, and also of coals, which contain variable quantities of iron sulfide minerals and elemental sulfur.

Sulfidic ores are the main sources of many commercially valuable base and semi-precious transition metals, such as copper, zinc, cobalt, nickel and silver, either as single metal sulfides (e.g. millerite; NiS) or in association with other metals (e.g. pentlandite; $(Fe,Ni)_9S_8$). Some metalloids such as arsenic, also form sulfide minerals, such as arsenopyrite (FeAsS) and realgar (As_4S_4). Native fine-grain refractory gold is also often intimately associated with sulfide minerals (arsenopyrite and pyrite, FeS_2), as is the uranium(IV) mineral uraninite (UO_2). Pyrite is the most ubiquitous of all sulfide minerals and is often present in ore bodies in greater quantities than the mineral(s) bearing the target metal(s). Since pyrite (and another commonly encountered iron sulfide, pyrrhotite; $Fe_{(1-x)}S$, where $x = 0–2$) has relatively little commercial value, though it has been, and continues to be, a source of sulfur and sulfuric acid, it often ends up in mineral wastes, such as fine-grain tailings, that are produced during ore processing (Dold, 2010). The oxidative dissolution of pyrite by acidophilic bacteria and its role in generating acidic, metal rich pollution, such as acid mine drainage waters, is among the most intensively studied processes in acidophile microbiology (e.g. Vera *et al.*, 2013). In brief, this involves a primary attack on the mineral by ferric iron, a reaction (Equation 1.4) that is both abiotic and independent of oxygen:

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+ \quad (1.4)$$

Ferric iron has to be regenerated for this reaction to continue. This is a very slow abiotic reaction at pH < 3, though it can be catalysed by numerous species of iron-oxidizing acidophiles in a proton- and oxygen-consuming reaction (Equation 1.5):

$$Fe^{2+} + H^+ + 0.25O_2 \rightarrow Fe^{3+} + 0.5H_2O \quad (1.5)$$

Thiosulfate, generated in Equation 1.4, is unstable in acidic ferric iron-rich liquors and is rapidly transformed to elemental sulfur and various polythionates. These in turn are oxidized by sulfur-oxidizing acidophiles, producing sulfuric acid (e.g. as in Equation 1.3). The reaction summarizing the oxidative dissolution of pyrite at low pH in which all of the products are in their most oxidized forms is therefore:

$$FeS_2 + 3.75O_2 + 0.5H_2O \rightarrow Fe^{3+} + 2SO_4^{2-} + H^+ \quad (1.6)$$

Equation 1.6 indicates that the oxidative dissolution of pyrite is indeed a net acid-generating reaction. However, this is more pronounced when secondary reactions involving the hydrolysis of ferric iron are taken into consideration, such as that shown in Equation 1.7 which depicts the formation of schwertmannite, a common ferric iron mineral that forms in moderately acidic (pH 2 to 4) mine waters:

$$8Fe^{3+} + 14H_2O + SO_4^{2-} \rightarrow Fe_8O_8(SO_4)(OH)_6 + 22H^+ \quad (1.7)$$

Combining Equations 1.6 and 1.7 it can be seen that the microbially mediated transformation of the ferrous iron mineral pyrite to the ferric iron mineral schwertmannite generates 3.75 moles of proton acidity per mole of pyrite oxidized. The hydrolysis and dissolution of ferric iron acts as a powerful pH buffer in oxidized, iron-rich waters, as exemplified in the Rio Tinto in south-west Spain, which maintains a pH of between ~ 2.2 and 2.8 throughout its 100 km length.

Water bodies impacted by mining activities are not only often moderately to extremely acidic, but also typically contain far more elevated concentrations of dissolved transition metals, aluminium and metalloids than non-impacted waters. The former include subterranean water bodies housed within abandoned deep mines (Johnson, 2012), pit lakes that form in opencast voids (e.g. Falagan *et al.*, 2013) and acidic streams than drain mines and mine spoils (e.g. Blowes *et al.*, 2003). Because metal mining was one of the earliest technologies developed by *Homo sapiens* (albeit on small scales in the Iron and Bronze Ages) and, along with coal mining, has been and in many countries remains an important industry, acidic sites caused by this human activity have widespread global distribution.

A brief history of acidophile microbiology

Acidophile microbiology (Fig. 1.2) began with the discovery, by Waksman and Joffe nearly a century ago, of a bacterium that grew on elemental sulfur and which was able to thrive in the dilute sulfuric acid (pH as low as 0.6) that it generated (Waksman and Joffe, 1922). They named this bacterium *Thiobacillus* (*T.*) *thiooxidans*, though it was later reclassified as *Acidithiobacillus* (*At.*) *thiooxidans* by Kelly and Wood (2000) along with other acidophilic species of *Thiobacillus*. Waksman and Joffe (1922) reported that 'sulfur is the all important energy source' for *At. thiooxidans*, and also that is was a strict autotroph and obligate aerobe, and a mesophile.

Some 30 years later, investigations carried out by Arthur Colmer, Kenneth Temple and colleagues in acidic ferruginous waters draining coal mines in eastern USA (Colmer and Hinkle, 1947) led to the isolation of another acidophilic sulfur-oxidizing *Thiobacillus*, though uniquely (at the time) this bacterium could also grow autotrophically using ferrous iron as sole electron donor. Following the description and naming of *Thiobacillus* (later *Acidithiobacillus*) *ferrooxidans* (Temple and Colmer, 1951) other, supposedly related, but novel species of acidophilic iron-oxidizers were isolated from coal mine drainage waters. '*Ferrobacillus ferrooxidans*' was differentiated from *At. ferrooxidans* on the basis that it appeared not to use reduced sulfur as electron donors (Leathen *et al.*, 1956) while '*Ferrobacillus sulfooxidans*' was claimed to grow on elemental sulfur but not on thiosulfate (Kinsel, 1960). Kelly and Tuovinen (1972) later suggested that these claimed differences in physiologies were invalid and that all three were strains of the same species (*T/At. ferrooxidans*). Interestingly, '*Ferrobacillus ferrooxidans*' later assumed the role of type species of *At. ferrooxidans* (ATCC 23270/DSM 14882) though its original description by Leathen *et al.* (1956) (actively motile, pH minimum ~2, and temperature optimum of 20–25°C) is more akin to that of *Acidithiobacillus ferrivorans* (Hallberg *et al.*, 2010) than to *At. ferrooxidans*^{T}, which is non-motile and grows at pH < 1.5 and optimally at ~30°C, suggesting that some cross-over of strains (possibly due to mixed cultures) might have occurred. *At. ferrooxidans* remains by far the most widely studied and reported of all acidophilic bacteria, though iron-oxidizing acidithiobacilli currently includes four classified species.

The 1970s saw some major breakthroughs in isolating new species of acidophiles with contrasting physiological characteristics. These included the first heterotrophic (and moderately thermophilic) acidophile, *Thermoplasma* (*Tp.*) *acidophila* (later renamed *Tp. acidophilum*) which was isolated from a coal refuse pile that had undergone self-heating (Darland *et al.*, 1970). *Tp. acidophilum* was considered at the time to be a *Mycoplasma* (a genus of bacteria that lacks a cell wall) but later shown to be a euryarchaeote, and was therefore was first acidophilic archaea to be formally described. A related genus, *Picrophilus*, contains two species (both heterotrophic) that can grow at pH < 0, making them the most acidophilic of all currently known life forms (Schleper *et al.*, 1995). The first acidophilic bacterium shown to grow on organic carbon was described a few years later (Guay and Silver, 1974).

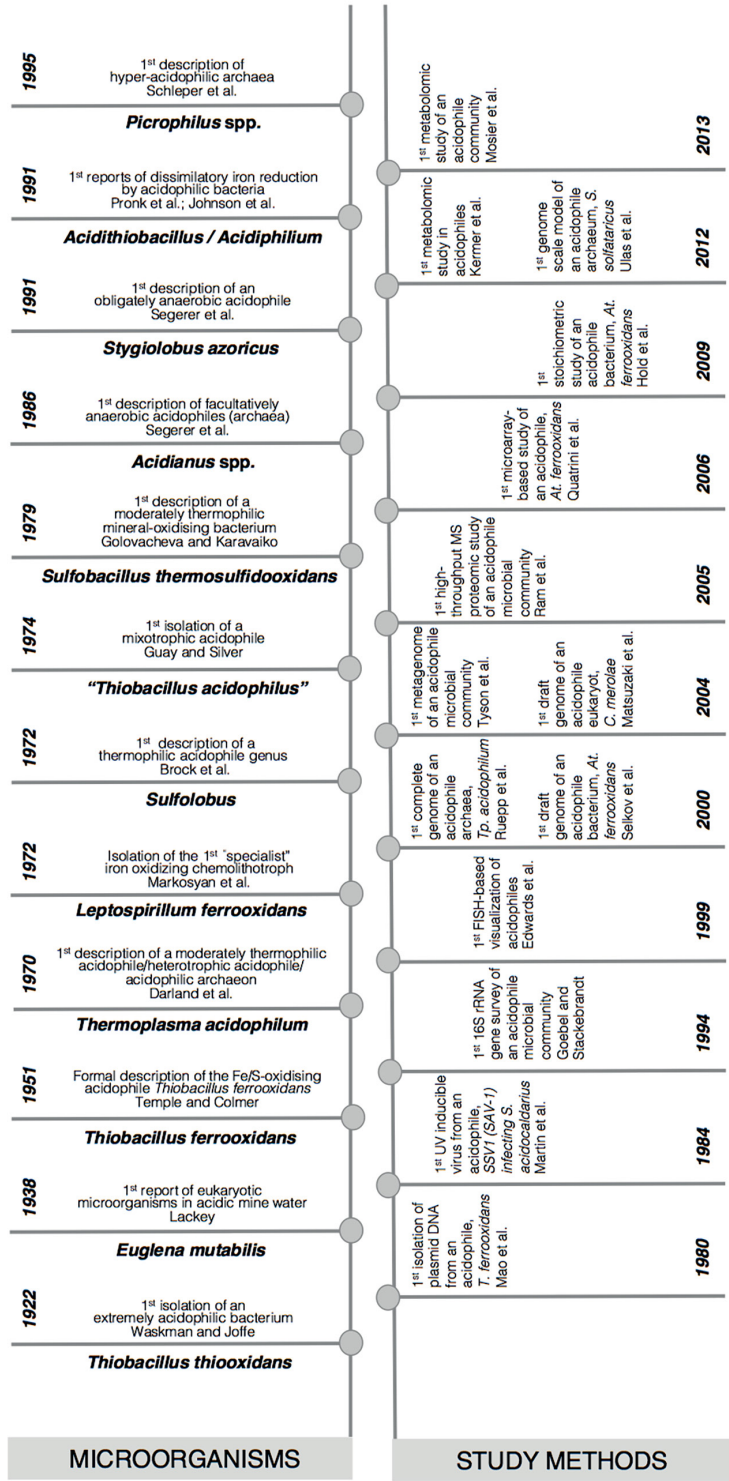

Figure 1.2 Major milestones in the development of acidophilic microbiology.

Named originally as *Thiobacillus acidophilus* since it could grow autotrophically on sulfur, as well as heterotrophically on sugars and some other small molecular weight organic compounds, it was later shown (from its 16S rRNA gene sequence) to be a member of the *Acidiphilium* genus. It remains the sole facultative autotroph in this genus; other classified species, the first of which were described by Arthur Harrison Jr in 1981, are all obligate heterotrophs.

The first extremely thermophilic acidophiles were isolated from Yellowstone National Park, Wyoming, and some other geothermal sites in Italy, Dominica and El Salvador, in the late 1960s and early 1970s. Tom Brock at colleagues described isolates obtained from acidic hot springs and thermal acid soils that grew optimally on sulfur and a variety of simple organic compounds at 70–75°C and at pH 2–3 (Brock *et al*., 1972). One strain was noted to grow at 85°C. *Sulfolobus* (*S*.) was described as 'a new genus of bacteria' and later confirmed as one of the first genus of the *Crenarchaeota* subdomain. The type strain of first species to be described, *S. acidocaldarius*, is an obligate heterotroph and does not oxidize elemental sulfur, though it was originally described as doing so. The very first description of an extremely thermophilic acidophile had earlier been reported in the 1966 doctoral thesis of James Brierley. This Yellowstone isolate was noted to oxidize ferrous iron and elemental sulfur, and had a lower temperature limit (~70°C) than *S. acidocaldarius* (Brierley and Brierley, 1973), suggesting that it was probably a strain of *S. metallicus*. While all *Sulfolobus* spp. are obligate aerobes, other extremely thermo acidophilic crenarchaeotes include genera such as *Acidianus* spp. that are facultative anaerobes (and oxidize or reduce sulfur) and the obligate anaerobe *Stygiologus azoricus*.

The 1970s also saw the first reports of iron-oxidizing acidophilic bacteria other than *At. ferrooxidans*. A second bacterial genus/species (*Leptospirillum ferrooxidans*) was isolated from a copper mine in Armenia (Markosyan, 1972). *Leptospirillum* spp. form a distinct clade within the *Nitrospira* phylum and appear to use only ferrous iron as an electron donor and are therefore obligate aerobes, since oxygen is the only thermodynamically feasible electron acceptor that can be coupled to ferrous iron oxidation at low pH. *Leptospirillum* spp. in general, and the thermo-tolerant species *L. ferriphilum* in particular (Coram and Rawlings, 2002) are now recognized to be the primary mineral-oxidizing acidophiles in biomining operations, including both heap operations and stirred tanks.

In the 1970s, bacteria that grew autotrophically on both ferrous iron and reduced sulfur but at much higher temperatures (~50°C) than previous chemolithotrophic acidophiles were isolated from mine sites and geothermal areas. These were also originally described as *Thiobacillus* spp. (e.g. Brierley *et al*., 1978) though many of these isolates were later confirmed to be related to a new genus of Gram-positive spore-forming bacteria, *Sulfobacillus* (Golovacheva and Karavaiko, 1979). One of the notable differences between *Sulfobacillus* spp. and a second genus of moderately thermophilic iron-oxidizing Gram-positive bacteria, *Acidimicrobium* (the earliest isolates of which were also isolated in the 1970s), and *Acidithiobacillus* and *Leptospirillum* spp. is that the former are facultative rather than obligate autotrophs, and their growth is enhanced by organic compounds, such as yeast extract.

Since the 1980s, many new genera and species of acidophilic prokaryotes have been described. These include specialized species that, like *Leptospirillum* spp., have limited metabolic potential and generalist species that, like *Sulfobacillus*, are able to use a wide range of electron donors and acceptors. In addition, species of obligately heterotrophic acidophilic archaea and bacteria have been described that catalyse the dissimilatory oxidation of ferrous iron (e.g. '*Ferroplasma acidarmanus*' and *Ferrimicrobium acidiphilum*) or reduced sulfur (e.g. *Acidicaldus organivorans*) and which, therefore, at least in theory, contribute to mineral dissolution in extremely acidic environments.

Because of the relative ease of observing and, in many cases, identifying them in environmental samples, eukaryotic microorganisms have been known to thrive in many moderately to extremely acidic environments for many years. In a pioneering report published in 1938, James Lackey noted that protozoa and micro-algae were abundant in acidic mine waters that formed in the vicinity of coal mine in eastern USA. He noted that rotifers were present, but prokaryotic blue-green 'algae' were absent, and also that fungi were relatively scarce in the streams he examined. In the same report, Lackey described stones, sticks and leaves in mine water streams

being extensively colonized by the phototrophic protozoan *Euglena mutabilis*. *Euglena* spp. in general, and *E. mutabilis* is particular, are now known to be among the most widely distributed acidophilic phototrophs in streams draining metal mines as well as coal mines. Joseph (1953) also reported that euglenoids were abundant in the acid streams he examined, as were fungi (e.g. *Trichoderma*) and the diatom *Navicula viridis*. As with prokaryotic microorganisms, later studies have both confirmed these first reports and expanded the known biodiversity of acidophilic eukaryotes.

The introduction of the polymerase chain reaction and associated DNA- and RNA-based methodologies revolutionized the study of acidophiles as elsewhere in microbiology. In one of the first applications of this developing area of molecular biology, Lane *et al.* (1992) established the phylogenetic relationships between 37 species and strains of iron- and sulfur-oxidizing bacteria, including 19 acidophiles, based on their partial 16S rRNA sequences. They highlighted the important fact that acidophilic prokaryotes have a widespread distribution in the 'tree of life', and also that classification based on physiological trends alone had resulted in some inaccuracies (e.g. '*T. acidophilus*', as referred to above, and '*T. ferrooxidans*' m1, which was noted to be not a *Thiobacillus/Acidithiobacillus* sp., and was reclassified almost 20 years later as the new genus/species *Acidiferrobacter thiooxydans* by Hallberg *et al.*, 2011). Classification and identification of acidophiles based on their 16S rRNA (gene) sequences has since become routine, though the use of multiple locus sequence analysis and, increasingly, whole-genome sequences, are emerging as more powerful and accurate approaches to determine the phylogeny of acidophilic microorganisms.

In the 1990s the cloning and sequencing of 16S rRNA genes from pure cultures and environmental samples begun to be used to study the microbial diversity in natural acidic environments, commercial bioleaching processes and acid mine drainages (e.g. Edwards *et al.*, 1999; Goebel and Stackebrandt, 1994). These molecular diversity inventories revealed that the acidic econiches had far less diverse populations than marine, soil and hot spring environments, with both smaller numbers of species and less clonal sequence heterogeneity. Low biodiversity was attributed to the selective nature of acidic biotopes (in terms of pH, redox potential, concentrations of dissolved metals, etc.). Evidence obtained from these and other studies using restriction profiles of 16S rRNA genes and denaturing gradient gel electrophoresis analysis of 5S rRNA genes, revealed that the sequence clones were closely related to known cultured acidophiles and very few if any 'unknown' microorganisms appeared to be present in these acidic econiches. Since then greater understanding of the diversity of organisms present in acidic environments and of their variations in time and space has been obtained through other types of biomolecular studies, including fluorescent *in situ* hybridization (FISH), terminal/restriction enzyme fragment length polymorphism (T-RFLP) analysis, real-time PCR and microarray analysis.

The 2000s saw the advent of next generation sequencing technologies and the radical change in the data generation opportunities brought about by them. The metagenomic analysis of acidic econiches begun with the seminal work of Jill Banfield and colleagues on the microbial communities in AMD in the Richmond Mine tunnels (Tyson *et al.*, 2004). Over the last two decades, and thanks to the relatively low biological and geochemical complexity of this ecosystem, an in-depth characterization and quantitative analysis of the associated microbial communities was achieved. Not only have complete or near-complete genomes of dominant AMD microbes been successfully reconstructed (e.g. Goltsman *et al.*, 2009) but multiple levels of variation between and within the microbial consortia have been revealed and characterized (e.g. Simmons *et al.*, 2008). Community genomics of the Iron Mountain system went far beyond previous gene marker surveys to provide for the first time evidence on the virus–host interactions and the patterns of viral and host distribution over time and space (Andersson and Banfeld, 2008). In addition, several novel taxa were identified and characterized in varying extent thanks to this approach, including a new group of acidophilic nanoarchaea referred to as 'ARMAN' and completely overlooked in other environmental studies before the advent of metagenomics (Baker *et al.*, 2006). Several other metagenomic studies of acidic econiches have followed over the years, including recent integrated community and organism-wide metagenomic and transcriptome analyses. These studies have begun

to explain the emerging patterns of variation in the microbial communities controlling geochemical cycling and acid generation in these biotopes, and their correlations with seasonal and spatial fluctuations in geochemical and environmental conditions.

Tandem mass spectrometry-based proteomic (metaproteomic) approaches were also first applied to the study of the natural microbial communities at the Richmond Mine site (Ram *et al.*, 2005). The proteomic research conducted allowed the assessment of the protein inventories of the member organisms in the AMD communities and helped identify relevant differences in physiology of genotypic variants as a function of environment type. These studies were foundational, demonstrating that it was possible to track variations in the proteomic responses of multiple coexisting microorganisms *in situ* and that clues to the functional contribution of the community members could be readily identified (e.g. Wilmes *et al.*, 2009). Also, in the early 2010s, a number of metabolomic studies using stable isotope labelling coupled with targeted or untargeted high-resolution mass spectrometry have been performed on acidophilic communities or acidophile bacterial models, offering further insights into the adaptation strategies and the biology of the organisms that drive geochemical cycles in acidic econiches and related bioprocesses. These studies have enabled, for example, tracking of the carbon flux between a heterotrophic and an autotrophic bacterial species in an artificial mixed culture (Kermer *et al.*, 2012) and provided hints of the metabolites that constitute the sulfur, nitrogen and carbon currencies in natural AMD communities (Moisier *et al.*, 2013).

In parallel, genomic sequencing of a large number of culturable acidophiles covering the three domains of life has provided a robust framework upon which to address fundamental questions related to the biology, the ecology and the evolution of microbial acidophiles. After the public release of the first complete genome of an acidophile archaeon *Thermoplasma acidophilum* (Ruepp *et al.*, 2000), the first draft genome of an acidophile bacteria *At. ferrooxidans* (Selkov *et al.*, 2000) and the first draft genome of an acidophile eukaryote *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004) in the early 2000s, a growing number of metabolic reconstructions and physiological studies were performed yielding valuable insight into different aspects of the metabolism and physiology of extreme acidophiles (reviewed in Cárdenas *et al.*, 2010). In the late 2000s and the beginning of the 2010s the first stoichiometric model of an acidophile model bacteria, *At. ferrooxidans*, was obtained (Hold *et al.*, 2009), and the first genome-scale model (GEM) of an acidophile archaea, *Sulfolobus solfataricus*, was reconstructed (Ulas *et al.*, 2012). Use of these models in conjunction with constraint-based methods has begun to allow the simulation of metabolic fluxes induced by different environmental and genetic conditions and the prediction of different cellular metabolic properties. Beyond their relevance for fundamental reasons, both acidithiobacilli bacteria and the sulfolobales archaea are of high interest for industry and biotechnology, and thus these models may prove useful for optimization of biomass production and the generation of relevant enzymes or metabolites.

The application of acidophilic microorganisms in the main biotechnology in which they are involved, the bio-processing of mineral ores and concentrates – generally referred to as biomining – has paralleled that of more fundamental research (Brierley and Brierley, 2013). Although the first purposeful application of mineral-oxidizing bacteria to extract a metal (copper) from a low-grade run-of-mine ore was set up around 15 years after the description of the first iron-oxidizing acidophile (*At. ferrooxidans*), bacterial leaching of metal ores had unknowingly been harnessed as a means of extracting metals since at least the middle ages (Johnson, 2014). Biomining has developed from a relatively basic technology for leaching metals from waste rocks to much more complex engineered bio-heaps and stirred tanks. Although most applications use consortia of mesophilic and thermo-tolerant acidophiles, there has been one successful pilot-scale demonstration using a thermoacidophilic archaeal consortium to bioleach the recalcitrant, but abundant, copper mineral chalcopyrite at ~80°C (Batty and Rorke, 2006). Future developments in biotechnologies that utilize acidophiles will quite probably harness their abilities in areas beyond oxidative mineral oxidation, such as bio-mineralization, organic matter metabolism and reductive bio-processing.

Looking forward: challenges and opportunities

Whilst knowledge of the microbiology of acidic environments has advanced greatly in recent decades, there are still many areas that are poorly understood and questions that remain to be answered. Some are fundamental, such as why most acidophiles are unable to grow in circumneutral pH and, in many cases, mildly acidic waters, and why the upper temperature limit for extremely acidophilic prokaryotes is some 30–40°C lower than those of their neutrophilic counterparts. Cultivation-independent studies have shown that there are unknown species of bacteria and very many unknown species of archaea, living in aerobic and anaerobic low-pH environments (e.g. Baker *et al.*, 2006). The roles of these uncultivated prokaryotes in low-pH environments can only be speculated upon (e.g. Justice *et al.*, 2012). In many cases, the very distant relationships between these uncultivated prokaryotes and characterized species of acidophilic bacteria and archaea (from comparison of their 16S rRNA gene sequences) means that it is not possible to imply or sometimes even give a reasonable guess at the physiological traits of these uncultivated acidophiles. Improved techniques and probably radically novel approaches are needed in order to isolate acidophilic archaea, in particular, from environmental samples, and to cultivate them *in vitro*. This was illustrated in the case of the iron-oxidizing chemolithotroph '*Ferrovum myxofaciens*' (Johnson *et al.*, 2014). Although this bacterium has global distribution in acidic ferruginous waters of pH 2–4, and is frequently the dominant bacterium in large-scale 'acid streamer' growths in these waters, it was not detected until the late 1990s and was not isolated until a modified 'overlay' solid medium was devised, in 2006. Novel acidophilic isolates with hitherto unknown physiological characteristics could open up new opportunities in technologies that use these microorganisms, such as the halotolerant species that are also effective at degrading minerals, which could be harnessed for biomining in areas where only brackish or saline waters are available. There is also the ongoing challenge to isolate and characterize species of hyper-acidophilic prokaryotes, to encompass genera other than *Picrophilus*, and possibly setting new limits for the lowest pH at which organisms can grow.

Genomic analysis of isolates and whole communities, together with other 'omic' approaches, have provided new resources to overcome some of these limitations, whilst providing an in-depth look at the biology of acidophiles and potential for interaction with other microorganisms and with extreme acidic environments. Comprehensive gene lists and catalogues of metabolic pathways have been collected for a number of model acidophiles and acidophilic communities, providing blueprints of their functional potential and task partitioning. Yet, compared with other ecosystems, genomic information of acidophiles is still 'scarce'. Some branches of the tree of life are undersampled (e.g. Johnson *et al.*, 2014), and, perhaps with the exception of *Sulfolobus islandicus* (e.g. Reno *et al.*, 2009) and the group II leptospirilli, few genomes are resolvable at the strain level with the currently existing genomic data. Additional sequencing efforts are required to enable comparative genomic studies that may yield valuable insight into genome architecture, dynamics and evolution. Despite the utility of single cell genomics to study unculturable or 'difficult to culture' microorganisms, no efforts of the kind have been reported in the literature to date, in the case of acidophiles.

Only a few model acidophiles have been thoroughly analysed using metabolic reconstruction and metabolic modelling tools, despite the clear biotechnological applications of many of these microorganisms and an obvious interest in modelling their metabolic fluxes. This is partly due to scarcity of biochemical and metabolic information available on acidophiles in the general and specific literature, compared to well-studied neutrophilic counterparts (e.g. *Escherichia coli*). Much room is still available for proteomic, metabolomic, lipidomic and glycomic analysis of model acidophiles. With the notable exceptions of the Iron Mountain system in the USA (Denef *et al.*, 2010), the Cae Coch abandoned mine system in northern Wales (Kimura *et al.*, 2011) and the Rio Tinto in Spain (Amils *et al.*, 2014), general understanding of the role of prevailing geochemical and environmental factors in shaping diversity patterns in acidophilic communities is still lacking. Further efforts to comprehensively characterize microbial communities inhabiting other acidic econiches are bound to reveal broad trends of microbial distribution and rules of adaptation to acidic environments.

Signatures of acidophilia have been clearly identified in archaea (e.g. high ratio of secondary over primary transport systems; Angelov and Lieb, 2006), some of which are shared with bacteria. In both domains of life, adaptation to extreme acidic conditions is achieved by the concerted action of several mechanisms that contribute to active pH homeostasis through proton exclusion, consumption, exchange and neutralization and by mitigation strategies including cytoplasmic buffering and repair of the damage produced by proton influx (Slonczewski *et al.*, 2009). However, minimal common strategies and specific adaptations of acidophilic bacteria and archaea are poorly explored. More genomes and further comparative genomic analyses are required to search for minimal signatures of acidophilia and to uncover the evolutionary forces acting on microorganisms present in acidic environments. Relevance of these studies extends beyond the understanding of the physiology of extreme acidophiles, touching upon two fields of fundamental interest, the early evolution of life on earth and the study of astrobiology. Further understanding of the transmissible, integrative and translocative elements that populate the genomes of most sequenced acidophiles is also required to understand, the impact of horizontal gene transfer and flexible gene pools on genome evolution and adaptation to extremely acidic econiches. An overwhelming example of the relevant role of horizontal gene transfer in the adaptation to these extreme conditions comes from comparative genomic analysis of the *Thermoplasmatales* and the *Sulfolobales* archaea. *Thermoplasma acidophilum* and *Picrophilus torridus* share approximately 65% of their genes between them, as they do with *S. solfataricus*, a very distant relative thermoacidophile, whereas only 35% of their genes are present in *Pyrococcus furiosus*, a closer relative (Fütterer *et al.*, 2004). Frequent genetic input via horizontal gene transfer during evolution of these archaea seems to have contributed to the evolution of the microorganisms enduring some of most extreme conditions in our planet. From a different perspective, this line of research is likely to be conductive to a more comprehensive understanding of the limitations for genetic manipulation of acidophiles and to open new opportunities for the development of well necessitated tools to transform and further advance functional investigation of acidophiles.

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