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The Periplasmic α-Carbonic Anhydrase Activity of *Helicobacter pylori* Is Essential for Acid Acclimation

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The role of the periplasmic α-carbonic anhydrase (α-CA) (HP1186) in acid acclimation of *Helicobacter pylori* was investigated. Urease and urea influx through UreI have been shown to be essential for gastric colonization and for acid survival in vitro. Intrabacterial urease generation of NH_3 has a major role in regulation of periplasmic pH and inner membrane potential under acidic conditions, allowing adequate bioenergetics for survival and growth. Since α-CA catalyzes the conversion of CO_2 to HCO_3^-, the role of CO_2 in periplasmic buffering was studied using an α-CA deletion mutant and the CA inhibitor acetazolamide. Western analysis confirmed that α-CA was bound to the inner membrane. Immunoblots and PCR confirmed the absence of the enzyme and the gene in the α-CA knockout. In the mutant or in the presence of acetazolamide, there was an ~3 $\log_{10}$ decrease in acid survival. In acid, absence of α-CA activity decreased membrane integrity, as observed using membrane-permeant and -impermeant fluorescent DNA dyes. The increase in membrane potential and cytoplasmic buffering following urea addition to wild-type organisms in acid was absent in the α-CA knockout mutant and in the presence of acetazolamide, although UreI and urease remained fully functional. At low pH, the elevation of cytoplasmic and periplasmic pH with urea was abolished in the absence of α-CA activity. Hence, buffering of the periplasm to a pH consistent with viability depends not only on NH_3 efflux from the cytoplasm but also on the conversion of CO_2, produced by urease, to HCO_3^- by the periplasmic α-CA.

Helicobacter pylori is a neutralophile with a bioenergetic profile suited for growth at neutral pH (16, 24, 35). However, it has developed unique adaptive mechanisms to regulate both its cytoplasmic and periplasmic pH necessary for survival and growth in the acid environment of the stomach (10, 35). The maintenance of a near neutral periplasmic pH in acid appears to be unique to gastric *Helicobacter* species and thus we use the term "acid acclimation" for this phenomenon.

One of the best-studied processes in *H. pylori* which maintains periplasmic and cytoplasmic pH near neutrality when the organism is exposed to acidity is the urease system (33). *H. pylori* expresses very high levels of urease with a pH optimum of pH 7.5 (26, 35). Expression of urease is essential for gastric colonization (3, 13, 40). Urease is present in the bacterial cytoplasm, and its activity is regulated by a proton-gated urea channel, UreI (32, 37, 44). Acidic pH activation of UreI increases urea diffusion into the cytoplasm by at least 300-fold (44). This rapid UreI-mediated penetration of substrate leads to maximal urease activity in an acidic environment. Urease activity at neutral pH is rate limited by urea entry, which depends solely on the passive diffusion of urea across the inner membrane. This diffusion-mediated urea entry is insufficient for acid survival at gastric juice urea and hydrogen ion concentrations. Thus, acidic pH activation of UreI-dependent transport of urea allows maximal production of ammonia and carbon dioxide at acidic pH. The absence of UreI activity at neutral pH avoids alkalinization to a pH ≥ 8.2, which is lethal to *H. pylori* (10).

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Other microorganisms can survive transit through the acidic gastric lumen by maintaining a cytoplasmic pH of ~5.0 by acid resistance or tolerance mechanisms (18). Some of the transport systems used for this purpose include the glutamate decarboxylase-glutamate-γ-aminobutyrate antiporter system, which exchanges external glutamate for the intracellular decarboxylation product (γ-aminobutyric acid), or the *adiA*-encoded, arginine decarboxylase-arginine-agmatine antiporter (AdiA), both of which consume protons and form CO_2. These buffer the cytoplasm. Proton transporters such as the F_1F_o ATPase and the $Na^+/2H^+$ antiporter export H^+ and also elevate cytoplasmic pH (6, 24, 28). However, these processes do not elevate the pH of the periplasm. These bacteria cannot colonize the stomach because they cannot regulate periplasmic pH or elevate cytoplasmic pH to a level where protein synthesis can occur. *H. pylori* also expresses acid resistance and tolerance genes that contribute to acid survival but on their own may be insufficient for acid acclimation (1, 21, 22, 23, 39, 42, 45). However, these genes may contribute to maintenance of gastric colonization under certain conditions such as abnormally low urea concentrations. For example, the arginase gene *rocF*, a gene that is upregulated in acidic conditions (45), generates intracellular urea and is required for in vitro survival of the organism in acid in the absence of urea but not when urea is present (21).

Measurement of the inner membrane potential at a fixed medium pH between 3.0 and 6.0 in vitro showed that *H. pylori* maintains an inner membrane potential ($\Delta\Psi$) of −101 mV (35) only in the presence of physiological urea concentrations. The organism also maintains a relatively neutral cytoplasmic pH under these conditions (45). In the absence of urea at acidic pH, the $\Delta\Psi$ falls to values predicted by the proton motive force

(PMF) equation to compensate for the increase in the inward pH gradient (35). The finding of a constant inner membrane potential between pH 3.0 and 6.0 in the presence of urea was interpreted as being due to acid activation of urea entry through UreI and urea hydrolysis in the cytoplasm, followed by NH_3 diffusion into the periplasm, neutralizing the acid entering from the medium (35). Opening and closing of the UreI channel with a half-maximal activation at a pH of about 5.9 (43) was thought to be sufficient to account for maintenance of a constant inner membrane potential (for a recent review, see reference 33). However, the NH_4^+/NH_3 couple has a pK_a of ~9.2, and therefore, while the NH_3 produced can neutralize entering acid, it is too weak a buffer at acidic pH to maintain the neutral periplasmic pH required for gastric colonization during periods of high acidity in the gastric environment, indicating the requirement for an additional buffering process.

Comparative microarray analysis of *H. pylori* genes showed a nearly fivefold increase in expression of a periplasmic α-type carbonic anhydrase (α-CA), HP1186 (45), upon exposure to acidic pH. The increase of gene expression was confirmed by real-time PCR analysis. *H. pylori* α-CA is ~40% homologous to the membrane-anchored mammalian type IV CA isoform. Similar to the type IV vertebrate CA, the *H. pylori* α-CA contains an N-terminal cytoplasmic retention signal (Lys-Lys) preceding a membrane insertion signal anchor sequence that should result in binding of the enzyme to the bacterial inner membrane and exposure of its active site in the periplasm. Its activity is strongly inhibited by acetazolamide (2-acetylamido-1,3,4-thiadiazole-5-sulfonamide) (7).

Deletion of HP1186 results in >3.3 $\log_{10}$ killing of *H. pylori* at pH 4.0 even in the presence of urea with, however, no change in growth at neutral pH (Y.-C. Lee, T. Takata, H. Y. Shan, B. Grandjean, A. N. Charney, T. Ando, G. I. Perez-Perez, and M. Blaser, Gastroenterology **122**(Suppl)**:**A423, 2002.). Treatment of some peptic ulcer patients with acetazolamide resulted in not only ulcer healing but also reduction of peptic ulcer recurrence to about 6% 2 years after therapy was stopped (41). The combination of healing of ulcers and low recurrence of *H. pylori* is suggestive of *H. pylori* eradication, as found with triple therapy (29). Both the experimental and clinical studies indicate that periplasmic α-CA is important for the survival of *H. pylori* in the acid environment of the stomach.

The hypothesis investigated in this work is that, while NH_3 production neutralizes acid entering the periplasm, the CO_2 produced by urea hydrolysis diffuses into the periplasm and is converted to HCO_3^- by the periplasmic α-CA. HCO_3^- then acts as an essential periplasmic buffer, maintaining periplasmic pH close to the effective pK_a of HCO_3^-, namely 6.1, close to the pH range of regulation of UreI. This is also the periplasmic pH calculated from the membrane potential measurement (−101 mV) when urea is added to *H. pylori* maintained over a pH range of 3.0 to 6.0 (35). The results of these studies show that UreI and urease expression are necessary but may not be sufficient for colonization of the mammalian stomach and that both products of urease activity, NH_3 and CO_2, are required by *H. pylori* for acid acclimation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strain ATCC 43504 was obtained from the American Type Culture Collection. A nonpolar ATCC 43504 α-CA deletion mutant was constructed by allelic exchange using a kanamycin resistance gene as described below. Bacteria were grown under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) either on TSA plates supplemented with 5% sheep blood (Becton Dickinson) or in brain heart infusion (BHI) medium (Difco Laboratories) supplemented with 7% horse serum (Gibco BRL-Life Technologies Inc.) and 0.25% yeast extract (Difco Laboratories). All bacteria were grown in media in the presence of Dent selective supplement (Oxoid Limited), and the α-CA deletion mutant was always grown in the presence of 20 μg of kanamycin (Sigma Chemical Co.)/ml.

Construction of the α-CA deletion mutant. A genomic knockout of HP1186 was constructed by homologous recombination (α-CA knockout mutant, α-CAKO*)*. pBluescript (Stratagene) containing a kanamycin resistance gene in the multicloning site flanked by SalI (5′) and BglII (3′) was used to generate the knockout plasmid. Primers were designed to flank the regions approximately 600 bp upstream of the 5′ end of the gene and 400 bp downstream from the 3′ end. The 635-bp upstream segment was amplified with a 5′ primer containing a site for digestion by XbaI (5′-GGACCAAAATCTAGAGTGGGCAAAAGCGATACC-3′) and a 3′ primer containing a site for digestion by SalI (5′-GCGATCAAAAAAGTTGTCGACATTTTAATTATCC-3′). The 400-bp downstream segment was amplified with a 5′ primer containing a site for digestion by BglII (5′-CCATTTAACGGAGATCTCACCGCTCCTCCTTGc-3′) and a 3′ primer containing a site for digestion by KpnI (5′-GCAGAAAAAGCGGGTACCGGTTATAGTAAAAGGATG-3′). The purified PCR products were sequentially ligated into pBluescript around the kanamycin resistance gene. The construct was introduced into *H. pylori* strain ATCC 43504 by natural transformation, and colonies were selected in the presence of kanamycin (20 μg/ml). Knockouts were confirmed by a series of PCRs.

Western blot analysis. (i) Antibody Production. Polyclonal antibodies against the synthetic peptide GHDYVLDNVHFHAPME were raised in rabbits by Sigma/Genosys. The 16-mer peptide used for antibody production contains two of the three histidine residues thought to be involved in zinc binding of the metalloenzyme. A Blastp search of the genomes of *H. pylori* strains 26695 and J99 did not detect any homologous peptide sequences other then α-CA.

(ii) Protein preparation. *H. pylori* strain 43504 and the α-CA deletion mutant were grown overnight on their respective agar plates. The bacteria were harvested from the plates, suspended in ice-cold 25 mM sodium phosphate buffer, pH 7.4, and passed three times through a French press at 20,000 lb/in^2. The lysate was centrifuged at 3,000 × *g* for 10 min to pellet any remaining whole cells. The supernatant was removed and centrifuged at 10,000 × *g* for 10 min. This supernatant was layered onto 5 ml of 20% sucrose and centrifuged at 100,000 × *g* for 1 h. The sucrose layer prevented soluble cytoplasmic proteins from contaminating the membrane pellet. The supernatant was removed from the top of the sucrose layer and concentrated by using a centrifugal filter device with a 10-kDa cutoff (Centricon, Inc.). The membrane pellet was resuspended in 25 mM sodium phosphate buffer, pH 7.4. Protein concentration was determined by the BCA (Pierce) and Lowry methods (17).

(iii) SDS-PAGE and immunoblotting. The proteins from the membrane and soluble fractions were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4 to 12% NuPage Bis-Tris gradient gels (Invitrogen) and transferred onto nitrocellulose (Bio-Rad). Western blot analysis was carried out on the membrane and soluble fractions using α-CA antibodies. Immunolabeling was detected with the SuperSignal West Pico chemiluminescence detection kit (Pierce).

Survival studies. *H. pylori* wild type and the α-CAKO mutant were grown overnight on agar plates. The bacteria were removed from the plates and resuspended in 10 ml of BHI medium, pH 7.4 or 2.0, with 10 mM urea and incubated for 30 min in a microaerobic environment at 37°C. Tenfold serial dilutions of the bacterial suspension were plated on TSA plates supplemented with 5% sheep blood and incubated for 3 to 5 days in a microaerobic atmosphere at 37°C. To determine the effect of inhibition of periplasmic CA activity on survival, the BHI medium was supplemented with 1 mM acetazolamide prior to the addition of wild-type bacteria. Acetazolamide does not penetrate the inner membrane; therefore, the activity of the intrabacterial CA was not inhibited in this series of experiments (12). Survival at pH 2.0 was determined for each condition by comparison to pH 7.4 controls. All experiments were performed in triplicate.

Membrane integrity. The integrity of the inner membrane of the bacteria was observed by confocal microscopy using membrane-permeant and -impermeant fluorescent DNA probes (Live/Dead; Molecular Probes). A compromised inner cell membrane is probably predictive of cell death. The membrane-permeant fluorescent DNA probe SYTO9, when bound to DNA and excited at 488 nm, emits green light (emission, >505 nm). Propidium iodide, which is membrane impermeant, emits red light (excitation, 543 nm; emission, >633 nm) when bound to DNA and quenches SYTO9 fluorescence. Therefore, viable bacteria

will emit green light but bacteria with their inner membrane integrity compromised will fluoresce red.

Wild-type *H. pylori* and the CAKO mutant were grown overnight on TSA plates at 37°C in a microaerobic atmosphere. The bacteria were scraped off the plates and suspended in BHI medium pH 7.4 or 3.0 containing the Live/Dead indicators. In some experiments 1 mM acetazolamide was added to the wild-type strain to inhibit CA activity. The bacteria were incubated for 30 min at 37°C in a microaerobic environment at the two pH values and then mounted on glass slides. Fluorescence was observed by confocal microscopy (LSM 510; Carl Zeiss, Inc.).

Urease activity. Urease activity was measured radiometrically (20, 35). Wild-type and α-CAKO mutant bacteria were added to 100 mM sodium phosphate buffer containing 5 mM KCl, 138 mM NaCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 1 mM glutamine, and 5 mM [^{14}C]urea with a specific activity of 10 μCi/μmol. Acetazolamide (1 mM) was added to the reaction medium to determine the effect of CA inhibition on urease activity in wild-type *H. pylori*. The range of pH of the buffers used was between 4.0 and 8.5. The pH of the buffer between 4.5 and 8.5 was achieved by mixing various amounts of 100 mM monobasic and dibasic sodium phosphate to the desired pH. Below pH 4.5, the desired pH was achieved by the addition of HCl. The pH of the buffer during the course of the experiment did not change by more than 0.1 pH unit, as measured with a pH electrode. Plastic wells containing 0.5 M KOH-soaked filter paper hung from rubber stoppers were used to collect the liberated $^{14}CO_2$ that resulted from the hydrolysis of urea by urease. Urease activity was measured for 30 min at 37°C with constant agitation. The reaction was terminated by the addition of 5 N H_2SO_4, which liberates all labeled CO_2 from the incubation medium. The wells containing the filter paper were placed in scintillation cocktail (HiIonicFluor; Packard Instruments), and the radioactivity was measured by scintillation counting (1216 RackBeta; LKB Instruments).

Measurement of membrane potential. Membrane potential was determined as previously described (24). Briefly, the bacteria were harvested from the plates in 300 μl of HP buffer (1 mM phosphate buffer containing 5 mM KCl, 138 mM NaCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM glutamine, and 10 mM glucose). The fluorescent membrane-potential-sensitive dye $diSC_3(5)$ was dissolved in dimethyl sulfoxide, and 3 μl was added to 3 ml of appropriate buffer to give a final concentration of 1 μM. The bacterial suspension was then added to 3 ml of the dye solution at different pH_{out} (external pH) values in a fluorimeter cuvette to reach an optical density of 0.160 at 600 nm (usually 15 to 20 μl).

Fluorescence quenching due to potential-dependent uptake of the dye was measured in a fluorimeter set at an excitation wavelength of 600 nm and emission wavelength of 665 nm. The dye solution was added 5 min before addition of the bacteria to allow temperature equilibration. With addition of the bacteria, the fluorescence quenched due to dye uptake dependent on the interior negative potential. After the fluorescence reached a steady state, 5 mM urea was added and the change in fluorescence was measured. All experiments were done at 37°C.

The calibration of the membrane potential was carried out as previously described by the addition of valinomycin followed by the addition of K^+ until no further change in fluorescence was observed (24). This enables calculation of the K^+ equilibrium potential found with the addition of the K^+ selective ionophore valinomycin, using the Nernst equation: potential difference = 61 log $[K^+]_{in}/5$, where $[K^+]_{in}$ is equal to the external K^+ concentration at which the potential difference becomes zero in the presence of valinomycin, i.e., where $[K^+]_{out} = [K^+]_{in}$, and 5 mM is the medium concentration when valinomycin is added. The membrane potential in the absence of valinomycin can then be calculated. No change in medium pH was found in these strong buffers before or after the addition of urea over the time course of measurement.

Membrane potential is displayed based on the calibration once the dye reaches equilibrium. The initial fluorescence quench depends on accumulation of the dye inside the bacteria, and the fluorescence present prior to equilibrium does not necessarily imply a positive interior potential.

Measurement of intrabacterial pH. (i) BCECF loading. *H. pylori* strain ATCC 43504 was grown overnight on TSA plates supplemented with 5% sheep blood. The bacteria were removed from the plate and resuspended in 5 ml of BHI medium (Difco). The membrane-permeant, pH-sensitive, fluorescent probe, 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was added to the bacterial suspension to a final concentration of 10 μM. The bacterial suspension was then incubated in a microaerobic (5% O_2, 10% CO_2, 85% N_2) environment at 37°C for 30 min. The bacteria were pelleted by gentle centrifugation (3,000 × *g*, 5 min) (19) and resuspended in 300 μl of Hp medium (140 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 10 mM glucose, 1 mM glutamine) buffered with 1 mM phosphate buffer at pH 7.4. Experiments were performed without and with 5 mM urea. To measure pH_{in} (cytoplasmic pH), 20 μl of BCECF-loaded *H. pylori* was added to 3 ml of 50 mM homopiperazine-*N*,*N*′-bis-2(ethanesulfonic acid) (HpHOMOPIPES) at pH 5.0 and fluorescence was monitored in a dual-excitation (Ex1 = 502 nm, Ex2 = 436 nm) single-emission (Em = 530 nm) fluorimeter.

(ii) Calibration of pH_{in}. Each experiment was independently calibrated. Once the fluorescence of the internal BCECF had been measured, a 150 nM concentration of the protonophore 3,3′,4′,5-tetrachlorosalicyanilide was added to equilibrate the internal pH with that of the medium. HCl was then added to obtain minimum fluorescence of the dye, followed by the addition of NaOH to obtain maximum fluorescence of the dye. The internal pH was calculated by the equation $pH_{in} = pK_a + \log[(R - R_A)(R_B - R) \times F_A(\lambda 2)/F_B(\lambda 2)]$, where $pK_a = pK_a$ of BCECF = 7.0, R is the absorbance at 502 nm/absorbance at 436 nm for each data point, R_A is the ratio at minimum fluorescence, R_B is the ratio at maximum fluorescence, $F_A(\lambda 2)$ is the minimum fluorescence at 436 nm, and $F_B(\lambda 2)$ is the maximum fluorescence at 436 nm.

Changes of environmental pH due to urease activity. BCECF in its impermeant, free-acid form was used at a final concentration of 10 μM in Hp medium (pH 5.0) to monitor medium and periplasmic pH. BCECF free acid can penetrate the outer but not the inner membrane (4). The dye was excited at 480 nm and detected at 515 nm by using a Spex fluorimeter. *H. pylori* was added to the medium at a final concentration equivalent to an optical density at 600 nm of 0.160. After steady baseline fluorescence was established, 5 mM urea was added and measurement was continued for 10 more minutes. Data points were collected every 200 ms and integrated over 1 s.

Visualizing changes of periplasmic pH due to urease activity. A Zeiss LSM 510 confocal microscope was used to observe pH changes revealed by BCECF fluorescence in the periplasm of *H. pylori* and the medium. *H. pylori* strain ATCC 43504 was grown overnight on TSA plates supplemented with 5% sheep blood. The *H. pylori* α-CA knockout mutant was grown overnight on BHI plates supplemented with 20 μg of kanamycin/ml. The bacteria were removed from the plate and resuspended in 0.3 ml of Hp medium (pH 7.0). Bacteria were immobilized onto collagen-coated coverslips by mixing a 20-μl aliquot of *H. pylori* with an equal amount of 0.6% low-melting agarose and spreading the mixture evenly over the surface of the coverslip.

BCECF in its impermeant, free-acid form was used at a final concentration of 5 μM in Hp medium (pH 5.0) at the beginning of the pH range at which fluorescence increases with increasing pH. The dye was excited at 488 nm and detected at 515 to 545 nm. At the start of the experiment, BCECF-containing medium was added to the bacteria, and images were collected every 5 s. After 2 min, 5 mM urea was added, and image collection continued at the same rate for 5 more minutes.

RESULTS

Deletion of H. pylori α-CA (HP1186). The *H. pylori* α-CA deletion mutant was constructed by homologous recombination. The gene deletion was confirmed with a series of PCRs using primers to the kanamycin resistance cassette and the upstream sense and downstream antisense primers used to construct the original knockout plasmid. The kanamycin resistance cassette used is 844 bp, from pUC4K (Amersham), and its presence was confirmed in the genomic DNA of the knockout. The remaining primers were used in combination with the kanamycin resistance gene primers to confirm that the resistance gene was in the proper position. The knockout is likely to be nonpolar since the upstream and downstream genes are oriented in opposite directions.

Western blot analysis of H. pylori α-CA. Polyclonal *H. pylori* α-CA antibodies were used for Western blot analysis. No bands were detected in the soluble protein fraction from the wild-type bacteria. A band with a predicted molecular mass of ~28 kDa was detected only in the membrane fraction of the wild-type organism (Fig. 1). This is very close to the calculated molecular mass of 28.116 kDa for the full-length protein encoded by HP1186, which includes the putative signal anchor sequence, showing that the membrane signal sequence is not cleaved following membrane insertion. The retention of the

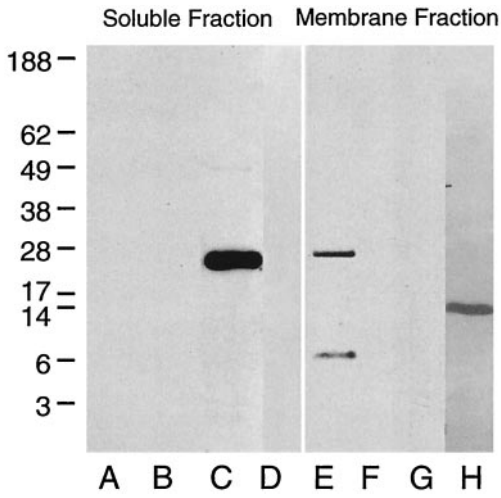

FIG. 1. Western blot analysis of α-CA. Membrane and soluble proteins were prepared from wild-type *H. pylori* and the *H. pylori* α-CA deletion mutant as described in Materials and Methods. Immunoblot analysis failed to detect α-CA in the soluble protein fraction from either the wild-type or mutant bacteria (lanes A and B, respectively). A band with an apparent molecular mass of 28 kDa was detected in the wild-type *H. pylori* membrane preparation (lane E) but not in the membrane fraction prepared from the α-CA deletion mutant (lane F). UreA was detected in the soluble fraction (lane C) but not in the membrane fraction (lane G). UreI was detected in the membrane fraction (lane H) but not in the soluble fraction (lane D).

signal anchor peptide with an N-terminal cytoplasmic anchor (Lys-Lys) indicates that this CA is likely tethered to the outside (2) face of the inner membrane with its active site in the periplasm. Routinely, a band with a molecular mass of ~10 kDa was also detected in the membrane fraction and may be a result of protein degradation. No protein bands were detected in the membrane or soluble fractions of the α-CA knockout, additional confirmation that HP1186 was deleted and the enzyme is not expressed. UreA was not detected in the membrane fraction, showing that adherent proteins were removed by our washing procedure. UreI, a known inner membrane protein, was found in the membrane fraction but not the soluble fraction.

α-CA activity is important for acid survival. After 30 min of exposure to pH 2.0 in the presence of 10 mM urea there was no change in survival in the wild-type organism (8.06 ± 0.10 $\log_{10}$ CFU). A significant ~3 $\log_{10}$ order loss of survival under these conditions was found in the α-CAKO mutant (5.43 ± 0.07 $\log_{10}$ CFU) and also in wild-type *H. pylori*, where the α-CA was inhibited by acetazolamide (5.69 ± 0.78 $\log_{10}$ CFU).

Similarly, deletion of CA or the presence of acetazolamide (data not shown) compromised the integrity of the inner membrane at pH 3.0, as shown by the enhanced uptake of propidium iodide (Fig. 2B), whereas membrane integrity was maintained at pH 7.4 in the mutant bacteria (Fig. 2A).

The absence of α-CA activity has no effect on acid gating of UreI. One possible explanation for the decreased survival of the α-CAKO mutant could be inactivation of UreI (32, 36). However, testing urease activity as a function of pH revealed a normal acid activation curve for the α-CAKO mutant and wild-type *H. pylori* in the presence of 1 mM acetazolamide.

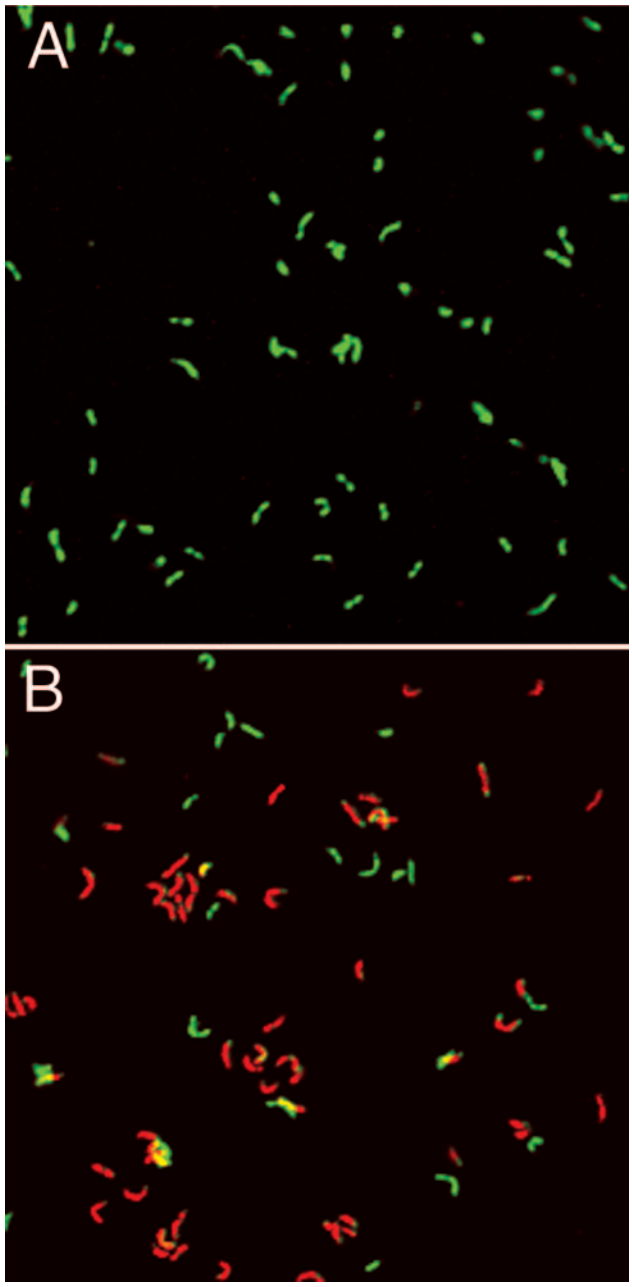

FIG. 2. Periplasmic CA activity is required for inner membrane integrity in acidic medium. α-CA deletion mutant bacteria were incubated for 30 min with urea in the presence of Syto9 (Live/Dead; Molecular Probes) and propidium iodide (Live/Dead) at pH 7.4 and pH 3.0. (A) No uptake of the positively charged propidium iodide was observed at pH 7.4. (B) At pH 3.0 the deletion mutant showed a large increase in propidium iodide uptake (red fluorescence), indicating loss of membrane in integrity.

There was about a 10-fold increase in intrabacterial urease activity between pH 7.0 and 5.0, with half-maximal activation at ~pH 6.0, as previously found for wild-type organisms (Fig. 3).

Membrane potential. Aerobic bacteria survive because of the generation of an electrochemical gradient of hydrogen ions across their cytoplasmic membrane. This electrochemical gradient or PMF is the algebraic sum of the pH gradient and

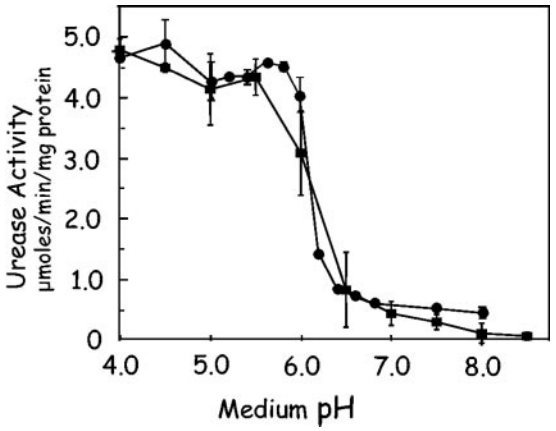

FIG. 3. α-CA expression is not required for UreI activation of urea entry. Steady-state urease activity as a function of different fixed medium pHs revealed a normal acid activation curve in the α-CAKO mutant (■). There was about a 20-fold increase in intrabacterial urease activity between pH 7.0 and 5.0, with half-maximal activation at ~pH 6.0, as previously found for wild-type organisms (●).

membrane potential across the inner membrane (25). When *H. pylori* is exposed to acidic media, the membrane potential falls, as expected from the increase in the inward pH gradient (35). For example, at pH 3.0, the contribution of the inward ΔpH to the PMF is much greater than that of the ΔΨ. The inner membrane potential under these conditions falls from −180mV at neutral pH to −10mV at pH_{out} 3.0, as expected from an increase of the inward acid gradient by 3 pH units (ca. −180mV). The addition of urea increases membrane potential from −10mV to −101mV (Fig. 4A). This increase in membrane potential was interpreted as being due to alkalinization of the periplasm by ammonia efflux, which then would decrease the ΔpH across the inner membrane, thus raising inner membrane potential to maintain a relatively constant PMF (35). In the α-CAKO mutant, urea addition had no effect on membrane potential, where it remained at −10mV, suggesting that the presence of a periplasmic CA is also required for maintaining urease-mediated periplasmic buffering (Fig. 4B). The addition of 1 mM acetazolamide reversed the increase in membrane potential observed with the addition of urea at pH 3.0 in the wild-type organism. These data suggest that in the absence of α-CA activity, the periplasm is not buffered after urea addition despite normal levels of urease activity and presumably NH_3 efflux into the periplasm (Fig. 4C).

Cytoplasmic pH. Cytoplasmic pH was determined by using the pH-sensitive, fluorescent dye BCECF-AM. At a pH_{out} of 5.0, in the absence of urea, the cytoplasmic pH of both the wild-type and α-CAKO mutant *H. pylori* was <6.0. The addition of 5 mM urea at pH_{out} 5.0 increased the cytoplasmic pH of the wild-type bacteria to 6.5, a pH at which protein synthesis and growth can occur (35) (Fig. 5A). Subsequent addition of acetazolamide to the wild-type organisms reversed the urea-induced increase in cytoplasmic pH. In contrast, the cytoplasmic pH of the α-CAKO mutant and acetazolamide-treated wild-type *H. pylori* was unchanged at pH 5.5 with urea addition at medium pH 5.0 (Fig. 5B).

Medium pH changes. When urea was added to wild-type organisms in the presence of BCECF free acid to monitor

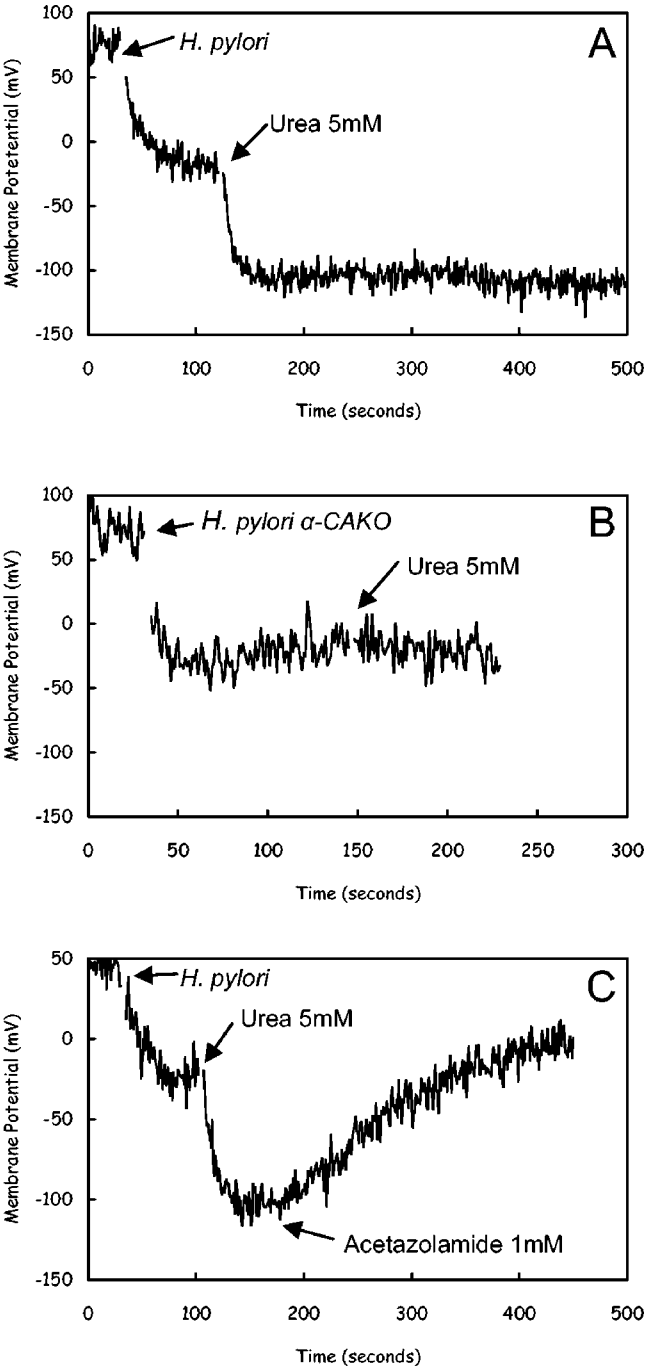

FIG. 4. α-CA activity is required to increase membrane potential following urea addition in acid. (A) At pH_{out} 3.0, the addition of urea increases membrane potential from −10 to −101 mV in wild-type *H. pylori* due to alkalization of the periplasm. (B) Urea addition had no effect on the membrane potential of the α-CAKO mutant. (C) The increase in membrane potential of the wild-type *H. pylori* following urea addition was reversed by the addition of 1 mM acetazolamide.

changes in external pH (periplasm and medium) at pH 5.0, fluorescence increased biphasically. There was a transient, rapid increase in fluorescence, followed by a slow sustained increase in fluorescence (Fig. 6A). With either the α-CA KO (Fig. 6B) or acetazolamide-inhibited organisms (data not

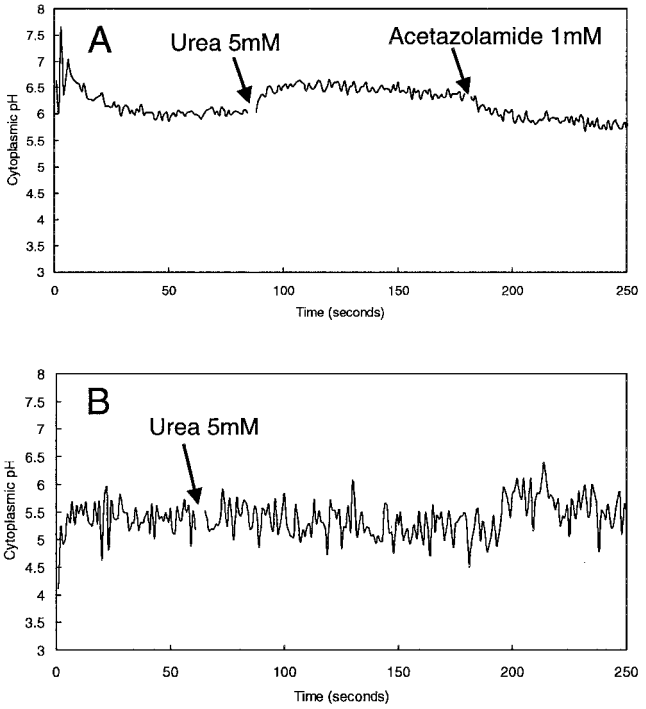

FIG. 5. Cytoplasmic pH in the wild type and α-CAKO mutant. Cytoplasmic pH was determined with the pH-sensitive, fluorescent dye BCECF-AM. (A) In acidic medium (pH 5.0), urea addition raises the cytoplasmic pH of wild-type *H. pylori*. (B) In the absence of α-CA activity, either by gene deletion or by enzyme inhibition, urea fails to increase intrabacterial pH.

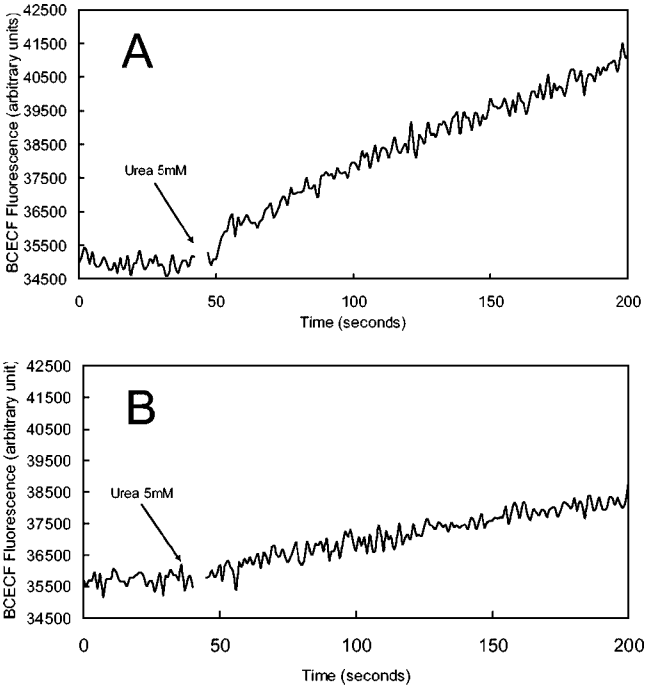

FIG. 6. Fluorimetric measurement of the rate of change of medium pH following the addition of urea to wild-type or mutant organisms. Medium pH was measured at pH 5.0 in weak buffer by the change of fluorescence of BCECF free acid. (A) In wild-type *H. pylori*, a transient increase in fluorescence followed by a steady increase was observed. In the HP1186 knockout mutant, the transient increase was not observed and the rate of medium alkalinization was slowed due to decreased cytoplasmic pH observed at pH 5.0 in the absence of periplasmic CA activity.

shown), the transient phase was not observed and the rate of fluorescence increase was slower than that of the wild type. The transient, rapid increase in fluorescence observed in the wild-type strain was interpreted as being due to elevation of periplasmic pH and the slower increase was interpreted as being due to elevation of medium pH. The loss of the transient rapid increase in fluorescence observed in the mutant and in the presence of acetazolamide is likely due to a loss of periplasmic buffering. Likewise, the decreased rate in the change of fluorescence observed in the mutant and in the presence of acetazolamide is likely due to the larger decrease in cytoplasmic pH in the absence of CA activity in acidic medium, as discussed above. Intrabacterial urease activity will decrease as the cytoplasm acidifies, lowering the pH from the enzyme pH optimum. These findings were confirmed using confocal microscopy.

Periplasmic pH. Using confocal microscopy in the presence of BCECF free acid, it is possible to visualize changes in periplasmic pH directly, since BCECF penetrates the outer, but not the inner, membrane and its fluorescence increases markedly with an increase of pH above 5.0 (4). The use of weak buffers in this set of experiments also allowed observation of alkalization of the external medium by a rise in general BCECF fluorescence.

At pH_{out} 5.0, an increase in periplasmic pH was observed in the wild-type organism 90 s after the addition of 5 mM urea. The increase in periplasmic pH is seen as green halos around the bacteria (Fig. 7A). Ninety seconds after urea addition to the α-CAKO mutant, no increase in periplasmic pH was observed (Fig. 7B). However, within 5 min, the BCECF fluorescence of the medium increased, indicating increased medium pH due to efflux of NH_3 from urease activity, resulting in neutralization of the weakly buffered medium for both the wild type and mutant (Fig. 7D and E). *H. pylori* organisms treated with acetazolamide also failed to increase their periplasmic pH 90 s after the addition of urea but did increase the pH of the medium after 5 min (Fig. 7C and F). These results are in accord with the changes in periplasmic pH predicted by the membrane potential measurements and with fluorimetric observation of total BCECF fluorescence (Fig. 6).

DISCUSSION

The results of the present study demonstrate that periplasmic CA is essential for survival of *H. pylori* in an acid environment studied in vitro. The acidity of the gastric niche occupied by *H. pylori* remains controversial.

The pH of the environment of *H. pylori* on the gastric surface. The gastric mucus is an aqueous gel and is unlikely to form a barrier to proton diffusion. The inability of mucus to act as a barrier to diffusion of even a relatively large molecule was elegantly demonstrated using two-photon uncaging of fluorescein close to the gastric surface in the mucus layer. Fluorescein diffusion in mucus was not slowed as compared with diffusion in free solution (5). It is therefore unlikely that there is any

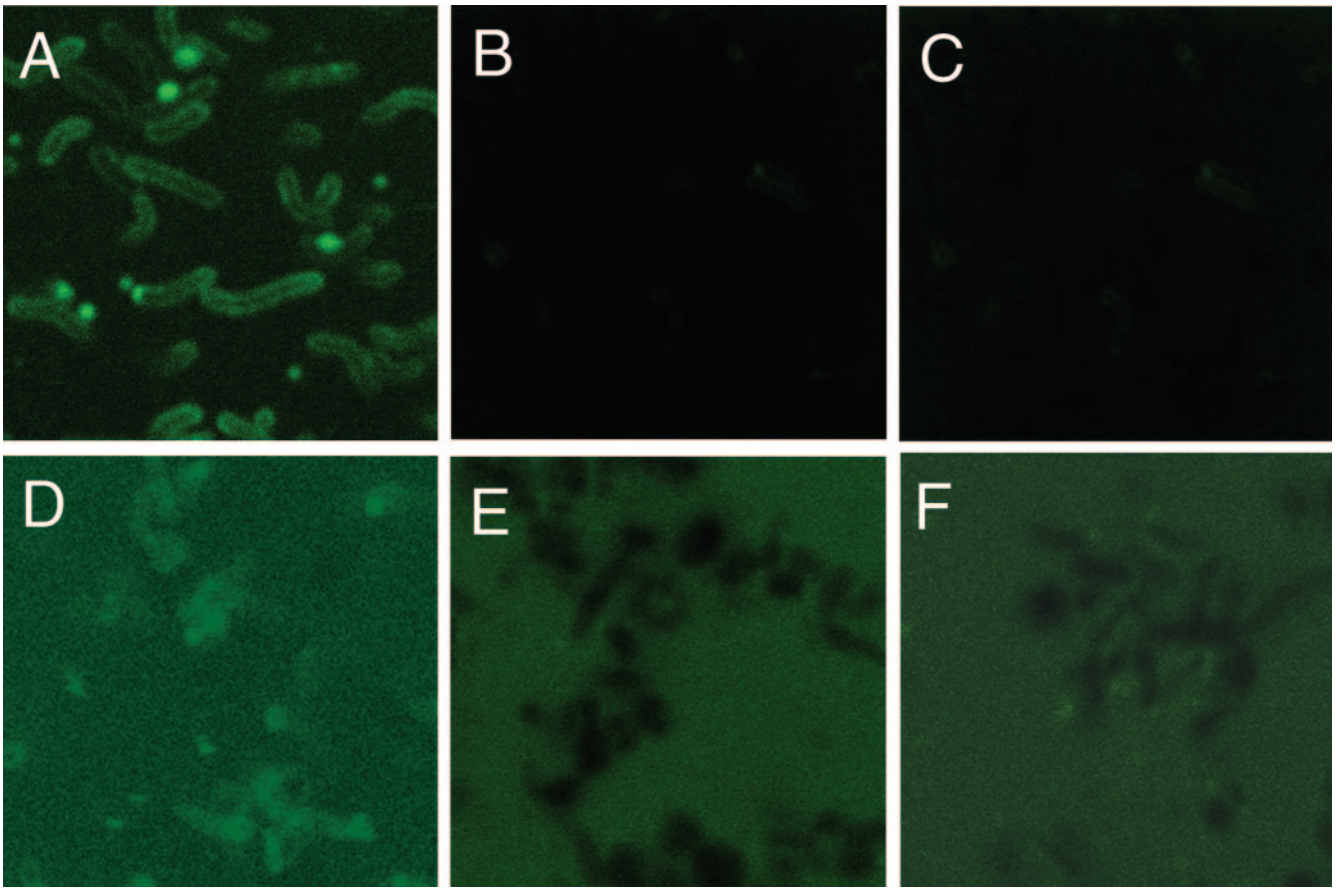

FIG. 7. Visualization of periplasmic alkalinization in *H. pylori*. Using confocal microscopy in the presence of BCECF free acid, it is possible to visualize changes in periplasmic pH since BCECF penetrates the outer, but not the inner, membrane. BCECF fluorescence increases with increasing pH. Ninety seconds after the addition of 5 mM urea the pH of the periplasm of wild-type *H. pylori* increased (A) but not that of the α-CAKO mutant (B) or with inhibition of α-CA by acetazolamide (C). After 5 min, the pH of the medium increased in all conditions, indicating retention of urease activity in the absence of α-CA activity (D, E, and F).

barrier to proton diffusion. The median pH in the human gastric mucosa is 1.4 (38), and given that the gastric mucus does not impede H^+ diffusion, it is unlikely that the human gastric epithelial surface pH will be close to neutral for much of the day. A neutral pH at the site of colonization of *H. pylori* would also imply that its acid acclimation mechanisms are required only for transit to its site of colonization but not for colonization. Also, neutralophiles with acid resistance and tolerance mechanisms allowing gastric transit would likely occupy the same niche.

Evidence for a gastric barrier to acidity was first found with pH-sensitive microelectrodes, where it was shown that at luminal pH values >3.0, the gastric surface was able to maintain an almost neutral pH (34). This pH gradient disappeared at a pH of ~2.0. The neutralization of luminal pH at the gastric surface is thought to be due to HCO_3^- secretion. Since this secretion is only 10% of maximal acid output, which is ~100 mM HCl, full neutralization is not to be expected at acid concentrations ≥10 mM HCl (pH 2.0 or less) (14). Indeed, most of the pH-sensitive microelectrode studies have confirmed that there is no pH gradient at the epithelial surface when the luminal pH falls below ~2.0 (14, 34).

Recently, the use of pH-sensitive fluorescent dyes has shown directly the absence of a pH gradient when the luminal pH is ≤3.0 (9). Also, the surface of the gastric epithelium maintains a surface pH of approximately 4.0 when the stomach lumen is perfused with weak buffer solutions. In contrast, surface pH equaled the pH of the perfusate with luminal perfusion with strong buffer (5). Hence, neutralization of the gastric surface is limited by the quantity of acid presented in the lumen, some of which can be neutralized by HCO_3^- efflux across the gastric epithelium. Secretion of HCl by the stomach at a pH ≤ 2.0 is equivalent to perfusion with strong buffer.

A significant finding that provides direct evidence for the acidic pH of the environment of the organism in vivo is the observation that *ureI*-negative mutants are able to infect the gerbil stomach only when acid secretion is inhibited but are eradicated with the return of acid secretion, in contrast to wild-type organisms (27). This finding shows that the organisms are exposed to an acidic pH in their natural habitat in the gerbil stomach and that this acidity is sufficient for eradication in the absence of stimulation of urea entry of intrabacterial urease via UreI. *ureI*-negative mutants do not survive pH 2.5 in vitro in the presence of 5 mM urea; however, wild-type bacteria can survive under the same conditions. Therefore, the pH at the gastric surface is likely to be at least as low as pH 2.5 to

explain the eradication of the *ureI*-negative *H. pylori* with the return of acid secretion.

Acid acclimation of *H. pylori*. *H. pylori* and other gastric *Helicobacter* spp. colonize the mammalian stomach but nevertheless are neutralophiles. This ability to inhabit the stomach implies that not only do they survive in a relatively acidic environment but they have also developed mechanisms to allow growth at acidic pH. This mechanism, acid acclimation, is defined here as periplasmic pH homeostasis close to neutrality in acidic media, to distinguish it from the more common acid resistance or acid tolerance mechanisms used by other neutralophiles to maintain cytoplasmic pH at a viable level.

(i) Intrabacterial urease and UreI. Bacterial genomic analysis suggests that expression of UreI is the dominant acid-adaptive feature of *H. pylori* and other gastric *Helicobacter* spp. (36) and acid activation of urea flux through this channel is essential for gastric colonization (27, 37). The reaction catalyzed by urease is $CO(NH_2)_2 + H_20 \rightarrow NH_2COOH + NH_3$. The product, carbamic acid, undergoes spontaneous hydrolysis as follows: $NH_2COOH + H_20 \rightarrow H_2CO_3 + NH_3$ and then $H_2CO_3 \rightleftarrows H_20 + CO_2$.

The products of urease activity in the cytoplasm are $2NH_3 + CO_2$. Both of these are gases and are able to diffuse very rapidly across the inner membrane. In the presence of periplasmic CA the reaction $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ is accelerated several thousand fold and is followed by the reaction $H^+ + NH_3 \rightarrow NH_4^+$, which neutralizes the proton produced by CA during the formation of HCO_3^-. The additional NH_3 formed by urease activity can neutralize acid entering the periplasm or diffuse into the medium and raise its pH if the medium is weakly buffered. However, the bicarbonate can buffer the periplasm to its effective pK_a of 6.1 to give an inner membrane potential of −101mV (35).

With the discovery of proton-gated urea transport by UreI, it was shown that the increased access of urea to intrabacterial urease resulted in increased urease activity in acidic conditions. The measurement of cytoplasmic pH and inner membrane potential as well as visualization of the increase of pH in the periplasmic space showed that increased urease activity was able to elevate periplasmic pH (4, 36, 44, 45).

(ii) Periplasmic CA. The HP1186 gene encodes a CA with a molecular mass of 28.116 kDa and a predicted N-terminal signal anchor sequence. Immunoelectron microscopy data were interpreted as showing that this CA was attached to the outer surface of the bacterium. However, the immunostaining was performed on intact organisms, precluding detection of periplasmic CA, and morphometric analysis was not performed (8). Here, we isolated *H. pylori* membrane and cytosolic proteins and used Western analysis to determine the localization of α-CA, UreI, and UreA. Full-length CA and UreI were found only in the membrane fraction, while UreA was found only in the cytosolic fraction. UreA was used in these experiments to show that under our conditions for producing membranes, proteins adhering to the outer membrane are removed (19). The retention of the signal anchor peptide indicates that this CA is likely tethered to the outside face of the inner membrane with its active site in the periplasm, with the Lys-Lys N-terminal sequence acting as a cytoplasmic anchor.

It was first suggested that the increased NH_3 production and its diffusion across the inner membrane into the periplasm, allowing consumption of entering protons, was sufficient to maintain the periplasm at pH 6.1 (35). It was hypothesized that the opening and closing of the UreI channel were sufficient to maintain the pH at close to the half-maximal pH of channel opening, a pH of ~5.9. The data presented here show that the mechanism is more subtle than previously thought.

The finding that a relatively constant inner membrane potential of −101mV was generated by the addition of 5 mM urea between a fixed medium pH of 3.0 and 6.0, with the assumption of a constant cytoplasmic pH of ~8.0 and an invariant PMF, led to a calculation of a fixed periplasmic pH of 6.1 from the PMF equation. This was thought to be due only to the effect of pH on opening and closing of the UreI channel (43, 44). The pK_a of the NH_4^+/NH_3 pair is 9.2, outside the pH range of viability of the organism. Therefore, only ammonia-efflux-dependent regulation of periplasmic pH in acidic medium would be unstable, with an unpredictable periplasmic pH and hence inner membrane potential and growth ability.

The data presented here, a lack of change in membrane potential with urea addition in the absence of or with inhibition of the periplasmic α-CA, show that not only NH_3 but also HCO_3^- is important in regulation of periplasmic pH. The width of the periplasm is ≤0.1 μm, and it is conceivable that the rate of NH_3 diffusion ($\sim 2.0 \times 10^{-5}$ cm^2/s) across the narrow space of the periplasm and out through the outer membrane is so fast that adequate generation of NH_4^+ does not occur in the periplasm. Further, NH_3 efflux would be able to elevate the periplasmic pH only if acid entry did not exceed NH_3 production. However, in the presence of periplasmic CA, the formation of HCO_3^- from the CO_2 diffusing from the interior of the organism as a result of urea hydrolysis would be extremely rapid. This would then provide buffering in the periplasm at the calculated pH of ~6.1, the effective pK_a of the HCO_3^-/CO_2 system, and within a range where the organism can still synthesize protein and grow (35).

At one time, CA activity was thought to be essential for gastric acid secretion by allowing intracellular buffering of the alkali produced in the cell by acid extrusion (11). It seemed reasonable, in the era before H_2 receptor antagonists, to treat peptic ulcer disease with CA inhibitors in order to reduce acid secretion. In one study, ulcer patients treated with acetazolamide for 30 days had a threefold decrease in basal acid output and a fourfold decrease in maximal acid output, showing that inhibition of CA inhibited gastric acid secretion (41). Normal acid secretion returned shortly after cessation of treatment. Additionally, there was a 94% healing rate of gastric ulcers with acetazolamide treatment as compared to 48% for antacid-treated ulcer patients. Similar data were found in other studies (15, 30, 31). An interesting and consistent finding in these studies was that after 2 years, the recurrence rate of ulcers in acetazolamide-treated patients was only 6.2% while that of the antacid-treated patients was 34%. The low rate of recurrence with acetazolamide treatment is surprisingly similar to that following triple therapy (a proton pump inhibitor and two antibiotics) for the eradication of *H. pylori* (29). These data preceded the use of histamine 2 receptor antagonists or proton pump inhibitors, where healing rates were much improved but recurrence rates reached ~60% within 1 year of cessation of treatment (29). Since these studies were conducted before the discovery of *H. pylori*, the healing effects of acetazolamide were

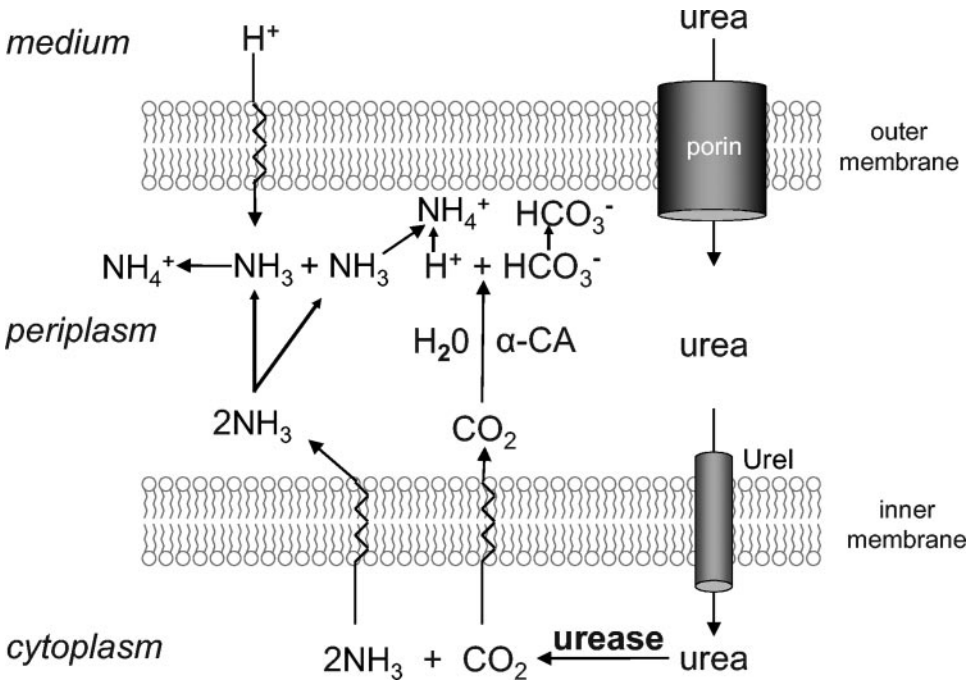

FIG. 8. A model for the role of urease and α-CA in maintenance of periplasmic pH in medium acidity. Urea enters from the medium into the periplasm presumably by diffusion across outer membrane porins. Under acidic conditions, urea moves into the cytoplasm due to acid gating of urea entry through the UreI channel. CO_2 and $2NH_3$ are then produced by urease activity in the cytoplasm. These gases diffuse rapidly into the periplasm. In the periplasm, CO_2 is hydrated rapidly to H_2CO_3 by α-CA and dissociates to H^+ and HCO_3^-, with the proton being consumed by $1NH_3$ to form NH_4^+. The additional NH_3 produced by urease can neutralize some of the acid entering the periplasm. This model accounts for the ability of intrabacterial urease activity to neutralize entering acid and at the same time maintain a pH of ~6.1 in the periplasm.

attributed solely to its acid-inhibitory actions. In light of the data presented here, showing that the periplasmic α-CA is required for acid acclimation of *H. pylori*, treatment of ulcer patients with acetazolamide could have led to eradication of the gastric pathogen.

In conclusion, the role of periplasmic CA is to generate HCO_3^- in the periplasm to both neutralize entering acid and buffer the periplasm to a level where a viable inner membrane potential is maintained. Activity of this enzyme could be an additional requirement for gastric colonization by the organism. A scheme showing the generation of $2NH_3$ and CO_2 from urease activity in the cytoplasm followed by their efflux into the periplasm is illustrated in Fig. 8. Following urea entry into the periplasm, urea enters the cytoplasm via activated UreI. Then intrabacterial urease produces $2NH_3$ and CO_2, gases that diffuse rapidly into the periplasm. One of the NH_3 molecules can neutralize entering acid, and the other forms $NH_4HCO_3^-$ due to the rapid production of HCO_3^- by CA, with a pK_a of 6.1, which is close to the gating pH of UreI. Another possibility is that NH_3 efflux across the 0.1-μM width of the periplasm is too fast to allow adequate protonation and neutralization of the periplasmic space and that HCO_3^- production traps NH_4^+. Another possibility is that the large amount of CA protein in the periplasm provides a framework restricting free proton diffusion, facilitating its neutralization by the NH_3 effluxing from the inner membrane.

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