Acid Acclimation by Helicobacter pylori

Helicobacter pylori is a Gram-negative neutralophile associated with peptic ulcers and gastric cancer. It has a unique ability to colonize the human stomach by acid acclimation. It uses the pH-gated urea channel, UreI, to enhance urea access to intrabacterial urease and a membrane-anchored periplasmic carbonic anhydrase to regulate periplasmic pH to ~6.1 in acidic media, whereas other neutralophiles cannot regulate periplasmic pH and thus only transit the stomach.

Warren and Marshall (38) first reported the key role of gastric infection by Helicobacter pylori in the development of peptic ulcer disease. Subsequently, in 1991 Parsonnet et al. (46) showed that this organism could also be a factor in the pathogenesis of gastric cancer. This hypothesis has been supported by another study (47). Indeed, persistent infection with H. pylori always produces gastritis and increases the risk of developing gastric cancer ~20-fold (54). Infection with H. pylori has also been suggested as a possible factor in the pathogenesis of other disorders, such as coronary occlusion, wherein stimulation of an immune response to the pathogen contributes to the development of cardiovascular disease (19).

Many pathogenic neutralophiles can survive in acidic environments by maintaining cytoplasmic pH at ~5.0. However, although they can survive in an acidic environment, they do not grow. Hence, they can transit but not colonize the stomach. Examples of such organisms are Salmonella typhimurium, Vibrio cholera, and pathogenic strains of Escherichia coli and Yersinia enterocolitica (16, 36).

Although H. pylori is also a neutralophile, it is able not only to survive in but also to colonize (i.e., grow within) the human stomach (27, 30). Other Helicobacter spp. have evolved specialized processes that allow maintenance of the pH of their cytoplasm at a level that enables the organisms to survive and grow. We have termed these properties of gastric Helicobacter spp. “acid acclimation” to differentiate them from the acid-resistance (acute) or acid-tolerance (chronic) mechanisms of neutralophiles in general.

Acid Resistance/Tolerance in Neutralophiles

The energy source in aerobic bacteria is derived from synthesis of ATP driven by proton movement down an electrochemical gradient for protons (occasionally sodium). The inward-driving force for protons forms the basis of the chemiosmotic theory of bioenergetics (38). ATP synthesis depends on the flux of protons across the F_{1}F_{0} ATP synthase energized by the pH and membrane-potential gradient. The electrogenic redox reactions are generated across the inner membrane in Gram-negative organisms or across the surface membrane in the case of Gram-positive bacteria and result in the formation of both a proton and an electrical gradient. The combination of these is termed the proton-motive force (pmf).

The pmf is calculated from the equation defining the electrochemical gradient for protons as

\[
\Delta \mu_{p} = \Delta \mu_{p}^{\text{H+}} = -RT/nF \ln[H^+]/[H^+]_0 = \Delta \phi = -n\Delta \phi + PD
\]

where \(\Delta \mu_{p}\) is the electrochemical gradient for protons, \(R\) is the gas constant, \(T\) is the temperature in kelvins, \(F\) is the Faraday constant, \(n\) is the valence of the ion, and \(\Delta \phi\) is the transmembrane potential. The pmf equation predicts that there will be a reciprocal relationship between the pH gradient and the potential difference across the relevant bacterial membrane.

In the case of neutralophiles, the pmf is maintained across their cytoplasmic membrane in a range between ~160 and ~280 mV at neutral extracellular pH. Generally, Gram-negative neutralophiles do not regulate their periplasmic pH in acidic media. Hence, for the neutralophiles that survive but do not grow in an acid environment, exposure to an acid pH causes their periplasmic pH to fall, increasing the inward proton gradient and decreasing the membrane potential or even causing development of a positive potential that could hinder proton influx. These organisms have pH-regulatory systems preventing cytoplasmic pH from falling below ~4.5 or ~5.0, a level sufficient for survival but not for replication. Not only do these neutralophiles have to tolerate a continuous acid load from their acidic environment during gastric transit, they also are exposed to several permeant organic acids present in the intestine. Entry of the unprotonated acid is followed by acidification of...
The cytoplasmic membrane of bacteria and eukaryotes is designed to regulate intracellular pH under acidic conditions. This regulation is achieved through the expression of acid transporters and amino acid decarboxylases. The Na+/H+ exchanger (NHE) is the primary transporter involved in maintaining the pH gradient across the plasma membrane. It functions to maintain a lower intracellular pH by exchanging Na+ for H+. NHEs are found in both prokaryotes and eukaryotes and are essential for acid resistance.

Amino acid decarboxylases, such as glutamate decarboxylase (GAD), are involved in the synthesis of amino acids and neurotransmitters. GAD catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA). This process is important for maintaining intracellular pH, especially in acidic environments.

In E. coli, the expression of GAD is regulated by the pH-cytoplasmic sensor ToxR. ToxR binds to the gadA promoter and activates its transcription. The gadA gene encodes glutamic acid decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA.

Similarly, in Y. enterocolitica, the expression of GAD is regulated by the pH-cytoplasmic sensor YaeU. YaeU binds to the gadB promoter and activates its transcription. The gadB gene encodes glutamic acid decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA.

The NHE transporters in E. coli are electroneutral, while those in Y. enterocolitica are electrogenic. This difference in stoichiometry is crucial for acid adaptation. The electroneutral NHEs in E. coli can only decrease intracellular pH, while the electrogenic NHEs in Y. enterocolitica can maintain a lower intracellular pH by exchanging Na+ for H+.

In conclusion, the expression of acid transporters and amino acid decarboxylases is crucial for maintaining intracellular pH under acidic conditions. The regulation of these systems is complex and involves pH-sensing transducers and transcriptional activators. The understanding of these mechanisms is essential for the development of strategies to combat acid resistance in bacteria.
Acid Acclimation in H. pylori

H. pylori is Gram-negative neutralophile with outer and inner membranes, and it shares some of the pH-cytoplasmic, pH-regulatory systems with other neutralophiles. However, the acid-acclimation pathways developed by this organism are unique, enabling H. pylori to maintain its periplasmic pH in acidic medium at levels at which both the cytoplasmic pH and membrane potential are compatible with growth (29). The several systems used to maintain periplasmic pH form the basis of acid acclimation and growth in the gastric environment. Several such processes have been explored in some detail, but much remains to be learned.

Acid Urease

Y. enteroxolitica expresses an intrabacterial urease with a pH optimum of 5.0 and a sharp pH activity curve. It is activated with the production of NH₃ and CO₂ when the cytoplasmic pH falls to 5.0 (63). At this pH, sufficient buffer is generated to allow the organism to survive with a cytoplasmic pH of 5.0 but not to grow. In contrast, the urease of H. pylori does not express any of the amino acid decarboxylases or the antiporers, so its response to acidic media is clearly different from these other neutralophiles.

The urease system

H. pylori expresses a neutral pH optimum urease at higher levels than any other known bacteria (39), and the generation of NH₃ and CO₂ provides both acid-neutralizing and acid-buffering capacity. The urease gene cluster of this organism consists of seven genes, an upstream promoter sequence, then ureA and ureB, the structural subunits of the enzyme, followed by a second promoter region, and then five other genes: ureC, ureD, ureG, and ureI (1, 11). The structural subunits and the last four genes show considerable homology to other urease cluster genes such as those in Klebsiella aerogenes (39). UreA and UreB form a complex that requires Ni²⁺ insertion for activity. Ni²⁺ insertion is mediated by a pair of complexes (UreE/UreG and UreF/UreH) that have been defined both biochemically in Klebsiella (41, 42) and by yeast two-hybridization methods in H. pylori (57). The UreA/UreB complex constitutes as much as 15% of the protein content of active H. pylori. Urease-negative mutants are unable to colonize animal models, showing that this urease is essential for gastric colonization (15). Hence urease activity is vital for gastric colonization by H. pylori, and regulation of urease occurs at several levels: biosynthesis of the structural genes, insertion of Ni²⁺ into the apoenzyme, and regulation of urease access to the urease itself.

Regulation of H. pylori content of active urease

There is evidence for upregulation of ureA and ureB expression under acidic conditions, perhaps due to activation of a two-component histidine kinase, HP0165/HP0166 (1, 48). The urease promoter is modular, contains a σ⁷⁰-responsive region responsible for basal transcription, and contains upstream sequences responsible for further regulation of transcription of these two subunit genes (12).
UreI and UreII are present largely as apoenzymes; therefore, regulation of Ni²⁺ concentration inside the organism plays a vital role in acid survival. S. typhimurium has a specific Ni²⁺ transporter with eight transmembrane segments that exists in the inner membrane of H. pylori and functions as a monomer (20). Deletion of this gene reduces but does not prevent infection of the mouse stomach (43), but many other genes may also be implicated not only in uptake but also in storage of this essential cation. H. pylori also expresses nitF, which functions as a global regulator of several genes, including ureI and ureII, in the presence of high Ni²⁺ concentrations (9).

Δfur, a gene involved in flagellar expression, was identified as a gene reducing urease activity in E. coli expressing the H. pylori urease locus and nitF gene (33). HP0548, a gene that may encode a DNA helicase in the cag pathogenicity island, and HP0511, a gene that potentially encodes a lipoprotein, enhance urease expression in this foreign host (32). Their role in H. pylori itself has not been examined.

hypA and hypB genes are involved in assembly of the hydrogenase complex, perhaps by assembly of Ni²⁺ into hydrogenase. It was found that deletion of these assembly genes not only affected hydrogenase activity but sharply reduced urease activity as well, by ~200-fold. This is attributed to a role for these genes in Ni²⁺ incorporation into urease (44).

The fur gene plays a role in both iron regulation and acid resistance in a variety of different bacteria. The Fur gene product is a transcriptional repressor that decreases iron uptake in the presence of a high-iron environment (53). In addition to a role in iron regulation in Gram-negative bacteria, the fur gene also appears to play a role in the acid-resistance mechanisms of several different organisms. The most widely studied organism in terms of fur and acid resistance is S. typhimurium, a bacteria that must survive transit of the stomach. The S. typhimurium fur mutant is acid sensitive (16). fur, like spoT and pchA, controls a distinct set of acid-shock proteins (3).

Like S. typhimurium, the H. pylori fur mutant is acid sensitive, but its role is unrelated to iron regulation (56). The amount of iron available to the bacteria has no effect on acid sensitivity (6). Fur does not affect H. pylori survival to acid shock, but it does play a role in growth in acid.

The role of urease in acid acclimation by H. pylori

The earliest hypothesis to explain the gastric acid-protective effect of this neutral-pH-optimus urease was that, following lysis of individual organisms, urease was bound to the surface of neighboring bacteria and the NH₃ produced generated a neutral pH environment around them (49). There are several difficulties in accepting this "altruistic lysis" hypothesis (14). Firstly, the neutral-pH optimum of the urease of H. pylori would prevent any activity in the acidic environment presumably inhabited by the organism, which must be at least as low as pH 5.0. Secondly, the methods used to demonstrate binding of urease were such that the urease produced by lysis of the bacteria was not separated from the intact organisms, hence resulting in an artifact exaggerating the quantity of surface urease (28). Removal of the urease genes, including ureI and ureII, in an artifact exaggerating the quantity of surface urease (28).

The key experiment showing that the crucial urease compartment was cytoplasmic and not surface bound was measurement of urease activity in intact bacteria by ¹⁴CO₂ release from labeled urea at neutral pH and in progressively acidic buffered media, as shown in FIGURE 2A. At neutral pH, little urease activity was measurable in intact organisms in contrast to lysed bacteria. But as the pH in the medium was reduced from pH 6.5 to 5.5 and down to pH 2.5, ¹⁴CO₂ release sharply increased in intact bacteria. The simplest explanation for these observations was that acidic pH activated a urea transporter in the inner membrane, increasing urea access to cytoplasmic urease. The stable activity down to pH 2.5 also shows that even down to this medium pH, cytoplasmic pH does not fall at the 5-mM urea concentration used in these experiments; otherwise urease activity would be inhibited (58). Deletion of ureI, whose sequence predicted an integral membrane protein, prevented the pH-dependent increase of urease activity (50). Hence it is ureI acting as an acid-activated urea transporter providing urea to the cytoplasmic urease that allows buffering of the periplasm of the organism to allow at least survival in acidic medium. Expression of UreI is essential for colonization in mice or gerbils (40, 51).

The ureI gene appears to be uniquely expressed by gastric Helicobacter spp. (50). Topographic analysis showed that this protein is a six-transmembrane segment polytopic membrane protein, and fractionation studies localized it to the inner membrane of the organism (58). Expression of UreI in Xenopus oocytes was used to determine its urea-transport properties, as had been done for the discovery of mammalian urea transporters (62). UreI displayed characteristics of a pH-gated urea channel (non-saturable, voltage independent), having low open probability at neutral pH and high open probability at pH 5.0 and below, with half-maximal opening at pH 5.9. It was very selective for urea,
Neutral pH optimum for the distal urea transporter (UreI) was at least as important as the presence of thiourea to maintain urea permeability, with even thiourea being impermeant (58). The protein is arranged with its NH₂ and COOH terminal domains, with three periplasmic domains, two loops connecting transmembrane segments 2 and 3 to 4 and 5, and the COOH terminus also exposed to the periplasm. The pH profile of urea uptake into oocytes was virtually identical to the pH activation of urease in intact H. pylori (FIGURE 2B) (58).

To explore the amino acids responsible for proton gating of the channel, site-directed mutagenesis was carried out on the protonatable exoplasmic amino acids of the UreI of H. pylori and H. hepaticus, a simpler pH-activated urea channel with half-maximal opening at pH 6.8, one pH unit higher than that of H. pylori. Mutations and chimeras were also generated with the UreI of S. salivarius, an even simpler exoplasmic urea channel variant that lacks pH-dependent activation (60). There is strong homology in the membrane domain, significant homology in the cytoplasmic domain, and none in the periplasmic loops, but a protonatable or protonated residue is present for any acidic amino acids of the UreI of H. pylori (59). Hence it was predicted that the pH optimum for urease was at least as important as the presence of thiourea to maintain urea permeability, with even thiourea being impermeant (58). The protein is arranged with its NH₂ and COOH terminal domains, with three periplasmic domains, two loops connecting transmembrane segments 2 and 3 to 4 and 5, and the COOH terminus also exposed to the periplasm. The pH profile of urea uptake into oocytes was virtually identical to the pH activation of urease in intact H. pylori (FIGURE 2B) (58).

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resulted in pH-independent urea uptake; hence the structure of the membrane domain of *S. salivarius* is dominant and unresponsive to the pH-dependent conformational changes in the heterologous periplasmic domain (60). It seems likely that with acidification, protonation of the histidines or even the carboxylates results in a change of hydrogen bonding in the periplasmic domain, resulting in a conformational change in the membrane segments allowing urea entry across the channel.

**Gastric juice urea**

The urea in gastric juice is maintained at the same level as in blood, between 1 and 3 mM. There is evidence for some expression of the urea-transporter gene, *ut1*, in the rat stomach based on microarray analysis, but the cell type expressing this transporter is not known (Lambrecht N, personal communication). However, it is generally thought that urea moves into the gastric juice by free diffusion largely via a paracellular pathway. The leakiness of the paracellular pathway in the antrum, the usual site of *H. pylori* infection, is greater than that of the fundic region (52). This may be due to a combination of greater urea access to the organism and a higher pH in this absorptive region of the stomach. Microscopic observations show a larger number of organisms at the apex of the tight junction than at the cell surface, perhaps explained by a higher urea concentration in this region (22).

**Consequences of UreI activation**

*H. pylori*, perhaps due to the structure of its outer membrane, is suitable for the study of its inner membrane potential and cytoplasmic pH using fluorescent dyes without the need for generation of a membrane potential and cytoplasmic pH using fluorescent dyes. However, it is extremely difficult to use tracer methods to investigate passive diffusion into bacteria since the surface-to-volume ratio is so high. It is possible to determine uptake of tracers that are concentrated by the pmf, however, and this has been done for measurement of gradients in *H. pylori* (31). This approach provided data similar to those found with fluorescent probes as discussed above.

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FIGURE 3. Demonstration of periplasmic alkalization due to UreI-catalyzed urea entry into H. pylori, and the action of the periplasmic carbonic anhydrase and the association of urease with UreI following acidification of the medium

A periplasmic pH control by intrabacterial urease activity requires UreI. Using the pH-sensitive fluorescent dye BCECF to monitor changes in periplasmic pH, intrabacterial urease activity following urea addition is shown to increase pH of the periplasm (arrows). Increased fluorescence of the periplasm is further emphasized by the high-magnification image (inset). UreI deletion mutants show no periplasmic pH elevation. B. periplasmic pH control by H. pylori also requires periplasmic carbonic anhydrase. The effect of urea addition at pH 5.5 to wild-type (left) and α-carbonic anhydrase knockout organisms (right) again in the presence of BCECF-free acid. In the absence of α-carbonic anhydrase, periplasmic buffering is abolished, demonstrating that periplasmic carbonic anhydrase activity is also essential for acid acclimation by H. pylori. Similar data are obtained in the presence of Diamox added to wild-type organisms. C: post section immunogold electron micrograph with staining with anti-UreB at neutral pH, showing mostly cytoplasmic location of this protein; and staining with anti-UreI, showing the expected membrane location of this protein, and staining with anti-UreB at a medium pH of 5.5, showing the large increase of UreB at the inner membrane, consistent with the association seen on blue native gel electrophoresis.

H. pylori urease is regulated in acidic medium. Although the above data clarified the role of UreI in the acid-acclimation processes of H. pylori, it would be extremely unlikely that this protein, along with urease, would be the only means of combating gastric acidity by the organism. Hence gene regulation due to acid exposure was studied by using microarray analysis at pH 7.4, 6.5, 5.5, and 4.5 in the absence or presence of urea (61). About 187 genes were upregulated by exposure to acidic pH down to 4.5, and 100 were downregulated. Other laboratories have also used microarray data, some in agreement with the data from this laboratory and others not, but thus far, the data presented here are unique in investigating the effect of urea on gene expression in different pH media.

Changes in gene expression that occur at a medium pH of 6.2 in the absence, but not in the presence, of urea are likely responses to periplasmic pH since the addition of urea reverses the gene regulation, indicating that a normal periplasmic pH and membrane potential have been restored. Under these conditions, there are several genes that might be considered pH homeostatic genes able to produce NH₃, such as asparaginase, aspartate ammonia lyase, and the periplasmic α-carbonic anhydrase (HP1186) able to produce HCO₃⁻ as a periplasmic buffer. There was also upregulation in this cluster of three hydrogenase expression/forming genes, hypA, hypB, and hypC, the first two considered to have a role in N₃⁻ uptake, essential for adequate urease enzyme activity, although these are actually hydrogenase-related genes (5, 44).

Carbonic anhydrase. Since urease produces 2NH₃ + CO₂ from urea, this carbonic anhydrase was the first of these pH homeostatic genes that we investigated in some detail. Since an inhibitor, acetazo-
urnI had been found with the 3-log decrease in survival in the knockout restoring it to baseline (29). Furthermore, there was organism, after regeneration of the membrane decreasing pH. There was also complete loss of the NH₃ that effluxes into the periplasm can neutralize entering acidity and the CO₂ due to periplasmic buffering in the carbonic anhydrase activity, producing H+ and HCO₃⁻. A second NH₄⁺ is formed along with HCO₃⁻ in two ways: either by the exoplasmic domain of this sensor, which would acidification of UreI as we had previously thought, resulting in the model of FIGURE 4. Although the NH₃ produced by urease activity is able to neutralize entering protons, the pKₐ of the NH₃/NH₄⁺ couple is 9.2 and would not buffer the periplasm effectively at a relatively neutral pH, allowing potentially lethal transient acidification of the periplasm. Therefore, NH₃ alone would not account for the finding that the periplasmic pH is relatively constant at 6.1 in the presence of urea, down to at least pH₃ 3.0 based on measurement of membrane potential and constancy of the pmf. On the other hand, pH 6.1 is the effective pKₐ of HCO₃⁻, whereas cytoplasmic carbonic anhydrase generates H⁺ and CO₂ from intrabacterial urease activity enable acid tolerance at pH 6.2. Since the response regulator HP0166 (4). It was found that there was upregulation of HP0165 at all external pH values (61) and that there was even retention upregulation of this gene with the addition of urea at pH 6.2. There are several histidines in the exoplasmic domain of this sensor, which would change protonation at pH ~6.2. Since the response regulator for HP0165 is HP0166, we decided to examine some of the 32 genes regulated by HP0166 at all external pH values (61). It is likely that many of the wild-type properties of the wild type, were lost, similar to the mutants. However, UreI was still functional in that there was the normal increase of bacterial urease activity with decreasing pH. There was also complete loss of periplasmic buffering in the carbonic anhydrase mutant as shown in FIGURE 3B, which stands in sharp contrast to the images shown in FIGURE 3A. The addition of acetzolamide to the wild-type organism, after regeneration of the membrane potential by urea, inhibited membrane potential, restoring it to baseline (29). Furthermore, there was a 3-log decrease in survival in the knockout mutants or in the presence of acetazolamide at pH 2.0 in the presence of urea. Recent in vivo data demonstrate a loss of infectivity in mice in the absence of carbonic anhydrase activity (13). These data, along with the gene analysis, show that the acid-acclimation response of H. pylori is significantly more complicated than simply the activation of urease by acid activation of UreI as we had previously thought, resulting in the model of FIGURE 4.

**FIGURE 4.** A model of two of the acid-acclimation mechanisms in H. pylori. The other membrane context provides a urea and proton gradient. With acidification, UreI opens and urea moves into the cytoplasm, increasing intrabacterial urease activity. This produces 2NH₃ and CO₂, which is a stronger buffer at neutral pH than NH₃. Similarly, the NH₄⁺ effluxes into the periplasm can neutralize entering acidity and the CO₂ due to periplasmic carbonic anhydrase activity, producing H⁺ and HCO₃⁻. A second NH₄⁺ is formed along with HCO₃⁻, the latter providing buffering in the range of pH 6.1.
lamine acid at pH responses at lower pH values will also be important for acid acclimation and their role in the acid acclimation of H. pylori holds out promise for an exciting future for further analysis of its gastric biology. Our work was supported in part by United States Veterans Affairs and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-44917, DK-53462, and DK-58333.

References


