

Cytoplasmic pH Measurement and Homeostasis in Bacteria and Archaea

Joan L. Slonczewski¹, Makoto Fujisawa², Mark Dopson³ and Terry A. Krulwich⁴

¹*Department of Biology, Kenyon College, Gambier, OH, USA*

²*Department of Pharmacology and Systems Therapeutics, New York, NY, USA*

³*Department of Molecular Biology, Umeå University, Umeå, Sweden*

⁴*Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA*

ABSTRACT

Of all the molecular determinants for growth, the hydronium and hydroxide ions are found naturally in the widest concentration range, from acid mine drainage below pH 0 to soda lakes above pH 13. Most bacteria and archaea have mechanisms that maintain their internal, cytoplasmic pH within a narrower range than the pH outside the cell, termed “pH homeostasis.” Some mechanisms of pH homeostasis are specific to particular species or groups of microorganisms while some common principles apply across the pH spectrum. The measurement of internal pH of microbes presents challenges, which are addressed by a range of techniques under varying growth conditions. This review compares and contrasts cytoplasmic pH homeostasis in acidophilic, neutralophilic, and alkaliphilic bacteria and archaea under conditions of growth, non-growth survival, and biofilms. We present diverse mechanisms of pH homeostasis including cell buffering, adaptations of membrane structure, active ion transport, and metabolic consumption of acids and bases.

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ABBREVIATIONS

CFAs	cyclopropane fatty acids
cFSE	carboxyfluorescein diacetate succinimidyl ester
GFP	green fluorescent protein
OMPs	outer membrane porins
PMF	protonmotive force
RSO	right-side-out
SCWPs	secondary cell wall polymers

1. INTRODUCTION

All microbes have evolved to grow within a particular range of external pH. Historically, pH has played many key roles in the development of

microbiology. Since ancient times, fermentation has produced storable food products containing inhibitory acids, such as dairy products and vinegar (Buckenhüskes, 2001; Johnson and Steele, 2001) or foods containing alkali, such as *natto* from soybeans and *dadawa* from locust beans (Wang and Fung, 1996). Acidophiles have been exploited for thousands of years for the recovery of valuable minerals (Olson *et al.*, 2003; Rohwerder *et al.*, 2003) but they also cause pollution and corrosion (Johnson and Hallberg, 2003). Alkaliphiles have been exploited for natural products, especially enzymes with high pH optima (Horikoshi and Akiba, 1982; Horikoshi, 1999).

The external pH partly determines the cytoplasmic or intracellular pH, which affects enzyme activity and reaction rates, protein stability, structure of nucleic acids, and many other biological molecules. However, most microbes maintain some degree of pH homeostasis such that the cytoplasmic pH is maintained within a narrower range than external pH, usually closer to neutrality (Fig. 1). For example, *Escherichia coli* during optimal growth conditions maintains its cytoplasmic pH within a range of pH 7.4–7.8 over an external pH range of 5.0–9.0 (Slonczewski *et al.*, 1981; Zilberstein *et al.*, 1984; Wilks and Slonczewski, 2007), and a similar range is observed for *Bacillus subtilis* (Shioi *et al.*, 1980). On the other hand, the alkaliphile *Bacillus pseudofirmus* OF4 shows a range of cytoplasmic pH 7.5–8.3 over the range of external pH 7.5–10.6 (Sturr *et al.*, 1994; Krulwich, 1995) and resting cells of *Acidithiobacillus ferrooxidans* maintain an internal pH between 6.0 and 7.0 over an external pH range of 1.0–8.0 (Cox *et al.*, 1979).

Microbes grow in different ranges of environmental pH, from pH 0 (Nordstrom and Alpers, 1999; Nordstrom *et al.*, 2000) to above pH 13 (Roadcap *et al.*, 2006). It is intriguing that over the range of microbes studied, the crossover point where cytoplasmic pH equals external pH lies between pH 7 and 8, although not all species can actually grow at this point. Actual pH ranges vary in breadth and transmembrane pH difference (ΔpH) but, in general, acidophiles are defined as organisms growing optimally within the pH range 0.5–5, neutralophiles within pH 5–9, and alkaliphiles within pH 9–12. Bacteria and archaea adapted to different habitats in different ranges of environmental pH have evolved diverse mechanisms of pH homeostasis, while some common principles of homeostasis apply across the pH spectrum. In this review, we compare and contrast the mechanisms of cytoplasmic pH homeostasis in acidophilic, neutralophilic, and alkaliphilic bacteria and archaea. We aim to synthesize patterns of cytoplasmic pH behavior across the range of pH environments, seeking connections that may be overlooked when only one range is considered. For example, the relationship between the ΔpH and the transmembrane electrical potential ($\Delta\Psi$) in maintaining pH homeostasis shows a striking pattern of pH dependence across many species, as discussed below.

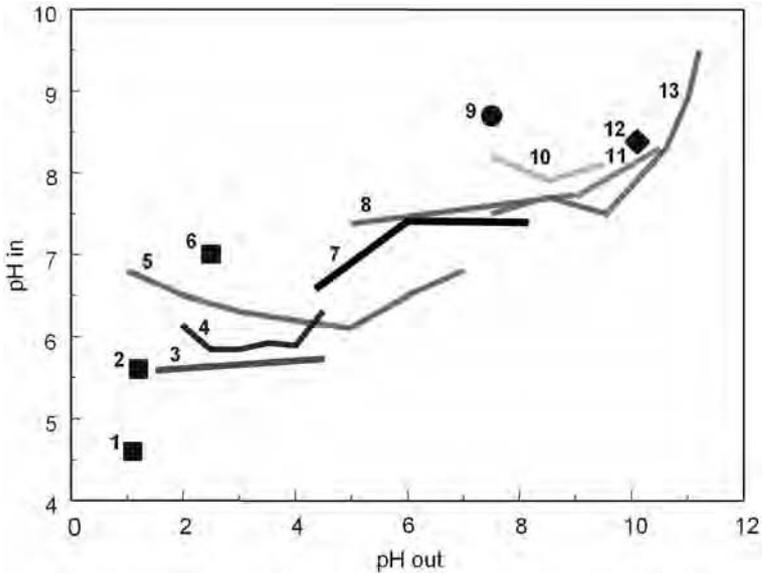


Figure 1 Cytoplasmic pH as a function of the external pH among acidophiles (■), neutralophiles (●), and alkaliphiles (◆). Acidophiles: (1) *Picrophilus torridus* (Fütterer *et al.*, 2004); (2) *Ferroplasma acidarmanus* (Baker-Austin and Dopson, 2007); (3) *Acidiphilium acidophilum* (dark purple; external pH 1–4.5; Matin *et al.*, 1982); (4) *Bacillus acidocaldarius* (dark blue; pH 2–4.5; Krulwich *et al.*, 1978); (5) *Acidithiobacillus ferrooxidans* (red; pH 1–7; Cox *et al.*, 1979); and (6) *Acidithiobacillus thiooxidans* (Baker-Austin and Dopson, 2007). Neutralophiles: (7) *Bacillus subtilis* (black; pH 4.5–8; Shioi *et al.*, 1980); (8) *Escherichia coli* (green, pH 5–9; Slonczewski *et al.*, 1981); and (9) *Bacillus licheniformis* (Hornbæk *et al.*, 2004). Alkaliphiles: (10) *Bacillus cohnii* (orange; pH 7.5–9.5; Sugiyama *et al.*, 1986); (11) *Bacillus pseudofirmus* RAB (turquoise; pH 9–10.5; Kitada *et al.*, 1982); (12) *Bacillus alcalophilus* (Hoffmann and Dimroth, 1991); and (13) *B. pseudofirmus* OF4 (brown; pH 7.5–11.2; Sturr *et al.*, 1994). (See plate 1 in the color plate section.)

1.1. Perturbation of Cytoplasmic pH

The cytoplasmic pH is buffered by small organic molecules such as amino acids, as well as by ionizable groups on proteins and inorganic polymers such as polyphosphate; typical buffering capacities for different species range from 50 to 200 mM protons per pH unit shift (Slonczewski *et al.*, 1982; Zychlinsky and Matin, 1983b; Krulwich *et al.*, 1985a; Rius *et al.*, 1995; Rius and Lorén, 1998; Leone *et al.*, 2007). As discussed in detail below, the

approximate degree of cytoplasmic buffering appears remarkably similar for most bacteria across the range of environmental pH.

Despite the various sources of buffering, in the absence of active mechanisms of pH homeostasis, ionophores or permeant acids or bases rapidly shift the cytoplasmic pH via influx or efflux of protons. Membrane-permeant acids such as fermentation acids become concentrated within the cytoplasm. This concentrative effect is observed when there is a pH difference across the membrane with the cytoplasmic pH higher than the external pH. Such a pH difference is generally small in neutralophiles but is very large in acidophiles, thus making membrane-permeant acids correspondingly more toxic in acidophiles. The accumulation of acids in the cytoplasm results in partial or complete failure of pH homeostasis, and may increase the concentration of an organic acid that can retard growth (Kihara and Macnab, 1981; Salmond *et al.*, 1984; White *et al.*, 1992). Similarly, the accumulation of membrane-permeant bases such as polyamines is favored under conditions in which the cytoplasmic pH is lower than the external pH. Under these conditions, the uncharged base that enters the cell is protonated in the cytoplasm and this consumption of cytoplasmic protons can impair pH homeostasis (Repaske and Adler, 1981; Yohannes *et al.*, 2005).

In growth media with high organic amine content, alkaliphilic bacteria are at special risk for cytoplasmic accumulation of ammonium at the expense of cytoplasmic protons, thus compromising pH homeostatic mechanisms. In alkaliphilic *B. pseudofirmus* OF4, an ammonium efflux system has a role in facilitating growth under these conditions (Wei *et al.*, 2003). Müller *et al.* (2006) studied the effect of high ammonium concentrations on several neutralophilic bacteria and concluded that high ammonium concentrations were not generally detrimental. However, *B. subtilis* mutants lacking one of the cation/proton antiporters important for alkaline pH homeostasis show a large decrease in expression of ammonium transport system, for example, 17-fold decrease in *nrgA* (Wei *et al.*, 2006). The down-regulation of the ammonia transport in the antiporter mutant implies that ammonia is detrimental to the cell under alkali stress. It would be of interest to re-visit the issue of general ammonium toxicity for neutralophiles under alkaline conditions.

Depending upon whether the cytoplasmic pH is higher or lower than the external pH, the ΔpH contributes or detracts from the total protonmotive force (PMF) that drives bioenergetic work. The PMF is an electrochemical or chemiosmotic gradient of protons that is established by active proton pumping and modulated by secondary ion movements (Mitchell, 1961; West and Mitchell, 1974; Booth, 1985; Macnab and Castle, 1987). The PMF

comprises the chemical gradient of protons (ΔpH) and the transmembrane electrical component ($\Delta\Psi$). The PMF in millivolts (negative) is equal to $\Delta\Psi - 60\Delta\text{pH}$ at 25 °C; thus, a ΔpH (alkaline inside) contributes to the PMF. The PMF energizes most active processes that contribute to bacterial pH homeostasis; drives ATP synthesis and ion-coupled solute uptake or efflux; and motility. The PMF must be sufficiently persistent to drive energy requiring processes and therefore, the membrane is proton impermeable such that the PMF is not rapidly dissipated by proton and/or charge movements across the membrane. While there are mechanisms for maintaining homeostasis of membrane ion permeability, especially proton permeability (Albers *et al.*, 2001), certain membrane lipids (such as the tetraethers in acidophilic archaea) confer greater proton impermeability on some species than others.

Other conditions can deplete the PMF such as the presence of an ionophore. For example, dinitrophenol or combination of permeant ions can cause a failure of cytoplasmic pH homeostasis (Khan and Macnab, 1980; MacLeod *et al.*, 1988). Another factor to consider in connection with PMF generation by proton-pumping complexes is that bacteria and archaea exhibit an amazing range of electron donors used for energy generation. Interestingly, use of some substrates mandates an extreme lifestyle. An example noted by Ferguson and Ingledew (2008) is that oxidation of ferrous iron by microorganisms such as *A. ferrooxidans* occurs at low pH because low pH minimizes auto-oxidation and maximizes solubility but also because the mid-point potential of the oxygen/water couple is higher at pH 2 than at pH 7. Since the mid-point potential for the ferrous/ferric couple does not change concomitantly, more energy is available from ferrous oxidation, which improves the energetics at low pH.

The balance between ΔpH and $\Delta\Psi$ across many species shows distinctive patterns that reflect dependence upon pH. Acidophiles, as well as neutralophiles that survive under extremely acidic conditions (below pH 3) maintain a cytoplasmic pH that is much less acidic than the external pH, and they exhibit an inverted $\Delta\Psi$ (inside positive) which subtracts from the PMF while helping to maintain a cytoplasmic pH that is only mildly acidic (Foster, 2004; Baker-Austin and Dopson, 2007). Thus, in extreme acid, the ΔpH solely contributes to the PMF in the chemiosmotically productive direction. Conversely, at high external pH, alkaliphiles as well as neutralophiles have an inverted ΔpH (acid inside) that subtracts from the PMF but results in a cytoplasmic pH that is only mildly alkaline even when the external pH is above pH 9 (Yumoto, 2002; Saito and Kobayashi, 2003; Padan *et al.*, 2005). Under these conditions, the $\Delta\Psi$ is the only chemiosmotically productive component of the bulk PMF. However, there

is evidence that proton sequestration occurs near the outside of the bacterial membrane and/or proton translocation near the outer membrane surface occurs faster than the equilibration of pumped protons with the bulk liquid phase outside the cell (Heberle *et al.*, 1994; Mulkidjanian *et al.*, 2006; Brändén *et al.*, 2006). During growth under alkaline conditions, the concentration of such surface-associated protons relative to the cytoplasmic proton concentration could constitute a ΔpH that is more chemiosmotically favorable than the bulk ΔpH (Krulwich, 1995; Mulkidjanian *et al.*, 2006).

Outside the range of pH permitting growth, pH homeostasis gradually or abruptly fails. Nevertheless, many species can survive (remain viable) for extended periods at pH values outside their growth range, ready to grow again when the pH returns to the optimum. For instance, clinical isolates of *E. coli* can survive several hours of exposure below pH 2 (Gorden and Small, 1993; Buchanan and Edelson, 1999; Price *et al.*, 2000). *E. coli* as well as *Vibrio cholerae* also survive but do not grow in some of the alkaline-saline waters into which they are released during their passage from animal hosts (Colwell and Huq, 1994; Rozen and Belkin, 2001). Acid- or base-resistant strains generally express inducible mechanisms of “acid resistance” or “base resistance,” enabling them to survive without growth at extreme pH (Small *et al.*, 1994; Foster, 2004). These mechanisms have some factors in common with the pH homeostasis mechanisms of extreme acidophiles and alkaliphiles. It is also evident that different bacteria vary not only in their capacity for pH homeostasis, but also in their ability to survive or even grow when their cytoplasmic pH is significantly below or above the pH range tolerated by most bacteria (see Fig. 1).

1.2. Mechanisms of pH Homeostasis

Diverse mechanisms that maintain active pH homeostasis greatly supplement the contribution of passive cytoplasmic buffering. Major categories of active pH homeostasis mechanisms include: coupling transmembrane proton movements to an energetically favorable exchange with cations (K^+ , Na^+) or anions (Cl^-), a strategy that is the central active component of alkaline pH homeostasis (Macnab and Castle, 1987; Padan *et al.*, 2005; Krulwich *et al.*, 2007); metabolic switching to generate acidic or neutral end-products (Stancik *et al.*, 2002; Wei *et al.*, 2006); acid-induced amino acid decarboxylases, and base-induced amino acid deaminases (Blankenhorn *et al.*, 1999; Foster, 2004; Richard and Foster, 2004); use of urease activity, sometimes working together with carbonic anhydrase activity, to regulate cytoplasmic and periplasmic pH (Stingl *et al.*, 2001, 2002; Sachs *et al.*, 2005,

2006); synthesis of acid-resistant membrane structures such as cyclopropane fatty acids (CFAs; Cronan, 2002) and tetraether lipids (Baker-Austin and Dopson, 2007) or increased synthesis of anionic phospholipids or specific neutral lipids at high pH (Clejan *et al.*, 1986); and chaperone protection from temporary damage due to pH shift (Stancik *et al.*, 2002). Some mechanisms are regulated as components of larger regulons, such as the RpoS-dependent acid resistance Gad regulon (Ma *et al.*, 2004). Others respond to pH with a combination of transcriptional responses together with a substantial component of activity control by pH. For example, the major Na^+/H^+ antiporter of *E. coli*, NhaA, is transcriptionally regulated by both sodium and by an RpoS-mediated response to the growth phase (Karpel *et al.*, 1991; Dover and Padan, 2001) but antiport activity is also dramatically and directly enhanced by alkaline pH activation (Padan *et al.*, 2004; Padan, 2008). High-resolution structural information recently obtained for NhaA (Hunte *et al.*, 2005) has made it possible to integrate extensive biochemical and genetic data into a detailed model for the mechanism of activity control of the antiporter by pH (Padan, 2008). Structural biological studies will similarly be central to understanding the specific adaptations in the proton-translocating complexes of the proton cycles that support pH homeostasis in acidophiles and alkaliphiles.

Some extremophile adaptations were found in functional assays, but additional examples are emerging as more extremophile genome data becomes available. Examples of adaptations in proton pumps include: the sequence-based proposal that the cytochrome oxidase of two iron-oxidizing acidophiles lacks one of the proton channels found in homologues from neutralophiles (Ferguson and Ingledew, 2008); adaptations of extremely alkaliphilic *Bacillus* species in cytochromes, including cytochrome *c*, that greatly reduce the mid-point potentials of the alkaliphile proteins relative to neutralophile homologues (Lewis *et al.*, 1981; Yumoto *et al.*, 1991; Hicks and Krulwich, 1995; Goto *et al.*, 2005); alkaliphile-specific sequence motifs in both the *caa*₃-type cytochrome oxidases (Quirk *et al.*, 1993); and alkaliphile-specific motifs in the proton-translocating *a*- and *c*-subunits of the ATP synthase that are important in proton capture and retention during ATP synthesis at high pH (Ivey and Krulwich, 1992; Arechega and Jones, 2001; Wang *et al.*, 2004; Liu *et al.*, 2009).

The multiplicity of mechanisms and adaptations observed for bacterial pH homeostasis is perhaps to be expected, given that in principle, every macromolecule with pH-titratable residues is a potential “pH sensor.” Thus, evolution has generated numerous pH-detecting devices that operate independently. Some categories of pH protection mechanisms are ubiquitous, such as cytoplasmic buffering or almost ubiquitous, such as

transmembrane proton transport of some type that is observed in all but a few bacteria that confine themselves to a narrow range of pH for example, *Clostridium fervidus* (Speelmans *et al.*, 1993). Other mechanisms are associated with groups of microorganisms that are adapted to particular ranges of environmental pH (for reviews see Slonczewski and Foster, 1996; Padan *et al.*, 2005; Baker-Austin and Dopson, 2007). Acidophiles include iron and sulfur bacteria such as *A. ferrooxidans*, as well as archaea such as *Ferroplasma acidiphilum* that grow under the most extreme acid conditions (close to pH 0). Archaeal acidophiles possess tetraether membranes that are highly impermeable to protons. Neutralophiles include the majority of organisms that grow in association with human bodies as well as a majority of those that inhabit soil and most freshwater habitats. Neutralophiles show a wide range of pH-regulating mechanisms that involve heterotrophic metabolism and inorganic ion exchange. Alkaliphilic bacteria that grow well at pH values up to 10.5, for example, the extensively studied *B. pseudofirmus* OF4 or *Bacillus halodurans* C-125, were isolated from soil or marine environments that are not consistently extremely alkaline (Krulwich and Guffanti, 1989; Takami *et al.*, 1999). Even more extreme alkaliphiles have been isolated from natural enrichments such as soda lakes that typically have a pH above 10 or industrial enrichments, for example, indigo dye plants (Jones *et al.*, 1998; Wiegel, 1998; Roadcap *et al.*, 2006). In the alkaliphiles studied to date, a Na^+ transport cycle that is coupled to cytoplasmic proton accumulation plays a major and indispensable role in pH homeostasis (Padan *et al.*, 2005; Krulwich *et al.*, 2007). However, extreme alkaliphiles have been isolated from non-saline groundwater with a pH of 11.4. Some of these bacteria, for example, *Bacillus foraminis* CV53T, grow better in the absence than in the presence of sodium (Tiago *et al.*, 2004, 2006). Therefore, net proton uptake cycles that are based on coupling ions other than sodium are likely to be employed in some environments.

2. CYTOPLASMIC pH MEASUREMENT

The study of cytoplasmic pH requires careful means of controlling and measuring pH, both inside and outside the cell. The pH of the medium must be maintained either in batch culture, through use of buffers that are pH-appropriate and non-metabolized, or through continuous culture (chemostat). Cytoplasmic pH is measured either by membrane-permeant radiolabeled probes of ΔpH , or by indicators of cytoplasmic pH that are

independent of external pH, such as fluorimetry and phosphorus NMR. The relative merits and limitations of these approaches are discussed below.

2.1. Buffered Batch Culture Versus Chemostat Culture

The first requirement for any study of cytoplasmic pH is to establish effective means of maintaining a constant pH of the extracellular medium or to incorporate changes in external pH into the study. In either case, accurate measurements of external and cytoplasmic pH are essential. Most commonly, pH homeostasis is assessed under conditions in which the pH is held constant. This establishes the homeostatic capacity under a specific condition in studies of different mutants or inhibitory conditions. However, there is sometimes merit in studying pH homeostasis during either acidification or alkalization of the medium caused by bacterial metabolism. Such conditions mimic the niches of bacteria that have relatively small volumes and/or buffering capacities, or that grow in biofilms and in high-density habitats such as the colon.

When the external pH is fixed, the external medium must have sufficient buffering to overcome the effects of metabolism on external pH. The most common approaches to this problem involve batch culture with non-metabolizable buffers, or the use of chemostat cultures. Usually, changes in absorbance and/or cell protein measurements are used to monitor growth. However, there is enormous value in tracking the viable count since this provides an indication of the proportion of cells in the population that retains the capacity for colony formation (Padan *et al.*, 2005). Limitations of batch culture are that (1) the buffer(s) must have appropriate pK_a for the pH range of interest but not be inhibitory to the bacteria in question; (2) concentrations as high as 50–100 mM are required for extended culture of rapidly growing cells; and (3) metabolic breakdown of buffers may occur when working with organisms whose full spectrum of degradative capacities are unknown, generating unknown byproducts (for discussion see Slonczewski and Foster, 1996). Limitations of chemostat culture include (1) the difficulty of establishing biologically independent replicate cultures; (2) the need to sample the chemostat in order to assess the cytoplasmic pH of the culture at intervals until it becomes feasible to use continuous measurements routinely, for example, by fluorimetry. In a review that highlights recent applications of chemostat cultures to studies of bacterial populations, mutations, and evolution, Ferenci (2008) points out that these cultures do not achieve a true steady state for bacterial populations with fixed characteristics that can be readily compared.

2.2. Radiolabeled Membrane-Permeant Probes

Radiolabeled membrane-permeant weak acids and weak bases are used to measure the bulk transmembrane ΔpH , which is added to the extracellular pH to yield cytoplasmic pH. Cells or membrane vesicles of either the right-side-out (RSO) orientation or inside-out orientation can be used for the assays (Padan and Schuldiner, 1986). The cells are collected by filtration or by centrifugation; the cytoplasmic volume must be determined simultaneously by a different radiolabeled probe (Small *et al.*, 1994; van den Vossenberg *et al.*, 1998b; Richard and Foster, 2004).

The weak acid or weak base probe must not be actively transported by the bacteria under study, as this would confound the measurement. Rather, the principle behind use of weak acid and weak base probes depends upon the relative impermeability of the membrane to the charged species of the acid or base in the absence of a transporter. In the absence of a transporter, the charged species do not significantly enter the cell or RSO vesicle and it is the uncharged species that is presumed to diffuse. If the cytoplasmic or intravesicular pH is higher than the external pH, the uncharged protonated weak acid that enters will dissociate more to the charged form than the probe that remains outside. The charged form that is generated inside is then trapped there and so its concentration relative to the probe concentration outside can be assayed. Weak base probes also enter in the uncharged form if the internal pH is lower than the outside pH, and more of the internalized probe than the probe remaining outside will become protonated. The charged protonated form of the basic probe traps the probe inside the cell and facilitates measurement of the inside versus outside probe concentration. If the pH gradient is small, it is advisable to conduct separate assays with a weak acid and weak base on the same preparations. The results should be the inverse of one another and such results help to validate findings of small gradients.

In all instances, a major challenge is correcting the total non-internalized cell- or vesicle-associated probe. The challenge is especially great because the amount of binding may be different among preparations from different mutants or may be affected by different treatments. Usually binding controls are conducted by permeabilization with either solvent, for example, toluenized or butanol-treated cells or membranes or with treatment of the preparation with an ionophore or combination of ionophores that abolishes the pH gradient (Rottenberg, 1979). To assess the optimal binding control with different cells and preparations, it is best to conduct preliminary assays with pH-equilibrated cells or vesicles in which pH gradients of known dimension are established by a sudden shift in external

pH. The other parameter that can be established in such preliminary experiments is the lowest concentration of probe that will be sufficient to saturate the non-specific binding sites while providing a measurement of the imposed pH gradient. It is important to ascertain this value because if the probe concentration is too low, it does not measure the actual gradient but is largely bound non-specifically. If the concentration is too high, the weak acid or base probe will change the pH gradient too much.

Calculation of the intracellular or cytoplasmic pH value (pH_{int}) requires the following equation:

$$\text{pH}_{\text{int}} = \log \left\{ \left(\frac{[A_{\text{in}}]}{[A_{\text{out}}]} \right) (10^{\text{p}K_{\text{a}}} + 10^{\text{pH}_{\text{out}}}) - 10^{\text{p}K_{\text{a}}} \right\}$$

where $[A]$ represents the concentration of the radiolabeled permeant-acid probe, such as benzoate ($\text{p}K_{\text{a}} = 4.2$) or salicylate ($\text{p}K_{\text{a}} = 3.0$). At extracellular pH values higher than the pH_{int} , an analogous equation applies to a permeant base such as methylamine ($\text{p}K_{\text{a}} = 10.6$). For acid conditions, the $\text{p}K_{\text{a}}$ of the probe should be sufficiently low that the protonated concentration is small (preferably well below the cytoplasmic pH) but not so low that the membrane permeation rate of the anion becomes comparable to that of the protonated species. Thus, for example, benzoate is an appropriate probe for use at external pH 5–7, whereas salicylate is optimally used at pH 3–6 (Small *et al.*, 1994; van den Vossenberg *et al.*, 1998b; Richard and Foster, 2004). At pH values below pH 3, the sensitivity of salicylate decreases because most of the probe is protonated.

As permeant acids and bases provide only indirect measures of the pH gradient, the results should be validated to the extent possible with other data about the system. The advantage of permeant-acid and -base probes is that a large number of samples can be measured conveniently. These measurements are reasonably sensitive, that is, over a large pH range, ΔpH values can be reproducibly assessed within 0.2 unit and such measurements correlate with fluorescence-based assays (Ito and Aono, 2002). A limitation is that these are steady-state type measurements and thus do not support kinetic analysis so that studies of pH homeostasis over time are limited to relatively long time intervals.

2.3. Fluorimetry and Fluorescence Microscopy

Fluorescence of a pH-titratable fluorophore yields a direct measure of cytoplasmic pH that is independent of extracellular pH (Olsen *et al.*, 2002; Wilks and Slonczewski, 2007). Fluorescent proteins provide highly sensitive

detection, do not require indicator loading, and lack phototoxicity. The most effective methods use pH-sensitive derivatives of green fluorescent protein (GFP) such as GFPmut3* and YFP (Andersen *et al.*, 1998; Kneen *et al.*, 1998; Llopis *et al.*, 1998; Robey *et al.*, 1998; McAnaney *et al.*, 2005). The pH dependence of fluorescence intensity is based on protonation of the phenolate moiety of the fluorophore derived from tryptophan, an exchange reaction occurring in less than a millisecond (Dickson *et al.*, 1997; McAnaney *et al.*, 2005). Thus, GFP observation allows assessment of the cellular rate of pH change in response to a rapid shift in extracellular pH, or to addition of a permeant acid. Fluorescence spectroscopy or fluorimetry of *E. coli* and *B. subtilis* cell suspensions yields kinetic data on a timescale as short as 4 seconds (Fig. 2A) (Wilks and Slonczewski, 2007; Kitko and Slonczewski, unpublished data). For optimal observation of cytoplasmic pH, the GFPmut3* is best expressed from a high-copy plasmid using a constitutive promoter (Kitko and Slonczewski, unpublished data). Use of current GFP probes is limited to cytoplasmic pH values above pH 5, as the protein denatures in acid. The optimal range of pH sensitivity of current proteins is about pH 5.5–8.5.

GFP fluorimetry can also be used to measure the periplasmic pH independently from the cytoplasmic and external pH values (Wilks and Slonczewski, 2007). The GFPmut3* fusion strain is expressed with a TorA signal peptide that can either retain the fusion protein in the cytoplasm or direct its transport to the periplasm via *tat* transport, a system in which the pre-folded GFP is transferred across the membrane (Thomas *et al.*, 2001; Barrett *et al.*, 2003; Mullineaux *et al.*, 2006). Arabinose incubation induces GFP transport, and eliminates GFP from the cytoplasm; thus, the periplasmic signal can be isolated. This method was used to show that periplasmic pH (unlike cytoplasmic pH) shifts according to external pH, with no apparent homeostasis (Fig. 2B).

pH-dependent fluorescence of cells is also observed by microscopy. For example, Olsen *et al.* (2002) used microscopy to observe GFP fluorescence as a function of pH in isolated cells of *E. coli* and *Lactococcus lactis*. The advantage of microscopy over fluorimetry is that it enables observation of individual cells within a population. Low noise level relative to signal permits ratiometric analysis, in which the ratio is obtained between fluorescence intensity at two excitation wavelengths, one of which increases with pH, and one of which decreases. The ratiometric method improves accuracy by eliminating intensity differences not associated with pH. On the other hand, microscopy necessitates cumbersome quantitation procedures that introduce error and limit the timescale of observable signal such that kinetic observations are limited compared to fluorimetry.

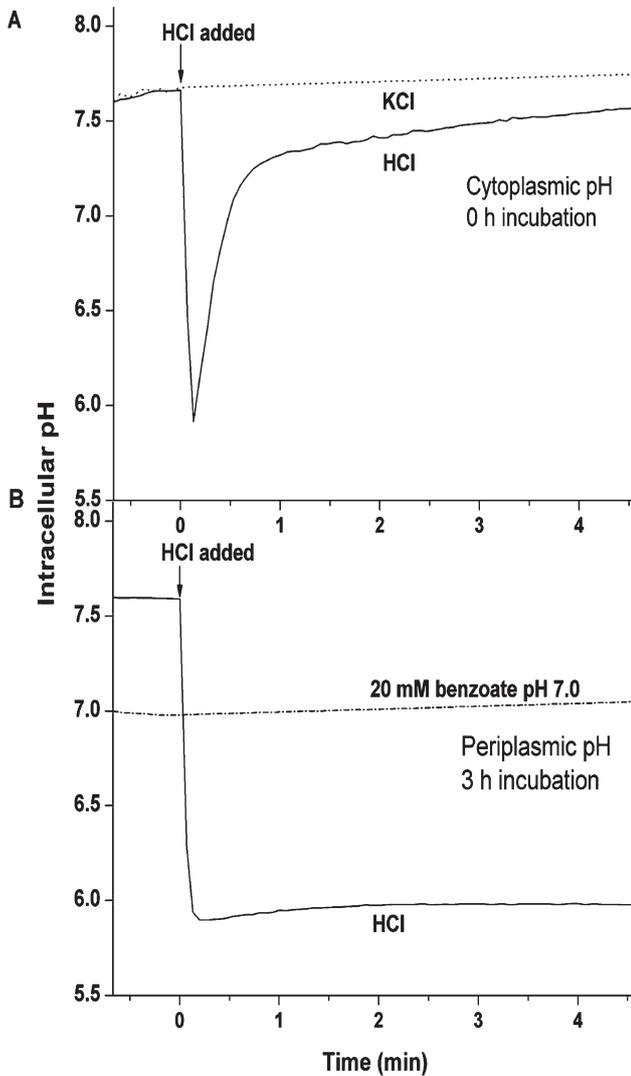


Figure 2 Cytoplasmic pH and periplasmic pH of *E. coli* K-12 observed by fluorimetry. Strain MC4100AR TorA-GFPmut3*. Cultures suspended in pH 7.5 M63 medium (5 mM HOMOPIPES) were tested (A) without further incubation and (B) after 3 h of incubation in the absence of arabinose. At time zero, the external pH was shifted from pH 7.5 to 5.5 by adding 8.5 mM HCl. For the control, at time zero, (A) 8.5 mM KCl (pH 7.5) was added or (B) 20 mM sodium benzoate (pH 7.0) was added to cultures suspended in pH 7.0 M63 medium (50 mM HOMOPIPES). The fluorescence signals from three independent cultures were averaged for each condition, and pH values determined from a standard curve based on cells with Δ pH collapsed by 20 mM sodium benzoate. [Adapted from Fig. 8 of Wilks and Slonczewski (2007).]

In Gram-positive bacteria, pH has been measured by microscopy using exogenous fluorophores such as derivatives of carboxyfluorescein. For an exogenous fluorophore, the challenge is to introduce a non-toxic probe into the cells that has an appropriate pK_a near that of cytoplasmic pH, and that remains in the cell without breakdown or excretion. Breeuwer *et al.* (1996) devised a method based on the use of carboxyfluorescein diacetate succinimidyl ester (cFSE) in *L. lactis* and *B. subtilis*. Within the cell, cFSE becomes hydrolyzed to carboxyfluorescein succinimidyl ester and is subsequently conjugated to aliphatic amines. The conjugated form appears to remain stable within the cell. Fluorescence is observed ratiometrically based on excitation wavelengths of 490 and 440 nm, with an approximate pK_a of pH 7.0. This method has been used to measure the internal pH of *Bacillus cereus* (Ultee *et al.*, 1999; Thomassin *et al.*, 2006) and *Lactobacillus* species (Siegumfeldt *et al.*, 1999), including dynamic changes on a timescale of 30–60 seconds (Siegumfeldt *et al.*, 2000).

Fluorescence microscopy can reveal the pH of a bacterial cellular compartment, such as the forespore compartment of *Bacillus megaterium* (Fig. 3) (Magill *et al.*, 1994, 1996). The fluorophore used is 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein (BCECF). Cells are loaded with the acetomethoxyester of BCECF (BCECF-AF), which is membrane-permeant and non-fluorescent. The probe becomes hydrolyzed by cellular esterases, releasing the pH-dependent fluorophore. Fluorescence excitation

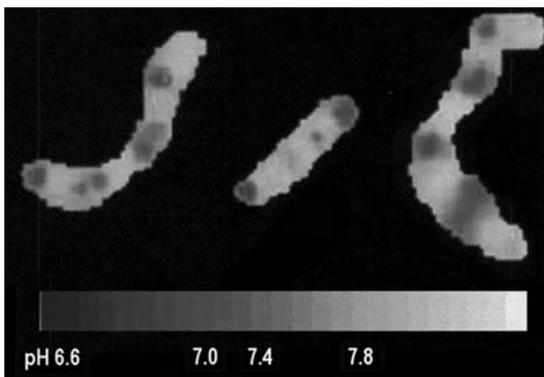


Figure 3 Fluorescent ratio image analysis of intracellular pH in sporulating *Bacillus megaterium*. Sporulating cells were loaded with BCECF-AM. Fluorescence ratios were measured and converted to pseudocolor images calibrated to pH as shown. Bacteria appear as chains of four to eight cells, in which the forespore components show lowered pH. The figure was adapted from Fig. 4 of Magill *et al.* (1994) with permission from American Society for Microbiology. (See plate 2 in the color plate section.)

ratios between 500 and 440 nm are calibrated to pH and represented by false color in the micrograph. During forespore development in the mother cell, the pH declines from pH 8.1 in the mother cell to pH 7.0 in the forespore.

2.4. Phosphorus NMR

Cytoplasmic pH can be measured simultaneously with extracellular pH using ^{31}P NMR observation of titratable phosphate resonances (Fig. 4) (Slonczewski *et al.*, 1981, 1982). NMR shifts are observed for inorganic phosphate (pK_a 7.1) and methylphosphonate (pK_a 7.5), which is taken up from the medium by *E. coli* cells. The observation of cytoplasmic pH requires highly concentrated cell suspensions, typically 20–200 optical density units at 600 nm, as the relative signal intensities of intracellular and extracellular phosphates depend on the cell volume. Cell suspensions are

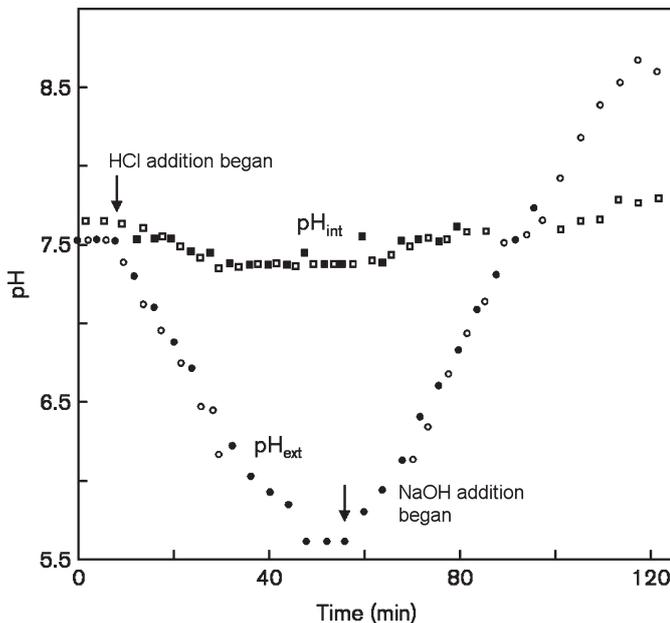


Figure 4 Cytoplasmic pH (pH_{int}) of *E. coli* K-12, and extracellular pH (pH_{ext}), measured by ^{31}P NMR. The chemical shifts were observed for inorganic phosphate (closed symbols) and methylphosphonate (open symbols). The pH was altered continuously by addition of acid (1 M HCl), then base (1 M NaCl), starting at the times indicated. [Adapted from Fig. 2 of Slonczewski *et al.* (1981).]

observed with aeration in a wide-bore tube of an NMR spectrometer, with field strength of at least 145 MHz. The use of two phosphorus probes with different pK_a values provides an intracellular confirmation of the pH calibration scale. Another advantage of NMR is that phosphorus resonances can be measured simultaneously for nucleotide phosphoryl groups and glycolytic intermediates, enabling assessment of the energetic state of the cell (Slonczewski *et al.*, 1981; Lohmeier-Vogel *et al.*, 2004).

^{31}P NMR studies can yield kinetic data on rapid perturbation of cytoplasmic pH, with a 12-second time resolution (Slonczewski *et al.*, 1982). However, in these experiments the cytoplasmic pH does not begin to recover from an external pH shift until approximately 2 minutes after HCl addition. By contrast, the fluorimetry data show a dip and initiation of recovery within 10–20 seconds (Fig. 2). A likely reason for this difference may be the relatively stressed condition of the cells in the NMR experiment, which are harvested in late log phase and/or are re-suspended at high density so that aeration comparable to optimal growth conditions is not feasible.

3. pH HOMEOSTASIS DURING GROWTH

pH homeostasis is required during growth of acidophiles (Fig. 5), neutralophiles (Figs. 6 and 7A), and alkaliphiles (Fig. 7B), in part due to the production and consumption of protons during metabolism. In most bacteria and archaea, primary pumps produce a PMF, although some marine bacteria and specialized fermentative bacteria also generate and drive bioenergetic work with a sodium motive force (Dimroth and Schink, 1998; Häse and Barquera, 2001; Müller *et al.*, 2001; Hayashi *et al.*, 2001; Dimroth, 2004). The PMF is generated by respiration, by other primary proton pumps, or by PMF-generating solute efflux cycles (Mitchell, 1961; Konings *et al.*, 1997; Schäfer *et al.*, 1999; Friedrich and Scheide, 2000). The modulation of the PMF and specific mechanisms to achieve pH homeostasis differ among acidophiles, neutralophiles, and alkaliphiles. The centrality of pH homeostasis mechanisms for most bacteria and archaea also has secondary effects on the choice of protons versus sodium used to power ion-coupled solute uptake and flagellar-based motility, as described below.

3.1. Acidophiles

Acidophiles maintain a more alkaline cytoplasmic pH that is typically 4–5 pH units above the external pH (Fig. 1). Additional acidophile cytoplasmic

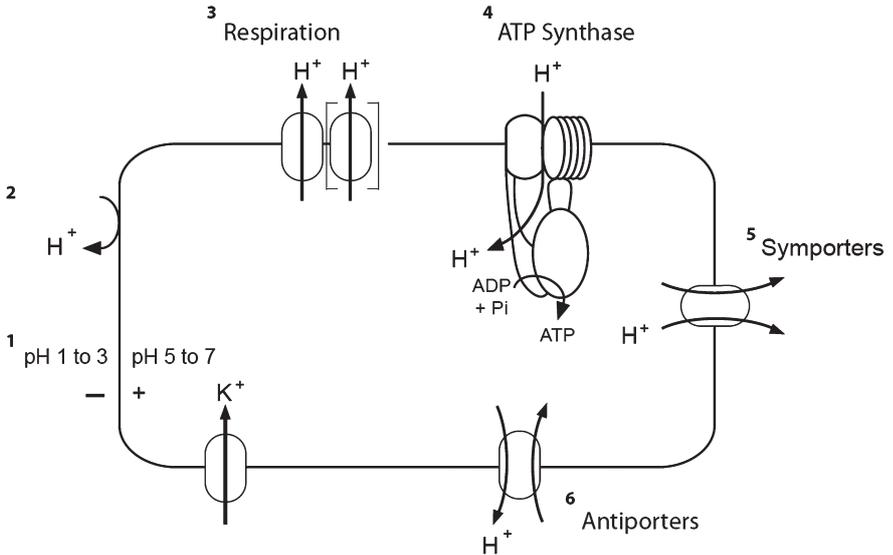


Figure 5 Schematic of general acidophile pH homeostatic mechanisms creating a near-neutral cytoplasmic pH despite an acidic external environment. ¹The internal positive membrane potential (potentially generated by potassium uptake) creates a chemiosmotic gradient that protons have to be transported against to enter the cytoplasm. ²The cytoplasmic membrane is extremely resistant to the influx of protons. ³Respiration-dependent primary proton pumps remove protons from the cytoplasm that ⁴re-enter to generate ATP via the F_0F_1 -ATPase. ^{5,6}Secondary symporters and antiporters can be used to remove excess protons from the cytoplasm. [Adapted from Fig. 1 of Baker-Austin and Dopson (2007).]

pH values to those given in Fig. 1 include *Thermoplasma acidophilum* that maintains an internal pH around pH 5.5–6.5 (Hsung and Haug, 1977), *Picrophilus oshimae* has an internal pH 4.6 at external pH values between 0.8 and 4.0 (van den Vossenberg *et al.*, 1998b), and *Sulfolobus solfataricus* grows at pH 2–4 with a cytoplasmic pH \approx 6.5 (Moll and Schafer, 1988). Acidophiles maintain an inverted transmembrane potential ($\Delta\Psi$, discussed below); thus, the pH gradient across the cytoplasmic membrane is the only chemiosmotic productive parameter of the PMF. pH homeostasis requires that any influx of protons for support of bioenergetic work such as ATP synthesis and proton-coupled solute uptake (as well as proton-coupled motility for some eubacterial acidophiles) must be balanced by proton extrusion by electron transport or by an alternative primary pump. Consistent with this expectation, inhibition at any point of electron transport halts metabolism in *Acidithiobacillus caldus* (Dopson *et al.*, 2002),

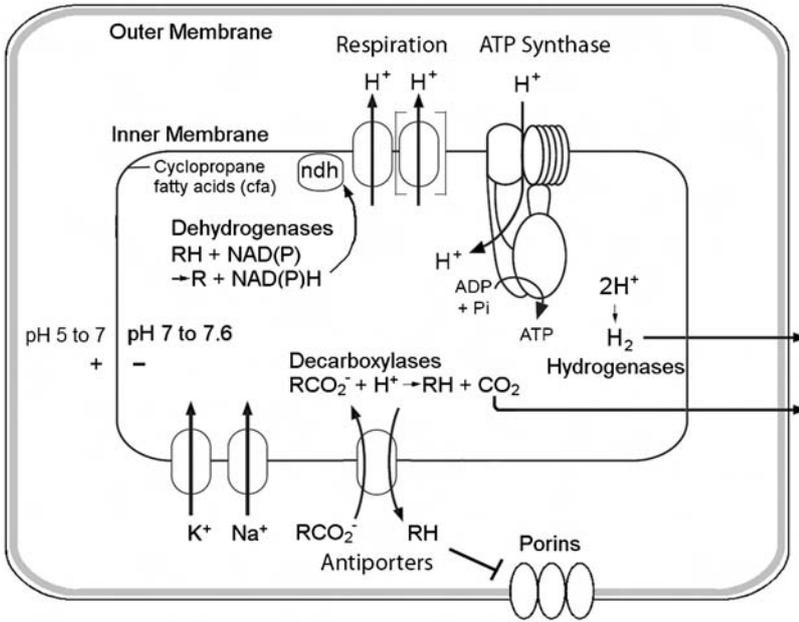
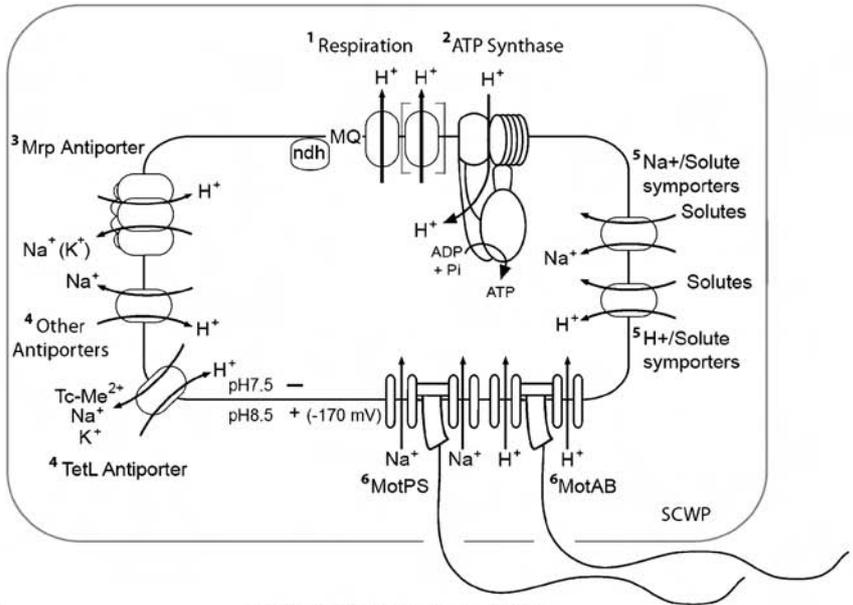


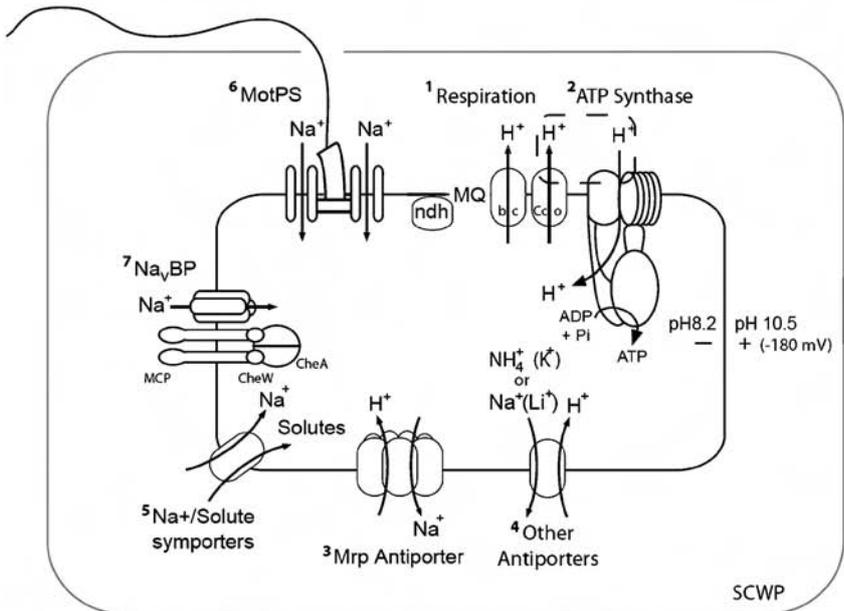
Figure 6 Acid stress during growth: mechanisms contributing to pH homeostasis in respiratory neutralophiles *E. coli* and *B. subtilis*. Acid up-regulates NAD(P)H-dependent substrate dehydrogenases, which enhance proton export. The F_1F_0 -ATP synthase is partly down-regulated to limit proton re-entry. K^+ and Na^+ flux balances the charge flow. Hydrogenases convert protons to H_2 , which diffuses from the cell. Amino acid and other substrate decarboxylases consume protons and generate polyamines, which are exported from the cell. In *E. coli*, polyamines block outer membrane porins from uptake of antimicrobial agents, including permeant organic acids. Acid up-regulates *cfa*, which synthesizes cyclopropane fatty acids (CFAs) in the inner membrane. (Based on Neely and Olson, 1996; Chang and Cronan, 1999; Tanner and Bornemann, 2000; Foster, 2004; Hayes *et al.*, 2006; Pagès *et al.*, 2008; Wilks *et al.*, 2009; others cited in text.)

Acidiphilium acidophilum (Matin *et al.*, 1982), and *Sulfolobus acidocaldarius* (Lübben and Schäfer, 1989). It was also demonstrated that metabolically active *A. acidophilum* cells maintained a stable external pH, consistent with the anticipated balance between proton consumption and proton pumping (Matin *et al.*, 1982).

Although the cytoplasmic membrane must allow flow of ions and molecules to support ATP synthesis, transport, and metabolism, the acidophile membrane must hinder the entry of protons (Fig. 5). Indeed, acidophile cell membranes (and in particular archaeal membranes) are



(A)

Neutralophilic *Bacillus subtilis*

(B)

Alkaliphilic *Bacillus pseudofirmus* OF4

highly impermeable to passive influx of protons down the concentration gradient from the outside to the inside of the cell (Konings *et al.*, 2002). This impermeability is vital as protons are more able to cross the membrane than other monovalent cations (van den Vossenberg *et al.*, 1995). It has been demonstrated that the proton permeability of archaeal membranes increases by only a factor of 10 from pH 1 to 11 (Nichols and Deamer, 1980; Nagle *et al.*, 2008), whereas there is a linear increase in the permeability of other ions. Proton translocation is suggested to be mediated by chains of hydrogen-bonded water (Deamer and Nichols, 1989) and therefore, archaeal membranes also have a low influx of water (Dannenmuller *et al.*, 2000). The cytoplasmic membrane of most bacteria consists of a lipid bilayer containing fatty acids ester-linked to a glycerol moiety whereas, archaea have ether linkages (Gliozzi *et al.*, 2002). Bipolar



Figure 7 Interacting proton and sodium cycles in neutralophilic *B. subtilis* and alkaliphilic *B. pseudofirmus* OF4. The pH_{in} , pH_{out} , and $\Delta\Psi$ (inside negative) are shown for an alkaline pH that supports growth. ¹Both species have exclusively proton-pumping respiratory chains; the *caa*₃-type cytochrome *c* oxidase (Cco) of the alkaliphile plays a major role in oxidative phosphorylation in the alkaliphile but not in the neutralophile. ²Both organisms have proton-coupled ATP synthases. The alkaliphile synthase has specific adaptations of the proton-translocating *a*- and *c*-subunits that are required for ATP synthesis and/or cytoplasmic pH homeostasis at pH 10.5 (Ivey and Krulwich, 1992; Wang *et al.*, 2004; Liu *et al.*, 2009). The dashed lines connecting the cytochrome oxidase of the alkaliphile respiratory chain and the ATP synthase indicate the existence of incompletely elucidated mechanisms for sequestered proton transfer between the respiratory chain and the synthase in the alkaliphile (Krulwich *et al.*, 2007). ³The multi-subunit Mrp Na^+/H^+ antiporter has a major role in Na^+ resistance in *B. subtilis* (Kosono *et al.*, 1999; Ito *et al.*, 1999) and a major role in pH homeostasis and in Na^+ resistance in alkaliphilic *B. halodurans* C-125 (Hamamoto *et al.*, 1994). ⁴The TetL antiporter plays a major role in pH homeostasis of *B. subtilis* strains that have this tetracycline and monovalent cation/proton antiport system and additional antiporters with supportive roles in pH homeostasis are found in both organisms (Padan *et al.*, 2005). ^{5,6}Ion-coupled solute uptake is exclusively coupled to sodium in the alkaliphile whereas *B. subtilis* can couple these processes to either protons or sodium. Sodium entry by these routes supports ongoing Na^+/H^+ antiport at high pH. ⁷A polarly localized voltage-gated sodium channel, Na_vBP is found in the alkaliphile that contributes to sodium entry to support pH homeostasis at high pH. Its function is required for normal chemotaxis and it co-localizes with chemotaxis receptors (shown as an MCP-CheW, CheA complex) (Ito *et al.*, 2004b). The SCWPs indicated by the line surrounding both cells indicates the presence of secondary cell wall polymers. In alkaliphilic *B. pseudofirmus* OF4, the S-layer protein SlpA supports pH homeostasis (Gilmour *et al.*, 2000) and in alkaliphilic *B. halodurans* C-125, teichuronic acid components of the SCWPs have a major role in pH homeostasis and alkaliphily (Aono and Ohtani, 1990; Aono *et al.*, 1999).

tetraether archaeal lipids have a monolayer organization with cyclopentane rings and a network of hydrogen bonds between the sugar residues on the outer face of the membrane (Elferink *et al.*, 1994) and tight lipid packing (Komatsu and Chong, 1998). Most acidophilic archaea contain tetraether-linked lipids (Macalady *et al.*, 2004) that have been identified in *F. acidiphilum* Y^T and Y2 (Batrakov *et al.*, 2002; Golyshina *et al.*, 2000; Pivovarova *et al.*, 2002), “*Ferroplasma acidarmanus*” (Macalady *et al.*, 2004), *P. oshimae* (van den Vossenberg *et al.*, 1998b), *S. solfataricus* (van den Vossenberg *et al.*, 1998a), and *T. acidophilum* (Shimada *et al.*, 2002). These results are supported by data from *S. acidocaldarius* liposomes (Elferink *et al.*, 1994; Komatsu and Chong, 1998) that were less proton permeable than liposomes from *E. coli* and *Bacillus stearothermophilus*.

Related to pH homeostasis and maintaining a pH gradient across the membrane is the size and permeability of membrane channels in either the outer or inner membrane that allow the influx of protons. The response of *A. ferrooxidans* to acid stress—identified up-regulated genes coding for putative lipoproteins and outer membrane proteins, suggesting that this bacterium may alter its outer membrane structure to reduce proton permeability (Chao *et al.*, 2008). In another study, the *A. ferrooxidans* outer membrane protein Omp40 was up-regulated as a result of a pH drop from pH 3.5 to 1.5 in a similar response to heat shock (Amaro *et al.*, 1991). Outer membrane porins (OMPs) from Gram-negative neutralophiles are trimeric structures that form water filled channels through which nutrients may pass (Nikaido, 2003). The *A. ferrooxidans* Omp40 has a large external L3 loop that may control the size and ion specificity at the entrance of the porin (Guiliani and Jerez, 2000). It also has an estimated isoelectric point of 7.21 and the L3 loop has a charge of +2 at pH 2.5 (compared to −4 for the *E. coli* homologue at pH 7) that may be adaptations to limit the passage of protons into the cytoplasm (Guiliani and Jerez, 2000). Omp40 has been shown to be potentially involved in Fe²⁺ oxidation by interaction with the substrate (Castelle *et al.*, 2008) although its role in response to a sudden drop in pH has not been elucidated. *A. ferrooxidans* is thus far the only acidophile in which the membrane is known to change in response to acid stress. There is a need for further studies of the porins of acidophile membranes in relation to acid stress, especially in an acidophilic archaea.

A further feature of pH homeostasis in acidophiles is the generation of an inverted transmembrane potential ($\Delta\Psi$), positive inside relative to outside (Fig. 5). This is opposite to the orientation of the $\Delta\Psi$, inside negative, of neutralophiles and alkaliphiles. The $\Delta\Psi$ has been measured in a number of acidophiles including +10 mV in *A. ferrooxidans* (Cox *et al.*, 1979), +73 mV in *A. acidophilum* (Matin *et al.*, 1982), and approximately

+20 mV in *T. acidophilum* (Michels and Bakker, 1985). The inside-positive $\Delta\Psi$ in acidophiles helps to maintain the large ΔpH and hence pH homeostasis by impeding entry of protons down their chemical gradient. A small internal positive potential is also observed in de-energized cells that counterbalance the pH gradient (ΔpH) across the membrane such that the PMF is zero in *T. acidophilum* (Hsung and Haug, 1977), *Bacillus acidocaldarius* (Oshima *et al.*, 1997), *A. caldus* (Dopson, 2001), *A. ferrooxidans* (Cox *et al.*, 1979), and *A. acidophilum* (Goulbourne *et al.*, 1986). Problems of accurately measuring such small potentials and correcting for probe binding were noted (Michels and Bakker, 1985). However, as recently described by Ferguson and Ingledew (2008), the inside-positive $\Delta\Psi$ in acidophiles is necessary to maintain a cytoplasmic pH much closer to neutrality than the outside pH, and this is clearly critical for cell metabolism and growth. Further, they note that under survival – only conditions such as de-energization or after treatments that increase proton permeability, the pattern of a counterbalanced inside-positive $\Delta\Psi$ and a ΔpH , acid outside, would be expected to persist, as has been reported, unless a permeant anion is present. A permeant anion would collapse the inside-positive $\Delta\Psi$ and that in turn would lead to collapse of the ΔpH .

There have been suggestions that potassium ions play a role in generation of the acidophile PMF and its inside-positive $\Delta\Psi$ (Fig. 5). For example, proton translocation during respiration and PMF generation in *Sulfolobus* spp. is dependent on the presence of K^+ ions (Schäfer, 1996) and generation of the $\Delta\Psi$ in *Acidithiobacillus thiooxidans* depends upon the presence of cations with K^+ being most effective (Suzuki *et al.*, 1999). Some of the effects of K^+ could result from the stimulation of respiration-dependent PMF generation secondary to PMF depletion during K^+ uptake as described by Bakker and Mangerich (1981). Richard and Foster (2004) describe a series of reactions, including amino acid carboxylases and electrogenic substrate/product exchange during acid adaptation of *E. coli* that could contribute to formation of the inside-positive $\Delta\Psi$ generated in this neutralophile (see Section 4.1). The nature and origin of the inside-positive $\Delta\Psi$ merits further investigation across a spectrum of acidophiles and acid-tolerant neutralophilic bacteria, as this potential plays a key role in maintaining pH homeostasis.

3.2. Neutralophiles: pH Homeostasis in Acid

Bacteria growing at near-neutral pH show fewer membrane adaptations than do acidophiles. At near-neutral values of external pH, both

respiratory neutralophiles and neutralophiles that lack respiratory chains are able to grow without pH homeostasis when the PMF is diminished or eliminated by an uncoupler. *E. hirae* grows at pH 7.1–7.8 with ΔpH and the proton potential eliminated by 1 $\mu\text{g/ml}$ gramicidin D (Harold and Van Brunt, 1977). Respiratory bacteria such as *E. coli* grow at pH 7.4–7.6 with the proton potential collapsed by 50 μM of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (Kinoshita *et al.*, 1984) or at pH 7.0–8.0 in the presence of benzoate, which diminishes or eliminates ΔpH (White *et al.*, 1992). In these cases, growth requires rich medium, with energy obtained through substrate-level phosphorylation on catabolites such as glucose or amino acids. Some organisms may in fact sacrifice pH homeostasis in order to avoid low pH-driven uptake of fermentation acids (discussed below, Section 3.3). For instance, the pathogen *E. coli* O157:H7 allows its internal pH to decrease below pH 7 in order to avoid acetate accumulation (Diez-Gonzalez and Russell, 1997). However, in most cases the loss of pH homeostasis allows growth only when the external pH is at or near the optimal value of cytoplasmic pH.

Exposure of neutralophiles to low pH requires adjustments, as discussed below, and induces structural changes in membranes, most notably an increase in production of CFAs (Grogan and Cronan, 1997). The means by which CFAs strengthen the membrane are unclear, but the lowering of membrane proton conductance may play a role. *E. coli* cells defective for the enzyme CFA synthase (*cfa*) lose the ability to survive an acid shift from neutral pH to pH 3 (Chang and Cronan, 1999). In *E. coli*, *cfa* is up-regulated both by low pH (Hayes *et al.*, 2006) and by acetate, which acidifies the cytoplasm at neutral pH (Rosenthal *et al.*, 2006). Up-regulation of *cfa* is RpoS-dependent, as are major mechanisms of acid resistance such as the Gad regulon (discussed below). The *cfa* enzyme is similarly up-regulated by acid in *Salmonella enterica* (Kim *et al.*, 2005), *L. lactis* (Budin-Verneuil *et al.*, 2005), and *Oenococcus oeni* (Grandvalet *et al.*, 2008). External acid and base also differentially regulate a number of outer membrane proteins, whose role in acid resistance remains unclear (Stancik *et al.*, 2002; Maurer *et al.*, 2005; Hayes *et al.*, 2006). As in the acidophile *A. ferrooxidans*, the OmpR/EnvZ regulation of porins enhances *E. coli* growth at low pH (Sato *et al.*, 2000). Some amino acid decarboxylases maintain pH homeostasis in enteric bacteria surviving at pH values below their growth range, in part through generation of an inverted electrical potential (Foster, 2004; see Section 5.1).

Two general mechanisms of acid pH homeostasis occur among neutralophiles: (1) ATPase-dependent proton extrusion in non-respiratory bacteria and (2) ATPase-independent ion transport and catabolic acid

consumption in respiratory bacteria. The first class includes bacteria lacking a respiratory chain, such as *Enterococcus hirae* (formerly *Streptococcus faecalis*), which inhabits animal intestines (Kakinuma, 1998). *E. hirae* primarily uses the F_1F_0 -ATPase to expend ATP to expel excess protons (Kobayashi and Unemoto, 1980; Kobayashi *et al.*, 1982; Shibata *et al.*, 1992). Both the expression and activity of the ATPase are increased at low external pH; and mutants in the ATPase fail to grow below pH 7 (Suzuki *et al.*, 1988). The ATPase similarly serves as the primary means of acid reversal for lactococci (Hutkins and Nannen, 1993) and for oral streptococci (Kuhnert and Quivey, 2003), although supplemented by catabolic mechanisms such as amino acid decarboxylases (Curran *et al.*, 1995).

In most respiratory neutralophiles, no one mechanism is known to be essential for growth below pH 7 (Fig. 6). The F_1F_0 -ATPase acts primarily as the ATP synthase coupled to electron transport. In fact, the *atp* operon is up-regulated at high external pH, suggesting a need to compensate for the lower PMF under conditions of inverted Δ pH (in *E. coli* K-12, Maurer *et al.*, 2005; in *B. subtilis*, Wilks *et al.*, 2009; in *Desulfovibrio vulgaris*, Stolyar *et al.*, 2007). In *E. coli*, potassium transport has been associated with pH homeostasis (Bakker and Mangerich, 1981; Roe *et al.*, 2000; Buurman *et al.*, 2004). Nevertheless, no one potassium transport system is essential for growth at low pH. A triple mutant for potassium transport systems, deleted for *kdpABC trkA trkD*, requires high extracellular K^+ for pH homeostasis and growth below pH 7.5 (White *et al.*, 1992). In standard laboratory strains, K^+ is not required for reversal of transient effects of acid exposure on cytoplasmic pH, even when the exposure is an abrupt acid shift (Wilks and Slonczewski, unpublished data).

A diverse collection of catabolic enzymes and substrate transporters are transcriptionally regulated so as to favor acid consumption and base production at low pH (and the reverse at high pH). The best characterized example is that of lysine decarboxylase, *cadAB*, and the regulator *cadC* (Slonczewski *et al.*, 1987; Meng and Bennett, 1992a, b; Dell *et al.*, 1994; Neely *et al.*, 1994; Neely and Olson, 1996). Amino acid decarboxylases consume an acidic group, usually with release of a polyamine (for details, see Section 4.4). Low pH also up-regulates hydrogenase complexes, which may consume protons to release as hydrogen gas (King and Przybyla, 1999; Hayes *et al.*, 2006). Both decarboxylases and hydrogenases show acid-enhanced expression primarily under anaerobiosis. Aerobic conditions lead to acid-up-regulation of proton pumps such as the NADH dehydrogenases and cytochrome *d* oxidase (Maurer *et al.*, 2005). In *B. subtilis*, acid also up-regulates numerous NAD(P)H-dependent substrate dehydrogenases, which could channel electrons into respiration and accelerate proton export

(Wilks *et al.*, 2009). The mechanism of the acid-stress decarboxylase induction response of *B. subtilis* is beginning to be unraveled with the finding that the σ -like protein YvrI works with a pair of co-regulators to control levels of the oxalate carboxylase OxdC that is implicated in the acid-stress response (Tanner and Bornemann, 2000; MacLellan *et al.*, 2009).

A special case is that of *Helicobacter pylori*, a neutralophilic epsilon proteobacterium that has the ability to persistently colonize the epithelium of the extremely acidic human stomach, where it is associated with peptic ulcers and gastric cancer (Fig. 8). Under laboratory culture conditions in the absence of urease, *H. pylori* behaves like a neutralophile in that it grows primarily between external pH 5 and 7.5 (Bauerfeind, *et al.*, 1997; Slonczewski *et al.*, 2000). By contrast, other pathogenic neutralophiles survive while passing through the stomach but do not grow in highly acidic environments or colonize them (Blaser and Atherton, 2004; Sachs *et al.*, 2005). The median pH of the gastric lumen in the absence of food is about pH 2; although there is controversy about the pH at the gastric surface, consistency between genes induced upon an *in vitro* acid shift and exposure to the *in vitro* environment support the conclusion that the acid pH challenge to *H. pylori* is formidable (Wen *et al.*, 2003; Scott *et al.*, 2007). *H. pylori* differs from both *bona fide* acidophiles and other neutralophiles that can survive an acid challenge in not exhibiting the inside-positive $\Delta\Psi$ found in these other organisms. Instead, *H. pylori* maintains a small $\Delta\Psi$ (~ -25 mV) in the chemiosmotically productive orientation (inside negative) while also maintaining a very large Δ pH (alkali inside), and a cytoplasmic pH in the near-neutral range during colonization of the acidic gut (Sachs *et al.*, 2005, 2006).

Not surprisingly, *H. pylori* use a unique pH homeostasis strategy. An important initial clue was the finding of particularly high constitutive levels of urease (Mobley *et al.*, 1995; van Vliet *et al.*, 2001), which is increased to even higher levels during growth in acid (Slonczewski *et al.*, 2000). In acidic media containing urea, urea passes through an OMP and is taken up by the UreI inner membrane urea channel that is proton gated and is associated on the cytoplasmic side with the cytoplasmic urease (Sachs *et al.*, 2005, 2006). The combined actions of the cytoplasmic urease and a cytoplasmic carbonic anhydrase release ammonia and carbon dioxide. Some ammonia may scavenge protons in the cytoplasm, but it is largely expected to diffuse into the more acidic periplasm along with the carbon dioxide. Some of the ammonia may escape into the outside medium and scavenge protons there but much of the ammonia and carbon dioxide remain in the periplasm where they mediate pH homeostasis of that compartment (Fig. 8). The regulation of periplasmic pH is in contrast with other neutralophiles such as

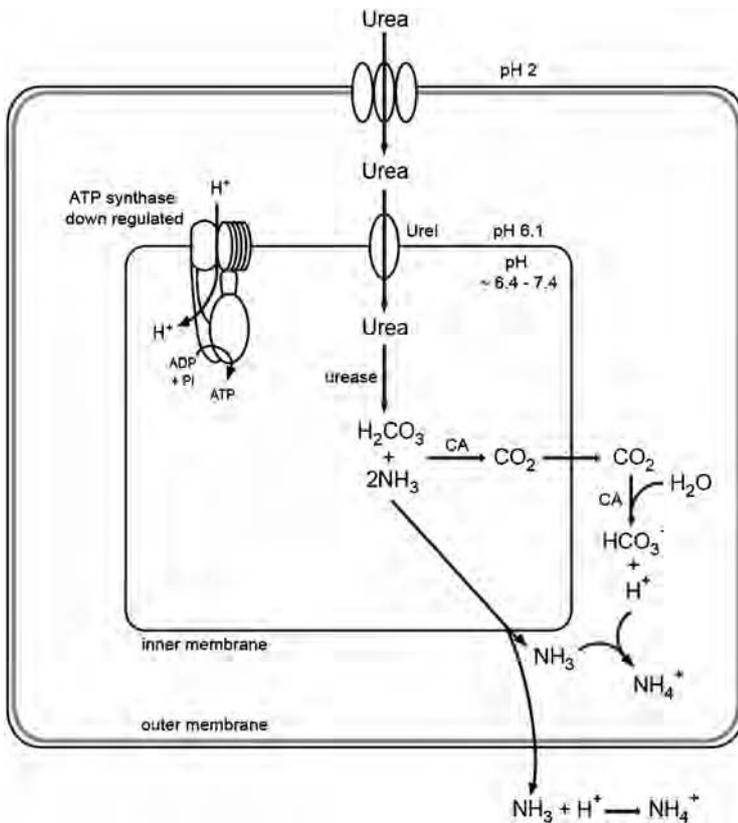


Figure 8 pH homeostasis in *H. pylori* growing in extreme acid. Urea-dependent pH homeostasis as an adaptive strategy in *H. pylori* requires passage of urea into the cytoplasm via the UreI channel and production of ammonia and carbon dioxide by the actions of cytoplasmic urease and carbonic anhydrase (CA). The abundant carbon dioxide and ammonia diffuse into the periplasm where a periplasmic carbonic anhydrase produces bicarbonate; the ammonia scavenges the protons released in the carbonic anhydrase reaction so that a “cloud” of buffering bicarbonate remains to support the periplasmic pH 6.1 and protect the cytoplasm within (After Sachs *et al.*, 2005). Among the strategies that are employed to limit proton uptake is the down-regulation of the ATP synthase (Wen *et al.*, 2003).

E. coli, in which the periplasmic pH appears to be continuous with that of the exterior (Wilks and Slonczewski, 2007). In *H. pylori*, a periplasmic pH ~ 6.1 is maintained through the proton capture by ammonia and the generation of a large amount of buffering bicarbonate from the carbon dioxide by the activity of a periplasmic carbonic anhydrase. The

well-buffered periplasmic compartment thus produced adds an extra layer of protection for the cytoplasmic compartment.

Induction of urease, as well as additional ammonia-generating enzymes, and of carbonic anhydrase was observed in an *in vitro* experiment; motility and chemotaxis genes were also induced, consistent with mutational evidence that these activities are necessary for colonization (Scott *et al.*, 2007). Mutational loss of urease, the urea channel and the carbonic anhydrase all result in defective pH homeostasis (Eaton *et al.*, 1991; Mollenhauer-Rektorschek *et al.*, 2002; Marcus *et al.*, 2005; Bury-Moné *et al.*, 2008). Potassium uptake by the HpKchA channel of the RCK domain family is also necessary for gastric colonization by *H. pylori*, providing a specific biological function for a prokaryotic potassium channel (Stingl *et al.*, 2007). The ATP synthase that brings protons inward as it functions is down-regulated (Wen *et al.*, 2003). This down-regulation of the ATP synthase in *Helicobacter* is consistent with the observations of up-regulation of ATP synthase expression in several neutralophiles under alkaline challenge (Maurer *et al.*, 2005; Stolyar *et al.*, 2007; Wilks *et al.*, 2009).

3.3. Neutralophiles: pH Homeostasis in Base

In alkaline pH homeostasis, both non-respiratory and respiratory neutralophiles show a central role for cation/proton antiporters. These secondary membrane transporters use the PMF developed by primary pumps such as the proton-pumping respiratory chain components of respiring bacteria or ATP-dependent proton pumping by the hydrolytic activity of F₁F₀- or H⁺-coupled P-ATPases. In order to support net proton accumulation in the cytoplasm, a Na⁺/H⁺ antiporter would have to transport more protons inward than the number of sodium ions transported outward during each turnover. Such an electrogenic antiport whose exchange involves a H⁺:Na⁺ ratio >1, and thus translocates net positive charge inward, could be energized by the $\Delta\Psi$ component of the PMF. Such an activity could thus achieve cytoplasmic proton accumulation (Booth, 1985; Macnab and Castle, 1987; Padan *et al.*, 2005). A K⁺/H⁺ antiporter could achieve acidification of the cytoplasm even if it functioned via a 1:1 electroneutral exchange, since it could use both the ΔpH component of the PMF and get the added energy required to reverse that gradient from the outwardly directed gradient of the efflux substrate, K⁺, which exists under most physiological circumstances (Booth, 1985; Padan *et al.*, 2005). A detailed characterization of a typical bacterial complement of cation/proton antiporters (often more than 5) has yet to be reported for any species and is a

challenging task; selected examples from the bacteria that have been most completely characterized will be discussed but even these bacteria have predicted antiporters whose activities and roles are not known. It is further likely that some of the membrane proteins of unknown function will turn out to have (novel) activities that contribute to pH homeostasis.

With respect to adaptation to alkaline pH, *E. hirae* is among the most intensively studied non-respiratory neutralophiles (Kakinuma, 1998). In the presence of bicarbonate, *E. hirae* grows at pH values as high as 10.5, and exhibits a cytoplasmic pH of 7.8 at an external pH 8.4, and a cytoplasmic pH of 8.2 at an external pH 9.5 (Kakinuma, 1987a). Kakinuma (1987b) proposed that alkaline pH homeostasis is supported by a constitutive Na^+/H^+ antiporter energized by the PMF produced by the H^+ -pumping F_1F_0 -ATPase that is up-regulated by alkali. It was further predicted that *E. hirae* also supports sodium extrusion with an inducible sodium ATPase. This ATPase activity would also increase the inwardly directed sodium gradient available to drive sodium-coupled transport activities at high pH. Evidence was also found for the involvement of a K^+/H^+ antiporter in *E. hirae* pH homeostasis since a mutant with deficient pH homeostasis was also deficient in K^+ /methylamine exchange (Kakinuma and Igarashi, 1999). Subsequently, a Na^+/H^+ antiporter from *E. hirae* was identified and characterized. It was designated NapA (Waser *et al.*, 1992) and it became one of the founding transporters of the Cation:Proton Antiporter-2 (CPA2) family in the Transporter Classification System (Saier *et al.*, 1999; Saier, 2000).

The anticipated Na^+ -coupled ATPase has also been identified and studied in detail. Encoded by the *ntp* operon, the ATPase product is a V(vacuolar)-type ATPase that uses a rotary mechanism. It works only in the hydrolytic, Na^+ extruding direction and is composed of nine NTP proteins (Kakinuma, 1998; Murata *et al.*, 2005). Consistent with roles in support of alkaline pH homeostasis and Na^+ -resistance, expression from the *ntp* promoter is increased by elevated pH or elevated Na^+ (Ikegami *et al.*, 1999). Another special feature of the *ntp* operon underscores the relationship between Na^+ cytotoxicity, K^+ requirements, and alkali stress. This relationship is as follows: at high pH, cells become more sensitive to growth inhibition by Na^+ , but the K^+ status strongly influences cytoplasmic Na^+ toxicity. Even at very high pH, the toxicity of Na^+ is lessened if cytoplasmic K^+ is elevated (Padan *et al.*, 2005; Wei *et al.*, 2007). In *E. hirae*, induction of V-ATPase-mediated Na^+ extrusion at high pH allows the bacterium to address the increased toxicity at alkaline pH even when Na^+ concentrations are not substantially elevated. In addition, the *ntpJ* gene encodes a protein that is not part of the V-ATPase but, rather, is a subunit

of an *E. hirae* KtrII-type K^+ uptake system. Induction of the *ntp* operon, by either high pH or high sodium, addresses the threat of increased Na^+ cytotoxicity both by increasing extrusion of the toxic ion but also by lessening its cytotoxicity through increasing the ameliorating K^+ levels (Kawano *et al.*, 2000).

Some lactic acid bacteria, of which *E. hirae* is often considered a relative or member, do not grow at alkaline pH, but many members of the genus *Lactobacillus* are capable of growth up to pH 8.5 or even 8.9. These strains, as well as some of those that are less able to grow at elevated pH, acidify their cytoplasm relative to the external medium during growth (Sawatari and Yokota, 2007). One of the most efficacious strains, *L. acidophilus* JCM 1132^T exhibited greater cytoplasmic proton accumulation in the presence of Na^+ than in its absence, suggestive of Na^+/H^+ antiporter involvement.

Among the respiratory neutralophiles, alkali-adaptation mechanisms to support pH homeostasis have been most intensively studied in the model Gram-negative and -positive organisms *E. coli* and *B. subtilis*, respectively. In both of these bacteria, the properties and roles of many, but still not all, of the cation/proton antiporters predicted in the genome have been studied (Padan *et al.*, 2005). In *E. coli*, NhaB is a modestly electrogenic Na^+/H^+ antiporter (1.5 H^+ :1 Na^+) that appears to have a housekeeping role (Pinner *et al.*, 1993, 1994). In contrast, NhaA has a $H^+:Na^+$ coupling stoichiometry of 2 (Taglicht *et al.*, 1993) and is a high-affinity, high-turnover antiporter that is highly regulated and tremendously activated by elevated pH (Padan *et al.*, 2004; Padan, 2008). Its properties suggest that it has a major role in Na^+ extrusion and proton capture at high pH. The high-resolution structure of NhaA offers insights into one of the problems of cation/proton antiporter function in alkaline pH homeostasis, that is, the problem of capturing protons from a highly alkaline bulk phase with kinetics that support an activity level that, in turn, achieves pH homeostasis. The NhaA structure contains two negatively charged funnels, one on each side of the membrane, that are proposed to foster efflux of the cytoplasmic Na^+ and entry of the periplasmic H^+ (Hunte *et al.*, 2005).

In spite of the efficacy of NhaA and supporting role of NhaB, it was clear from mutant studies that there must be additional contributors to pH homeostasis since double *nhaA*, *nhaB* deletion strains were very sensitive to Na^+ but still grew well in media at $pH > 8$ if the Na^+ concentration was kept low (Pinner *et al.*, 1993). Plack and Rosen (1980) present evidence that *E. coli* has a K^+/H^+ antiporter that also has a role in pH homeostasis. A third *E. coli* antiporter, designated ChaA because of its Ca^{2+}/H^+ antiport activity as well as Na^+/H^+ (Ivey *et al.*, 1993), was shown to support not only sodium and calcium circulation (Ohyama *et al.*, 1994; Shijuku *et al.*,

2002) but also to have K^+/H^+ antiport activity. ChaA functions best as a K^+/H^+ antiporter at and just above pH 9.0, where NhaA is less efficacious (Radchenko *et al.*, 2006). There are two well-studied two-component transporters of the CPA2 family that have a role in electrophile resistance in *E. coli*, KefFC and KefGB (Booth *et al.*, 2003). These transporters have recently been shown to carry out K^+/H^+ antiport (Fujisawa *et al.*, 2007) but KefFC and KefGB have not been shown to play a role in pH homeostasis. Nor have two members of the CPA-1 family predicted in *E. coli* to have such a role. The one additional antiporter that has been shown to support alkalitolerance via low affinity $(Na^+)K^+/H^+$ antiport above the pH range for NhaA is a known multi-drug antiporter of *E. coli*, MdfA (Lewinson *et al.*, 2004). It is interesting that a drug/ H^+ antiporter has cation/proton activity in both *E. coli* and *B. subtilis*. These findings may have implications with respect to pathways by which drug/ H^+ antiporters evolve and also with respect to how they sometimes persist even without selective pressure of the drug (Krulwich *et al.*, 2005). The neutralophile *B. subtilis* can grow at pH values about 8.5–9.0, and in pH 8.5 media, it maintains a cytoplasmic pH ~ 7.5 (Cheng *et al.*, 1994, 1996). Elements of the *B. subtilis* active ion-transport cycles that support pH homeostasis include a diverse array of antiporters (Fig. 7A). These antiporters include a chromosomal tetracycline–metal/proton antiporter TetL that has a major role in alkaline pH homeostasis by virtue of its additional capacities for Na^+/H^+ and K^+/H^+ antiport activities (Cheng *et al.*, 1994, 1996). However, wild-type *B. subtilis* keeps its cytoplasmic pH at 7.5 after a shift of external pH 7.5 to 8.5, if either Na^+ or K^+ is present (Table 1). However, *B. subtilis* cannot maintain a cytoplasmic pH at 7.5 in their absence or in the absence of a functional *tetL*; nor can it regulate its pH if subjected to the much greater challenge of a pH between 8.5 and 10.5 that can be handled by an alkaliphilic *Bacillus*. Although *tetL* deletion mutants are viable, these mutants exhibit a complex pattern of changes in the transcriptome that are apparent adjustments to a more sodium- and alkali-challenged state; most of these changes are not reversed upon re-introduction of *tetL* to the chromosome (Wei *et al.*, 2006). The 14-TMS drug/ H^+ antiporters are hypothesized to have evolved from the more common 12-TMS bacterial antiporters that had a housekeeping function. Sequence analyses suggest that the two middle TMS were inserted late and the subsequent selection ultimately led to the final constellation of capacities (Griffith *et al.*, 1992). This is supported by the fact that when the two middle TMS of TetL were removed to create a 12-TMS TetL, this engineered transporter was still incorporated into the membrane and retained the Na^+ - and K^+ -related transport functions but could no longer carry out tetracycline–metal/proton antiport (Jin *et al.*, 2001).

Table 1 pH homeostasis in neutralophilic *Bacillus subtilis* and alkaliphilic *Bacillus pseudofirmus* OF4: effects of mutations in transporters or a secondary cell wall polymer.

(A)			Cytoplasmic pH (10 minutes after shift)				
			pH 7.5→8.5		pH 8.5→10.5		
			Choline Cl	NaCl	KCl	NaCl	KCl
<i>B. subtilis</i>	WT	Malate	8.5	7.5	7.6	10.5	10.5
	$\Delta telL$	Malate	8.5	8.5	8.5	–	–
	$\Delta mrpA$	Malate	8.5	7.6	7.6	–	–
<i>B. pseudofirmus</i> OF4	WT	Glucose	–	–	–	8.4	9.2
	WT	Malate	8.5	7.5	8.4	8.2	10.5
	$\Delta slpA$	Malate	–	–	–	9.1	10.5

(B)		Cytoplasmic pH (10 minutes after shift)	
		pH 8.5→10.5	
<i>B. pseudofirmus</i> OF4 strain		100 mM Na ⁺	2.5 mM Na ⁺
Wild type		8.46	9.03
SC34, NavBP deletion ($\Delta ncbA$)		8.66	9.43
SC34R-NavBP restored ($\Delta ncbA$, $ncbA$ restored)		8.48	9.02
Mot6, non-motile ($\Delta motPS$)		8.52	9.03
SC34/Mot6, double NavBP, MotPS mutant ($\Delta ncbA$, $\Delta motPS$)		8.72	9.51

The indicated strains of neutralophilic *B. subtilis* or *B. pseudofirmus* OF4 were grown at pH 7.5 or pH 10.5, respectively in malate-containing media, washed and equilibrated at the starting pH for particular shift experiments in buffers to which no choline, sodium, or malate were added but which contained the indicated carbon source. A sudden alkaline shift was then imposed and the cytoplasmic pH was determined 10 minutes after the shift. (A) Left: Cells were equilibrated at pH 7.5 and shifted to pH 8.5 in the presence of malate in buffer containing added choline chloride, NaCl, or KCl (50 mM). Right: Cells were equilibrated at pH 8.5 and shifted to 10.5 in malate- or glucose-containing buffer with either NaCl or KCl (100 mM) (Cheng *et al.*, 1996; Gilmour *et al.*, 2000). (B) Cells of alkaliphilic strains were subjected to an alkaline shift from pH 8.5 to 10.5 as described above except that the shift buffer contained no carbon source and efficacy of two different concentrations of NaCl were compared (Ito *et al.*, 2004b). The strains were: wild type; SC34 gene encoding NavBP deleted; SC34R, NavBP gene restored; Mot6, deletion of *motPS*; SC34/Mot6, double NavBP and MotPS mutant.

Another important and unusual *B. subtilis* antiporter, the Mrp antiporter, was first discovered as the major Na⁺/H⁺ antiporter of alkaliphilic *B. halodurans* C-125 (Hamamoto *et al.*, 1994). This widespread antiporter is part of a unique type of cation/proton antiporter and hence

classified in its own family, CPA-3 family (Saier *et al.*, 1999; Saier, 2000). Mrp antiporters are encoded in operons that have six or seven genes whose products are all very hydrophobic membrane proteins and are all required for activity (Hiramatsu *et al.*, 1998; Ito *et al.*, 1999, 2000). All seven *B. subtilis* Mrp proteins form a hetero-oligomeric complex presumed to be the active form of the antiporter (Kajiyama *et al.*, 2007). The Mrp antiport is electrogenic, although the precise stoichiometry is not yet known for any Mrp system since the complexity of these systems makes purification and functional reconstitution especially challenging (Swartz *et al.*, 2007). In *B. subtilis*, Mrp only plays a significant role in pH homeostasis if *tetL* is absent or disrupted (Ito, unpublished data; see Table 1 for the absence of an effect of a *mrp* deletion in a homeostasis assay in *B. subtilis*). However, *mrp* mutants of *B. subtilis* are particularly sensitive to Na^+ inhibition (Ito *et al.*, 1999; Kosono *et al.*, 1999). Additional antiporters include two members of the NhaC antiporter family that play modest roles in pH homeostasis or growth at low PMF (Wei *et al.*, 2000) and NhaK, that exhibits $\text{Na}^+(\text{Li}^+)(\text{K}^+)/\text{H}^+$ antiport but has no established function (Fujisawa *et al.*, 2005) (Fig. 7A). Active proton uptake by the Na^+/H^+ antiporters is energized by the PMF generated by respiration and is critical for alkaline pH homeostasis in which the cytoplasmic pH is lower than the external pH. The PMF also energizes ATP synthesis and H^+ -coupled solute transport systems (H^+ /solute symporters) so protons also enter by these routes in support of pH homeostasis. The ongoing availability of cytoplasmic sodium to act as an efflux substrate for the Na^+/H^+ antiporters requires re-uptake routes for sodium which are comprised of Na^+ -coupled solute uptake systems (Na^+ /solute symporters) as well as a second motility system that uses Na^+ -coupled MotPS, an alternative motility system that is particularly active at elevated pH, sodium, and viscosity (von Blohn *et al.*, 1997; Ito *et al.*, 2004a).

3.4. Alkaliphiles

Studies of non-respiratory alkaliphiles that grow well at pH values above 9 have demonstrated a variety of interesting ATPases, some of which are discussed in a later section. This group of anaerobic or facultatively anaerobic alkaliphiles exhibits pH homeostasis that, as in respiratory alkaliphiles, depends upon Na^+ . For example, *Exiguobacterium aurantiacum* sustains a cytoplasmic pH that is ~ 0.3 pH units higher than the starting cytoplasmic pH 8.1 after a shift in external pH to 9.4 (McLaggan *et al.*, 1984). The thermophilic, anaerobic alkaliphile *Clostridium paradoxum* maintains a

Δ pH (acid inside) of as much as 1.3 units during increases of pH from 7.6 to 9.8 (Cook *et al.*, 1996). At present, much less is known about the specific transporters or other factors that help sustain the cytoplasmic pH in the non-respiratory alkaliphiles but this should change as more genomic data is obtained and comparisons with extensively studied respiratory alkaliphiles can be conducted.

The extreme respiratory alkaliphiles whose pH homeostasis has been studied most are *Bacillus* species (Kitada *et al.*, 2000; Krulwich *et al.*, 2007) (Fig. 7B). Extreme alkaliphiles such as *B. halodurans* C-125 and *B. pseudofirmus* OF4 and many other distinct strains, maintain a cytoplasmic pH that is ≥ 2 pH units lower than the external medium (Hamamoto *et al.*, 1994; Goto *et al.*, 2005; Padan *et al.*, 2005; Krulwich *et al.*, 2007). For example, *B. pseudofirmus* OF4 maintains a cytoplasmic pH 8.2–8.3 when growing on malate at pH 10.5 (Guffanti and Hicks, 1991; Sturr *et al.*, 1994) (Fig. 1) and can even do so after an abrupt pH 8.5→10.5 shift (Table 1A). This remarkable ability for pH homeostasis is dependent upon Na^+ , which cannot be replaced by K^+ as apparently is the case in neutralophilic *E. coli* and *B. subtilis* (Padan *et al.*, 2005; Krulwich *et al.*, 2007) and has been suggested for acidophiles (see Section 3.1). In the pH shift assays, non-fermentable malate is usually present as an energy source to support respiration and antiport. When glucose is added instead, overall homeostasis is not quite as good as with malate but there is a Na^+ -independent capacity for modest pH homeostasis that is not observed with non-fermentable malate (i.e., the cytoplasmic pH is kept below 10.5 in the presence of K^+ when glucose but not malate is present during a pH 8.5→10.5 shift). This capacity is presumed to reflect metabolic acid generation in the cytoplasm when glucose is present (Table 1A).

The Mrp antiporter is critical for alkaliphily and alkaline pH homeostasis in extremely alkaliphilic respiratory *Bacillus* species. This was shown in *B. halodurans* C-125 which was viable but non-alkaliphilic when *mrp* was mutated (Hamamoto *et al.*, 1994). Thus far, attempts to make deletions in the more extremely alkaliphilic *B. pseudofirmus* OF4 have not succeeded, probably because the deletion is lethal (Ito, unpublished data). As with *B. subtilis*, *B. pseudofirmus* OF4 Mrp forms hetero-oligomeric complexes containing all 7 Mrp proteins (Morino *et al.*, 2008); such complexes include a species that is approximately the expected size of a complex containing one of each protein, similar to the species observed for *B. subtilis* Mrp by Kajiyama *et al.* (2007). In the alkaliphile preparations, there are also larger hetero-oligomers that could be a dimer of a full complex (Morino *et al.*, 2008). It has been hypothesized that the multiple Mrp proteins may have several different transport activities that share synergies and

present a large protein surface to the outside by forming a complex. The Mrp complex surface could be engineered to effectively funnel protons into the antiporter(s) of the complex from a very alkaline medium (Swartz *et al.*, 2005). If a larger size distribution of alkaliphile Mrp complexes compared to neutralophile complexes is confirmed, it could represent an adaptation that promotes adequate proton gathering in the more alkaline milieu. Like *B. subtilis*, alkaliphilic *Bacillus* species also express additional Na^+/H^+ antiporters (Fig. 7B), one of which, NhaC, has been shown to make a small but discernible contributions to pH homeostasis and sodium-resistance (Ivey *et al.*, 1991; Ito *et al.*, 1997). There is also a two-component $\text{K}^+(\text{NH}_4^+)/\text{H}^+$ whose only demonstrated physiological role is in ammonium efflux that prevents interference with pH homeostasis during growth in media with high amine contents (Wei *et al.*, 2003; Fujisawa *et al.*, 2007).

Other aspects of the intersecting proton and sodium cycles of respiratory alkaliphiles are quite distinct from those in neutralophilic *B. subtilis* (Fig. 7A). Oxidative phosphorylation is proton-coupled in all respiratory alkaliphiles in spite of the low-bulk PMF that directly results from pH homeostasis (i.e., the inside-acidic ΔpH detracts from the PMF) (Krulwich, 1995; Krulwich *et al.*, 2007). Some sort of sequestration of protons is widely thought to be used for proton-coupled alkaliphile oxidative phosphorylation, but the relative contributions of specific membrane lipids, particular acidic protein segments near the membrane surface, and specific adaptations in the proton pumps and ATP synthase still need to be defined (Mulkidjanian *et al.*, 2006; Krulwich *et al.*, 2007). All other ion-coupled bioenergetic work, solute transport, and motility are sodium coupled (Ito *et al.*, 2004a; Krulwich *et al.*, 2007; Fujinami *et al.*, 2007b). Use of sodium is observed even among alkaliphiles that live in environments with low sodium concentrations. Its use as the major coupling ion for bioenergetic work is clearly an adaptation to the low PMF that exists at high pH. Even if the sodium concentration is not high, the sodium motive force is generally much higher than the PMF at very high pH. In addition to facilitating solute uptake, the sodium that enters the cells with solutes during symport has been shown to be a major source of the cytoplasmic sodium. This is in turn required to sustain continued antiporter activity in the alkaliphile in support of pH homeostasis (Krulwich *et al.*, 1985b). In the presence of sodium and a solute that can help it cycle, there is no discernible rise in the cytoplasmic pH or growth arrest as seen with neutralophiles directly after a large alkaline shift (Wang *et al.*, 2004; Padan *et al.*, 2005).

If solutes that enter *B. pseudofirmus* OF4 with sodium are largely omitted from the medium, sodium can re-enter via the sodium-coupled MotPS, in which it is the only stator-force generator for alkaliphile motility

(Ito *et al.*, 2004a), or via a voltage-gated sodium channel shown to be an alkali-activated voltage-gated sodium channel that was named NaChBac (Ren *et al.*, 2001; Ito *et al.*, 2004b) (Table 1B). Since *B. halodurans* C-125 is not genetically accessible, a deletion strain (SC34) of the *ncbA* gene that encodes the *B. pseudofirmus* OF4 homologue, Na_vBP was constructed. The *B. pseudofirmus* OF4 Na_vBP channel is required for full motility, for normal as opposed to inverse chemotaxis, and for fully normal growth at high pH (Ito *et al.*, 2004b; Fujinami *et al.*, 2007a). In pH shift experiments in the media with greatly decreased levels of substrates that enter with sodium, the Na_vBP mutant shows a deficit in pH homeostasis that increases if MotPS is also deleted (Table 1B) (Ito *et al.*, 2004b). The channel co-localizes with the polar chemotaxis receptors (MCPs) and deletion of the channel reduces the polar localization of the channel and vice versa (Fujinami *et al.*, 2007a). The mechanism and nature of the channel effect on chemotaxis will be of interest in other organisms in which channels may also interact with the chemotaxis pathway.

Finally, we note that properties of the membrane and secondary cell wall polymers (SCWPs; see Fig. 7) of alkaliphiles appear to play a role in alkaliphily that still needs more intensive investigation. Alkaliphilic *Bacillus* species have particularly high cardiolipin contents that are highest at very alkaline pH and there is also an interesting complement of neutral lipids (Clejan *et al.*, 1986). For example, it is possible that the high cardiolipin content of alkaliphile membranes fosters closeness of respiratory chain elements with the ATP synthase. Evidence for dynamic physical interaction between the terminal oxidase and ATP synthase of *B. pseudofirmus* OF4 has been shown under conditions in which the two complexes were present together in artificial phospholipid preparations (Krulwich *et al.*, 2007; Liu *et al.*, 2007).

In addition to the membrane lipids, a characteristic of the extreme alkaliphiles is that the hydrophilic loops of membrane proteins that are exposed on the outer surface have a much higher content of acidic residues than their homologues in neutralophiles, thus further increasing the negative charge near the membrane surface (Krulwich *et al.*, 2007). Figure 9 depicts a segment of the CtaC protein of *caa*₃-type cytochrome oxidase that is close to the outside of the membrane in proximity to the subunit, CtaD, from which pumped protons emerge. In alkaliphilic *Bacillus* species, this region is much more acidic than the same segment of neutralophile homologues (Quirk *et al.*, 1993; Hicks and Krulwich, 1995).

Secondary cell wall teichuronic acids are essential for alkaliphily in *B. halodurans* C-125 (Aono and Ohtani, 1990; Aono *et al.*, 1999). This is not the case with the S-layer polymer (SlpA) of *B. pseudofirmus* OF4. SlpA

		# acidic residues	# basic residues	pI
<i>B. pseudofirmus</i> OF4	E R D E Y D A W V E G M S A E V E E P - - T E	9	1	3.3
<i>B. halodurans</i> C-125	E R D D Y D A W V E G M M E A D A E P D T D D	11	1	3.0
<i>B. clausii</i>	E R D E Y D A W V E D M L A V E Q E A T - - A	8	1	3.3
<i>B. amyloliquefaciens</i>	P S K E F K Q W T K A M K N Y K H - T T D S G	2	6	10.8
<i>B. pumilus</i>	S Q D E F L G W T K K M A D Y K K P T S T K D	4	5	10.1
<i>B. subtilis</i>	S A K E F Q G W T K E M K N Y K S - T A E S D	4	4	7.5
<i>B. thuringiensis</i>	D E S E Y K K W L A D M K K I D G K K E V A S	6	6	7.4
<i>G. kaustophilus</i>	P R T E F D A W V E K M Q N A K K P V V T D P	4	4	7.5

Figure 9 Alkaliphile-specific sequence motif (or two) in the cytochrome oxidase. The protein sequences of subunit II of *Bacillus* sp. cytochrome *caa*₃ oxidases were aligned using the ClustalW program. Shown is the alignment of the region corresponding to residues 207–227 of the mature *B. pseudofirmus* OF4 protein. Acidic residues are boxed and basic residues are circled. The predicted isoelectric point (pI) of the region was taken from the DS gene program. The accession number and residue numbers for each species is as follows (residues are given for the mature protein): *B. pseudofirmus* OF4, Q04441, and 207-227; *B. halodurans* C-125, NP_243481, and 206-228; *B. clausii*, YP_175889 and 205-225; *B. amyloliquefaciens*, YP_001421069 and 216-237; *B. pumilus*, YP_091295 and 216-238; *B. subtilis*, NP_389372 and 216-237; *B. thuringiensis*, YP_896312 and 209-231; and *G. kaustophilus*, YP_146935 and 215-237.

is not essential for growth of *B. pseudofirmus* OF4 at pH 10.5. However, the cells exhibit a distinctly greater lag at pH 10.5 and even more so at pH 11; a deficiency in pH homeostasis by the *slpA* mutant is also observable in pH shift experiments (Table 1A) (Gilmour *et al.*, 2000). A deletion mutant lacking the *slpA* gene grows better than the wild type at pH 7.5. Nonetheless, high levels of the major cell surface polymer are present in both pH 7.5- and 10.5-grown cells (Gilmour *et al.*, 2000). This indicates that the organism is “hard-wired” for alkaliphily, ready to support pH homeostasis and grow optimally if there is a sudden alkaline shift even at the expense of growth at near-neutral pH.

3.5. pH Perturbation and Recovery

When growing cells are exposed to environmental pH near the acidic or alkaline limits of their growth range, especially if the transition is abrupt, this is respectively termed “acid shock” or “base shock.” Under such conditions, the growth rate is suboptimal, presumably due to partial loss of pH homeostasis. Acid or base shock typically leads to up-regulation of gene products that enhance growth under the pH stress condition. Such shock also leads to generation of compounds that increase survival at pH values above or below the pH range for growth. Responses that increase survival

in extreme acid are known as “acid tolerance” or “acid resistance” (Foster and Hall, 1991; Lin *et al.*, 1995; Foster, 2004). Acid resistance and base resistance are discussed in Section 5.

Acidophiles, particularly extreme acidophiles (growing below pH 3) need to maintain the largest Δ pH of any known organisms. Acidophiles use this large Δ pH across the cytoplasmic membrane to generate ATP, drive proton-coupled solute transporters and, in some cases, energize motility, but re-extrusion of the protons is critical, as noted above. There are relatively few studies of acidophiles subjected to acid shock. One such study of acid-stress of *A. ferrooxidans* (Chao *et al.*, 2008) involved “acid shock” as defined here. In this study, an organism that grows optimally at pH 2.3 was exposed to pH 1.3. Interestingly, the acidophile exhibited up-regulation of many of the same key acid-response genes observed in neutrophiles: RpoS and other stress sigma subunits, the Fur iron-acid regulator, the EnvZ/OmpR envelope stress regulator, and a number of OMPs.

Neutrophiles exhibit the same sorts of responses in challenges that are mediated either by direct pH shifts or by mutations in transporters with important roles in pH homeostasis. The SOS response, a prototypic stress response of neutrophilic *E. coli*, is induced by cytoplasmic alkalization in cells that lack the full complement of active pH homeostasis mechanisms (Schuldiner *et al.*, 1986) and mutants in particular two-component systems of *E. coli*, including an ArcAB deletion strain, show an alkaline-sensitive growth phenotype (Zhou *et al.*, 2003). In neutrophilic *B. subtilis*, alkali shock results in induction of a constellation of genes that significantly overlapped with the σ^W regulon that is involved in the cell wall stress response (Wiegert *et al.*, 2001; Cao *et al.*, 2002).

The actual process of pH perturbation and recovery has been observed with maximal time resolution in *E. coli* and in *Bacillus* species. In well-energized cultures of *E. coli* K-12, a rapid shift of external pH from pH 7.5 to 5.5 causes the cytoplasmic pH to decrease by more than 1.5 units (Fig. 1A, Wilks and Slonczewski, 2007) with recovery beginning within 10 seconds of acid addition. The recovery is biphasic, with a rapid recovery of most of the cytoplasmic pH value occurring within half a minute, followed by more gradual recovery approaching pH 7.4 over the next 4 minutes. By contrast, addition of 20 mM sodium benzoate permanently depresses cytoplasmic pH without recovery. Lower concentrations of benzoate allow partial, slow recovery, without the initial rapid phase. The measurement of pH recovery ultimately should provide clues as to the mechanism. So far, pH recovery rates show no correlation with flux of ions such as K^+ or Na^+ (Wilks and Slonczewski, unpublished data). An alkaline shift of *E. coli* from pH 7.2 to 8.3 results in a rapid and transient

alkalinization during which the cytoplasmic pH briefly reaches the new external pH. Growth arrests after the shift and does not resume until the cytoplasmic pH is about 7.9, about 15 minutes after the shift and complete restoration of the pre-shock cytoplasmic pH is observed about 30 minutes after the shock (Zilberstein *et al.*, 1984). In *B. subtilis*, a general stress response that depends upon σ^B is involved in acid adaptation (Hecker and Volker, 2001; Hecker *et al.*, 2007). As noted by Earl *et al.* (2008), *B. subtilis* strains survive passage through the human gastrointestinal track and acid fermentation conditions. At the alkaline side, *B. subtilis* cells have been shifted from a medium pH 6.3 to either pH 8.8 or 9.0 resulting, respectively, in a growth arrest of about an hour and 5 hours followed by restoration of growth. Shifts to pH 9.3 or higher led to growth arrest that was not reversed after 20 hours (Wiegert *et al.*, 2001).

3.6. Membrane-Permeant Organic Acids and Bases

Membrane-permeant weak acids that primarily cross the cell membrane as the hydrophobic protonated form can depress cytoplasmic pH (Kihara and Macnab, 1981; Salmond *et al.*, 1984; Russell and Diez-Gonzalez, 1998). Their net uptake is driven by ΔpH ; thus, at low external pH, a high concentration of protons is released upon deprotonation of the weak acid that is internalized. This can exhaust the cell's buffering and proton export capacity that leads to depression of the internal pH. Low molecular weight organic acids with a $\text{p}K_a$ in the range of pH 3–5 are toxic to neutralophiles, and even more so to acidophiles. These organic acids of moderately low $\text{p}K_a$ are fully protonated at the low pH typically used for growth of acidophiles. Due to their small size and neutral charge, they easily pass the cytoplasmic membrane; hence the term “permeant weak acids.” Once the permeant weak acid is inside the cell, the cytoplasmic pH is above the $\text{p}K_a$ for the dissociation of the proton that results in acidification of the cytoplasm (Alexander *et al.*, 1987; Ciaramella *et al.*, 2005). Of the tested organic acids in *A. ferrooxidans*, propionic acid was the least effective at lowering the pH and the chloroacetic acids were the most effective; this difference corresponds with decreasing $\text{p}K_a$ values with the time course of the drop in ΔpH over the first 10 minutes (Alexander *et al.*, 1987). A further study of *A. ferrooxidans* demonstrated that Fe^{2+} and sulfur oxidation as well as growth were inhibited by low molecular weight organic compounds including organic acids (Tuttle and Dugan, 1976). It was shown that the monocarboxylic organic acids completely inhibited growth on S^0 whereas other organic acids increased the lag phase

from 1 day in the absence of organic acid to 5–6 days during growth on S^0 but they did not completely inhibit growth (Tuttle and Dugan, 1976). However, some of the inhibitory action was due to direct inhibition of Fe^{2+} oxidation.

Compared to acidophiles, neutralophiles tolerate higher levels of permeant acids, which induce various means of adaptation. The depression of internal pH by permeant acids induces acid resistance genes in the neutralophilic Gram-negative pathogen *S. enterica* (formerly *S. typhimurium*) (Bearson *et al.*, 1998). In *E. coli*, permeant acids such as benzoic acid and acetic acid mediate pH taxis (Kihara and Macnab, 1981; Repaske and Adler, 1981). In lactococci, buildup of lactic acid limits the growth of competing organisms (Hutkins and Nannen, 1993). At low concentration, radiolabeled permeant acids serve as tools to measure internal pH (discussed above). Higher concentrations of a permeant acid that depress internal pH are useful to distinguish cytoplasmic effects from external pH (Slonczewski *et al.*, 1987; Kannan *et al.*, 2008). Exposure to a permeant acid or an uncoupler has been used to select for acid-sensitive mutants of *E. coli* (Slonczewski *et al.*, 1987), *S. enterica* (Foster and Bearson, 1994), and *Mycobacterium smegmatis* (Tran *et al.*, 2005).

The effect of permeant organic acids on cytoplasmic pH is complicated by several other kinds of effects. The most common naturally occurring organic acids are fermentation products, such as lactic, acetic, and formic acids, all of which have specific metabolic roles in cells (Russell and Diez-Gonzalez, 1998). Where ΔpH is maintained, the conjugate base accumulates in the cell in proportion to the ΔpH and specific compounds may elicit specific cellular responses. For example, in *E. coli*, acetate and formate up-regulate expression of completely different protein complements in proteome studies (Kirkpatrick *et al.*, 2001). Even a substance not metabolized by *E. coli*, such as benzoic acid, up-regulates a specific drug resistance regulon, Mar (Rosner and Slonczewski, 1994). In addition, compounds such as benzoic acid and salicylic acid are sufficiently hydrophobic to cross the membrane in the deprotonated form, thus partly collapsing $\Delta\Psi$ as well as ΔpH , and acting as uncouplers, that is, able to equilibrate protons across the membrane, resulting in depletion of the PMF. The degree of uncoupler effect will depend upon the pK_a and the membrane solubility of the compound (Salmond *et al.*, 1984).

Permeant bases cross the membrane in the deprotonated form, picking up protons inside the cell; at high concentration and high external pH, polyamines may raise cytoplasmic pH. In *E. coli*, exogenous spermine enhances bacterial survival in extreme acid, but diminishes survival in extreme base (Yohannes *et al.*, 2005). Production of polyamines can

counteract acidity (see Section 4.4). A number of commonly used biological buffers, such as tris(hydroxymethyl)aminomethane (Tris) and triethanolamine, act as permeant bases, even causing a pH-dependent chemotactic response (Repaske and Adler, 1981). The sensitivity of bacteria to inhibition by permeant bases can be used. Hsieh *et al.* (1998) noted an enormous increase in sensitivity of *Staphylococcus aureus* to cationic and weakly basic antimicrobials at alkaline pH, an effect that was synergistic with mutation of the multi-drug resistance gene *norA*. They suggested that such a strain would be useful for natural product screening.

4. pH CORRECTION MECHANISMS

If an excess of protons and hydroxide ions bypass the cell's pH homeostatic mechanisms and enter the cytoplasm, then the cell requires means by which to ameliorate the potential damage to the cell, and provide tolerance to cytoplasmic pH above or below its optimum. Some of these mechanisms are common between the neutralophiles, acidophiles, and alkaliphiles, such as cytoplasmic buffering that acts either to decrease or increase the cytoplasmic pH to regain optimum pH. Other mechanisms are found in particular species, such as catabolism that eliminates the uncoupling action of organic acids (discussed below).

4.1. Cytoplasmic Buffering

One method by which microorganisms may counteract changes in their internal pH is via the buffering capacity of the cytoplasm, in which pH-titratable cell components sequester or release protons according to the flux in pH. Cytoplasmic buffering molecules include proteins, polyamines, polyphosphates, and inorganic phosphate. Protein amino acid side chains offer potential buffering over a wide range of pH. Polyphosphates as well as inorganic phosphate have pK_a values around 7.2; thus, in principle, they could offer good buffering capacity near the optimal internal pH of neutralophiles. Polyphosphates are involved in many extreme stress adaptations (Seufferheld *et al.*, 2008) including acid exposure of *Burkholderia* (Moriarty *et al.*, 2006). Polyamines are also associated with acid resistance (Wortham *et al.*, 2007). Both polyphosphates and polyamines contribute to biofilm formation, a context in which passive buffering might provide particularly useful protection from pH shift.

Cytoplasmic buffering capacity is challenging to measure; in one example, the buffering capacity of non-growing *E. coli* cells (i.e., cells unlikely to accomplish significant enzymatic consumption of acids or bases) was estimated at 50 mM per pH unit, based on titration of intracellular pH by addition of the permeant acid sodium benzoate (Slonczewski *et al.*, 1982). Subsequently, Zychlinsky and Matin (1983) used the acid titration of unpermeabilized and permeabilized cells, as developed by others (Scholes and Mitchell, 1970; Maloney, 1979), to compare the buffering capacity of metabolically compromised *A. acidophilum* and *E. coli* giving values of 97 and 85 nmol H⁺ per mg protein, respectively. Measurement of the cytoplasmic buffering capacity of an acidophilic bacterium (strain PW2) also gave a buffering capacity of 85 nmol H⁺ per mg protein from a proton influx of 14.4 nmol H⁺ per mg protein (Goulbourne *et al.*, 1986). This amount of proton influx had little effect on the cytoplasmic pH although the anticipated effect would have been reduction of the cytoplasmic pH to 2 in the absence of cytoplasmic buffering capacity.

In a comparative study of acidophilic, neutralophilic, and alkaliphilic *Bacillus* species conducted using the same acid titration methodology on whole and permeabilized cells, the most striking finding was that high pH-grown alkaliphiles exhibited an especially high cytoplasmic buffering capacity at pH 8–9.5, the highest pH used in the study, whereas the highest buffering capacity for *B. subtilis* was at ~pH 5 (Krulwich *et al.*, 1985a). A more refined methodology was subsequently developed by Rius *et al.* (1995) and applied to a group of Gram-negative and later to Gram-positive bacteria (Rius and Lorén, 1998); decay of an acid pulse was measured in a protocol that avoided the use of permeabilized preparations. They confirmed the high cytoplasmic buffering capacity in pH 10.5-grown cells of *Bacillus alcalophilus* and found that pH 8.5-grown cells of the alkaliphile had much lower cytoplasmic buffering, lower than that of neutralophiles *B. subtilis* and *S. aureus*. The cytoplasmic buffering capacity of diverse Gram-negative bacteria was in a comparable range to those found in other studies, in general showing little correlation with growth optima. Cytoplasmic pH buffering is a ubiquitous component of pH homeostasis in microorganisms at all pH levels although the capacity for growth at very high or very low pH does not appear to directly correlate with the level of buffering, consistent with the importance of active mechanisms. Specific buffering molecules of particular importance have not been identified in acidophiles or alkaliphiles; thus there may be many nuanced variations in the poising of cytoplasmic buffering.

Many studies have calculated cytoplasmic buffering capacity (B_i) from the difference between whole intact cell buffering (B_o) and total buffering

of permeabilized preparations (B_1). These studies consistently showed that the buffering of the cell surface is a significant proportion of the total, as expected from the presence of numerous charged components of the outer cell membrane(s), cell wall, and SCWPs; this outer buffering is also likely to be protective and changes in the total surface buffering by increased synthesis of particular polymers is a strategy that can supplement active processes, for example, as with the secondary cell wall teichuronic acid polymers of alkaliphilic *B. halodurans* C-125 (Aono and Ohtani, 1990; Aono *et al.*, 1999).

4.2. Primary Proton Pumps

A key mechanism for acid pH homeostasis is transport of protons out of the cytoplasm by primary proton pumps. Primary proton pumps of acidophile electron transport chains for which descriptions encompassing experimental evidence have been presented include pumps in: *A. ferrooxidans* (Chen and Suzuki, 2005; Ferguson and Ingledew, 2008); “*F. acidarmanus*” Fer1 (Dopson *et al.*, 2005); and a number of thermoacidophilic archaea (reviewed in Schäfer *et al.*, 1999). In addition, primary proton pumps have been identified by sequencing data in *Leptospirillum* group II (*L. ferriphilum*) and *Ferroplasma* type II (Tyson *et al.*, 2004). Based on the Δ pH decrease during anaerobiosis and the addition of a protonophore, it was concluded that the Δ pH is maintained in *B. acidocaldarius* and *Thermoplasma acidiphilum* by active PMF generation (Michels and Bakker, 1985). All of the available genomes from acidophiles that respire have elements that could be involved in PMF generation in support of pH homeostasis. Interestingly, cytochrome *c* oxidase into which the electrons from ferrous iron directly enter in *A. ferrooxidans* exhibits a deviation from the consensus that eliminates one of the two putative proton pathways found in neutralophile homologues from the cytoplasm into the hydrophobic core of the oxidase (Ferguson and Ingledew, 2008). Models have been presented to account for PMF generation in this and related acidophiles. *A. ferrooxidans* also has two putative proton-efflux P-type ATPases (Valdés *et al.*, 2008) and putative proton pumps are also found in genomes of *A. caldus* and *A. thiooxidans* (Holmes, unpublished data).

As noted earlier, the F_1F_0 -ATP synthase is proton-coupled in non-marine respiratory alkaliphiles in spite of the low-bulk PMF, even though much of the other bioenergetic work of the aerobic alkaliphiles is coupled to sodium (Fig. 7B). The ion-translocating complexes of the respiratory chains of these organisms are proton pumping, for example, the Complex III (bc_1 complex)

and *caa3*-type cytochrome oxidase of *B. pseudofirmus* OF4 (Krulwich *et al.*, 2007). Like neutralophilic *B. subtilis*, the alkaliphilic *Bacillus* species for which there are genomic data lack a proton pumping, Complex I type NADH dehydrogenase. For electron input into the respiratory chain, they possess multiple type-II NADH dehydrogenases that carry out transfer of electrons from NADH to (mena)quinone but do not pump ions as well as additional non-pumping electron input enzymes such as succinate dehydrogenase and malate:quinone oxidoreductase (Gilmour and Krulwich, 1996; Liu *et al.*, 2008). There are several indications of a special role for the proton-pumping terminal oxidases of alkaliphiles. In *B. pseudofirmus* OF4, the *caa3*-type cytochrome oxidase encoded by the *cta* operon is up-regulated at high pH (Quirk *et al.*, 1993) and disruption of *cta* results in an inability of the alkaliphile to grow non-fermentatively even though alternative terminal oxidases are retained and even up-regulated (Gilmour and Krulwich, 1997). A characteristic noted earlier, that has consistently been observed with respiratory chain components of different alkaliphilic *Bacillus* species, is that many of the species, especially of cytochrome *c*, have been found to have much lower mid-point potentials than their homologues from neutralophiles (Lewis *et al.*, 1981; Yumoto *et al.*, 1991; Hicks and Krulwich, 1995; Goto *et al.*, 2005). Goto *et al.* (2005) and Muntyan and Bloch (2008) reported that the cytochrome *a* mid-point potential in terminal oxidases of alkaliphilic *Bacillus* species is normal and thus the lower than normal mid-point potential of cytochrome *c* species that donate to the terminal oxidase may permit an unusually high capture of energy at this critical segment of the respiratory chain.

4.3. Inorganic Ion Fluxes: Co-Transport and Antiport

Acidophiles use secondary transporters such as ion-coupled co-transporters (symporters) that utilize the ΔpH to energize solute transport (Albers *et al.*, 2001; Ferguson and Ingledew, 2008). In *B. acidocaldarius*, the uptake of a lactose homologue is inhibited by uncouplers that reduce the ΔpH suggesting that lactose is coupled to proton uptake (Krulwich *et al.*, 1978). The uptake of glucose is also suggested to be coupled to proton uptake as its transport collapses the ΔpH by 20% (Matin *et al.*, 1982). The potential importance of K^+ uptake has also been noted, and is reflected in the large number of secondary cation transporters in the sequenced genomes of *Picrophilus torridus* (Fütterer *et al.*, 2004), “*F. acidarmanus*” (<http://genome.ornl.gov/microbial/faci/>), *S. solfataricus* (She *et al.*, 2001), and *Leptospirillum* group II (Tyson *et al.*, 2004). The *S. solfataricus* genome contains a predicted Trk-like K^+ transporter that might contribute to

production of the inside-positive $\Delta\Psi$, symporters that are expected to use the ΔpH for uptake of sugars and peptides (15 encoded proteins), and high-affinity ABC-type transporters coded by 11 operons that are probably uptake systems as they include both permeases and extra cytoplasmic-binding proteins (She *et al.*, 2001). The genome sequence of *A. ferrooxidans* also contains a large number of secondary transporters including an Na^+/H^+ antiporter, several K^+ transporters including a voltage-gated channel, K^+ uptake and efflux proteins, and two copies of an ABC K^+ import system (Valdés *et al.*, 2008). The *H. pylori* homologue of the *E. coli* NhaA Na^+/H^+ antiporter has been extensively studied and found to have lower pH range for activity than *E. coli* NhaA, a range that suggests a role for the antiporter at the upper edge of its range of pH for growth (Inoue *et al.*, 1999). Perhaps the *A. ferrooxidans* Na^+/H^+ antiporter provides protection against “alkali shock” or against the increased toxicity of cytoplasmic Na^+ when the organism finds itself above its very acidic growth range. The establishment of roles for the other cation transporters and the special contribution of K^+ in particular, requires further investigation in the acidophiles.

In respiratory alkaliphilic *Bacillus* species, the only process other than oxidative phosphorylation that requires proton uptake from the highly alkaline external *milieu* by these alkaliphiles is cytoplasmic pH homeostasis itself, since it is dependent upon active inward transport of protons by antiporters (Hamamoto *et al.*, 1994). For the alkaliphilic *Bacillus* species, use of the Mrp complex as the major antiporter has been hypothesized to provide a large protein surface on the outside that could be adapted to act as a proton-concentrating and funneling element to support antiport-based pH homeostasis (Swartz *et al.*, 2005; Morino *et al.*, 2008). However, the other ion-dependent bioenergetic work in the alkaliphilic *Bacillus* species is driven by a sodium motive force established by the vigorous efflux due to Na^+/H^+ antiport activities (see Fig. 7B). The sodium motive force is constituted by an inwardly directed Na^+ gradient and the large chemiosmotically productive $\Delta\Psi$ (inside negative); it is much larger than the PMF because it is not adversely affected by the acid-inside ΔpH . Both ion-coupled solute transport and motility of the extreme alkaliphiles is coupled to sodium, even when growth is at near-neutral pH and it has been noted that these processes of extreme alkaliphiles require higher sodium ion concentrations at near-neutral pH than at high pH (Gilmour *et al.*, 2000; Fujinami *et al.*, 2007b). This apparently reflects an optimization of the sodium-coupled processes of alkaliphiles to function at high pH. The optimization is apparently accompanied by an inhibitory effect of the increased proton concentration at near-neutral pH. Other, more moderate alkaliphiles and alkali-adaptable neutralophiles either possess distinct sodium- and

proton-coupled transporters and motility channels (see Fig. 7B) or have transporters or motility channels that can use either a sodium motive force or a PMF, depending upon the conditions (Pourcher *et al.*, 1995; Franco and Wilson, 1996; Terahara *et al.*, 2008). The pH-dependent sodium versus proton preference of a bi-functional motility channel of alkaliphilic *Bacillus clausii* can be altered in either direction by pairs of mutations in the MotB component of the channel (Terahara *et al.*, 2008).

Genomes from aerobic alkaliphiles are predicted to encode a large number of ATP-dependent transporters, especially ABC-type transporters, as well as ion-coupled transporters (Takami *et al.*, 2000). In non-respiratory alkaliphiles, several P-type ATPases have been intensively studied (Suzuki *et al.*, 2005). In view of the prediction by Niggli and Sigel (2007) that antiport is a mechanistic feature of P-type ATPases it would be interesting to probe whether P-type ATPases that pump Na⁺ contribute to pH homeostasis by taking up H⁺.

4.4. Production and Consumption of Acids and Bases

As noted in Section 3.3, acidophiles are especially sensitive to uncoupling by organic acids and their production during fermentation as terminal electron acceptors would be highly toxic to the cell. It is possibly due to this that no fermentative acidophiles have been identified. It is also interesting to note that the most extreme acidophiles such as *Ferroplasma* spp. are chemoorganotrophs (Dopson *et al.*, 2004) that are capable of metabolizing organic acids and thus, ameliorating their toxic effects. Due to the fact that the acidophiles are able to gain organic carbon and energy from the organic acids, it is impossible to discern if the expression of proteins for their degradation is a pH homeostasis response. Examples of genes involved in organic acid degradation are found in acidophile genomes including *P. torridus* (Angelov and Liebl, 2006) and “*F. acidarmanus*” Fer1 (<http://genome.ornl.gov/microbial/faci/>). The enzymes encoded include propionyl-CoA synthase, two acetyl-CoA synthetases, and lactate-2-monooxygenase that convert lactate to pyruvate (Ciaramella *et al.*, 2005).

In neutralophilic heterotrophs, a large number of different amino acid decarboxylases and related degradative enzymes are up-regulated at low pH (Table 2). These enzymes offer the opportunity to remove a proton from the cytoplasm through outward diffusion of CO₂ while retaining an alkaline product such as an amine, or exporting it through a cognate transporter (Fig. 6). In most cases, the decarboxylase is co-expressed with an antiporter that exchanges the substrate with a decarboxylated product,

Table 2 Enzymes consuming acids or bases.

Enzyme substrate	Gene(s)	Organism(s)	Reference(s)
<i>Decarboxylases and other enzymes up-regulated at low pH</i>			
2-Acetolactate	<i>alsD</i>	<i>B. subtilis</i>	Wilks <i>et al.</i> (2009)
Agmatine (deiminase)	<i>aguAI</i>	<i>Lactococcus brevis</i>	Lucas <i>et al.</i> (2007)
Arginine	<i>speA</i>	<i>E. coli, B. subtilis</i>	Gong <i>et al.</i> (2003), Stim and Bennett (1993), Wilks <i>et al.</i> (2009)
Arginine (deiminase)	<i>arcA</i>	<i>Streptococcus spp., Lactococcus spp.</i>	Dong <i>et al.</i> (2002), Cotter and Hill (2003)
CO ₂ (carbonic anhydrase)	<i>aac, aphA</i>	<i>H. pylori</i>	Bury-Moné <i>et al.</i> (2008)
Glutamate	<i>gadA, B</i>	<i>E. coli, L. lactis</i>	Castanie-Cornet <i>et al.</i> (1999), Nomura <i>et al.</i> (1999), Richard and Foster (2004)
Glycine	<i>gcvPA, PB</i>	<i>B. subtilis</i>	Wilks <i>et al.</i> (2009)
Histidine		<i>Lactococcus</i> spp.	Fernández and Zúñiga (2006)
Lysine	<i>cadA</i>	<i>E. coli</i>	Auger and Bennett, 1989, Watson <i>et al.</i> (1992)
Malate	<i>maeA</i>	<i>B. subtilis</i>	Wilks <i>et al.</i> (2009)
Ornithine	<i>speF</i>	<i>E. coli</i>	Kashiwagi <i>et al.</i> (1992)
Oxalate	<i>yvrK</i>	<i>B. subtilis</i>	Tanner and Bornemann (2000)
Phosphatidylserine	<i>psd</i>	<i>B. subtilis</i>	Wilks <i>et al.</i> (2009)
Tyrosine	<i>tyrDC</i>	<i>Lactococcus</i> spp.	Fernández and Zúñiga (2006), Wolken <i>et al.</i> (2006)
Urea (urease)	<i>ureA</i>	<i>Helicobacter pylori, Yersinia enterocolitica</i>	Stingl <i>et al.</i> (2002), Young <i>et al.</i> (1996)
<i>Deaminases up-regulated at high pH</i>			
Arginase (removes urea)	<i>rocF</i>	<i>B. subtilis</i>	Wilks <i>et al.</i> (2009)
Serine	<i>sdaA</i>	<i>E. coli</i>	Yohannes <i>et al.</i> (2004)
Tryptophan, cysteine, and serine	<i>tnaA</i>	<i>E. coli</i>	Blankenhorn <i>et al.</i> (1999), Bordi <i>et al.</i> (2003)

under regulation of one or more regulators sensitive to external pH and anaerobiosis (Meng and Bennett, 1992a, b). The net result of these decarboxylases is release of CO₂ and an amine, with net removal of a proton from the system. For example, in the case of lysine decarboxylase,

cadAB, low external pH is detected by the regulator *cadC* (Slonczewski *et al.*, 1987; Meng and Bennett, 1992a, b; Neely *et al.*, 1994; Neely and Olson, 1996). CadC is a fused ToxR-type two-component regulator whose extracellular domain detects pH (Dell *et al.*, 1994). Through CadC, low pH induces expression of both the lysine decarboxylase (CadA) and the lysine-cadaverine antiporter (CadB). The decarboxylase converts lysine into CO₂ and cadaverine, a basic polyamine. The polyamines generated by amino acid decarboxylases have an alkalinizing effect that may raise the external pH. In addition, they block OMPs thus retarding the influx of some organic permeant acids (Pagès *et al.*, 2008).

Different species express different sets of acid-dependent decarboxylases. *E. coli* expresses the catabolic decarboxylases for lysine and arginine (Stim and Bennett, 1993), ornithine (Kashiwagi *et al.*, 1992), and glutamate (Hersh *et al.*, 1996; Castanie-Cornet *et al.*, 1999). Expression is maximal anaerobically at low pH. Lactococci express the glutamate and arginine systems (Nomura *et al.*, 1999; Cotter and Hill, 2003) as well as decarboxylases for tyrosine, histidine, and agmatine (Cotter and Hill, 2003; Fernández and Zúñiga, 2006). In *B. subtilis*, proteins up-regulated at low pH include decarboxylases for arginine, 2-acetolactate, glycine, malate, phosphatidylserine (Wilks *et al.*, 2009), and oxalate (Tanner and Bornemann, 2000).

The assessment of metabolic contributions to pH homeostasis is complicated by the fact that these systems are subject to multiple layers of regulation. For instance the lysine decarboxylase and cognate lysine-cadaverine antiporter are co-induced by acid, anaerobiosis, and lysine (Meng and Bennett, 1992a, b). The Gad system, including glutamate decarboxylase and antiporter plus an associated acid fitness island (Mates *et al.*, 2007) are induced by the stationary-phase RpoS regulon at low pH under aerobic conditions (Lin *et al.*, 1995; Masuda and Church, 2003; Foster, 2004), but RpoS also enables Gad induction at high pH under anaerobiosis (Hersh *et al.*, 1996). In *H. pylori*, the urease extreme-acid protection system is maximally expressed in the presence of urea, nickel, and low pH (Slonczewski *et al.*, 2000; van Vliet *et al.*, 2001).

In most cases the actual contribution of acid-up-regulated metabolism to pH homeostasis has been difficult to demonstrate in growing cells, because such a large number of systems are available. However, the requirement for several specific decarboxylases has been documented for pH homeostasis during non-growth survival (see Section 5.1). Other enzymes that provide protection against acid during bacterial survival at extreme low pH include urease and carbonic anhydrase that function in *H. pylori* as discussed earlier, and that are also used by

bacteria that only survive but do not colonize the acid regions of the gut (see below).

At high external pH, neutralophiles tend to catabolize amino acids and related substrates by different pathways that release ammonia rather than CO₂ (Blankenhorn *et al.*, 1999; Bordi *et al.*, 2003; Yohannes *et al.*, 2004; Hayes *et al.*, 2006). Like the decarboxylases, the amino acid deaminases show enhanced expression under anaerobiosis when the absence of oxygen limits metabolic options. The tryptophan deaminase *tnaA* is of particular interest as it catabolizes serine and cysteine as well as tryptophan. The base-up-regulated *roc* pathway of *B. subtilis* generates two or three NH₃ plus TCA cycle acids, differing markedly from the acid-up-regulated *arc* pathway of streptococci (Chen *et al.*, 2002) which generates one NH₃ plus polyamines. The alkali-adaptive activities of the *B. subtilis roc* pathway are consistent with its induction in *tetL*-deficient mutants (Wei *et al.*, 2006). Nevertheless, some ambiguous cases remain to be explained; for example, glutamate decarboxylase is also induced at high pH, particularly under anaerobiosis and stationary phase (Blankenhorn *et al.*, 1999; Hayes *et al.*, 2006). The effects of amino acid catabolism at high pH are poorly characterized.

Acid and base modulate several other pathways of anaerobic catabolism. In *Leuconostoc lactis*, low pH shunts fermentation to the neutral product acetoin (Cogan *et al.*, 1981) and in *Lactobacillus plantarum* acetoin production is associated with improved pH homeostasis (Tsau *et al.*, 1992). In *B. subtilis*, low pH favors production of lactate (Schilling *et al.*, 2007). *E. coli* limits internal acidification by producing lactate instead of acetate plus formate (Bunch *et al.*, 1997) and by conversion of formate to H₂ and CO₂ (Rossman *et al.*, 1991). Low pH up-regulates several hydrogenases, which interconvert protons with H₂ (Hayes *et al.*, 2006). Low pH appears to enhance catabolism of sugar derivatives whose fermentation minimizes acid production, including sorbitol, glucuronate, and gluconate (Hayes *et al.*, 2006). Above pH 7, the favored fermentation products are acetate and formate (Wolfe, 2005) and the favored pathways of sugar catabolism are those related to glucose (Hayes *et al.*, 2006). High pH also up-regulates amino acid deaminases that remove ammonia and direct carbon into the TCA cycle, such as tryptophan deaminase and serine deaminase (Stancik *et al.*, 2002).

5. pH HOMEOSTASIS UNDER NON-GROWTH CONDITIONS

The ability of microorganisms to remain viable during conditions not permitting growth is essential for persistence in a changing environment.

Many neutralophiles possess inducible means of maintaining limited pH homeostasis for several hours under “extreme” pH conditions, enabling survival below pH 3 or above pH 10. Such microorganisms are commonly said to be “acid resistant” or “base resistant,” respectively. The phenomena of extreme-acid survival in neutralophiles are highly important for microbial ecology, such as nitrogen-fixing rhizobia (Dilworth *et al.*, 1999; Tiwari *et al.*, 2004), as well as for human and animal pathogens such as *E. coli* O17:H7 (Lin *et al.*, 1996) and *Yersinia enterocolitica* (de Koning-Ward and Robins-Browne, 1995). In extreme base, survival of pathogens such as *Listeria monocytogenes* is important for food treatment (Giotis *et al.*, 2008).

5.1. Extreme Acid: pH Homeostasis Without Growth

The terminology for various conditions of extreme-acid survival has shifted over the years (Gorden and Small, 1993; Slonczewski and Foster, 1996; Foster, 2004). Generally, the term “acid survival” or “acid resistance” refers to the ability of a substantial portion of cells (>10%) to retain colony-forming potential at pH 7 following 2–4 hours exposure at an external pH value below pH 3. Acid resistance factors may be constitutive, such as the low H⁺ conductance of membranes, or the buffering capacity of the cell, or the presence of constitutive ion transporters. Alternatively, acid resistance may be increased under various environmental conditions such as stationary phase, mediated by the RpoS regulon. Acid resistance “inducible” by growth in moderate acid is also called “acid tolerance.” The distinction between constitutive and inducible is blurred, however, as (1) “constitutive” acid resistance may turn out to require unidentified inducible factors; (2) “inducible” acid resistance of a laboratory strain may turn out to be constitutive in wild strains or clinical isolates; and (3) the transition between the ranges of growth and survival may be unclear, and may be strain-dependent.

A growing number of metabolic systems are known to help bacteria survive at pH below their growth range (Young *et al.*, 1996; Richard and Foster, 2003; Foster, 2004; Sachs *et al.*, 2005). Some of these systems are acid-independent, such as the RpoS-dependent acid tolerance response of *E. coli*, whereas others such as the Gad regulon are induced during growth at low pH and/or by other factors such as anaerobiosis. Some components help raise the pH homeostasis; whereas others help cells cope with lower pH for example by chaperones removing misfolded proteins. Here we focus

on systems that enhance pH homeostasis while maintaining a relatively large ΔpH .

In *E. coli*, several metabolic systems governed by overlapping regulons contribute to acid resistance. The stationary-phase expression of RpoS confers acid resistance through a poorly understood mechanism involving cAMP activation and the F_0F_1 -ATP synthase (Castanie-Cornet *et al.*, 1999; Ma *et al.*, 2003; Richard and Foster, 2004). RpoS also enhances expression of the glutamate-dependent Gad system (Lin *et al.*, 1995; Richard and Foster, 2004). The glutamate decarboxylases (GadA and GadB) and the glutamate-2-ketoglutarate antiporter (GadC) consume protons through production of polyamines and CO_2 , enabling *E. coli* to survive for many hours at pH 2.5 (Fig. 10). Survival at pH 2.5 in minimal medium requires the presence of glutamate. Analogous acid survival mechanisms have been demonstrated for arginine and lysine (Lin *et al.*, 1995; Gong *et al.*, 2003; Iyer *et al.*, 2003). Besides the polyamines that buffer pH, the CO_2 generated by decarboxylases and other metabolism enhances acid resistance by an unknown mechanism (Sun *et al.*, 2005). RpoS also amplifies the acid induction of cyclopropane fatty acid synthesis, which increases survival in extreme acid (Chang and Cronan, 1999; Kim *et al.*, 2005).

The *E. coli* survival mechanism at pH 2.5 involving glutamine or arginine (and possibly lysine) requires maintenance of cytoplasmic pH at a minimum of pH 4, with the $\Delta\Psi$ inverted (inside positive) (Iyer *et al.*, 2002; Richard and Foster, 2004). The source of the inside-positive $\Delta\Psi$ remains unclear, although the ClC-type proton-chloride antiporter may play a role (Fig. 10). The inverted $\Delta\Psi$ enables cells to sustain a ΔpH for an extended period without ongoing energy expenditure. The ClC H^+/Cl^- antiporter may also help the cell recover its inside-negative $\Delta\Psi$ after the external pH recovers above a critical acid threshold, at about pH 4–5. Thus, cytoplasmic pH homeostasis in extreme acid extends strategies used during growth in moderate acid, such as the amino acid decarboxylases, while adding a mechanism found in extremophiles, the inversion of $\Delta\Psi$.

An analogous form of acid resistance is also exhibited by *H. pylori*, which can survive below its growth range of gastric acid by maintaining a cytoplasmic pH ~ 4.9 at an outside pH ~ 1.3 (Stingl *et al.*, 2001). As discussed above for growth of *H. pylori* (Section 3.2), survival below external pH 2 requires uptake of urea into the cytoplasm by UreI, cytoplasmic urease and carbonic anhydrase and a periplasmic carbonic anhydrase to maintain a periplasmic pH that is much higher than the external pH and a cytoplasmic pH that is even higher (Fig. 8) (Marcus *et al.*, 2005; Stähler *et al.*, 2005; Bury-Moné *et al.*, 2008). At pH 2, these mechanisms may support sufficiently robust pH homeostasis to ensure

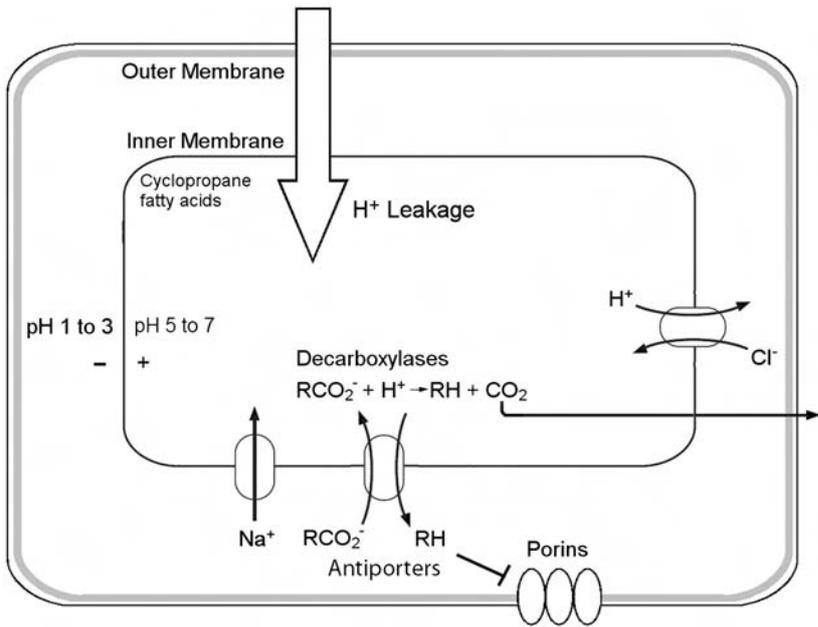


Figure 10 Acid resistance mechanisms in *E. coli* surviving at pH values below its growth range. Proton leakage into the cell is limited by the inverted $\Delta\psi$ (inside positive). The Cl⁻/Cl⁻ chloride-proton antiporter may help cells restore the inside-negative $\Delta\psi$ following neutralization of external acid (Iyer *et al.*, 2002). Sodium transport contributes to the inside-positive charge. Decarboxylases of glutamate, arginine, and lysine consume protons, exporting amines plus CO₂ (Iyer *et al.*, 2002; Foster, 2004; Richard and Foster, 2004). CO₂ enhances acid resistance by an unknown mechanism (Sun *et al.*, 2005). CFAs in the inner membrane increase acid resistance, possibly by lowering proton conductance (Chang and Cronan, 1999).

survival with growth arrest, while they can support colonization and growth in somewhat less extremely acidic surface of the gut.

Acid-induced urease similarly enhances the acid resistance of enteric pathogens *Yersinia enterocolitica* and *Morganella morganii* (Young *et al.*, 1996). Like *H. pylori*, *Y. enterocolitica* employs urease to survive passage through the very acidic stomach; it differs from *H. pylori* in that it does not colonize a very acidic niche, but moves on to colonize the more hospitable intestine (de Koning-Ward and Robins-Browne, 1995, 1997; Young *et al.*, 1996). The cytoplasmic urease of *Y. enterocolitica* is activated 780-fold by low-pH conditions and exhibits a lower pH optimum than most ureases, in a range of 4.5–5.5 (Young *et al.*, 1996; De Koning-Ward and

Robins-Browne, 1997). This is lower than the pH optimum for the *H. pylori* urease, consistent with the *Yersinia* use of urease for survival rather than to facilitate growth (Sachs *et al.*, 2006). Mutants of *Y. enterocolitica* with a disrupted urease gene exhibited a 1,000-fold decrease in acid survival *in vitro* relative to wild type and a 10-fold reduction in viability after passage through the stomach of mice (de Koning-Ward and Robins-Browne, 1995).

Acid resistance mechanisms, both constitutive and acid-induced, are now known to be widespread among neutralophiles. Other examples not discussed above include the enteric organisms *Shigella flexneri* and *S. enterica* (Gorden and Small, 1993; Lin *et al.*, 1995); *Lactobacillus brevis*, involving agmatine deiminase and tyrosine decarboxylase (Lucas *et al.*, 2007); *L. lactis*, involving chloride transport (Sanders *et al.*, 1998) as well as mycobacteria, staphylococci, and other Gram-positive organisms (reviewed by Cotter and Hill, 2003). In some bacteria, acid resistance requires increased tolerance to metals. Adaptation for acid survival of *Lactobacillus bulgaricus* includes up-regulation of metal-transporting CPX-Type ATPases, believed to contribute to copper homeostasis (Penaud *et al.*, 2006). Similar protection from copper toxicity contributes to acid resistance of *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Reeve *et al.*, 2002).

Like the neutralophiles, even extreme acidophiles need to survive in environments at extreme pH values below their growth range, such as Iron Mountain, CA where pH values have been measured as low as pH -3.6 , termed “super acids” (Nordstrom and Alpers, 1999). The reporting of negative pH values is controversial as by the classical definition, only pH values between 1 and 13 are possible and a new model based on sulfuric acid solutions is used (Nordstrom and Alpers, 1999). The extremely low-pH mine waters are formed due to the rock consisting of 95% pyrite, the temperature and humidity being near optimal for net proton producing acidophile catalyzed mineral dissolution, concentration of protons by evaporation, and the formation of soluble, efflorescent salts containing acidity that can be re-dissolved during periods of higher water levels whereby, the acidity is released (Nordstrom *et al.*, 2000).

Microorganisms are found in the Iron Mountain site at pH values ranging from 0 to ≈ 2.5 (Robbins *et al.*, 2000) including eukarya, bacteria, and archaea (Edwards *et al.*, 1999). Based on fluorescent *in situ* hybridization, “*F. acidarmanus*” Fer1 constituted up to 85% of the population with low numbers of *Leptospirillum* spp. at a pH 0.3–0.7 area of the mine suggesting the microorganisms were active and therefore, able to balance their internal pH (Bond *et al.*, 2000; Edwards *et al.*, 2000). At a second extremely acidic site between pH 0.6 and 0.8, *Ferroplasma* spp. constituted

52% of the microbial population once again with low numbers of *Leptospirillum* spp. (España *et al.*, 2008). “*F. acidarmanus*” Fer1 (Edwards *et al.*, 2000), *Ferroplasma thermophilum* (Zhou *et al.*, 2008), *Ferroplasma cupricumulans* (Hawkes *et al.*, 2006), and other species from the genus *Picrophilus* (Schleper *et al.*, 1995) are capable of growth at between pH 0 and 0.4. Although “*F. acidarmanus*” Fer1 cannot grow at pH -2 , it has been shown to survive at this pH though it cannot survive at pH -3.6 (Edwards, personal communication). A study of the extremely acidophilic archaeon *P. oshimae* cytoplasmic pH shows that it has an internal pH around 4.6 up to an external pH 4.0, and above this value the cells rapidly lysed (van den Vossenberg *et al.*, 1998b). The ΔpH of *P. oshimae* was found to be greater than 4 pH units, rapidly declining as the external pH is raised, since internal pH remains constant (van den Vossenberg *et al.*, 1998b). Over the same external pH range, the $\Delta\Psi$ decreases from 100 to 45 mV giving a PMF of -175 mV at pH 1.0 that decreases upon increasing external pH. Therefore, the study suggests that extreme acidophiles utilize similar pH homeostatic mechanisms and, as a consequence of their low internal pH, their enzymes may show unusually low optimum pH (Golyshina *et al.*, 2006).

5.2. Extreme Base: pH Homeostasis Without Growth

Survival of neutralophiles in extreme base has been less studied than acid survival. Interestingly, in *E. coli* base survival requires RpoS, as does acid survival (Small *et al.*, 1994; Bhagwat *et al.*, 2006). Survival of an RpoS-positive strain at pH 10 requires prior growth to stationary phase at pH 8. In *L. monocytogenes*, an alkali-inducible base resistance has been demonstrated, involving glutamine and phosphate transporters, among other components (Giotis *et al.*, 2008). The role of cytoplasmic pH homeostasis in these cases has not been characterized.

5.3. Biofilms

An intriguing question for further study is the role of pH homeostasis in biofilms. In principle, biofilm formation should enhance pH homeostasis since the ratio of cytoplasmic to extracellular volume is increased by the close proximity of cells. The increased relative volume of cytoplasm should offer greater opportunities for pH maintenance, while also posing greater challenges for expulsion of excess acids and bases. Biofilms are associated with various kinds of stress protection, including acid resistance. In the oral

bacterium *Streptococcus mutans*, surface adhesion and biofilm development enhance survival at pH 3–3.5 (Zhu *et al.*, 2001; Welin *et al.*, 2003; McNeill and Hamilton, 2004; Welin-Neilands and Svensäter, 2007). In oral biofilms, arginine catabolism to polyamines may contribute to caries formation (Burne and Marquis, 2000). Maintenance of pH homeostasis is important for *S. mutans* biofilms under acid stress. Biofilm formation may also contribute to acid resistance of *H. pylori* (Stark *et al.*, 1999). Biofilms of nitrifying bacteria appear to balance acid-producing with acid-consuming forms of metabolism (Gieseke *et al.*, 2006).

In extremely acidic environments, acidophiles often exist as biofilms such as the “*F. acidarmanus*” Fer1 dominated site at Iron Mountain, CA (Edwards *et al.*, 2000) (discussed in Section 5.2) and the *Acidithiobacillus* spp. dominated snottites in the Frasassi cave system, Italy (Macalady *et al.*, 2007). Further analysis of the approximately pH 1 Iron Mountain biofilms revealed various defined stratified structures with the less acid-tolerant *Leptospirillum* group II in a dense layer at the bottom of the biofilm and the archaea in the surface areas in a mature pool pellicle biofilm (Wilmes *et al.*, 2008). However, a community proteomics analysis of an Iron Mountain biofilm did not reveal any particular pH homeostatic mechanisms (Ram *et al.*, 2005). The pH 0–1 environment in the Frasassi cave system is generated by H₂S oxidation and is extremely low for the acidithiobacilli identified that habitually inhabit environments around pH 2–3 (Macalady *et al.*, 2007). The acidic biofilms were much lower in diversity than in neutral areas of the cave system and they are one of the simplest biofilm communities known. The biofilm was unusual in that it was dominated by bacteria rather than archaea that are usually prevalent at very low-pH values and this may be reflected by the electron donor being sulfide (and the lack of Fe²⁺) that is less commonly oxidized by the extremely acidophilic archaea. That the bacteria were growing in a biofilm may provide some degree of protection against the low pH in the cave.

6. CONCLUSIONS

The balance of protons and hydroxyl ions is of universal importance for microbial growth and for survival outside the growth range of pH. Bacteria and archaea adapted to acid maintain a cytoplasmic pH higher than that of the exterior, whereas those growing in base maintain a lower pH (Fig. 1). To maintain cytoplasmic pH at the more neutral value, cells accept a substantial energy loss through inversion of $\Delta\psi$ in extreme acid and

expenditure of $\Delta\psi$ in extreme base. Intriguingly, over the range of microbes studied, the crossover point where cytoplasmic pH equals external pH lies between pH 7–8, although not all species can actually grow at this point.

The observation and study of microbial pH is challenging for several reasons. Observation of pH within the cell requires use of probes labeled by radioactivity, fluorescence, or NMR, all of which can only be used for particular ranges of pH and culture conditions. Numerous organic and inorganic molecules within the cell can generate or consume protons. Multiple mechanisms contribute to cytoplasmic pH, often preventing isolation of mutants with a defective phenotype. Cells include various compartments such as periplasm and forespore whose pH may or may not equal that of the cytoplasm.

The mechanisms of pH homeostasis are manifold and often redundant; thus, multiple K^+ and Na^+ transporters contribute ion fluxes that balance proton flow, and multiple catabolic enzymes consume and generate protons. Membrane and envelope adaptations are most pronounced in acidophiles, although lipid and porin adaptations are also seen in neutralophiles and alkaliphiles. While diverse mechanisms predominate in particular species, such as urease/carbonic anhydrase-dependent protection from acid in *H. pylori* and Na^+/H^+ antiporters in alkaliphilic *Bacillus* species, general classes of metabolic, transport flux, and membrane-based mechanisms appear throughout the range of pH-adapted microorganisms. Thus, the study of pH homeostasis in any given species may yield clues as to mechanisms in very different species.

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REFERENCES

- Albers, S.V., Van Den Vossenberg, J.L., Driessen, A.J. and Konings, W.N. (2001) Bioenergetics and solute uptake under extreme conditions. *Extremophiles* **5**, 285–294.

- Alexander, B., Leach, S. and Ingledew, W.J. (1987) The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile *Thiobacillus ferrooxidans*. *J. Gen. Microbiol.* **133**, 1171–1179.
- Amaro, A.M., Chamorro, D., Seeger, M., Arredondo, R., Peirano, I. and Jerez, C.A. (1991) Effect of external pH perturbations on *in vivo* protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. *J. Bacteriol.* **173**, 910–915.
- Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjorn, S.P., Givskov, M. and Molin, S. (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl. Environ. Microbiol.* **64**, 2240–2246.
- Angelov, A. and Liebl, W. (2006) Insights into extreme thermoacidophily based on genome analysis of *Picrophilus torridus* and other thermoacidophilic archaea. *J. Biotechnol.* **126**, 3–10.
- Aono, R., Ito, M. and Machida, T. (1999) Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *Bacillus lentus* C-125. *J. Bacteriol.* **181**, 6600–6606.
- Aono, R. and Ohtani, M. (1990) Loss of alkaliphily in cell-wall-component-defective mutants derived from alkaliphilic *Bacillus* C-125. *Biochem. J.* **266**, 933–936.
- Arechaga, I. and Jones, P.C. (2001) The rotor in the membrane of the ATP synthase and relatives. *FEBS Lett.* **494**, 1–5.
- Auger, E.A. and Bennett, G.N. (1989) Regulation of lysine decarboxylase activity in *Escherichia coli* K-12. *Arch. Microbiol.* **151**, 466–468.
- Baker-Austin, C. and Dopson, M. (2007) Life in acid: pH homeostasis in acidophiles. *Trends Microbiol.* **15**, 165–171.
- Bakker, E.P. and Mangerich, W.E. (1981) Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. *J. Bacteriol.* **147**, 820–826.
- Barrett, C.M.L., Ray, N., Thomas, J.D., Robinson, C. and Bolhuis, A. (2003) Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **304**, 279–284.
- Batrakov, S.G., Pivovarova, T.A., Esipov, S.E., Sheichenko, V.I. and Karavaiko, G.I. (2002) β -D-glucopyranosyl caldarchaetidylglycerol is the main lipid of the acidiphilic, mesophilic, ferrous iron-oxidising archaeon *Ferroplasma acidiphilum*. *Biochim. Biophys. Acta* **1581**, 29–35.
- Bauerfeind, P., Garner, R., Dunn, B.E. and Mobley, H.L. (1997) Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. *Gut* **40**, 25–30.
- Bearson, B.L., Wilson, L. and Foster, J.W. (1998) A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J. Bacteriol.* **180**, 2409–2417.
- Bhagwat, A.A., Tan, J., Sharma, M., Kothary, M., Low, S., Tall, B.D. and Bhagwat, M. (2006) Functional heterogeneity of RpoS in stress tolerance of enterohemorrhagic *Escherichia coli* strains. *Appl. Environ. Microbiol.* **72**, 4978–4986.
- Blankenhorn, D., Phillips, J. and Slonczewski, J.L. (1999) Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J. Bacteriol.* **181**, 2209–2216.
- Blaser, M.J. and Atherton, J.C. (2004) *Helicobacter pylori* persistence: biology and disease. *J. Clin. Invest.* **113**, 321–333.

- Bond, P.L., Druschel, G.K. and Banfield, J.F. (2000) Comparison of acid mine drainage microbial communities in physically and geochemically distinct ecosystems. *Appl. Environ. Microbiol.* **66**, 4962–4971.
- Booth, I.R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49**, 359–378.
- Booth, I.R., Ferguson, G.P., Miller, S., Li, C., Gunasekera, B. and Kinghorn, S. (2003) Bacterial production of methylglyoxal: a survival strategy or death by misadventure?. *Biochem. Soc. Trans.* **31**, 1406–1408.
- Bordi, C., Théraulaz, L., Méjean, V. and Jourlin-Castelli, C. (2003) Anticipating an alkaline stress through the Tor phosphorelay system in *Escherichia coli*. *Mol. Microbiol.* **48**, 211–223.
- Brändén, M., Sandén, T., Brzezinski, P. and Widengren, J. (2006) Localized proton microcircuits at the biological membrane–water interface. *Biochim. Biophys. Acta* **103**, 19766–19770.
- Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5- (and 6-) carboxyfluorescein succinimidyl ester. *Appl. Environ. Microbiol.* **62**, 178–183.
- Buchanan, R.L. and Edelson, S.G. (1999) pH-dependent stationary phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. *J. Food Prot.* **62**, 211–218.
- Buckenhuskes, H.J. (2001) Fermented vegetables. In: *Food Microbiology: Fundamentals and Frontiers* (M.P. Doyle, L.R. Beuchat and T.J. Montville, eds), Chapter 32, pp. 665–679. ASM Press, Washington, DC.
- Budin-Verneuil, A., Maguin, E., Auffray, Y., Ehrlich, S.D. and Pichereau, V. (2005) Transcriptional analysis of the cyclopropane fatty acid synthase gene of *Lactococcus lactis* MG1363 at low pH. *FEMS Microbiol. Lett.* **250**, 189–194.
- Bunch, P.K., Mat-Jan, F., Lee, N. and Clark, D.P. (1997) The *IdhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology* **143**, 187–195.
- Burne, R.A. and Marquis, R.E. (2000) Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiol. Lett.* **193**, 1–6.
- Bury-Moné, S., Mendz, G.L., Ball, G.E., Thibonnier, M., Stingl, K., Ecobichon, C., Avé, P., Huerre, M., Labigne, A., Thiberge, J.M. and De Reuse, H. (2008) Roles of alpha and beta carbonic anhydrases of *Helicobacter pylori* in the urease-dependent response to acidity and in colonization of the murine gastric mucosa. *Infect. Immun.* **76**, 497–509.
- Buurman, E.T., McLaggan, D., Naprstek, J. and Epstein, W. (2004) Multiple paths for nonphysiological transport of K⁺ in *Escherichia coli*. *J. Bacteriol.* **186**, 4238–4245.
- Cao, M., Kobel, P.A., Morshedi, M.M., Wu, M.F., Paddon, C. and Helmann, J.D. (2002) Defining the *Bacillus subtilis* σ^W regulon: a comparative analysis of promoter consensus search, run-off transcription/microarray analysis (ROMA), and transcriptional profiling approaches. *J. Mol. Biol.* **16**, 443–457.
- Castanie-Cornet, M.P., Penfound, T.A., Smith, D., Elliot, J.F. and Foster, J.W. (1999) Control of acid resistance in *Escherichia coli*. *J. Bacteriol.* **181**, 3525–3535.
- Castelle, C., Guiral, M., Malarte, G., Ledgham, F., Leroy, G., Brugna, M. and Giudici-Ortoni, M.T. (2008) A new iron-oxidizing/O₂-reducing supercomplex

- spanning both inner and outer membranes, isolated from the extreme acidophile *Acidithiobacillus ferrooxidans*. *J. Biol. Chem.* **283**, 25803–25811.
- Chang, Y.Y. and Cronan, J.E., Jr. (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. *Mol. Microbiol.* **33**, 249–259.
- Chao, J., Wang, W., Xiao, S.M. and Liu, X.D. (2008) Response of *Acidithiobacillus ferrooxidans* ATCC 23270 gene expression to acid stress. *World J. Microbiol. Biotechnol.* **24**, 2103–2109.
- Chen, Y.Q. and Suzuki, I. (2005) Effects of electron transport inhibitors and uncouplers on the oxidation of ferrous iron and compounds interacting with ferric iron in *Acidithiobacillus ferrooxidans*. *Can. J. Microbiol.* **51**, 695–703.
- Chen, Y.Y., Betzenhauser, M.J. and Burne, R.A. (2002) cis-Acting elements that regulate the low-pH-inducible urease operon of *Streptococcus salivarius*. *Microbiology* **148**, 3599–3608.
- Cheng, J., Guffanti, A.A. and Krulwich, T.A. (1994) The chromosomal tetracycline-resistance locus of *Bacillus subtilis* encodes a Na⁺/H⁺ antiporter that is physiologically important at elevated growth pH. *J. Biol. Chem.* **269**, 27365–27371.
- Cheng, J., Guffanti, A.A., Wang, W., Krulwich, T.A. and Bechhofer, D.H. (1996) Chromosomal *tetA(L)* gene of *Bacillus subtilis*: regulation of expression and physiology of *tetA(L)* deletion strain. *J. Bacteriol.* **178**, 2853–2860.
- Ciaramella, M., Napoli, A. and Rossi, M. (2005) Another extreme genome: how to live at pH 0. *Trends Microbiol.* **132**, 49–51.
- Clejan, S., Krulwich, T.A., Mondrus, K.R. and Seto-Young, D. (1986) Membrane lipid composition of obligately and facultatively alkaliphilic strains of *Bacillus*. *J. Bacteriol.* **168**, 334–340.
- Cogan, T.M., O'Dowd, M. and Mellerick, D. (1981) Effects of pH and sugar on acetoin production from citrate by *Leuconostoc lactis*. *Appl. Environ. Microbiol.* **41**, 1–8.
- Colwell, R.R. and Huq, A. (1994) In: *Vibrio cholerae and Cholera: Molecular to Global Perspectives* (I.K. Wachsmuth, P.A. Blake and O. Olsvik, eds). ASM Press, Washington, DC.
- Cook, G.M., Russell, J.B., Reichert, A. and Wiegel, J. (1996) The intracellular pH of *Clostridium paradoxum*, an anaerobic, alkaliphilic and therophilic bacterium. *Appl. Environ. Microbiol.* **62**, 4576–4579.
- Cotter, P.D. and Hill, C. (2003) Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* **67**, 429–453.
- Cox, J.C., Nicholls, D.G. and Ingledew, W.J. (1979) Transmembrane electrical potential and transmembrane pH gradient in the acidophile *Thiobacillus ferrooxidans*. *Biochem. J.* **178**, 195–200.
- Cronan, J.E., Jr. (2002) Phospholipid modification in bacteria. *Curr. Opin. Microbiol.* **5**, 202–205.
- Curran, T.M., Lieou, J. and Marquis, R.E. (1995) Arginine deiminase system and acid adaptation of oral streptococci. *Appl. Environ. Microbiol.* **61**, 4494–4496.
- Dannenmuller, O., Arakawa, K., Eguchi, T., Kakinuma, K., Blanc, S., Albrecht, A.M., Schmutz, M., Nakatani, Y. and Ourisson, G. (2000) Membrane properties of archaeal macrocyclic diether phospholipids. *Chem. Europ. J.* **6**, 645–654.

- Deamer, D.W. and Nichols, J.W. (1989) Proton flux mechanisms in model and biological-membranes. *J. Membr. Biol.* **107**, 91–103.
- de Koning-Ward, T.F. and Robins-Browne, R.M. (1995) Contribution of urease to acid tolerance in *Yersinia enterocolitica*. *Infect. Immun.* **63**, 3790–3795.
- de Koning-Ward, T.F. and Robins-Browne, R.M. (1997) A novel mechanism of urease regulation in *Yersinia enterocolitica*. *FEMS Microbiol. Lett.* **147**, 221–226.
- Dell, C.L., Neely, M.N. and Olson, E.R. (1994) Altered pH and lysine signalling mutants of *cadC*, a gene encoding a membrane-bound transcriptional activator of the *Escherichia coli cadBA* operon. *Mol. Microbiol.* **14**, 7–16.
- Dickson, R.M., Cubitt, A.B., Tsien, R.Y. and Moerner, W.E. (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature* **388**, 355–358.
- Diez-Gonzalez, F. and Russell, J.B. (1997) Effects of carbonylcyanide-*m*-chlorophenylhydrazine (CCCP) and acetate on *Escherichia coli* O157:H7 and K-12: uncoupling versus anion accumulation. *FEMS Microbiol. Lett.* **151**, 71–76.
- Dilworth, M.J., Rynne, F.G., Castelli, J.M., Vivas-Marfisi, A.I. and Glenn, A.R. (1999) Survival and exopolysaccharide production in *Sinorhizobium meliloti* WSM419 are affected by calcium and low pH. *Microbiology* **145**, 1585–1593.
- Dimroth, P. (2004) Bacterial sodium ion-coupled energetics. *Antonie Van Leeuwenhoek* **65**, 381–395.
- Dimroth, P. and Schink, B. (1998) Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. *Arch. Microbiol.* **170**, 69–77.
- Dong, Y., Chen, Y.Y., Snyder, J.A. and Burne, R.A. (2002) Isolation and molecular analysis of the gene cluster for the arginine deiminase system from *Streptococcus gordonii* DL1. *Appl. Environ. Microbiol.* **68**, 5549–5553.
- Dopson, M. (2001) Potential role of *Acidithiobacillus caldus* in bioleaching: a study of its sulfur metabolism, energetics and arsenic resistance. PhD Thesis, Department of Microbiology, Umeå University, Umeå, 85 pp.
- Dopson, M., Baker-Austin, C. and Bond, P.L. (2005) Analysis of differential protein expression during growth states of *Ferroplasma* strains and insights into electron transport for iron oxidation. *Microbiology* **151**, 4127–4137.
- Dopson, M., Baker-Austin, C., Hind, A., Bowman, J.P. and Bond, P.L. (2004) Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. *Appl. Environ. Microbiol.* **70**, 2079–2088.
- Dopson, M., Lindstrom, E.B. and Hallberg, K.B. (2002) ATP generation during reduced inorganic sulfur compound oxidation by *Acidithiobacillus caldus* is exclusively due to electron transport phosphorylation. *Extremophiles* **6**, 123–129.
- Dover, N. and Padan, E. (2001) Transcription of *nhaA*, the main Na⁺/H⁺ antiporter of *Escherichia coli*, is regulated by Na⁺ and growth phase. *J. Bacteriol.* **183**, 644–653.
- Earl, A.M., Losick, R. and Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol.* **16**, 269–275.
- Eaton, K.S., Brooks, D.L., Morgan, D.R. and Krakowka, S. (1991) Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.* **59**, 2470–2475.

- Edwards, K.J., Bond, P.L., Gihring, T.M. and Banfield, J.F. (2000) An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science* **287**, 1796–1799.
- Edwards, K.J., Gihring, T.M. and Banfield, J.F. (1999) Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. *Appl. Environ. Microbiol.* **65**, 3627–3632.
- Elferink, M.G.L., de Wit, J.G., Driessen, A.J.M. and Konings, W.N. (1994) Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. *Biochim. Biophys. Acta* **1193**, 247–254.
- Espana, J.S., Toril, E.G., Pamo, E.L., Amils, R., Ercilla, M.D., Pastor, E.S. and Martin-Uriz, P.S. (2008) Biogeochemistry of a hyperacidic and ultraconcentrated pyrite leachate in San Telmo mine (Iberian pyrite belt, Spain). *Water, Air, Soil Pollut.* **194**, 243–257.
- Ferenci, T. (2008) Bacterial physiology, regulation and mutational adaptations in a chemostat environment. *Adv. Microb. Physiol.* **53**, 169–229.
- Ferguson, S.J. and Ingledew, W.J. (2008) Energetic problems faced by microorganisms growing or surviving on parsimonious energy sources and at acidic pH: I. *Acidithiobacillus ferrooxidans* as a paradigm. *Biochim. Biophys. Acta* **1777**, 1471–1479.
- Fernández, M. and Zúñiga, M. (2006) Amino acid catabolic pathways in lactic acid bacteria. *Crit. Rev. Microbiol.* **32**, 155–183.
- Foster, J.W. (2004) *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.* **2**, 898–907.
- Foster, J.W. and Bearson, B. (1994) Acid-sensitive mutants of *Salmonella typhimurium* identified through a dinitrophenol lethal screening strategy. *J. Bacteriol.* **176**, 2596–2602.
- Foster, J.W. and Hall, H.K. (1991) Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* **173**, 5129–5135.
- Franco, P.J. and Wilson, T.H. (1996) Alteration of Na⁺-coupled transport in site-directed mutants of the melibiose carrier of *Escherichia coli*. *Biochim. Biophys. Acta* **1282**, 240–248.
- Friedrich, T. and Scheide, D. (2000) The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. *FEBS Lett.* **479**, 1–5.
- Fujinami, S., Sato, T., Trimmer, J.S., Spiller, B.W., Clapham, D.E., Krulwich, T.A., Kawagishi, I. and Ito, M. (2007a) The voltage-gated Na⁺ channel Na_vBP co-localizes with methyl-accepting chemotaxis proteins at cell poles of alkaliphilic *Bacillus pseudofirmus* OF4. *Microbiology* **153**, 4027–4038.
- Fujinami, S., Terahara, N., Lee, S. and Ito, M. (2007b) Na⁺ and flagella-dependent swimming of alkaliphilic *Bacillus pseudofirmus* OF4: a basis for poor motility at low pH and enhancement in viscous media in an “up-motile” variant. *Arch. Microbiol.* **187**, 239–247.
- Fujisawa, M., Ito, M. and Krulwich, T.A. (2007) Three 2-component transporters with channel-like properties have monovalent cation/proton antiport activity. *Proc. Natl. Acad. Sci. USA* **104**, 13289–13294.
- Fujisawa, M., Kusumoto, A., Wada, Y., Tsuchiya, T. and Ito, M. (2005) NhaK, a novel monovalent cation/H⁺ antiporter of *Bacillus subtilis*. *Arch. Microbiol.* **183**, 411–420.

- Fütterer, O., Angelov, A., Liesegang, H., Gottschalk, G., Schleper, C., Schepers, B., Dock, C., Antranikian, G. and Liebl, W. (2004) Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proc. Natl. Acad. Sci. USA* **101**, 9091–9096.
- Gieseke, A., Tarre, S., Green, M. and de Beer, D. (2006) Nitrification in a biofilm at low pH values: role of in situ microenvironments and acid tolerance. *Appl. Environ. Microbiol.* **72**, 4283–4292.
- Gilmour, R. and Krulwich, T.A. (1996) Purification and characterization of the succinate dehydrogenase complex and CO-reactive *b*-type cytochromes from the facultative alkaliphile *Bacillus firmus* OF4. *Biochim. Biophys. Acta* **1276**, 57–63.
- Gilmour, R. and Krulwich, T.A. (1997) Construction and characterization of a mutant of alkaliphilic *Bacillus firmus* OF4 with a disrupted *cta* operon and purification of a novel cytochrome *bd*. *J. Bacteriol.* **179**, 863–870.
- Gilmour, R., Messner, P., Guffanti, A.A., Kent, R., Scheberl, A., Kendrick, N. and Krulwich, T.A. (2000) Two-dimensional gel electrophoresis analyses of pH-dependent protein expression in facultatively alkaliphilic *Bacillus pseudofirmus* OF4 lead to characterization of an S-layer protein with a role in alkaliphily. *J. Bacteriol.* **182**, 5969–5981.
- Giotis, E.S., Muthaiyan, A., Blair, I.S., Wilkinson, B.J. and McDowell, D.A. (2008) Genomic and proteomic analysis of the alkali-tolerance response (AITR) in *Listeria monocytogenes* 10403S. *BMC Microbiol.* **8**, 102.
- Gliozzi, A., Relini, A. and Chong, P.L.G. (2002) Structure and permeability properties of biomimetic membranes of bolaform archaeal tetraether lipids. *J. Membr. Sci.* **206**, 131–147.
- Golyshina, O.V., Golyshin, P.N., Timmis, K.N. and Ferrer, M. (2006) The ‘pH optimum anomaly’ of intracellular enzymes of *Ferroplasma acidiphilum*. *Environ. Microbiol.* **8**, 416–425.
- Golyshina, O.V., Pivovarova, T.A., Karavaiko, G.I., Kondrat’eva, T.F., Moore, E.R.B., Abraham, W.R., Lunsdorf, H., Timmis, K.N., Yakimov, M.M. and Golyshin, P.N. (2000) *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the Archaea. *Int. J. Syst. Evol. Microbiol.* **50**, 997–1006.
- Gong, S., Richard, H. and Foster, J.W. (2003) YjdE (AdiC) is the arginine:agmatine antiporter essential for arginine-dependent acid resistance in *Escherichia coli*. *J. Bacteriol.* **185**, 4402–4409.
- Gorden, J. and Small, P.L. (1993) Acid resistance in enteric bacteria. *Infect. Immun.* **61**, 364–367.
- Goto, T., Matsuno, T., Hishinuma-Narisawa, M., Yamazaki, K., Matsuyama, H., Inoue, N. and Yumoto, I. (2005) Cytochrome *c* and bioenergetic hypothetical model for alkaliphilic *Bacillus* spp. *J. Biosci. Bioeng.* **100**, 365–379.
- Goulbourne, E.J., Matin, M. and Zychlinsky, E. (1986) Mechanism of delta pH maintenance in active and inactive cells of an obligately acidophilic bacterium. *J. Bacteriol.* **166**, 59–65.
- Grandvalet, C., Assad-García, J.S., Chu-Ky, S., Tollot, M., Guzzo, J., Gresti, J. and Tourdot-Maréchal, R. (2008) Changes in membrane lipid composition in ethanol- and acid-adapted *Oenococcus oeni* cells: characterization of the *cfa* gene by heterologous complementation. *Microbiology* **154**, 2611–2619.

- Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G., Skurray, R.A., Paulsen, I.T., Chater, K.F., Baldwin, S.A. and Henderson, P.J. (1992) Membrane transport proteins: implications of sequence comparisons. *Curr. Opin. Cell Biol.* **4**, 684–695.
- Grogan, D.W. and Cronan, J.E., Jr. (1997) Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol. Mol. Biol. Rev.* **61**, 429–441.
- Guffanti, A.A. and Hicks, D.B. (1991) Molar growth yields and bioenergetic parameters of extremely alkaliphilic *Bacillus* species in batch cultures, and growth in a chemostat at pH 10.5. *J. Gen. Microbiol.* **137**, 2375–2379.
- Guiliani, N. and Jerez, C.A. (2000) Molecular cloning, sequencing, and expression of *omp-40*, the gene coding for the major outer membrane protein from the acidophilic bacterium *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **66**, 2318–2324.
- Hamamoto, T., Hashimoto, M., Hino, M., Kitada, M., Seto, YI., Kudo, T. and Horikoshi, K. (1994) Characterization of a gene responsible for the Na⁺/H⁺ antiporter system of alkaliphilic *Bacillus* species strain C-125. *Mol. Microbiol.* **14**, 939–946.
- Harold, F.M. and Van Brunt, J. (1977) Circulation of H⁺ and K⁺ across the plasma membrane is not obligatory for bacterial growth. *Science* **197**, 372–373.
- Häse, C.C. and Barquera, B. (2001) Role of sodium bioenergetics in *Vibrio cholerae*. *Biochim. Biophys. Acta* **1505**, 169–178.
- Hawkes, R.B., Franzmann, P.D., O'Hara, G. and Plumb, J.J. (2006) *Ferroplasma cupricumulans* sp. nov., a novel moderately thermophilic, acidophilic archaeon isolated from an industrial-scale chalcocite bioleach heap. *Extremophiles* **10**, 525–530.
- Hayashi, M., Nakayama, Y. and Unemoto, T. (2001) Recent progress in the Na⁺-translocating NADH-quinone reductase from the marine *Vibrio alginolyticus*. *Biochim. Biophys. Acta* **1505**, 37–44.
- Hayes, E.T., Wilks, C., Yohannes, E., Tate, D.P., Radmacher, M., BonDurant, S.S. and Slonczewski, J.L. (2006) pH and anaerobiosis coregulate catabolism, hydrogenases, ion and multidrug transporters, and envelope composition in *Escherichia coli* K-12. *BMC Microbiol.* **6**, 89.
- Heberle, J., Riesle, J., Thiedemann, G., Oesterhelt, D. and Dencher, N.A. (1994) Proton migration along the membrane surface and retarded surface to bulk transfer. *Nature* **370**, 379–382.
- Hecker, M., Pane-Farre, J. and Volker, U. (2007) σ^B -dependent general stress response in *Bacillus subtilis* and related Gram-positive bacteria. *Annu. Rev. Microbiol.* **61**, 215–236.
- Hecker, M. and Volker, U. (2001) General stress response of *Bacillus subtilis* and other bacteria. *Adv. Microb. Physiol.* **44**, 35–91.
- Hersh, B.M., Farooq, F.T., Barstad, D.N., Blankenhorn, D.L. and Slonczewski, J.L. (1996) A glutamate-dependent acid resistance gene in *Escherichia coli*. *J. Bacteriol.* **178**, 3978–3981.
- Hicks, D.B. and Krulwich, T.A. (1995) The respiratory chain of alkaliphilic bacteria. *Biochim. Biophys. Acta* **1229**, 303–314.
- Hiramatsu, T., Kodama, K., Kuroda, T., Mizushima, T. and Tsuchiya, T. (1998) A putative multisubunit Na⁺/H⁺ antiporter from *Staphylococcus aureus*. *J. Bacteriol.* **180**, 6642–6648.

- Hoffmann, A. and Dimroth, P. (1991) The electrochemical proton potential of *Bacillus alcalophilus*. *Eur. J. Biochem.* **201**, 467–473.
- Horikoshi, K. (1999) Alkaliphiles: some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.* **63**, 735–750.
- Horikoshi, K. and Akiba, T. (1982) *Alkaliphilic Microorganisms: A New Microbial World*. Springer-Verlag KG, Heidelberg, Germany.
- Hornbæk, T., Jakobsen, M., Dynesen, J. and Nielsen, A.K. (2004) Global transcription profiles and intracellular pH regulation measured in *Bacillus licheniformis* upon external pH upshifts. *Arch. Microbiol.* **182**, 467–474.
- Hsieh, P.C., Seigel, S.A., Rogers, B., Davis, D. and Lewis, K. (1998) Bacteria lacking a multi-drug pump: a sensitive tool for drug discovery. *Proc. Natl. Acad. Sci. USA* **95**, 6602–6606.
- Hsung, J.C. and Haug, A. (1977) Membrane potential of *Thermoplasma acidophilum*. *FEBS Lett.* **73**, 47–50.
- Hunte, C., Screpanti, M., Venturi, M., Rimon, A., Padan, E. and Michel, H. (2005) Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature* **534**, 1197–1202.
- Hutkins, R.W. and Nannen, N.L. (1993) pH homeostasis in lactic acid bacteria. *J. Dairy Sci.* **76**, 2354–2365.
- Ikegami, M., Kawano, M., Takase, K., Yamato, I., Igarashi, K. and Kakinuma, Y. (1999) *Enterococcus hirae* vacuolar ATPase is expressed in response to pH as well as sodium. *FEBS Lett.* **454**, 67–70.
- Inoue, H., Sakurai, T., Ujike, S., Tsuchiya, T., Murakami, H. and Kanazawa, H. (1999) Expression of functional Na^+/H^+ antiporters of *Helicobacter pylori* in antiporter-deficient *Escherichia coli* mutants. *FEBS Lett.* **443**, 11–16.
- Ito, M. and Aono, R. (2002) Decrease in cytoplasmic pH-homeostatic activity of the alkaliphile *Bacillus lentus* C-125 by a cell wall defect. *Biosci. Biotechnol. Biochem.* **66**, 218–220.
- Ito, M., Guffanti, A.A., Oudega, B. and Krulwich, T.A. (1999) *mnp*, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and Na^+ and in pH homeostasis. *J. Bacteriol.* **181**, 2394–2402.
- Ito, M., Guffanti, A.A., Wang, W. and Krulwich, T.A. (2000) Effects of nonpolar mutations in each of the seven *Bacillus subtilis mnp* genes suggest complex interactions among the gene products in support of Na^+ and alkali but not cholate resistance. *J. Bacteriol.* **182**, 5663–5670.
- Ito, M., Guffanti, A.A., Zemsky, J., Ivey, D.M. and Krulwich, T.A. (1997) Role of the *nhaC*-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4. *J. Bacteriol.* **179**, 3851–3857.
- Ito, M., Hicks, D.B., Henkin, T.M., Guffanti, A.A., Powers, B., Zvi, L., Uematsu, K. and Krulwich, T.A. (2004a) MotPS is the stator-force generator for motility of alkaliphilic *Bacillus* and its homologue is a second functional Mot in *Bacillus subtilis*. *Mol. Microbiol.* **53**, 1035–1049.
- Ito, M., Xu, H., Guffanti, A.A., Wei, Y., Zvi, L., Clapham, D.E. and Krulwich, T.A. (2004b) The voltage-gated Na^+ channel Na_vBP has a role in motility, chemotaxis and pH homeostasis of an alkaliphilic *Bacillus*. *Proc. Natl. Acad. Sci. USA* **101**, 10566–10571.
- Ivey, D.M., Guffanti, A.A., Bossewitch, J.S., Padan, E. and Krulwich, T.A. (1991) Molecular cloning and sequencing of a gene from alkaliphilic *Bacillus firmus*

- OF4 that functionally complements an *Escherichia coli* strain carrying a deletion in the *nhaA* Na⁺/H⁺ antiporter gene. *J. Biol. Chem.* **266**, 23483–23489.
- Ivey, D.M., Guffanti, A.A., Zemsy, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S. and Krulwich, T.A. (1993) Cloning and characterization of a putative Ca²⁺/H⁺ antiporter gene from *Escherichia coli* upon functional complementation of Na⁺/H⁺ antiporter-deficient strains by the overexpressed gene. *J. Biol. Chem.* **268**, 11296–11303.
- Ivey, D.M. and Krulwich, T.A. (1992) Two unrelated alkaliphilic *Bacillus* species possess identical deviations in sequence from those of other prokaryotes in regions of F₀ proposed to be involved in proton translocation through the ATP synthase. *Res. Microbiol.* **143**, 467–470.
- Iyer, R., Iverson, T.M., Accardi, A. and Miller, C. (2002) A biological role for prokaryotic ClC chloride channels. *Nature* **419**, 715–718.
- Iyer, R., Williams, C. and Miller, C. (2003) Arginine-agsmatine antiporter in extreme acid resistance in *Escherichia coli*. *J. Bacteriol.* **185**, 6556–6561.
- Jin, J., Guffanti, A.A., Beck, C. and Krulwich, T.A. (2001) Twelve-transmembrane-segment (TMS) version (Δ TMS VII-VIII) of the 14-TMS Tet(L) antibiotic resistance protein retains monovalent cation transport modes but lacks tetracycline efflux capacity. *J. Bacteriol.* **183**, 2667–2671.
- Johnson, D.B. and Hallberg, K.B. (2003) The microbiology of acidic mine waters. *Res. Microbiol.* **154**, 466–473.
- Johnson, M.E. and Steele, J.L. (2001) Fermented dairy products. In: *Food Microbiology: Fundamentals and Frontiers* (M.P. Doyle, L.R. Beuchat and T.J. Montville, eds), Chapter 31, pp. 651–664. ASM Press, Washington, DC.
- Jones, B.E., Grant, W.D., Duckworth, A.W. and Owenson, G.G. (1998) Microbial diversity of soda lakes. *Extremophiles* **2**, 191–200.
- Kajiyama, Y., Otagiri, M., Sekiguchi, J., Kosono, S. and Kudo, T. (2007) Complex formation by mutants defective in the *mrpABCDEF* gene products, which constitute a principal Na⁺/H⁺ antiporter in *Bacillus subtilis*. *J. Bacteriol.* **189**, 7511–7514.
- Kakinuma, Y. (1987a) Lowering of cytoplasmic pH is essential for growth of *Streptococcus faecalis* at high pH. *J. Bacteriol.* **169**, 4403–4405.
- Kakinuma, Y. (1987b) Sodium/proton antiporter in *Streptococcus faecalis*. *J. Bacteriol.* **169**, 3886–3890.
- Kakinuma, Y. (1998) Inorganic cation transport and energy transduction in *Enterococcus hirae* and other streptococci. *Microbiol. Mol. Biol. Rev.* **62**, 1021–1045.
- Kakinuma, Y. and Igarashi, K. (1999) Isolation and properties of *Enterococcus hirae* mutants defective in the potassium/proton antiport system. *J. Bacteriol.* **181**, 4103–4105.
- Kannan, G., Wilks, J.C., Fitzgerald, D.M., Jones, B.D., Bondurant, S.S. and Slonczewski, J.L. (2008) Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. *BMC Microbiol.* **8**, 37.
- Karpel, R., Alon, T., Glaser, G., Schuldiner, S. and Padan, E. (1991) Expression of a sodium proton antiporter (*NhaA*) in *Escherichia coli* is induced by Na⁺ and Li⁺ ions. *J. Biol. Chem.* **266**, 21753–21759.
- Kashiwagi, K., Miyamoto, S., Suzuki, F., Kobayashi, H. and Igarashi, K. (1992) Excretion of putrescine by the putrescine-ornithine antiporter encoded by the *potE* gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89**, 4529–4533.

- Kawano, M., Abuki, R., Igarashi, K. and Kakinuma, Y. (2000) Evidence for Na⁺ influx via the NtpJ protein of the Ktr II K⁺ uptake system in *Enterococcus hirae*. *J. Bacteriol.* **182**, 2507–2512.
- Khan, S. and Macnab, R.M. (1980) Proton chemical potential, proton electrical potential and bacterial motility. *J. Mol. Biol.* **138**, 599–614.
- Kihara, M. and Macnab, R. (1981) Cytoplasmic pH mediates pH taxis and weak-acid repellent taxis of bacteria. *J. Bacteriol.* **145**, 1209–1221.
- Kim, B.H., Kim, S., Kim, H.G., Lee, J., Lee, I.S. and Park, Y.K. (2005) The formation of cyclopropane fatty acids in *Salmonella enterica* serovar Typhimurium. *Microbiology* **151**, 209–218.
- King, P.W. and Przybyla, A.E. (1999) Response of *hya* expression to external pH in *Escherichia coli*. *J. Bacteriol.* **181**, 5250–5256.
- Kinoshita, N., Unemoto, T. and Kobayashi, H. (1984) Proton motive force is not obligatory for growth of *Escherichia coli*. *J. Bacteriol.* **160**, 1074–1077.
- Kirkpatrick, C., Maurer, L.M., Oyelakin, N.E., Yontcheva, Y., Maurer, R. and Slonczewski, J.L. (2001) Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. *J. Bacteriol.* **183**, 6466–6477.
- Kitada, M., Guffanti, A.A. and Krulwich, T.A. (1982) Bioenergetic properties and viability of alkaliphilic *Bacillus firmus* RAB as a function of pH and Na⁺ contents of the incubation medium. *J. Bacteriol.* **176**, 1096–1104.
- Kitada, M., Kosono, S. and Kudo, T. (2000) The Na⁺/H⁺ antiporter of alkaliphilic *Bacillus* sp. *Extremophiles* **4**, 253–258.
- Kneen, M., Farinas, J., Li, Y. and Verkman, A.S. (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* **74**, 1591–1599.
- Kobayashi, H., Murakami, N. and Unemoto, T. (1982) Regulation of the cytoplasmic pH in *Streptococcus faecalis*. *J. Biol. Chem.* **257**, 13246–13252.
- Kobayashi, H. and Unemoto, T. (1980) *Streptococcus faecalis* mutants defective in regulation of cytoplasmic pH. *J. Bacteriol.* **143**, 1187–1193.
- Komatsu, H. and Chong, P.L.G. (1998) Low permeability of liposomal membranes composed of bipolar tetraether lipids from thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *Biochemistry* **37**, 107–115.
- Konings, W.N., Albers, S.V., Koning, S. and Driessen, A.J.M. (2002) The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. *Antonie van Leeuwen. Int. J. Gen. Mol. Microbiol.* **81**, 61–72.
- Konings, W.N., Lolkema, J.S., Bolhuis, H., van Veen, H.W., Poolman, B. and Driessen, A.J.M. (1997) The role of transport processes in survival of lactic acid bacteria. *Antonie van Leeuwen* **71**, 117–128.
- Kosono, S., Morotomi, S., Kitada, M. and Kudo, T. (1999) Analyses of a *Bacillus subtilis* homologue of the Na⁺/H⁺ antiporter gene which is important for pH homeostasis of alkaliphilic *Bacillus* sp. C-125. *Biochim. Biophys. Acta* **1409**, 171–175.
- Krulwich, T.A. (1995) Alkaliphiles: “basic” molecular problems of pH tolerance and bioenergetics. *Mol. Microbiol.* **15**, 403–410.
- Krulwich, T.A., Agus, R., Schneier, M. and Guffanti, A.A. (1985a) Buffering capacity of bacilli that grow at different pH ranges. *J. Bacteriol.* **162**, 768–772.
- Krulwich, T.A., Davidson, L.F., Filip, S.J., Jr., Zuckerman, R.S. and Guffanti, A.A. (1978) The protonmotive force and β-galactoside transport in *Bacillus acidocaldarius*. *J. Biol. Chem.* **253**, 4599–4603.

- Krulwich, T.A., Federbush, J.G. and Guffanti, A.A. (1985b) Presence of a nonmetabolizable solute that is translocated with Na^+ enhances Na^+ -dependent pH homeostasis in an alkalophilic *Bacillus*. *J. Biol. Chem.* **260**, 4055–4058.
- Krulwich, T.A. and Guffanti, A.A. (1989) Alkalophilic bacteria. *Annu. Rev. Microbiol.* **43**, 435–463.
- Krulwich, T.A., Hicks, D.B., Swartz, T. and Ito, M. (2007) Bioenergetic adaptations that support alkaliphily. In: *Physiology and Biochemistry of Extremophiles* (C. Gerday and N. Glansdorff, eds), pp. 311–329. ASM Press, Washington, DC.
- Krulwich, T.A., Lewinson, O., Padan, E. and Bibi, E. (2005) Do physiological roles foster persistence of drug/multidrug-efflux pumps? A case study. *Nat. Rev. Microbiol.* **3**, 566–572.
- Kuhnert, W.L. and Quivey, R.G., Jr. (2003) Genetic and biochemical characterization of the F-ATPase operon from *Streptococcus sanguis* 10904. *J. Bacteriol.* **185**, 1525–1533.
- Leone, L., Ferri, D., Manfredi, C., Persson, P., Shchukarev, A., Sjöberg, S. and Loring, J. (2007) Modeling the acid-base properties of bacterial surfaces: A combined spectroscopic and potentiometric study of the Gram-positive bacterium *Bacillus subtilis*. *Environ. Sci. Technol.* **41**, 6465–6471.
- Lewinson, O., Padan, E. and Bibi, E. (2004) Alkalitolerance: a biological function for a multidrug transporter in pH homeostasis. *Proc. Natl. Acad. Sci. USA* **101**, 14073–14078.
- Lewis, R.J., Prince, R., Dutton, P.L., Knaff, D. and Krulwich, T.A. (1981) The respiratory chain of *Bacillus alcalophilus* and its non-alkalophilic mutant derivative. *J. Biol. Chem.* **256**, 10543–10549.
- Lin, J., Lee, I.S., Frey, J., Slonczewski, J.L. and Foster, J.W. (1995) Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. *J. Bacteriol.* **177**, 4097–4104.
- Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N. and Foster, J.W. (1996) Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.* **62**, 3094–3100.
- Liu, J., Fujisawa, M., Hicks, D.B. and Krulwich, T.A. (2009) Characterization of the functionally critical AXAXAXA and PXXEXXP motifs of the ATP synthase c-subunit from an alkaliphilic *Bacillus*. *J. Biol. Chem.* **284**, 8714–8725.
- Liu, J., Krulwich, T.A. and Hicks, D.B. (2008) Purification of two putative type II NADH dehydrogenases with different substrate specificities from alkaliphilic *Bacillus pseudofirmus* OF4. *Biochim. Biophys. Acta* **1777**, 453–461.
- Liu, X., Gong, X., Hicks, D.B., Krulwich, T.A., Yu, L. and Yu, C.-A. (2007) Interaction between cytochrome *caa3* and F_1F_0 -ATP synthase of alkaliphilic *Bacillus pseudofirmus* OF4 is demonstrated by saturation transfer electron paramagnetic resonance and differential scanning calorimetry assays. *Biochemistry* **46**, 306–313.
- Llopis, J., McCaffery, M., Miyawaki, A., Farquhar, M.G. and Tsien, R.Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95**, 6803–6808.
- Lohmeier-Vogel, E.M., Ung, S. and Turner, R.J. (2004) In vivo ^{31}P nuclear magnetic resonance investigation of tellurite toxicity in *Escherichia coli*. *Appl. Environ. Microbiol.* **70**, 7342–7347.

- Lübben, M. and Schäfer, G. (1989) Chemiosmotic energy conversion of the archaeobacterial thermoacidophile *Sulfolobus acidocaldarius*: oxidative phosphorylation and the presence of an F_0 -related N,N' -dicyclohexylcarbodiimide-binding proteolipid. *J. Bacteriol.* **171**, 6106–6116.
- Lucas, P.M., Blancato, V.S., Claisse, O., Magni, C., Lolkema, J.S. and Lonvaud-Funel, A. (2007) Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. *Microbiology* **153**, 2221–2230.
- Ma, Z., Masuda, N. and Foster, J.W. (2004) Characterization of EvgAS-YdeO-GadE branched regulatory circuit governing glutamate-dependent acid resistance in *Escherichia coli*. *J. Bacteriol.* **186**, 7378–7389.
- Ma, Z., Richard, H. and Foster, J.W. (2003) pH-dependent modulation of cyclic AMP levels and GadW-dependent repression of RpoS affect synthesis of the GadX regulator and *Escherichia coli* acid resistance. *J. Bacteriol.* **185**, 6852–6859.
- Macalady, J.L., Jones, D.S. and Lyon, E.H. (2007) Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. *Environ. Microbiol.* **9**, 1402–1414.
- Macalady, J.L., Vestling, M.M., Baumler, D., Boekelheide, N., Kaspar, C.W. and Banfield, J.F. (2004) Tetraether-linked membrane monolayers in *Ferroplasma* spp: a key to survival in acid. *Extremophiles* **8**, 411–419.
- MacLellan, S.R., Helmann, J.D. and Antelmann, H. (2009) The YvrI alternative σ factor is essential for acid-stress induction of oxalate decarboxylase in *Bacillus subtilis*. *J. Bacteriol.* **191**, 931–939.
- MacLeod, R.A., Wisse, G.A. and Stejskal, F.L. (1988) Sensitivity of some marine bacteria, a moderate halophile, and *Escherichia coli* to uncouplers at alkaline pH. *J. Bacteriol.* **170**, 4330–4337.
- Macnab, R.M. and Castle, A.M. (1987) A variable stoichiometry model for pH homeostasis in bacteria. *Biophys. J.* **52**, 637–647.
- Magill, N.G., Cowan, A.E., Koppel, D.E. and Setlow, P. (1994) The internal pH of the forespore compartment of *Bacillus megaterium* decreases by about 1 pH unit during sporulation. *J. Bacteriol.* **176**, 2252–2258.
- Magill, N.G., Cowan, A.E., Leyva-Vazquez, M.A., Brown, M., Koppel, D.E. and Setlow, P. (1996) Analysis of the relationship between the decrease in pH and accumulation of 3-phosphoglyceric acid in developing forespores of *Bacillus* species. *J. Bacteriol.* **178**, 2204–2210.
- Maloney, P.C. (1979) Membrane H^+ conductance of *Streptococcus lactis*. *J. Bacteriol.* **140**, 197–205.
- Marcus, E.A., Moshfegh, A.P., Sachs, G. and Scott, D.R. (2005) The periplasmic alpha-carbonic anhydrase activity of *Helicobacter pylori* is essential for acid acclimation. *J. Bacteriol.* **187**, 729–738.
- Masuda, N. and Church, G.M. (2003) Regulatory network of acid resistance genes in *Escherichia coli*. *Mol. Microbiol.* **48**, 699–712.
- Mates, A.K., Sayed, A.K. and Foster, J.W. (2007) Products of the *Escherichia coli* acid fitness island attenuate metabolite stress at extremely low pH and mediate a cell density-dependent acid resistance. *J. Bacteriol.* **189**, 2759–2768.
- Matin, A., Wilson, B., Zychlinsky, E. and Matin, M. (1982) Proton motive force and the physiological basis of delta pH maintenance in *Thiobacillus acidophilus*. *J. Bacteriol.* **150**, 582–591.

- Maurer, L.M., Yohannes, E., Bondurant, S.S., Radmacher, M. and Slonczewski, J.L. (2005) pH regulates genes for flagellar motility, catabolism and oxidative stress in *Escherichia coli* K-12. *J. Bacteriol.* **187**, 304–319.
- McAnaney, T.B., Zeng, W., Doe, C.F., Bhanji, N., Wakelin, S., Pearson, D.S., Abbyad, P., Shi, X., Boxer, S.G. and Bagshaw, C.R. (2005) Protonation, photobleaching, and photoactivation of yellow fluorescent protein (YFP 10C): a unifying mechanism. *Biochemistry* **44**, 5510–5524.
- McLagan, D., Selwyn, M.J. and Dawson, A.P. (1984) Dependence on Na⁺ of control of cytoplasmic pH in a facultative alkalophile. *FEBS Lett.* **165**, 254–258.
- McNeill, K. and Hamilton, I.R. (2004) Effect of acid stress on the physiology of biofilm cells of *Streptococcus mutans*. *Microbiology* **150**, 735–742.
- Meng, S.Y. and Bennett, G.N. (1992a) Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. *J. Bacteriol.* **174**, 2659–2669.
- Meng, S.Y. and Bennett, G.N. (1992b) Regulation of the *Escherichia coli cad* operon: location of a site required for acid induction. *J. Bacteriol.* **174**, 2670–2678.
- Michels, M. and Bakker, E.P. (1985) Generation of a large, protonophore-sensitive proton motive force and pH difference in the acidophilic bacteria *Thermoplasma acidophilum* and *Bacillus acidocaldarius*. *J. Bacteriol.* **161**, 231–237.
- Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature* **191**, 144–148.
- Mobley, H.L., Island, M.D. and Hausinger, R.P. (1995) Molecular biology of microbial ureases. *Microbiol. Rev.* **59**, 451–480.
- Moll, R. and Schafer, G. (1988) Chemiosmotic-H⁺ cycling across the plasma-membrane of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *FEBS Lett.* **232**, 359–363.
- Mollenhauer-Rektorschek, M., Hanauer, G. and Sachs, G. (2002) Expression of UreI is required for intragastric transit and colonization of gerbil gastric mucosa by *Helicobacter pylori*. *Res. Microbiol.* **153**, 659–666.
- Moriarty, T.F., Mullan, A., McGrath, J.W., Quinn, J.P., Elborn, J.S. and Tunney, M.M. (2006) Effect of reduced pH on inorganic polyphosphate accumulation by *Burkholderia cepacia* complex isolates. *Letts. Appl. Microbiol.* **42**, 617–623.
- Morino, M., Natsui, S., Swartz, T.H., Krulwich, T.A. and Ito, M. (2008) Single gene deletions of *mrpA* to *mrpG* and *mrpE* point mutations affect activity of the Mrp Na⁺/H⁺ antiporter of alkaliphilic *Bacillus* and formation of hetero-oligomeric Mrp complexes. *J. Bacteriol.* **190**, 4162–4172.
- Mulkidjanian, A.Y., Heberle, J. and Cherepanov, D.A. (2006) Protons @ interfaces: implications for biological energy conversion. *Biochim. Biophys. Acta* **1757**, 913–930.
- Müller, T., Walter, B., Wirtz, A. and Burkovski, A. (2006) Ammonium toxicity in bacteria. *Curr. Microbiol.* **52**, 400–406.
- Müller, V., Aufferth, S. and Rahifs, S. (2001) The Na⁺ cycle in *Acetobacterium woodii*: identification and characterization of a Na⁺-translocating F₁F₀-ATPase with a mixed oligomer of 8 and 10 kDa proteolipids. *Biochim. Biophys. Acta* **1505**, 108–120.
- Mullineaux, C.W., Nenninger, A., Ray, N. and Robinson, C. (2006) Diffusion of green fluorescent protein in three cell environments in *Escherichia coli*. *J. Bacteriol.* **188**, 3442–3448.

- Muntyan, M.S. and Bloch, D.A. (2008) Study of redox potential in cytochrome *c* covalently bound to terminal oxidase of alkaliphilic *Bacillus pseudofirmus* FTU. *Biochemistry (Moscow)* **73**, 107–111.
- Murata, T., Yamato, I. and Kakinuma, Y. (2005) Structure and mechanism of vacuolar Na⁺-translocating ATPase from *Enterococcus hirae*. *J. Bioenerget. Biomemb.* **37**, 411–413.
- Nagle, J.F., Mathai, J.C., Zeidel, M.L. and Tristram-Nagle, S. (2008) Theory of passive permeability through lipid bilayers. *J. Gen. Physiol.* **131**, 77–85.
- Neely, M.N., Dell, C.L. and Olson, E.R. (1994) Roles of LysP and CadC in mediating the lysine requirement for acid induction of the *Escherichia coli* *cad* operon. *J. Bacteriol.* **176**, 3278–3285.
- Neely, M.N. and Olson, E.R. (1996) Kinetics of expression of the *Escherichia coli* *cad* operon as a function of pH and lysine. *J. Bacteriol.* **178**, 5522–5528.
- Nichols, J.W. and Deamer, D.W. (1980) Net proton-hydroxyl permeability of large unilamellar liposomes measured by an acid–base titration techniques. *Proc. Natl. Acad. Sci. USA* **77**, 2038–2042.
- Niggl, V. and Sigel, E. (2007) Anticipating antiport in P-type ATPases. *Trends Biochem. Sci.* **33**, 156–160.
- Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67**, 593–656.
- Nomura, M., Nakajima, I., Fujita, Y., Kobayashi, M., Kimoto, H., Suzuki, I. and Aso, H. (1999) *Lactococcus lactis* contains only one glutamate decarboxylase gene. *Microbiology* **145**, 1375–1380.
- Nordstrom, D.K. and Alpers, C.N. (1999) Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the iron mountain superfund site, California. *Proc. Natl. Acad. Sci. USA* **96**, 3455–3462.
- Nordstrom, D.K., Alpers, C.N., Ptacek, C.J. and Blowes, D.W. (2000) Negative pH and extremely acidic mine waters from Iron Mountain, California. *Environ. Sci. Technol.* **34**, 254–258.
- Ohyama, T., Igarashi, K. and Kobayashi, H. (1994) Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J. Bacteriol.* **176**, 4311–4315.
- Olsen, K.N., Budde, B.B., Siegmundfeldt, H., Rechinger, K.B., Jakobsen, M. and Ingmer, H. (2002) Noninvasive measurement of bacterial intracellular pH on a single-cell level with green fluorescent protein and fluorescence ratio imaging microscopy. *Appl. Environ. Microbiol.* **68**, 4145–4147.
- Olson, G.J., Brierley, J.A. and Brierley, C.L. (2003) Bioleaching review part B. Progress in bioleaching: applications of microbial processes by the minerals industries. *Appl. Microbiol. Biotechnol.* **63**, 249–257.
- Oshima, T., Arakawa, H. and Baba, M. (1997) Biochemical studies on an acidophilic, thermophilic bacterium, *Bacillus acidocaldarius*: isolation of bacteria, intracellular pH, and stabilities of biopolymers. *J. Biochem.* **81**, 1107–1113.
- Padan, E. (2008) The enlightening encounter between structure and function in the NhaA Na⁺–H⁺ antiporter. *Trends Biochem. Sci.* **9**, 435–443.
- Padan, E., Bibi, E., Ito, M. and Krulwich, T.A. (2005) Alkaline pH homeostasis in bacteria: new insights. *Biochim. Biophys. Acta* **1717**, 67–88.
- Padan, E. and Schuldiner, S. (1986) Intracellular pH regulation in bacterial cells. *Methods Enzymol.* **125**, 337–352.

- Padan, E., Tzuberly, T., Herz, K., Kozachkov, L., Rimon, A. and Galili, L. (2004) NhaA of *Escherichia coli*, as a model of a pH-regulated Na⁺/H⁺ antiporter. *Biochim. Biophys. Acta* **1658**, 2–13.
- Pagès, J.M., James, C.E. and Winterhalter, M. (2008) The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat. Rev. Microbiol.* **6**, 893–903.
- Penaud, S., Fernandez, A., Boudebouze, S., Ehrlich, S.D., Maguin, E. and van de Guchte, M. (2006) Induction of heavy-metal-transporting CPX-Type ATPases during acid adaptation in *Lactobacillus bulgaricus*. *Appl. Env. Microbiol.* **72**, 7445–7454.
- Pinner, E., Kotler, Y., Padan, E. and Schuldiner, S. (1993) Physiological role of *nhaB*, a specific Na⁺/H⁺ antiporter in *Escherichia coli*. *J. Biol. Chem.* **268**, 1729–1734.
- Pinner, E., Padan, E. and Schuldiner, S. (1994) Kinetic properties of NhaB: a Na⁺/H⁺ antiporter from *Escherichia coli*. *J. Biol. Chem.* **269**, 2674–2679.
- Pivovarova, T.A., Kondrat éva, T.F., Batrakov, S.G., Esipov, S.E., Sheichenko, V.I., Bykova, S.A., Lysenko, A.M. and Karavaiko, G.I. (2002) Phenotypic features of *Ferroplasma acidiphilum* strains Y-T and Y-2. *Microbiology (Russian)* **71**, 698–706.
- Plack, R.H., Jr. and Rosen, B.P. (1980) Cation/proton antiport systems in *Escherichia coli*. Absence of potassium/proton antiporter activity in a pH-sensitive mutant. *J. Biol. Chem.* **255**, 3824–3825.
- Pourcher, T., Leclercq, S., Brandolin, G. and Leblanc, G. (1995) Melibiose permease of *Escherichia coli*: large scale purification and evidence that H⁺, Na⁺ and Li⁺ sugar symport is catalyzed by a single protein. *Biochemistry* **34**, 4412–4420.
- Price, S.B., Cheng, C., Kaspar, C.W., Wright, J.C., DeGraves, F.C., Perfound, T.A., Castaine-Cornet, M. and Foster, J.W. (2000) Role of rpoS in acid resistance and fecal shedding of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **66**, 632–637.
- Quirk, P.G., Hicks, D.B. and Krulwich, T.A. (1993) Cloning of the *cta* operon from alkaliphilic *Bacillus firmus* OF4 and characterization of the pH-regulated cytochrome *caa*₃ oxidase it encodes. *J. Biol. Chem.* **268**, 678–685.
- Radchenko, M.V., Tanaka, K., Waditee, R., Oshimi, S., Matsuzaki, Y., Fukuhara, M., Kobayashi, H., Takabe, T. and Nakamura, T. (2006) Potassium/proton antiport system of *Escherichia coli*. *J. Biol. Chem.* **281**, 19822–19829.
- Ram, R.J., VerBerkmoes, N.C., Thelen, M.P., Tyson, G.W., Baker, B.J., Blake, R.C., Shah, M., Hettich, R.L. and Banfield, J.F. (2005) Community proteomics of a natural microbial biofilm. *Science* **308**, 1915–1920.
- Reeve, W.G., Tiwari, R.P., Kale, N.B., Dilworth, M.J. and Glenn, A.R. (2002) ActP controls copper homeostasis in *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* preventing low pH-induced copper toxicity. *Mol. Microbiol.* **43**, 981–991.
- Ren, D., Navarro, B., Xu, H., Yue, L., Shi, Q. and Clapham, D.E. (2001) A prokaryotic voltage-gated sodium channel. *Science* **294**, 2372–2375.
- Repaske, D. and Adler, J. (1981) Change in intracellular pH of *Escherichia coli* mediates the chemotactic response to certain attractants and repellents. *J. Bacteriol.* **145**, 1196–1208.

- Richard, H. and Foster, J.W. (2004) *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. *J. Bacteriol.* **186**, 6032–6041.
- Richard, H.T. and Foster, J.W. (2003) Acid resistance in *Escherichia coli*. *Adv. Appl. Microbiol.* **52**, 167–186.
- Rius, N. and Lorén, J.G. (1998) Buffering capacity and membrane H⁺ conductance of neutrophilic and alkalophilic Gram-positive bacteria. *Appl. Environ. Microbiol.* **64**, 1344–1349.
- Rius, N., Solé, M., Francia, A. and Lorén, J.G. (1995) Buffering capacity and H⁺ membrane conductance of Gram-negative bacteria. *FEMS Microbiol. Lett.* **130**, 103–110.
- Roadcap, G.S., Sanford, R.A., Jin, Q., Pardinias, J.R. and Bethke, C.M. (2006) Extremely alkaline (pH > 12) ground water hosts diverse microbial community. *Ground Water* **44**, 511–517.
- Robbins, E.I., Rodgers, T.M., Alpers, C.N. and Nordstrom, D.K. (2000) Ecogeochemistry of the subsurface food web at pH 0–2.5 in Iron Mountain, California, USA. *Hydrobiologica* **433**, 15–23.
- Robey, R.B., Ruiz, O., Santos, A.V.P., Ma, J., Kear, F., Wang, L.J., Li, C.J., Bernardo, A.A. and Arruda, J.A.L. (1998) pH-dependent fluorescence of a heterologously expressed *Aequorea* green fluorescent protein mutant: *in situ* spectral characteristics and applicability to intracellular pH estimation. *Biochemistry* **37**, 9894–9901.
- Roe, A.J., McLaggan, D., O'Byrne, C.P. and Booth, I.R. (2000) Rapid inactivation of the *Escherichia coli* Kdp K⁺ uptake system by high potassium concentrations. *Mol. Microbiol.* **35**, 1235–1243.
- Rohwerder, T., Gehrke, T., Kinzler, K. and Sand, W. (2003) Bioleaching review part A. Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Appl. Microbiol. Biotechnol.* **63**, 239–248.
- Rosenthal, A.Z., Hu, M. and Gralla, J.D. (2006) Osmolyte-induced transcription: –35 region elements and recognition by sigma38 (rpoS). *Mol. Microbiol.* **59**, 1052–1061.
- Rosner, J.L. and Slonczewski, J.L. (1994) Dual regulation of *inaA* by the multiple antibiotic resistance (Mar) and superoxide (SoxRS) stress response systems of *Escherichia coli*. *J. Bacteriol.* **176**, 6262–6269.
- Rossmann, R., Sawers, G. and Bock, A. (1991) Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. *Mol. Microbiol.* **5**, 2807–2814.
- Rottenberg, H. (1979) The measurement of membrane potential and ΔpH in cells, organelles and vesicles. *Methods Enzymol.* **55**, 547–569.
- Rozen, Y. and Belkin, S. (2001) Survival of enteric bacteria in seawater. *FEMS Microbiol. Rev.* **25**, 513–539.
- Russell, J.B. and Diez-Gonzalez, F. (1998) The effects of fermentation acids on bacterial growth. *Adv. Microb. Physiol.* **39**, 205–234.
- Sachs, G., Kraut, J.A., Wen, Y., Feng, J. and Scott, D.R. (2006) Urea transport in bacteria: acid acclimation by gastric *Helicobacter* spp. *J. Membr. Biol.* **212**, 71–82.
- Sachs, G., Weeks, D.L., Wen, Y., Marcus, E.A., Scott, D.R. and Melchers, K. (2005) Acid acclimation by *Helicobacter pylori*. *Physiology* **20**, 429–438.

- Saier, M.H., Jr. (2000) A functional-phylogenetic classification system for transmembrane solute transporter. *Microbiol. Mol. Biol. Rev.* **64**, 354–411.
- Saier, M.H., Eng, B.H., Fard, S., Garg, J., Haggerty, D.A., Hutchinson, W.J., Jack, D.L., Lai, E.C., Liu, G.J., Nusinew, D.P., Omar, A.M., Pao, S.A., Paulsen, I.T., Quan, J.A., Siwinski, M., Tseng, T.-T., Wachi, S. and Young, G.B. (1999) Phylogenetic characterisation of novel transport protein families revealed by genome analyses. *Biochim. Biophys. Acta* **1422**, 1–56.
- Saito, H. and Kobayashi, H. (2003) Bacterial responses to alkaline stress. *Sci. Prog.* **86**, 277–282.
- Salmond, C.V., Kroll, R.G. and Booth, I. (1984) The effect of food preservatives on pH homeostasis in *Escherichia coli*. *J. Gen. Microbiol.* **130**, 2845–2850.
- Sanders, J.W., Leenhouts, K., Burghoorn, J., Brands, J.R., Venema, G. and Kok, J. (1998) A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. *Mol. Microbiol.* **27**, 299–310.
- Sato, M., Machida, K., Arikado, E., Saito, H., Kakegawa, T. and Kobayashi, H. (2000) Expression of outer membrane proteins in *Escherichia coli* growing at acid pH. *Appl. Environ. Microbiol.* **66**, 943–947.
- Sawatari, Y. and Yokota, A. (2007) Diversity and mechanisms of alkali tolerance in lactobacilli. *Appl. Environ. Microbiol.* **73**, 3909–3915.
- Schäfer, G. (1996) Bioenergetics of the archaebacterium *Sulfolobus*. *Biochim. Biophys. Acta* **1277**, 163–200.
- Schäfer, G., Engelhard, M. and Muller, V. (1999) Bioenergetics of the Archaea. *Microbiol. Mol. Biol. Rev.* **63**, 570–620.
- Schilling, O., Frick, O., Herzberg, C., Ehrenreich, A., Heinzle, E., Wittmann, C. and Stülke, J. (2007) Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: transcription regulation is important but not sufficient to account for microbial adaptation. *Appl. Environ. Microbiol.* **73**, 499–507.
- Schleper, C., Puehler, G., Holz, I., Gambacorta, A., Janekovic, D., Santarius, U., Klenk, H.P. and Zillig, W. (1995) *Picrophilus* Gen. Nov., Fam. Nov.: a novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around pH 0. *J. Bacteriol.* **177**, 7050–7059.
- Scholes, P. and Mitchell, P. (1970) Acid–base titration across plasma membrane of *Micrococcus denitrificans*: factors affecting the effective proton conductance and respiratory rate. *Bioenergetics* **1**, 61–72.
- Schuldiner, S., Agmon, V., Brandsma, J., Chohen, A., Friedman, E. and Padan, E. (1986) Induction of SOS functions by alkaline intracellular pH in *Escherichia coli*. *J. Bacteriol.* **168**, 936–939.
- Scott, D.R., Marcus, E.A., Wen, Y., Oh, J. and Sachs, G. (2007) Gene expression *in vivo* shows that *Helicobacter pylori* colonizes an acidic niche on the gastric surface. *Proc. Natl. Acad. Sci. USA* **104**, 7235–7240.
- Seufferheld, M.J., Alvarez, H.M. and Farias, M.E. (2008) Role of polyphosphates in microbial adaptation to extreme environments. *Appl. Environ. Microbiol.* **74**, 5867–5874.
- She, Q., Singh, R.K., Confalonieri, F., Zivanovic, Y., Allard, G., Awayez, M.J., Chan-Weiher, C.C.Y., Clausen, I.G., Curtis, B.A., De Moors, A., Erauso, G., Fletcher, C., Gordon, P.M.K., Heikamp-de Jong, I., Jeffries, A.C., Kožera, C.J., Medina, N., Peng, X., Thi-Ngoc, H.P., Redder, P., Schenk, M.E., Theriault, C., Tolstrup, N., Charlebois, R.L., Doolittle, W.F., Duguet, M., Gaasterland, T.,

- Garrett, J., Ragan, M.A., Sensen, C.W. and Van der Oost, J. (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proc. Natl. Acad. Sci. USA* **98**, 7835–7840.
- Shibata, C., Ehara, T., Tomura, K., Igarashi, K. and Kobayashi, H. (1992) Gene structure of *Enterococcus hirae* (*Streptococcus faecalis*) F₁F₀-ATPase, which functions as a regulator of cytoplasmic pH. *J. Bacteriol.* **174**, 6117–6124.
- Shijuku, T., Yamashino, T., Ohashi, H., Saito, H., Kakegawa, T., Ohta, M. and Kobayashi, H. (2002) Expression of *chaA*, a sodium ion extrusion system of *Escherichia coli*, is regulated by osmolarity and pH. *Biochim. Biophys. Acta* **1556**, 142–148.
- Shimada, H., Nemoto, N., Shida, Y., Oshima, T. and Yamagishi, A. (2002) Complete polar lipid composition of *Thermoplasma acidophilum* HO-62 determined by high-performance liquid chromatography with evaporative light-scattering detection. *J. Bacteriol.* **184**, 556–563.
- Shioi, J.-C., Matsuura, S. and Imae, Y. (1980) Quantitative measurements of proton motive force and motility in *Bacillus subtilis*. *J. Bacteriol.* **144**, 891–897.
- Siegmundfeldt, H., Björn Rechinger, K. and Jakobsen, M. (2000) Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Appl. Environ. Microbiol.* **66**, 2330–2335.
- Siegmundfeldt, H., Rechinger, K.B. and Jakobsen, M. (1999) Use of fluorescence ratio imaging for intracellular pH determination of individual bacterial cells in mixed cultures. *Microbiology* **145**, 1703–1709.
- Slonczewski, J.L. and Foster, J.W. (1996) pH-regulated genes and survival at extreme pH. In: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (F.C. Neidhardt, eds), Chapter 96, pp. 1539–1549. ASM Press, Washington, DC.
- Slonczewski, J.L., Gonzalez, T.N., Bartholomew, F.M. and Holt, N.J. (1987) Mud-directed *lacZ* fusions regulated by acid pH in *Escherichia coli*. *J. Bacteriol.* **169**, 3001–3006.
- Slonczewski, J.L., Macnab, R.M., Alger, J.R. and Castle, A.M. (1982) Effects of pH and repellent tactic stimuli on protein methylation levels in *Escherichia coli*. *J. Bacteriol.* **152**, 384–399.
- Slonczewski, J.L., McGee, D.J., Phillips, J., Kirkpatrick, C. and Mobley, H.L.T. (2000) pH-dependent protein profiles of *Helicobacter pylori* analyzed by two-dimensional gels. *Helicobacter* **5**, 240–247.
- Slonczewski, J.L., Rosen, B.P., Alger, J.R. and Macnab, R.M. (1981) pH homeostasis in *Escherichia coli*: measurement by ³¹P nuclear magnetic resonance of methylphosphonate and phosphate. *Proc. Natl. Acad. Sci. USA* **78**, 6271–6275.
- Small, P.L.C., Blankenhorn, D., Welty, D., Zinser, E. and Slonczewski, J.L. (1994) Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: the role of *rpoS* and growth pH. *J. Bacteriol.* **176**, 1729–1737.
- Speelmans, G., Poolman, B., Abee, T. and Konings, W.N. (1993) Energy transduction in the thermophilic anaerobic bacterium *Clostridium fevidus* is exclusively coupled to sodium ions. *Proc. Natl. Acad. Sci. USA* **90**, 7975–7979.
- Stähler, F.N., Ganter, L., Lederer, K., Kist, M. and Bereswill, S. (2005) Mutational analysis of the *Helicobacter pylori* carbonic anhydrases. *FEMS Immunol. Med. Microbiol.* **44**, 183–189.

- Stancik, L.M., Stancik, D.M., Schmidt, B., Barnhart, D.M., Yoncheva, Y.N. and Slonczewski, J.L. (2002) pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J. Bacteriol.* **184**, 4246–4258.
- Stark, R.M., Gerwig, G.J., Pitman, R.S., Potts, L.F., Williams, N.A., Greenman, J., Weinzweig, I.P., Hirst, T.R. and Millar, M.R. (1999) Biofilm formation by *Helicobacter pylori*. *Lett. Appl. Microbiol.* **28**, 121–126.
- Stim, K.P. and Bennett, G.N. (1993) Nucleotide sequence of the *adi* gene, which encodes the biodegradative acid-induced arginine decarboxylase of *Escherichia coli*. *J. Bacteriol.* **175**, 1221–1234.
- Stingl, K., Brandt, S., Uhlemann, E.-M., Schmid, R., Altendorf, K., Zeilinger, C., Ecobichon, C., Labigne, A., Bakker, E.P. and de Reuse, H. (2007) Channel-mediated potassium uptake in *Helicobacter pylori* is essential for gastric colonization. *EMBO J.* **26**, 232–241.
- Stingl, K., Uhlemann, E.M., Deckers-Hebestreit, G., Schmid, R., Bakker, E.P. and Altendorf, K. (2001) Prolonged survival and cytoplasmic pH homeostasis of *Helicobacter pylori* at pH 1. *Infect. Immun.* **69**, 1178–1180.
- Stingl, K., Uhlemann, E.M., Schmid, R., Altendorf, K. and Bakker, E.P. (2002) Energetics of *Helicobacter pylori* and its implications for the mechanism of urease-dependent acid tolerance at pH 1. *J. Bacteriol.* **184**, 3053–3060.
- Stolyar, S., He, Q., Joachimiak, M.P., He, Z., Yang, Z.K., Borglin, S.E., Joyner, D.C., Huang, K., Alm, E., Hazen, T.C., Zhou, J.-Z., Wall, J.D., Arkin, A.P. and Stahl, D.A. (2007) Response of *Desulfovibrio vulgaris* to alkaline stress. *J. Bacteriol.* **189**, 8944–8952.
- Sturr, M.G., Guffanti, A.A. and Krulwich, T.A. (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. *J. Bacteriol.* **176**, 3111–3116.
- Sugiyama, S., Matsukura, H., Koyama, N., Nosoh, Y. and Imae, Y. (1986) Requirement of Na⁺ in flagellar rotation and amino acid transport in a facultatively alkaliphilic *Bacillus*. *Biochim. Biophys. Acta* **852**, 38–45.
- Sun, L., Fukamachi, T., Saito, H. and Kobayashi, H. (2005) Carbon dioxide increases acid resistance in *Escherichia coli*. *Lett. Appl. Microbiol.* **40**, 397–400.
- Suzuki, I., Lee, D., Mackay, B., Harahuc, L. and Oh, J.K. (1999) Effect of various ions, pH, and osmotic pressure on oxidation of elemental sulfur by *Thiobacillus thiooxidans*. *Appl. Environ. Microbiol.* **65**, 5163–5168.
- Suzuki, T., Unemoto, T. and Kobayashi, H. (1988) Novel streptococcal mutants defective in the regulation of H⁺-ATPase biosynthesis and in F₀ complex. *J. Biol. Chem.* **263**, 11840–11843.
- Suzuki, Y., Ueno, S., Ohnuma, R. and Koyama, N. (2005) Cloning, sequencing and functional expression in *Escherichia coli* of the gene of a P-type Na⁺-ATPase of a facultatively anaerobic alkaliphile, *Exiguobacterium aurantiacum*. *Biochim. Biophys. Acta* **1727**, 162–168.
- Swartz, T.H., Ikewada, S., Ishikawa, O., Ito, M. and Krulwich, T.A. (2005) The Mrp system: a giant among antiporters? *Extremophiles* **9**, 345–354.
- Swartz, T.H., Ito, M., Ohira, T., Natsui, S.S., Hicks, D.B. and Krulwich, T.A. (2007) Catalytic properties of *Staphylococcus aureus* and *Bacillus* members of the secondary Cation-Proton Antiporter-3 family are revealed by an optimized assay in an *Escherichia coli* host. *J. Bacteriol.* **189**, 3081–3090.

- Taglicht, D., Padan, E. and Schuldiner, S. (1993) Proton-sodium stoichiometry of NhaA, an electrogenic antiporter from *Escherichia coli*. *J. Biol. Chem.* **268**, 5382–5387.
- Takami, H., Kobata, K., Nagahama, T., Kobayashi, H., Inoue, A. and Horikoshi, K. (1999) Biodiversity in deep-sea sites located near the south part of Japan. *Extremophiles* **3**, 97–102.
- Takami, H., Nadasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S. and Horikoshi, K. (2000) Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res.* **28**, 4317–4331.
- Tanner, A. and Bornemann, S. (2000) *Bacillus subtilis* YvrK is an acid-induced oxalate decarboxylase. *J. Bacteriol.* **182**, 5271–5273.
- Terahara, N., Krulwich, T.A. and Ito, M. (2008) Mutations alter the sodium versus proton use of a *Bacillus clausii* flagellar motor and confer dual ion use on *Bacillus subtilis* motors. *Proc. Natl. Acad. Sci. USA* **105**, 14359–14364.
- Tiago, I., Chang, A.P. and Verissimo, A. (2004) Bacterial diversity in a nonsaline alkaline environment: heterotrophic aerobic populations. *Appl. Environ. Microbiol.* **70**, 7378–7387.
- Tiago, I., Pires, C., Mendes, V., Morais, P.V., da Costa, M.S. and Verissimo, A. (2006) *Bacillus foraminis* sp. nov., isolated from a non-saline alkaline groundwater. *Int. J. Syst. Evol. Microbiol.* **56**, 2571–2574.
- Tiwari, R.P., Reeve, W.G., Fenner, B.J., Dilworth, M.J., Glenn, A.R. and Howieson, J.G. (2004) Probing for pH-regulated genes in *Sinorhizobium medicae* using transcriptional analysis. *J. Mol. Microbiol. Biotechnol.* **7**, 133–139.
- Thomas, J.D., Daniel, R.A., Errington, J. and Robinson, C. (2001) Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. *Mol. Microbiol.* **39**, 47–53.
- Thomassin, S., Jobin, M.P. and Schmitt, P. (2006) The acid tolerance response of *Bacillus cereus* ATCC14579 is dependent on culture pH, growth rate and intracellular pH. *Arch. Microbiol.* **186**, 229–239.
- Tran, S.L., Rao, M., Simmers, C., Gebhard, S., Olsson, K. and Cook, G.M. (2005) Mutants of *Mycobacterium smegmatis* unable to grow at acidic pH in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. *Microbiology* **151**, 665–672.
- Tsai, J.L., Guffanti, A.A. and Montville, T.J. (1992) Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **58**, 891–894.
- Tuttle, J.H. and Dugan, P.R. (1976) Inhibition of growth, iron, and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds. *Can. J. Microbiol.* **22**, 719–730.
- Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., Solovyev, V.V., Rubin, E.M., Rokhsar, D.S. and Banfield, J.F. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**, 37–43.
- Ultee, A., Kets, E.P.W. and Smid, E.J. (1999) Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* **65**, 4606–4610.

- Valdés, J., Pedroso, I., Quatrini, R., Dodson, R.J., Tettelin, H., Blake, R., Eisen, J.A. and Holmes, D.S. (2008) *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. *BMC Genomics* **9**, 597, DOI:10.1186/1471-2164-9-597.
- van den Vossenberg, J., UbbinkKok, T., Elferink, M.G.L., Driessen, A.J.M. and Konings, W.N. (1995) Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea. *Mol. Microbiol.* **18**, 925–932.
- van den Vossenberg, J.L.C.M., Driessen, A.J.M. and Konings, W.N. (1998a) The essence of being extremophilic: the role of the unique archaeal membrane lipids. *Extremophiles* **2**, 163–170.
- van den Vossenberg, J.L.C.M., Driessen, A.J.M., Zillig, W. and Konings, W.N. (1998b) Bioenergetics and cytoplasmic membrane stability of the extremely acidophilic, thermophilic archaeon *Picrophilus oshimae*. *Extremophiles* **2**, 67–74.
- van Vliet, A.H., Kuipers, E.J., Waidner, B., Davies, B.J., de Vries, N., Penn, C.W., Vandenbroucke-Grauls, C.M., Kist, M., Bereswill, S. and Kusters, J.G. (2001) Nickel-responsive induction of urease expression in *Helicobacter pylori* is mediated at the transcriptional level. *Infect. Immun.* **69**, 4891–4897.
- von Blohn, C., Kempf, B., Kappes, R.M. and Bremer, E. (1997) Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternate transcription factor sigma B. *Mol. Microbiol.* **25**, 175–187.
- Wang, J. and Fung, D.Y.C. (1996) Alkaline-fermented foods: a review with emphasis on pidan fermentation. *Crit. Rev. Microbiol.* **22**, 101–138.
- Wang, Z., Hicks, D.B., Guffanti, A.A., Baldwin, K. and Krulwich, T.A. (2004) Replacement of amino acid sequence features of *a*- and *c*-subunits of ATP synthases of alkaliphilic *Bacillus* with *Bacillus* consensus sequence results in defective oxidative phosphorylation and non-fermentative growth at pH 10.5. *J. Biol. Chem.* **297**, 26546–26554.
- Waser, M., Hess-Bienz, D., Davies, K. and Solioz, M. (1992) Cloning and disruption of a putative NaH-antiporter gene of *Enterococcus hirae*. *J. Biol. Chem.* **268**, 26334–26337.
- Watson, N., Dunyak, D.S., Rosey, E.L., Slonczewski, J.L. and Olson, E.R. (1992) Identification of elements involved in transcriptional regulation of the *Escherichia coli* *cad* operon by external pH. *J. Bacteriol.* **174**, 530–540.
- Wei, Y., Deikus, G., Powers, B., Shelden, V., Krulwich, T.A. and Bechhofer, D.H. (2006) Adaptive gene expression in *Bacillus subtilis* strains deleted for *tetL*. *J. Bacteriol.* **188**, 7090–7100.
- Wei, Y., Guffanti, A.A., Ito, M. and Krulwich, T.A. (2000) *Bacillus subtilis* YqkI is a novel malic/Na⁺-lactate antiporter that enhances growth on malate at low protonmotive force. *J. Biol. Chem.* **275**, 30287–30292.
- Wei, Y., Liu, J., Ma, Y. and Krulwich, T.A. (2007) Three putative cation/proton antiporters from the soda lake alkaliphile *Alkalimonas amylolytica* N10 complement an alkali-sensitive *Escherichia coli* mutant. *Microbiology* **153**, 2168–2179.
- Wei, Y., Southworth, T.W., Kloster, H., Ito, M., Guffanti, A.A., Moir, A. and Krulwich, T.A. (2003) Mutational loss of a K⁺ and NH₄⁺ transporter affects

- the growth and endospore formation of alkaliphilic *Bacillus pseudofirmus* OF4. *J. Bacteriol.* **185**, 5133–5147.
- Welin, J., Wilkins, J.C., Beighton, D., Wrzesinski, K., Fey, S.J., Mose-Larsen, P., Hamilton, I.R. and Svensäter, G. (2003) Effect of acid shock on protein expression by biofilm cells of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **227**, 287–293.
- Welin-Neilands, J. and Svensäter, G. (2007) Acid tolerance of biofilm cells of *Streptococcus mutans*. *Appl. Environ. Microbiol.* **73**, 5633–5638.
- Wen, Y., Marcus, E.A., Matrubutham, U., Gleeson, M.A., Scott, D.R. and Sachs, G. (2003) Acid-adaptive genes of *Helicobacter pylori*. *Infect. Immun.* **71**, 5921–5939.
- West, I.C. and Mitchell, P. (1974) Proton/sodium ion antiport in *Escherichia coli*. *Biochem. J.* **144**, 87–90.
- White, S., Tuttle, F.E., Blankenhorn, D., Dosch, D.C. and Slonczewski, J.L. (1992) pH dependence and gene structure of *inaA* in *Escherichia coli*. *J. Bacteriol.* **174**, 1537–1543.
- Wiegel, J. (1998) Anaerobic alkalithermophiles, a novel group of extremophiles. *Extremophiles* **2**, 257–267.
- Wiegert, T., Homuth, G., Versteeg, S. and Schumann, W. (2001) Alkaline shock induces the *Bacillus subtilis* σ^W regulon. *Mol. Microbiol.* **41**, 59–71.
- Wilks, J.C., Kitko, R.D., Cleeton, S.H.G.E., Ugwu, C.S., Jones, B.D., BonDurant, S.S. and Slonczewski, J.L. (2009) Acid and base stress and transcriptomic responses in *Bacillus subtilis*. *Appl. Env. Microbiol.* **75**, 981–990.
- Wilks, J.C. and Slonczewski, J.L. (2007) pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by GFP fluorimetry. *J. Bacteriol.* **189**, 5601–5607.
- Wilmes, P., Remis, J.P., Hwang, M., Auer, M., Thelen, M.P. and Banfield, J.F. (2008) Natural acidophilic biofilm communities reflect distinct organismal and functional organization. *ISME J.* **3**, 266–270.
- Wolfe, A.J. (2005) The acetate switch. *Microbiol. Mol. Biol. Revs.* **69**, 12–50.
- Wolken, W.A., Lucas, P.M., Lonvaud-Funel, A. and Lolkema, J.S. (2006) The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis*. *J. Bacteriol.* **188**, 2198–2206.
- Wortham, B.W., Patel, C.N. and Oliveira, M.A. (2007) Polyamines in bacteria: pleiotropic effects yet specific mechanisms. *Adv. Exp. Med. Biol.* **603**, 106–115.
- Yohannes, E., Barnhart, D.M. and Slonczewski, J.L. (2004) pH-dependent catabolic protein expression during anaerobic growth of *Escherichia coli* K-12. *J. Bacteriol.* **186**, 192–199.
- Yohannes, E., Thurber, A.E., Wilks, J.C., Tate, D.P. and Slonczewski, J.L. (2005) Polyamine stress at high pH in *Escherichia coli* K-12. *BMC Microbiol* **5**, 59.
- Young, G.M., Amid, D. and Miller, V.L. (1996) A bifunctional urease enhances survival of pathogenic *Yersinia enterocolitica* and *Morganella morganii* at low pH. *J. Bacteriol.* **178**, 6487–6495.
- Yumoto, I. (2002) Bioenergetics of alkaliphilic *Bacillus* spp. *J. Biosci. Bioeng.* **93**, 342–353.
- Yumoto, I., Fukumori, Y. and Yamanaka, T. (1991) Purification and characterization of two membrane-bound c-type cytochromes from a facultative alkalophilic *Bacillus*. *J. Biochem.* **110**, 267–273.

- Zhou, L., Lei, X.H., Bochner, B.R. and Wanner, B.L. (2003) Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J. Bacteriol.* **185**, 4956–4972.
- Zhou, H., Zhang, R., Hu, P., Zeng, W., Xie, Y., Wu, C. and Qiu, G. (2008) Isolation and characterization of *Ferroplasma thermophilum* sp. nov., a novel extremely acidophilic, moderately thermophilic archaeon and its role in bioleaching of chalcopyrite. *J. Appl. Microbiol.* **105**, 591–601.
- Zhu, M., Takenaka, S., Sato, M. and Hoshino, E. (2001) Influence of starvation and biofilm formation on acid resistance of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **16**, 24–27.
- Zilberstein, D., Agmon, V., Schuldiner, S. and Padan, E. (1984) *Escherichia coli* intracellular pH, membrane potential and cell growth. *J. Bacteriol.* **158**, 246–252.
- Zychlinsky, E. and Matin, A. (1983) Cytoplasmic pH homeostasis in an acidophilic bacterium, *Thiobacillus acidophilus*. *J. Bacteriol.* **156**, 1352–1355.