ION TRANSPORT IN ACIDOPHILES

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Summary

Acidophilic organisms grow in environments of high acidity and are found in all three major biological lineages, that is, archaea, bacteria, and eukarya. This article focuses on acidophilic bacteria and archae. These constitute an environmentally important group that includes pathogens, such as *Helicobacter pylori,* which causes gastric ulcer disease. Existence in strongly acid environments necessitates the ability to generate a large pH gradient so that the cytoplasm can be maintained at near-neutral pH and acid labile cell constituents can be protected. This in turn requires the capacity to extrude protons

across the thermodynamic barrier imposed by the large pH gradient, as well as to resist proton influx into the cell in response to this gradient. The pivotal mechanism by which acidophiles meet these requirements is by generating a positive inside membrane potential that mitigates the chemical gradient of protons. Several mechanisms, both passive and active, underlie the generation of the membrane potential. The passive mechanisms include proton diffusion potential as well as a Donnan potential arising from the existence of non-diffusible positive charges inside the cells. The active mechanisms involve not only the respiratory chain mediated proton extrusion, but also an electrogenic proton antiporter that appears to exchange two or more potassium ions per cellular proton. In addition, several kinds of chloride conductances exist in these bacteria that evidently facilitate rapid proton extrusion without the generation of charge imbalance. These organisms need special transport proteins that are likely to possess special features to be able to function in an acidic environment and have unique roles. These provide useful targets for the control of these organisms.

1. Introduction

The capacity to tolerate acidic conditions is widespread in the biological world. A large number of pathogenic bacteria, for example, must survive acidic conditions in order to establish infection. Enteric pathogens have to pass through the stomach where the average pH on a 24 h basis is ca. 1.4, and *Helicobacter pylori,* the causative agent of gastric ulcer disease, inhabits the stomach. Intracellular pathogens, such as *Salmonella*, *Listeria, Mycobacterium,* and *Brucella* can also replicate under acid conditions, albeit less extreme ones, since they are able to recruit macrophages (internal pH of ca. 5) for propagation. For non-pathogens also, the ability to tolerate acid is important since acidic environments are wide-spread, encompassing low lying soils, many aquatic environments, mining regions, and sulfur rich geothermal areas.

In relation to external acid conditions, organisms fall into three categories:

1. The neutrophiles, that grow best at neutral pH, but many of which have the capacity to withstand exposure to acid conditions.
2. The acidophiles that require extreme acid conditions (pH of 1 – 4) for optimal growth.
3. *H. pylori* which requires a neutral pH for growth, at least in the laboratory, but which inhabits the stomach.

This article will focus on the latter two groups, although acid tolerance mechanisms in neutrophiles will also be briefly considered to provide context.

Biological entities able to withstand acidic conditions have been objects of curiosity, since acid conditions destroy vital cell components such as DNA, proteins, and lipids. The general acceptance of the Mitchellian framework of proton circulation as the basis of energy generation added a new dimension to this curiosity. Did bacteria pump H^+ more rapidly out of the cell under acid conditions and if so, what co- or counter-ionic movement prevented charge imbalance? What role did membrane permeability to ions play in this phenomenon and did H^+ permeability decrease under acid conditions? Within the broad framework of Mitchell's hypothesis, acid resistance appeared to

provide a magnified perspective on a general property of all living cells, namely their capacity to maintain a different ionic environment inside the cell compared to the outside. Thus, a study of mechanisms underlying acid resistance did not only promise practical benefits. These included prospects for better control of pathogens and of environmental pollution resulting from acid mine drainage and acid rain, as well as of effective technologies for biodesulfurization of fossil fuels, but the study also offered a magnifying lens through which to view fundamental life processes.

2. Acid Resistance Mechanisms in Neutrophiles

Many of the mechanisms responsible for acid resistance in all organisms are linked to chemiosmotic processes and it is therefore relevant to consider the chemiosmotic hypothesis. Proton motive force, according to this hypothesis, is the driving force for many of the cell's energy-consuming reactions, such as ATP synthesis, motility, nutrient uptake, and maintenance of an appropriate cellular ionic milieu. Based on studies with neutrophiles and mitochondria, the hypothesis originally described the components of the proton motive force in these systems. It proposed that oxidation of reducing equivalents such as NADH through the respiratory chain leads to the extrusion of H^+ from the cell into the external medium. This results in the generation of a ca. 1 unit alkaline-inside pH gradient (ΔpH), and a membrane potential ($\Delta\Psi$), negative inside of over 100 mV. Both the chemical H^+ gradient as well as $\Delta\Psi$ exert an inward pull on H^+, the combined force, expressed in mV, being given by the Nernst equation:

$$\Delta p = \Delta\psi - \frac{2.3RT}{F}\Delta pH, \text{ where } \frac{2.3RT}{F} = 58.8 \text{ mV at room temperature.}$$

A Δp of ca. –200 mV is generated in mitochondria and neutrophilic bacteria.

Since H^+ is the commonly involved ion in chemiosmotic processes, chemiosmotic mechanisms are also intimately involved in cytoplasmic pH (pH_i) regulation and acid resistance. When a neutrophilic bacterium, like *Escherichia coli*, experiences lowered pH_i, it accelerates its extrusion of cellular H^+, thereby restoring pH homeostasis. The increased extrusion is achieved by uptake into the cells of K^+, which compensates for the charge imbalance that H^+ extrusion generates. Several active systems have been found in this bacterium for taking up K^+: Kup, Trk, and Kdp. In addition, proteins KefB and KefC are concerned with K^+ efflux and are utilized if K^+ uptake leads to over-alkalinization of the cytoplasm.

Another mechanism for acid tolerance in these bacteria appears to be alteration in membrane permeability to H^+. In *Clostridium acetobutylicum* exposure to low pH changes the membrane lipid composition. The unsaturated to saturated fatty acid ratio is decreased and cyclopropane fatty acid content is increased; these changes are believed to decrease H^+ permeability. Increased phospholipids with amino acid head groups may decrease H^+ permeability in *Staphylococcus aureus.*

Amino acid decarboxylases provide another means for acid tolerance. A well-studied system in this respect is lysine decarboxylation. This reaction removes CO_2 from lysine, generating cadaverine, which by picking up H^+, buffers the internal pH. The protonated

cadaverine is exchanged for the medium lysine by a protein called CadB. This system is induced only in the presence of lysine and low pH_o. These conditions are sensed by special membrane proteins: LysP/CadR (lysine) and CadC (acidity). A protein called Fur is also involved in sensing acidity. The enzyme urease, which hydrolyzes urea, is yet another mechanism for buffering the cytoplasm as will be discussed below.

Broad protection against acid conditions is afforded also by the general stress response. Like myriad other stresses that constantly face bacteria in their habitats, the acid stress is countered by responses at two levels. One is to neutralize the stress itself, and the examples mentioned above refer to this aspect of the acid-stress response. The other level at which stresses are countered is concerned with repair of damage that the stress might cause. Because the damage that different stresses inflict has common ingredients, similar mechanisms are activated in response to different stresses. These make the cell more resistant overall and are termed the general stress response.

The ability to both escape and resist stresses is achieved by the synthesis of a special class of proteins termed the stress proteins, which in turn is attained by the activation under stress of complex regulatory mechanisms involving transcriptional, translational, and posttranslational control. Upon exposure to acid stress, for example, *E. coli* induces some 50 proteins. Many of these are concerned with buffering the cytoplasm as discussed above; others are general stress proteins concerned with repairing damage to cell macromolecules that may result from exposure to a low cytoplasmic pH. Among the proteins in this category are molecular chaperones like DnaK, GroELS, GrpE, and HtpG. Three global regulatory systems have been implicated in the synthesis of acid stress proteins: the stress sigma factor, σ^s, the response regulator protein OmpR, and the two-component system PhoPQ. Mutants of *S. typhimurium* deficient in σ^s, or PhoPQ are highly sensitive to acid stress, and are less virulent.

As stated, this article will focus on bacteria that either require a low pH (1–4) for optimal growth or inhabit a low pH environment. All of these bacteria maintain a near-neutral cytoplasmic pH. Studies so far conducted have dealt with the role of chemiosmotic mechanisms that are involved in protecting the cell cytoplasm from the external acid, and in one of them with enzymatic reactions that consume H^+ in the cytoplasm. No studies have as yet dealt with the role of the stress response in acid tolerance of these bacteria.

3. Mechanism of Growth Under Extreme Acid Conditions

3.1. Proton Motive Force

This has been measured in a large number of acidophiles (Table 1). In respiring cells pH_i ranges between 5.5 and 6.8 in different bacteria, representing a ΔpH of up to 4.5 units, that is, an inward chemical gradient of H^+ of approximately –270 mV.

Unlike the neutrophiles, the acidophiles do not possess an inside-negative $\Delta\psi$, instead the membrane potential is positive inside in these bacteria. Thus, instead of augmenting the chemical pull on H^+ back into the cell, the $\Delta\psi$ in these bacteria subtracts from this force, resulting in a Δp, ranging between –75 to –256 mV. The lower measured values

of Δp could be an artifact of methodologies in which high-density cell suspensions were used. These suspensions may have been oxygen limited, exhibiting only a part of their full capacity to generate Δp.

The fact that the Δp values in acidophiles are by and large comparable to the neutrophiles probably points to the reason why Δψ in these bacteria is of an opposite orientation. Such orientation at once reduces both the energetic span across which H^+ must be extruded, as well as the force that pulls the H^+ into the cells. The capacity of the respiratory chain to extrude H^+ is constrained by thermodynamic factors. Were the acidophiles maintaining a pH gradient of 4 or more (Table 1) to generate a negative-inside Δψ, their H^+ pumps would have to extrude H^+ against a prohibitively large energetic barrier. Similarly, the force impelling H^+ into the cells would acquire a magnitude that the biological membranes are not usually called upon to resist. Indeed, as will be discussed below, as ΔpH increases, there is a parallel increase in Δψ, so that the Δp value is kept within a narrow range, presumably commensurate with the capacity of the proton pumps to extrude H^+ and that of the membrane to resist their influx. For example, in *Thiobacillus acidophilus* Δψ has a value of +35 at pH_o of 4 (ΔpH, 1.8), and +95 at pH_o of 2 (ΔpH, 3.6).

Organism	pH_o	pH_i	ΔpH	$\Delta\psi^{\alpha}$	Δp^{a}
Thermoplasma acidophilum	2.0	6.6	4.6	+120	-160
Bacillus acidocaldarius	3.5	6.8	3.3	+30	-170
Bacillus coagulans	3.0	6.6	3.6	+50	-173
Thiobacillus ferrooxidans	2.0	6.5	4.5	+10	-256
Thiobacillus acidophilus	3.0	5.5	2.5	+60	-90
Sulfolobus acidocaldarius	3.5	6.8	3.3	+30	-170
Acidophilum facile	3.0	5.7	2.7	+90	-75

[a]Millivolts

Table 1. Bioenergetic parameters in active cells of acidophiles

Initial measurements of the above parameters in these bacteria employed radio labeled probes, which accumulate inside the cell in response to ΔpH, or Δψ, and accurately measure these parameters only if they remain in solution inside the cell and do not bind to cell components. Since this aspect can be difficult to establish with certainty, especially at the low pH values at which experiments must be done, there was considerable skepticism about these measurements in acidophiles. However, use of a variety of techniques has given similar results. These techniques include: external pH

change upon cell lysis, fluorescent probes, and nuclear magnetic and electron spin resonance methods.

The last mentioned—electron spin resonance (ESR) technique—proved especially definitive. It makes use of nitroxide spin probes that can be a weak acid, a weak base, or a permeable ion, and exploits the phenomenon of exchange broadening to differentiate the spin signal on the two sides of the membrane. Exchange broadening of an ESR signal occurs upon collision of paramagnetic molecules resulting in an exchange of electrons between paramagnetic species, which quenches the spin-label signal. Thus, addition of a membrane impermeant paramagnetic molecule will quench the signal only outside of the cell, permitting quantification of the probe inside the cells. What is of particular importance is that this methodology permits direct determination of probe binding in a system. A bound probe produces a characteristic distortion of the normal ESR signal and is not quenched when brought into contact with an impermeable quenching agent. This is illustrated in Figure 1 for the probe 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid (PECU) present in an unbound state in cells of the acidophile *B. coagulans*, and for CAT-16 that binds to the cell membrane. The distorted signal generated by the latter probe is evident; furthermore, sonication, which destroys the membrane permeability barrier to the quenching agent, quenches the PECU signal, but not that of CAT-16.

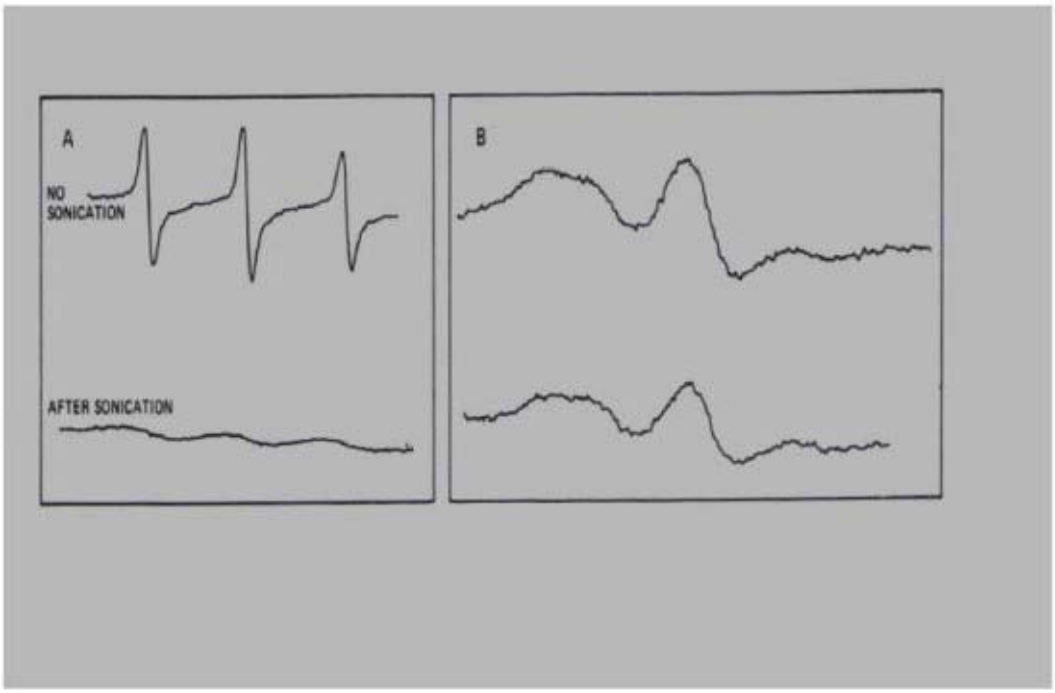

Figure 1. Comparison of ESR probe signal before and after disruption of bacterial cells in the presence of $NiSO_4$. Cells of *B. coagulans* were suspended in the medium at pH_o 3.15, containing nigericin and mixed with quenching agent $NiSO_4$. The cell suspension was split into two aliquots, one of which was sonicated. The ΔpH probe PECU (trace A), or the surface potential probe CAT-16 (trace B), was added to sonicated (lower trace) on nonsonicated (upper trace) cell suspension and the electron spin resonance measurements of the probes were obtained.

3.2. Effect of Protonophores

Protonophores are weak acids that are permeable across the biological membranes in their associated (uncharged) as well as dissociated (charged) state and can therefore bring about equilibration of H^+ gradients across membranes. The Δp components, ΔpH and Δψ, are generally completely abolished in respiring neutrophiles in the presence of this class of compounds, and since neutrophiles and mitochondria were the only entities that had been studied, this was the result expected also in acidophiles. The intuitive feeling that acidophiles must be highly impermeant to H^+ reinforced this notion, as it seemed logical to expect that abolishing H^+ permeability barrier would rapidly collapse the pH gradient.

The reality turned out to be different however. In all the acidophiles studied, a ΔpH of 1–2 units remained in the presence of a protonophore. The experiments included protonophores with different pK_a values and it was shown that they did indeed abolish H^+ impermeability of these bacteria. Moreover, the various techniques for ΔpH, and Δψ measurement mentioned above gave the same results. Protonophore treatment also increased the positive-inside Δψ: in *T. ferroxidans*, it increased from +10 to +192 mV; in *B. coagulans*, from +50 to +90 mV; in *T. acidophilus*, from +60 to +95 mV; and in *A. facile*, from 0 to +90 mV. Within the conceptual framework derived from studies on neutrophiles, this finding also generated considerable initial skepticism.

However, the findings were entirely consistent with what the chemiosmotic hypothesis, applied to a low pH_o environment, would predict. Protonophores, when used at an appropriately low concentration, only introduce an H^+ conductance into the membrane. The resulting H^+ influx into the cells would be expected to rapidly increase the positive-inside Δψ, unless another conductance is simultaneously introduced to compensate for the H^+ influx. Barring this compensation, the increased Δψ would constitute a counterforce limiting further influx of H^+. Indeed in nearly all situations, it was observed that the pH gradient that remained in protonophore-treated cells was matched by an equal positive-inside Δψ and thus represented a thermodynamic near-equilibrium between the two forces, one impelling H^+ into the cells (ΔpH), the other opposing its entry (Δψ). Respiration-inhibited cells also maintained a sizable ΔpH, exhibiting a similar thermodynamic near-equilibrium (Table 2).

Organism	$\Delta pH^{b,c}$	$\Delta\psi^{b}$	Δp^{c}
Bacillus coagulans	-102 (4.7)	+95	-10
Thermaoplasma acidophilum	-126 (5.1)	+100	-26
Acidophilium facile	-132 (5.2)	+107	-25

[a]Inactivation due to inhibition of respiration
[b]Millivolts
[c]Numbers in parentheses indicate intracellular pH

Table 2. Proton gradients in inactive cells of selected acidophiles at pH_o of 3

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-
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(pp. 131-151, focusing on archaebacteria, and the other by A. Matin (pp. 152-166), dealing with acidophiles in general. Figure 10 is taken from the article by G. Schafer, and Table 2 and Figures 6 and 13 are taken from the article by Matin. Besides the articles the discussion is also included and is very useful.]

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Biographical Sketch

A. Matin was born in Delhi, India and received his early education in Karachi, Pakistan. He came to the United States in 1964 and acquired his Ph.D. with honors from University of California at Los Angeles. His thesis subject was intermediary metabolism of the thiobacilli. He then went to the State University of Groningen in the Netherlands and worked over the following four years on two topics: membrane transport and bacterial cellular response to nutrient deprivation. In 1975 he joined Stanford University, where he is a full professor of Microbiology and Immunology. His research interests are the molecular dissection of the bacterial stress response, the bioenergetics of acidophiles, molecular bioremediation, and the molecular basis of enhanced resistance to antimicrobial agents of bacterial biofilms. He has published close to 200 original papers, review articles, and book chapters, and has been a widely invited participant in international symposia and seminars. He has been a member of several program evaluating committees, grant study sections, and editorial boards of journals. He was an American Society Foundation lecturer, is recipient of a Fulbright Fellowship and an elected Fellow of the American Academy of Microbiology. He has consulted widely as an expert witness and for drug companies. He resides at Stanford, California and his hobbies are hiking, walking, and reading.