

Structure, Function and Localization of *Helicobacter pylori* Urease

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Helicobacter pylori is the causative agent of most cases of gastritis. Once acquired, *H. pylori* establishes chronic persistent infection; it is this long-term infection that, in a subset of patients, leads to gastric or duodenal ulcer, gastric cancer or gastric MALT lymphoma. All fresh isolates of *H. pylori* express significant urease activity, which is essential to survival and pathogenesis of the bacterium. A significant fraction of urease is associated with the surface of *H. pylori* both *in vivo* and *in vitro*. Surface-associated urease is essential for *H. pylori* to resist exposure to acid in the presence of urea. The mechanism whereby urease becomes associated with the surface of *H. pylori* is unique. This process, which we term "altruistic autolysis," involves release of urease (and other cytoplasmic proteins) by genetically programmed autolysis with subsequent adsorption of the released urease onto the surface of neighboring intact bacteria. To our knowledge, this is the first evidence of essential communal behavior in pathogenic bacteria; such behavior is crucial to understanding the pathogenesis of *H. pylori*.

INTRODUCTION

Since the discovery of *Helicobacter pylori* in 1982 [1], its critical role in many gastroduodenal diseases has become clear. *H. pylori* is the causative agent of most cases of gastritis [2]. Once acquired, *H. pylori* establishes chronic persistent infection; it is this long-term infection that, in a subset of patients, leads to gastric or duodenal ulcer, gastric cancer or gastric MALT lymphoma [2-5].

H. pylori colonizes the human stomach, which, due to the highly acidic pH, is a hostile environment for most microorganisms and as a result, is considered the first line of defense against most gastrointestinal pathogens. It has been estimated that exposure to gastric acid kills more than 99.9 percent of ingested *Salmonella* and *Vibrio* sp. [6]. *H. pylori* survives in this acidic environment by producing abundant quantities of urease, which hydrolyses urea into ammonia and CO₂, thought to result in generation of a pH-neutral micro-environment.

Urease is not only an essential factor for colonization of *H. pylori*, but contributes to pathogenesis by diverse mechanisms. In addition, urease-based vaccines have proven efficacious against *Helicobacter* infection in animals and are now in clinical trials in humans.

In this review, we highlight available information concerning the structure, function and subcellular localization of *H. pylori* urease. We place particular emphasis on the unique mechanisms whereby urease becomes associated with the surface of *H. pylori*, since surface-association of urease is crucial to survival of this important gastroduodenal pathogen.

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^bAbbreviations: LT, labile enterotoxin.

GENETICS AND ENZYMOLOGY OF *H. PYLORI* UREASE

All fresh isolates of *H. pylori* express significant urease activity, which is essential to survival and pathogenesis of the bacterium [7-9]. Urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia and carbamate, which, in turn, spontaneously decomposes to yield another molecule of ammonia and carbonic acid. In solution, the released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms, respectively. The net effect of these reactions is an increase in pH [10]. Ammonium, not ammonia, is the toxic form of the molecule [10, 11]. Within the gastric lumen, it is thought that hydrolysis of urea generates ammonia to counterbalance gastric acid, presumably forming a neutral microenvironment surrounding *H. pylori*.

H. pylori urease has been purified to homogeneity; the enzyme is composed of a heavy (60-62 kDa) and a light (27-30 kDa) subunit in a 1:1 molar ratio [8, 9]. With a K_m of 0.2-0.5 mM [8, 9], *H. pylori* urease is well adapted to the low urea concentrations normally present within the stomach.

The specific activity of urease in whole cell lysates of *H. pylori* is higher than in other urease-positive bacteria, in part because the specific activity of purified *H. pylori* urease (1100-1500 U/mg) is relatively high, and because urease constitutes a large amount (6 to 10 percent) of the total protein of *H. pylori* [8, 9].

Nine genes have been identified in the urease gene cluster, similar to other urease-producing bacterial species (reviewed in [10]). Only seven of these are thought to be essential for urease expression and functional activity, however. The structural genes *ureA* and *ureB* and accessory genes *ureF*, *ureG* and *ureH* are essential for enzyme activity [10]. By analogy with homologous genes in *Klebsiella aerogenes* and *Protease mirabilis* [12-14], the accessory genes likely play a role in activation of the apoenzyme and insertion of Ni^{2+} ions into the active site of the nascent enzyme during assembly.

Recombinant urease is optimally expressed when Ni^{2+} uptake is not inhibited and when sufficient synthesis of urease subunits *UreA* and *UreB* is permitted [15]. A high-affinity Ni^{2+} transport protein (*NixA*), which provides Ni^{2+} transport at low metal concentrations, is necessary for full activity of recombinant *H. pylori* urease [16, 17]. However, isogenic *nixA* deficient strains of *H. pylori* do exhibit urease activity, although at reduced levels compared with strains possessing functional *NixA* protein [17], suggesting that at least one additional Ni^{2+} uptake system is operational.

ROLE OF *H. PYLORI* UREASE IN COLONIZATION

Urease activity is essential for initiating colonization of the stomach of animal models by *Helicobacter* sp. Isogenic urease-negative mutants were unable to colonize gnotobiotic piglets regardless of whether the piglets had normal acid output [7] or had been rendered achlorhydric [18]. In addition, isogenic urease-negative mutants of *H. pylori* failed to colonize the stomach of nude mice [19], and isogenic urease-negative mutants of *H. mustelae* failed to colonize ferrets [20].

Lack of colonization by urease-negative mutants is not due to inability to adhere to the underlying mucosa, since urease-negative mutants are able to colonize gastric mucosa biopsies at neutral pH *in vitro* [21], and urease *per se* is not thought to function as an adhesin [22]. Further supporting the essential role of urease activity in colonization, urea protects wildtype (urease-positive) *H. pylori* against acid (pH 2) *in vitro*. In contrast, *H. pylori* exposed to the same pH levels in the absence of urea fails to survive [23, 24].

DIRECT TOXICITY TO THE HOST

Ammonium generated by hydrolysis of urea by *H. pylori* urease is toxic to cultured cells [25-27]. To demonstrate the cytotoxic effects of urease, cell cultures of a human gastric adenocarcinoma cell line were incubated with *H. pylori* and supplemented with various concentrations of urea [25]. Cell viability was found to be inversely proportional to ammonia concentrations generated by urea hydrolysis. Cell viability was improved when the urease inhibitor acetohydroxamic acid was added to the culture before exposure to *H. pylori* [25].

HOST DAMAGE INDUCED BY THE IMMUNE RESPONSE

Urease activity has been implicated in immune-mediated injury to the gastric mucosa. *H. pylori* whole cells and sonicates stimulate an oxidative burst in human neutrophils [28, 29]. The urease enzyme *per se* can induce activation and recruitment of monocytes and neutrophils, resulting in indirect damage to the gastric epithelium [30]. Water extracts of *H. pylori*, known to contain urease in high concentration, can activate monocytes by a lipopolysaccharide-independent pathway [31]. *In vitro*, stimulation of human monocytes by *H. pylori* leads to secretion of inflammatory cytokines and reactive oxygen intermediates, all of which may be involved in mediating the inflammatory response in the gastric epithelium [28, 30, 31]

Urease from *H. pylori* can act as a chemotactic factor for human monocytes and neutrophils, causing local inflammation [32, 33]. Such chemotactic activity was present in purified urease samples and could be inhibited by specific antibody to the UreB urease subunit. Further, a 20-amino-acid peptide, based on the amino terminus of the UreB subunit protein, also exhibited similar levels of chemotaxis in a micro-chamber test system [31, 33]. By immunocytochemical staining, urease is closely associated with the crypt cells in the lamina propria of individuals infected with *H. pylori*. Thus, urease released from *H. pylori* may become adsorbed into the mucosa where it attracts leukocytes and promotes mucosal inflammation [32, 33].

SUBCELLULAR LOCALIZATION OF UREASE AND HSPB

H. pylori appears unique in that both urease and HspB, a homolog of the GroEL heat shock protein [34, 35], are major surface-associated proteins in late log phase bacteria as has been documented by numerous investigators. These observations can be summarized as follows. First, from 50 to 80 percent of the intact cell urease activity is recovered simply by washing the cells with deionized, distilled water [8] without adversely affecting bacterial viability [8, 36]. Similarly, approximately 90 percent of the whole-cell urease activity can be recovered by extraction with the detergent n-octylglucoside [37]. In addition, surface radioiodination demonstrates that urease and HspB are the major surface-exposed proteins of *H. pylori* [38]. Finally, immunogold and immunohistochemical localization studies have also demonstrated surface-association of urease [39, 40]. However, despite clear evidence that urease is located on the surface of late log phase *H. pylori*, the mechanisms whereby surface-association occurs are not immediately obvious.

UREASE AS A VACCINE AGAINST *HELICOBACTER* INFECTION

Because of its association with the surface of *H. pylori* and abundant quantity, urease has been studied as a vaccine candidate against *Helicobacter* sp. by several groups [41-

45]. *H. pylori* urease, when administered with appropriate adjuvants, such as cholera toxin and the heat labile enterotoxin (LT)^b of *E. coli*, is able to protect mice from infection with *H. felis*, a species closely related to *H. pylori*; the mouse-*H. felis* model mimics many features of *H. pylori*-related human gastritis [45]. Orogastric administration of urease (with adjuvant) can be used to eradicate existing infection or to prevent infection by experimental challenge with *H. felis* [41-45]. Similarly, orogastric administration of *H. pylori* urease (with adjuvant) can be used to eliminate existing infection with *H. mustelae* in the ferret *H. mustelae* model [46]. These experiments indicate that in the mouse and ferret models, antibodies to *H. pylori* urease have access to and are able to cross-react with *H. felis* and *H. mustelae* urease. They further indicate that surface-exposed urease is essential for colonization of mice and ferrets by *H. felis* and *H. mustelae*, respectively.

Urease is a promising candidate for human use. A double-blind placebo-controlled clinical trial evaluated the safety of urease in human volunteers infected naturally with *H. pylori* [47]. In this phase I trial, orally-administered recombinant *H. pylori* urease was well tolerated by the volunteers. However, since a mucosal adjuvant was not included, *H. pylori* infection was not cured [47, 48]. A phase II clinical trial designed to monitor the safety and immunogenicity of recombinant urease in the presence of LT as mucosal adjuvant is in progress [48].

There is indirect evidence that urease is surface-associated *in vivo*. First, the strong immunological response mounted against urease and HspB in humans naturally infected with *H. pylori* is consistent with a surface-associated or extracellular localization [11,49]. Second, pre-treatment of *H. felis* with monoclonal IgA against urease prevents infection [50]. Finally, immunization of animals and humans induces mucosal secretory IgA directed against urease epitopes [44, 51].

MECHANISMS FOR SURFACE-ASSOCIATION OF UREASE

There are several reasons to suspect that a novel mechanism is responsible for surface association of *H. pylori* urease. First, urease is a cytoplasmic protein in all other bacteria in which its subcellular location has been studied. Second, the nucleotide sequences of the *ureA* and *ureB* structural genes of *H. pylori* urease do not contain an N-terminal signal sequence [10, 52, 53]. Third, not only urease, but other proteins such as HspB, superoxide dismutase and catalase (which are typical cytoplasmic proteins in other gram negative bacteria), have been shown to be surface-associated in *H. pylori* [49, 54, 55]. The gene clusters encoding urease, HspB and catalase do not display the features normally associated with exported proteins [10, 35, 56]. Finally, *H. pylori* urease genes cloned in *E. coli* produce active urease located in the cytoplasm. The production of recombinant urease is enhanced by cloning a high affinity Ni²⁺ transporter gene, *nixA*, from *H. pylori*, indicating that enzymatically active recombinant urease can be assembled in the cytoplasm of *E. coli*. Thus, the urease of *H. pylori* can be fully active when present strictly within the cytoplasmic compartment of bacteria.

To obtain adequate bacterial yields for most experiments, *H. pylori* must be subcultured in the laboratory. Using cryo-immunolocalization techniques, we have examined the age- and culture condition-dependence of the subcellular localization of urease [49]. We have demonstrated for the first time that urease is located strictly within the cytoplasmic compartment in early log (one day) and mid log phase (two days) of growth on agar plates. Only in the late log phase of growth (three days) is some urease associated with the surface of *H. pylori*. In contrast, after one day of growth in repeated subculture (the type of culture typically used to inoculate animals in experimental challenges), significant amounts of surface-associated urease are present. This surface-association of urease is

accompanied by the appearance of extracellular wall fragments and amorphous debris indicative of bacterial autolysis. In these analyses, urease is not found within a single compartment, but is present associated with the surface, free in the extracellular space and within the cytoplasm of intact cells [49]. A variety of additional evidence supports the occurrence of autolysis in *H. pylori* [49]. First, protein profiles of "water wash" preparations from whole cells are identical to those of intentionally-lysed bacteria. Second, surface radioiodination of whole cells generates a profile similar to that observed in radioiodinated bacterial lysate preparations, suggesting that major cytoplasmic proteins liberated due to autolysis have adsorbed to intact bacteria and, hence, mask the underlying intrinsic outer membrane proteins. Third, by using flurofamide, a potent, poorly-diffusible inhibitor of urease activity, we have shown that both surface-associated and cytoplasmic urease molecules are active enzymatically. The time course of localization of HspB and catalase, two other typical cytoplasmic proteins, is essentially identical to that of urease. These observations are incompatible with known mechanisms of protein export in bacteria.

There are three known mechanisms of protein export in bacteria. The general secretory pathway requires that the exported protein possess an N-terminal signal sequence, which is removed during the process of secretion [57]. The DNA sequence data for urease, HspB and catalase do not reveal any N-terminal signal sequence [10, 35, 56]. The general secretory pathway is the only protein export mechanism known to assemble and export complex multimeric proteins; it is well known that proteins exported by this mechanism are assembled in the periplasm, not within the cytoplasm [57].

The ABC transporter pathway requires the presence of two accessory proteins, an ATPase and a specific transport protein that spans both the inner and outer membranes; the genes for these accessory proteins are usually located within the same operon as is the gene encoding the exported protein [58]. To our knowledge, no multi-subunit proteins are secreted by this pathway.

The type III pathway of protein export has recently attracted much attention. This pathway may be induced by bacterial contact with eukaryotic cells; the exported proteins (which lack a typical N-terminal signal sequence) are often delivered directly to the target cells. This pathway requires the presence of 12 or more accessory proteins that are highly conserved across diverse bacterial species; such proteins are usually located in the bacterial membrane [59]. The exported protein, almost always comprised of a single polypeptide chain, is complexed with a unique molecular chaperone which promotes proper conformational change only after the exported protein is released by the bacteria. There are no examples of transmembrane transport of multi-subunit proteins using this pathway.

Transporters analogous to the ABC pathway or type III accessory proteins are not present in either the urease, HspB or catalase gene clusters. The strict cytoplasmic location of active urease in early log phase *H. pylori* and in recombinant *E. coli* strongly argues against the presence of individual molecular chaperones for urease structural proteins, since such molecular chaperones would not allow assembly of active urease in the cytoplasm.

While there is no evidence to suggest that urease and HspB are translocated actively onto the surface of *H. pylori*, if a secretion system were to exist, it would require a variety of features that would be unique compared with other bacterial transport systems, as we have discussed in detail previously [49, 60]. Such a secretion system would be non-specific in nature, be induced only in late log-phase bacteria, be induced asynchronously within individual bacteria and be capable of transporting large, fully assembled multi-subunit proteins. Finally, the accumulation of cytoplasmic protein, as observed both *in vivo* and *in vitro*, would suggest either that the hypothetical transport system can discriminate between two populations of proteins (those destined to remain within the cytoplasm

versus those destined to be secreted) or that the rate of secretion is limited by the number of transporter molecules (resulting in “back-up” of protein in the cytoplasm prior to secretion). Given the strong evidence supporting the bacterial autolysis theory, and the tremendous constraints required for such a hypothetical secretion system, we believe that bacterial autolysis followed by adsorption is the mechanism for surface association of urease [49, 60].

We have recently performed immunolocalization studies of urease and HspB within *H. pylori* present in gastric biopsies obtained from naturally-infected humans [60]. Urease and HspB are present both on the surface and within the cytoplasm of *H. pylori*, similar to the subcellular location of these proteins in late log phase bacteria *in vitro*. Thus, we presume that the identical mechanisms responsible for surface association of urease and HspB *in vitro* are also operative *in vivo*.

THE PATHOGENIC ROLE OF SURFACE-ASSOCIATED UREASE

Despite the fact that *H. pylori* colonize and grow in the human stomach where luminal pH may be 2.0 or less, the bacteria are not intrinsically resistant to acid. *In vitro*, it has been shown that *H. pylori* are resistant to low pH only in presence of urea [23, 24]. Thus, urease activity is essential for bacterial survival. We have recently assessed the relative contributions of cytoplasmic and surface-associated urease to acid resistance [61]. Early log phase *H. pylori* (which possess only cytoplasmic urease) are unable to survive at low pH despite the presence of physiological concentrations (5 mM) of urea; such bacteria are acid sensitive. In contrast, late log phase bacteria (which possess both cytoplasmic and surface-associated urease activity) are resistant to acid in the presence of 5 mM urea [61]. From these and related experiments, we conclude that surface-associated urease is essential to protect *H. pylori* from the low pH it normally encounters in the stomach.

ALTRUISTIC AUTOLYSIS

As noted above, surface-associated urease *per se* is essential to acid resistance of *H. pylori* [61]. We believe that surface-association of urease occurs by a novel mechanism. Specifically, urease (and other cytoplasmic proteins) are released as a result of genetically programmed autolysis affecting a fraction of the bacteria. Released urease (and other cytoplasmic proteins, notably HspB) then adsorb onto the surface of neighboring intact bacteria, thereby permitting survival of the population at the cost of individual cell death. We have coined the term “altruistic autolysis” to describe this process [62]. Since surface-associated urease is essential for resistance of *H. pylori* to acid, and since bacterial autolysis is a prerequisite for surface-association of urease, “altruistic autolysis” is an essential feature of the pathogenesis of *H. pylori* (Figure 1).

In addition to urease and HspB, several typical cytoplasmic proteins are associated with the surface of late log phase *H. pylori*. Using standard biochemical techniques to recover membrane proteins, a variety of proteins have been recovered from *H. pylori* that ultimately have proven to be cytoplasmic proteins. Four such proteins include catalase, originally described as an exoenzyme S-like adhesin [56], a hemagglutinin [63], bacterioferritin [64] and a 26-kDa species-specific protein [65]. These observations support the notion that multiple cytoplasmic proteins are released and bind to the surface of intact *H. pylori*.

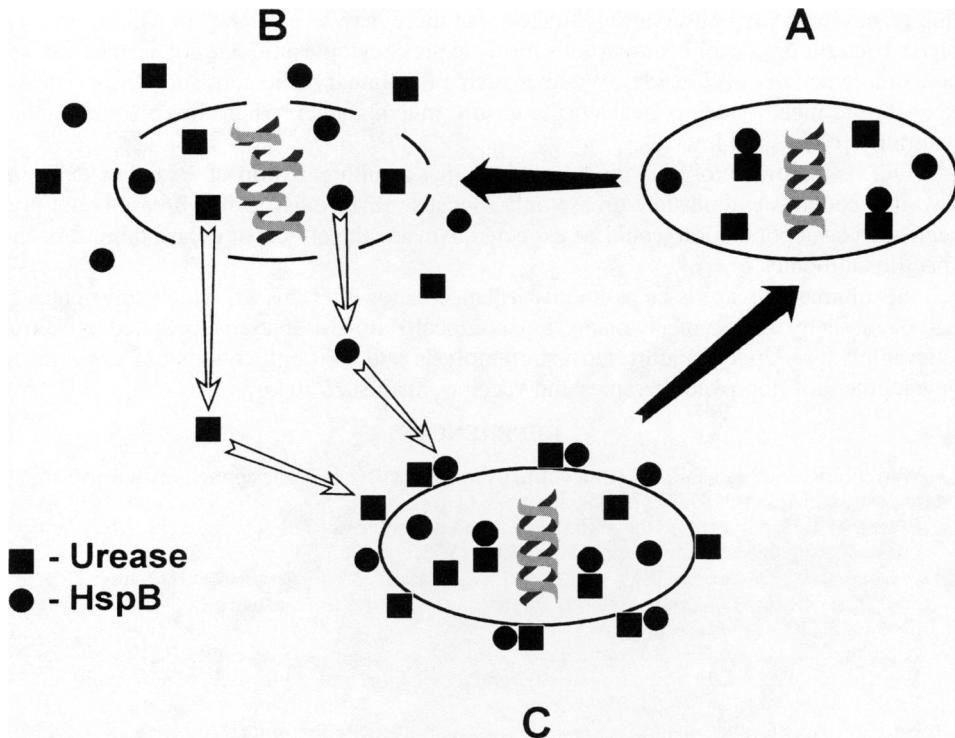


Figure 1. Key steps involved in surface-association of urease. A) Early log phase bacteria contain only cytoplasmic urease and HspB; B) bacteria undergo genetically-programmed autolysis, resulting in the release of urease and HspB into the surrounding environment; C) some of the released urease and HspB adsorbs to the surface of neighboring intact bacteria, which, as a result, possess both surface-adsorbed and cytoplasmic urease. The latter forms (C) can convert to coccoid forms (not shown) or to bacillary forms in which urease and HspB are present within the cytoplasm only (A).

IMMUNE EVASION

Continual release of urease (and HspB) from bacteria undergoing autolysis *in vivo* may provide a form of camouflage by binding to specific secretory IgA directed against *H. pylori*, thereby allowing intact *H. pylori* to evade the immune system. By such a mechanism, secretory IgA that binds to free urease and HspB released from *H. pylori* would be unable to bind to intact bacteria [49, 62].

IS THERE A FUNCTIONAL OR ANATOMIC NICHE FOR EARLY LOG PHASE *H. PYLORI*?

Since surface-associated urease is required for resistance of *H. pylori* to acid, inactivation of surface-associated urease by specific inhibitors might be expected to clear *Helicobacter* infection. In studies using the ferret-*H. mustelae* model, treatment of animals with flurofamide, a potent, poorly-diffusible urease inhibitor have failed to clear the infection [66]. Inhibition of urease activity seems to eliminate most bacteria from the stomach as evidenced by the marked decrease in urease activity detected post-treatment

using a urea breath test. However, soon after treatment with urease inhibitors, the infection recurs [66]. Such observations suggest that there may be a "niche" in which early log phase bacteria or coccoid forms (both forms express cytoplasmic but not surface-associated urease activity [49] reside). Whether such a functional niche corresponds to a specific anatomic niche (such as beneath the gastric mucous layer, where the pH approaches neutrality [67]) is not known.

Our "altruistic autolysis" model predicts that a subpopulation of *H. pylori* exists *in vivo* that contains cytoplasmic urease only. Because surface-associated urease is not present, such a subpopulation would be expected to evade the effects of urease inhibitors and specific antibodies *in vivo*.

In summary, urease is an essential virulence factor of *H. pylori*, which contributes to pathogenesis by diverse mechanisms. Surface localization of urease is mediated by "altruistic autolysis." Understanding altruistic autolysis and its ramifications are essential to development of improved therapies and vaccines against *H. pylori*.

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