Published in final edited form as:
Nat Rev Microbiol. 2011 May ; 9(5): 330–343. doi:10.1038/nrmicro2549.

Molecular aspects of bacterial pH sensing and homeostasis

Terry A. Krulwich, PhD[*], **George Sachs, MD, DSc**[†], and **Etana Padan, PhD**[‡]

Terry A. Krulwich: terry.krulwich@mssm.edu; George Sachs: gsachs@ucla.edu; Etana Padan: etana@vms.huji.ac.il

[*]Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, Box 1603, 1 Gustave L. Levy Place, New York, NY 10029, USA; Tel. 212-241-7280; Fax. 212-996-7214

[†]Departments of Physiology and Medicine, David Geffen School of Medicine at UCLA, 405 Hilgard Ave., Los Angeles, California 90024, USA Tel. 310-268-3923, Fax 310-312-9478

[‡]Alexander Silberman Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel, Tel. 972 2 6585094, Fax 972 2 658947

Abstract

Diverse mechanisms for pH-sensing and cytoplasmic pH homeostasis enable most bacteria to tolerate or grow at external pH values that are outside the cytoplasmic pH range they must maintain for growth. The most extreme cases are exemplified by the extremophiles that inhabit environments whose pH is below 3 or above 11. Here we describe how recent insights into the structure and function of key molecules and their regulators reveal novel strategies of bacterial pH-homeostasis. These insights may help us better target certain pathogens and better harness the capacities of environmental bacteria.

Introduction

Living cells are critically dependent upon pH homeostasis because most proteins have distinct ranges of pH for function. Also, the proton concentration is intricately involved in cellular bioenergetics. The proton motive force (PMF) is a central energy currency and the pH gradient (ΔpH) across the bacterial cell membrane is one of the two PMF components [BOX 1]. Higher eukaryotes typically exhibit strict cytoplasmic pH homeostasis at a pH_{in} of 7.3 and rely on a strongly controlled external pH (pH_{out}) of 7.4[1]. In contrast, neutralophilic bacteria can grow at pH_{out} values from ~5.5–9.0 but generally maintain their cytoplasmic pH in a narrow range ~7.5–7.7[2,3]. Therefore, almost all neutralophiles have strategies for maintaining a significantly more alkaline cytoplasmic pH relative to the outside pH at the low end of their growth pH range. For example, caries-producing *Streptococcus mutans* grow in dental plaque niches at pH ~ 4.8 [4,5] [FIGURE 1]. Neutralophiles also maintain a significantly more acidic cytoplasmic pH than the external pH at the high end of their pH range. Bacteria have additional strategies for surviving without growth during periods of exposure to pH values outside their growth range. Survival without growth is assessed by resumption of growth upon return of the bacteria to a permissive pH, i.e., neutral pH for neutralophiles. For example, enteric bacteria such as *Escherichia coli* and *Salmonella* species survive passage through the stomach but do not grow in that niche [6,7] and *E. coli* survives exposure to alkaline seawater but does not grow [8]. Survival as well as growth under acid or alkaline stress involves changes in cell structure, metabolic and transport patterns.

Box 1

The protonmotive force (PMF) and diverse PMF patterns of bacteria

The proton motive force (PMF) is an electrochemical gradient of H^+ (protons) across the bacterial cell membrane or specific organelle membrane, e.g. mitochondria and thylakoid. Typically, the PMF of bacteria consists of two components, a transmembrane pH gradient (ΔpH), alkaline inside the cell relative to outside, and a transmembrane electrical potential (Δψ), negative inside the cell relative to outside[139,140,141]. Exceptions to this pattern are found in connection with specific demands of cytoplasmic pH homeostasis as shown below and discussed in the text.

$$\mathrm{PMF\,(mV)} = \Delta\psi - 2.3\mathrm{RT/F} \cdot \Delta\mathrm{pH}$$

where:

R is the gas constant, T is the absolute temperature, F is the Faraday constant

$$\Delta\mathrm{pH} = \mathrm{pH_{in}} - \mathrm{pH_{out}}$$

$\Delta\psi = \psi_{in} - \psi_{out}$ with the convention being that Δψ is negative when the inner membrane surface is negative

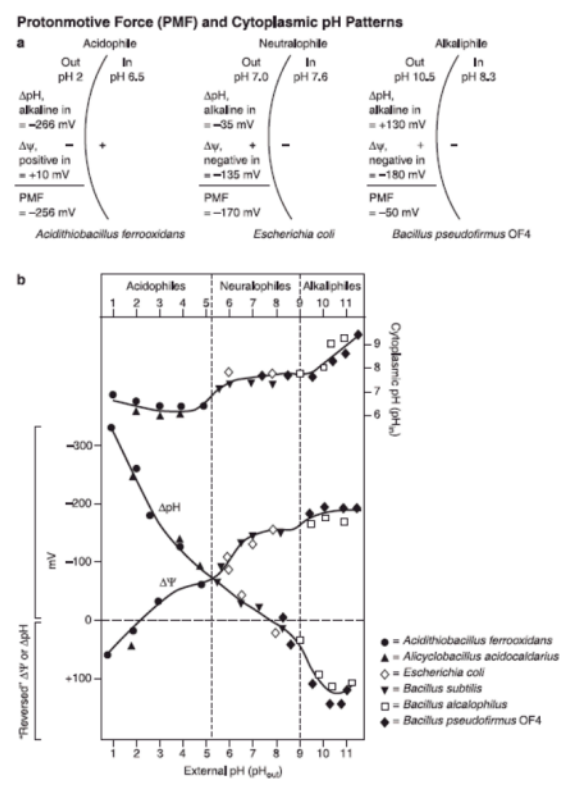
Under standard conditions, the following approximation holds: PMF (mV) = Δψ − 59 ΔpH

Primary proton pumps generate the PMF in bacteria as well as in mitochondria and photosynthesis[141]. They include respiratory or other redox potential-driven pumps (e.g. respiratory chain pumps), light-driven pumps (e.g. bacteriorhodopsin) or bond-energy driven pumps (e.g. proton-pumping ATPases). Secondary active transporters as well as rotary nano-machines such as the ATP synthase and the flagellar machinery of bacteria can harness the energy of the PMF to energize active transport, synthetic and mechanical processes[141]. Examples of how these processes play roles in pH homeostasis of specific bacteria are discussed in the text.

The PMF patterns of bacteria growing in different ranges of pH reflect accommodation of pH homeostasis, as shown in **a** for acidophile *Acidithiobacillus ferrooxidans* growing at pH 2[9], neutralophile *E. coli* growing at pH 7.0 [142], and alkaliphile *Bacillus pseudofirmus* OF4 growing at pH 10.5 [120,143]. The extremely large ΔpH of acidophiles ($pH_{in} > pH_{out}$) is partially offset by a Δψ that is "reversed" i.e. inside-positive relative to outside. *E. coli* has only a small ΔpH ($pH_{in} > pH_{out}$) when growing at pH ~7.0, accompanied by a significant Δψ (negative-inside relative to outside). The total PMF is lower than in the acidophiles and the cytoplasmic pH is higher. *B. pseudofirmus* OF4, maintains a cytoplasmic pH of 8.3, significantly lower than the external pH of 10.5 but above the cytoplasmic pH at which neutralophiles can grow. The Δψ of the alkaliphile is higher than the neutralophile Δψ, but only partially offsets the effect of the large "reverse" ΔpH in reducing the total PMF[13,119].

Diagram **b** shows the patterns of Δψ, ΔpH and cytoplasmic pH, pH_{in}, over a range of external pH values, for acidophilic *A. ferrooxidans*[113] and *Alicyclobacillus acidocaldarius*[115], neutralophilic *E. coli* [142] and *Bacillus subtilis*[144], and alkaliphilic *B. pseudofirmus* OF4 [120] and *Bacillus alcalophilus* [145]. The Δψ and ΔpH patterns illustrate:

(i) the contrasting relationship of between the magnitude of these two PMF components as a function of external pH: and (ii) the different cytoplasmic pH levels and patterns.



Extremely acidophilic and alkaliphilic bacteria grow, respectively, at pH 1–3 and pH 10–13 [BOX 1]. Environments of extreme acidophiles include mining and geothermal areas and acid soils in which sulfate is the major anion [9]. Environments of extreme alkaliphiles include natural environments such as highly alkaline segments of the hindgut of certain insects and alkaline soda lakes as well as industrial settings such as indigo dye plants, sewage plants and geochemically unusual ground waters with pH values > 12 [10,11]. Extremophiles use many of the same strategies observed in neutralophiles, further adapting them to respond to more extreme challenges. Typically, major pH homeostatic mechanisms of extremophiles are constitutively expressed, so that these bacteria are prepared for sudden shifts to the extreme end of the pH range. This preparedness often negatively impacts growth at near neutral pH because of the energetic cost of expressing proteins that are not useful at neutral pH and because some essential proteins are adapted to work at extreme pH values but function sub-optimally at neutral pH [12,13].

Bacterial pH homeostasis is important for physiology, ecology and pathogenesis. Unraveling the phenomenology of bacterial pH homeostasis has depended upon continued refinement of techniques that accurately measure internal and external pH (recently reviewed in [3][see BOX 2]). Here, we will first present an overview of pH homeostasis mechanisms in bacteria (see other reviews for discussions of archaea[3,14]) and will then focus on recent structure-function insights into specific molecules from three different bacteria that have pivotal pH homeostasis roles.

Box 2

Measuring cytoplasmic pH, ΔpH and Δψ

The small size of bacterial cells has generally precluded use of direct measurements of cytoplasmic pH and membrane potential with microelectrodes.

Measurement of ΔpH and cytoplasmic pH

Weak permeant acids (e.g. benzoic acid, 5, 5-dimethyl-2,4-oxazolidinedione (DMO)) that are radioactive or fluorescent have been widely used to assess a ΔpH in the alkali-inside relative to the outside. Weak bases (e.g. methylamine and related amines) have been used for the acid-inside relative outside orientation. The probes used must enter the cells by diffusion and have a pK_a in the pH range of the assay. The principle behind the ΔpH measurement is that the cytoplasmic accumulation of the probe relative to the outside is directly related to the magnitude of the ΔpH[3,139]. This principle holds because the uncharged, protonated acid probe or de-protonated basic probe will cross the membrane, while the charged forms will not. Once the probe is exposed to the cytoplasmic pH, its charged form becomes more abundant and is trapped within the cytoplasm[3]. The cytoplasmic pH can then be calculated from the ΔpH and the external pH.[31]P-NMR methods have also been applied to assess cytoplasmic pH but high cell densities are required, which is problematic for aerobic cells[3].

There are several fluorescent pH probes including 2′,7′-*bis*-(2-carboxyethyl)-5 and 6-carboxyfluorescein (BCECF) and Oregon Green whose spectra directly reflect pH[146]. With these, the pH is determined after dye uptake and spectral recording. Standard curves from which to calculate the cytoplasmic pH can be generated from spectra recorded from preparations in which the PMF is collapsed followed by equilibration at pH values in the range of interest. In 2007, Wilks and Slonczewski[89] introduced use of pH-sensitive green fluorescent protein fluorimetry, directly monitoring of the *E. coli* periplasmic and cytoplasmic pH.

Measurement of Δψ

Measurement of the Δψ, is often included in studies of cytoplasmic pH homeostasis since the orientation and magnitude of the Δψ impacts homeostasis strategies. Δψ measurements are most often conducted using membrane permeant radioactive probes (such as triphenylphosphonium and thiocyanate (^{14}C-SCN$^-$)) or fluorescent dyes (such as the carbocyanine dye 3,3′-dipropryl-2,2′ thiadicarbocyanine (DiS-C_3-5) or oxonol dyes)[139,141]. Distribution of radioactive cations or anions is studied in an analogous way to that described above for weak acids or bases using appropriate calibration of the relationship of the signal to concentration.

Active strategies for bacterial pH homeostasis

A major unifying principle of bacterial pH homeostasis that is depicted in BOX 1 is that the demands of pH homeostasis for particular bacteria determine the relative magnitudes of the two PMF components, the transmembrane electrical potential Δψ and the transmembrane ΔpH. Under significant pH stress conditions, both neutralophiles and extremophiles exhibit “reversal” of the orientation of a PMF component from the usual negative- and alkaline-inside orientation relative to outside.

Strategies in which pH challenges are met by direct active uptake or efflux of protons

A major strategy for bacterial pH homeostasis is use of transporters that catalyze active proton transport. Such transporters include primary proton pumps such as the proton-

pumping respiratory chain complexes or proton-coupled ATPases and secondary active transporters such as cation/proton antiporters, which use the PMF generated by respiration or ATPases to energize active proton uptake in exchange for cytoplasmic cations such as Na^+ or K^+. In respiratory bacteria the PMF is generated by the respiratory chain. Under conditions of acid challenge [FIGURE 1a], the neutralophile *E. coli* increases expression of respiratory chain complexes that pump protons out of the cell. Expression of the ATP synthase that brings protons into the cell during ATP synthesis is decreased[3]. In non-respiratory neutralophiles, such as *S. mutans*, up-regulation of the hydrolytic activity of F_1F_o-ATPase promotes ATP-dependent H^+ extrusion under acidic conditions[15]. For pH homeostasis under alkaline conditions, active transport of protons inward is a crucial adaptation, which usually involves activation and transcriptional up-regulation of key cation/proton antiporters [FIGURE 1b]. In addition, *E. coli* increases expression of non-proton pumping cytochrome *bd* and decreases expression of proton-pumping respiratory chain complexes to minimize proton loss from the cytoplasm during PMF generation. Meanwhile proton capture is further enhanced by increased expression of the F_1F_o ATP synthase[3,16,17]. In Gram-positive *Enterococcus hirae* the F_1F_o ATPase works entirely in the hydrolytic direction, in which protons are pumped outward. Under alkaline conditions, expression and activity of the F_1F_o ATPase complex are both greatly reduced while activity of a Na^+-pumping V_1V_o ATPase is up-regulated and plays the major role in $\Delta\psi$-generation[15,18,19].

Generation of a substantial $\Delta\psi$ at high pH is crucial to support the central role of monovalent cation/proton antiporters in alkaline pH homeostasis by both respiratory and non-respiratory bacteria[20]. Most free-living bacteria have multiple Na^+/H^+ and K^+/H^+ antiporters that probably make different contributions to pH homeostasis, cation and osmotic homeostasis[2,21]. Antiporters with roles in alkaline pH homeostasis catalyze electrogenic antiport in which the ratio of H^+ entering the cell in exchange for Na^+ or K^+ moving out of the cell is unequal[22], e.g. the stoichiometry for *E. coli* NhaA is $2H^+/1Na^+$ [22,23] enabling proton entry driven by the transmembrane potential, $\Delta\psi$, component of the PMF[2,22] [BOX 1]. Na^+/H^+ antiporters often have dominant roles in alkaline pH homeostasis, e.g. in *E. coli* and aerobic alkaliphilic *Bacillus* strains[2]; however, under Na^+-poor conditions or when there is a large inwardly directed Na^+ gradient, K^+/H^+ antiporters assume dominance. In *E. hirae*, whose Na^+-pumping V_1V_o ATPase is activated at high pH, a critical role in alkaline pH homeostasis is played by a K^+/H^+ antiporter while a Na^+/H^+ antiporter, NapA, appears to be more involved in Na^+ homeostasis at less alkaline pH values[24,25] [FIGURE 1b].

pH homeostasis strategies involving regulation of proton consumption or generation by metabolic enzymes

A second major strategy for pH homeostasis is re-modeling of metabolic patterns to support pH homeostasis. Under acidic conditions [FIGURE 1a], there is increased expression of enzymes whose reactions consume cytoplasmic protons, including specific hydrogenases and amino acid decarboxylases[3,16,17]. During anaerobic acid challenge, *E. coli* up-regulates hydrogenase-3 that catalyzes H_2 production from cytoplasmic protons, contributing to survival at pH 2–2.5 [26]. In non-respiratory *Streptococcus mutants*, use of the proton-consuming malolactic fermentation is proposed to support acid-tolerance[27]. Glutamate and arginine decarboxylases are a corner-stone of the acid-resistance response of *E. coli* and other enteric bacteria as they pass through the stomach. Glutamate decarboxylase (GadB, shown in FIGURE 1a) is activated by gastric chloride ions, and then consumes a proton during decarboxylation to γ-aminoglutarate (GABA). GadB partners with an antiporter that catalyzes efflux of the resulting GABA in exchange for more substrate glutamate for continued decarboxylation[7,28]. The consumption of the proton supports acid pH homeostasis. In addition, the conversion of cytoplasmic glutamate (with a net charge of −1) to GABA (with a net charge of 0) is proposed to contribute to a “reversed $\Delta\psi$” [BOX 1] that

helps prevent proton leakage into the cells[7,28]. Conversely, challenges by alkaline conditions [FIGURE 1b] lead to up-regulation of amino acid deaminases or catabolic pathways that produce organic acids[3] as shown for tryptophan deaminase in *E. coli*[29,30].

Passive support of bacterial pH homeostasis

Passive mechanisms of pH homeostasis are proposed to support active mechanisms. While no strong correlation has emerged between cytoplasmic buffering capacity and pH homeostasis capacity in bacteria[3,31], strategic changes in membrane proton permeability and cell surfaces charges are proposed to delay proton entry into or loss from the cytoplasm. For example, extremophile proteomes have unusual p*I* profiles that may address functional needs but are also thought to provide a passive adjunct to the active mechanisms for pH homeostasis. Surface proteins of acidophiles such as *Acidithiobacillus ferrooxidans* and of *Helicobacter pylori* which acclimates to acidity have high p*I* values relative to neutralophile homologues, so that their positive charges could act as a transient proton repellent at the cell surface[32,33]. The p*I* of the outer membrane OmpA-like protein of *A. ferrooxidans* is 9.4 while that of *E. coli* OmpA is 6.2. Conversely, the surface exposed proteins of alkaliphiles such as *Bacillus pseudofirmus* OF4 generally have a low p*I* relative to those of neutralophiles, potentially contributing to proton capture and surface retention under proton-scarce conditions of high pH[21,34,35]. The p*I* of CtaC, a respiratory protein that has a large domain just outside the cell membrane, is 4.4 in *B. pseudofirmus* OF4 and 8.6 in neutralophilic *Bacillus subtilis*.

Adjustments of membrane lipid[36,37,38,39] and porin[3,40] composition are also used to minimize inward proton leakage during acid stress [see examples in FIGURE 1a]. In acidophilic *A. ferrooxidans*, changes in membrane lipids are observed in response to a shift to pH 1.3 from the optimal pH 2.3[41,42]. In alkaliphiles, acidic secondary cell wall polymers such as teichuronic acids and an acid S-layer protein, SlpA, contribute to pH homeostasis at high pH. They may bind protons, perhaps enhancing proton uptake by increasing the proton concentration near the surface[12,43]. Deletion of *slpA* from alkaliphilic *B. pseudofirmus* OF4 results in reduced ability to adapt to a sudden shift from pH 7.5 to 11. The *slpA* mutant grows better than wild type at pH 7.5, presumably because the S-layer is energetically costly to synthesize but plays no role at pH 7.5 except readiness for an alkaline shift[12].

pH sensors and signaling elements

A myriad of networks of sensors, signaling molecules and regulatory proteins are involved in pH homeostasis as well as in overlapping homeostatic responses to sodium, osmotic, cell wall, and reactive oxygen stresses[44,45,46,47,48]. These networks typically involve multiple transcription regulators, alternate sigma factors and DNA binding proteins. For example, proteins involved specifically in the glutamate decarboxylation-based acid response of *E. coli* are regulated by ≥15 proteins that include alternate sigma factors, AraC-like and LuxR-related gene regulators, the cyclic AMP receptor protein CRP, an Era-like GTPase (TrmE), the histone-like protein HNS, and at least two two-component signaling systems (TCSs)[7,49]. TCSs have major roles in pH homeostasis-related signaling. Some TCSs sense external pH or cytoplasmic pH. In *H. pylori* the TCS HP0165/HP0166, ArsRS, directly senses the pH of the medium[50] while HP0244, an orphan histidine kinase sensor likely responds to cytoplasmic pH [51]. Other TCSs sense changes that are presumably secondary to a change in pH. For example, the TorSR TCS of *E. coli* detects the presence of TMAO (Trimethylamine N-oxide) whose reduction forms trimethylamine that would alkalinize the cytoplasm. Instead, TorSR is anticipatory in inducing Tna, tryptophanase, which produces acid[52]. Evidence has also emerged for pH-sensing roles for transporters that are directly involved in

proton transport in support of pH homeostasis[53]. A paradigm for such a dual pH-sensing effector is the NhaA antiporter of *E. coli*.

The sections that follow focus on transporters and enzymes of three different bacteria that have key roles in pH homeostasis: (i) structure-function insights into the major cation/proton antiporter, NhaA, of *E. coli* that reveal the basis for its essential role in meeting dual challenges of alkali and elevated sodium; (ii) acclimation of *H. pylori* to the intense acidity of the stomach using a strategy that depends upon pH homeostasis of the periplasm; (iii) and unusual features of the key cation/proton antiporter and ATP synthase of extremely alkaliphilic *B. pseudofirmus* OF4 that are needed for growth at pH >10. These examples illustrate different patterns of adaptation in bacteria for which genomic data, physiological studies of pH homeostasis and detailed structure-function studies of one or more key molecules are available.

The *E. coli* NhaA antiporter

Na^+/H^+ antiporters were first described by West and Mitchell[54] [BOX 1] and are found in the cytoplasmic membranes of almost all cells and in many organellar membranes[55]. The NhaA Na^+/H^+ antiporter is essential for adaptation of the enteric pathogen *E. coli* to alkaline pH in the presence of Na^+ but not in the absence of added Na^+ [56]. The K^+/H^+ antiport activity of a $K^+(Na^+)(Ca^{2+})/H^+$ antiporter, ChaA[57], and of the MdfA antiporter, which has both multi-drug/H^+ and $K^+(Na^+)/H^+$ antiport capacities[58], could support pH homeostasis under those conditions. Homologues and orthologues of NhaA with related functions are present in other bacteria as well as in humans[59,60,61]. For example, the human genome has 9 NHE type antiporters of which hNHE1 defects lead to heart hypertrophy, ischemia, and reperfusion[62,63]. hNHA2 may be involved in human essential hypertension[61].

Purification of *E. coli* NhaA and its reconstitution in a functional form in proteoliposomes made it possible to unravel the properties that determine its role in *E. coli* pH homeostasis[53,64]. First, NhaA catalyzes a very fast rate of Na^+/H^+ antiport activity (10^5/minute)[64]. Second, NhaA catalyzes electrogenic antiport that facilitates maintenance of a pH_{in} below an alkaline pH_{out} by consuming electrical potential to maintain a "reversed" ΔpH [2,53]. Third, NhaA exhibits dramatic pH-dependence, increasing its activity by three-orders of magnitude between a pH_{out} of 6.5 and 8.5[64]. Mutations of *nhaA* that disrupt any of these three properties result in the inability to grow at high pH[53,55].

Structural, functional organization of NhaA

The elucidation of the atomic structure of NhaA was a critical breakthrough[65]; it facilitated understanding of the functional organization of the antiporter in a 3-dimensional context; and it provided the basis for combining computational, biophysical and biochemical approaches to determine functional dynamics. NhaA is composed of 12 transmembrane segments which form a cytoplasmic funnel and periplasmic funnel with a barrier separating them [FIGURE 2a]. The pH "sensor" is a cluster of ionizable residues that, when mutated, change the pH profile but not Na^+/H^+ antiport capacity. Most of these mutations are located at the orifice of the cytoplasmic funnel[53] while the active site is at the bottom of the cytoplasmic funnel [FIGURE 2b,c][65].

A new protein fold that is central to the translocation

The NhaA new fold is comprised of inverted topology repeats. Each repeat is composed of three transmembrane segments (TMS) of which one is interrupted by extended chain in the middle of the membrane (the TMS IV/XI assembly)[65] [FIGURE 2c]. The active site of NhaA is at the middle of the extended chains. This structure is delicately electrostatically balanced, a property that is thought to allow the rapid ion translocation observed with NhaA

after a pH-induced conformational change that is initiated at the pH "sensor" and transduced to the TMS IV/XI assembly[53,66] The conformation of the TMS IV/XI assembly of NhaA is very sensitive to perturbations as revealed by mutations[67] or binding by conformational monoclonal antibodies (mAbs)[68,69]. Since inverted repeats and discontinuous helices were found in NhaA, a similar fold with interrupted helices has been observed in structures of diverse secondary[55,70,71] and primary transporters[72,73].

Residues crucial for pH sensing and signal transduction

Na^+/H^+ antiporter-deficient *E. coli* strains, which require restoration of a functional NhaA antiporter for growth on selective media, have been useful for cloning antiporter genes from *E. coli* and other bacteria, expressing them and characterizing both native and mutant antiport properties[2,3,74,75]. Two types of mutations affecting the NhaA pH response are isolated: mutations that affect the apparent K_M for the cation but do not shift the pH-dependence of NhaA antiport under conditions of saturating ion concentrations; and mutations that affect both the apparent K_M and/or alter the pH-dependence of the antiporter even at saturating concentrations of the ions[67,76]. Mutations that change only pH-dependence are expected to affect the pH sensor. Mutations that change both K_M and pH-dependence are expected to affect the pH sensor and/or the pH-signal transduction[76,77,78]. By projecting the data on the 3D crystal structure it is possible to locate the pH sensor at the orifice of the cytoplasmic funnel separated by 15 Å from the binding site which is located in the middle of the TMS IV/XI assembly[53] [FIGURE 2a,b].

pH induced conformational changes in NhaA

At least two types of conformational changes, pH-induced and Na^+-induced, are expected for NhaA; here the focus is on changes induced by pH. pH-induced conformational changes in TMS I and IX were identified by trypsin digestion of native NhaA[79], NhaA-binding of anti-NhaA mAbs[80] and by cryo-electron microscopy of 2D crystals[81].

An established approach to identify conformational changes uses accessibility of Cys replacements in membrane proteins to various thiol reagents from either side of the membrane[82]. This approach, as a function of pH, identified pH-induced conformational changes in NhaA. Cysteine residues of NhaA were first replaced with serine, with minor effect on activity[83]. Then single Cys-replacements in the Cys-less (CL) NhaA protein were tested for their accessibility to various thiol reagents. Membrane permeant probes that ethylate Cys in the presence of water[77] revealed water-filled pockets in the protein[84]. Charged and membrane impermeant probes identified residues that are exposed on the protein surface or via water-filled funnels connected to the environmental water space[84,85]. Distances between two Cys replacements as a function of pH were estimated by bifunctional cross linking reagents of known length[85]. Changes in these distances as a function of pH indicated pH-induced conformational changes in the antiporter[86].

The crystal structure opened the way to experiments driven by structure-based computation. The protonation state of ionizable residues in NhaA was investigated by Multiconformation Continuum Electrostatics analysis (MCCE). Four clusters of electrostatically tightly interacting residues were identified in a trans-membrane arrangement[87]; two encompassing the pH sensor at the orifice of the cytoplasmic funnel, one at the active site and another at the periplasmic funnel [FIGURE 2d]. Computational predictions further predict a number of residues with extreme p*K* values, including several of the pH sensors. The sensors can only undergo protonation/deprotonation reactions subsequent to conformational changes. The importance of these NhaA residues in pH-induced conformational changes has been validated by characterization of site-directed mutants at Lys300[86,88], E252[84] and D65[77]. How the pH-induced conformational changes are integrated to yield the NhaA response is an

open question whose answer will be sought by combining crystallographic models of NhaA conformers with experimental and computational analyses.

Alkaline pH homeostasis of *E. coli* depends upon cation/proton antiporters and is augmented by increased expression of amino acid dehydrogenases and ATP synthase. This is a model for many other neutralophiles[13,16,17]. The next section describes a distinct, intricate acid acclimation strategy used by neutralophile *H. pylori*.

H. pylori acclimation to an acidic niche

The key to gastric colonization by *H. pylori* is periplasmic pH homeostasis. The periplasmic pH is maintained at ~6.1 at pH_{out} as low as 2.0. This facilitates maintenance of a cytoplasmic pH > 7.0 and a $\Delta\psi$ of −100 mV, allowing growth in the gastric niche. Periplasmic pH homeostasis of *H. pylori* contrasts with the patterns in *E coli*[89] and Gram negative acidophiles[33] in which the periplasmic pH is thought to be in equilibrium with the medium and not regulated in concert with the cytoplasm. Several key processes contribute to *H. pylori* periplasmic pH homeostasis.

H. pylori buffers its periplasm by using the products of the urease reaction

Urease is a key component of periplasmic pH homeostasis. *H. pylori* expresses the highest level of urease of any known microorganism[90]. The urease gene cluster consists of 7 genes: *ureA* and *ureB*, the constituents of urease apoenzyme; *ureE/G F/H*, required for nickel insertion into the apoenzyme to produce active urease; and *ureI*, which encodes a pH-gated inner membrane urea channel[91]. The UreI channel opens in acid, allowing access of urea to cytoplasmic urease and leading to greatly increased production of intrabacterial NH_3 and H_2CO_3. The NH_3 produced buffers the cytoplasm by the $H^+ + NH_3 \rightarrow NH_4^+$ reaction. However, to maintain a relatively neutral periplasmic pH, the $NH_3 \rightarrow NH_4^+$ couple is inadequate since, with a pK_a of 9.23, it is a very weak buffer at pH~ 6.0. The periplasmic pH of 6.1 is generated by the action of cytoplasmic β-carbonic anhydrase, which accelerates conversion of H_2C0_3 to CO_2 that enters the periplasm and is converted to $HC0_3^- + H^+$ by membrane bound α-carbonic anhydrase[92] [see model in FIGURE 3a]. The organism has further adapted to its environment by ensuring that the products of urease exit directly to the periplasm thereby avoiding excess NH_4^+ accumulation in the cytoplasm.

UreI regulation of urease activity

UreI is an acid-gated, electroneutral channel that is highly selective for urea and does not even permit transit of thiourea. The UreI channel, which is essential for infection, begins to open at about pH 6.2 and is fully open at pH ≤ 5 [93,94,95]. Its properties were deduced from a comparison of urease activity in intact and lysed bacteria as a function of pH [96]. Urease activity of the intact wild type strain increases with decreasing medium pH, the converse of the pH curve of free urease, and there is no such increase in the *ureI* knockout strain. Confirmation that UreI acts as a pH-gated urea channel was obtained in *Xenopus* oocytes injected with *ureI* cRNA[95]. Study of mutations and of chimeras of *ureI* homologues with different pH-dependence reveals that the most likely mechanism for opening the channel of *H. pylori* is a change of hydrogen bonding due to histidine protonation in a periplasmic loop and in the C-terminal domain[97].

UreI can also transport CO_2 and NH_4^+ providing their rapid access to the periplasm. Thus, in wild type organisms, addition of CO_2 rapidly decreases pH_{in} due to UreI permeation. Export of CO_2, NH_3 and NH_4^+ through UreI avoids excessive alkalization of the cytoplasm while buffering the periplasm[98].

Regulation of urease expression

Although urease activity is essential for infection and for acid survival of the organism below external pH 3.5, the presence of urea at pH > 3.5 results in loss of *H. pylori* survival because of rapid elevation of pH of the medium above 8.0 [99]. This observation suggests that while urease activity must be up-regulated in acid, it also must be down-regulated at more neutral pH levels during the gastric digestion phase. Evidence has emerged for both scenarios.

Two TCSs in *H. pylori* up-regulate expression of the urease gene cluster. The first, HP0165/HP0166 (ArsRS), has a membrane bound auto-phosphorylating histidine kinase sensor, HP0165, and a phosphorylatable response regulator, HP0166. *HP0165* mRNA expression is up-regulated *in vitro* at pH 6.2 and increases 5-fold at pH 4.5. *HP0166* mRNA is also up-regulated 3-fold at pH 4.5 [51]. Deletion of HP0165 abolishes infectivity but has little effect on growth *in vitro* at pH 4.5 [100,101]. Deletion of His94, one of 7 His residues in the periplasmic domain, abrogates the increased gene expression during acidification[102]. This TCS is also involved in the regulation of most of the genes listed in BOX 3 and another ~100 genes[103].

Box 3

Habitat of *Helicobacter pylori*

The major site of colonization for *H. pylori* is the antrum of the stomach, which is an absorptive rather than a secretory epithelium. However, the organism also colonizes, to a lesser extent, the gastric fundus where acid is secreted. Researchers have provided different explanations for the ability of *H. pylori* to colonize the stomach. Perhaps dominant in the field is the notion that the pH of the gastric surface is relatively neutral so that specialized acid survival mechanisms are required only for transit to the gastric surface. Measurement of the surface pH of the stomach using glass tipped or open tip pH microelectrodes suggested a relatively neutral pH layer until luminal pH dropped to < 3.0 [147]. However, more recently, pH fluorimetric studies suggest that the surface pH is ~4.0 independent of luminal pH until it falls below pH 2.0 when there is no lumen to surface pH gradient[148]. Further, measurement of the surface pH of the *H. pylori* infected mouse stomach suggests disappearance of the putative gastric barrier to acid[149].

Given the conflicting results of the direct measurements, a transcriptome was generated from mRNA of *H. pylori* infecting the gerbil stomach and compared to an i*n vitro* microarray transcriptome generated either at pH 7.4 and 4.5 without or with urea to mimic the gastric surface where there is a urea concentration of 3mM[51,150]. Since the pH of the gerbil stomach is similar to the human stomach and there are also similar consequences of infection, i.e. gastritis, ulcers and carcinoma, this seems an appropriate animal model. The Table below indicates the gastric or pH-dependence of expression of those genes that are potentially pH homeostatic. Most of these genes are more highly up-regulated in the stomach *in situ* than at pH 4.5 *in vitro*, in particular the genes responsible for UreA and UreB. These data strongly suggest an acidic habitat for *H. pylori* in the stomach with a pH < 4.5.

Gene #	In vivo	pH 4.5 + U	pH 4.5	Description
HP0072	29.90±8.23	1.2	1.2	urease beta subunit (*ureB*)[1]
HP0073	21.96±4.38	1.15	1.34	urease alpha subunit (*ureA*)
HP0071	5.04±2.06	1.06	2.11	urease accessory protein (*ureI*)

Gene #	In vivo	pH 4.5 + U	pH 4.5	Description
HP0070	1.79± 0.53	1.10	1.67	urease accessory protein (*ureE*)
HP0068	3.64±1.22	0.85	1.39	urease accessory protein (*ureG*)
HP0900	10.16±1.39	1.19	1.35	hydrogenase expression (*hypB*)
HP0869	2.57±0.19	1.45	10.42	hydrogenase expression/(*hypA*)
HP1186	2.68±0.98	2.15	4.64	carbonic anhydrase
HP0294	5.31±1.78	1.72	2.28	aliphatic amidase (*amiE*)
HP1238	1.97±0.25	1.09	1.32	formamidase (*amiF*)
HP0649	2.42±0.60	1.37	2.56	Aspartase (*aspA*)
HP1399	15.94±2.90	1.81	7.11	arginase (*rocF*)

Relative fold changes as compared to levels at pH 7.4 *in vitro*, in mRNA levels of putative pH homeostatic genes of *H. pylori* when infecting the gerbil stomach and of *H. pylori* grown at pH 4.5 *in vitro* with and without 5 mM urea. Genes in bold text are members of the HP0165/HP0166 regulon (see text).

A second cytoplasmic histidine kinase, HP0244, is required for survival at pH 2.5 but not at pH 4.5. Its response regulator for acid survival remains unknown[104]. HPO244 also regulates many of the acid acclimation genes in the table in BOX 3. We hypothesize that HP0165/HP0166 responds to changes in periplasmic pH and HP0244 supports cytoplasmic and periplasmic pH homeostasis when HP0165 is overwhelmed.

Post transcriptional recruitment of urease to the membrane supports the acidic pH acclimation

Membrane-bound UreA and UreB levels increase at acidic pH as observed by post-sectioning immunogold staining of organisms incubated at pH 7.0 or 5.5 with anti-UreI and anti-UreB antibodies. When the distribution of UreA, UreB and UreE is compared at pH 7.4 and 4.5 in isolated membranes from wild type *H. pylori*, there is a clear increase in membrane association of these proteins at pH 4.5. Consistent with acid activation of UreI, membrane-bound urease activity increases two-fold at pH 4.5 relative to pH 7.4. This membrane recruitment is dependent on expression of UreI[105,106] and is not found in the absence of HP0244[98]. Association of urease with UreI in the membrane is thought to permit immediate access of entering urea to urease, resulting in a more rapid response of periplasmic pH without obligatory elevation of cytoplasmic urease. Deletion of a TCS responsible for recruitment of urease to the inner membrane leads to loss of periplasmic buffering[98] [FIGURE 3b].

Transcriptional down-regulation of urease activity when leaving an acidic environment

Unphosphorylated HP0166, the response regulator of HP0165, regulates the expression of a *cis*-acting sRNA (*ureB*-sRNA). This results in truncation of *ureB*, with production of a 1.4 kb mRNA transcript instead of the full length 2.7 kb *ureA/ureB* mRNA transcript [107]. A mutant of HP0166 that cannot be phosphorylated exhibits specific binding to *ureB*-sRNA in electrophoretic mobility-shift assays. Expression of this mutated HP0166, in which an aspartate is replaced by asparagine, also results in a large increase of the truncated form of *ureB* in *H. pylori.* Since the level of phosphorylated HP0166 decreases with elevation of pH, the amount of full length *ureB* probably also decreases. Over-expression of *ureB*-sRNA results in a large reduction of urease activity associated with truncation of *ureB.* These data are illustrated in FIGURE 3c.

In summary, *H. pylori* achieves pH homeostasis by buffering its periplasm using the products of the urease reaction and recruiting urease to the inner membrane in association with UreI, permitting rapid access of urea to urease. Export of CO_2, NH_3 and NH_4^+ through UreI avoids excessive alkalization of the cytoplasm while buffering the periplasm. The membrane recruitment of the urease gene products depends upon activity of two pH-dependent TCSs *(Hp0244* and *Hp0165/Hp0166*). These acid acclimation processes appear unique to gastric *Helicobacter spp.* but there are many other natural environments that oscillate between acid and neutral, making periplasmic buffering a distinct possibility in other Gram-negative bacteria.

Adaptations for homeostasis in extremophiles

Extreme acidophiles

Extremely acidophilic bacteria include chemolithotrophs such as *A. ferrooxidans* that play roles in geochemistry and bioleaching processes in mines[9,108]. Adaptations of the respiratory chain complexes of *A. ferrooxidans* have been suggested to be important for addressing problems that arise from the interplay of its physiology and the environment[109,110,111]. *A. ferrooxidans* derives energy from oxidation of ferrous ions by oxygen. While auto-oxidation of the substrate ion is decreased at very acid pH and the energetics of this oxidation are also more favorable, this energy source is still a "parsimonious" living that complicates the challenge of extreme acidity[109]. Some extreme acidophiles are heterotrophic bacteria, including thermoacidiphilic *Alicyclobacillus acidocaldarius*, which contains an unusual ω-alicyclic fatty acid as a major membrane component[9,112]. These acidophiles all maintain a cytoplasmic pH at ~6.0, lower than that of neutralophiles, while growing at pH < 3 [BOX 1][113,114,115].

The large ΔpH of extreme acidophiles, inside-alkaline relative to the acidic outside, is maintained by active mechanisms and is supported by the reversed $\Delta\psi$, inside-positive relative to outside [see BOX 1] [109,113,114,115]. An acidophile F_1F_o-ATP synthase with the typical eight subunits has a pH optimum of 8.5 for its hydrolytic activity in assays of the membrane-associated enzyme; the proton-translocating *a*- and *c*-subunits of the enzyme have some deviations from neutralophile synthases but whether these are adaptive to the unusual PMF pattern is not yet known[116]. The acidophile PMF pattern results in sensitivity of these bacteria to organic acids, because of the large ΔpH, and to toxic anions, because of the inside-positive $\Delta\psi$ [9].

Extreme alkaliphiles

The pH homeostasis of extreme alkaliphiles has been most extensively studied in *Bacillus* species such as *B. pseudofirmus* OF4 and *Bacillus halodurans* C-125 that can grow non-fermentatively, i.e. without fermentative acid generation, at pH ≥ 10 [117,118,119]. In pH-controlled continuous cultures on malate-containing media at a series of pH values, alkaliphilic *B. pseudofirmus* OF4 only maintains complete pH homeostasis, i.e. a cytoplasmic pH of ~7.5, at external pH values from 7.5 to 9.5, but it grows optimally up to a pH_{out} of ~10.5 at which its pH_{in}=8.3 [120]. By contrast, a cytoplasmic pH of 8 causes growth arrest of neutralophiles[47,121]. The alkaliphile still grows, although more slowly, with a cytoplasmic pH ≥ 9.5 at external pH ≥ 11 [BOX 1]. It is not yet known what properties underpin their capacity for growth at such unusually high cytoplasmic pH values.

Na^+/H^+ antiporter-dependent pH homeostasis is the major strategy for pH homeostasis of extremely alkaliphilic *Bacillus* species. Although these bacteria have multiple Na^+/H^+ antiporters, the unusual hetero-oligomeric Mrp antiporter has an indispensible role at high pH[2,3]. A point mutation in the *mrpA* gene of alkaliphilic *B. halodurans* C-125 leads to a non-alkaliphilic phenotype accompanied by loss of alkaline pH homeostasis and loss of Na^+/

H^+ antiport measured in whole cells[117]. *Bacillus* Mrp antiporters are encoded in operons that contain genes for seven hydrophobic proteins [FIGURE 4a]. The two largest Mrp proteins, MrpA and MrpD share homology with each other and with three membrane-embedded subunits of proton-pumping NADH oxidoreductases (Complex I) of the respiratory chain[122,123]. MrpA also has an "MrpB" domain that shares homology with the independent MrpB protein of this type of Mrp system. These domains are functionally important, but like other aspects of the Mrp antiporter complexity, the role of the MrpB domain is yet to be deciphered [124,125,126]. All the Mrp proteins are required to form a hetero-oligomeric complex and are required for Mrp antiport activity[125,126,127], in contrast to the majority of bacterial antiporters, e.g. NhaA, which are single gene products[2,123]. Perhaps a large Mrp protein surface on the external face of the cytoplasmic membrane forms a large proton funnel that helps support the observed kinetic competence of Mrp antiporters in low proton environments[123,125].

The ongoing requirement for cytoplasmic Na^+ to support high levels of alkaliphile antiport activity is met by numerous Na^+/solute symporters and two Na^+ channels, the flagella-associated MotPS channel and a voltage-gated sodium channel (Na_vBP)[FIGURE 4b][128,129,130,131]. Less is known about the antiporters that have major roles in anaerobic alkaliphiles or Gram-negative alkaliphiles, but the poly-extremophilic anaerobe *Natranaerobius thermophilus*, a halophilic, thermophilic alkaliphile, has a large complement of both Na^+/H^+ and K^+/H^+ antiporters[21,132].

The proton uptake that accompanies ATP synthesis by the F_1F_0-ATP synthase contributes to alkaliphile pH homeostasis in the aerobic alkaliphilic *Bacillus* species. Anaerobic alkaliphiles such as *N. thermophilus* and *Clostridium paradoxum* use their F_1F_0-ATPases in the hydrolytic direction to generate a $\Delta\psi$ but they avoid proton loss by using Na^+-coupled instead of H^+-coupled F_1F_0-ATPases[21,133]. The ATP synthases of aerobic alkaliphilic *Bacillus* species function in the synthetic direction. They have specific sequence motifs in proton-translocating subunit-*a* and subunit-*c* that support function at high pH and guard against cytoplasmic proton loss during ATP synthesis [134,135,136,137]. Mutations of these motifs to the non-alkaliphile consensus sequence leads to reduced ATP synthase activity, usually with a greater effect at pH 10.5 than at pH 7.5. The magnitude of the defect in ATP synthase activity correlates with a loss of the mutants' capacities for pH homeostasis during a sudden alkaline shift in pH_{out}[134,137]. Some ATP synthase motif mutations also lead to proton leakiness[134,136,137]. Thus the alkaliphile synthase is adapted to promote both function in pH homeostasis and ATP synthesis at high pH.

Recently, the atomic structure of the rotor from the *B. pseudofirmus* OF4 ATP synthase, a homo-oligomeric ring composed of 13 hairpin-like *c*-subunits, was revealed by 3-dimensional X-ray crystallography[138]. Two major alkaliphile-specific motifs, AxAxAxA in the N-terminal helix and PxxExxP in the C-terminal helix that are functionally important at high pH[136,137] appear to influence properties of the ion binding site that include the presence of a water molecule (shown in red in the bottom, right of FIGURE 4c). Together, those features of this *c*-ring are proposed to support high affinity of the binding sites for protons[138] and some of these features decrease growth capacity of *B. pseudofirmus* OF4 near neutral pH[13].

Perspectives

Much has been learned about individual strategies for bacterial pH homeostasis and the molecules involved, but bacterial pH homeostasis is a cell-wide physiological process that deploys and integrates these strategies differently depending upon other environmental factors, e.g. oxygen and salinity. Development of systems-level models will depend upon

further efforts to gather broad-based quantitative "omics" information as a function of pH under different conditions. Such models will also require detailed molecular information about the stoichiometry, kinetic and mechanistic properties of key transporters, as has been obtained for *E. coli* NhaA. Data of both types are particularly scarce for extremophiles, for which systems models would enhance our understanding of extremophile adaptations and facilitate application of that understanding to ecological settings, e.g. for bioleaching and bioremediation. For pathogenic bacteria, the insights that may emerge from detailed understanding of the integrated process of pH homeostasis should lead to identification of new antibiotic targets and strategies.

Acknowledgments

T.A.K. is supported by a research grant from the National Institutes of Health, USA, G.S. is supported by grants from the National Institutes of Health, USA and the United States Veterans Administration. E.P. is supported by grants from the USA-Israel BiNational Science Foundation and from the European Drug Initiative on Channels and Transporters. We thank colleagues L. Kozachkov for Figure 2, M. Ito, J. Liu, M. Morino and L. Preiss for their assistance with panels of Figure 4, and D.B. Hicks, H.R. Kaback, J. Kraut, T. Meier, L. Preiss, D.R. Scott, O. Vagin and Y. Wen for critically reviewing sections of the manuscript.

Glossary

Protonmotive force (PMF)	A transmembrane electrochemical gradient across the bacterial cell membrane (see Box 1)
Extremophile	An organism that grows under conditions (e.g. of pH, temperature, salt concentration, pressure, etc) that are incompatible with growth of most organisms
Acidophilic bacteria	Extremely acidophilic bacteria grow at external pH < 3 whereas the lowest growth pH for moderate acidophiles is in the pH 3–5 range
Alkaliphilic bacteria	Extremely alkaliphilic bacteria grow at external pH ≥ 10 whereas moderate alkaliphiles grow in the pH 9–10 range. The pH range for growth of facultative alkaliphiles extends down to pH 7.0–7.5
Alkaline soda lake	Found in many parts of the world, these are highly alkaline lakes that are rich in dissolved sodium salts, especially sodium carbonate, sodium chloride, and sodium sulfate
Ground water	Water that collects below the surface of the Earth, filling the porous spaces in soil, rocks and sediment
Cytoplasmic buffering capacity	The capacity of cytoplasmic contents to resist changes in pH upon exposure to acid or base, i.e. possessing acidic or basic groups with p*K* values in the range of the challenge pH
p*I* profile of a proteome	The relative representation of proteins predicted by the genome sequence with different p*I* values (isoelectric points, at which the protein has no net charge)
K_M	Michaelis constant, an important characteristic of enzyme-substrate interaction. It reflects the concentration of substrate at which half the active sites of an enzyme are filled. For transport reactions, the term apparent K_M is used

p*K*	This property of a basic or acidic group is related to the equilibrium constant of its ionization and is defined as the pH at which it is half dissociated
Discontinuous helices	Membrane-spanning helical protein segments that are interrupted in the middle by an extended non-helical region
Cys-less (CL) transporter mutants	CL mutants have all the native cysteines replaced with other residues. If active, CL transporters are useful for a variety of structure-function experiments
Multiconformation Continuum Electrostatics (MCCE)	A simulation program that uses a combination of biophysical approaches to calculate properties that facilitate development of functional models
Two Component Systems (TCSs)	TCSs generally are composed of two multi-domain proteins, a sensory histidine kinase and a response regulator that, respectively, sense and initiate a response to a stimulus such as a pH change. Localization of the sensory domain can support sensing of external pH or cytoplasmic pH
Acid Acclimation	The ability of a Gram-negative neutralophile to maintain periplasmic pH close to neutrality in highly acidic media permitting both survival and growth
$pH_{0.5}$	pH at which an enzyme exhibits half of its maximum activity
Chemolithotrophic bacteria	Bacteria that can apply oxidation-reduction reactions to inorganic compounds to provide all the energy needed for cell growth and maintenance processes
ω-Alicyclic fatty acids	Fatty acids with rings at the end

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Biographies

Terry Ann Krulwich

Terry Ann Krulwich is the Sharon & Frederick A. Klingenstein–Nathan G. Kase, M.D. Professor of Pharmacology and Systems Therapeutics at Mount Sinai School of Medicine in New York, USA. She received her Ph.D. in bacteriology from the University of Wisconsin, Madison, Wisconsin, USA, followed by postdoctoral studies in molecular biology at the Albert Einstein College of Medicine, Bronx, New York, USA. Among her awards are a DSc from Goucher College and the William A. Hinton Award from the American Society for Microbiology. Her research interests include structure-function and physiology of cation/proton and antibiotic/proton antiporters in Gram-positive bacteria, strategies for bacterial oxidative phosphorylation at high pH and structure-function of an alkaliphile ATP synthase.

George Sachs

George Sachs is Professor of Medicine and Physiology in the David Geffen School of Medicine, UCLA, Los Angeles and holds the Wilshire Chair of Medicine. Among numerous

awards, he was awarded the Gairdner Foundation Award in 2004. He obtained his MB ChB and DSc at the University of Edinburgh, Scotland and then spent 19 years at UAB in Birmingham Alabama focused on the mechanism of gastric acid secretion with particular reference to the gastric H,K ATPase that continued when he moved to UCLA with emphasis on structure-function and targeted inhibitors of acid secretion. In addition, his work has investigated the means whereby the gastric pathogen, *Helicobacter pylori* is able to colonize the human stomach elucidating its gastric biology.

Etana Padan

Etana Padan is a Professor of Biological Chemistry at the Institute of Life Sciences of the Hebrew University of Jerusalem, Israel. She is incumbent of the Massimo and Adelina Della Pergolla in Life Sciences, the Sarov Prize of the Israeli Society of Microbiology and the Landau Prize. She received her PhD in Biochemistry and molecular biology from the Hebrew University, Jerusalem followed by postdoctoral studies in bioenergetics and membrane molecular biology at the Weizmann Institute of Science, Israel and The Roche Institute of Molecular Biology, Nutley, NJ, USA. She then gained expertise in membrane structure biology in the Max Planck Insitut? fur Biophysics, Frankfurt, Germany. Her research interests include, the structure and function of NhaA, the main Na^+/H^+ antiporter that is involved in homeostasis of pH and Na^+ in *Escherichia coli* and other enterobacteria and its orthologues in humans.

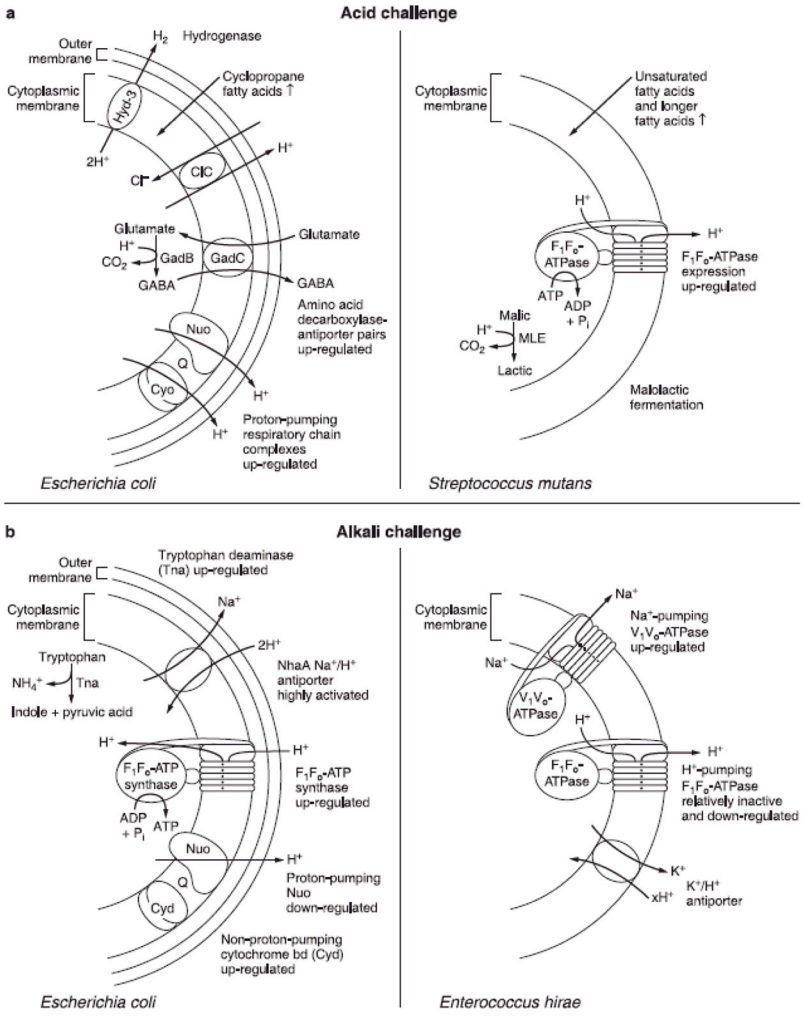

Figure 1. Examples of adaptations by neutralophilic bacteria to manage acid or alkali challenge

a Left, Selected *E. coli* adaptations supporting acid tolerance during passage through the stomach. Right, Strategies proposed for the non-respiratory oral bacterium *Streptococcus mutans.* **b** Left, *E. coli* adaptations supporting alkali-tolerance. Right, Adaptations of non-respiratory *Enterococcus hirae* (formerly *Streptococcus faecalis*) supporting alkali-tolerance. See text for description of these adaptations and of additional examples.

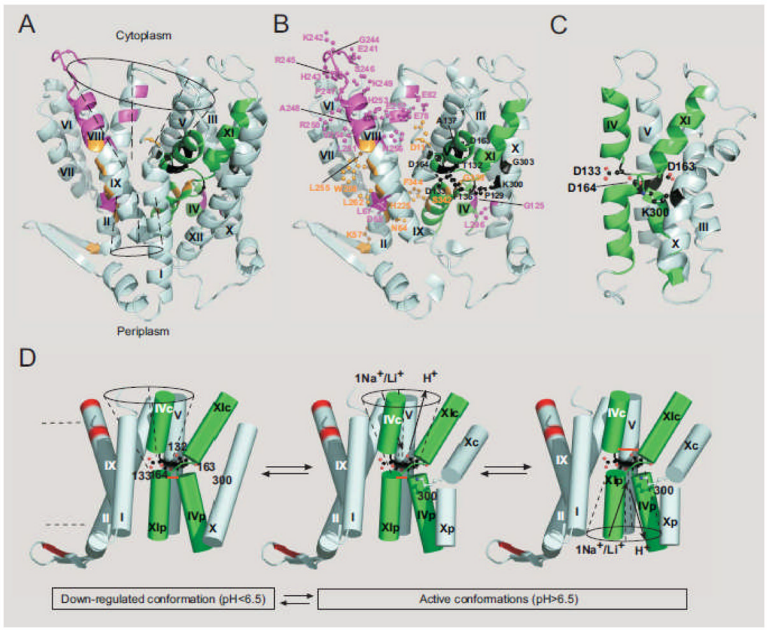

Figure 2. Functional organization of the *E. coli* Na^+/H^+ antiporter NhaA

a The overall architecture of NhaA with its 12 trans-membrane segments (TMS) and funnels (black line) is shown[65]. **b** The residues whose mutation affects the pH response (magenta), the pH response and the translocation (yellow) or the translocation alone (black) are shown on the structure. **c** The inverted repeat including TMS III, IV, V and TMS X,XI,XII are shown and the discontinuous helices (IV and XI assembly) are colored green. **d** Schematic cartoon illustrating the conformational changes caused by pH activation and ion transport. The pH sensor on TMS IX (double red lines) is marked.

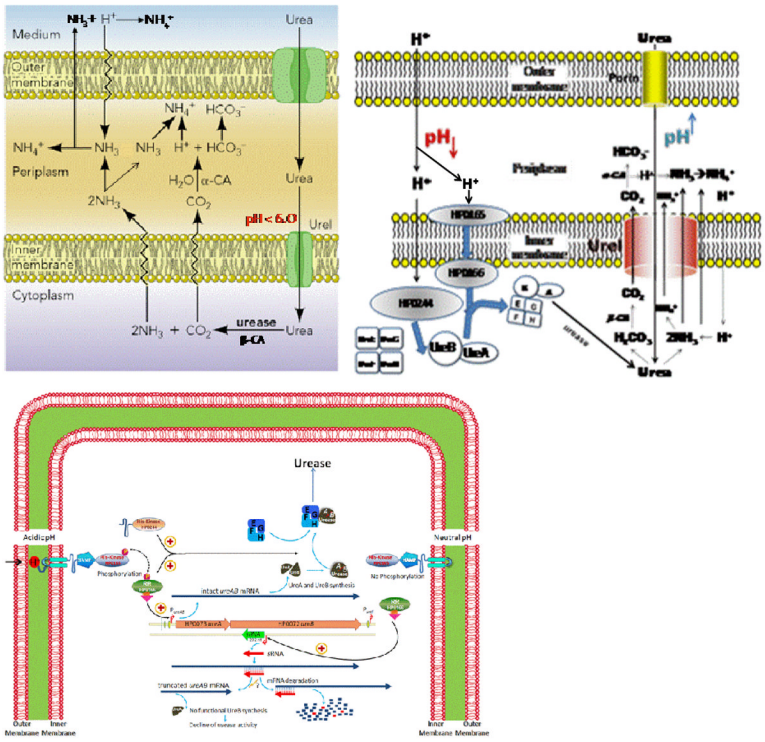

Figure 3. Periplasmic buffering by *H. pylori* and its regulation

a Periplasmic buffering by *H. pylori*. Urea crosses the outer membrane and then the inner membrane via UreI at pH < 6.0. Cytoplasmic urease forms $2NH_3 + H_2CO_3$. The latter is converted to CO_2 by cytoplasmic β-carbonic anhydrase. These gases cross the inner membrane and the CO_2 is converted to $HCO_3^-$by the membrane bound α-carbonic anhydrase thereby maintaining periplasmic pH at ~6.1, the effective pK_a of the CO_2/ HCO_3^-couple. Exiting NH_3 neutralizes the H^+ produced by carbonic anhydrase and entering H^+ and can also exit the outer membrane to alkalize the medium. This allows maintenance of periplasmic pH much higher than medium pH [51,95,96]. **b** The role of the pH-responsive TCS HP0244 in acid acclimation by *H. pylori*. Activation of this TCS results in recruitment of the urease proteins to UreI with immediate access of urea to urease and outward transport of CO_2 and NH_3 and NH_4^+ through UreI increasing the rate of periplasmic buffering and disposal of cytoplasmic NH_4^+ [51,94,95,96]. **c** A simplified model representing the HP0165-HP0166 TCS regulation of *ureAB* gene expression by unphosphorylated HP0166 binding to 5′ureB-sRNA at neutral pH and by phosphorylated HP0166 binding to P_{ureA} at acidic pH. At neutral pH, HP0165 is not activated and the response regulator HP0166 is not phosphorylated. The unphosphorylated HP0166 binds to the 5′*ureB*-sRNA promoter, leading to transcription of 5′ureB-sRNA and consequent truncation of *ureB* resulting in a decline of urease activity. This reflects adaptation to non-acidic pH. At acidic pH, HP0165 is activated with phosphorylation of HP0166 and the phosphorylated HP0166 then binds to the P_{ureA} promoter to positively regulate the transcription of *ureAB* genes which results in up regulation of *ureA* and *ureB* with a consequent increase of urease activity, reflecting acid acclimation. Antisense sRNAs are shown in red, mRNAs in dark blue. Red bent arrows denote promoters, orange and green bars with arrows denote *ureAB* gene and antisense 5′*ureB*-sRNA gene, respectively. A bold lightning sign indicates action of RNase III or RNase E. Positive signs '+' denote positive regulation, Question mark '?' denotes not yet experimentally confirmed. Yellow ovals represent HP0166 binding sites [107].

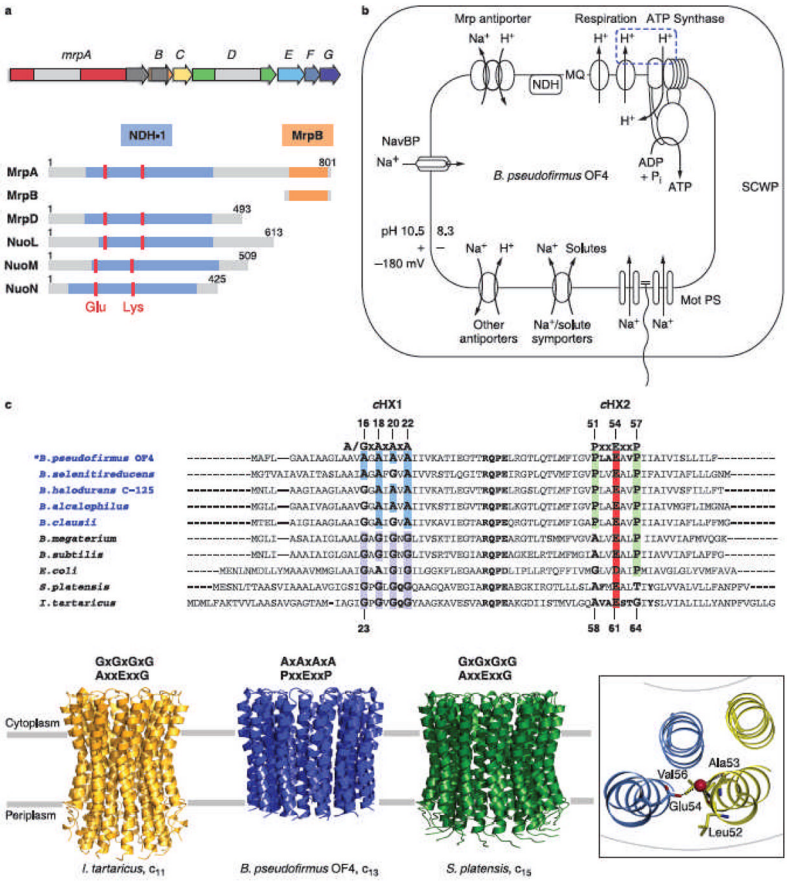

Figure 4. The hetero-oligomeric Mrp antiporter and other major pH homeostasis strategies of extremely alkaliphilic B. pseudofirmus OF4

a Top: Diagram of the 7-gene *mrp* operon of alkaliphilic *Bacillus* sp. indicates oxidoreductase NDH-1 domains in MrpA and MrpD (light gray) and MrpB domains in MrpA and MrpB (dark gray). **Bottom**: Schematic illustration of the oxidoreductase NDH-1 domains in MrpA, MrpD, and Complex I NuoL, M and N and the MrpB domains in MrpA and MrpB. Red vertical lines mark locations of conserved, functionally important glutamates and lysines[72,73,124,125]. **b** Schematic diagram of *B. pseudofirmus* OF4 showing external and cytoplasmic pH and $\Delta\psi$, secondary cell wall polymers (SCWP, e.g. S-layer in *B. pseudofirmus* OF4) and Na^+ and H^+ cycles that support pH homeostasis. Active proton uptake occurs via the critical Mrp antiporter, other antiporters and ATP synthase. The energetic driving force is from two proton-pumping respiratory complexes. The dashed lines indicate hypothesized capture of protons by the ATP synthase near the membrane surface, before they fully equilibrate with the outside liquid phase. Na^+ re-entry, which supports continuous antiport, occurs through Na^+/solute symporters, the voltage-gated Na_VBP channel and flagellar-associated MotPS channel[119]. **c Top:** Two motifs of *c*-subunits in alkaliphilic *Bacillus* species (names shaded in blue) exemplify adaptations of the ATP synthase required for function at high pH. **Bottom:** The *c*-subunit motifs reduce the pronounced hour-glass shape of the overall *c*- rotor observed for other *c*-rotors (left) and promote tight proton binding in the ion binding site (right) (see text).