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Characterization of *Helicobacter pylori* Urease Mutants

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The association between *Helicobacter pylori*, gastritis, and peptic ulcer is well established, and the association of infection with gastric cancer has been noted in several developing countries. However, the pathogenic mechanism(s) leading to disease states has not been elucidated. The *H. pylori* urease is thought to be a determinant of pathogenicity, since the enzyme is produced by all *H. pylori* clinical isolates. Evidence indicates that some *H. pylori* strains are more cytotoxic than others, with a correlation between the activity of the urease and the presence of a vacuolating cytotoxin having been made. However, the number of cytotoxins remains unknown at this time. The relationship between the urease and cytotoxicity has previously been examined with chemical inhibitors. To examine the role of the urease and its relationship to cytotoxicity, urease-deficient mutants were produced following ethyl methanesulfonate mutagenesis of *H. pylori* 87A300. Two mutants (the *ure1* and *ure5* mutants) which were entirely deficient in urease activity (Ure$^-$) were selected. Characterization of the isolates at the protein level showed that the urease subunits lacked the ability to complex and form the active urease enzyme. The *ure1* mutant was shown to be sensitive to the effects of low pH in vitro and exhibited no cytotoxicity to eucaryotic cells, whereas the parental strain (Ure$^+$) produced a cytotoxic effect in the presence of urea. Interaction between the *H. pylori* Ure$^+$ and Ure$^-$ strains and Caco-2 cells appeared to be similar in that both bacterial types elicited pedestal formation and actin condensation. These results indicate that the *H. pylori* urease may have many functions, among them (i) protecting *H. pylori* against the acidic environment of the stomach, (ii) acting as a cytotoxin, with human gastric cells especially susceptible to its activity, and (iii) disrupting cell tight junctions in such a manner that the cells remain viable but an ionic flow between the cells occurs.

Helicobacter pylori is a microaerophilic, spiral, gram-negative rod first isolated from human gastric antrum epithelium in 1983 (26). Clinical, histological, and bacteriological investigations have shown *H. pylori* to be the etiological agent of active chronic gastritis and to be associated with the development of peptic and gastric ulcers. Most recently, the presence of *H. pylori* has been directly correlated with intestinal-type gastric cancer (20–22). Several properties associated with *H. pylori* are believed to play a role in the pathogenic processes of gastritis and ulcer formation. These include adhesion to the gastric epithelial layer (7), the production of proteases capable of degrading glycoproteins (23), and the production of cytotoxins (13, 26, 28). It is unclear whether these traits are expressed by all *H. pylori* isolates, although it is clear that all clinical isolates of *H. pylori* and other *Helicobacter* species produce urease activity. Urease catalyzes the hydrolysis of urea to carbon dioxide and ammonia and has been postulated to play a major role in the ability of *H. pylori* to colonize and subsequently damage the gastric mucosa (19). The mechanisms by which the enzyme might act include urea hydrolysis, with an increase of the local pH of the mucosal surface subsequent to the formation of urea breakdown products (NH_3 and CO_2), preventing the normal passage of hydrogen ions from gastric glands to gastric lumen and enhancing back-diffusion of hydrogen ions (6). High concentrations of ammonia may exert a toxic effect on the tight cell junctions of gastric mucosal epithelial cells, resulting in an alteration of gastric mucosal permeability and providing a nutrient gradient. Urease may also be needed to provide a free nitrogen source from urea. By generating ammonia in the immediate bacterial microenvironment, urease activity may protect *H. pylori* from the effects of gastric acid and the low pH of the stomach (pH 1.5) and thus might be important in colonization, since *H. pylori* is extremely acid sensitive when urea is not present (1).

Urease production in many bacterial species has been shown to be regulated in conjunction with the nitrogen regulatory system, although the *H. pylori* urease appears to be produced constitutively in vitro. The genes encoding the *H. pylori* urease have recently been cloned in *Campylobacter jejuni* and analyzed (11). Four open reading frames encoding polypeptides with predicted molecular weights of 26,500 (UreA), 61,600 (UreB), 49,200 (UreC), and 15,000 (UreD) were found. UreA and UreB correspond to the two structural subunits of the enzyme. No role has been assigned to the UreC or UreD polypeptide, although they have both been mapped to a DNA region shown to be required for urease activity in a *C. jejuni* recipient strain. On the basis of sequence analysis, the *H. pylori* urease appears phylogenetically closer to the single subunit jack bean urease than to the 3-subunit bacterial ureases (11) and is biochemically and genetically distinct from ureases produced by species commonly associated with urinary tract infections, such as *Proteus*, *Providencia*, and *Morganella* species (11, 18, 19). The *H. pylori* urease has a much higher affinity for substrate than those of the last three bacterial species, with a K_m of 0.3 mM for urea. This property allows *H. pylori* to scavenge urea from serum at physiological blood urea concentrations of 1.7 to 3.4 mM, whereas uropathogens are exposed to saturation urea concentrations of 400 to 500 mM (19).

To understand the role of urease in pathogenesis, genetic mutants must be made and analyzed in appropriate models. Due to the lack of a genetic exchange system for *H. pylori*, the more desirable mechanisms of molecular mutagenesis

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are not currently applicable. The data presented below result from the production of *H. pylori* mutants by the classical method of chemical mutagenesis. Characterization of *H. pylori* urease mutants identified two which were urease negative. One of these urease-negative isolates, the *ure1* mutant (formerly the *ura1* mutant) (23a), was shown to be sensitive to low pH, did not produce a cytotoxic effect on HEp-2, KatoIII, or AGS cells, and was unable to disrupt a monolayer of polarized Caco-2 cells in vitro. The Ure^+ parental isolate, 87A300, with the addition of urea, was able to survive at low pH, was cytotoxic to HEp-2, KatoIII, and AGS cells, and caused a reduction in the transepithelial resistance of polarized Caco-2 cells.

MATERIALS AND METHODS

Bacterial strains and cell lines. *H. pylori* 87A300 is a human clinical isolate obtained from the State of California Department of Health Services, Berkeley. It was passaged in the laboratory on either 5% sheep blood plates (TSA II; BBL, Cockeysville, Md.) or on brucella agar (Difco) plates with 5% fetal bovine serum (FBS; GIBCO) added. Cultures were grown in a BBL GasPak jar containing an anaerobic gas pack (without catalyst) or in a 5% CO_2 incubator. The *ure1*, *ure5*, *ure10*, *ure27*, and *ure39* mutants were ethyl methanesulfonate (EMS)-derived mutants of *H. pylori* 87A300.

All eucaryotic cell lines were obtained from the American Type Culture Collection and grown as recommended. HEp-2 cells (ATCC CCL 23) were grown in Eagle's minimal essential medium (MEM) plus 10% FBS, AGS cells (ATCC CRL 1739) were grown in Ham's F12 plus 10% FBS, KatoIII cells (ATCC HTB 103) were grown in Dulbecco MEM plus 20% FBS, and Caco-2 cells (ATCC HTB 37) were grown in Eagle's MEM with nonessential amino acids plus 10% FBS.

Production of *H. pylori* urease mutants. The chemical mutagen EMS (Sigma) was selected as a mutagen, since it produces single-base changes in DNA at a frequency of approximately 1 nucleotide base change per genome (17). *H. pylori* 87A300 was grown in a liquid culture (brucella broth plus 5% FBS) overnight in a gas pack jar without catalyst by using an Anaerobic System GasPak (BBL) with shaking (120 rpm) at 37°C. The cells were spun down (2.2 krpm [1,000 × *g*], 20 min), washed once with phosphate-buffered saline (PBS), and suspended in one-half of their original volume in PBS; 2 ml of this suspension was added to 7.6 ml of 1 M Tris (pH 7.4). One hundred microliters of EMS was added, and the culture was placed at 37°C for 5 min with constant shaking. Ninety milliliters of brucella broth (plus 5% FBS) was added to the culture, which was then grown overnight in a gas pack jar as described above. Following overnight incubation, the cells were spun down, resuspended in 2 ml of PBS, plated onto sheep blood plates at the appropriate dilutions, and placed in a 5% CO_2 water-jacketed 37°C incubator. Individual colonies could be distinguished after 3 to 4 days of growth; these were picked and restreaked onto brucella agar (plus 5% FBS) plates. After 2 days of growth, colonies were assayed for urease activity by replica streaking onto brucella-urea agar plates (urea agar base; Difco). Urease-positive colonies gave a pink signal almost immediately; isolates that gave a negative or a decreased signal were studied further.

Urease activity assay. For urease assays, *H. pylori* cultures were grown for 48 h on sheep blood agar plates at 5% CO_2 and the bacteria were harvested in 0.6 ml of PBS (pH 7.4) by centrifugation at 1,000 rpm (180 × *g*) for 5 min. The bacterial pellet was suspended in PBS at concentrations appropriate for the assays. Protein concentrations were determined by using a modified biuret assay (Sigma). The urease activity of *H. pylori* 87A300 and those of the mutants were initially measured by incubation of 5×10^8 intact cells in Christensen broth (urea agar base; Difco). A color change from orange to red, caused by the production of ammonia and the resulting increase in pH, was measured spectrophotometrically (optical density at 560 nm [OD_{560}]). Since the urease activities of the *ure1* and *ure5* mutants were undetectable with this assay, the more sensitive Bertholet (indophenol) reaction (19) was used to perform further kinetic studies. Whole cells were incubated at room temperature in PBS (pH 7.4) containing urea at a saturating concentration (100 mM). The ammonia released by the urease was recorded by measuring the OD_{570} over several time points (up to 30 min) with ammonium chloride as a standard. The *H. pylori ura1* and *ura5* mutants had additional indophenol urease assays which used up to 1,000 times the amount of protein used for *H. pylori* 87A300 and were incubated for a period of 4 h. All urease values are reported as the mean and standard error of at least four separate trials. To obtain urease activity values, intact cells were incubated at room temperature with PBS (pH 7.4) containing urea at a saturating concentration (100 mM). The ammonia released by the urease was measured by recording the OD_{570} with ammonium chloride as a standard. Additional indophenol urease assays were performed on the *ure1* and *ure5* mutants by using higher concentrations of protein and incubating for 4 h. All urease values are reported as the mean and standard error of at least four separate trials. Reagents for all assays were purchased from Sigma.

SDS-PAGE, PAGE, and immunoblot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using a modified Laemmli gel system (12) with a 1-mm-thick gel, a 7.5% or 15% separating gel, and a 4.5% stacking gel (29.2 acrylamide:0.8 bisacrylamide). Whole *H. pylori* isolates were removed from brucella agar plates (plus 5% FBS) with a cotton swab and were suspended in PBS. An OD_{600} reading was done to determine cell density, and the volume was adjusted accordingly to standardize the samples. Bacteria were lysed by the addition of one-third the volume of 3× sample buffer (0.375 M Tris [pH 6.8], 12% SDS, 30% glycerol, 0.15% bromophenol blue). 2-Mercaptoethanol (BME) was added to a final concentration of 5%, and the samples were heated at 95°C for 5 min prior to loading. Electrophoresis was carried out at 100 to 150 V by using a Bio-Rad Protean II Vertical Gel System.

Nondenaturing PAGE was done as suggested by Hoeffer Scientific by using a 5.5% separating gel and a 3.0% stacking gel. The separating-gel buffer had a final concentration of 236.75 mM Tris–72.5 mM HCl (pH 8.48), and the stacking-gel buffer had a final concentration of 39.5 mM Tris–0.25 N phosphoric acid (pH 6.9). Samples were prepared as described above but without the addition of BME, and they were not heated. The lower-tank running buffer was 63 mM Tris–50 mM HCl (pH 7.47), and the upper-tank running buffer was 37.6 mM Tris–40 mM glycine (pH 8.9). Electrophoresis was carried out as described above.

After electrophoresis, a portion of the gel was stained with Coomassie brilliant blue R250; the remaining sample was electrotransferred at 180 mA for 12 to 18 h to a nitrocellulose membrane (Schleicher and Schuell) by using a Bio-Rad Trans Blot apparatus. The transfer buffer consisted of 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Transfer efficiency was monitored by using prestained low-molecular-weight protein markers (Bio-Rad). Upon the completion of transfer, the proteins were reacted with the appropriate

antibody by following the instructions of the Immun-Blot Assay Kit (Bio-Rad).

pH sensitivity assay. Plate-grown *H. pylori* isolates were harvested in 0.6 ml of PBS (pH 7.4), and cell densities were measured by OD_{600}. To test the effects of pH, 5×10^8 CFU/ml were incubated for 15 min at 37°C in PBS of varying pH (7.4, 3.0, and 1.5) with and without 5 mM urea. After incubation, dilutions were plated onto brucella agar (plus 5% FBS) plates and placed in a 5% CO_2 incubator until colony counts could be performed (approximately 3 to 4 days).

Cytotoxicity assay. *H. pylori* 87A300 and the *ure1* mutant were used to determine whether the *H. pylori* urease had cytotoxic effects on eucaryotic epithelial cells. For this assay, monolayers of tissue culture cells were grown overnight in 24-well flat-bottom dishes. Approximately 10^7 *H. pylori* cells were added to each well in the presence and the absence of 5 mM urea. The dish was placed in a 5% CO_2 incubator, and eucaryotic cell death was measured at various time points by using Eosin Y (Sigma), which stains nonviable cells red. Prior to counting, a stock of 10× Eosin was added to each well to give a final concentration of 1%. The monolayer was removed from the well by dislocating cells with fluid, and the cells were counted by using a hemocytometer. All cell lines were grown in a 5% CO_2 37°C incubator. All experiments were done in duplicate, and four fields from each well were counted.

Polarized cells. Approximately 10^5 viable Caco-2 cells per membrane were seeded (Costar Transwell; 3-μm-pore-size filter) and fed (Eagle's MEM plus 10% FBS plus penicillin-streptomycin) every 2 to 3 days until a polarized monolayer was formed (at days 10 to 18) as measured with a resistance meter (Millicell-ERS; Millipore, Bethesda, Md.). One hour before the *H. pylori* was added, Eagle's MEM plus 10% FBS plus 5 mM urea was added to half of the wells; urea was omitted from the remainder. *H. pylori* strains were removed from 2-day-old plates and suspended in PBS, and 10^7 bacteria were added to the inner well. Resistance was measured at time zero and at the appropriate intervals thereafter. The area resistance (Ω/cm^2) was calculated by multiplying the measured resistance by the area of the filter.

TEM. To prepare the Caco-2 polarized monolayers for transmission electron microscopy (TEM), the cells were washed twice in cold 0.1 M $NaPO_4$ (pH 6.3) and fixed for 40 min at 4°C in 1% gluteraldehyde–1% OsO_4–0.05 M $NaPO_4$ (pH 6.3). The cells were then washed three times with double-distilled water (ddH_2O) at 4°C for 5 min each time and were postfixed in 0.5% uranyl acetate at 4°C overnight. After the cells were washed with ddH_2O (4°C, 10 min), the filters were removed from the Transwell by using a scalpel and were dehydrated two times each in a series of ethanol washes (25, 50, 75, 85, 95, 100, and 100%) for 5 min on ice, with a final 100% wash at room temperature. The filters were embedded in BEEM polyethylene capsules by using Poly/Bed 812 (Polysciences, Inc.) according to the manufacturer's instructions and were baked at 70°C for 2 days to allow for complete polymerization.

Statistics. The confidence coefficient was calculated by using Student's *t* distribution test.

RESULTS

Characterization of *H. pylori* urease mutants. A total of 600 colonies taken from the bacterial culture exposed to EMS were screened on brucella-urea agar plates; 5 did not cause a color reaction or caused a decreased color reaction on urea media and were picked for further analysis. These five were quantitatively characterized for urease activity by the indophenol (Bertholet) reaction (19); the amount of urease activity was determined by spectrophotometry (OD_{570}). The urease activities of all isolates were equivalent in viable-cell assays and cell lysates. Two mutants, the *ure1* and *ure5* mutants, were shown to be urease negative, while the *ure10*, *ure27*, and *ure39* mutants expressed decreased activities. A summary of the results is presented in Table 1.

TABLE 1. Urease activities of *H. pylori* 87A300 and the urease mutants

H. pylori strain or mutant	Activity[a] (%)
87A300	23.265 ± 2.106 (100.00)
ure1	0.00 (0.00)
ure5	0.00 (0.00)
ure10	0.751 ± 0.075 (3.23)
ure27	0.099 ± 0.022 (0.43)
ure39	0.159 ± 0.015 (0.68)

[a] Activities were determined as nanomoles of urea per minute per microgram (mean ± standard deviation).

Urease subunit expression was analyzed by comparing denatured and nondenatured whole-cell protein preparations run through SDS-PAGE or nondenaturing PAGE gels, followed by Western blot (immunoblot) analysis with either anti-UreA or anti-UreB antiserum (a gift from H. Mobley). As can be seen in Fig. 1A and B, the *ure1*, *ure5*, and *ure10* mutants produced wild-type amounts of both 26.6- and 61.5-kDa urease subunits, whereas the *ure39* mutant produced extremely low amounts of both subunits. The *ure27* mutant produced results identical to those for the *ure39* mutant (data not shown). The relative decrease in the amount of each subunit suggests that they were coordinately expressed. This is in agreement with the genetics of the *Helicobactor* urease operon, in which the coding regions for the *ureA* and *ureB* genes are separated by 3 bp, with a single putative sigma 54 promoter upstream of the start of the *ureA* coding region (11). Analysis of these same proteins by using a nondenaturing gel system (Fig. 1C and D) showed that while the *ure1* and *ure5* mutants produced normal amounts of the urease subunits, the active urease enzyme [proposed to have the configuration $(A_3B_3)_2$ or A_6B_6] (10) was not formed. The urease subunits produced by the *ure10* mutant formed a complex comparable to that of 87A300 but had decreased urease activities. The *ure27* and *ure39* mutants produced urease subunits that formed an active complex but at much reduced amounts.

All other characteristics assayed in the urease mutants were unchanged. All five mutants were catalase and oxidase positive and had the same growth rates as the parental 87A300 strain (data not shown), indicating that the EMS mutagenesis resulted in a single hit per genome, although more than one mutation per isolate could not be ruled out.

Viability at low pH. It has been shown that *H. pylori* is able to survive at low pH in buffer only when urea is present (1). Therefore, the *ure1* mutant was tested for the ability to survive at low pH in the presence and the absence of 5 mM urea. Compared with those of the wild-type strain 87A300, *ure1* cells were quickly killed when exposed to low pH (1.5) in both the presence and the absence of urea (Table 2). In the presence of 5 mM urea at pH 7.4, the urease of strain 87A300 produced enough ammonia to kill the bacterium; the *ure1* mutant was viable in the presence of urea, implying that its urease could not produce the toxic effect.

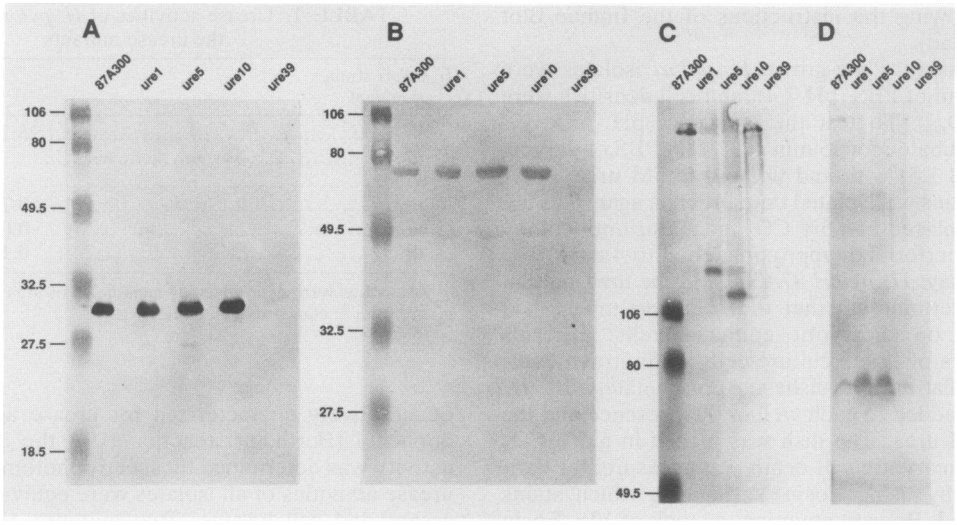

FIG. 1. Immunoblot analysis of *H. pylori* 87A300 and the *ure1*, *ure5*, *ure10*, and *ure39* mutants. (A and B) Total cellular proteins were denatured and electrophoresed through either a 15% (A) or a 7.5% (B) SDS-polyacrylamide gel. (C and D) Nondenatured total cellular proteins were electrophoresed through a 5.5% nondenaturing polyacrylamide gel. After the proteins were transferred to nitrocellulose, blotting was done with either anti-UreA (A and C) or anti-UreB (B and D) antiserum.

Cytotoxicity. It has been postulated that urease can act as a cytotoxin to eucaryotic cells through its ability to produce ammonia. This hypothesis has previously been able to be tested only by using the urease inhibitor acetohydroxamic acid (24, 28). Therefore, the abilities of the urease mutants and the wild-type parent to produce a cytotoxic effect were tested. An in vitro cytotoxicity assay was carried out by using three different human cell lines incubated with *H. pylori* 87A300 or the *ure1* mutant in the presence or the absence of 5 mM urea. Cytotoxicity was measured as the percent eucaryotic cell death. Along with inducing vacuolization, the *H. pylori* parental strain, 87A300, produced a cytotoxic effect on HEp-2 and KatoIII cells when urea was present; in the absence of urea, no significant cytotoxic activity was observed until the 48-h time point. *ure1* mutant cells did not damage the monolayers, whether or not urea was present (Table 3).

The *H. pylori* urease had a statistically significantly greater cytotoxic effect on the KatoIII cells (gastric carcinoma) than on the HEp-2 cells (Table 3). To determine whether gastric tissue was more susceptible than tissue of other origins to the effects of *H. pylori*, a second human gastric cell line, AGS, was used as described above. Within 24 h of exposure of the AGS cells to *H. pylori* 87A300 and 5 mM urea, the AGS cells became heavily vacuolated (Fig. 2A), whereas cells incubated with strain 87A300 in the absence of urea or the *ure1* mutant with or without urea did not produce vacuolization of the AGS cells (Fig. 2B).

To investigate the relationship between urease and the disruption of intercellular tight cell junctions, Caco-2 polarized cells (a human colonic epithelial cell line) were used as a substrate, and the integrity of the junctions was monitored with a resistance meter. This cell line has been amply characterized and is known to form polarized monolayers with well-defined tight cell junctions when grown on an appropriate surface. The addition of *H. pylori* 87A300 and 5 mM urea to a polarized monolayer of Caco-2 cells caused a 50.68% drop in its resistance compared with the resistance of Caco-2 cells after *H. pylori* 87A300 in the absence of urea was added. The *H. pylori ure1* mutant did not alter the resistance levels of the Caco-2 cells under either incubation condition. The resistance levels (in centimeters squared) of Caco-2 cells under various conditions were as follows: Caco-2 plus urea, 592.68; Caco-2 plus *H. pylori* 87A300, 539.96; Caco-2 plus *H. pylori* 87A300 plus urea, 292.30; Caco-2 plus the *H. pylori ure1* mutant, 641.03; and Caco-2 plus the *H. pylori ure1* mutant plus urea, 528.17.

TEM of the polarized cells under various experimental conditions showed that *H. pylori* 87A300 (Ure^+) in the absence of urea and the *H. pylori ure1* mutant (Ure^-) in the presence or the absence of urea did not have any grossly visible effect on the epithelial monolayer. In some electron microscopic sections, it appeared that *H. pylori* 87A300 incubated with urea affected the tight junction and adherence zones, producing a thickening in the plaque material surrounding the adherence regions (data not shown).

DISCUSSION

Following EMS chemical mutagenesis of an *H. pylori* urease-producing clinical isolate (87A300), five urease-deficient mutants were identified and characterized into three groups. Two isolates (the *ure1* and *ure5* mutants) were

TABLE 2. Effect of pH on *H. pylori* viability in vitro with and without urea

Buffer pH and contents	No. of viable *H. pylori* cells per ml	
	87A300	*ure1* mutant
	1.02×10^7	2.90×10^7
7.4		
5 mM urea	1.82×10^4	9.30×10^7
No urea	7.20×10^6	8.05×10^7
3.0		
5 mM urea	7.10×10^6	1.67×10^3
No urea	2.62×10^4	9.45×10^2
1.5		
5 mM urea	2.40×10^5	0.00
No urea	0.00	0.00

TABLE 3. Effect of *H. pylori* on epithelial cell viability in vitro with or without urea

Cell line	Time of exposure (h)	% of cells viable				
		Control	*H. pylori* 87A300		*H. pylori ure1* mutant	
			With 5 mM urea	Without urea	With 5 mM urea	Without urea
HEp-2	2	69.97	69.21	65.36	69.34	70.72
	6	75.44	67.07	70.44	66.29	66.78
	24	75.80	50.17[a,b]	67.68	71.78	63.50
	48	59.62	36.93[a,b]	35.98[a,b]	58.56	65.79
KatoIII	2	65.50	60.72	60.92	63.12	58.00
	6	56.74	58.04	57.41	62.58	59.81
	24	73.70	40.93	59.50	64.56	67.66
	48	64.08	38.94[a,c]	54.97	46.41	50.90

[a] Statistically significant eucaryotic cell death calculated by using Student's *t* distribution test.
[b] $P < 0.01$ for cytotoxic activity.
[c] $P < 0.001$ for cytotoxic activity.

shown to produce no urease activity (Table 1), as determined by the indophenol urease assay. While both urease subunits were produced by the *ure1* and *ure5* mutants at wild-type levels as judged by immunoblot analysis, examination of nondenatured total proteins showed that the subunits were not able to complex to form an active urease enzyme (Fig. 1). The second class, consisting of the *ure10* mutant, produced only 3.23% of wild-type urease activity, although it produced wild-type levels of the urease subunits. Since the urease subunits appeared to be able to complex, one possible explanation for the decrease in urease activity could be that the mutation affected the active site. The third class, exemplified by the *ure27* and *ure39* mutants, consisted of mutants that produced equivalent decreased amounts of the UreA and UreB subunits, resulting in a proportional decreased amount of urease activity and suggesting that a down mutation in the promoter region upstream of the *ureA* gene had occurred.

The *ure1* mutant was studied further as a representative of the first class of mutants having no detectable urease activity. In vitro, the *ure1* mutant was shown to be exquisitely susceptible to an acidic environment; exposure at pH 1.5 for 15 min was lethal to the organism, even with the addition of a physiologic concentration of urea (Table 2), whereas the parental strain survived at this low pH when urea was added. These results suggest that urease may not act as a colonization factor in a mechanical sense but rather may enable *H. pylori* to survive the environment of the stomach long enough to be able to subsequently colonize. Paradoxically, the inability to produce urease was also shown to be beneficial to *H. pylori*, since 15 min of exposure at pH 7.4 in the presence of urea was lethal to ~99% of the wild-type *H. pylori* cells, whereas the *ure1* mutant showed no decrease in viability (Table 2), indicating that in vitro accumulation of ammonia was toxic to the bacteria. This observation indicates that it might be beneficial for *H. pylori* to be able to regulate the production of its urease, initially producing urease to combat the lethal effects of acidity and in order to colonize, with down regulation of urease production once infection was established, allowing survival in a more neutral environment. The similarities between the presumed *H. pylori* urease promoter and the promoters of the nitrogen-regulatory systems found in other bacteria indicate the potential for regulation by environmental stimuli (11). At present, *H. pylori* urease expression is thought to be constitutive, although recently Cussac and coworkers have shown that a cloned copy of the *H. pylori* urease gene can be regulated by nitrogen when expressed in *Escherichia coli* (2).

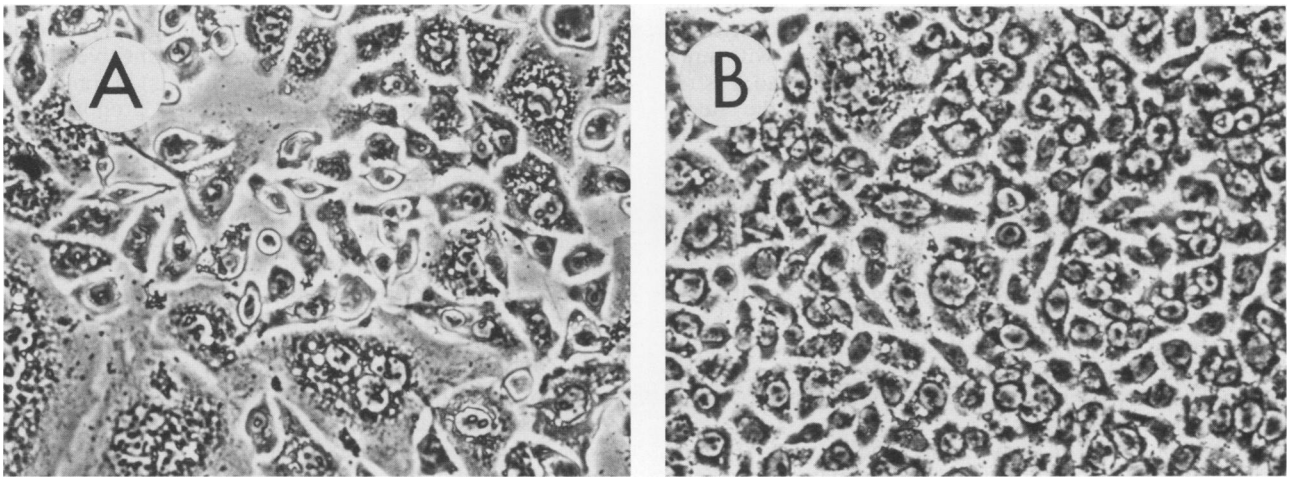

FIG. 2. AGS cell line after 72 h of exposure to *H. pylori* 87A300 and 5 mM urea (A) or the *H. pylori ure1* mutant and 5 mM urea (B). Magnification, ×50.

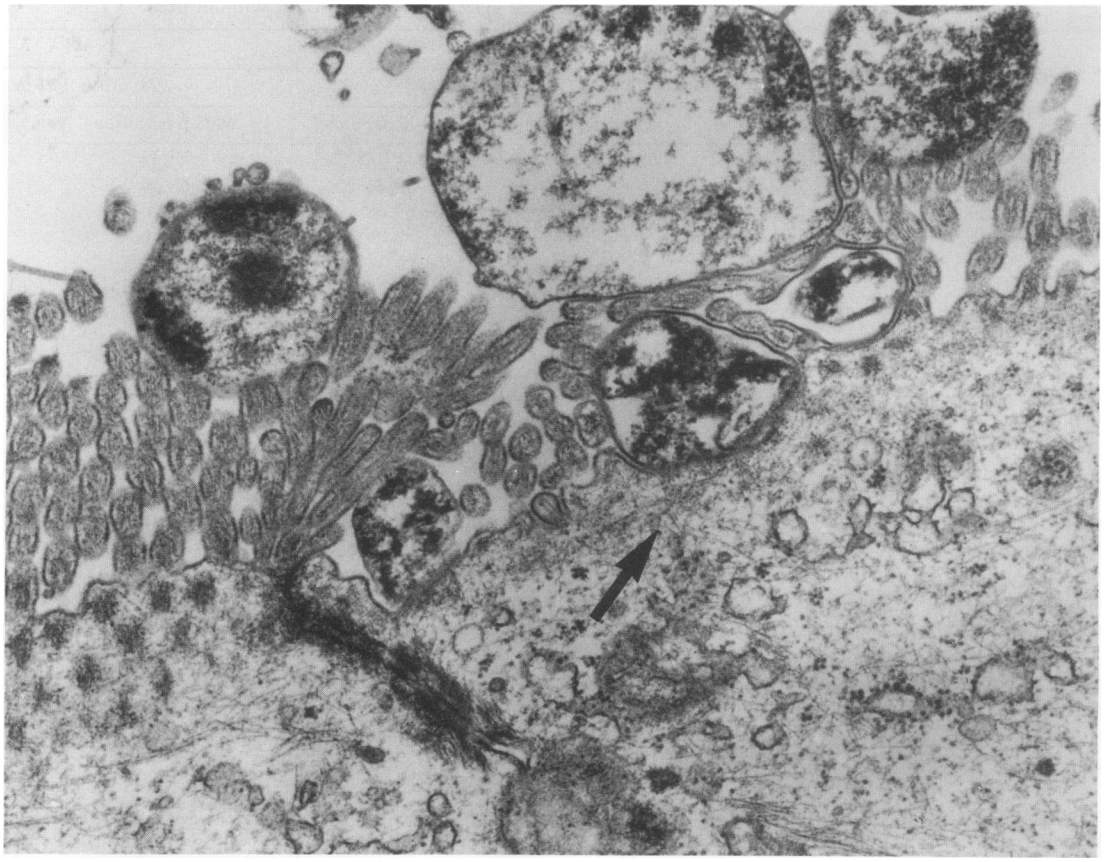

FIG. 3. TEM of polarized Caco-2 cells exposed to *H. pylori* 87A300 plus 5 mM urea for 64 h. Arrow points to a region of cytoskeletal filament condensation. Magnification, ×20,000.

The cytotoxicity of *H. pylori* products on eucaryotic cells was investigated in two experimental systems, by the examination of *H. pylori*-induced vacuolization and cell death and by the examination of the effect of *H. pylori* on polarized cells. The comparison of the effects that the parent strain and the Ure$^-$ mutant had on three tissue culture cell lines was helpful in elucidating possible roles for urease. First, eucaryotic cell death was observed only when *H. pylori* 87A300 was present with urea; in the absence of urea, neither the parent strain nor the *ure1* mutant produced a lethal environment, nor did the presence of the *ure1* mutant with urea cause a decrease in eucaryotic cell viability. Second, gastric cells (KatoIII and AGS) were more susceptible to the effects of urease than HEp-2 cells, indicating the relative tissue specificity of the cytotoxic effect (Table 3). Lastly, vacuolization was directly correlated with the ability of *H. pylori* to produce urease (Fig. 2). It has been debated whether the cytotoxin responsible for causing vacuolization is urease or another *H. pylori* product. The data presented here strongly indicate that the urease indirectly induces the vacuolization observed in vitro through the production of ammonia and, as recently shown by Eaton et al. (3), is most probably responsible for the cellular damage observed in vivo. These results do not necessarily exclude the possibility of another vacuolating cytotoxin, however.

Examination by TEM showed that adherence of *H. pylori* to Caco-2 polarized cells was associated with a localized condensation of cytoskeletal filaments in the apical portion of the cytoplasm directly beneath the region of attachment (Fig. 3). This morphology was apparent with both the parental Ure$^+$ strain and the Ure$^-$ mutant. Examinations of gastric biopsies have shown binding of *H. pylori* to be associated with cuplike structures and adherence pedestals (2, 9), and in vitro studies using a human gastric cell line (AGS) demonstrated the association of actin polymerization with adherence by *H. pylori* (25).

In vivo, *H. pylori* has been observed to localize in close proximity to the tight junctions of gastric epithelial cells and has also been found deep within the junctions; surrounding cells remain intact. The simplest and most sensitive measure of the tight cell junctions (zonula occludens) is transepithelial electrical resistance, which is directly proportional to inorganic ion permeability (5, 14). Although there was a 50.68% decrease in resistance produced upon infection with *H. pylori* 87A300 in the presence of 5 mM urea, the Caco-2 monolayer remained intact as examined by TEM (Fig. 3), with the tight cell junctions appearing basically unchanged. The interaction of *H. pylori* isolates and Caco-2 cells may not necessarily be representative of in vivo processes, however. The lack of any gross change to the zonula occludens is similar to the effects of *Clostridium difficile* toxin A on a polarized monolayer of T_{84} cells (a human intestinal epithelial cell line) (8). Monolayer transepithelial resistance was nearly abolished, yet the monolayers remained confluent, with no evidence of cell damage at the time the maximum resistance response was observed. It was determined that toxin A affected the barrier function by selectively enhancing tight junction permeability, concurrent

with an alteration of the cytoskeletal structure. Studies examining the affects of the cytokine interferon on T_{84} cells likewise showed that although a cytotoxic effect was not observed, monolayer resistance was substantially diminished, most likely by its increasing the permeability of tight junctions (15). Further studies investigating the exact effect by *H. pylori* on the cytoskeletal structure need to be done to determine the mechanisms responsible for the decrease in transepithelial resistance. In addition, the results obtained with polarized gastric cells might provide more relevant information about the precise effect of *H. pylori* infection on tight junction function in the stomach.

The results obtained with Caco-2 polarized monolayers indicate that the *H. pylori* urease was responsible for disruption of the tight cell junctions. The addition of *H. pylori* 87A300 plus urea was followed by decreased resistance of the polarized monolayer over time; the *ure1* mutant plus urea did not have this effect. It appears that *H. pylori* was able to affect the tight junctions in vitro, resulting in an ionic flow across the monolayer possibly equivalent to a nutrient flow being created in vivo. Increases in the permeability resulting from leaky tight junctions could prevent the cell monolayer from functioning as a normal epithelium. It is interesting that studies have demonstrated that carcinomas either lack or have structurally altered tight junctions (16, 27). While the presence of *H. pylori* has been correlated with intestinal-type gastric carcinoma (20–22), a relationship between the alteration of the epithelial monolayer and carcinoma has not yet been established. These observations suggest that experiments to further delineate the mechanisms by which *H. pylori* perturbs the integrity of polarized epithelia may elucidate the pathogenesis of diseases associated with chronic infection.

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