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Cation Flux-Mediated Activation of P-Type ATPase in *Helicobacter pylori*

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Abstract The production and cation flux-mediated activation of the P-type ATPase in *Helicobacter pylori* was investigated. Using the polymerase chain reaction (PCR), the proton pump genotype of *H. pylori* was found to be positive for both F-type and P-type ATPases. Yet, their production in terms of enzyme specific activity varied substantially depending on *H. pylori* strains, ranging over 3-fold. Its main constituent appeared to be the P-type ATPase pool, in contrast to other common bacterial compositions. Interestingly, the F-type ATPase was observed only when intact *H. pylori* cells were exposed to pH 4.5 or above (37°C for 1 h). In contrast, significant amounts of the P-type ATPase still remained after 1 h of cell treatment even at pH below 4.5. By enriching the acidic medium with RPMI (pH 3.0), the P-type ATPase was stabilized, accompanied by inactivation of the F-type ATPase. Using *H. pylori* membrane vesicles, it was found that ammonia-mediated cation flux increased the rate of ATP hydrolysis by the P-type ATPase. Accordingly, these data strongly suggest that the P-type ATPase is involved or functions as an effective regulator for the cation flux across the *H. pylori* membrane, thereby reducing the risk of excess proton influx.

Key words: *Helicobacter pylori*, proton pump, F-type ATPase, P-type ATPase, cation flux, ammonia

H. pylori is etiologically associated with gastritis or peptic ulcer, and is believed to be highly contagious [4, 7]. Failure to eliminate this organism usually results in the chronicity or recurrence of the diseases [4, 7, 20]. Moreover, prolonged pathogenesis may give rise to the development of gastric cancer [6, 26]. Thus, eradicating *H. pylori* at an early stage is now a matter of worldwide concern [21, 22]. Promising results have recently been made using triple therapy, for example, by the combination of omeprazole with either amoxicillin and clarithromycin, or metronidazole and clarithromycin [19, 34]. Unfortunately, the increasing occurrence of multi-resistant *H. pylori* strains not only exacerbates an incurable burden but also causes various unwanted side effects [5, 9]. To avoid such counterproductive chemotherapy, eradication by single dose seems to be the prudent solution. Aiming to develop such a rational therapeutic trial, the investigation of the essential machinery in *H. pylori* that protects it against sudden milieu acidity is considered to be of great significance.

The cell's function that controls the internal pH against ambient acidity seems to be one of the most attractive areas for study. Despite the considerable controversy over the pH of the surface of gastric mucosa, whether in the fundic or antral region, *H. pylori* is believed to be occasionally exposed to acid [29]. Yet, intact *H. pylori* cells are markedly sensitive to acid, being killed within 1 h at pH 3.0 *in vitro* [32]. Furthermore, a relatively narrow pH range (6.8–7.5) is required for *in vitro* growth [33]. Accordingly, the mystery is how and why *H. pylori* occupies such a hostile environment as the stomach as its ecological niche. This organism supposedly interacts with its host in such a way as to optimize the gastric environment to maintain its own physiological niche. This, in turn, may indicate that *H. pylori* possesses a specialized machinery that can regulate the cytoplasmic concentration of protons or metal ions [1]. A mechanism to resist against the toxic effect of heavy metals has been proposed in some intracellular parasites [31]. Similar machinery in which a P-type (or E_1E_2-type) ATPase may closely participate has also been identified in *H. pylori* [12]. Nonetheless, the mechanism of metal (mono- or di-valent cations) influx in this bacterium has not yet been elucidated.

Using *H. pylori* membrane, it was previously found that ammonium ion-dependent transport of nickel ions was strongly inhibited by sodium-orthovanadate [17]. It is conceivable that some of the ammonia internally produced by urease [10] was transformed to cationic form, entailing the facile flux of cations across the *H. pylori* membrane. Based on this supposition, it would appear that the nature of the acid-tolerance of *H. pylori* may be accessed by

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focusing on P-type ATPase. Accordingly, this study was first attempted to explicate the main significance of the high proportion of P-type ATPase pool in *H. pylori*. When the stability of the P-type ATPase was compared with other enzyme activities (after the exposure of intact *H. pylori* cells to acid), it would imply that the P-type ATPase is one of the essential factors enabling this organism to survive in an acidic environment. To demonstrate this, the effect of ammonium ions on the P-type ATPase activity was examined using cation-saturated membrane vesicles, and the physiological significance of the enzyme-motive cation flux in this organism is discussed.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

H. pylori type strains ATCC 43504, 43526, and 49503 were supplied from the American Type Culture Collection (Rockville, U.S.A.). *H. pylori* 51-1, 331-1, G88017-1, and G88022-1 were kindly provided by the Medical School, KyungSang University (Korea). *H. pylori* P-3 and O-4 were gifts from the Center for Ulcer Research, VAMC (Los Angeles, U.S.A.). As a standard, *H. pylori* ATCC 49503 was used in this study. Other strains (*Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, and *Salmonella typhymurium*) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Korea). The *H. pylori* cells were grown for 2 days at 37°C in a brain heart infusion medium (Difco; pH 6.8) supplemented with 5% horse serum (GibcoBRL; Life Technologies, U.S.A.). Microaerobic conditions were constantly maintained with a GasPak-enveloped jar (Difco). If necessary, a CO_2 incubator (10% CO_2 with high humidity) was used [38]. The other bacteria were aerobically grown overnight at 37°C using the Luria medium (1% bacto peptone, 0.5% yeast extract, and 1% NaCl, pH 7.0), as described previously [17].

PCR-Based Genotyping of Membrane ATPases in *H. pylori*

The synthetic primers were from Korea Biotech. Co. (Korea) and derived from the conserved sequences in the bacterial F-type ATPase β-subunit [23] and phosphorylating site of the P-type ATPase [24] as follows: For the former, 5'-GCT-GGCGTAGGCAAAAC-3' (sense primer) and 5'-TCGTC-TGCTGGCACATA-3' (antisense primer); for the latter, 5'-GCTGGCGTAGGCAAAAC-3' (sense primer) and 5'-TC-GTCTGCTGGCACATA-3' (antisense primer). A PCR was carried out with a DNA thermal cycler (Biometra, Germany) under conditions of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, elongation for 1 min at 72°C, and post-elongation for 10 min at 72°C. The cycle was repeated 30 times to obtain enough DNA fragments for determination.

Subcellular Fractionation

Unless otherwise stated, all of the following treatments of the membrane vesicles were carried out on an ice-bath. Fresh cells (grown until the mid- or late-logarithmic phase) were harvested by centrifugation (9,000 ×g, 10 min) and washed three times by resuspending in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES)-Tris buffer containing 0.25 M sucrose (HT buffer; pH 7.4). The resulting cells were swirled by thermal change (freezing & thawing in distilled water), and 10 mM [ethylenedinitrilo] tetraacetate (EDTA) and 0.5 mg lysozyme/ml were added, followed by incubation for 30 min at room temperature. After incubation, the suspension was centrifuged at 9,000 ×g for 10 min to separate the spheroplasts. The resulting supernatant was ultracentrifuged (190,000 ×g for 1 h) to isolate the outer membrane and periplasmic components, respectively. Thereafter, the spheroplasts were resuspended in the HT buffer (A_{660}=0.1), allowed to swirl again by thermal change as above, and then subsequently exposed to ultrasonic waves (Sonic & Materials Inc., U.S.A.; VCX 400, frq. 20 KHz). The cleared mixture was centrifuged (9,000 ×g, 10 min) to remove any cell debris. The resulting supernatant was ultracentrifuged as above to separate an aqueous cytoplasm and inner membrane.

ATPase Assay

To remove any unwanted soluble ATPases, the membranes were thoroughly washed until no ATP hydrolytic activity was observed in the supernatant fraction after ultracentrifugation (190,000 ×g, 1 h). The resulting membranes were concentrated to give about 10 mg/ml of 20 mM HT buffer, pH 7.4. For a typical ATPase assay, 1 ml of the reaction mixture containing 0.1 mg of the membrane vesicles, 1 mM ATP, and 20 mM 3-[N-morpholino]-2-hydroxy-propane sulfonic acid (MOPSO; Sigma product, pH 6.1) was incubated for 30 min at 37°C. If necessary, the membrane vesicles were pretreated with 0.1 mM azide (F-type ATPase inhibitor) or vanadate (P-type ATPase inhibitor) for 5 min before the addition of ATP. Upon terminating the enzyme reaction with perchloric acid, the liberated inorganic phosphate was reacted with ammonium molybdate. The ATPase activity was then quantified by measuring the amount of molybdophosphate adduct at 320 nm [37]. One unit of enzyme (U) was defined as 1 µmoles of ATP hydrolyzed per milligram of protein per minute.

Miscellaneous Assays

H. pylori cells exposed to various pH regions were subjected to ultrasonication, followed by centrifugation at 9,000 ×g for 10 min to remove cell debris. The resulting supernatant was cleaned by ultracentrifugation (190,000 ×g, 1 h), concentrated by filtering with a polysulfone membrane (10,000 NMWC), and used for following enzyme assays: Urease content was quantified by the Berthelot method by which the ammonia

liberated upon urea hydrolysis was consecutively reacted with alkaline hypochlorite and phenol-nitroprusside (Sigma reagent kit) to yield colored indophenol blue (560 nm) [35]. The leucine aminopeptidase (LAP) was determined using L-phenylalanyl-L-3-thia-phenylalanine (PSP) as a substrate (412 nm) [14]. The amidase activity was monitored with L-leucyl-*p*-nitroanilide (Leu-pNA) (405 nm) [23]. The γ-glutamyl transpeptidase (γ-GTP) assay was done with L-γ-glutamyl-*p*-nitroanilide (γ-GTP) (405 nm) [13]. The mucinase was determined by measuring the amount of pycryl adduct using mucine (Sigma product) as a substrate (420 nm) [3]. The cytotoxin activity found in the culture filtrate was determined by measuring the extent of vacuolar uptake of neutral red in HeLa cells (540 nm) [8].

Determination of Cation-Motive P-Type ATPase Activity

The membrane vesicles from *H. pylori* were suspended in 20 mM MOPSO buffer, pH 6.1, and the vesicles were saturated with mono- or di-valent metal cations by overnight incubation with 100 mM of each cation (agitated gently on an ice-bath). After incubation, the cation-saturated vesicles were harvested by ultracentrifugation (190,000 ×g, for 1 h at 4°C), resuspended in the pre-chilled TB buffer (pH 7.4) containing 3 mM EDTA, and then carefully washed by ultrafiltration at 4°C, as described above. The vesicle deposits on the filter membranes were harvested and homogenized on ice-bath with a Wheaton homogenizer. The resulting vesicles were directly used for the analysis of the cation motive P-type ATPase in the presence or absence of 10 mM NH_4Cl.

Protein Determination

The protein concentration was determined using a bicinchoninic acid assay kit according to the instructions of Pierce's Manual [36].

RESULTS

Unique Production of Membrane ATPases from *H. pylori*

In bacterial cell membranes, the accurate assessment of the ratio of proton pump contents (F-type and P-type ATPases) is extremely difficult, since substantial amounts of the F_1-domain containing the catalytic site are often found in the aqueous fraction during membrane isolation. Even though the F-type ATPase is generally regarded to be one of the most abundant proton pumps in living systems, no discernable contents of this enzyme have seemingly been observed in the *H. pylori* membrane. In contrast, the P-type ATPase activity has consistently been quantified in this organism (S. K. Yun *et al.* 1998. *Abstr. Annu. Meet. Kor. Soc. Microbiol.* p20). To demonstrate the potential of the ATPase expression in *H. pylori*, a PCR amplification was attempted using the primers described above. From this experiment, the existence

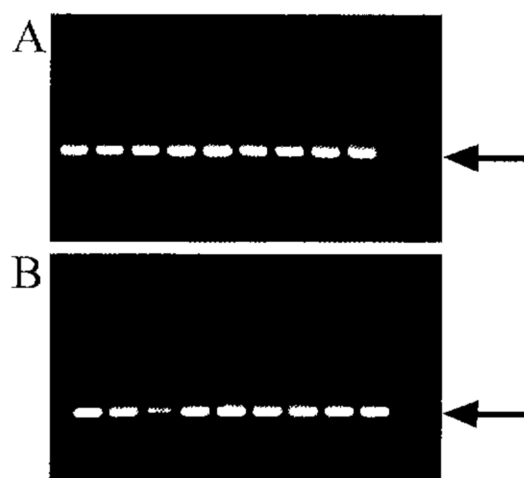

Fig. 1. Determination of *H. pylori* proton pump genotype by PCR technique.

Lanes from left to right: *H. pylori* ATCC 43504, ATCC 43526, ATCC 49503, G88017-1, G88022-1, 51-1, 333-1, P-3, O-4, and size marker (100 bp DNA ladder; GibcoBRL). Arrows indicate the corresponding PCR products of *H. pylori* proton pumps. A, F-type ATPase, B P-type ATPase. For detailed PCR conditions, see 'Materials and Methods'.

of uniform DNA base-paired fragments of 480 (for F-type ATPase) and 324 (for P-type ATPase) was ascertained throughout the *H. pylori* strains tested (Fig. 1).

The F-type and P-type ATPase contents of the *H. pylori* strains were independently determined using ATPase inhibitors. Their percentage amounts relative to the whole ATPase activity were computed, and the results are summarized in Table 1. In terms of enzyme specific activity, the productivity of proton pumps by *H. pylori* differed greatly, varying over 3-fold. Moreover, the amounts of the F-type ATPase were found to be far lower than the P-type ATPase in all cases. It

Table 1. Determination of membrane ATPases in *H. pylori*

H. pylori Strains	Enzyme quantity sensitive to[a]		Specific activity[b] (U)
	Azide	Vanadate	
ATCC 43504	25±4	52±10	0.17±0.03
ATCC 43526	4±0.05	75±15	0.07±0.01
ATCC 49503	22±5	68±12	0.04±0.007
G88017-1	15±3	70±12	0.16±0.02
G88022-1	7±2	44±9	0.06±0.005
51-1	28±6	65±13	0.06±0.006
333-1	25±5	82±11	0.03±0.003
P-3	10±2	80±16	0.06 0.006
O-4	4±1	72±14	0 03 0.004

Membrane vesicles were preincubated for 5 min at 37°C with or without 0.1 mM of the above ATPase inhibitors before the addition of ATP.

[a]Each value indicates the percentage inhibition of ATP hydrolysis by the whole membrane.

[b]Refer to 'Materials and Methods' for unit (U) definition. Data shown are the mean±standard error of every triplet assay, performed twice

Table 2. Comparison of the cellular distribution of ATPases in bacteria.

Strain	OM[a]		Periplasm		IM[b]		Cytoplasm	
	F[c]	P[d]	F	P	F	P	F	P
H. pylori	34±5	70±8	37±6	63±9	30±5	74±11	8±6	49±21
E. coli	75±10	30±2	58±8	41±9	48±6	52±2	0	48±7
K. pneumonia	65±4	38±11	12±2	42±7	51±8	60±10	8±3	8±8
P. mirabilis	42±2	56±1	26±4	14±1	55±7	38±11	42±6	68±12
S. typhimurium	70±11	0	10±2	41±3	39±10	58±7	0	7±1

Data values indicate the percentage degree of inhibition of each ATPase pool, with 0.1 mM of ATPase inhibitors, for F-type ATPase and P-type ATPase, azide and vanadate was used respectively.
[a]OM; outer membrane
[b]IM; inner membrane
[c]F; F-type ATPase.
[d]P; P-type ATPase Refer to 'Materials and Methods' for the detailed procedure of the cell fractionation.

is noteworthy that individual strains showed highly divergent ratios of ATPases, thus indicating the redundancy of the production of proton pumps by *H. pylori*. In contrast to the case of *H. pylori*, the principal ATPase in common bacteria was apparently the F-type ATPase (Table 2). Coincidently, the growth of these bacteria was highly susceptible to azide treatment. When determining the minimum inhibitory concentrations (MICs: μmoles/disk) estimated by using the semi-logarithmic slopes [17], the potential concentrations of azide on the bacterial strains were as follows: for *B. subtilis*, 0.32; for *E. coli*, 0.7; for *P. mirabilis*, 0.64; for *S. typhimurium*, 0.9; for *K. aeruginosa*, 0.3, and the MIC values of the bacteriostatic zones are underlined. Under the same conditions, *H. pylori* strains were generally tolerant against the azide treatment, requisitioning 10-fold or more than the mean value of the above MICs. Interestingly, omeprazole [18], known to inhibit P-type ATPase, showed a strong activity against *H. pylori*, yet not towards any of the other bacteria above.

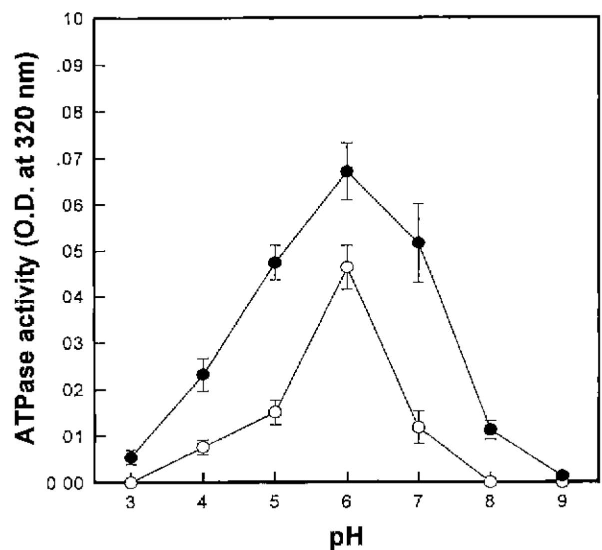

Fig. 2. Effect of pH on membrane ATPase activities from *H. pylori*.
Symbols used. (○) F-type ATPase; (●), P-type ATPase

Effect of pH on Proton Pumps in *H. pylori* Membrane

Using the everted membrane vesicles from *H. pylori*, the effect of pH on the ATPase activities was determined. The data shown in Fig. 2 reveals that the ATP hydrolytic activities of both the F-type and P-type ATPases appeared to be the highest at pH 6.0±0.1, however, they exhibited a different pH-spectrum to each other. For example, at pH one or two units lower than the above optimal pH, the relative inactivation of the F-type ATPase was far more severe than that of the P-type ATPase. From this observation, it was presumed that the virtual proton pump enabling *H. pylori* cells to resist against milieu acidity was not the F-type ATPase but rather the P-type ATPase. Despite its conceivable role in proton extrusion, the enzyme activity all but disappeared at pH 3.0 or below, thus indicating that the pH encompassing these ATPases would never decrease to such a low pH region unless the *H. pylori* cells failed to combat the acid. To verify whether this could happen in a cellular state, intact *H. pylori* cells were exposed to buffers of various pHs (microaerobically for 1 h at 37°C), and the residual ATPase activities were measured after isolation of the membrane vesicles. Coincidently, as with their optimal pH described above, the highest contents of the ATPases were maintained after the *H. pylori* cells were incubated at near pH 6.0. When taking the enzyme specific activity into account, however, the above pH was shifted to pH 4.5, wherein only the P-type ATPase could be determined. Perhaps, the acid-tolerant property of this ATPase may be associated with the *H. pylori* cell's adaptability to acid. It was noteworthy that the F-type ATPase activity was only observed at pH above 5.0, known to be the border pH for the *in vitro* growth of *H. pylori* (Fig. 3). We had earlier found that, being a cationic form, ammonia efflux in the membrane vesicles was considerably enhanced at such acidic conditions (S. K. Yun *et al.* 1999. *Abstr. Annu. Meet. Kor. Soc. Appl. Microbiol.* p. 29). These observations would

suggest that the persistent viability of *H. pylori* under acidic conditions may be attributed to the P-type ATPase function that supports the cells not to be acidified.

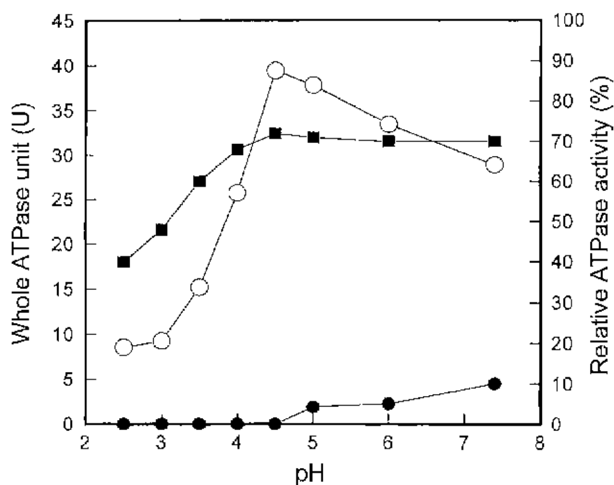

Fig. 3. Effect of medium pH on cellular proton pumps of *H. pylori*.

The *H. pylori* cell suspension (A_{600}=1.0 in 0.7% NaCl and 0.25 M sucrose) was diluted 10-fold with buffers (pH adjusted by 1 N HCl) containing 20 mM of acetate, HEPES, and Tris, and incubated for 1 h at 37°C. After incubation, the cells were harvested by centrifugation (9,000 ×g, 10 min) and washed twice. The resulting cells were disrupted for membrane preparation (refer to 'Materials and Methods' for detailed procedure). Membrane suspensions containing 0.25 M sucrose and 20 mM MOPSO (pH 6.0) were used for the ATPase assay. Symbols used are as follows: ○, whole ATPase activity (presented as unit); ● and ■, percent proportions of F-type and P-type ATPases relative to the whole ATPase activity in the vesicles, respectively.

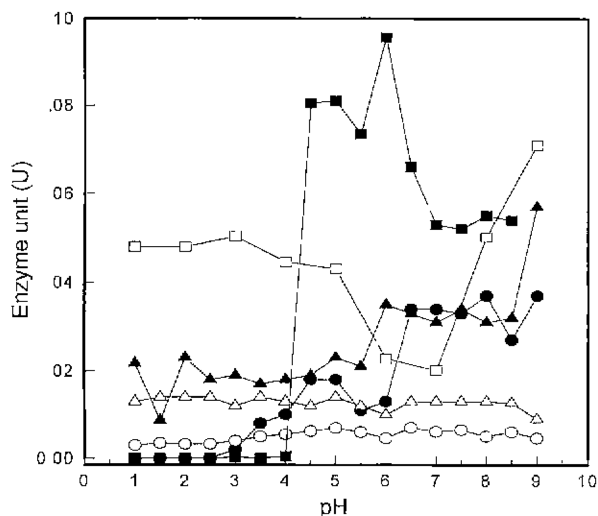

Fig. 4. Effect of medium pH on miscellaneous enzymes of *H. pylori*.

The conditions for the *H. pylori* cell treatment are presented in Fig. 3. After the cells were disrupted, the internal enzymes were determined using the relevant substrates as introduced in 'Materials and Methods'. Symbols used: γ-GTP (●), mucinase (○), urease (■), cytotoxin (□), APM (△), LAP (▲).

To further establish what would be caused internally by such an acidic environment, we also tested certain intracellular enzymes (urease, peptidases, etc.) in the different medium pHs, as above. As shown in Fig. 4, all the internal enzymes, except urease and γ-GTP, were found to be tolerant against acidic conditions (pH<3.0), exemplifying the metabolic potential of *H. pylori* under the non-growing state. It was interesting that the total protein content was reduced with an increase in the acidity, even though relevant amounts of proteins by either secretion or lytic event were not determined in the medium (data not shown) [30, 35].

Stabilization of *H. pylori* P-type ATPase by Enriching Acidic Medium

H. pylori is readily killed within 1 h at pH 3.0, unless an adequate amount of urea is provided in the buffer. An unanswered question, however, is whether urea is the only component that enables *H. pylori* to survive under acidic conditions. Therefore, to access how *H. pylori* can overcome a surrounding acidity, intact *H. pylori* cells were suspended in an acidic medium containing 1% RPMI 1640 [27] and 20 mM citric acid (pH 3.0), and incubated in the presence or absence of urea at 37°C, where the pH of the cell suspension was maintained constant by adjusting with 1 N HCl. A time course analysis showed that the P-type ATPase pool in the *H. pylori* membrane remained constant and enhanced with the aid of urea (Fig. 5). Ammonium chloride

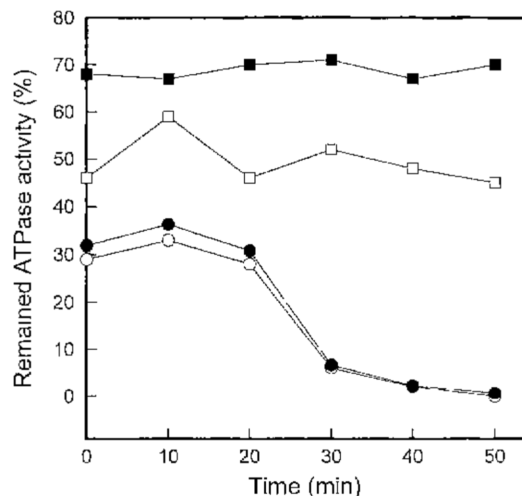

Fig. 5. Time course determination of the acid-susceptibility of membrane proton pumps in intact *H. pylori* cells.

Intact *H. pylori* cells (A_{600}=0.2) were carefully suspended in a defined medium containing RPMI and 20 mM citric acid (pH 3.0), and an aliquot was initially taken. Portions of the cell suspension were then placed on an ice-bath during microaerobic incubation at 37°C with or without 5 mM urea. Upon preparing the membrane vesicles, each ATPase activity was estimated using 0.1 mM of azide or vanadate. The relative contents of ATPases are shown as the percentage degree of inhibition out of the whole ATP hydrolytic activity. Symbols used: ○/□, F-type/P-type ATPase activity in the absence of urea; ●/■, F-type/P-type ATPase activity in the presence of urea.

also showed an effect similar to urea, indicating the importance of ammonium ions on the P-type ATPase under acidity (data not shown). In contrast, the F-type ATPase pool gradually decreased, suggesting that only the P-type ATPase functions to keep *H. pylori* cells in a dormant state when in contact with acid in their habitat. (Gang *et al.* 1999. *Abstr. Annu. Meet. Kor. Soc. Appl. Microbiol.* p30).

Effect of Ammonium Ion on Cation-Motive P-type ATPase Activity in *H. pylori* Membrane

If the *H. pylori* resistance against acid was due to the presence of urease and urea, the *H. pylori* cells must then effectively eliminate ammonium ions from their cells. Accordingly, ammonium ions as a cation antiporter may be involved in the function of the P-type ATPase, therefore, the influence of the cation flux that is the cation gradient across the membrane on the enzyme activity was investigated. To do this, membrane vesicles saturated with Ni^{2+} were prepared, and then the effect of pH or ammonium ions on the P-type ATPase activity was determined. The result showed that the enzyme activity in the presence or absence of NH_4Cl was the highest at or near pH 6.0, where an increase of over 20% in enzyme activity was observed. Astonishingly, this same inductive effect by NH_4Cl did not occur at pH 7.0. It is noteworthy that the additive effect of NH_4Cl was specifically apparent at the acidic pH region. For example, in the pH range of 3.0 to 4.0, the basal activity increased up to 5-fold with NH_4Cl, thus strongly suggesting the existence of a P-type ATPase which regulates the cation flux with ammonium ions in the *H. pylori* cells. To elucidate the cation specificity of this ATPase, the membrane vesicles were prepared, saturated with various cations, and the additive effect of NH_4Cl was examined as above. The data summarized in Table 3 reveals that the

Table 3. Activation of *H. pylori* P-type ATPase via ammonium ion-mediated cation flux.

Monovalent cation	Induction ratio (%)*
Li^+	14±3
Na^+	27±5
K^+	16±6
Rb^+	9±1
Cs^+	10±2
Divalent cation	**Induction ratio (%)***
Zn^{2+}	55±10
Cd^{2+}	26±2
Ni^{2+}	25±5
Mn^{2+}	20±5
Ca^{2+}	17±2
Mg^{2+}	15±5

Everted cation-saturated vesicles were treated with 0.1 mM azide for 5 min at room temperature before assessing the P-type ATPase activity. *Induction ratio indicates the percentage value of the net increase in the enzyme activity with the aid of 10 mM NH_4Cl.

ammonium ion could be the universal effector for cation-motive P-type ATPase. Likewise, NaCl or KCl also accelerated the enzyme activity, indicating that the ammonium ion acts as an important alternate of these monovalent cations, thereby rendering the *H. pylori* cells resistance against excess influx of protons and maintaining the cation concentrations across their membranes (data not shown).

DISCUSSION

Based on a knowledge of the fundamental function of the proton pumps in prokaryotes, the role of the membrane P-type ATPase on the acid-tolerance of *H. pylori* was investigated, albeit certain acidic conditions resulted in cell death. By evaluating various factors affecting the expression, stability, and activity of the enzyme, the role of the P-type ATPase was established, especially when *H. pylori* cells are in an acidic environment.

Dormancy under an acidic environment is supposed to be one of the most distinct properties of *H. pylori*. Since this bacterium perpetually inhabits stomach where a diurnal cycle of gastric acidity commonly yields a pH range of 1 to 2.5 [15], the repeated cycles of a dormant state would be presumed. However, unfortunately, this unique lifestyle of *H. pylori* often precludes the *in vivo* potency of antibiotics. Therefore, understanding how and why this organism prefers such an extremely hostile locus as its ecological niche is important for developing a novel anti-*H. pylori* agent that is lethal to dormant cells. With regard to its *in vitro* adaptability against pH, this organism would not appear to be a facultative acidophile, unless urea is provided [16]. Despite such a presupposition, their potential of quiescency without death in the acidic environments seems to be the most unique characteristic of the *H. pylori* physiology. Nevertheless, clear understanding of the determinants enabling this bacterium to survive in such a way is still elusive. The results in this study suggest that the P-type ATPase appears to be the factor, based on the following observations: First, as a constitutional proton pump in the *H. pylori* membrane, the P-type ATPase could tolerate acid. Second, its activity was activated by ammonium ions and further activated by counteracting cations. Accordingly, it is possible that this enzyme cooperatively maintains the internal potential of *H. pylori* (i.e., internal maintenance of enzymes; refer Fig. 4), thereby avoiding death from lethal acidity.

Mechanisms to control the intracellular pH may include reduced acid production from glycolysis and increased proton export *via* proton pumps [11]. In *H. pylori*, however, it has been generally accepted that the regulation of the internal pH is occasioned by the action of urease to produce ammonia from urea [25]. However, the machinery pertaining to the ammonia extrusion in this bacterium has not yet been elucidated. Using both intact *H. pylori* cells and membrane vesicles, it

was found that the P-type ATPase activity may be closely involved in the ammonia extrusion, especially at an acidic pH (Fig. 5 and Table 3). At a pH below 5.0, there was P-type ATPase activity yet no F-type, strongly suggesting that the F-type ATPase might not be necessary when *H. pylori* cells were in a non-growing state. No F-type ATPase activity was found until the medium pH reached to 5.0 or over (Fig. 3), which was necessary for the cells to grow. It was interesting, when comparing to other bacteria, that *H. pylori* was markedly resistant against azide treatment [17], emphasizing that this bacterium is a typical parasite which depends exclusively on energy sources from the host.

The expression of P-type ATPase is thought to be regulated environmentally. In bacteria, some of them are controlled either by a two-component system, consisting of a sensor kinase and a response regulator or by cytoplasmic cation binding proteins. Recently, a P-type ATPase encoded by the *cop*A operon of *H. pylori* was cloned and characterized [2]. Unfortunately, the corresponding enzyme, in its natural form in *H. pylori*, has not yet been studied. In the current study, vanadate-sensitive ATPase activities could easily be determined from all the cellular fractions, suggesting that a PCR-based screening technique pursuing a certain P-type ATPase would be insufficient to determine its physiological significance. Meanwhile, a preferential appearance of P-type ATPase activity in the *H. pylori* membrane was noted, thus suggesting its putative role in the cell physiology [17, 38, 39]. By using an intact cell system, it was observed that the stability of the P-type ATPase in *H. pylori* at pH 3.0 was greatly increased by enriching the system. A further increase in its activity was observed with urea or ammonium chloride (Fig. 5). D-Glucose and some mono- or di-valent cations, but not amino acids, appeared to enhance the enzyme specific activity (data not shown). As indicated in Fig. 3, the enzyme specific activity of P-type ATPase was the highest at around pH 4.5. In addition, the P-type ATPase production was closely related with the growth stage of the organism. That is, the production of the F-type ATPase was significantly induced in the exponentially growing state, followed by a decrease when the cell growth reached the static phase (S. K. Yun *et al.* 1998. *Abstr. Ann. Meet. Kor. Soc. Microbiol.* p20). Nevertheless, *H. pylori* coccoids still possessed significant amounts of the P-type ATPase, but no F-type ATPase. This observation, together with its property in the operations of the cation flux using ammonium ions, suggested that the P-type ATPase is essential for *H. pylori* survival in both a vegetative and a dormant state.

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