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Surface Localization of *Helicobacter pylori* Urease and a Heat Shock Protein Homolog Requires Bacterial Autolysis

SUHAS H. PHADNIS,[1,2] MARY H. PARLOW,[2] MARILYN LEVY,[3] DAG ILVER,[4] CASEY M. CAULKINS,[2] JAMES B. CONNORS,[2] AND BRUCE E. DUNN[1,2]*

Department of Pathology, Medical College of Wisconsin,[1] and Pathology and Laboratory Medicine Service, Department of Veterans Affairs, Clement J. Zablocki Medical Center,[2] Milwaukee, Wisconsin; Department of Cell Biology and Physiology, Washington University, St. Louis, Missouri[3]; and Department of Microbiology, University of Umea, Umea, Sweden[4]

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Helicobacter pylori **is a gram-negative bacterium which causes chronic gastritis and is associated with peptic ulcer disease, gastric carcinoma, and gastric lymphoma. The bacterium is characterized by potent urease activity, thought to be located on the outer membrane, which is essential for survival at low pH. The purpose of the present study was to investigate mechanisms whereby urease and HspB, a GroEL homolog, become surface associated in vitro. Urease, HspB, and catalase were located almost exclusively within the cytoplasm in fresh log-phase cultures assessed by cryo-immunoelectron microscopy. In contrast, significant amounts of surface-associated antigen were observed in older or subcultured preparations concomitantly with the appearance of significant amounts of extracellular antigen, amorphous debris, and membrane fragments. By use of a variety of biochemical methods, a significant fraction of urease and HspB was associated with the outer membrane in subcultured preparations of *H. pylori*. Taken together, these results strongly suggest that *H. pylori* cells undergo spontaneous autolysis during culture and that urease and HspB become surface associated only concomitant with bacterial autolysis. By comparing enzyme sensitivity to flurofamide (a potent, poorly diffusible urease inhibitor) in whole cells with that in deliberately lysed cells, we show that both extracellular and intracellular urease molecules are active enzymatically. Autolysis of *H. pylori* is an important phenomenon to recognize since it likely exerts significant effects on the behavior of *H. pylori*. Furthermore, the surface properties of *H. pylori* must be unique in promoting adsorption of cytoplasmic proteins.**

Helicobacter pylori is a gram-negative bacterium which causes chronic gastritis and is associated with peptic ulcer disease, gastric carcinoma, and gastric lymphoma (4, 41, 43, 44).

All fresh isolates of *H. pylori* express significant urease activity, which appears to be essential to survival and pathogenesis of the bacterium. In vivo, it is thought that hydrolysis of urea generates ammonia to counterbalance gastric acid, presumably forming a neutral microenvironment surrounding bacteria within the gastric lumen. Isogenic mutants of *H. pylori* and of the related bacterium *Helicobacter mustelae*, which fail to express urease activity, are unable to colonize the gastric mucosa of gnotobiotic piglets and ferrets, respectively (1, 17). In vitro, *H. pylori* cells are resistant to acid only in the presence of urea, whereas urease-negative bacteria are not resistant to acid (32). The generation of ammonium ions in close proximity to gastric epithelium may have a variety of pathogenic effects, including toxicity to gastric mucosal cells, weakening of intracellular junctions, and reduction of transmucosal potential difference (2, 55).

H. pylori appears unique in that three proteins, urease, HspB, i.e., a homolog of the GroEL family of heat shock proteins, and superoxide dismutase (SOD), which are found exclusively within the cytoplasm in other bacteria, are associated with the outer membrane (5, 18, 24, 56). The strong immune response to urease and HspB observed in humans infected with *H. pylori* (13, 30) and the promising results obtained with purified recombinant *H. pylori* urease apoenzyme as a vaccine candidate against *Helicobacter* infection in animal models (6, 22, 39) suggest that urease and HspB are readily accessible to the immune system in vivo.

We show in vitro that urease, HspB, and catalase are intrinsic cytoplasmic proteins in freshly subcultured bacteria. These cytoplasmic proteins are released by autolysis and become adsorbed to the surface of intact bacteria, reflecting unique characteristics of the outer membrane of *H. pylori*. Bacterial autolysis and surface characteristics likely have a significant influence on the behavior of *H. pylori* in vitro and are essential to understanding the pathogenesis of this widespread pathogen.

(Portions of this work were presented at the 1993 and 1995 meetings of the European *H. pylori* Study Group in Brussels, Belgium, and Edinburgh, Scotland [14, 47], respectively.)

MATERIALS AND METHODS

Bacterial culture. *H. pylori* 84-183 (13) and NCTC 11638 were grown on Trypticase soy agar containing sheep blood (Remel, Lenexa, Kans.) at 37°C in an atmosphere containing 10% CO_2, 5% O_2, and 85% inert gas (N_2 or Ar). Cultures on agar plates were established in one of two ways. Freshly subcultured (fresh sub) bacteria were prepared either by repeated subculture at 24-h intervals or by culturing previously frozen stock preparations immediately from storage vials, with a single subculture at 24 h prior to analysis. Cultures termed repeat sub were subcultured at least once at 72-h intervals (as performed routinely in our laboratory to obtain sufficient material for protein characterization) and then harvested for analysis.

Enzyme assays. Urease activity was assayed with a coupled enzyme assay as described previously (11). Activities of catalase and SOD were measured by published methods (25, 29, 33).

Enzyme purification and specific antiserum. Purification of *H. pylori* urease and HspB and generation of urease- and HspB-specific rabbit antiserum have been described previously (11, 15). Purification of *H. pylori* catalase and gener-

* Corresponding author. Mailing address: Pathology and Laboratory Medicine Service (113), Department of Veterans Affairs, Clement J. Zablocki Medical Center, 5000 W. National Ave., Milwaukee, WI 53295-1000. Phone: (414) 384-2000, ext. 1285. Fax: (414) 382-5319.

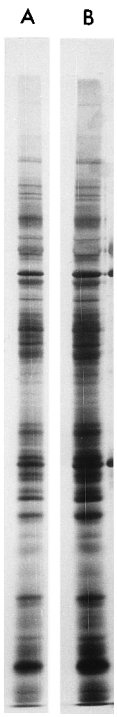

FIG. 1. Silver-stained SDS-PAGE profiles of water wash (A) and whole-cell (B) preparations of *H. pylori* 84-183 at 48 h (repeat sub). Each lane contains approximately 2 μg of protein.

ation of specific goat antiserum were performed as described before (12). Immunoblotting was performed as described previously (11, 15).

Release of enzyme activity from *H. pylori* during culture. Bacterial cells grown on agar plates were collected in ice-cold 20 mM phosphate buffer containing 0.15 M NaCl (pH 7.0; PBS) and then harvested by centrifugation (5,000 × *g* for 30 min at 4°C). The cells were resuspended in 30 ml of distilled water, vortex mixed for 45 to 60 s, and then sedimented by centrifugation (10,000 × *g* for 20 min at 4°C), and enzyme activity in the pellet and supernatant (i.e., water wash) fractions were determined. Protein profiles of water wash and whole-cell preparations were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11).

Radioiodination of whole bacterial cells and lysates. Whole cells and bacterial lysates produced by passing cells through a French pressure cell at 20,000 lb/in^2 (in the presence of 0.1 μM phenylmethylsulfonyl fluoride and 1 μM leupeptin) were subjected to radioiodination, two-dimensional gel electrophoresis, and autoradiography, as described previously (13).

Sarkosyl-insoluble membrane preparations. Sarkosyl-insoluble membrane preparations were prepared as described previously (13).

Preparation of bacterial outer membranes by sucrose gradient centrifugation. Isolation of outer membrane preparations was performed by methods described by Osborn and Munson (42). Briefly, bacteria grown on agar plates for 48 h (repeat sub) were harvested in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4) and lysed in a French pressure cell as described above. Whole cells were removed by centrifugation (10,000 × *g* for 10 min at 4°C). A 5-ml portion of the lysate was then layered onto a 15 to 60% (wt/vol) sucrose step gradient prepared in an SW41 centrifuge tube. All sucrose solutions were prepared in 10 mM HEPES buffer (pH 7.4). Gradient tubes were then centrifuged (134,000 × *g* for 4 h at 4°C) with a Beckman L8 ultracentrifuge. After centrifugation, the cytoplasmic and periplasmic fractions were removed from the top 15% fraction. The total membrane fraction, which migrated to the 60% sucrose cushion, was then removed. A 1.5-ml aliquot of the total membrane fraction was then layered onto a 40 to 60% discontinuous sucrose gradient prepared in a separate SW41 centrifuge tube. This preparation was centrifuged at 134,000 × *g* for 36 h at 4°C. After centrifugation, the outer membrane fraction that had migrated to the 55% sucrose cushion was harvested and stored at −20°C until analyzed.

Ultrastructural localization of bacterial antigens. *H. pylori* cultures were fixed in 2% paraformaldehyde–0.2% glutaraldehyde–PBS (pH 7.2) for 2 h at room temperature. Bacteria were then embedded in 10% gelatin (54) but without fixation of the gelatin. After the bacteria were pelleted, the gelatin was solidified on ice. Blocks for ultracryotomy were prepared and immunolabeled with 10% goat serum in the blocking buffer (53). Immunolabeling with primary antibodies (diluted 1:400 to 1:800) was carried out for 2 h. Incubation with secondary antibodies (Jackson ImmunoResearch Labs, West Grove, Pa.; 12 nM goat anti-rabbit immunoglobulin G-colloidal gold, diluted 1:25) was carried out for 1 h. Sections were stained with uranyl acetate and embedded in methylcellulose by a modification of the Tokuyasu method (59) introduced by Griffiths et al. (23). Specimens were viewed and photographed with a Zeiss 902 electron microscope. Urease, HspB, and catalase were localized in *H. pylori* 84-183 and NCTC 11638 grown on agar plates at 24, 48, 72, and 96 h (fresh sub) and after 24 and 72 h (repeat sub). Control sections were incubated with preimmune serum and then secondary antibody or with secondary antibody only.

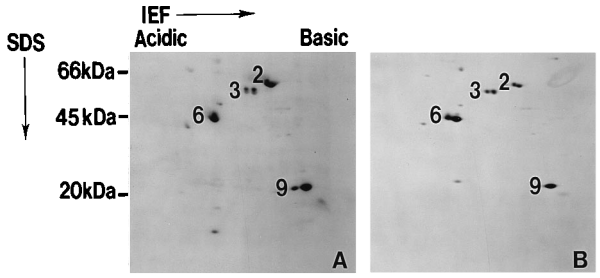

FIG. 2. Autoradiographs of radioiodinated *H. pylori* proteins separated by two-dimensional gel electrophoresis. All gels are oriented with the acidic side of the isoelectric focusing (IEF) gel to the left and high-molecular-mass proteins at the top. Labeled proteins are identified as follows as described previously (13): 2, heavy subunit of urease (UreB); 3, HspB; 6 and 9, identity not known. (A) Surface-radioiodinated whole cells of *H. pylori* 84-183 at 48 h (repeat sub); (B) radioiodinated proteins of intentionally lysed *H. pylori* 84-183 at 48 h (repeat sub).

Urease inhibition studies. Flurofamide (kindly provided by R. Leunk), a potent urease inhibitor (50% inhibitory concentration, 60 nM), was used at a final concentration of 1 μM. Flurofamide stock (100 μM) was prepared by dissolving in water. Acetohydroxamic acid, known to diffuse across bacterial membranes, was used at a final concentration of 7 μM (36). Briefly, whole-cell suspensions of *H. pylori* 84-183 were incubated for 30 min at room temperature with the inhibitor prior to assessing urease activity. Suspensions were washed twice in PBS and then lysed by passage through a French pressure cell at 20,000 lb/in^2. After measurement of urease activity, lysates were then incubated with urease inhibitors for 30 min at room temperature prior to measuring urease activity.

RESULTS

Release of enzyme activity. To measure the relative amounts of urease, catalase, and SOD released from *H. pylori* 83-184 grown on agar plates, we briefly subjected repeatedly subcultured bacteria to vortex mixing in distilled water after culture for 48 to 96 h. The amounts of enzyme activity recovered in the supernatant, expressed as percentages of the total enzyme activity at 48, 72, and 96 h, respectively, were 35% ± 12%, 62% ± 21%, and 52% ± 18% for urease, 29% ± 14%, 72% ± 31%, and 46% ± 19% for SOD, and 16% ± 12%, 21% ± 10%, and 16% ± 11% (mean ± standard deviation; n = 3 to 5) for catalase. Silver-stained protein profiles of water wash preparations and of whole cells of *H. pylori* 84-183 at 48 h (repeat sub) adjusted for protein content showed similar protein bands (Fig. 1).

Radioiodination of whole bacterial cells and lysates. At 48 h (repeat sub), the major proteins of whole-cell preparations surface radioiodinated with immobilized lactoperoxidase (Fig. 2) were very similar to those radioiodinated by identical techniques in preparations of *H. pylori* 84-183 deliberately subjected to lysis by passage through a French pressure cell (Fig. 2B). The major radiolabeled proteins in both whole cells and lysates were UreB (protein 2) and HspB (protein 3) and proteins 6 and 9 as described previously (13).

Sarkosyl-insoluble membrane preparations. In 48-h (repeat sub) preparations of *H. pylori* 84-183, the amounts of urease and SOD activity recovered in the cytoplasmic, Sarkosyl-insoluble (outer membrane), and Sarkosyl-soluble (inner mem-

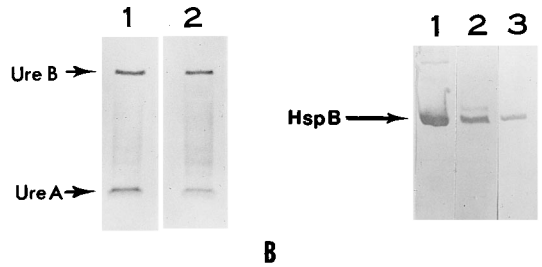

FIG. 3. Immunoblot of outer membrane proteins of *H. pylori* with anti-urease and anti-HspB. (A) Outer membrane proteins labeled with anti-urease (diluted 1:1,000). Lanes: 1, 1 μg of Sarkosyl-insoluble membrane proteins; 2, 1 μg of outer membrane proteins prepared by sucrose gradient centrifugation. UreB (62 kDa) and UreA (30 kDa) are the two subunits of *H. pylori* urease. (B) Outer membrane proteins labeled with anti-HspB (diluted 1:500). Lanes: 1, 0.5 μg of purified HspB; 2, 1 μg of Sarkosyl-insoluble membrane proteins; 3, 1 μg of outer membrane proteins prepared by sucrose gradient centrifugation.

brane) fractions, expressed as percentages of the total enzyme activity, were 54% ± 23%, 18% ± 9%, and 28% ± 14%, and 94% ± 27%, 1.5% ± 2%, and 5% ± 2% (mean ± standard deviation; $n = 3$ to 4), respectively. Separation of Sarkosyl-insoluble membrane proteins by SDS-PAGE followed by immunoblotting with anti-urease and anti-HspB hyperimmune sera demonstrated that both proteins were present (Fig. 3).

Sucrose gradient membrane preparations. Separation of outer membrane preparations isolated by sucrose gradient centrifugation followed by immunoblotting with anti-urease and anti-HspB hyperimmune sera demonstrated the presence of both proteins (Fig. 3).

Cryo-immunolocalization of bacterial antigens. Cryo-immunolabeling was used to preserve the antigenicity of the proteins. It was deemed necessary to localize proteins in thin sections of bacteria (rather than in intact bacterial cells) to assess the relative distribution of antigens within both the cytoplasmic, periplasmic, and surface-associated compartments.

In general, localization of urease did not differ between the two strains, which were grown under comparable conditions. Numerous colloidal gold particles were observed in bacteria by use of antisera against urease and HspB. In contrast, many fewer particles were observed when antiserum against catalase was used (data not shown).

All three antigens were localized almost exclusively within the cytoplasm at 24 h (fresh sub) (Fig. 4a and data not shown; also see Fig. 6a). Urease had a similar distribution at 48 h (fresh sub) (Fig. 4b). Of interest, rare bacteria showing surface association of urease and HspB were noted at 24 h (fresh sub) (Fig. 4a and data not shown). Extracellular debris and free antigen (not associated with debris) were minimal at 24 or 48 h (fresh sub). In contrast, at 72 h (fresh sub), urease was not only present within the cytoplasm but also associated with the surface of many bacteria and associated with extracellular debris (Fig. 4c). At 96 h (fresh sub), only coccoid forms (40), in which urease appeared almost exclusively within the cytoplasm (Fig. 4d), were observed. Very little extracellular debris or free urease antigen was observed between the coccoid forms.

At 24 to 72 h (repeat sub), there was significantly more extracellular debris, which appeared as amorphous electron-dense material, occasionally associated with distinct fragments of trilaminar cellular membranes, than was observed in 24- to 48-h (fresh sub) preparations (compare Fig. 5a and 5b with 4a and 4b). In some cases, such bacterial debris was associated with the surface of intact bacterial cells. Three patterns of distribution of urease, HspB, and catalase were noted within apparently intact cells: (i) primarily cytoplasmic, (ii) cytoplasmic plus surface associated, and (iii) primarily surface associated (Fig. 5a, 5b, 6b, and data not shown). Abundant antigen was associated with extracellular debris and was free within extracellular spaces. Bacterial morphology and antigen localization as a function of culture type and age are summarized in Fig. 7.

Urease inhibition studies. The rationale for these experiments was to determine whether both cytoplasmic and extracellular or surface-associated forms of urease are active enzymatically. We reasoned that exposure of intact cells to a nondiffusible inhibitor would inhibit only extracellular or surface-associated enzyme; deliberately lysing cells after washing away external inhibitor would reveal a second (cytoplasmic) compartment of enzyme activity. At 48 h (fresh sub), 30 min of exposure to 1 μM flurofamide inhibited 48% of the urease activity apparent in whole-cell preparations grown on agar plates. However, after bacterial cells were washed twice and then lysed by passage through a French pressure cell, urease activity consisting of 26% of that present initially within whole cells was detectable. The latter urease activity was completely inhibited by reexposure to 1 μM flurofamide (Table 1). Thus, the inability of flurofamide to inhibit cytoplasmic urease was not due to intrinsic resistance to this inhibitor but to compartmentalization of active enzyme within intact bacteria. In bacteria repeatedly subcultured prior to analysis at 48 h, exposure of whole cells to 1 μM flurofamide inhibited over 90% of the detectable urease activity. In this case, only 8% of the initial urease activity was recoverable in lysates of washed cells. The latter enzyme activity was completely inhibited by reexposure to 1 μM flurofamide. In contrast, 7 μM acetohydroxamic acid, a diffusible inhibitor of urease activity, inhibited >99% of urease activity in both fresh and repeatedly subcultured bacteria at 48 h; no additional enzyme activity was recovered after cells were washed and lysed (Table 1).

DISCUSSION

Biochemical and ultrastructural localization studies by a variety of investigators have demonstrated that urease, HspB, and SOD are associated with the surface of *H. pylori* grown with both solid and liquid substrates (5, 13, 18, 24, 56). *H. pylori* appears unique in possessing surface-associated urease; in other bacteria, urease appears strictly within the cytoplasmic compartment (35, 38). In fact, the surface association of urease is a significant factor in testing recombinant *H. pylori* urease apoprotein as a vaccine against *Helicobacter* infection (6, 22). Studies to confirm prophylactic and therapeutic activities of the urease vaccine against *H. pylori* infection in mice, monkeys, and cats are under way; a phase I clinical trial to assess toler-

TABLE 1. Effects of flurofamide (1 μM) and acetohydroxamic acid (7 μM) on urease activity in 48-h (fresh sub) whole cells and lysates of *H. pylori*

Prepn	Urease activity (total U) after treatment with[a]:	
	Flurofamide	AHA[b]
Whole cells (no inhibitor)	1,454	792
Whole cells + inhibitor	759 (52)	5.6 (0.7)
Washed, lysed cells	381 (26)	4.5 (0.6)
Lysed cells + inhibitor	3 (0.2)	4.3 (0.5)

[a] Values in parentheses are percentages of initial activity present in whole cells.
[b] AHA, acetohydroxamic acid.

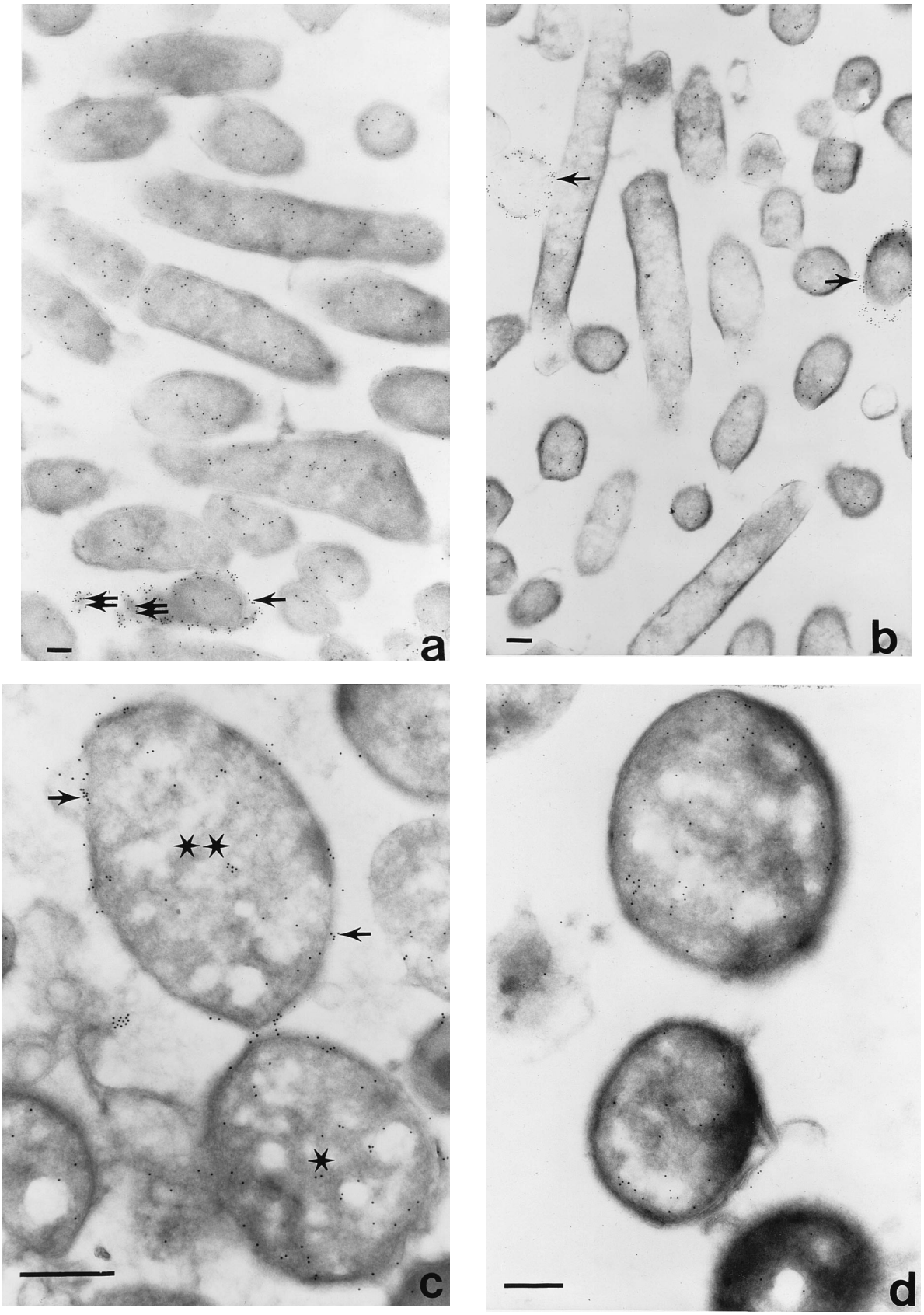

FIG. 4. Cryo-immunoelectron microscopic localization of urease in *H. pylori* 84-183 at 24 to 96 h (fresh sub). (a) At 24 h, urease is almost exclusively cytoplasmic in distribution, although a single cell (arrow) demonstrates surface-adsorbed urease. Urease is also adsorbed to extracellular debris (double arrows). Bar, 0.5 μm. (b) At 48 h, urease appears primarily within the cytoplasm. Very little surface-adsorbed or extracellular antigen is observed (arrows). Bar, 0.5 μm. (c) At 72 h, urease is distributed within the cytoplasm only in some cells (asterisk) and within the cytoplasm and absorbed to the surface (arrows) of others (double asterisks). Bar, 0.2 μm. (d) At 96 h, only coccoid forms in which urease is strictly cytoplasmic in distribution are identified. Bar, 0.2 μm.

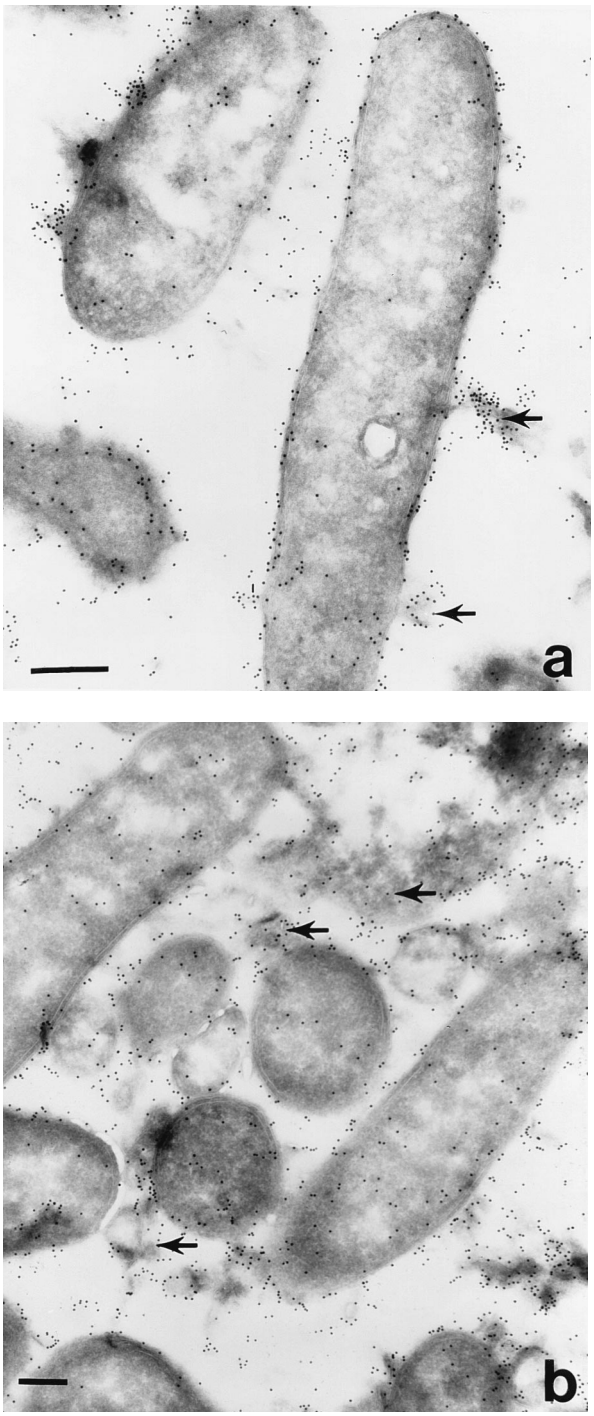

FIG. 5. Cryo-immunoelectron microscopic localization of urease in *H. pylori* 84-183 at 24 and 72 h (repeat sub). (a) At 24 h, there is extracellular debris and membrane fragments (arrows) and surface-adsorbed urease. Cytoplasmic enzyme is also noted. Bar, 0.5 μm. (b) At 72 h, extracellular debris (arrows) is more prominent than at 24 h, and urease is extracellular, surface adsorbed, and cytoplasmic in nature. Bar, 0.5 μm.

ability of the vaccine in humans is also under way (39). However, the mechanisms whereby urease, HspB, and SOD become associated with the outer membrane of *H. pylori* are not known.

Taken together, our results suggest that urease, HspB, and catalase are intrinsic cytoplasmic proteins in log-phase bacteria; these proteins become associated with the outer membrane of *H. pylori* only concomitantly with autolysis. We propose that cytoplasmic proteins are released from autolyzed bacteria; such proteins accumulate in repeatedly subcultured preparations of *H. pylori* and become adsorbed onto the outer membrane of remaining intact bacteria. The amount of adsorbed protein is variable and dependent upon culture age and conditions. Figure 8 outlines the proposed life cycle of *H. pylori* in vitro.

The evidence supporting these conclusions is summarized as follows. First, the major radioiodinated proteins (including UreB, HspB, and the unidentified proteins 6 and 9) of whole cells and intentionally lysed preparations were essentially identical, suggesting that autolysis of a significant proportion of *H. pylori* cells occurred spontaneously prior to harvesting. Supporting this hypothesis, SDS-PAGE analysis of proteins in supernatant fractions obtained after washing repeatedly subcultured 48- to 72-h whole-cell preparations of *H. pylori* with water revealed complex protein patterns similar to those observed in pellet fractions. Further, the relative amounts of urease, SOD, and catalase recoverable in water wash preparations were dependent upon culture age and were enzyme specific. In all cases, the amount of recoverable enzyme (presumably extracellular at the time of recovery) was greater at 72 h than at 48 h. These results are unlikely to be due to water-induced lysis alone, since brief vortex mixing of *H. pylori* in water does not significantly decrease bacterial survival (11, 46). Of interest, water-extractable proteins of *H. pylori* have been used previously as starting material from which to purify urease, since it is possible to recover up to 75% of urease activity in water-soluble form from 72-h-old bacteria, repeatedly subcultured to maximize protein yield (11). In addition, urease and HspB copurified with Sarkosyl-insoluble membrane fractions and outer membranes prepared by sucrose gradient centrifugation. Similarly, Doig and Trust (10) reported that urease is a major immunogenic component of Sarkosyl-insoluble membrane preparations. Using cryo-immunogold labeling in two separate strains, we demonstrated that urease, HspB, and catalase are located within the cytoplasmic compartment in most bacteria in 24- to 48-h-old fresh preparations of *H. pylori*. In contrast, in 24- to 72-h (repeat sub) preparations, large amounts of surface-associated antigen were observed in conjunction with significant amounts of extracellular antigen, amorphous debris and membrane fragments, strongly suggestive of autolysis. The observation that catalase also becomes associated with the surface of *H. pylori* in vitro suggests that the mechanisms for surface adsorption of cytoplasmic proteins are not specific for urease or HspB.

Both the surface-associated and cytoplasmic forms of urease exhibit enzyme activity. On the basis of our inhibition studies with flurofamide, approximately 50% of urease activity was surface associated in 48-h (fresh sub) *H. pylori*, while approximately 90% was surface associated in 72-h (repeat sub) bacteria.

An alternative hypothesis to explain surface association of urease and HspB in *H. pylori* is that an active export system(s) exist for these proteins. Three basic pathways are recognized whereby proteins are exported onto the outer membrane or secreted into the extracellular space of bacteria. First, the general secretory pathway requires that the secreted protein possess a hydrophobic N-terminal signal sequence which is cleaved during secretion (49). The *H. pylori* genes for urease (9, 21, 37), HspB (30, 57), SOD (56), and catalase (46a) all lack an N-terminal signal sequence, making it unlikely that these proteins are exported by the general secretory pathway. Sec-

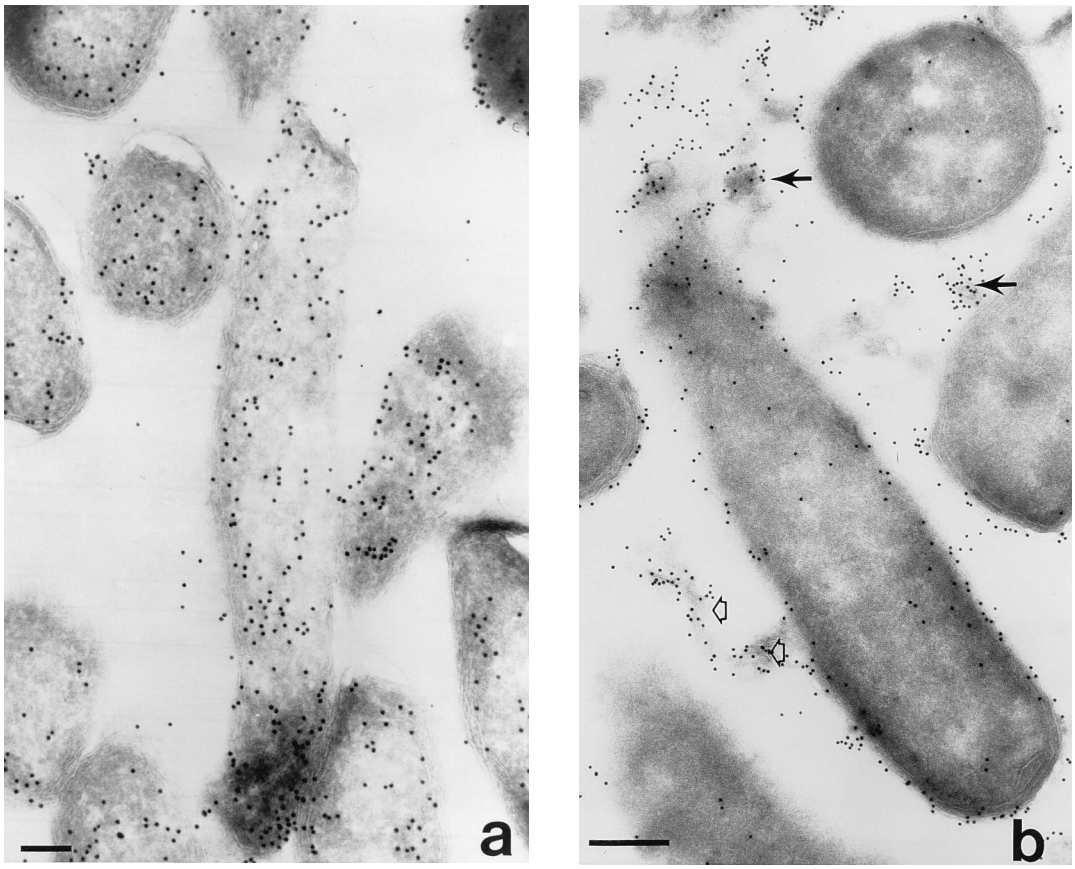

FIG. 6. Cryo-immunoelectron microscopic localization of HspB in *H. pylori* 84-183 at 24 (fresh sub) and 72 (repeat sub) h. (a) At 24 h (fresh sub), HspB is located almost exclusively within the cytoplasm. Bar, 0.5 μm. (b) At 72 h (repeat sub), extracellular debris (arrows) is prominent, and HspB is extracellular, surface adsorbed, and cytoplasmic in nature. Open arrows show a portion of a flagellum with associated HspB. Bar, 0.5 μm.

ond, transporters in the ABC export system have a conserved C-terminal ATP-binding motif (20). Typically, the gene encoding the secreted protein is located within an operon containing exported protein, accessory proteins, and modification enzymes (20). Finally, proteins secreted by the type III secretion system lack typical signal sequences and require the products of large gene clusters consisting of at least 12 genes for their secretion (27). Accessory protein transporters analogous to those in the ABC and type III export systems have not been identified in the operons or gene clusters of the *H. pylori* genes encoding urease, HspB, catalase, or SOD, despite the existence of extensive sequence data (9, 21, 30, 56, 57). It is well-known, however, that the vacuolating cytotoxin of *H. pylori* possesses an N-terminal sequence, which is processed, and appears to be secreted during culture (7, 48, 51, 58). Thus, *H. pylori* does possess conventional protein secretory mechanisms; autolysis is not the sole means by which proteins are released from this bacterium.

Can surface localization of urease and HspB be explained by invoking one (or more) active export mechanisms? To account for the observations reported herein, such an export mechanism(s) should have a variety of unique characteristics. First, an export mechanism(s) should be repressed in early-log-phase cells and be induced in late-log-phase cells and again be repressed in coccoid cells. Second, to account for the observation that individual bacteria in the late log phase demonstrate antigen which is located within the cytoplasm only, on the surface only, or in both surface and cytoplasmic compartments, the export mechanism(s) must be induced asynchronously within individual bacteria. Third, the export mechanism(s) would apparently overshoot the bacterial surface, resulting in secretion of urease and HspB into the extracellular space. Finally, since both cytoplasmic and surface-associated forms of urease are enzymatically active, the presumed export mechanism(s) must have the capacity to export a large, fully assembled, active enzyme; alternatively, *H. pylori* has the capacity to assemble active urease in both the cytoplasmic and periplasmic space or surface compartments. Urease is a nickel-dependent, hexam-

Fresh *H. pylori*	→ 1 day	→ 2 day	→ 3 day	→ 4 day	
Morphology	—	Rod and Spiral shapes		—	Coccoid
Localization	Cytoplasmic	Cytoplasmic	Surface and Cytoplasmic		Cytoplasmic
Subcultured *H. pylori*	→ 1 day	→ 2 day	→ 3 day	→ 4 day	
Morphology	—	Rod and Spiral shapes		—	Coccoid
Localization	Surface and Cytoplasmic	Surface and Cytoplasmic	Surface and Cytoplasmic		ND

FIG. 7. Summary of *H. pylori* morphology and antigen localization assessed by cryo-immunoelectron microscopy as a function of culture type and age.

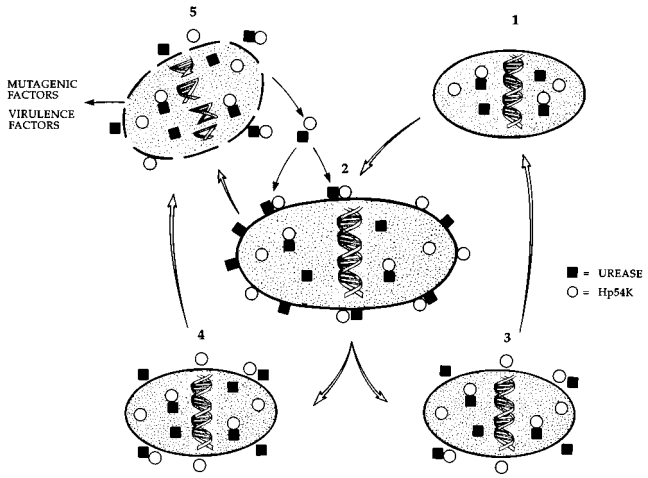

FIG. 8. Proposed life cycle of *H. pylori* in vitro. Open arrows show proposed changes from one bacterial form to another. Linear arrows represent pathways followed by cytoplasmic proteins released from autolyzed bacteria. First, log-phase *H. pylori* bacteria express urease and HspB (Hp54K) strictly within the cytoplasm (form 1). Under appropriate environmental and/or genetic impetus, autolysis occurs within a fraction of the bacterial population (form 5), resulting in release of cytoplasmic proteins, including urease, HspB, and putative virulence or mutagenic factors; adsorption of urease and HspB onto the surface of intact bacteria then follows (form 2). *H. pylori* with surface-adsorbed urease and HspB (form 2) may either divide to form daughter cells with surface-adsorbed proteins (forms 3 and 4) or may undergo autolysis (form 5). Some daughter cells (form 3) presumably convert to coccoid forms (data not shown) or to bacillary forms in which urease and HspB are present only within the cytoplasm (form 1).

eric molecule with a molecular size of approximately 540 kDa consisting of equal numbers of large (UreB, approximately 62-kDa) and small (UreA, approximately 30-kDa) subunits (11, 16, 19, 26). The structural genes *ureA* and *ureB* are present in a cluster of seven genes essential for expression of enzymatic activity (9, 37, 38). In the face of strong evidence supporting autolysis and surface adsorption, the occurrence of protein export in *H. pylori* by the hypothetical events outlined above appears extremely unlikely.

The present observations demonstrate that urease is not an intrinsic component of the surface of *H. pylori*. If the infective form of *H. pylori* for humans lacks surface-associated urease, then the distinct possibility exists that urease-based vaccines, now under development, will not prove efficacious against the naturally infective form of *H. pylori*. We recognize that urease-based vaccines have proven efficacious in mice infected experimentally with *Helicobacter felis* (6, 22, 34). However, it is likely that the *H. felis* preparations used to challenge mice possess significant surface-associated urease, as a result of in vitro culture conditions, and hence may not represent the naturally infective form of the bacterium.

H. pylori is not unique in exhibiting autolysis. For example, autolysis of *Streptococcus pneumoniae* occurs in vivo and in vitro and is regulated genetically (45); inactivation of the autolysin gene decreases virulence of *S. pneumoniae* in an animal model (3). Autolysis of *Neisseria gonorrhoeae* leads to liberation of DNA, which is taken up by viable cells, accounting for transformation leading to antigenic variation of pilin (28, 52). Although not analyzed directly, it does not appear likely that release of proteins by autolysis leads to significant adsorption of cytoplasmic proteins onto the bacterial surface in either *S. pneumoniae* or *N. gonorrhoeae* (45, 52). Therefore, the surface properties of *H. pylori* must be unique in promoting the adsorption of cytoplasmic proteins onto its surface.

Autolysis of *H. pylori* is an important phenomenon to recognize since it likely exerts significant effects on the behavior of *H. pylori* in vivo as well as in vitro. First, autolysis would help to explain how the noninvasive bacterium *H. pylori* can present virulence factors and antigens to the gastric mucosa and immune system in vivo. In this regard, both urease and HspB induce significant humoral immune responses in infected humans (13, 30), and urease, per se, can activate neutrophils (31, 50) and may be a mediator of inflammation (8, 31). Second, autolysis may play an important role in immune evasion by overwhelming the immune system with released cytoplasmic proteins. Supporting this proposal is the observation that free urease has been detected in the lamina propria of infected humans (31). In a similar vein, surface-adsorbed urease and HspB would help to conceal intrinsic outer membrane proteins of *H. pylori* from immune surveillance. Third, autolysis with subsequent surface adsorption of cytoplasmic proteins likely influences the specificity and magnitude of adherence of *H. pylori* to eukaryotic cells in vitro. Finally, masking of integral outer membrane proteins of *H. pylori* in vitro helps to explain the recognized difficulty of identification of such proteins (10, 13). As a result, the choice of antigens for oral immunization against *H. pylori* is greatly influenced, since few definitive intrinsic outer membrane proteins have been identified to date. Studies to further characterize autolysis of *H. pylori* both in vitro and in vivo are in progress in this laboratory.

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ADDENDUM IN PROOF

Since the manuscript was reviewed, cytoplasmic location of urease in *H. pylori* has been confirmed by R. Leunk and C. Catrenich at Procter and Gamble, Cincinnati, Ohio.

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