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# 2 Microbial evolution: the view from the acidophiles

## 2.1 Introduction

Acidophilic organisms have provided a highly fertile ground for research into micro-bial evolution. Their low-biodiversity communities have allowed for extensive metagenomic, metatranscriptomic, and metaproteomic analysis [1]. A wealth of data from comparative genomics of closely related strains is beginning to reveal the evolutionary processes that allow for genotypic change, and how they relate to selective pressures. In the last decade it has become evident that the genetic diversity available in bacterial communities is vast and in constant flow. DNA is constantly mobilized by plasmids and phage, and recombination occurs at high rates. Recent studies in acidophiles have described not only the type of events that are taking place, but also to begin to make a quantitative assessment of their predominance and rates.

The acidophiles have been mainly studied in two scenarios. The first one, acid mine drainage (AMD) environments, are areas in which organisms rely on chemoautotrophic production mainly based on iron and sulphur oxidation. In addition to very low pH, there are often high concentrations of heavy metals such as iron, zinc or arsenic [1–3]. Well-characterized examples of these environments include the Río Tinto in Southern Spain [4] and Iron Mountain in California, USA [5]. The main actors of these studies have been *Leptospirillum* (*Nitrospira*), *Acidithiobacillus* (*Gammaproteobacteria*) and *Ferroplasma* (Archaea, *Thermoplasmata*). The second scenario is the volcanic springs or "mud pots" generated by geothermal activity, in which, in addition to extreme acidity, organisms must contend with temperatures that can reach 80 °C. These environments are dominated by thermoacidophilic Archaea and the main actor of evolutionary studies has been the genus *Sulfolobus* (*Crenarchaeota*).

Although it had been assumed that extremely acidic environments could prove hostile or limit DNA exchange, there is no evidence that mechanisms of gene transfer or genomic change are different from those operating in other less-extreme habitats. Numerous phage, plasmids, and mobile elements have been described in association with acidophilic communities or as part of the genomes of acidophiles, as well as mechanisms for DNA uptake, DNA secretion, or CRISPR (clusters of regularly interspaced short palindromic repeats) defense systems [see reviews in 6–9]. Indeed, phage are abundant and diverse in all environments where acidophilic prokaryotes have been found. For example, the optimal growth conditions of the *Sulfolobus* turreted icosahedral virus (STIV) are pH 3.3 and 80 °C [10]. There is some evidence, however, that DNA exchange among acidophilic organisms, even when not closely related phy-

logenetically, is more frequent than DNA exchange from organisms from other habitats, pointing to the relative isolation of these extreme ecosystems. Further, the conditions endured by acidophiles were thought initially to impose special requirements for DNA repair or damage tolerance, but no significant differences have been found if compared with organisms living in less-extreme habitats. For example, the mutation rate in the hyperthermophilic archaeon *Sulfolobus acidocaldarius* was shown to be equal to mesophilic organisms [11].

Interest in the evolutionary mechanisms present in acidophiles is twofold. First, acidophiles living in AMD environments are relevant in biomining (bioleaching and bio-oxidation) for the extraction of copper and gold [12]. However, the manipulation of cultures of acidophilic organisms for use in biomining is still in its infancy. Since microbial consortia are involved, often consisting of one or few dominant species accompanied by low-abundant but diverse bacteria, the problem to be addressed is the understanding of how communities change given a set of environmental parameters. Second, *Sulfolobus* and other thermoacidophilic Archaea have been studied for their potential in production of thermostable enzymes and processes of interest in biotechnology [13]. However, despite recent advances, the genetic engineering of *Sulfolobus* is also limited [14, 15].

In this review we describe the major advances of recent years in the study of the evolutionary mechanisms that shape the genomes of acidophilic organisms, as well as the ecological scenarios in which these changes take place. Finally, we suggest future avenues of research into this fascinating group of microorganisms.

## 2.2 Horizontal gene transfer

Horizontal gene transfer (HGT), the transference of genetic material between organisms not directly related genealogically, is pervasive in bacterial communities. It has been widely documented among the acidophiles, a phenomenon that could point to the fact that acidic environments are probably relatively closed habitats. For example, the sequencing of the *Picrophilus* genome has shown that it contains almost as many genes in common with the phylogenetically close organism *Thermoplasma* (*Euryarchaeota*) as with the phylogenetically distant *Sulfolobus solfataricus* (*Crenarchaeota*) [16]. Also, *Sulfolobus islandicus* strains often share genes with other *Sulfolobus* species [17], possibly highlighting the fact that these environments are quite refractory to foreign genetic input.

An extraordinary example of HGT has been demonstrated recently with the sequencing of the genome of acidophilic red algae *Galdieria* [18]. Comparative genomics have shown that adaptation to the acidic environment, heavy-metal resistance, and metabolic versatility can be directly attributed to at least 75 separate gene acquisitions from Archaea and Bacteria. For example, the bacterial arsenic membrane protein pump ArsB likely was acquired from thermoacidophilic Bacteria, and the acetate

permeases and polyamine transporters present in *G. sulphuraria*'s genome originate from Bacteria and Archaea, respectively [18]. Interestingly, genes recruited by HGT into the algae's genome are specially enriched in those from extremophilic Bacteria, again suggesting the closed ecosystem idea. Typically eukaryotes evolve via gene duplications and neofunctionalizations, but this alga has adapted the prokaryotic way, by appropriation of genes by HGT.

HGT seems the key to explain fast adaptation and evolution of genomes in short evolutionary time-scales. Genes which may provide an ecological advantage or are estimated to be physiologically relevant have often been observed in recently transferred blocks of DNA in the genomes of acidophiles. These include, for example, quorum sensing genes of the LuxIR system in *Leptospirillum* [19], metabolic genes (toluene monooxygenase and nitrate reductase) in *S. islandicus* [20], or genes allowing adaptation to metal tolerance and acidity (arsenic-specific operons *ars2* and *aox*, biofilm formation, and motility) in *Thiomonas* [21] and *Acidithiobacillus* [22]. Many of these genes are likely niche-specific which could contribute to adaptation in restrictive ecosystems.

Of considerable interest is to ascertain the vehicles for genetic flow in natural populations, and to quantify their relative importance. Of the three classical mechanisms, natural transformation, conjugation (plasmid-mediated), and transduction (phage-mediated), transformation by free DNA is probably limited among the acidophiles due to the hydrolysis of DNA in acidic conditions with high metal content [7]. However, the analysis of the half-life of free DNA in acidic environments clearly requires further quantitative studies. Wide host-range phage could be responsible for some limited HGT but a recent study indicated that they could be poor vectors in prokaryotes due to the tight packing in their genomes [23]. However, the extraordinary abundance and variety of phage in natural ecosystems make any conclusions in this respect highly speculative. Conjugation, mediated by plasmids or ICEs (integrative conjugative elements), which require physical contact between donor and recipient cells, seems to be the main mechanism of HGT [24]. The host range is wider for conjugation than for transduction. In this regard, the genes required for DNA transfer and processing have been found in the genomes of most acidophiles.

## 2.3 The mobilome

The sequencing of full genomes of diverse strains of the same species has revealed the existence of a core genome that is common to all of them, and a variable or 'flexible' genome that is strain-specific and the product of genetic exchange and recombination. For example, a study of 7 strains of *S. islandicus* isolated from 3 different locations showed that their variable genome accounts for 20–30% of the genes [17]. A recent study comparing two strains of *Acidithiobacillus caldus* has shown that about 20% of the genes present in their genomes were strain-specific, and that a large num-

ber of mobile elements (including plasmids, transposons, and integrative elements) suggests a high degree of genetic flux [25].

The sequencing of various genomes from acidophiles have revealed the presence of genomic islands, segments of the chromosome of up to 200 kb in length and which are distinct from the core genome [26]. Genomic islands identified in most sequenced genomes in acidophiles are often associated with prophages, transposable elements, or plasmid mobilization elements. Many genomic islands contain a 'recombination module' containing at least an integrase, which can be used to track their evolutionary history, and its attachment sites [27]. Genomic islands have often been transferred via HGT in the recent evolutionary past, as revealed by their anomalous nucleotide composition (G+C content or codon usage). They can also be identified by comparative genomics of phylogenetically close relatives. Interestingly, genomic islands often encode genes that are directly selectable and environmentally relevant, such as those for heavy-metal resistance, DNA repair, biofilm formation, or motility. It is for this reason that they are considered critical during adaptation to changing environments, ecological differentiation, and, in general, genome evolution. For example, a comparative analysis of genomic islands found in two strains of *Acidithiobacillus ferrooxidans* showed major differences in gene content, with predominance of metal resistance (e.g., mercury detoxification, copper transport) and metabolic genes of ecological relevance [28].

## 2.4 Phages

Phages are essential players in microbial ecosystems and genome evolution, yet little is known about their role, dynamics, and impact in acidic environments. The study of phages is now being revolutionized by the use of high-throughput sequencing techniques and bioinformatics. Phages have been found in all environments where bacteria can be found and there are clearly no physical limitations to the maintenance of large and highly diverse phage communities in extreme acidic conditions. Phage predation is a major selective force for the evolution of bacterial populations, and some authors believe that they serve to preserve metabolic diversity by allowing the coexistence of multiple bacterial strains rather than just one [29]. On the other hand, it has been suggested that the huge phage 'metavirome' could serve as a genetic reservoir and allow the quick retrieval of advantageous genes under shifting environmental conditions. Another possible function of phage is as vehicles and facilitators for intra- and inter-species HGT. Often phage have broad host ranges, as has been shown for prophages found in the genomes of *Ferroplasma* and G-plasma, and in the genomes of *Leptospirillum* Groups II and III [30, 31]. Also, it has been suggested that phage transduction could promote the rise of phenotypic differences and fast and adaptive evolution in coexisting populations of *Ferroplasma* [32].

The study of several phages that infect *Sulfolobus* species has revealed two major forms that are genus-specific, the spindle-shaped viruses (SSVs) and the rod-shaped viruses (SIRVs), which could be specific to *S. islandicus* [33]. Local studies of these phages at specific hot spring locations throughout the world revealed high sequence diversity, suggesting that the combination of high isolation and mutation drove phage population evolution. Surprisingly, a recent study of SSV and SIRV population dynamics over a period of two years in the hot and acidic springs of Yellowstone National Park has demonstrated that phage migration from distant [global] locations contributes critically to maintaining local diversity [34]. Indeed, the rate of virus immigration and colonization, followed by extensive recombination, was significantly higher than mutation, and the reason for the high local genetic diversity. This study highlighted the fact that the fast-evolving phage populations must be studied from a temporal point of view, with repeated sampling. Further, this study demonstrated that phages, which could most likely travel long distances by air currents, could be critical in gene shuffling for bacterial populations at a global scale [34].

The highly complex relationship between phages and bacteria has been enhanced by the discovery and study of the CRISPR loci in bacterial genomes. These chromosomal regions consist of hypervariable arrays of short segments of phage, plasmid, or transposon DNA, and are thought to reflect the history of infections of that specific genome [35]. CRISPR loci serve as templates for the synthesis of antisense RNA that is used to target and destroy incoming foreign DNA, and have been used to analyze the dynamics of virus and hosts in AMD populations [36, 37]. CRISPR loci, whose study was pioneered in acidophiles, are currently being successfully developed into the newest-generation of tools for gene manipulation in eukaryotes [38].

## 2.5 Plasmids

Plasmids are the most important vehicles for HGT and critically contribute to host-cell adaptability and fitness. Many plasmids have been described for most acidophilic Bacteria [6] and thermoacidophilic Archaea [7]. They often carry niche-specific genes that point to their contribution to ecological adaptation as, for example, the arsenic-resistant genes contained in plasmids isolated from *A. caldus* [39], or genes mediating conjugation. The comparative analysis of two strains of *A. caldus* has shown the presence of a megaplasmid (> 150 Kb) in both strains, and a large number of metabolic genes also present in the chromosome, suggesting that the exchange of genes from chromosomes to plasmids and vice versa is fluid [25]. In *Leptospirillum*, however, a large plasmid was described containing a large number of proteins of unknown function, making the contribution of these elements to the metabolic potential difficult to deduce [40]. Plasmids isolated from *Acidiphilium symbioticum* have been reported to provide resistance to cadmium and zinc, and to carry a multi-drug efflux system [41]. Although the potential of plasmids as biotechnology tools is obvious, their use as ge-

netic tools for manipulation of organisms with bioleaching potential is still in development.

## 2.6 Transposons

Although phages and plasmids can ferry genes between cells in the bacterial population, another class of mobile elements, transposons, facilitate the flow of genes between replicons. When present in high copy number, transposons create instability in the chromosome by increasing recombination, deletions, and chromosomal rearrangements. The insertion sequences (ISs) are the smallest of transposons, often carrying only one gene encoding the transposase required for their movement. Since insertion sequences are ubiquitous and very abundant, they facilitate DNA exchange within species in bacterial communities [42, 43]. Transposon activity could be an indication of accelerated change in genomes, or of stress under fluctuating environmental conditions. However, studies of these small mobile elements have been limited by their high diversity and the difficulties in tracing their genealogies and behaviour at the population-level. On the other hand, since ISs are extremely variable, they are excellent bacterial strain-level genetic markers. For example, substantial differences in IS content patterns can be found in strains of *S. islandicus* [17] or in *A. caldus* [8, 25].

Transposon expression was detected by proteomics in AMD biofilms dominated by *Leptospirillum* in early-stage biofilms (see below, [44]) and by microarray hybridization in *Leptospirillum ferrooxidans* [45], highlighting the fast dynamics of these elements. Changes in IS composition have also been observed in laboratory settings [46] or during cultivation of *Ferroplasma* [32]. Further studies of changes in IS copy number and location in chromosomes will be required to examine the impact of ISs in adaptation.

In addition to their effects as catalysts of recombination and genetic change, ISs can be carriers of genes of ecological relevance. For example, an IS21-derived transposon was found to contain a nine-gene operon containing arsenic resistance genes [22] in a strain of *A. caldus* isolated in an arsenopyrite bio-oxidation tank, and later was found in a strain of *Leptospirillum ferriphilum* from the same tank [47]. Recent research has shown that ISs interact with their hosts via an essential and universal replication factor, thus facilitating their movement between cohabiting but phylogenetically distant microorganisms [48]. The importance of IS elements is highlighted by the fact that they are often abundant in genomic islands and plasmids, possibly facilitating integration and recombination events.

## 2.7 Evolution and ecology: long term studies of genetic variation

The study of the genomic mechanisms of change shows a picture of great plasticity and adaptive potential. Due to their relatively rare habitats and limited biodiversity, acidic environments have been excellent settings for the initial dissection of evolving microbial communities, as they can be readily analyzed using high-throughput techniques. These techniques have revolutionized ecological analysis because they allow for the relatively unbiased coverage of DNA, RNA, and proteins by metagenomics, metatranscriptomics, and metaproteomics, respectively.

Mueller and colleagues studied a large set of biofilms from an acid mine, with the objective of identifying physiological changes and ecological interactions among organisms [44]. These biofilms were analyzed at different stages of development, generating a detailed view of how metabolism and biodiversity change with time. Bacteria from the genus *Leptospirillum* were always the founders and dominant species within the biofilms but as they aged, the biodiversity increased greatly. Interestingly, the physiology of *Leptospirillum* also changed with the maturity of the biofilms, likely as a result of interactions with other organisms [44]. Mobile DNA elements were especially overrepresented in low-diversity biofilms, probably reflecting a lower selective pressure. Importantly, it also showed that mobile elements, facilitators of genetic diversity and evolutionary change, could be modulated environmentally. It is tempting to speculate that transposons and other mobile elements become active to provide genomic plasticity in a regulated fashion, perhaps as a result of stress.

In another landmark study, Denef and Banfield analyzed underground acidic biofilms in a nine-year period by metagenomics [49]. These biofilms were mostly dominated by six genotypes of *Leptospirillum*. The authors could assemble different genotypes and reconstruct their evolutionary history and relative abundance over time. The conclusion is that successive prevalence of one genotype over the others could happen relatively quickly and be determined by a major recombination event. Indeed, evolution of *Leptospirillum* consisted of a periodic succession of events of HGT, recombination, and selective sweeps. The authors suggest that the evolutionary advantage that determines dominance of a genotype over others could often be determined by just a few genetic changes. In addition, Denef and Banfield could derive for the fist time the single-nucleotide substitution rate of a free-living organism, in a cultivation-independent manner. This rate, $1.4 \times 10^{-9}$ ($\pm 0.2 \times 10^{-9}$) substitutions per nucleotide per generation, is consistent with other estimates of mutation rates in bacterial chromosomes [49].

Detailed metagenomic studies combined with comparative genomics have also been carried out for populations of *S. islandicus*. Whittaker and collaborators have studied two strains of *S. islandicus* that grow in isolated mud pots in the Mutnovsky Volcano in Russia [20]. The patterns of homologous gene flow among genomes of 12 strains show strong signs of sympatric speciation into two groups. These groups show a declining exchange of DNA among them, suggesting that divergence is increasing

with time. Multilocus sequence analysis of many strains collected in 2000 and 2010 show that the two groups are coexisting with no signs of competitive exclusion resulting in extinction of one of them, at least in this time-scale [20]. The nature of the barriers to genetic exchange are obscure, but ecological specialization due to large genomic islands (genomic continents) is most likely responsible. In this case the comparison between aligned genomes and geological records produced an average rate of single nucleotide substitution per site per year of $4.66 \times 10^{-9}$ ($\pm 6.76 \times 10^{-10}$) [17].

While studies of *S. islandicus* have shown that isolation and geographical distance between the volcanic springs correlates with genetic divergence, and that allopatric diversification is possible, the opposite pattern has been observed in studies of *S. acidocaldarius* growing in acidic springs separated by thousands of kilometres [50]. The analysis of these strains has shown near identical genotypes, suggesting rapid, global gene flow among them. These results have led to suggest that, somewhat surprisingly and contrary to what had been often argued, distance does not restrict gene flow among Bacteria or Archaea [17, 50].

## 2.8 Future directions

The study of acidophiles will continue to provide strong insights into evolutionary processes through the integration of high-throughput techniques with ecology studies. This will in turn allow the production of predictive models for community composition and change. Long-term studies aimed at the observation of change in natural settings are essential to address the impact of phage predation on bacterial populations, or the effect of fluctuating environmental conditions on community composition.

Detailed field studies can be complemented with results obtained in controlled laboratory conditions. For example, recombination has been extensively studied in the laboratory for *S. acidocaldarius* [51], and the study of recombination mechanisms of *S. islandicus* using genetic markers is starting to yield insights into their pathways of allopatric speciation [52]. Much remains to be learned about the mechanisms that promote or restrict gene flow on a global scale, and acidophiles could provide an excellent tool for these types of studies. While AMD or volcanic acidic springs could seem isolated from an anthropocentric point of view, genetic exchange between them, perhaps promoted by air-borne bacteria or phages, could be rapid and frequent. In this regard, sampling must be considered carefully when addressing questions of bacterial population dynamics.

The relatively low-biodiversity natural populations of acidophilic organisms could also be a fertile ground for the analysis of the dynamics of transposable elements. Most major questions remain unanswered regarding these critical actors of genomic evolution, such as their major routes of propagation, their capacity to expand explosively and cause lineage extinction, the correlation between their abundance and genomic evolvability, or their role in the growth and reduction of chromosomes.

Transposable elements thrive and evolve on host chromosomes in a fashion that could resemble that of viruses, but very little is known about their dynamics in natural environments, or about their impact on bacterial populations.

The described analysis of the genome of *G. sulphuraria* suggests that transfer of DNA between prokaryotic and eukaryotic organisms could be more frequent than previously expected. Very little is known about the mechanisms of transfer of this genetic material, the selective pressures that favour this exchange, or how the incorporated genes adapt to the host genome. Detailed knowledge of these processes could open new ways to genetic engineering of eukaryotic organisms to perform functions otherwise restricted to Bacteria and Archaea. A largely unexplored eukaryotic diversity is present in acidic environments [53, 53] and proteomic analysis in laboratory settings has shown possible adaptations of these organisms to fluctuating stress conditions [55].

Finally, an area of great interest is the study of acidic underground habitats. Little is known about the structure and biodiversity of the bacterial communities that thrive deep in the earth's crust [3]. Certainly the slow metabolism and growth, coupled to the restrictions in the movement of individual organisms and genetic material, impose a completely distinct evolutionary dynamics compared to organisms living in water-rich, open environments. Although there are major technological challenges in these types of studies, they will ultimately provide essential insights into the role of acidophiles in geochemical processes on Earth and, perhaps, in evolutionary potential beyond our planet [56].

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