J. Microbiol. Biotechnol. (2001), **11**(2), 317–325

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Significance of Urease Distribution across *Helicobacter pylori* Membrane

GANG, JIN-GU, SOON-KYU YUN, KYUNG-MIN CHOI, WANG-JIN LIM, JEONG-KYU PARK[1], AND SE-YOUNG HWANG*

Graduate School of Biotechnology, Korea University, 5-1 Anam-Dong, Seoul 136-701, Korea
[1]Department of Microbiology, College of Medicine, Chungnam National University, Taejon 301-131, Korea

Received: January 10, 2001
Accepted: March 6, 2001

Abstract For heuristic purposes, the relative ratio of urease contents inside and outside cells was surveyed using nine *ureB*$^+$ strains of *Helicobacter pylori*. The ratio of the enzyme specific activity appeared to vary greatly between the various *H. pylori* strains, ranging from 0.5 to 2.5. Besides the above compartments, urease was also richly found in the membrane fraction, especially in either peripheral or integral form. The urease distribution across the *H. pylori* membrane was significantly influenced by the ambient pH; the specific activity of external urease was highest at pH 5.5 with a narrow plateau, whereas the internal specific activity was highest within a pH range of 4.5 to 6.5 with a broad plateau. These findings strongly suggest that *H. pylori* urease is secretory and responded to the external pH. However, at pH 4.0 or below, no urease activity was detected in either the internal or external compartment, although an increase in the color development with 2,4,6-trinitrobenzene sulfonate (TNBS) was observed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that these phenomena may be related to a specific proteolysis in certain proteins, including urease or γ-glutamyl transpeptidase. Interestingly, the effect of ammonium ions on alleviating the enzyme inactivation inside the *H. pylori* cells was remarkably similar to that of D-glucose. In addition, it would appear that the cation acted as a surrogate of not only Na^+ but also K^+, thereby increasing the *H. pylori* P-type ATPase activity. This is of great interest, as it implies that the urease action in *H. pylori* is indispensible at any locus.

Key words: *Helicobacter pylori*, urease, *ureB*, ammonium ion, P-type ATPase

Urease (urea amidohydrolase: EC 3.5.1.5) is a unique enzyme that catalyzes the hydrolysis of urea, and formed exclusively by the urea cycle [2, 17, 24]. The enzyme is ubiquitously found in living systems, however, the productivities between species vary greatly [26]. Accordingly, the assessment of the enzyme activity is taxonomically valuable, especially in the clinical identification of bacterial species. Hydrolysis of urea by urease results in the formation of carbon dioxide and two equivalents of ammonia. Hence, an increase in the milieu pH is an immediate effect of this reaction [2]. The role of urease on bacterial pathogenesis is well recognized. Nevertheless, the mechanisms by which bacterial urease affects nearby host cells have not been fully established, probably because of the fact that their location is exclusively restricted to cytoplasmic space [6, 25, 27]. One exceptional case in which urease can be found in any locus of the cells is *Helicobacter pylori* [12, 23].

Helicobacter pylori is a spiral, Gram-negative rod which occupies the human stomach as an ecologic niche. This bacterium is generally considered to be an obligatory parasite, as no free-living form has yet been identified in nature [4, 35]. One of its characteristic features is the prodigious production of urease, a constitutive property of *H. pylori*, although urease-deficient *H. pylori* mutant strains have occasionally been found in gastric biopsy specimens [13]. In animal models, this enzyme appears to be essential for the *H. pylori* colonization of the gastric mucosa layer [23]. Also, many previously published reports suggest an attribution of urease to *H. pylori* pathogenesis [6, 15, 18]. Numerous hypotheses have been put forward concerning the mechanisms by which *H. pylori* colonization and persistence lead to mucosal inflammation that can, in turn, become incurable. Ammonia toxicities (e.g., monochloramine [11] or vacuolating cytotoxin [8, 9]) are perhaps among the most well-documented risk factors that derange host cells, raising the incidence of developing gastric cancer or lymphoma [13, 28]. In contrast, *H. pylori* is able to effectively exploit ammonia for the cell's energy expenditure and nitrogen assimilation [34, 41]. Until now, there appears to be no reports regarding the role of the ammonia produced inside and outside cells.

*Corresponding author
Phone: 82-2-3290-3420; Fax: 82-415-865-3339;
E-mail: shwang@tiger.korea.ac.kr

The environment of the human stomach is usually within a pH range of 1 to 3.5, with the most common pH being 1 to 2.5 with a diurnal cycle related to food consumption [16]. Therefore, it is likely that *H. pylori* cells are at least occasionally exposed to acidic conditions where the proton motive force becomes inert and fatal to this bacterium [30, 33]. Even though urease is believed to contribute to neutralization of the area surrounding *H. pylori*, the enzyme activity is actually vulnerable under *in vitro* acidic conditions (pH≤3.0). Accordingly, since it is unlikely that urease resident in the cell-free system plays a key role in protecting *H. pylori* in the presumed acidity *in vivo*, a question is raised as to the real significance of the ubiquity of urease in this bacterium. There exists a controversy over whether the enzyme is equally engaged both internally and externally in protecting *H. pylori* from the gastric environment [22, 33]. When taking the pK_b value of ammonia into account, most of the internal ammonia liberated by urea hydrolysis must be cationic, making the urea useless in neutralizing an acidic medium. Thus, the external fraction which is known to increase concomitantly with the cell's aging seems to be the probable factor involved in raising the medium pH. Nonetheless, whether such localization of urease is a transitory event in the course of the enzyme secretion or the result of occasional adsorption arising from the lysis of other cells is still not well defined.

In the present study, the concept of enzyme specific activity was examined to establish the pattern of urease secretion and its significance in *H. pylori*. The first half of this paper deals with an acid-caused initial event in *H. pylori* cells, resulting from the failure to keep the urease function, and the latter half demonstrates that the ubiquitous distribution of urease is indispensable for *H. pylori*.

MATERIALS AND METHODS

H. pylori Strains and Cultivation

The *H. pylori* ATCC type strains were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The *H. pylori* O-4 and P-3 were kind gifts from Dr. George Sachs, Department of Medicine at University of California, Los Angeles, CA, U.S.A. The *H. pylori* G88017-1, 22-1, 51-1, and 331-1 were authentic gifts from Dr. Kwang Ho Lee, Department of Microbiology, College of Medicine, Gyeong-Sang National University, Chinju, Kyung-Nam, Korea. The *H. pylori* cells were grown microaerobically (10% CO_2 incubator with saturated humidity) for 2 days at 37°C using a brain-heart infusion medium or Mueller Hinton medium (Difco), enriched with 5% horse serum. The cells were grown either by a solid or liquid cultivation method depending on their usage [44, 45]. For prolonged storage, the cells were suspended in a culture medium containing 10% glycerol and kept for months in a liquid nitrogen tank.

PCR Conditions to Determine *ureB* in *H. pylori*

5'-TGGGATTAGCGAGTATGT-3' (bp position: 1971-1988) and 5'-CCCATTTGACTCATG-3' (bp position: 2087-2102) were used as the sense and antisense primers, respectively [43]. Fresh *H. pylori* cells were washed twice by being resuspended in a phosphate-buffered saline solution (PBS) and centrifuged (9,000 ×g, 10 min). The resulting cell pellet was then suspended in a 10 mM Tris-HCl buffer containing 1 mM [ethylenedinitrilo]tetraacetate (EDTA), 1% sodium dodecyl sulfate (SDS), and 1 μg RNase A/ml (pH 8.0) followed by incubation for 1 h at 37°C. Three μg proteinase K/ml were then added and the mixture was incubated for 1 h at 37°C. DNA was extracted from the resultant lysate and concentrated using the phenol-ethanol method. A 0.2 μg aliquot of DNA was then taken and added to 50 μl of a PCR buffer (0.1 M Tris-HCl, 0.5 M KCl, 0.015 M $MgCl_2$, pH 8.0) containing 5 μl of 10× dNTP, 0.02 mM primers, 1 U Taq DNA polymerase, and distilled water. After denaturing for 1 min at 94°C, annealing for 1 min at 50°C, elongating for 1 min at 72°C, and post-elongating for 10 min at 72°C, 30 cycles of amplification were performed using a DNA thermal cycler (Biometra, Germany).

Urease Assay

To continuously monitor the urea hydrolysis, 1 ml of the reaction mixture containing a urease sample, 0.1 mM urea, 0.2 mM phenol red, and 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES)-Tris (pH 6.8) was placed in a 1-ml light-path cuvette. The linear change relative to time at 560 nm was observed up to a 0.5 absorbance unit [31]. When necessary, a modified Berthelot reaction [40] was also used. Briefly, portions (~0.1 ml) of the intact cells or cell-free extract were added to 0.1 ml of the above HEPES-Tris buffer (pH 6.8) containing ~1 mM urea. After 10 min of incubation (37°C), aliquots were taken, added to 0.4 ml of phenol-nitroprusside and alkaline hypochlorite (Sigma), and left for 10 min at room temperature to form indophenol blue. The absorbancy was then read at 560 nm. One unit (U) of enzyme specific activity was defined as 0.5 μmoles of urea hydrolyzed per microgram of protein per minute.

ATPase Assay

Using *H. pylori* cells, everted membrane vesicles were prepared, as described previously [45]. For the enzyme assay, the vesicles (ca. 0.1 mg proteins/0.1 ml) were carefully suspended in 0.9 ml of the reaction mixture containing 1 mM $MgCl_2$, 0.25 M sucrose, and 20 mM HEPES (pH 6.4). The enzyme reaction was then initiated at 37°C by adding 0.1 ml of 10 mM ATP. The membrane P-type ATPase was assessed by treating the membranes with 0.1 mM Na-azide before adding ATP. After 30 min of incubation, 1 ml of 12% perchloric acid containing 3.6% ammonium molybdate

was added to the reaction mixture and left at ambient temperature for 10 min to produce the pale yellow color of phosphomolybdate complex. To this, 2 ml of n-butylacetate was added, throughly mixed, and centrifuged at 1,500 ×g. The resulting organic layer was used to measure the absorbance at 320 nm [43].

Miscellaneous Assay

The leucine aminopeptidase and arylamidase were determined using L-phenylalanyl-L-3-thiaphenylalanine (PSP) and L-leucine-p-nitroanilide (Leu-pNA) as substrates, respectively [20, 21]. The γ-glutamyl transpeptidase (γ-GTP) was determined as described previously [19]. The esterase was assayed using benzyloxycarbonyl-L-alanyl-p-nitrophenyl ester (Z-Ala-ONp). For a protease assay, 1 mM mucin (Sigma) was used as a substrate; the newly appeared free amino group was reacted in a 5% borate buffer (pH 9.5) with 2,4,6-trinitrobenzene sulfonate (TNBS; Sigma) for 30 min at room temperature and the resulting yellow color of the pycryl adducts were quantified by measuring the absorbance at 420 nm [3]. Every enzyme assay was done three times and data given as the mean.

Protein Determination

The protein concentrations were estimated using a Bradford assay kit (Sigma St. Louis U.S.A.) with bovine serum albumin as a standard [5].

RESULTS

Urease Production from *H. pylori*

Under nitrogen-limiting conditions, the normal expression of the *ureB* gene is known to be essential for both the urease-positive phenotype and gastric colonization [10, 37]. Thus, a *ureB* genotype was made first using a PCR technique with the *H. pylori* strains being investigated. As seen in Fig. 1, all strains appeared to be *ureB*-positive, yielding an identical PCR product of 132 bp. In a separate

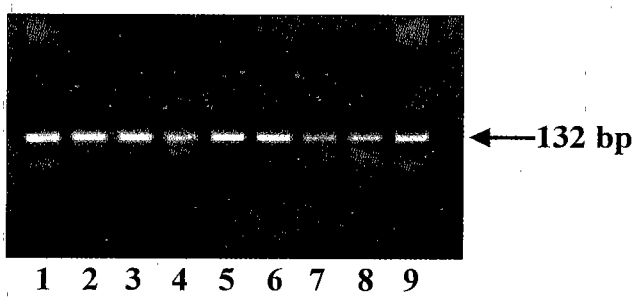

Fig. 1. PCR typing of *ureB* from *H. pylori* strains.
Lane 1, *H. pylori* ATCC 43504; 2, *H. pylori* ATCC 43526; 3, *H. pylori* ATCC 49503; 4, *H. pylori* G88017-1; 5, *H. pylori* G88022-1; 6, *H. pylori* G88051-1; 7, *H. pylori* G880333-1; 8, *H. pylori* P3; 9, *H. pylori* O4. Arrow designates the relevant PCR product pertaining to *ureB* of *H. pylori*.

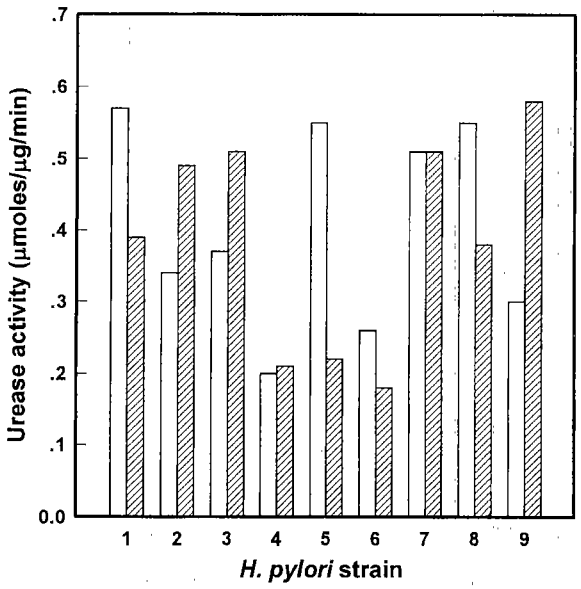

Fig. 2. Comparison of urease distribution between *H. pylori* strains.
Fresh cells of each strain of *H. pylori* were grown for 1 day at 37°C on an agar plate (see Materials and Methods for detailed culture conditions). The harvested cells were carefully suspended using a hand homogenizer into PBS (pH 7.0) and centrifuged at 9,000 ×g. The precipitated cell pellet was washed twice with distilled water (A_{660}=0.1), treated with ultrasonic waves, and centrifuged at 9,000 ×g. The resulting supernatant and previously obtained supernatant were used to determine the specific activity of the internal (□) and external (▨) urease, respectively. Strain No. 1, *H. pylori* ATCC 43504; 2, *H. pylori* ATCC 43526; 3, *H. pylori* ATCC 49503; 4, *H. pylori* G88017-1; 5, *H. pylori* G88022-1; 6, *H. pylori* G88051-1; 7, *H. pylori* G880333-1; 8, *H. pylori* P3; 9, *H. pylori* O4.

experiment, these same *H. pylori* strains were grown under identical culture conditions, and their production mode of urease was studied. By evaluating the enzyme specific activity, it was found that the urease distribution across the *H. pylori* membranes were considerably different and each strain exhibited a unique ratio of internal to external specific activity, ranging from 0.5 to 2.5 (Fig. 2). This variability could be attributed to the cells' flexibility in secreting urease. To elucidate the significance in *H. pylori*, the change of time course in the specific activity of the secreted enzyme was monitored during cell growth, and the results were compared with some other enzymes. Unexpectedly, urease was found to have the shortest life-span, concomitantly decreasing with an increased rate of coccoid formation (Fig. 3). This data suggested that the external urease activity in *H. pylori* was highly regulated.

Determination of Membrane Integrity of *H. pylori* Urease

To elucidate the mechanism by which urease was localized externally, the membrane capacity associated with the enzyme was determined using a consecutive method of cropping urease from the *H. pylori* membranes. Most of

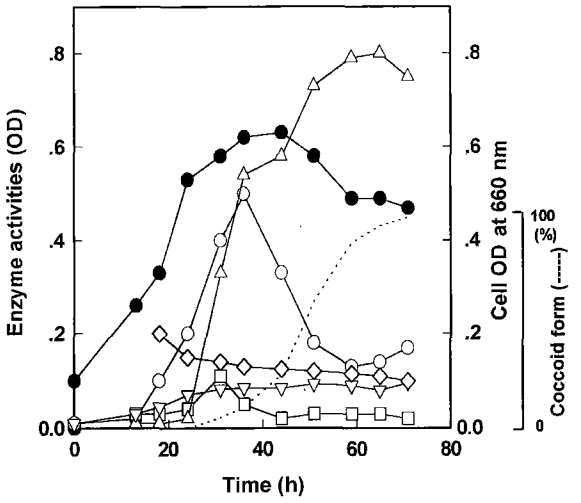

Fig. 3. Time course determination of external urease from *H. pylori*.

Using a *H. pylori* type strain, ATCC 49503, a time course of the cell growth and urease production was undertaken using an Erlenmeyer flask, during which, aliquots of culture broth were taken, centrifuged, and frozen. After thawing all the samples, the cells were removed by centrifugation at 9,000 ×g for 10 min, and the resultant supernatent was used to determine the urease specific activity. For comparison, a number of enzyme markers were also determined. Symbols used: (●) cell growth, (○) urease, (□) LAP, (△) γ-GTP, (▽) cytotoxin, (◇) ATPase. The percentage extent of *H. pylori* coccoids relative to that of bacilliform was microscopically estimated and is presented with a dotted line.

the urease was harvested by washing the *H. pylori* membranes with a buffer of low ionic strength, however, the amounts of urease in each crop greatly differed (Table 1). In some cases, considerable amounts of urease were found in the 3rd crop, likely membrane-integrated. Similarly, substantial portions of other enzymes were also detected in the 3rd cropping, except for the case of P-type ATPase, a typical transmembrane protein (Table 2). From these observations, the *H. pylori* membrane would seem to possess an unusually high capacity to contain proteins. Since proteins in a membrane must be configurated as a

Table 1. Determination of urease potential in relation to *H. pylori* membrane.

H. pylori	Urease contents (U)*		
	1st crop	2nd crop	3rd crop
ATCC 43504	0.027	0.040	0.166
ATCC 43526	0.055	0.246	1.050
ATCC 49503	0.214	2.317	0.633
G88017-1	0.095	0	0
G88022-1	0.251	0.633	0
G88051-1	0.347	0	0
G880333-1	0.245	0.769	0.300
P-3	0.862	3.333	1.138
O-4	0.904	6.831	1.582

*For unit (U) definition, see Materials and Methods. Note the discrepant and diversified contents in each crop through membrane sources.

Table 2. Determination of *H. pylori* membrane's potential to harboring enzyme proteins.

Enzyme	Enzyme contents (U)		
	1st crop	2nd crop	3rd crop
Arylamidase	0.016	0.159	0.334
ATPase	0.004	0.045	0
Esterase	0.016	0.042	0.1
γ-GTP	0.033	0.43	0.134
LAP	0.01	0.016	0.025
Protease	0.004	0.029	0.061
Urease	0.021	0.232	0.063

Cell membrane from *H. pylori* ATCC 49503 was used.

bulk complex, it was presumed that some of the integrated proteins were not active due to masking of their active sites. To confirm this idea, an experiment using a non-ionic detergent, n-octyl glucoside, was performed to find out if this detergent could unmask the enzyme active sites. The results, shown in Table 3, indicated that, regardless of the strain type of *H. pylori*, all the enzyme activities increased to some degree as a result of the detergent, thus confirming the above theory. It would appear that the enzyme active sites were transitionally masked in the course of enzyme traversing through the cell membrane.

Factors Affecting Urease Location Across *H. pylori* Membrane

Given that *H. pylori* urease is secretory, the surrounding pH of *H. pylori* cells is presumably associated with this action. To demonstrate the pH effect on urease secretion, an experiment with intact *H. pylori* cells was performed. After exposing the cells to various buffered pHs, the enzyme specific activity inside and outside the cells was determined. From a plot of the enzyme quantity as a function of pH, it was found that there was a clear-cut difference in the shape of the plateaus between the enzyme distributions: The external specific activity of urease was the highest at pH 5.5 with a narrow plateau, whereas the internal specific activity was within a pH range of 4.5 to 6.5 with a broad plateau. Furthermore, toward a neutral pH or over, approximately one-half of the specific activity was observed in both compartments. This data demonstrated that urease secretion was not related with urea but rather regulated by a change in the medium pH (Fig. 4). However, at the acidic limit, the activities seemingly fell off rapidly, followed by irreversible inactivation. Interestingly, the pH-dependent secretory mode of urease was somehow contrary to that of the total protein contents, indicating that a relevant acidity might account for the selective secretion of urease from *H. pylori*. The effect of some urease-related compounds (e.g., NH_4Cl, $NiCl_2$, and urea) on urease secretion was also examined, however, no recognizable effect was observed (data not shown).

Table 3. Effect of n-octyl glucoside on membrane-associated enzyme activities in *H. pylori*.

H. pylori membrane source	n-Octyl glucoside-caused activation (%)*					
	γ-GTP	Esterase	LAP	Amidase	Protease	Urease
ATCC 43504	227	218	59	47	48	31
ATCC 43526	21	30	8	ND	78	47
ATCC 49503	0	ND	ND	24	860	14
G88017-1	5	9	11	6	12	24
G88022-1	7	29	2	55	ND	17
G88051-1	0.5	116	32	ND	72	19
G880333-1	17	34	2	ND	ND	2
P-3	2	49	21	0	170	0
O-4	1	67	27	3	39	0

H. pylori membranes were isolated from the washed cells with distilled water.
*Reveals percent activation due to the presence of 1% n-octyl glucoside from the basal activity present in the cell membrane. ND, not detectable.

Acid-caused Inactivation of Internal Enzymes

To quantify the acid-susceptibility of *H. pylori*, the effect of acid on the internal enzyme activities was studied using a buffered system containing no urea. After incubating intact *H. pylori* cells for 1 h at different pH values, a cell-free extract was made by treating the cells with ultrasonic waves, then the enzyme activities were measured. As shown in Table 4, urease appeared to be among the most acid-susceptible, completely disappearing after treatment of the cells for 1 h at pH 3.5. Nevertheless, urease was the only one which retained most of its activity at pH 4.5, in which *H. pylori* cells grew. Taken together, the internal urease activity in particular would appear to be a criterion in assessing the potential of *H. pylori*'s viability under acidic environments. Alternatively, it might equally indicate that *H. pylori* should not be an acidophile, because of an inability to regulate the internal proton concentration without urea. In the current study, urease and γ-GTP were both prone to inactivation when the *H. pylori* cells were placed under conditions of a low pH (≤4.0), in which some other enzymes remained active. In this situation, an increase in the free amino groups occurred, indicating the occurrence of a proteolytic event (Fig. 5). In contrast, however, the total protein content was shown to decrease for an unknown reason. To confirm that this type of proteolysis did occur restrictively, an experiment using an SDS-PAGE was performed (Fig. 6). At pH 4.0, it was clear that only a few protein bands disappeared, evidenced by positions of molecular sizes of 77 kDa, urease A (66 kDa) and B (29.5 kDa) subunits, 40 kDa, and 21 kDa. The latter two bands were presumed to be γ-GTP heavy and light subunits, respectively [7]. Whether or not this phenomenon could implicate *H. pylori* death or dormancy was not further pursued in the current work.

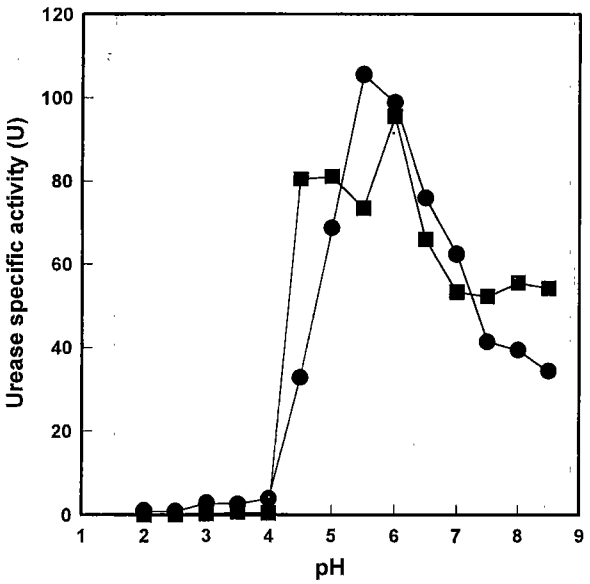

Fig. 4. Effect of pH on urease location across the *H. pylori* membrane.
Intact *H. pylori* cells were suspended to be 0.1 OD at 660 nm, in a buffer containing 50 mM of acetate, phosphate, and borate, respectively (pH was adjusted by 1 N HCl or NaOH). The cell suspension was incubated under microaerobic conditions for 1 h at 37°C. After incubation, the internal and external specific activities of the urease were assessed as described in Fig. 2. Symbols used: (●) external urease, (■) internal urease.

Factors Protecting *H. pylori* from Acidification

Albeit the mechanisms by which *H. pylori* persists *in vivo* are generally believed to be due to the neutralizing effect

Table 4. Effect of external pH on internal enzyme activities of *H. pylori*.

pH	Remaining enzyme activity (U)				
	Amidase	γ-GTP	LAP	Protease	Urease
3.5	0.14	0.14	0.49	0.267	0
4.5	0.211	0.561	0.784	0.417	100
7.0	0.281	1.298	1.471	0.567	108

Intact *H. pylori* cells (ATCC 49503) were incubated at 37°C for 1 h at a constant pH using a buffer containing 20 mM of acetate, phosphate, and borate. The resulting cells were disrupted with ultrasonication, and the resultant cell-free extract was used to assess the enzyme activities using the relevant substrates as described in Materials and Methods (refer also Table 5).

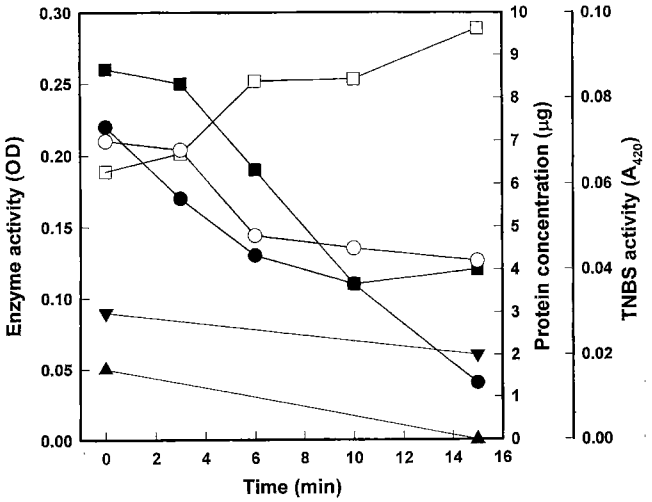

Fig. 5. Effect of acid on internal enzyme activities of *H. pylori*.
Intact *H. pylori* cells were incubated in a buffered pH of 3.5 (refer Fig. 4 for compositions) for 1 h (microaerobically at 37°C) and portions were taken and placed on an ice-bath. Sonic oscillation was applied to the resulting samples, followed by centrifugation at 9,000 ×g for 10 min to remove any cell debris. The resultant supernatants were then directly used for the enzyme assay. Symbols used: (▲) Amidase, (▼) LAP, (■) γ-GTP, (●) Urease, (○) Protein, (□) TNBS activity.

Table 5. Effect of additives on acid-caused inactivation of internal enzymes in *H. pylori*.

pH	Additives	Remaining hydrolytic activity			
		Z-Ala-ONp (A_{405})	γ-Glu-pNA (A_{412})	PSP (A_{412})	Urea (A_{625})
7	none	7.9	24.3	5.6	37.5
3	none	5.7	2.1	2.1	0.4
3	urea	7.6	17.0	2.6	4.0
3	urea+glucose	7.2	17.5	2.8	15.3
3	urea+amino acids	ND	ND	ND	0.5
3	urea+NH_4Cl	6.7	16.6	3.0	13.5
3	urea+metal ions	ND	ND	ND	0.2

H. pylori cells were suspended into prechilled 0.8% NaCl (A_{660}=0.1) containing 10 mM of each additive, then the pH was adjusted with 0.1 N HCl. The cell suspensions were incubated for 30 min (microaerobically at 37°C), during which the pH was maintained without any change, with the occasional infusion of 0.1 N HCl using a pH meter. After incubation, the cells were thoroughly washed by resuspending in the above saline solution, passed through sonication, and centrifuged at 9,000 ×g to remove any cell debris. The resulting supernatant was then used for determining the internal enzyme activities. Additives used: urea, 5 mM; D-glucose, 1%; NH_4Cl, 10 mM. Casamino acid (1 mg/ml; Sigma) was used as the amino acid mixture. The metal salts contained 0.1 mM of $ZnCl_2$, $NiCl_2$, $CuCl_2$, and $CdCl_2$, respectively.

of urease, there remain still some doubtful points. Since an earlier study reported that urease activity is not essential for the survival of the bacterium nor sufficient to fully neutralize its environment, a speculation that *H. pylori* possesses a bias to preferring a more acidic pH for survival and growth has arisen. The data in Table 5 seemed to substantiate this supposition. Even when intact *H. pylori* cells were exposed at a lethal acidity (pH 3.0), the presence of urea in conjunction with either D-glucose or ammonium chloride considerably relieved the enzyme inactivation, and the effect of ammonium chloride was remarkably similar to that of D-glucose. Several other minerals containing chloride ions were also tested, however, none of them exhibited such an effect as ammonium chloride (data not shown). From these observations, a possible role of ammonium ions, but not chloride ions, in maintaining the membrane potential (e.g., proton motive force) of *H. pylori* can be postulated.

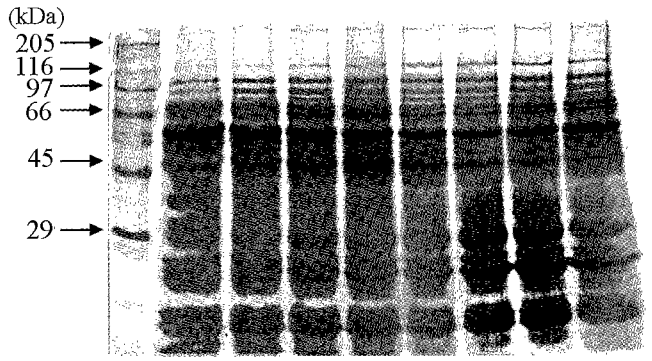

Fig. 6. Electrophoretic determination of acid-caused proteolysis in *H. pylori* cytoplasm.
Intact *H. pylori* cells were incubated in constant pH regions (refer Fig. 4 for compositions) for 1 h. Thereafter, the cells were harvested by centrifugation at 9,000 ×g, and washed twice before disruption. After sonic treatment, the resulting supernatants were used for electrophoresis upon treatment with 1% SDS. The leftmost lane shows the molecular marker proteins, and toward the right-hand side are for the protein profiles inside *H. pylori* after treating the cells for 1 h at pH values in the order of 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5.

Effect of Ammonium Ions on *H. pylori* P-type ATPase

As noted earlier, the free ammonia produced with urea hydrolysis was readily protonated, thereby increasing the milieu pH. However, because of the prodigious ammonia production by *H. pylori*, the *H. pylori* cells were born in an increasing concentration of ammonium ions. If we assume that ammonium ions acted as counter ions for the transport of other cations, such an antiport would most likely be mediated by the action of P-type ATPase [46]. To demonstrate this, an experiment was carried out using an everted membrane vesicle system from *H. pylori* cells. After saturating the vesicles with monovalent cations, such as Na^+ or K^+, the vesicle's P-type ATPase activity was measured in the presence and absence of 10 mM NH_4Cl, or vice versa. The data shown in Table 6 shows that, regardless of the location of the ammonium chloride, its presence resulted in the ATPase activation just like the above two cations. This observation strongly suggests that ammonium ions are an effective surrogate for either Na^+ or K^+, enabling a cation motive P-type ATPase, probably Kdp

Table 6. Cation specificity of membrane P-type ATPase in *H. pylori.*

Internal cation	External cation	P-type ATPase activity (A_{320})
Na^+	None	0.101
	NH_4^+	0.152
	Ni^{2+}	0.024
K^+	None	0.136
	NH_4^+	0.183
	Ni^{2+}	0.035
NH_4^+	None	0.148
	Ni^{2+}	0.042
	Na^+	0.181

Everted vesicles were saturated with various cations (internal) overnight on an ice-bath with gentle shaking. The vesicular P-type ATPase activity was then determined with or without 10 mM of cations (external). See Materials and Methods for detailed assay conditions.

K^+- or Na^+,K^+-ATPase [14], to function in maintaining the membrane potential that is supposed to be essential for *H. pylori* to control the proton concentration across cell membranes.

DISCUSSION

As a gastric pathogen, *H. pylori* is now clinically a matter of great concern. Because prolonged infection by this bacterium can account for the development of chronic gastric disease, thus predisposing to carcinoma [13, 28], elucidation of the mechanisms for *H. pylori* to respond to acid stress is of great significance in identifying a rational method of chemotherapy. A variety of evidence has suggested that urease is essential for initiating *H. pylori* colonization *in vivo* [23]. As mentioned earlier, this enzyme in bacteria is generally found only in the cytoplasmic compartment [6, 27]. In contrast, *H. pylori* urease is ubiquitous, present on both sides of the cell membrane. As for the location of the enzyme which is immediately responsible for resisting acidity, several recent reports have stressed the importance of only one side of urease location [22, 33]. The facile property of urease in traversing the *H. pylori* membrane is thought to be a clue in solving the role of the enzyme in this bacterium, in that the cell's ambient pH supposedly has a direct effect on the enzyme's translocation and/or activation.

Under this supposition, this paper introduced the concept of enzyme specific activity and investigated as follows; First, whether the ubiquitous nature of urease is a common feature in *H. pylori* was investigated. Next, studies on how and why *H. pylori* cells allow the enzyme to reside externally (including periplasm) were conducted. Finally, based on the finding that ammonium chloride has the same effect as D-glucose on *H. pylori*'s tolerance against acidity, an experiment using an everted membrane vesicle system was also performed. Through the current study, several valuable findings related to understanding the role of urease in *H. pylori* were made. First of all, the ubiquity of urease seemed to be a common mode in *H. pylori* strains, however, it varied in quantity between the compartments. Next, the external pH appeared to be the immediate cause that allowed the cells to change in their quantities between the compartments and even the extent of whole urease activity. Interestingly, in the membrane preparation, the urease activity was often markedly increased in the presence of n-octyl glucoside, thereby implying its integrated form on *H. pylori* membrane (Table 3). Accordingly, these observations would seem to suggest that the external location of urease did not result from the release due to the lysis of other cells [32] but rather as a result of selective secretion *per se* [38], particularly in the earlier life of *H. pylori*. However, there is still a question of whether this event is indispensible to *H. pylori*. In Figs. 5 and 6, it is clear that, when *H. pylori* cells encountered acid in the absence of urea, an intracellular proteolysis occurred, resulting in the diminution of certain proteins including urease. Yet, such a phenomenon was only slightly reduced with the supplementation of urea, whereas substantially reduced with either D-glucose or ammonium chloride, reflecting the importance of ammonium ions rather than ammonia itself for *H. pylori* to survive when the acidic pH of the surrounding cells remained constant (Table 5). This phenomenon may also reflect, as a teleologically satisfying exhibition, that such external location of urease is not just gratuitous but rather something eligible for this bacterium. The effect of ammonium ions in alleviating internal acidification was also addressed, postulating that ammonium ions may be transported by a P-type proton pump.

Among proton pumps, P-type ATPase is generally suggested to be involved in cation homeostasis across bacterial membranes [1, 14]. It has been previously demonstrated that, in the cell membrane of *H. pylori*, the vanadate-sensitive ATPase pool was preferentially found [44]. The fact that urea is normally abundant in the stomach (ca. 5 mM) together with the unusually high potential of *H. pylori* producing urease suggests that this bacterium must always encounter excess amounts of ammonia, thereby turning them beneficial for *H. pylori* survival. Otherwise, they would be toxic to the cells. In fact, it was previously suggested that ammonia in a cationic form may participate in cation antiport systems (J. G. Gang *et al.* 1999. *Abstr. Annu. Meet. Kor. Soc. Appl. Microbiol.*, Seoul, Korea, p. 189). In the case of gastric H^+,K^+-ATPase, the phospho-enzyme breaks down in the presence of K^+ and its surrogate NH_4^+ [39]. At an elevated pH, Na^+ is known to act as a surrogate for H^+ in terms of transport [29]. Furthermore, Na^+ has also been reported to behave like K^+ [36]. Perhaps, this interchangeable relationship

between monovalent cations is of particular importance for *H. pylori* since it suddenly encounters tremendous amounts of both H^+ and NH_4^+. The data in Tables 5 and 6 exemplify that *H. pylori* utilizes NH_4^+; on one hand, to protect itself from ambient acidity, and on the other hand, as a surrogate of either Na^+ or K^+, known as the fundamental elements for membrane energetics in nature. This signifies that the internal and external ammonium ions are both equally important in this bacterium, and the relevant distribution of urease through the inside and outside *H. pylori* membrane may be necessary to maintain the membrane potential, which is essential for this organism in controlling its proton concentration and for active transport.

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation with Specified Prime Research Grant 97-0403-0301-3.

References

1. Abee, T., K. J. Hellinhwerf, and W. N. Konings. 1988. Effects of potassium ions on proton motive force in *Rhodobacter sphaeroides*. *J. Bacteriol.* **170:** 5647-5653.
2. Andrews, R. K., R. L. Blakeley, and B. Zerner. 1984. Urea and urease. *In* Eichhorn, G. L. and L. G. Marzilli (eds.), *Advances in Inorganic Biochemistry.* vol. 6: pp. 245-283. Elsevier Sci. Pub. Inc., New York, U.S.A.
3. Binkley, F., F. Leibach, and N. King. 1968. A new method of peptidase assay and the separation of three leucyl-glycinases of renal tissues. *Arch. Biochem. Biophys.* **128:** 397-405.
4. Blaser, M. J. 1997. Ecology of *Helicobacter pylori* in the human stomach. *J. Clin. Invest.* **100:** 759-762.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
6. Braude, A. L. and J. Siemienski. 1960. Role of bacterial urease in experimental pyelonephritis. *J. Bacteriol.* **80:** 171-179.
7. Chevalier, C., J. M. Thiberge, R. L. Ferrero, and A. Labigne. 1999. Essential role of *Helicobacter pylori* γ-glutamyl transpeptidase for the colonization of the gastric mucosa of mice. *Mol. Microbiol.* **31:** 1359-1372.
8. Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and R. Rappuoli. 1993. Molecular characterization of the 128 kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc. Natl. Acad. Sci. USA* **90:** 5791-5795.
9. Cover, T. L. and M. J. Blaser. 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.* **267:** 10570-10575.
10. Cussac, V., R. L. Ferrero, and A. Labigne. 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia* grown under nitrogen-limiting conditions. *J. Bacteriol.* **174:** 2466-2473.
11. Davis, G. R., N. J. Simmonds, T. R. J. Stevens, and M. T. Sheaff. 1994. *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production *in vivo*. *Gut* **35:** 179-185.
12. Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser. 1990. Purification and characterization of urease from *Helicobacter pylori*. *J. Biol. Chem.* **265:** 9464-9469.
13. Dunn, B. E., H. Cohen, and M. Blaser. 1997. *Helicobacter pylori*. *Clin. Microbiol. Rev.* **10:** 720-741.
14. Epstein, W., M. O. Waldelhaug, J. W. Polarek, J. E. Hesse, E. Dorus, and J. M. Daniel. 1990. The bacterial Kdp K^+-ATPase and its relation to other transport ATPases, such as the Na^+/K^+- and Ca^{2+}-ATPases in higher organisms. *Philos. Trans. R. Soc. Lond. Biol. Sci.* **326:** 479-487.
15. Ferrelo, R. L. and A. Lee. 1996. The importance of urease in acid protection for the gastric colonizing bacteria, *Helicobacter pylori* and *Helicobacter felis*. *Microb. Ecol. Health Dis.* **4:** 121-134.
16. Florence, A. T. and D. Attwood. 1988. *Physicochemical Principles of Pharmacy*, 2nd ed. Chapman and Hall, New York, U.S.A.
17. Grisolia, S., R. Baguena, and F. Mayer. 1976. *The Urea Cycle*. Wiley-Liss, Inc., New York, U.S.A.
18. Hazell, S. L. and G. L. Mendz. 1993. The metabolism and enzymes of *Helicobacter pylori*: Function and potential virulence effects in *H. pylori*. *In* C. S. Goodwin and B. W. Worsley (eds.), *Biology and Clinical Practice*, CRC Press, Bosca Raton, FL. pp. 115-142.
19. Hwang, S. Y., J. H. Ryang, W. J. Lim, I. D. Yoo, and K. Oishi. 1996. Purification and properties of γ-glutamyl transpeptidase from *Bacillus* sp. KUN-17. *J. Microbiol. Biotechnol.* **6:** 238-244.
20. Hwang, S. Y., S. Y. Cho, and I. D. Yoo. 1995. Phenylalanyl-2-sulfanilylglycine as substrate for leucine aminopeptidase assay. *J. Microbiol. Biotechnol.* **5:** 319-323.
21. Hwang, S. Y., W. D. Kingsbury, N. M. Hall, D. R. Jakas, G. L. Dunn, and C. Gilvarg. 1986. Determination of leucine aminopeptidase using phenylalanyl-3-thiaphenylalanine as substrate. *Anal. Biochem.* **154:** 552-558.
22. Krishnamurthy, P., M. Parlow, J. B. Zitzer, N. B. Vakil, H. L. T. Mobley, M. Levy, S. H. Phadnis, and B. E. Dunn. 1998. *Helicobacter pylori* containing only cytoplasmic urease is susceptible to acid. *Infect. Immun.* **66:** 5060-5066.
23. Lee, A., J. Fox, and S. Hazell. 1993. Pathogenicity of *Helicobacter pylori*: A perspective. *Infect. Immun.* **61:** 1601-1610.
24. Mehler, A. H. 1992. The Urea Cycle: regulation of the urea cycle. *In* T. M. Devlin (ed.), *Textbook of Biochemistry with Clinical Correlations*. 3rd ed. Wiley-Liss, Inc. New York pp. 475-489.
25. Molby, H. L. T., R. M. Garner, and P. Bauerfeind. 1995. *Helicobacter pylori* nickel-transport gene *nixA*: Synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. *Mol. Microbiol.* **16:** 97-109.

26. Molby, H. L. T. and R. P. Hausinger. 1989. Microbial urease: Significance, regulation, and molecular characterization. *Microbiol. Rev.* **53:** 85–108.
27. Molby, H. L. T., M. D. Island, and R. P. Hausinger. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59:** 451–480.
28. Nomura, A., G. N. Stemmerman, P.-H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser. 1991. *Helicobacter pylori* infection and gastric carcinoma in a population of Japanese-Americans in Hawaii. *N. Engl. J. Med.* **325:** 1132–1136.
29. Polvani, C., G. Sachs, and R. Blostein. 1989. Sodium ions as substitutes for protons in the gastric H,K-ATPase. *J. Biol. Chem.* **264:** 17854–17859.
30. Rektorschek, M., D. Weeks, G. Sachs, and K. Melchers. 1998. Influence of pH on metabolism and urease activity of *Helicobacter pylori*. *Gastroenterol.* **115:** 628–641.
31. Ruiz-Herrera, J. and J. Gonzalez. 1969. A continuous method of urease activity. *Anal. Biochem.* **31:** 366–374.
32. Schraw, W., M. S. McClain, and T. L. Cover. 1999. Kinetics and mechanisms of extracellular protein release by *Helicobacter pylori*. *Infect. Immun.* **67:** 5247–5252.
33. Scott, D. R., D. Weeks, C. Hong, S. Postius, K. Melchers, and G. Sachs. 1988. The role of internal urease on acid resistance of *Helicobacter pylori*. *Gastroenterol.* **114:** 58–70.
34. Scouloubris, S., A. Labigne, and H. De Reuse. 1997. Identification and characterization of an aliphatic amidase in *Helicobacter pylori*. *Mol. Microbiol.* **25:** 989–998.
35. Smith, K. L. and J. Pansonnet. 1998. *Helicobacter pylori*. *In* A. S. Evans and P. S. Brachman (eds.), *Bacterial Infections of Humans: Epidemiology and control.* 3rd eds. Plenum Pub. Co. NY, U.S.A. chapt. 17, pp. 337–353.
36. Swarts, H. G. P., C. H. W. Klaassen, F. M. A. H. S. Stekhoven, and J. J. H. H. M. De Pont. 1995. Sodium acts as a potassium analog on gastric H,K-ATPase. *J. Biol. Chem.* **270:** 7890–7895.
37. Tsuda, M., M. Karita, M. G. Morched, K. Okita, and T. A. Nakazawa. 1994. Urease negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect. Immun.* **62:** 3586–3589.
38. Vanet, A. and A. Labigne. 1998. Evidence for specific secretion rather than autolysis in the release of some *Helicobacter pylori* proteins. *Infect. Immun.* **66:** 1023–1027.
39. Wallmark, B., H. B. Stewart, E. Rabon, G. Saccomani, and G. Sachs. 1980. The catalytic cycle of gastric $(H^+ + K^+)$-ATPase. *J. Biol. Chem.* **255:** 5313–5319.
40. Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39:** 971–974.
41. Williams, C. L., T. Preston, M. Hossack, C. Slater, and K. E. McColl. 1996. *Helicobacter pylori* utilizes urea for amino acid synthesis. *FEMS Immunol. Med. Microbiol.* **13:** 87–94.
42. Yamada, Y., M. Kita, T. Kodama, N. Sawai, and J. Imanishi. 1996. *Helicobacter pylori cagA* gene and expression of cytokine messenger RNA in gastric mucosa. *Gastroenterol.* **110:** 1744–1752.
43. Yoda, A. and L. E. Hokins. 1970. On the reversibility of binding of cardiotonic steroids to a partially purified (Na+K)-activated adenosinetriphosphatase from beef brain. *Biochem. Biophys. Res. Commun.* **40:** 880–886.
44. Yun, S. K. and S. Y. Hwang. 1997. Characteristics of ATPases present in everted membrane vesicles of *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **7:** 167–173.
45. Yun, S. K. and S. Y. Hwang. 1997. Expression of latent P-type ATPases and their presumptive roles in cell membrane of *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **7:** 378–385.
46. Yun, S. K., M. R. Ki, J. K. Park, W. J. Lim, and S. Y. Hwang. 2000. Cation flux-mediated activation of P-type ATPase in *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **10:** 441–448.