

Helicobacter pylori Requires an Acidic Environment To Survive in the Presence of Urea

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The aim of this work was to study the significance of the urease enzyme in promoting *Helicobacter pylori* survival in various environments. A urease-positive *H. pylori* isolate, strain N6, and an isogenic urease-negative strain, strain N6(*ureB*::TnKm), were incubated in phosphate-buffered saline at a pH ranging from 2.2 to 7.2 for 60 min at 37°C in both the presence and the absence of 10 mM urea. The number of CFU per milliliter in each solution, the pH of the bacterial supernatant, and the amounts of ammonia present in the solutions were measured. *H. pylori* N6 survived well in solutions with pH values ranging from 4.5 to 7.0 in the absence of urea but survived in solutions only with an initial pH below 3.5 in the presence of urea. Neither strain grew after incubation in an alkaline environment. The pH of an acidic solution (i.e., 3.5) rose rapidly to 8.45 in the presence of the wild-type strain and urea. The urease-negative mutant survived in solutions with pH values ranging from 4.5 to 7.2 irrespective of the presence of urea. Ammonia was present in significant amounts when *H. pylori* N6 was incubated in the presence of urea. Strain N6 survived exposure to concentrations of ammonia as high as 80 mM. The acid environment of the stomach may be crucial for *H. pylori* survival in the presence of urea. *H. pylori* does not survive in the normal environment in the presence of urea because of the subsequent rise in pH rather than ammonia toxicity.

Helicobacter pylori is a gram-negative, microaerophilic organism which colonizes the gastric mucosa of humans. It exhibits a strict trophism for gastric mucus-secreting cells in vivo and is found only in the intestine in association with areas of gastric metaplasia (45). The organism exhibits a similar tropism for gastric cells in vitro (3, 8). *H. pylori* has been shown to be the cause of chronic active gastritis, and the evidence that it is associated with peptic ulcer disease is overwhelming (13, 17, 35). Childhood acquisition of the organism is associated with an increased risk of developing gastric cancer in later life (25). Exactly how *H. pylori* causes disease is not known, but several pathogenic determinants have been proposed for the organism. These include adhesins (16), cytotoxins (4, 10, 21), and a range of different enzymes, including urease (6), catalase (15), lipase (39), superoxide dismutase (40), and protease (37, 38).

One of the most striking characteristics of *H. pylori* is its very potent urease activity. With a K_m for urea of 0.8 mM (26), the urease of *H. pylori* binds substrate with a much higher affinity than that bound by the ureases of other bacterial species. Urea has been found to be present in the stomach at concentrations ranging from 1 to 14 mM (19, 22). Because of the high specific activity of the urease enzyme, *H. pylori* is able to hydrolyze the limited amounts of urea present in the stomach. It is thought that the urease enzyme of *H. pylori* plays a role in protecting the organism from the harmful effects of gastric acid and that ammonia generated by the organism protects the organism by buffering gastric acid.

Mutants of *H. pylori* incapable of urease production have been generated by chemical mutagenesis. Experiments with such mutants have suggested that urease activity is essential to enable *H. pylori* to colonize gnotobiotic piglets (6). It has been

shown that at neutral pH, the organism cannot survive if urea is present. It has been postulated that at neutral pH in the presence of urea, the amount of ammonia generated is toxic to the organism (34, 36).

When chemical mutagenesis is used, it is not possible to exclude the possibility that more than one mutation occurred in the bacteria during the mutagenesis procedure. Recently, an isogenic urease-negative mutant of *H. pylori* has been constructed (9). This mutant, which is modified specifically in the gene which encodes for the large urease subunit *ureB*, has given us the opportunity to study the precise role of the urease enzyme in determining the survival of *H. pylori* in various pH environments and to study the effect of ammonia generated by *H. pylori* urease on the viability of the organism.

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MATERIALS AND METHODS

Bacterial strains. An isogenic urease-negative mutant of *H. pylori*, N6(*ureB*::TnKm) (isogenic *ureB* mutant), and the wild-type parent strain, N6, were used in this study. Strain N6 originated from a patient with gastritis, and strain N6(*ureB*::TnKm) was constructed by allelic replacement (9). A region of cloned DNA containing the structural genes *ureA* and *ureB* was disrupted by insertion of a mini Tn3 kanamycin transposon (9).

Culture conditions. Strains were grown in liquid culture and held in storage at –70°C as described previously (5). For each assay, a vial of bacterial cells was thawed and cultured on Columbia blood agar (GIBCO) plates containing 7% (vol/vol) defibrinated horse blood for 3 days at 37°C in an atmosphere of 5% O₂ and 10% CO₂. Bacteria were confirmed as *H. pylori* on the basis of colony morphology, Gram stain, and the production of oxidase and catalase. Bacteria were harvested by rinsing the surface of each plate with 5 ml of phosphate-buffered saline (PBS; Dulbecco's formula A, 0.137 M sodium chloride, 0.0026 M potassium chloride, 0.008 M disodium hydrogen phosphate, and 0.0014 M potassium dihydrogen phosphate [pH 7.3]) and removing growth by scraping with a sterile swab. Bacteria were washed once in PBS by centrifugation at 3,600 × g for 15 min. Quantitation of bacteria in suspension was determined by optical density measurement at 450 nm and by viable counts. Appropriate dilutions of the bacterial suspensions were spread on Columbia blood agar plates, and after incubation of plates at 37°C under microaerophilic conditions for 5 days, the CFU were enumerated.

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TABLE 1. Effect of pH and urea on the viability of *H. pylori*

Initial buffer pH	Osmolarity (mmol/kg)	Buffer contents	<i>H. pylori</i> CFU/ml ^a	
			N6	N6(<i>ureB</i> ::TnKm)
2.2	318	10 mM urea	$(5.09 \times 10^4) \pm (1 \times 10^4)$	— ^b
		No urea	—	—
3.5	314	10 mM urea	—	$(2.30 \times 10^3) \pm (1.83 \times 10^3)$
		No urea	—	$(6.97 \times 10^3) \pm (3.21 \times 10^3)$
4.5	293	10 mM urea	—	$(4.90 \times 10^8) \pm (3.09 \times 10^8)$
		No urea	$(3.20 \times 10^6) \pm (2.89 \times 10^5)$	$(5.15 \times 10^8) \pm (3.50 \times 10^8)$
7.2	292	10 mM urea	—	$(8.23 \times 10^8) \pm (5.40 \times 10^8)$
		No urea	$(3.20 \times 10^7) \pm (1.83 \times 10^7)$	$(2.30 \times 10^9) \pm (1.25 \times 10^9)$
8.0	298	No urea	$(3.61 \times 10^4) \pm (2.06 \times 10^4)$	$(4.00 \times 10^6) \pm (7.42 \times 10^5)$
9.0	301	No urea	—	—
10	293	No urea	—	—

^a Results are expressed as the mean result of at least three experiments \pm standard deviation.

^b —, no organisms viable under these conditions.

Urease activity. Growth from the agar cultures was transferred with a sterile loop into 100 μ l of urea solution containing 2% (wt/vol) urea and 0.001% (wt/vol) phenol red in 0.01 M PBS (pH 6.8). A positive reaction was indicated by a change in color from yellow to pink within 5 min.

pH sensitivity assay. To assess the effect of pH on the survival of *H. pylori*, agar-grown organisms were harvested in PBS as described above. The pellet of bacterial cells was resuspended in PBS. Bacteria were used at a concentration of approximately 2×10^8 CFU/ml. A 5-ml aliquot of bacterial suspension was pelleted by centrifugation and resuspended in 5 ml of PBS of various pH values (i.e., pH 2.2, 3.5, 4.5, 7.2, 8.0, 9.0, and 10.0) with or without 10 mM urea. The pH of each solution was adjusted by the addition of either HCl or NaOH. The osmolarity of each solution was measured after the pH had been adjusted. The bacterial suspensions were incubated at 37°C for 60 min in 7-ml bijoux containers with loose-fitting caps under microaerophilic conditions. A 3-ml aliquot of each bacterial suspension was centrifuged at $3,600 \times g$ for 15 min, the supernatant was removed, and the pH was measured with a Corning universal pH meter. Serial 10-fold dilutions of the cell suspensions in PBS (pH 7.2) were made, plated onto Columbia blood agar plates, and incubated for 5 days at 37°C under microaerophilic conditions to determine the number of CFU per milliliter.

Ammonia sensitivity assay. The concentration of ammonia present in the supernatants of each bacterial suspension was measured by an enzyme assay (Sigma diagnostic kits). A reductive amination is carried out with L-glutamate dehydrogenase as follows: 2-oxoglutarate + NH₃ + NADPH \rightarrow L-glutamate dehydrogenase \rightarrow glutamate + NADP.

The direct effect of ammonia on the viability of the urease-positive parent strain N6 was also assessed. The bacteria were harvested from agar plates as described above and resuspended in solutions of PBS containing different concentrations of ammonia (ranging from 0 to 100 mM). The pH of each solution was adjusted to 7.0 with HCl prior to addition of the bacteria. The bacterial suspensions were incubated for 60 min at 37°C. After the incubation period, the amount of ammonia present in each of the solutions was determined as described above, and the number of CFU per milliliter present in the bacterial suspensions was measured.

RESULTS

Urease activity. Strain N6 caused an immediate color change from yellow to pink when inoculated into 100 μ l of urea solution containing 0.001% phenol red. Strain N6(*ureB*::TnKm) did not cause a color change when inoculated into urea solution, demonstrating that this strain does not possess an active urease enzyme.

Effect of *H. pylori* urease activity on viability of the organism and on pH of the incubation medium. In the absence of urea, both the wild-type strain, N6, and the isogenic urease-negative mutant strain, N6(*ureB*::TnKm), survived well in solutions of PBS with pH values ranging from 4.5 to 7.2 (Table 1). They did not survive well in solutions of PBS with a pH of <4.5. Following the addition of 10 mM urea to the medium, the wild-type strain survived in solutions with an initial pH value of 2.2, but it did not grow in solutions with a pH of >2.2. The urease-negative mutant survived well only in solutions with pH values ranging from 4.5 to 7.2 irrespective of the presence of urea (Table 1).

There was a marked rise in the pH of each of the solutions containing the wild-type *H. pylori* strain following the addition of urea (Fig. 1A). This change in pH occurred within 10 min of incubation. When urea was absent, no such change in the pH of the solutions occurred (Fig. 1B). Likewise, there was no change in the pH of the solutions containing the urease-negative mutant irrespective of the presence of urea (Fig. 1C and D). The wild-type strain therefore appeared to be able to survive in the low-pH environment in the presence of urea as a result of the rapid rise in pH generated by its urease activity. In contrast, the wild-type strain did not survive in solutions with an initial pH of greater than 2.2 when urea was present since the pH of these solutions became alkaline under these conditions (Fig. 1A). We tested the direct effect of alkaline pH in the absence of urea on the viability of the strains. The viability of both strains decreased markedly if the pH was raised from 7.2 to 8.0. If the pH was raised above 8.0, the organisms were not able to survive (Table 1). Both the wild-type strain and the urease-negative mutant survived only in solutions in which the final pH ranged from approximately 5.0 to 7.2.

Production of ammonia by *H. pylori*. It has been postulated that the reason why *H. pylori* does not survive at pH 7.0 in the presence of urea is because the amount of ammonia generated by the organism at this pH is actually toxic to the bacteria (34, 36). We therefore measured the amount of ammonia present in solution when *H. pylori* was incubated in the presence of urea at various pHs. The amounts of ammonia present in solution containing the wild-type strain and urea were almost identical (20 mM) in acidic and neutral pH environments (Table 2). As expected, the amounts of ammonia present in solutions containing the urease-negative mutant and in solutions containing the wild-type strain in the absence of urea were very low.

Effect of ammonia on the viability of *H. pylori*. Our results indicated that the amount of ammonia generated at neutral pH in the presence of urea is not responsible for the lack of viability of the wild-type strain under these conditions. To substantiate this finding, we measured the direct effect of ammonia on the viability of the urease-positive strain, N6. The organism remained viable even when it was exposed to concentrations of ammonia as high as 85 mM (Table 3).

DISCUSSION

Investigators were surprised initially to find *H. pylori* growing in the acidic environment of the stomach, but the results of this

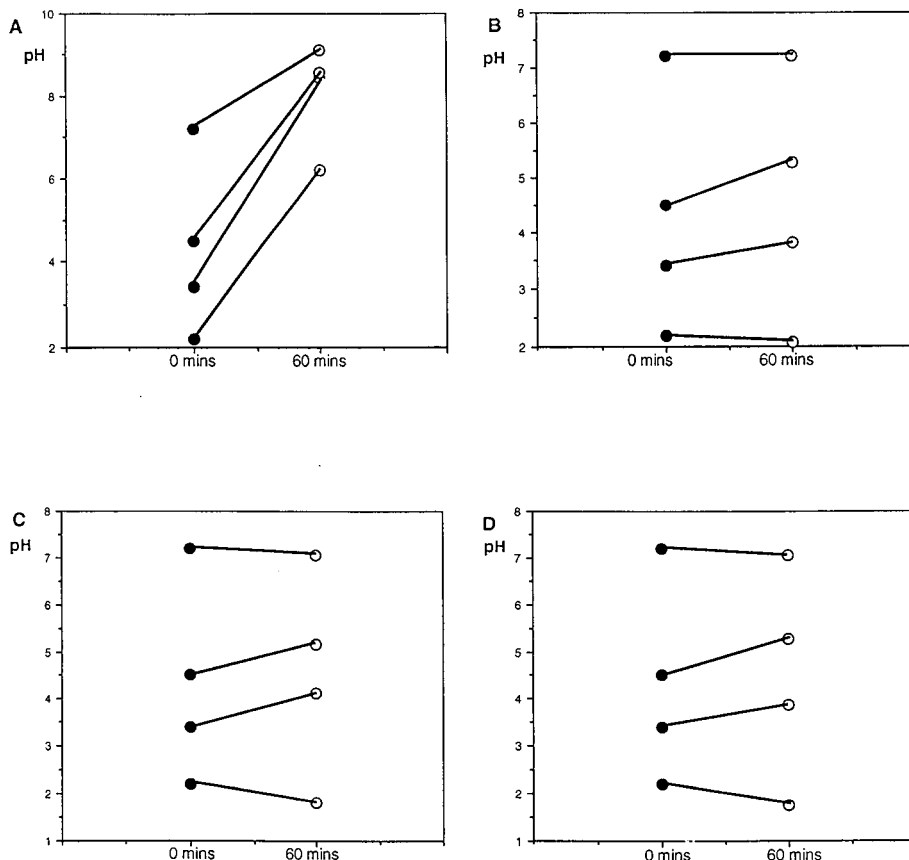


FIG. 1. Effect of *H. pylori* urease activity on pH of incubation medium. (A and B) *H. pylori* N6 was inoculated into PBS containing 10 mM urea (A) or no urea (B). (C and D) *H. pylori* N6(*ureB::TnKm*) was inoculated into PBS containing 10 mM urea (C) or no urea (D). Initial (0-min) (●) and end (60-min) (○) pH values of solutions inoculated with *H. pylori* are indicated. The results of a single experiment are shown. These results are typical of results obtained for several identical experiments.

study show that an acidic environment may be optimal for the survival of the organism in the presence of urea. This is the first report of the survival of *H. pylori* under different conditions in vitro with isogenic urease-negative mutants as controls.

Other workers using chemical mutants (36) and spontaneously occurring urease-negative mutants (34) speculated that the amount of ammonia generated by the organism at neutral pH is toxic to the bacteria (34, 36). However, we found that the amounts of ammonia generated by the bacteria were identical under acidic and neutral conditions. Furthermore, the effect of

ammonia on *H. pylori* survival has, to our knowledge, not been measured previously. Ammonia was not toxic to *H. pylori*, and the wild-type strain was able to survive even when exposed to concentrations of ammonia as high as 85 mM.

Conditions known to be associated with decreased gastric acidity have been found to result in a lower prevalence of *H. pylori* infection. O'Connor et al. (32) found a highly significant association between the absence of *H. pylori* and hypochlorhydria in patients who underwent surgery for peptic ulceration. Our findings suggest that the hypochlorhydria may be a major reason for the decreased survival of *H. pylori* in these patients. A subsequent study (31) demonstrated that colonized patients who cleared the infection following surgery became reinfected when alkaline reflux into the stomach was prevented. A report

TABLE 2. Production of ammonia by *H. pylori* urease

<i>H. pylori</i> strain	Initial pH	Concn of ammonia (mmol/liter) after incubation with <i>H. pylori</i> ^a	
		10 mM urea	No urea
N6	7.2	20.00 ± 1.41	0.0235 ± 0.016
	4.5	20.00 ± 0.00	<0.005
	3.5	19.75 ± 1.76	0.055 ± 0.038
	2.2	19.95 ± 0.035	<0.005
N6(<i>ureB::TnKm</i>)	7.2	0.1205 ± 0.0134	0.0445 ± 0.006
	4.5	0.1435 ± 0.0233	0.0440 ± 0.008
	3.5	0.1135 ± 0.0233	0.0220 ± 0.011
	2.2	0.1080 ± 0.0169	0.0255 ± 0.020

^a Results are expressed as the mean result of at least three experiments ± standard deviation.

TABLE 3. Effect of ammonia on the viability of *H. pylori* N6

Concn of ammonia (mmol/liter) in bacterial supernatant after incubation	<i>H. pylori</i> CFU/ml (mean ± SD)
0.00.....	(1.18 × 10 ⁷) ± (2.12 × 10 ⁴)
3.03 ± 1.39.....	(1.01 × 10 ⁷) ± (1.42 × 10 ⁶)
7.52 ± 1.75.....	(4.11 × 10 ⁷) ± (3.22 × 10 ⁷)
13.65 ± 0.95.....	(2.30 × 10 ⁶) ± (9.54 × 10 ⁵)
15.68 ± 3.05.....	(2.26 × 10 ⁶) ± (1.00 × 10 ⁶)
43.20 ± 4.80.....	(9.67 × 10 ⁶) ± (4.45 × 10 ⁵)
86.50 ± 9.19.....	(8.72 × 10 ⁶) ± (5.09 × 10 ⁵)

by Offerhaus et al. (33) concluded that alkaline reflux into the gastric remnant after gastrectomy resulted in the clearance of the organism. Similarly, in patients with pernicious anemia, gastric pH is raised, and even when antral gastritis is present, there is an almost total absence of *H. pylori* (11, 12, 30). Thus, situations in which the antral pH is raised are those in which *H. pylori* is likely to be absent.

Hypochlorhydria is one of the features of acute *H. pylori* infection in adults (18, 27), but this is caused presumably by the organism itself. This may occur by several mechanisms. Cave and Vargas (2) have described parietal cell inhibition by *H. pylori*. The present study suggests that *H. pylori* urease in the presence of urea is another mechanism which may contribute to the creation of hypochlorhydria.

Omeprazole is the first of a new class of drugs, the acid pump inhibitors which reduce basal and stimulated acid secretion. Omeprazole administered alone often appears to clear *H. pylori* from antral biopsies. However, it has been shown that clearance is not long lasting and that suppression rather than eradication of the organism has occurred (23, 42). The efficacy of omeprazole in suppressing *H. pylori* infection is not understood. There is in vitro evidence that omeprazole may inhibit urease activity (1, 28). However, both *H. pylori* and *Helicobacter mustelae* urease-negative strains are also susceptible to omeprazole (1). Our results suggest that if the secretion of acid is powerfully suppressed, *H. pylori* in the presence of urea will increase the pH of its local environment to alkaline values and will be unlikely to survive in a culturable form.

These results may explain why *H. pylori* has been isolated only rarely from other nonacidic sites in the body. *H. pylori* has been isolated from the feces of patients infected with *H. pylori* in the stomach (43). However, isolation of the organism from the feces of infected individuals is difficult, and often, only very low numbers of the organism are cultured. Furthermore, a recent study using PCR was not able to detect *H. pylori* in the feces of *H. pylori*-positive patients (44). There have also been reports of detection of *H. pylori* in dental plaque and saliva (14, 20, 29), but these studies suggest that if infection of the mouth by *H. pylori* occurs, it is transient. It is interesting to note that *Helicobacter canis*, a species of *Helicobacter* isolated recently from the feces of dogs, is urease negative and can survive in the presence of 1% bile (41). The fact that *H. canis* was isolated from feces, lacks urease activity, and can survive in bile suggests that this organism colonizes the lower bowel rather than the stomach.

Eaton and Krakowka (7) inoculated piglets which were treated with both omeprazole and ranitidine to raise the pH of the stomach to 7.0 prior to inoculation. Surprisingly, a urease-negative strain of *H. pylori* colonized only four of nine of these animals, and only very low numbers of the organism were recovered. The animals were, however, sacrificed after only 2 and 5 days postinfection. McColm et al. (24) found that ferrets infected with a urease-negative isolate of *H. mustelae* had lower mucosal *H. mustelae* numbers than those of ferrets infected with a urease-positive *H. mustelae* isolate on day 3 postchallenge but had attained numbers similar to those of urease-positive *H. mustelae*-colonized animals by day 21 postchallenge. Eaton and Krakowka (7) also found that the urease-positive strains colonized the stomach of the achlorhydric animals as well as they colonized the stomachs of normo-chlorhydric animals. However, the pH of these stomachs after infection was still only 7.0. The amount of urea present in the stomachs of these animals would be of interest since no change in pH could occur if urea was absent. Another possibility is that the omeprazole used to treat these animals may, to some extent, have inhibited the activity of the urease enzyme. In

summary, our results show that an acidic environment promotes the survival of *H. pylori* in the presence of urea and that the reason why *H. pylori* does not survive at a pH above 4.0 in the presence of urea is the generation of alkaline pH conditions and not ammonia toxicity. These findings may also explain the decrease in prevalence of *H. pylori* infection in patients with pernicious anemia and in duodenal ulcer patients following surgery.

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