Medium pH-dependent redistribution of the urease of Helicobacter pylori

Wu Hong, 1 Kouichi Sano, 1 Shinichi Morimatsu, 2 David R. Scott, 3 David L. Weeks, 3 George Sachs, 3 Toshiyuki Goto, 4 Sharad Mohan, 1 Fumiue Harada, 1 Norihiro Nakajima 1 and Takashi Nakano 1

1 Department of Microbiology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki-shi, Osaka 569-8686, Japan
2 Department of Nursing, Kobe Tokiwa College, Ohtani-cho 2-6-2, Nagata-ku, Kobe, Hyogo 653-0838, Japan
3 VA Greater Los Angeles Healthcare System and Department of Physiology and Medicine, University of California at Los Angeles, CA, USA

Helicobacter pylori is an aetiological agent of gastric disease. Although the role of urease in gastric colonization of H. pylori has been shown, it remains unclear as to where urease is located in this bacterial cell. The purpose of this study was to define the urease-associated apparatus in the H. pylori cytoplasm. H. pylori was incubated at both a neutral and an acidic pH in the presence or absence of urea and examined by double indirect immunoelectron microscopy. The density of gold particles for UreA was greatest in the inner portion of the wild-type H. pylori cytoplasm at neutral pH but was greatest in the outer portion at acidic pH. This difference was independent of the presence of urea and was not observed in the ureI-deletion mutant. Also, the eccentric shift of urease in acidic pH was not observed in UreI. After a 2 day incubation period at acidic pH, it was observed that the urease gold particles in H. pylori assembled and were associated with UreI gold particles. Urease immunoreactivity shifted from the inner to the outer portion of H. pylori as a result of an extracellular decrease in pH. This shift was urea-independent and UreI-dependent, suggesting an additional role of UreI in urease-dependent acid resistance. This is the first report of the intracellular transport of molecules in bacteria in response to changes in the extracellular environment.

INTRODUCTION

Helicobacter pylori is a Gram-negative microaerobic pathogen that causes chronic gastritis, peptic ulceration and gastric cancer (Warren & Marshall, 1983; Bohnen et al., 1986; Wadström, 1995). Bacterial infection occurs in young individuals and persists throughout life (Kurosawa et al., 2000). The urease of H. pylori is essential for gastric colonization and presumably neutralizes acidity, thereby allowing bacterial survival (McGowan et al., 1996). Most of the urease is in the bacterial cytoplasm and only a small amount is found on the surface of the bacterial cell (Nilius et al., 1991; Bode et al., 1993; Dunn & Phadnis, 1998; Marcus & Scott, 2001). Since the enzyme has an optimum pH between 7-5 and 8-0 and is irreversibly inactivated below pH 4-0 (Rektorschek et al., 1998; Scott et al., 1998) or pH 3-0 (Ha et al., 2001), it is difficult to explain how the enzyme acts on the surface of the bacterial cell for its protection in the stomach. Furthermore, UreI is essential for gastric colonization and survival in acidic media, and UreI does not affect surface but only intrabacterial urease (Scott et al., 2000; Weeks & Sachs, 2001; Skouloubris et al., 1998; Mollenhauer-Rektorschek et al., 2003). These characteristics show that it is the intracytoplasmic urease that is important for H. pylori colonization.

The unique gastric acid resistance of H. pylori may be due in part to an acid-regulated urea channel, UreI, which increases the access of urea to intrabacterial urease in acidic media (Weeks et al., 2000). That is, after acidity opens a urea channel in the inner membrane of the bacterium, urea is taken up passively and hydrolysed by the enzyme in the cytoplasm. If urea and ammonia are transported across the cytoplasm in large amounts, they may cause degeneration of cytoplasmic proteins and alkalinization of the bacterial cytoplasm. To overcome toxicity, the bacterium may have a protective mechanism or apparatus. It has been suggested that the urease antigen is localized near the bacterial surface (Dunn &
Phadnis, 1998), as shown by immunoelectron microscopy of cryoultramicrosections and acrylic resin-embedded bacterial cells. Since these methods did not provide sufficient contrast of the cell wall in micrographs, it was difficult to analyse their data by any quantitative methodology. On the other hand, the urease acts in the bacterial cytoplasma (Athmann et al., 2000) and localization of UreI at the inner membrane has been proven by biochemical methods (Weeks et al., 2000) and by morphological methods using contrast-enhanced immunoelectron microscopy (Hong et al., 2000). According to these findings, we hypothesized that a complex of urease and UreI is localized near the inner membrane and we attempted to determine the actual localization of H. pylori urease and UreI using contrast-enhanced immunoelectron microscopy (Hong et al., 2000). Urel has been shown to interact with UreA by a variety of methods and this would be consistent with our hypothesis (Voland et al., 2003; Rain et al., 2001).

METHODS

Bacteria and growth. The H. pylori strain ATCC 43504T and the ureI-deletion mutant described by Weeks et al. (2000) were used in this study. Bacteria were cultured on Pylori agar plates (bioMe´rieux) and Campyppack Microaerophilic System Envelopes (Becton Dickinson) at 37°C for 3 days. The ureI-deletion mutant exhibits normal total urease activity (Athmann et al., 2000). Colonies of both strains were collected, suspended and incubated in either McIlvaine buffer containing 0·1 M citric acid monohydrate and 0·2 M disodium hydrogen phosphate, pH 5·5 or 7, at 37°C for 15 min or Brucella broth (supplemented with 0·1 M MES buffer and 5 % horse serum, pH 5·5 or 7) at 37°C under microaerophilic conditions for 2 days. Some of the samples were incubated in McIlvaine and MES buffer containing 1 mM urea.

pH change of urease under different pH conditions. H. pylori cultured on agar was collected and resuspended in Brucella broth supplemented with 5 % horse serum and the turbidity was adjusted to McFarland standard 0·5. Two ml of this H. pylori suspension, and 1·0 ml of pH-adjusted urease assay buffer, which contained 1·3 M urea, 0·06 M NaNO3, 0·005 M phenol red and McIlvaine buffer (pH 5·0 and 7·0), were mixed in a 10 ml tube. Measurement of pH was performed in the tube at room temperature using a pH meter (Checker, HANNA).

Antibodies. The mouse monoclonal antibody against H. pylori urease subunit A (Austral Biologicals) and rabbit polyclonal anti-UreI antibody (Weeks et al., 2000) were used as primary antibodies, and 5 nm colloidal gold-labelled anti-mouse IgG and 10 nm gold-labelled anti-rabbit IgG-labelled goat antibodies (Amersham) were used as secondary antibodies.

Fixation and staining. H. pylori colonies were fixed with 1 % glutaraldehyde in 0·05 M cacodylate buffer (pH 7·2) at 4°C for 60 min. Fixed colonies were washed five times with 0·05 M cacodylate buffer and dehydrated in serially graded ethanol. Samples were then embedded in low-cryogen KEM resin (LKB Instruments, Piscataway, NJ, USA). Polymerization was allowed to occur in an ultraviolet irradiator (Dosaka EM) at 30°C for 2 days and then at room temperature for 2 days. Ultrathin sections were cut using a Porter Blum Ultramicrotome (Sorvall MT-5000, Du Pont) and mounted on a nickel grid (300 mesh) supported by a carbon-coated colloidal film.

Ultrathin sections on the grid were treated with 5 % normal goat serum in 0·15 M PBS (pH 7·2) to block nonspecific reactions. Sections were then double-stained with mouse antibody against H. pylori Urea and rabbit antibody against Urel (primary antibodies) and then 5 nm colloidal gold-labelled anti-mouse IgG and 10 nm gold-labelled anti-rabbit IgG-labelled goat antibodies (secondary antibodies). Sections were first reacted by adding drops of the primary antibodies at room temperature for 120 min and washed five times in PBS. Sections were then allowed to react with the secondary antibodies at room temperature for 60 min and were subsequently washed five times in PBS.

Immunostained sections were fixed with 1 % glutaraldehyde in 0·05 M cacodylate buffer for 15 min and washed five times in distilled water. Finally, sections were contrast-enhanced with a mixture of alizarin blue and osmium tetroxide solution and were stained with uranyl acetate aqueous solution as described previously (Hong et al., 2000). All procedures for contrast enhancement were performed at room temperature.

All sections were observed under a transmission electron microscope (H-300 and H-7100, Hitachi). Photomicrographs were taken at a magnification of ×15 000 and enlarged to a magnification of ×30 000.

Morphometric analysis. The number of immunogold particles associated with H. pylori cells was determined using the immunoelec- tron photomicrographs. Areas of bacterial cells in the photomicrographs were measured using an image analyser (MCID system, Imaging Res.) and the numbers of gold particles per unit area were compared. In order to quantify the distribution of gold particles, the densities of the particles per square micrometer in three portions of a bacterial cell were determined. The bacterial cell was divided into three portions: the outer portion is the area extending from a depth of one-sixth of the diameter to the surface of the cell; the medium portion is the area between the outer and inner portions extending from one- to two-sixths of the diameter; and the inner portion is the central area of the cell extending beyond two-sixths of the diameter to the centre of the cell. The principle of dividing portions of H. pylori is explained in Fig. 1. As shown, the outer portion that is between the surface and the depth of one-sixth of the shorter diameter (approx. 85 nm) is equal to the sum of the cell wall (approx. 30 nm) width, length of the antibody (approx. 25 nm) and the thickness of the cell (approx. 30 nm).

RESULTS AND DISCUSSION

In order to measure urease activity in whole bacterial cells cultured under different pH conditions, the pH of urease assay buffer was changed. In Fig. 2, the pH change of urease assay buffer mixed with bacterial suspension is shown. Results reveal that the level of pH change at pH 5 is greater than that at pH 7; this difference was not observed in urease assay buffer mixed with the suspension of the ureI-deletion mutant. That is, the difference in pH change between the wild-type and ureI-deletion mutant at pH 5 was not the same as that observed at pH 7. In contrast, a pH change of the assay buffer was not observed in the urease-free control. The pH change of the assay buffer can therefore be taken as an indirect measure of urease activity. Our results indicate that the urease activity of wild-type H. pylori increases rapidly as the pH of the medium decreases, but this does not happen in the ureI-deletion mutant, and these results are similar to Scott et al. (2000).

To clarify whether the number of gold particles associated with UreA and the distribution of urease are influenced by environmental factors, the localization and the number of gold particles associated with urease were determined in cells incubated for 15 min at different pHs. The overall densities...
of gold particles in the bacterial cells incubated at neutral pH in the presence and absence of urea were $105\cdot1$ $\mu$m $^2$ and $117\cdot4$ $\mu$m $^2$, respectively. These values were not found to be significantly different by Student’s $t$-test ($P$, 0.01). At acidic pH, the overall densities in the cell in the presence and absence of urea were $151\cdot2$ $\mu$m $^2$ and $161\cdot1$ $\mu$m $^2$, respectively. These values at the same pH were found to be statistically the same by Student’s $t$-test ($P$, 0.01). The distribution of gold particles seemed to be uniform in bacterial cells incubated in the presence of urea at neutral pH in wild-type (Fig. 3a) but eccentric in those incubated at acidic pH (Fig. 3b).

Although the presence of urea in the buffer did not influence the densities of gold particles in any portion of the bacterial cells, a definite change in the distribution of gold particles was observed in cells incubated at different pHs. At neutral pH, the density was highest in the inner portion and lowest in the outer portion (Student’s $t$-test paired analysis, $P$, 0.05). In contrast, at acidic pH, the density was highest in the outer portion and lowest in the inner portion (Student’s $t$-test paired analysis, $P$, 0.05) (Table 1).

To determine whether the eccentric distribution of urease at acidic pH is UreI dependent, the ureI- deletion mutant (Weeks et al., 2000) was also observed under the same experimental conditions (Table 1). The densities at each portion were similar to those of wild-type cells incubated at neutral pH (Fig. 3c, d); i.e. the eccentric shift of the distribution of urease molecules did not occur in the ureI- deletion mutant at acidic pH (Student’s $t$-test paired analysis, $P$, 0.05).

In order to determine whether the shift of urease distribution was due to nonspecific effects of the acidic condition, the localization of UreI was determined by similar methods (Fig. 3a, b). The difference in the density of gold particles for UreI (a) was greater than that at pH 7 and this difference was not observed in the urea-free control. Each assay was performed three times.

Fig. 1. Diagram showing the procedure and direction of sectioning of H. pylori. (a) A lateral view of H. pylori in a three-dimensional diagrammatic representation. The blade of the microtome is shown cutting a longitudinal section (LS) of the bacterium. (b) A lateral view of the LS obtained after sectioning. (c) A frontal view of the LS (d) The dorsal view of the LS, as observed through the microscope and seen in all photomicrographs presented in this paper. Dotted lines represent a frame of reference for the reader so as to give a clear picture of the area in discussion. 'a' and 'b' are two points on the same outer membrane of the LS of H. pylori but are separated from each other by a distance 'x' due to the curvature of the bacterial membrane. The distance 'x' is due to the limitation of two-dimensional electron microscopy, x. Maximum distance observed between any two points 'ab' owing to the curvature of the cell membrane obstructing the passage of electron wave/field of vision. S, Source of light/field of vision.

Fig. 2. Urease activity of H. pylori at different pHs. Wild-type (UreI $^+$) H. pylori incubated at pH 7 (○) and pH 5 (●); ureI deletion mutant (UreI $^-$) incubated at pH 7 (□) and pH 5 (■); wild-type (UreI $^+$) H. pylori incubated at pH 7 (◇) and pH 5 (◆) without urea. The pH change of urease assay mixture containing H. pylori in different pHs is shown. Urease activity of H. pylori at pH 5 was greater than that at pH 7 and this difference was not observed in the urea-free control. Each assay was performed three times.
between neutral pH (Fig. 3a) and acidic pH (Fig. 3b) was similar to that reported previously (Hong et al., 2000) and was not found to be statistically significant by Student’s t-test ($P < 0.01$). The densities of the gold particles in the outer portion of wild-type cells incubated at neutral and acidic pH were similar to those reported previously (Hong et al., 2000).

To determine whether the shift occurs in proliferating bacteria, the bacteria incubated for 2 days at reduced pH were observed in the experiments (Table 2). The urease immunogold particles were distributed in a similar manner to those incubated for 15 min at neutral pH (Fig. 4a) and acidic pH (Fig. 4b). It was also observed by immunoelectron microscopy that the urease gold particles in *H. pylori* incubated for 2 days at acidic pH were gathered and associated with UreI gold particles (Fig. 4c).

Recent reports have shown that it is cytoplasmic rather than surface-associated urease that is required for the acid resistance of *H. pylori* (Rektorschek et al., 1998; Scott et al., 1998; Weeks & Sachs, 2001). It is unclear where cytoplasmic urease neutralizes pH in a bacterial cell under acidic conditions. Urea enters the bacterial cytoplasm through the proton-gated urea channel, UreI, after exposure of the cell to a medium of pH 6·0 or less (Marcus & Scott, 2001; Rektorschek et al., 1998; Scott et al., 1998; Athmann et al., 2000). The urease of *H. pylori* catalyses the degradation of urea to ammonia to protect the bacterium from harmful effects of acid. However, if urea in large amounts is transported into the cytoplasm and ammonia is generated equally throughout the cytoplasm, there could be lethal protein degeneration due to alkalinization of the cytoplasm. The bacterium does not survive at pH$_{in}$<8·5. (Meyer-Rosberg et al., 1996).

**Fig. 3.** Electron photomicrographs of *H. pylori* incubated for 15 min and then double immunostained with anti-UreA and anti-UreI antibodies. Urease gold particles (the smaller gold particles) are distributed uniformly in the bacterial cell incubated at neutral pH in the wild-type strain (a). The urease gold particles are distributed more in the outer portion than in the inner portion of the bacterial cell incubated at acidic pH in the wild-type strain (b). UreI gold particles (the bigger gold particles) in the bacterial cell incubated at neutral pH (a) are distributed in a similar manner to those at acidic pH (b). Urease gold particles are distributed uniformly in the ureI-deletion mutant cell incubated at neutral pH in (c). Gold particles are distributed uniformly in the ureI-deletion mutant cell incubated at acidic pH (d). The UreI-deletion mutant was not labelled with the anti-UreI antibody-associated gold particles (c, d). Original magnification, ×15 000.

**Fig. 4.** Electron photomicrographs of *H. pylori* incubated for 2 days and then double immunostained with anti-UreA and anti-UreI antibodies. Immunogold particles are distributed in a similar manner to those in the bacterial cell incubated for 15 min at neutral pH (a) and acidic pH (b). (c) Representative of (b) at a higher magnification showing that the urease gold particles in *H. pylori* after 2 days of incubation assembled and were associated with UreI gold particles. Original magnification, ×15 000.
The catalytic expression of cytoplasmic urease is regulated by a mechanism that is closely associated with extracellular pH. Thus, we hypothesized that cytoplasmic urease molecules shift toward the inner membrane and assemble with UreI molecules at acidic pH. Assembly of urease and UreI has been demonstrated biochemically (Voland et al., 2003). To establish this hypothesis in intact bacteria using quantitative contrast-enhanced immunoelectron microscopy (Hong et al., 2000), we examined whether urease is redistributed to the bacterial inner membrane at low pH. The redistribution of urease was observed with a change in medium pH and was associated with the presence of UreI molecules. This was not observed in the ureI-deletion mutant and was independent of the presence of urea.

UreI is required for an acidic pH-induced increase in Table 1. Distribution of urease immunoreactivity in H. pylori after a 15 min incubation

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>pH</th>
<th>Urea</th>
<th>Number of gold particles (μm⁻²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Outer</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7</td>
<td>+</td>
<td>90.3 ± 45.8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>–</td>
<td>111.1 ± 64.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>205.0 ± 75.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>–</td>
<td>213.9 ± 51.3</td>
</tr>
<tr>
<td>ureI-deletion mutant</td>
<td>7</td>
<td>–</td>
<td>132.0 ± 28.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>–</td>
<td>114.6 ± 44.6</td>
</tr>
</tbody>
</table>

*The areas of visible bacterial cells were measured using photomicrographs taken at a magnification of ×30,000 using an optical image analyser. The number of gold particles (μm⁻²) was counted in each portion of 10 bacterial cells. Outer, area extending from a depth of one-sixth of the diameter to the surface of the cell; medium, area between the outer and inner portions extending from one- to two-sixths of the diameter; inner, central area of the cell extending beyond two-sixths of the diameter to the centre of the cells.

Table 2. Distribution of urease immunoreactivity in cells of H. pylori after 2 days of incubation

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>pH</th>
<th>Urea</th>
<th>Number of gold particles (μm⁻²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Outer</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7</td>
<td>+</td>
<td>52.3 ± 26.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>–</td>
<td>34.6 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>57.6 ± 19.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>–</td>
<td>72.5 ± 21.6</td>
</tr>
</tbody>
</table>

*The areas of visible bacterial cells were measured using photomicrographs taken at a magnification of ×30,000 using an optical image analyser. The number of gold particles (μm⁻²) was counted in each portion of 10 bacterial cells. Outer, area extending from a depth of one-sixth of the diameter to the surface of the cell; medium, area between the outer and inner portions extending from one- to two-sixths of the diameter; inner, central area of the cell extending beyond two-sixths of the diameter to the centre of the cells.

http://jmm.sgmjournals.org
cytoplasmic urease activity in *H. pylori* (Scott et al., 2000). The data presented here show that when UreI is activated by an acidic medium pH, urease moves closer to the source of urea and presumably ammonia production occurs at or near the inner membrane. This eliminates the necessity of generalized cytoplasmic alkalinization due to intra-bacterial urease.

In the protein–protein interaction map of *H. pylori* urease, the urease operon that encodes UreI may be associated with the presence of UreI, indicating that urease activity is membrane limited (Scott et al., 2000). In this study, we confirmed that the ureI-deletion mutant does not induce neutralization of an acidic medium. It was also observed that the urease gold particles in *H. pylori* were associated with Urea gold particles in immunoelectron microscopy. Urease activity was observed with the presence of UreL, and urease and UreI may assemble a supramolecular complex in an acidic medium.

This is the first report that cytoplasmic molecules in bacteria are trafficked to the membrane with a change in environmental conditions outside the cell. We propose that *H. pylori* possesses a proton-regulated urease redistribution mechanism, which may be a new target for anti-*H. pylori* therapy.

**ACKNOWLEDGEMENTS**

We thank Mr Yoshikichi Fujinaka in the Department of Microbiology, Osaka Medical College for his technical help.

**REFERENCES**


