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Microbiomes in extremely acidic environments: functionalities and interactions that allow survival and growth of prokaryotes at low pH

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Extremely acidic environments have global distribution and can have natural or, increasingly, anthropogenic origins. Extreme acidophiles grow optimally at pH 3 or less, have multiple strategies for tolerating stresses that accompany high levels of acidity and are scattered in all three domains of the tree of life. Metagenomic studies have expanded knowledge of the diversity of extreme acidophile communities, their ecological networks and their metabolic potentials, both confirmed and inferred. High resolution compositional and functional profiling of these microbiomes have begun to reveal spatial diversity patterns at global, regional, local, zonal and micro-scales. Future integration of genomic and other meta-omic data will offer new opportunities to utilize acidic microbiomes and to engineer beneficial interactions within them in biotechnologies.

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Introduction: extremely acidic environments, origins and diversities

Acidic environments with pH values of <5 (e.g. soils and some water bodies) are widely distributed in the lithosphere. However, extremely acidic (pH < 3) environments are far less common. These may be natural or anthropogenic (man-made) ecosystems, and are mostly associated with the biogenic formation and accumulation of sulfuric acid [1,2*]. Sulfur is one of the most abundant elements in planet Earth, and can occur in any one of its nine oxidation states, most commonly +6 (sulfate, e.g. in oceanic waters), 0 (zero-valent sulfur (ZVS) e.g. in volcanic and geothermal areas), and -1/-2 (in sulfide minerals such as pyrite (FeS₂) in rocks or ore bodies). In volcanic areas and geothermal areas, oxidation of ZVS

and other reduced sulfur compounds can generate extremely low pH ecosystems (e.g. pH ~ 0.7 in Lake Kawah Idjen, Indonesia [3] and pH ~ 0.2 in the Copahue volcano — Lake Caviahue area, Argentina [4]). Geothermal sites, found typically where the Earth's crust is relatively thin, occur in New Zealand, Iceland, the Azores, some Caribbean islands and elsewhere, with Yellowstone National Park (Wyoming) being the most well-known and documented area. Extensive cave systems formed where sulfide-rich waters interact with oxygen at the water table or at subterranean springs also give origin to sulfuric acid and the emergence of extreme acidic environments [5]. A similar scenario of sulfur cycling in sewage systems can cause the formation of zones of extreme acidity in the upper (aerobic) regions and severe corrosion and sometimes collapse of concrete pipes (e.g. [6]).

Although natural extremely acidic environments associated with the dissolution of sulfide minerals exist, they are relatively rare compared to those of anthropogenic origin that have arisen from mining metals and coals, which can be found in most (post)-industrialized countries [7*]. Again low pH derives mostly from elevated concentrations of sulfuric acid, though the pH of mine-impacted environments tends often to be marginally higher than those associated with sulfur oxidation due to the buffering influence of the ferrous/ferric couple (iron is generally the dominant transition metal in these environments). For example, the pH of the most famous sulfide mineral-derived water body, the Rio Tinto river in south-west Spain, is 2.3–2.7 throughout its entire 100 km length. Exceptions to this general trend do occur however, such as within the Richmond mine in Iron Mountain, California, where very warm temperatures cause extensive evaporation from the acidic iron-rich pools within it, resulting in the formation of 'acid-generating salts' and highly elevated concentrations of hydronium ions. Negative pH values as low as -3.6 have been recorded within this pyrite-rich mine [8].

Phylogenetic diversity of extremely acidophilic prokaryotes, and key functional features of acidophile communities

Acidophiles are distributed throughout all three Domains within the tree of life [1]. Extremely acidophilic prokaryotes include Alpha-proteobacteria, Beta-proteobacteria and Gamma-proteobacteria, as well as several species in

the class Acidithiobacillia (Figure 1). Other Gram-negative acidophiles are found within the deeply-branching bacterial phyla Nitrospirae (*Leptospirillum* spp.), Aquificae and Verrucomicrobia. Gram-positive extreme acidophiles currently fall within two phyla, Actinobacteria and Firmicutes, and include some of the most metabolically versatile of all extremophilic prokaryotes. Within the Domain Archaea, extreme acidophiles are found within the Euryarchaeota (which includes *Picrophilus* spp., the most acidophilic of all currently known life-forms) and the Crenarchaeota (mostly thermo-acidophiles) phyla. Extreme acidophiles can also be found in many branches of the Eukarya, including the fungi, green and red algae and diatoms, amoeba, flagellates and ciliates [9].

Extreme acidophiles are known to display a wide range of physiological traits, both individually (traits/species) and collectively (traits/microbiome). Some of these traits can be found in many species and are atypical in terms of their spread and abundance with respect to non-acidophilic prokaryotic counterparts as summarized below. The ability to tolerate and thrive in extremely acidic liquors defines them as a group, and the ability to (passively) maintain pH gradients of several orders of magnitude between themselves and the outer world (the internal pH of most acidophiles is ~ 6.5) is unique to these microorganisms. Diverse strategies, comprising both active (proton exclusion, exchange, pumping, consumption and neutralization) and passive (cytoplasmic buffering) mechanisms of pH homeostasis as well as damage mitigation strategies (DNA repair, synthesis of acid stable proteins) appear to underlie this general capacity [10,11]. However, the contributions of each of these strategies to enhancing acid tolerance at the community level are currently unknown.

In addition to having to cope with extreme acidity, and sometimes with significant temperature variations, indigenous life-forms frequently also have to tolerate elevated concentrations of various transition metals (e.g. copper) and metalloids (e.g. arsenic), high osmotic potentials deriving from the presence of large amounts of inorganic solutes, and variable temperatures and oxidation–reduction potentials [1]. Known strategies to cope with these many stresses entail responses by individual species, with the exception of biofilm formation which is frequently regarded as a collective response.

Commonly observed physiological traits of acidophiles are summarized in Figure 2. These include chemolithotrophy (the ability to use inorganic electron donors ferrous iron, hydrogen, ZVS and sulfur oxy-anions), ferric iron respiration, autotrophy (both obligate and facultative), diazotrophy, osmo-tolerance (though tolerance to NaCl salt is more variable), enhanced abilities to tolerate elevated concentrations of cationic transition metals, and propensity for extracellular polymeric substance (EPS)

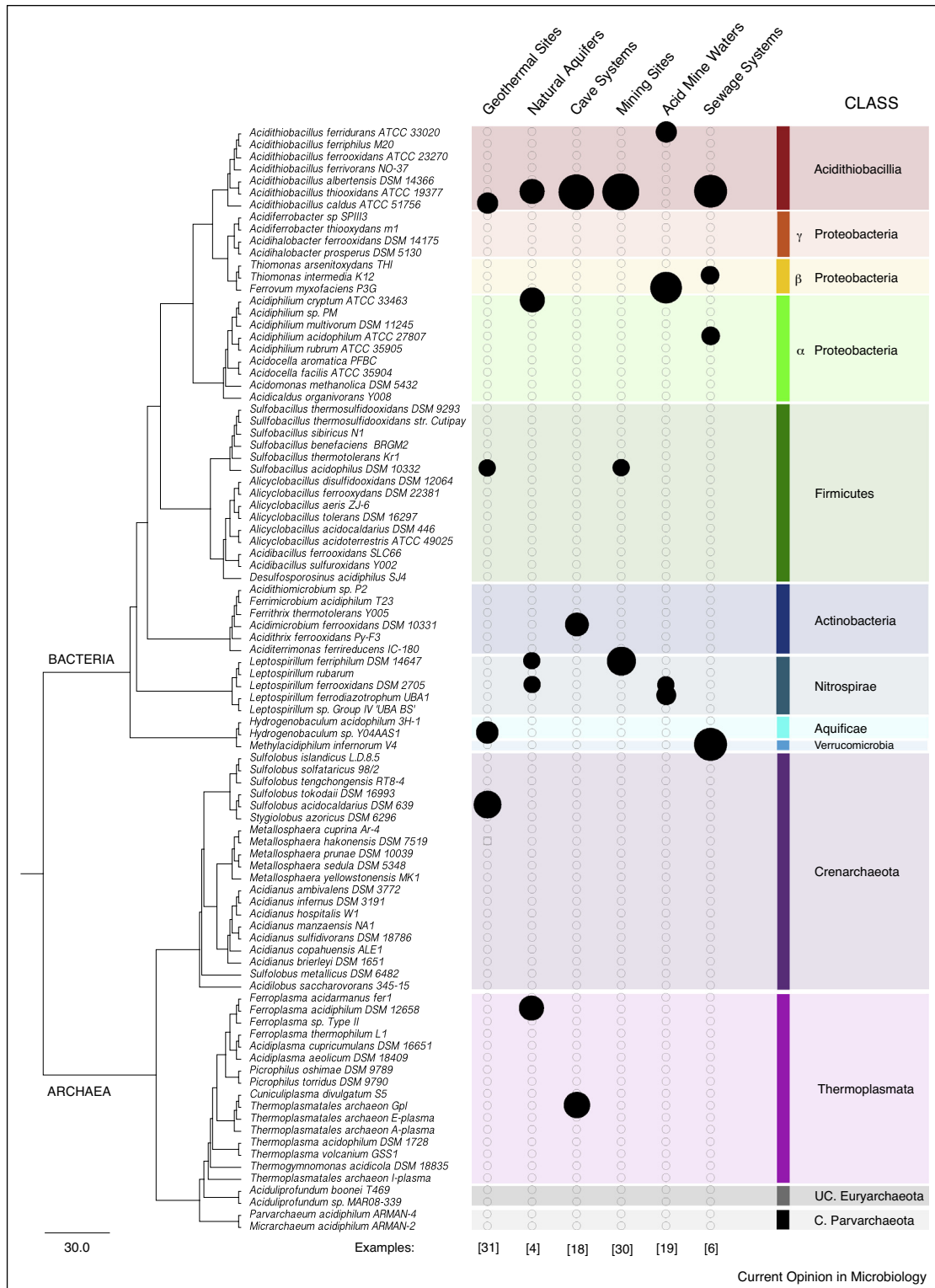
production. Thermo-tolerance (in archaea) and psychro-tolerance (some Gram-negative bacteria) is also characteristic of some extreme acidophiles. Several of these capacities (e.g. metal tolerance) are globally conserved across acidophilic lineages while others partition differentially between taxa (e.g. autotrophy–diazotrophy), and as such these tend to be features that underlie structuring of acidic microbiomes [12].

Acidic microbiomes: patterns of assembly and occurrence

In both natural and anthropogenic extremely acidic environments, levels of species richness are significantly less than those of non-extreme environments, making them highly tractable microbiome model systems (e.g. [13]). Understanding of the organizational principles of several acidic microbiomes has increased as a result of numerous cultivation-dependent and independent studies, and more recently also from a plethora of targeted environmental metagenomic studies (Table 1) ranging from bioprospecting analyses (e.g. [14]) to perturbation-based experiments (e.g. [15]). These studies have revealed that regardless of the specific habitat type, relatively few bacterial and archaeal species account for the vast bulk of acidic microbiomes. Core assemblages made up of a single dominant species or a couple of co-dominant species generally constitute 50–80% of the community, while a variable number (typically 3–10) of satellite species tend to be present at less than 20%. Occurrence and proportions of the different predominant acidophiles varies with the community concerned [16–19,20*,21]. For example, snottites dripping from the ceiling of acidic caves are either dominated by *Acidithiobacillus thiooxidans* populations [17] or *Leptospirillum ferrooxidans* populations [18] depending on the predominant energy source available (sulfide-rich springs vs pyrite-rich minerals). Other interesting and consistent patterns emerging from the increasingly available data, show the predominance of *Leptospirillum* and *Ferroplasma* spp. in warmer ($>30^\circ$) extremely acidic (pH < 2) ferruginous mine waters [13], and *Ferroplasma* spp. and *Acidithiobacillus ferrooxidans* in cooler ($<20^\circ\text{C}$) less acidic (pH 2–3) water bodies of similar chemical composition [22]. In addition to the core microbiome members, a variety of low-abundance species of both known (e.g. *Acidimicrobium ferrooxidans* [22]) and novel (e.g. ARMAN nanoarchaea [23]) acidophiles accompany the core-species and satellite-species in the assemblages.

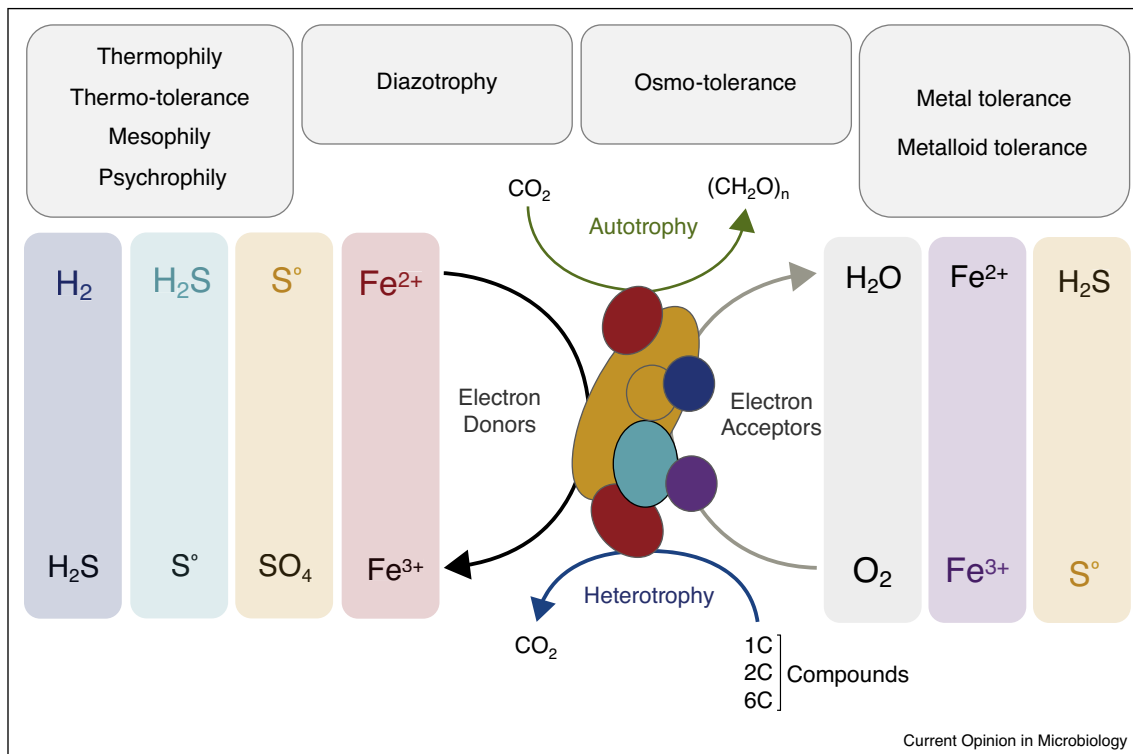
Despite increased knowledge of the microbial components of acidic microbiomes, detailed distribution maps for acidophiles are mostly unavailable, with the exception of a few model microorganisms that have been the object of biogeography-focused studies [24,25,26*,27]. In recent years however, high resolution compositional and functional profiling of microbial communities inhabiting acidic environments have begun to reveal spatial

Figure 1



Phylogenetic tree of bacterial and archaeal acidophiles (type strains or reference strains) based on the 16S small subunit rRNA gene sequence. The taxonomic groups, at class level, are colored as indicated at the right side of the figure. Black circles represent occurrence of a taxon in a given sample while empty circles represent absence. Relative abundance of the different taxa in natural and anthropogenic acidic environments from representative studies ([4,6,18,19,30,31]) are indicated by the size of the black circles. Tree construction details can be found in Ref. [1]. C: candidate; UC: unclassified.

Figure 2



Key physiological traits of acidophilic communities. Electron donors (left), electron acceptors (right), inorganic (top) and organic (bottom) carbon sources preferred by different acidophiles are depicted together with the general transformations driven by the community. Additional metabolic features of extreme acidophiles are also indicated in the upper boxes.

diversity patterns at several different scales: global (e.g. [25]), regional (e.g. [19,28]), local (e.g. [29]), zonal (e.g. [30]) and micro-scale (e.g. [17]). From these studies, various physico-chemical factors driving the observed ecological patterns have begun to be identified in a number of acidic ecosystems (Figure 3) and entail, in order of importance: temperature (differentiating archaea-dominated from bacteria-dominated communities; e.g. [31]), pH (e.g. [32]), oxygen availability (e.g. [33]) and redox potentials (dictated mostly by ratios of ferric to ferrous iron concentrations, e.g. [34]). The effect of other environmental factors, such as osmotic potentials, in shaping the phylogenetic structure of acidic microbiomes has been much less researched. Similarly, temporal patterns in the taxonomic and metabolic diversity of the majority of acidic habitats remain mostly unresolved, though some studies have profiled seasonal shifts in acidophile communities (both compositional and functional; e.g. [35]) while others have tracked longer-term temporal successions in geothermal springs (e.g. [36]), acid salt lakes (e.g. [37]), mine waters (e.g. [38]), biomining sites (e.g. [39]) and built environments susceptible to acidic corrosion (e.g. [40]). Additional efforts need to be made to integrate spatial, temporal and physico-chemical data in order to provide further understanding of the

patterns of occurrence and the rules of assembly of acidic microbiomes.

Ecophysiological interaction networks between acidophiles in natural and anthropogenic ecosystems

Growing evidence from different well-studied ecosystems indicates that the composition of the microbiomes is less important than the topology of their functional and ecological networks. Through diverse pathways for nutrient uptake and exchange, energy flow and horizontal transfer of genetic information, interacting partners in a given community acquire new and distinctive emergent properties, which in the case of acidic microbiomes, are only beginning to be understood.

Extensive cataloguing of the metabolic potential of acidophiles and their microbiomes has been achieved through genomics and metagenomics, and functional inferences derived from this data have been extensively covered in the literature (reviewed in Ref. [41,42]). However, only in relatively few acidic ecosystems has it been possible to link the occurrence of a gene or gene pathway to cognate transcripts, proteins and/or metabolites in order to ascertain the inferred contributions of given community

Table 1

Recent meta-omics studies providing insights into the compositional and functional diversity of extreme acidophile communities and their spatial and diversity patterns.

| Pattern | System | Origin | | Sample type | Meta-omics | | | | Other | Refs. | |
|-----------|---------------------|-------------------|---------------------|-------------------------------|--------------------------|----|----|----|-------|--------|----------|
| | | Country | Location | | TMG | MG | MT | MP | | | |
| Spatial | <i>Global</i> | Cave | Italy; Mexico | Marche region; Tabasco | Biofilm, snotittes | – | + | – | – | – | [26**] |
| | <i>Regional</i> | AMD | China | Southeast region | Water | + | – | – | – | – | [19,28*] |
| | | AMD | China | Guandong province | Water | – | + | + | – | – | [48] |
| | <i>Local</i> | Tailings | China | Fankou Mine | Mineral | + | – | – | – | – | [29] |
| | | Tailings | China | Shuimuchong | Mineral | + | – | – | – | – | [34] |
| | <i>Zonal</i> | AMD | Norway | Svalbard | Biofilm, sediment, water | + | – | – | – | – | [14] |
| | | AMD | USA | Richmond Mine | Biofilm | – | – | – | + | – | [16] |
| | | AMD | USA | Richmond Mine | Biofilm | – | – | + | – | – | [44] |
| | | AMD | Spain | Los Ruedos | Biofilm, streamers | – | + | – | – | – | [33] |
| | | AMD | Sweden | Kristineberg Mine | Biofilm, streamers | – | + | – | – | – | [22] |
| | | Heap | China | ZiJinShan Mine | Mineral, water | + | – | – | – | – | [30] |
| | | Heap | China | Dexing Mine | Mineral, water | – | + | – | – | – | [21] |
| | | Heap | China | Dexing Mine | Mineral, water | + | – | – | – | – | [20**] |
| | | Acid Salt Lake | Australia | Yigarn Craton | Water | – | + | – | – | – | [37] |
| | <i>Micro-scale</i> | Cave | Italy | Frasassi cave system | Biofilm, snotittes | – | + | – | – | – | [18] |
| Mine adit | | Germany | Drei Kronen und Ehr | Biofilm, snotittes | – | + | – | – | – | [17] | |
| AMD | | USA | Richmond Mine | Biofilms | – | – | – | – | FISH | [32] | |
| Temporal | <i>Seasonal</i> | AMD lake | China | Nanshan Mine | Water | + | – | – | – | [62] | |
| | | AMD | Australia | Mt Todd Mine; Rum Jungle Mine | Water | + | – | – | – | – | [35] |
| | | Mine adit; AMD | Wales | Dyffryn Adda | Biofilm, streamers | – | – | – | – | T-RFLP | [38] |
| | <i>Successional</i> | Geothermal spring | China | Yunnan, Tengchong | Sediments, water | + | – | – | – | – | [36] |
| | | Geothermal spring | New Zealand | Inferno Crater Lake | Water | + | – | – | – | – | [31] |
| | | Concrete | USA | Colorado Front Range | Biofilm, slime | + | – | – | – | – | [40] |

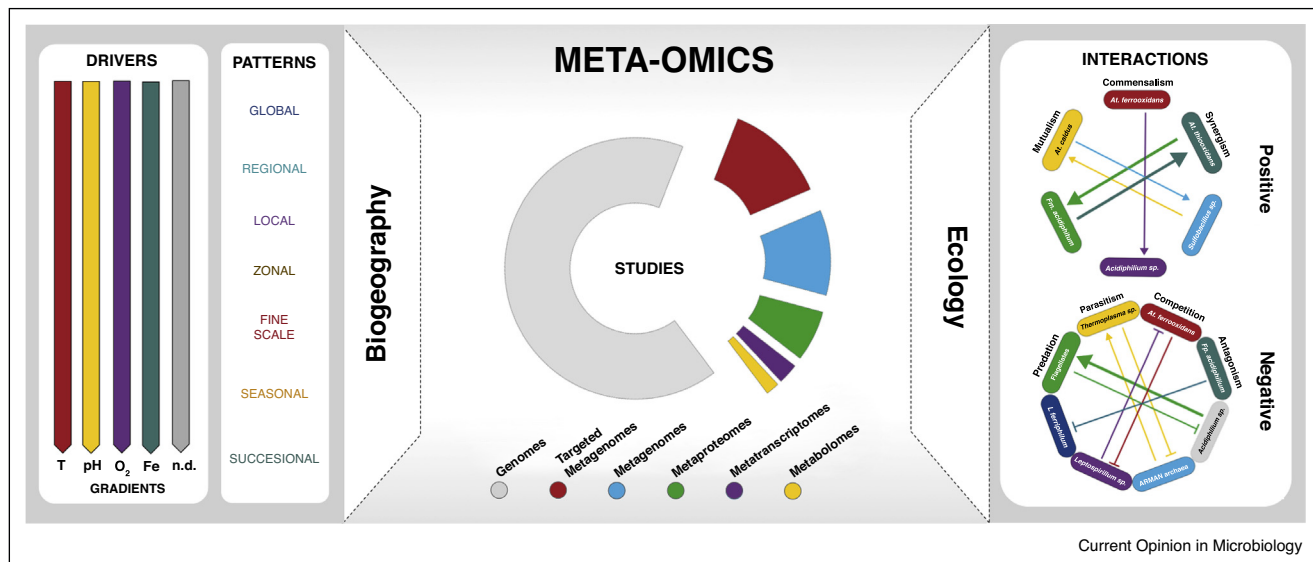
Abbreviations: TMG, targeted metagenomics; MG, metagenomics; MT, metatranscriptomics; MP, metaproteomics; T-RFLP, terminal restriction enzyme fragment length polymorphism; FISH, fluorescence *in situ* hybridization.

members to ecosystem processes. The first and most thoroughly system studied at this holistic level is the Richmond mine at Iron Mountain, California, where exhaustive genome (e.g. [43]), transcript (e.g. [44]), protein (e.g. [16]) and metabolite (e.g. [45]) data gathered using diverse high-throughput technologies has been used to build models of key cellular processes in acidic ecomiches. Community-wide changes in the activity and physiology of this [46**] and a few other mine water communities [47,48] have been correlated to qualitative and quantitative changes in emergent properties of the acidic microbiomes (such as the development of biofilms) or the local geochemistry. However, advanced molecular understanding of acidic communities other than those in mine waters is very limited, with studies mostly focusing on single meta-omics analysis of selected communities (e.g. [47]) or community members (e.g. [49]). This lacuna still limit further generalizations to be derived transversally on the shared and distinctive functionalities that warrant survival, adaptation and specialization of the microbiomes from diverse acidic habitats.

Despite this fact, a number of focused studies have been instrumental in advancing knowledge of the interactions

between microorganisms in acidic communities and are beginning to aid in deciphering the underlying organizational principles (rules of assembly) and integrated physiological capacities (rules of function) of these communities. Acidophiles interact with each other in ways which are as diverse as those reported for non-extremophiles. Positive symbioses (where one or more partners derive some benefit) include mutualistic interactions (where all partners benefit), commensalism (or facilitation, where only one partner benefits) and synergistic interactions (where complimentary activities of participants are more efficient than either operating alone). Negative symbioses (antagonism) are where activities of one or both partners sharing an environmental niche are impaired or suffer more serious negative impact, and include competition, amensalism (where one or more species repress others), predation, parasitism and virus-induced cell lysis. Many examples of microbial interactions listed above have been described, *in vivo* and *in vitro*, in low pH environments and defined laboratory cultures [50]. One that well illustrates interplay of carbon, iron and sulfur cycling and a binary culture acting with far greater efficiency than axenic cultures alone, is the case of *Acidithiobacillus thiooxidans* and *Ferrimicrobium acidiphilum* growing in

Figure 3



Advances and challenges in acidic microbiomes studies. High-throughput analyses (center) performed on extreme acidophiles and their microbiomes are heavily biased towards genome- and metagenome-based studies. The general scarcity of functional data (metatranscriptomic, metaproteomic and metabolomic) conditions further understanding of ecosystem processes in acidic environments other than acid mine drainages. Recent meta-omics studies have uncovered spatial and temporal patterns of variation in acidic niches (right) and helped identify the factors acting as principal drivers of such patterns. Ecological interactions (left) emerging from these and other studies (reviewed in Ref. [50]) are beginning to reveal the underlying organizational principles and the integrated capacities of these communities.

'inorganic' (i.e. free of any added organic carbon compounds) media with the mineral pyrite (FeS_2) as sole energy source [51]. *At. thiooxidans* is an autotroph that oxidizes reduced sulfur but not iron, while *Fm. acidiphilum* has opposite traits (a heterotroph that oxidizes iron but not reduced sulfur). Together, but not alone, they can unlock the energy available from oxidizing pyrite. *Fm. acidiphilum* initiates the process by generating ferric iron, which attacks the mineral and releases small amounts of reduced sulfur which in turn is used as electron donor by *At. thiooxidans*, fuelling its fixation of CO_2 into organic carbon, some of which is leaked and incorporated by *Fm. acidiphilum*, facilitating its continued oxidation of iron and dissolution of pyrite. Acidity produced from sulfur oxidation (by *At. thiooxidans*) is of mutual benefit to both acidophiles.

Negative interactions taking place in acidic environments have been far less documented. Virus–host interactions represent one notable exception. A rich diversity of viruses infecting acidophilic archaea from both high (e.g. *Sulfolobus* spp. and *Acidianus* spp.) and moderate temperature environments (e.g. ARMAN nanoarchaea and *Ferroplasma* spp.) have been described over the last 30 years [52]. Despite a general scarcity of known viruses infecting bacterial acidophiles, support for their existence has been obtained from genomics (e.g. [53]), metagenomics (e.g. [54]) and enrichment cultures (e.g. [55]). Also CRISPRs (segments of DNA containing short repetitions

of base sequences) which are known to protect microbial cells from invasion by microbial viruses and other foreign mobile genetic elements have been found in genomic and metagenomics datasets derived from acidic environments (e.g. [56]), and to encode diversified virus-directed spacer sequences (e.g. [57]) and prevent the establishment of productive infections in certain acidophilic model microorganisms (e.g. [58]). For instance, exposure of *Sulfolobus islandicus* to *Sulfolobus* spindle-shaped virus SSV9 causes virus-induced dormancy of the cells 24–48 hours post-infection, with a severe impact on host physiology and ecology [58]. Potentially, viruses cause mortalities of specific microorganisms within communities, perturbing their taxonomic and functional organization and dynamics. Such perturbations may even cause the crash of biologically-driven mineral leaching operations [41]. However, understanding of viral impact on the biology and ecology of acidophiles and acidic microbiomes is currently very limited. Similarly, other antagonistic interactions merit further attention.

Conclusions: outlook and applications

The biotechnological potential of acidophiles that mediate redox transformation of iron and sulfur has been exploited in commercial-scale mineral bio-processing operations for over 50 years, with new opportunities for developing 'biomining' technologies emerging more recently [59]. Incorporating different species of acidophiles in natural or synthetic (engineered) microbial

consortia, working in concerted action to perform complex tasks more efficiently, is considered the path to overcoming the limitations of bioleaching with single strains [60]. Despite this fact, engineered consortia have often turned out to be unstable (not robust), largely because synthetic assemblies and naturally-occurring consortia transferred to foreign environments can eliminate the ecological and evolutionary context in which the relevant interactions were forged and the inherent metabolisms selected. This realization has highlighted the need to better identify the ecophysiological principles that lay behind a community's assembly rules, and that are critical for synthetic microbial consortia design and, in particular, the design of microbial consortia that can be used for bioleaching technologies which are almost exclusively mediated by acidophilic prokaryotes.

'Meta-omic' studies of microbial communities that occur in man-made and natural extremely acidic environments are beginning to make this challenge approachable. However, except for a handful of studies that have actually used available tools to assess the ecological and evolutionary significance of observed variability patterns in ecosystem functioning (reviewed in Ref. [46**]), most community-genomic studies of these extreme systems are so far approximate assessments of the types of organisms present (phylum to genus level), the diversity of metabolic pathways inferred (and only occasionally linked to activity) and/or correlate to spatio-temporal variations (reviewed in Ref. [61]).

The ecological interactions that govern microbiome composition (diversity) and dynamics (stability) in acidic environments remain mostly unresolved. How interactions vary across communities, both in terms of the interacting partners and the nature and strength of the dependencies among microorganisms, requires further analyses. Genome-scale models of metabolism need to be integrated with meta-omics data to elucidate the underlying assembly rules. Such knowledge will offer new opportunities to synthetically engineer relevant interactions and harness acidic microbiomes in renewed biotechnological applications.

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- of outstanding interest

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