

Life in acid: pH homeostasis in acidophiles

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Microorganisms that have a pH optimum for growth of less than pH 3 are termed ‘acidophiles’. To grow at low pH, acidophiles must maintain a pH gradient of several pH units across the cellular membrane while producing ATP by the influx of protons through the F₀F₁ ATPase. Recent advances in the biochemical analysis of acidophiles coupled to sequencing of several genomes have shed new insights into acidophile pH homeostatic mechanisms. Acidophiles seem to share distinctive structural and functional characteristics including a reversed membrane potential, highly impermeable cell membranes and a predominance of secondary transporters. Also, once protons enter the cytoplasm, methods are required to alleviate effects of a lowered internal pH. This review highlights recent insights regarding how acidophiles are able to survive and grow in these extreme conditions.

Biotechnological and fundamental considerations of acidophiles

Both natural and man-made acidic environments occur in the biosphere, including sulfidic mine areas and marine volcanic vents; the microorganisms that inhabit them are termed ‘acidophiles’ and have a pH optimum for growth that is less than pH 3. Acidophiles are most widely distributed in the bacterial and archaeal domains [1] (Table 1) and contribute to numerous biogeochemical cycles including the iron and sulfur cycles [2]. In the production of acid mine drainage (AMD), the release of metal-rich, acidic effluents can cause considerable environmental damage such as the contamination of drinking water. One important effect of acidophiles lies in their biotechnological application for metal extraction from ores [3], and this sustainable biotechnological process is becoming increasingly important [4] because of its reduced and containable pollutant outputs [5]. Acidophiles could also be a source of gene products; for example, acid-stable enzymes with applications as lubricants and catalysts [6].

Acidophiles have potential importance in evolution because metabolic processes might have originated on the surface of sulfide minerals [7] and structuring of the genetic code could have taken place at acidic pH [8]. Also, the low pH and metal-rich conditions in which acidophiles grow might be similar to volcanic aqueous conditions during Archaean and early Proterozoic periods. Therefore, acidophiles could represent primordial relics from which

more complex life evolved. Many acidophiles are found in inaccessible and isolated environments such as AMD sites and geothermal vents. These environments can present an inaccessible physical barrier capable of reducing the colonization potential of neutralophiles (microorganisms that grow at or around neutral pH). Recent bioinformatic analysis of several thermoacidophile archaeal genomes indicates that the similarities between these organisms were greater than expected when compared with other more closely related organisms [9]. Therefore, acidic environments might form an old and genetically distinct niche of life in which (in these environments at least) ecological closeness overrides phylogenetic relatedness [9].

pH homeostasis is essential

Similarly to neutralophiles, acidophiles require a circumneutral intracellular pH (Table 1). However, acidophiles tolerate pH gradients [pH gradient (ΔpH) = $\text{pH}_{\text{in}} - \text{pH}_{\text{out}}$] several orders of magnitude greater than neutralophiles. The ΔpH across the cytoplasmic membrane is intrinsically linked to cellular bioenergetics because it is the major contributor to the proton motive force (PMF; see Glossary) in acidophiles. However, the influx of protons

Glossary

Proton motive force (PMF): A measurement of the energized state of the cell membrane as a result of a charge separation between the cytoplasm and external milieu created by the membrane potential ($\Delta\psi$) and pH gradient (ΔpH) across the membrane. In acidophiles, the PMF is primarily made up of the ΔpH . For example, an acidophile with a near neutral cytoplasm (pH 6) separated from an acidic environment (pH 2) will experience a net force (i.e. the PMF) across the cell membrane that can drive energy dependent processes.

Reversed membrane potential ($\Delta\psi$): Acidophiles have an inside positive $\Delta\psi$ potentially formed by the influx of potassium ions, which inhibits proton entry into the cell. The inside positive $\Delta\psi$ detracts from the large PMF formed by the ΔpH .

Donnan potential: A difference in electrical potential formed between two solutions separated by an ion-exchange membrane without any current flowing through the membrane. A stable Donnan potential or ‘Donnan equilibrium’ is formed when the diffusion of ions in one direction equals the electromigrational flux of ions in the opposite direction, resulting in net zero mass and charge transport.

Secondary transporters: Active secondary transporters are membrane proteins that use the transmembrane electrochemical gradient of protons or sodium ions to drive transport. As a result of the predominance of secondary transporters evident in acidophile genome sequences, they are believed to be an important functional adaptation to life at low pH.

Uncoupler (protonophore): Synthetic, uncharged compounds (such as protonated acids or a conjugate base) that have dissociable protons and permeate bilayers to easily pass across cell membranes. Uncouplers shuttle the net uniprot of protons and ‘uncouple’ proton transport from cellular processes. In acidophiles, uncouplers include organic acids that are protonated (and therefore neutral) at the low external pH and pass into the near-neutral cytoplasm where the proton dissociates, leading to cytoplasmic protonation.

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Table 1. Metabolic characteristics and genome details of selected acidophilic microorganisms^a

Microorganism	Metabolism ^b	pH optimum	pH _{in}	Genome (Mbp)	Genome URL and/or Refs
Acidophilic archaea					
<i>Thermoplasma acidophilum</i>	H	1.4	6.4	1.56	http://img.jgi.doe.gov/ [56]
<i>Thermoplasma volcanium</i>	H	1.58	NA ^c	1.58	http://img.jgi.doe.gov/ [57]
' <i>Ferroplasma acidarmanus</i> '	IO/H	1.2	5.6	1.97	http://genome.ornl.gov/microbial/faci/
<i>Ferroplasma acidiphilum</i>	IO/A	1.3	NA	NA	NA
<i>Ferroplasma</i> type II	NA	NA	NA	~1.97 ^d	http://www.jgi.doe.gov/ [21]
<i>Picrophilus torridus</i>	H	1.1	4.6	1.55	http://www.g2l.bio.uni-goettingen.de/ [9]
<i>Picrophilus oshimae</i>	H	1.1	~4.6	NA	NA
<i>Sulfolobus acidocaldarius</i>	H	1.8	6.5	2.23	http://img.jgi.doe.gov/ [58]
<i>Sulfolobus solfataricus</i>	H	2.5	~6.5	2.99	[24]
<i>Sulfolobus metallicus</i>	IO/SO/A	3.0	NA	NA	http://genomesonline.org/
<i>Sulfolobus tokodaii</i>	SO/H	2.5	NA	2.69	http://www.bio.nite.go.jp
<i>Acidianus brierleyi</i>	IO/SO/A/H	1.5	NA	1.80	http://www.ku.dk/english/
Acidophilic bacteria					
<i>Acidithiobacillus ferrooxidans</i>	IO/SO/A	1.8	6.5	2.9	http://www.tigr.org/
<i>Acidithiobacillus ferrooxidans</i> ATCC23270	IO/SO/A	1.8	6.5	1 ^e	http://www.integratedgenomics.com/
<i>Acidithiobacillus caldus</i>	SO/A	2.5	NA	1	NA
<i>Acidithiobacillus thiooxidans</i>	SO/A	2.5	~7	1	NA
<i>Leptospirillum</i> group II	IO/A	2.0	NA	~1.9 ^d	http://www.jgi.doe.gov/ [21]
<i>Acidiphilium acidophilum</i>	SO/A/H	1.8	6.0	NA	NA
<i>Acidiphilium multivorum</i>	H	3	NA	1	http://www.bio.nite.go.jp/
<i>Acidiphilium cryptum</i>	H	3	NA	3.9	http://genome.ornl.gov/microbial/acry/
<i>Acidocella faecalis</i>	H	2.3	NA	NA	NA

^aData adapted and updated from Refs [54,55].

^bA, autotrophic; H, heterotrophic; IO, iron oxidizer; SO, sulfur oxidizer.

^cNA, not available.

^dDerived from an environmental shotgun metagenome sequence, therefore approximate size.

^eCurrently incomplete.

through the F₀F₁ ATPase to produce ATP intensifies cellular protonation and, if left unchecked, will rapidly dissipate the ΔpH. The functions of proteins and nucleic acids are impaired by protonation, and interference caused by free intracellular protons can impair processes such as DNA transcription, protein synthesis and enzyme activities [10].

pH homeostasis in acidophiles is a poorly understood mechanism central to the growth and survival of an ecologically and biotechnologically important group of microorganisms. The aims of this article are to review several acidophile pH homeostatic mechanisms postulated in the 1980s and early 1990s [11,12] including the use of a reversed membrane potential (Δψ; see Glossary), an impermeable cell membrane, and cytoplasmic buffering. However, at that time, several of the theories lacked clear and rigorous evidence to support how they occurred. We also discuss how the recent sequencing of several acidophile genomes has provided further evidence to support the earlier theories, in addition to suggesting supplementary pH homeostatic mechanisms such as active proton extrusion and organic acid degradation. Recent research will also be reviewed that has identified additional mechanisms for coping with growth at low pH. These mechanisms are not truly 'homeostatic' in that they do not help maintain a near-neutral cytoplasmic pH but rather enable tolerance to low pH or alleviate its effects.

Mechanisms of pH homeostasis

Acidophiles use a variety of pH homeostatic mechanisms that involve restricting proton entry by the cytoplasmic membrane and purging of protons and their effects by the cytoplasm. These methods are described in the following sections.

The cell membrane is highly impermeable to protons

To help maintain ΔpH, acidophiles have a highly impermeable cell membrane to restrict proton influx into the cytoplasm [13] (Figure 1). Because the membrane proton permeability determines the rate at which protons leak inward, the balance between proton permeability, proton influx through energetic and transport systems, and the rate of outward proton pumping determines whether a cell can sustain an appropriate PMF. An example of a highly impermeable cell membrane is the archaeal-specific structures composed of tetraether lipids (as opposed to the ester linkages found in bacterial and eukaryal cell membranes), which have been identified in *Thermoplasma acidophilum* [14], *Ferroplasma acidiphilum* Y^T and Y-2 [15–17], '*Ferroplasma acidarmanus*' [18], *Sulfolobus solfataricus* [19] and *Picrophilus oshimae* [20]. Indeed, differences in lipid head-group structures and ion permeability between the extreme acidophiles '*F. acidarmanus*' and *F. acidiphilum* have been suggested as a reason for the different optimal growth pH of these acidophiles [18]. The low permeability of acidophile membranes (as demonstrated in liposomes derived from *P. oshimae* membrane lipids [19]) is a result of several factors including: (i) the fixed nature of the monolayer such that fracturing of these membranes does not cleave the two opposing lipid layers and opposing polar head groups [19]; (ii) a bulky isoprenoid core [20]; and (iii) the fact that ether linkages characteristic of these membranes are less sensitive to acid hydrolysis than ester linkages [5]. Recently, published results for thermophilic and mesophilic acidophilic archaea indicate that there might be a stronger association between tetraether lipids and tolerance to acid gradients than previously thought [18]. Whole shotgun sequencing and *in silico* genome reconstruction of an

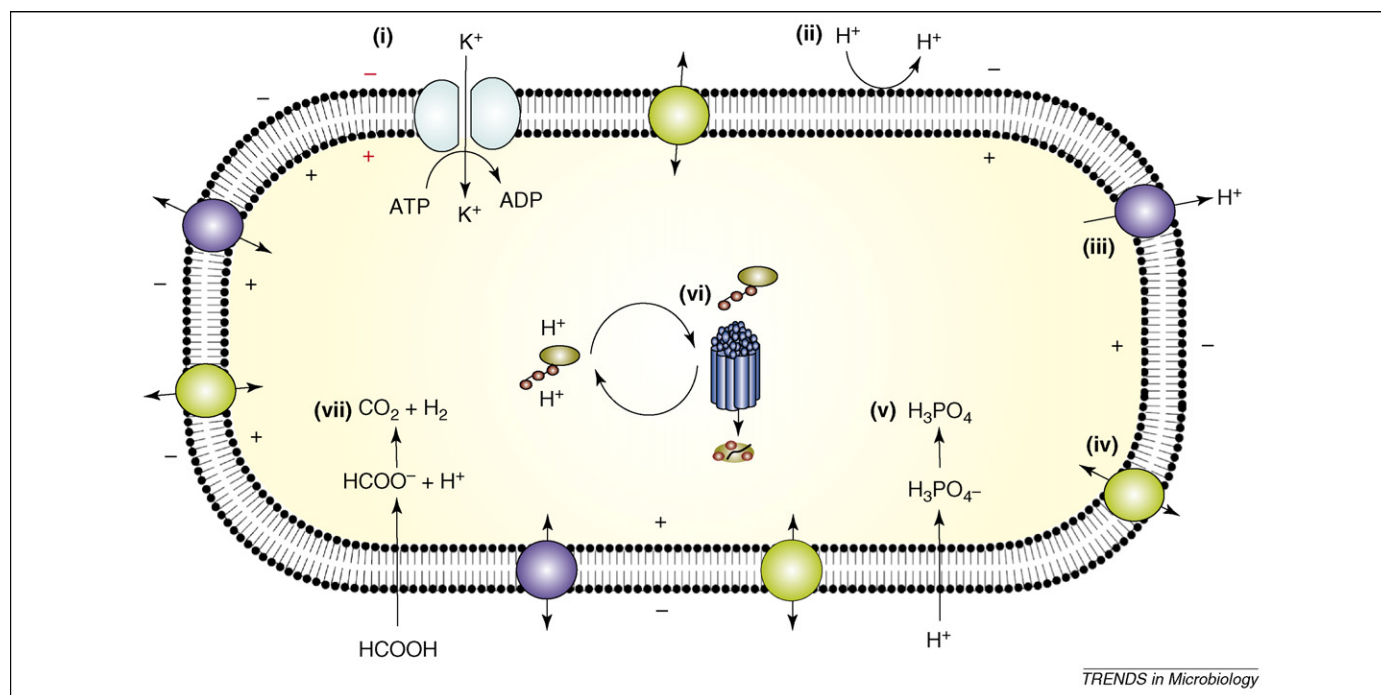


Figure 1. Processes associated with pH homeostasis in acidophiles. (i) Acidophiles reverse the $\Delta\psi$ to partially deflect the inward flow of protons. One potential mechanism of generating a reversed $\Delta\psi$ is by potassium transport – a predominance of potassium-transporting ATPases is found in acidophile genomes. (ii) Many acidophiles have evolved highly impermeable cell membranes to retard the influx of protons into the cell. (iii) ΔpH is maintained through active proton export by transporters (blue). (iv) The sequencing of several acidophile genome sequences has indicated that there is a higher proportion of secondary transporters (green) than in neutralophiles. Overall, they reduce the energy demands associated with pumping necessary solutes and nutrients into the cell. (v) The presence and availability of enzymes and/or chemicals capable of binding and sequestering protons might help to maintain pH homeostasis. (vi) Comparative genome analysis suggests that a larger proportion of DNA and protein repair systems might be present in acidophiles compared with neutralophiles and that this could be associated with the cellular demands of life at low pH. (vii) Organic acids that function as uncouplers in acidophiles might be degraded by heterotrophic acidophiles.

AMD biofilm revealed that *Leptospirillum* group II (subsequently named *Leptospirillum ferriphilum*) dedicates a comparatively large number and variety of genes for cell membrane biosynthesis [21]. The authors suggest that this wide repertoire of genes for the cell wall is indicative of a complex structure that probably has an intrinsic (if to date, largely unknown) role in bacterial acidophile acid tolerance. An alternative explanation could be because of different cell wall requirements that are dependent on differential growth conditions. In summary, a cell membrane impermeable to protons forms an important strategy for acidophile pH homeostasis by limiting the influx of protons into the cell.

Membrane channels have a reduced pore size

The size and permeability of membrane channels might also represent an important mechanism for pH homeostasis. Characterization of an *Acidithiobacillus ferrooxidans* outer membrane porin (Omp40) protein upregulated as a result of a pH shift from pH 3.5 to 1.5 [22] identified a large external L3 loop that could control the size of the entrance to the pore and ion selectivity at the porin entrance. The calculated charge of this loop at pH 2.5 is +2 (compared to -4 at neutral pH for a similar *Escherichia coli* porin) and this charge is postulated to control influx of protons across the outer membrane [23]. The adaptations of acidophile channel-forming proteins have only been addressed in a single study and therefore, they warrant further analysis across a representative range of obligate acidophiles.

Proton influx is inhibited by a chemiosmotic gradient created by a Donnan potential

A further mechanism used by acidophiles to reduce proton influx is the generation of an inside positive $\Delta\psi$ (which is opposite to the inside negative $\Delta\psi$ of neutralophiles). The $\Delta\psi$ is generated by a Donnan potential (see Glossary) of positively charged molecules and inhibits the influx of protons using a chemiosmotic barrier against the proton gradient. This potential is possibly produced by a greater influx of potassium ions than the outward flux of protons (Figure 1). The genomes of the acidophiles *Picrophilus torridus* [9], '*F. acidarmanus*' (<http://genome.ornl.gov/microbial/faci/>), *S. solfataricus* P2 [24] and *Leptospirillum* group II (*L. ferriphilum*) [21] all contain a disproportionately high number of putative cation transporters and it is possible that these transporters are involved in the generation of the Donnan potential. Further evidence for the putative importance of potassium in the generation of the PMF is that respiration-linked proton pumping in *Sulfolobus* spp. requires the presence of potassium ions [25] and the maintenance of $\Delta\psi$ in *Acidithiobacillus thiooxidans* directly relates to its ability to take up cations, with potassium being the most efficient for maintaining the $\Delta\psi$ [26]. It has previously been suggested that the Donnan potential is created by a passive mechanism such that even though the PMF is zero, a small residual inside positive $\Delta\psi$ and ΔpH (which counterbalance each other) are maintained in inactive cells of *T. acidophilum* [27], *Bacillus acidocaldarius* [28], *Acidithiobacillus caldus* (M. Dopson, PhD thesis, Umeå University, 2001), *At. ferrooxidans* [29]

and *Acidiphilium acidophilum* [30]. Recently, Krulwich *et al.* [31] reported that the ΔpH in *B. acidocaldarius* was completely abolished by a protonophore. Subsequently, it was suggested that at least a portion of the reported residual $\Delta\psi$ and ΔpH in metabolically compromised, non-viable cells is an experimental artifact due to binding of the radiochemical used to measure the $\Delta\psi$ and ΔpH within the cell [32,33]. The data suggest that an inside positive $\Delta\psi$ is a ubiquitous strategy in maintaining pH homeostasis [26]. However, recent data has supported the role of energy dependent cation pumps (rather than a passive mechanism) in the production and maintenance of the $\Delta\psi$. Therefore, the role of cation pumps in pH homeostasis warrants further analysis.

Excess protons are pumped out of the cell

To maintain pH homeostasis, acidophiles need to be able to remove excess protons from the cytoplasm and the ΔpH in *B. acidocaldarius* and *T. acidophilum* is created by active proton pumping [32] (Figure 1). Also, all of the available sequenced acidophile genomes have putative proton efflux systems (i.e. H^+ ATPases, antiporters and symporters). For example, the whole-community shotgun sequencing of an AMD biofilm from Iron Mountain identified a predominance of proton efflux systems present on the *Ferroplasma* type II and *Leptospirillum* group II (*L. ferriphilum*) sequenced genomes [21]. Several candidate proton efflux proteins have also been identified in the sequenced genomes of *At. ferrooxidans*, *At. thiooxidans* and *At. caldus* (D.S. Holmes *et al.*, unpublished). The numerous proton-driven secondary transporters (the overall ratio of secondary to primary transporters present on the *P. torridus* and *T. acidophilum* genomes are 10:1 and 5.6:1, respectively; see Glossary) [9] probably represent an adaptation of these organisms to survive in an extremely acidic environment [5] (Figure 1). Thus, by using a predominance of secondary transporters, the PMF can be harnessed for metabolic purposes. Intuitively, it would seem that acidophiles would be capable of using the ΔpH across the membrane to generate large amounts of ATP (demonstrated in *At. ferrooxidans* [34] and *At. caldus* [35] membrane vesicles). However, in viable cells, this would result in rapid acidification of the cytoplasm. Therefore, any protons that enter the cell through the F_0F_1 ATPase need to be balanced by extrusion during electron transport and reduction of molecular oxygen at the terminal oxidase [35], and interference at any point in electron transport results in cessation of metabolism in *At. caldus* [35] and *Sulfolobus acidocaldarius* [36]. The induction and enhanced expression of proton translocating systems to remove excess protons from the cytoplasm has not been clearly defined and represents a relatively unexplored mechanism of pH homeostasis.

Cytoplasmic buffering helps to maintain the intracellular pH

If protons manage to penetrate the acidophile cell membrane, a range of intracellular mechanisms help to ameliorate the ensuing biological damage. First, the buffering capacity of the cytoplasm to sequester or release protons can be used as a pH homeostasis mechanism

(Figure 1). All microbial cells contain a range of cytoplasmic buffer molecules that have basic amino acids (e.g. lysine, histidine and arginine) capable of sequestering protons. For example, the decarboxylation of glutamate and arginine in *E. coli* is involved in cell buffering by consuming protons, which are then transported out of the cell [37]. Other buffering molecules present within the cell include phosphoric acid (H_3PO_4) which has a pK_a of 7.2, and at near-neutral pH the addition or removal of protons has a negligible effect on the pH of this molecule. Zychlinsky and Matin postulated that amino acid side chains were primarily responsible for acidophile cytoplasmic buffering [38]. They compared the buffering capacity of the acidophile *A. acidophilum* and neutralophile *E. coli* as giving 97 and 85 $\text{nmol H}^+ \text{mg protein}^{-1}$, respectively [38]. Also, the cytoplasmic buffering in the acidophilic bacterium PW2 was 85 $\text{nmol H}^+ \text{mg protein}^{-1}$ and with the addition of azide, a net influx of 14.4 $\text{nmol H}^+ \text{mg protein}^{-1}$ occurred that would be sufficient to lower the pH_{in} to 2, although the cytoplasmic pH did not alter [30]. A comparison of the buffering capacity of bacilli with various pH optima did not find that *B. acidocaldarius* has a higher buffering capacity than other bacilli tested [39]. This suggests that although acidophiles are able to maintain pH homeostasis by buffering, their buffering capacity is not necessarily greater than neutralophiles.

Proton uncoupling by organic acids

Organic acids (such as acetic or lactic acid) are harmful to acidophiles because they function as uncouplers (see Glossary) of the respiratory chain at low pH by diffusion of the protonated form into the cell followed by dissociation of a proton [40–42]. Thus, active mechanisms of organic acid degradation might be a pH homeostatic mechanism that is used by heterotrophic acidophiles (Figure 1). An analysis of several extreme acidophile genomes [including *P. torridus* [43] and '*F. acidarmanus*' (<http://genome.ornl.gov/microbial/faci/>)] revealed genes encoding organic acid degradation pathways. These include genes encoding the enzymes propionyl-CoA synthase, two acetyl-CoA synthetases and lactate-2-monooxygenase that convert lactate to pyruvate [40]. Because these archaea are able to gain energy from organic acids, it is unclear if this potential pH homeostatic mechanism is specifically regulated in response to low intracellular pH. However, it is interesting to note that all acidophiles capable of growth at extreme acidic pH values (i.e. $\text{pH} < 0$) are heterotrophs that are potentially capable of degrading organic acids.

DNA and protein damage caused by low pH can be repaired by chaperones

The presence of a large number of DNA and protein repair genes evident on several extreme acidophile genome sequences might also be related to problems associated with pH homeostasis (Figure 1), whereby biomolecules damaged by low pH require fast and efficient repair. The *P. torridus* genome has been shown to contain a large number of genes determining DNA repair proteins [44]. Interestingly, chaperones involved in protein refolding were highly expressed in an environmental AMD biofilm community containing *Leptospirillum* group II (*L. ferriphilum*) and constituted

11% of the total expressed proteome [45]; chaperones were also highly expressed in *F. acidarmanus* cultured during aerobic [46] and anaerobic growth [47]. Also, a drop in the external pH from 3.5 to 1.5 resulted in upregulation of *At. ferrooxidans* proteins in a similar response to heat shock, but not with a shift from pH 1.5 to 3.5 [48]. The prevalence of chaperones in this wide range of acidophiles and conditions suggests that damage to DNA and proteins is a key challenge for survival under acidic conditions.

Intracellular enzymes might be stabilized by 'iron rivets'

Recent analysis of several genes from the obligate acidophile *F. acidiphilum* revealed that the corresponding enzymes were functional at a much lower pH (1.7–4.0) than the predicted intracellular pH of 5.6. The authors suggested several possibilities to account for this phenomenon, including intracellular compartmentalization of enzymes and pH gradients existing within the cytoplasm of this archaeon [49]. The ΔpH in acidophiles is typically 4 to 5 pH units and because *Ferroplasma* spp. are capable of growth at negative pH (the production of negative pH mine waters is described by Nordstrom *et al.* [50]), a further possibility for the role of the low pH optimum of cytoplasmic proteins could be that they need to be functional when the cells grow at such extreme pH values. Subsequently, it was found that the *F. acidiphilum* proteome contains a uniquely high proportion of iron proteins that might contribute to the pH stability of enzymes at low pH [51]. Removal of the iron from six purified *F. acidiphilum* proteins resulted in loss of secondary structure and, consequently, protein activity. This led the authors to suggest that the iron is crucial in maintaining the protein 3D structure and functions as an 'iron rivet' – a potentially ancient property that stabilizes proteins which has been retained in *F. acidiphilum* [51]. These possibilities remain to be confirmed but the low pH optimum of cytoplasmic proteins and the 'iron rivet' could be a mechanism associated with tolerance to growth at low pH.

Genome analysis has not identified ubiquitous DNA adaptations to growth at low pH

A recent comparative analysis of the isoelectric point distribution of several acidophile genomes failed to provide any convincing evidence of an acidophile-specific and proteome-wide adaptation to life in acidic conditions (i.e. in the predicted pI of protein-encoding genes). However, the authors noticed a slight increase in the isoleucine content of the acidophile *P. torridus* [9], which was suggested as contributing to acid stability [52].

Adding heat to the problem

Almost without exception, all obligate extreme acidophile genome sequences are relatively small, especially compared with their neutrophilic counterparts. Indeed, the smallest of these genomes belongs to the *Thermoplasmatales* (<2 Mb; Table 1), which are thermoacidophiles and contain the highest coding density of any free-living prokaryote [9]. It currently remains to be seen what advantages are allied with maintaining a streamlined genome and if the genome size is related to growth at low pH, high temperatures, or both. However, the constant threat of cellular protonation might

be a selective pressure that favors organisms with a relatively small complement of functional genes. Additionally, the DNA sequence could also be altered to reflect adaptations to a thermoacidophilic environment. At high temperature, microorganisms tend to use a higher proportion of purines in their codons, which are more resistant to heat denaturation than pyrimidines (purines have three hydrogen bonds, compared with two in pyrimidines). Unfortunately, at low pH purines are highly susceptible to acid hydrolysis. *P. torridus* (pH_{in} of 4.6 and temperature optimum >60 °C) has adapted to growth at high temperatures by a general increase in the concentration of purine-containing codons as a heat-stabilizing adaptation, while simultaneously reducing the concentration of purine-containing codons in long open reading frames that are more prone to acid-hydrolysis-associated mutations [53].

Concluding remarks and future directions

Acidophiles represent an ecologically and increasingly economically important group of microorganisms. The recent completion of several acidophile genome sequences has supported previous data suggesting that acidophile pH homeostasis requires a complex cell wall structure, that the reversed $\Delta\psi$ is generated by cation transport (while also suggesting the importance of potassium ions), and that proton transporters remove protons once they have entered the cytoplasm. In addition, genome sequencing has suggested a role in low pH growth adaptation for processes such as organic acid degradation, DNA and protein repair systems, and possibly through the maintenance of a small genome size. Using these genome sequences in a functional context through the application of high throughput transcriptomic and proteomic tools to scrutinize acid stress might elucidate further potential pH homeostasis mechanisms.

Far more research in acid homeostasis has been carried out in *E. coli* and *Helicobacter pylori* (which only survive in acid transiently during pathogenesis) than in all obligate acidophiles combined. A disadvantage of genomics (and proteomics or transcriptomics) is that the data are descriptive (hypothesis-generating) and therefore, to rebalance the discrepancy in the amount of research between obligate acidophiles and *E. coli* and *H. pylori*, more work is required to test the generated hypotheses (Box 1). One of the major problems with obligate acidophile research (and probably the explanation for the lack of data in this area) is that the genetic tools necessary for in-depth analysis are still lacking, and the ability to use mutational analyses is necessary to understand the genetic and biochemical basis of pH homeostasis in these microorganisms. Unique technological obstacles to this goal are posed by the acidophilic nature of these organisms, the transcriptional peculiarities of many acidophilic archaea, the lack of genetic elements for vector development, and in the construction of genetic markers in these largely uncharacterized isolates. Accordingly, the development of genetic, biochemical and structural approaches in acidophile microbiology will help to unravel many of the molecular mechanisms that enable life under extreme conditions, and will allow us to glean further insights into the biochemistry and physiology of these organisms.

Box 1. Future research directions

Several important questions remain regarding the mechanisms of acid resistance in acidophiles. These include:

- **Reversed membrane potential**

Do all acidophiles use the same strategy to generate a reversed membrane potential to deflect the inward flow of protons and hence facilitate pH homeostasis? A key area to be addressed is the functional mechanism underlying this resistance mechanism and, in particular, K^+ transport.

- **Membrane channels**

The role of membrane channels in facilitating acid homeostasis has only been defined in a single study. Further scrutiny of these systems across a range of acidophiles is required, including membrane channel protein characterization and mutation studies of their genes.

- **Proton pumps**

Proton pumping is necessary to remove excess intracellular protons, although little is known about how this process is achieved. Key questions include if or how protons are sequestered in the cell and the physical characteristics of membrane pumps.

- **Organic acid degradation**

Organic acids can quickly acidify the cytoplasm and genes responsible for their degradation have been identified in some acidophile genomes. It is probable that acidophiles generate energy or useful products from their degradation but it is not known if organic acid degradation contributes to pH homeostasis. Transcriptional analysis of organic acid degradation genes followed by mutational and phenotypic characterization of acid-challenged isolates might help to clarify their role in pH homeostasis.

- **Acid stable enzymes**

Proteins in the extreme acidophile *Ferroplasma acidiphilum* have an unusually high complement of iron proteins that might function as a 'rivet' at low pH. A comparative analysis of the protein-encoding complement from a representative number of acidophiles is required to address if other acidophiles contain similar machinery. Of special interest would be to test if the related archaeon '*Ferroplasma acidarmanus*', which is capable of heterotrophic growth (i.e. in the presence of only trace levels of iron), contains an equally high complement of iron proteins.

- **Protein and DNA repair systems**

Several reports suggest that adequate protein and DNA repair is an important aspect in cells dealing with acid stress. However, it is unknown if there are any differences in established acidophile protein and DNA repair systems compared with neutralophiles. Real-time and quantitative transcriptional analysis of these genes in conjunction with a structural understanding of the corresponding DNA and protein repair proteins is necessary to answer these questions.

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The WHO and six medical journal publishers have launched the Health InterNetwork Access to Research Initiative, which enables nearly 70 of the world's poorest countries to gain free access to biomedical literature through the internet.

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