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Potential and Significance of Ammonium Production from *Helicobacter pylori*

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Abstract Glutamine and urea, abundant in body fluids or plasma, yield net ammonium ions upon hydrolysis by γ-glutamyl transpeptidase (γ-GTP) and urease, respectively, and these two enzymes are largely produced from *Helicobacter pylori*. To investigate bacterial potential of ammonium production, we first quantified those in whole-cell systems and found that the relative ratio of their amounts varied greatly, especially with pH values and the cell's aging. During the *H. pylori* cultivation, the ratio appeared to be inversely proportional to each other, showing a progressive increase of the γ-GTP with decreasing of the urease. Under the urease-defective conditions due to low pH or coccoids, the bacterial cells still possessed a considerable amount of γ-GTP, which was found exclusively in the external compartment, therefore, the cell's ammonium production was found to be solely dependent upon glutamine, and the external ammonium concentration was constant without any contribution of urea concentration. Such ammonium constancy would definitely have an adverse effect on the host, because of its absolute requirement for vacuolar degeneration by *H. pylori* VacA, maximized at approximately 10 mM NH_4Cl. It was also found that, by using the metal-saturated membrane vesicles, ammonium ions were likely to be involved in the pH-dependent cation-flux across the *H. pylori* membrane, where the role of γ-GTP in ammonium homeostasis around cells was suggested, especially under the hostile conditions against *H. pylori*.

Key words: *Helicobacter pylori*, ammonia, ATPase, γ-glutamyl transpeptidase, urease

Helicobacter pylori is a pathogenic Gram-negative bacterium that specifically colonizes the human gastric mucosa and it is etiologically considered to be the main causative agent of gastric disease [3, 20]. Persistent infection by *H. pylori* thus raises a chance to exacerbate the gastroduodenal disease to be chronic and to develop gastric malignancy [7]. *H. pylori* eradication is, therefore, a prerequisite for patients, but no successful treatment has yet been established for eradicating this pathogen. Furthermore, it has become increasingly apparent that current medical therapy with complex combination induces side effect or occurrence of resistant strains. Accordingly, selective targeting based on the unique property of *H. pylori* will provide an ideal trial for the control of this bacterium [15, 16, 18, 24, 27].

H. pylori is unable to grow or survive *in vitro* under the low pH conditions of 4.0 or below [29]. In contrast, the bacterial cells are often found even at areas directly encountering with gastric acid such as the gastric pit or mucosal surface [25]. In these regards, it has been postulated that the bacterium may possess a unique property committing accidental and/or prolonged acidity [1, 14]. Many published reports attribute urease to contribute to neutralizing the milieu pH of *H. pylori* [6, 11, 28]. Astonishingly, however, under the acidic conditions below pH 4.0, *H. pylori* urease is readily inactivated, unless ammonium ions are provided appropriately [12]. For this reason, we have postulated that ammonium ions would be crucial for *H. pylori* resistance against incidental exposure to acid, until urease functions properly. On the other hand, because of the ammonium toxicity causing cell death in mammalian species [19, 26], ammonium productivity by infected *H. pylori* may be a significant factor in the etiology of the host's diseases. Therefore, it was interesting to investigate the reason how this bacterium produces ammonium ions.

Similar to urea, glutamine is abundantly present in the body fluids or plasma [2]. Interestingly, typical characteristics of *H. pylori* include the prodigious production of urease and γ-GTP, both of which are recognized to be associated with *H. pylori* virulence and colonization [4, 9, 10, 21]. In the light of a possible identity of these two enzymes in net ammonium production, there is every probability of ammonium accumulation to *H. pylori*-infected mucosa

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[19, 30]. In this report, we describe the potential production of external ammonium ions by *H. pylori* and discuss its impact towards the host and the pathogen.

MATERIALS AND METHODS

H. pylori Strain and Culture Condition

H. pylori ATCC 49503 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The *H. pylori* cells were grown microaerobically [35]; In brief, *H. pylori* colonies grown on the agar plate were scraped out, and then inoculated onto the Erlenmyer flask containing a brain heart infusion medium (Difco, Franklin Lakes, NJ, U.S.A.; pH 6.8) supplemented with 5% virus-free horse serum (GibcoBRL; Life Technologies, Carlsbad, CA, U.S.A.), followed by incubating for 1-2 days at 37°C in a 10% CO_2 incubator, by using a rotary shaker (90 rpm). Cells of the logarithmic growth phase were harvested by centrifugation for 10 min at 9,000 ×g and the resulting cell pellet was washed twice by resuspending into 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (HEPES)-Tris buffer (pH 7.4). The washed cells were directly used as intact *H. pylori* cells [18].

Isolation of the *H. pylori* Compartments

Typically, fresh *H. pylori* cells were incubated microaerobically for 30 min at 37°C in a buffered solution containing 20 mM HEPES-Tris, pH 7.4. After incubation, the cell suspension was centrifuged for 10 min, and the resultant cell pellet was thoroughly washed by repeating suspension and centrifugation, using a hand homogenizer with the above HEPES-Tris buffer. The washed cells were then swirled by thermal change (freezing/thawing in distilled water). To this mixture, ethylenediamine tetraacetate (EDTA) and lysozyme were added to give final concentrations of 10 mM and 0.5 mg/ml, respectively, and placed for 30 min at room temperature. After incubation, the reaction mixture was centrifuged (9,000 ×g, 10 min) to harvest spheroplasts, and the resultant supernatant was ultracentrifuged (190,000 ×g, 1 h) to isolate the outer membrane and soluble periplasmic compartment. The above spheroplasts were exposed to ultrasonic waves (Sonic & Materials Inc., Santa Rosa, CA, U.S.A.; VCX 400, frq. 20 KHz) and centrifuged for 10 min at 9,000 ×g to remove any cell debris. The resulting supernatant was then ultracentrifuged to isolate the inner membrane from the soluble cytoplasmic compartment. To obtain membrane vesicles, the membrane sediments were carefully washed by resuspending into 10 mM HEPES buffer containing 0.25 M sucrose and 2 mM $MgCl_2$, by using a glass-teflon homogenizer.

Enzyme Assay

ATPase. Using the *H. pylori* membrane, metal cation-saturated vesicles were prepared as described previously [36]. To the vesicle suspension containing 1 mM $MgCl_2$, 0.25 M sucrose, and 20 mM HEPES, pH 6.0 (ca. 0.1 mg proteins/ml), ATP was added to give a final concentration of 1 mM, incubated for 30 min at 37°C. Enzyme reaction was terminated with 12% perchloric acid containing 3.6% ammonium molybdate (1:1, v/v) and left at an ice-bath for 10 min. Next, yellow pale adduct of phosphomolybdate was extracted with *n*-butylacetate, and absorbance at 320 nm was read for ATPase activity [34].

γ-GTP. Typically, enzyme was reacted with 0.1 mM γ-glutamyl-*p*-nitroanilide (γ-glutamyl donor) in the presence or absence of 20 mM glycylglycine (γ-glutamyl acceptor), and the amount of liberated *p*-nitroaniline was measured at 405 nm. When necessary, the extent of transpeptidation was assessed by determining the relative ratio of the increased amount of *p*-nitroaniline by the acceptor [22].

Urease. The liberated ammonium ions upon hydrolysis of urea by urease were reacted with an indophenol blue-forming kit containing phenol-nitropruside and alkaline hypochlorite (Sigma), and the blue color was determined spectrophotometrically at 625 nm [33]. This method was also used to determine ammonium concentration throughout this study.

Determination of Ammonium-Involved Vacuolation with *H. pylori* VacA

VacA protein was purified according to the method described previously [5]. Briefly, to the seeded 96-well titration plate with HeLa cells (1×10^4 cells/well), VacA sample, NH_4Cl, and Eagle's modified minimum essential medium (MEM) containing 10% fetal bovine serum were added, followed by incubation in a 5% CO_2 incubator for up to 2 days at 37°C. After incubation, the extent of vacuolation was determined by a staining technique using neutral red: Each well was carefully washed with saline solution, and then the dye accumulated inside vacuoles was extracted with an acidified ethanol, adjusted to pH 3.0 by 0.1 N HCl, and the extract was quantified spectrophotometrically. One unit (U) of VacA was denoted as an optical density of 1.0 at 540 nm per milligram of proteins. In this study, a purified VacA sample with vacuolating specificity of about 6 U was used.

Protein Determination

Protein concentrations were estimated by using a Bradford assay kit (Bio-Rad Lab., Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

RESULTS

Change of γ-GTP and Urease Production During *H. pylori* Growth

During the *H. pylori* cultivation, the apparent activities of γ-GTP and urease were differentially changed showing a

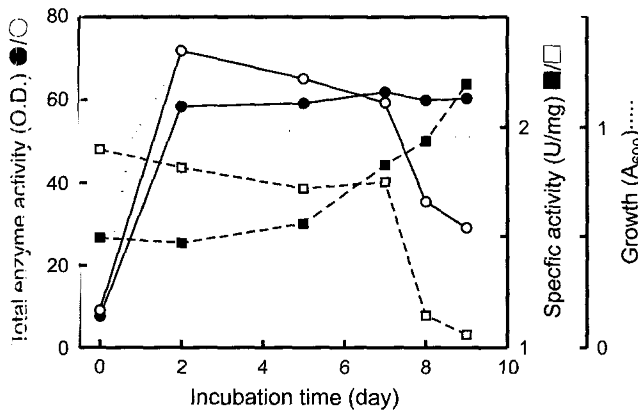

Fig. 1. Determination of γ-GTP and urease during *H. pylori* growth.

Cells were grown in an Erlenmyer flask by reciprocal shaking as described in Materials and Methods. Symbols used: ●, total activities of γ-GTP; ○, total activities of urease; ■/□, specific activities of γ-GTP and urease, respectively. One unit (U) defines O. D. 1.0/mg protein/min. Growth curve is represented as dotted line.

reverse relation to each other as a progressive increase in the γ-GTP with decrease of the urease (Fig. 1). Coincidently, a marked change of their quantity began almost equally after 6 days of cultivation; Microscopically, *H. pylori* cells at this point were ascertained to be exclusively coccoids (data not shown). It should be noted that such morphological alteration distinguishes this organism from other bacteria, occuring in the hostile environment with unknown mechanism. After a few more days of the cultivation of the coccoidal *H. pylori*, γ-GTP quantity was maximized while the urease was minimized, contrary to that shown by bacillary cells. Such an extreme change in their activities during cultivation suggested that these two enzymes interplay physiologically. According to their ability to produce net ammonium ions with substrates (glutamine, urea), we hypothesized that they would collaborate together to maintain ammonium homeostasis around the cells.

Effect of pH on *H. pylori* γ-GTP and Urease

When intact *H. pylori* cells were suspended in a buffered solution (20 mM sodium acetate-phosphate-borate, pH

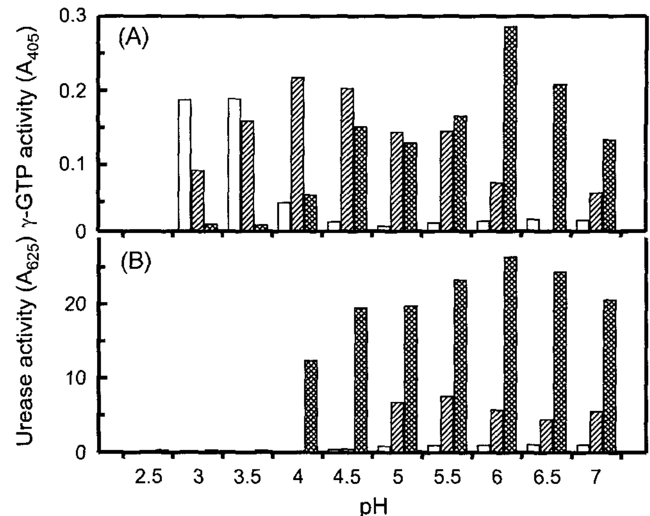

Fig. 2. Effect of pH on γ-GTP (A) and urease (B) in whole-cell suspension of *H. pylori*.

H. pylori cells were incubated at various pH values in 50 mM citrate-phosphate buffer, then centrifuged for 10 min at 9,000 xg. The resulting supernatants were denoted as 1st crops. Next, the resultant cell pellets were incubated again under the above same pH conditions for 1 h at 37°C. After incubation, the cell suspensions were centrifuged to obtain supernatants, and used as 2nd crops. Remaining enzymes of the cell pellets were also quantified. Symbols used: □, 1st crop; ▨, 2nd crop, and ▩, cellular amounts of indicated enzyme.

adjusted by 1 N HCl) of pH below 4.0, the cellular urease was largely inactivated within 1 h, whereas γ-GTP activity was still maintained without any significant loss. Most of the activities were detected in the cell-free extract; strictly speaking, activity was in the outer membrane vesicles, since all the enzyme activity was present in the sediment after the ultracentrifugation process. In contrast, most of the urease activity was in the cytoplasm [12]. After extracting by centrifugation, the resulting cells became void of γ-GTP. Nevertheless, the cells appeared to promptly restore the enzyme simply by successive exposure to acidity, yielding even larger amounts than that of the above 1st harvest (Fig. 2). Such a cropping with acid was able to repeat several times, and this raises a question of how this phenomenon was possible in such a nongrowing cell system. Meanwhile,

Table 1. Determination of γ-GTP distribution in *H. pylori*.

	Protein (mg)		γ-GTP		Specific activity[a]	
	Bacilli	Coccoids	Bacilli	Coccoids	Bacilli	Coccoids
Extracellular	4.850	3.276	28	157	0.006	0.048
Outer membrane	1.218	1.140	107	60	0.098	0.053
Periplasm	3.304	1.180	110	33	0.033	0.028
Cytoplasm	0.939	3.052	2	16	0.002	0.005
Inner membrane	1.115	4.260	11	33	0.010	0.010
Total	11.427	12.908	258	310	0.023	0.024

[a] Values of O.D. at 405 nm per micorgram of proteins.

For determination of γ-GTP distribution, 125 ml of *H. pylori* culture broth with a turbidity of 1.0 at 660 nm was used.

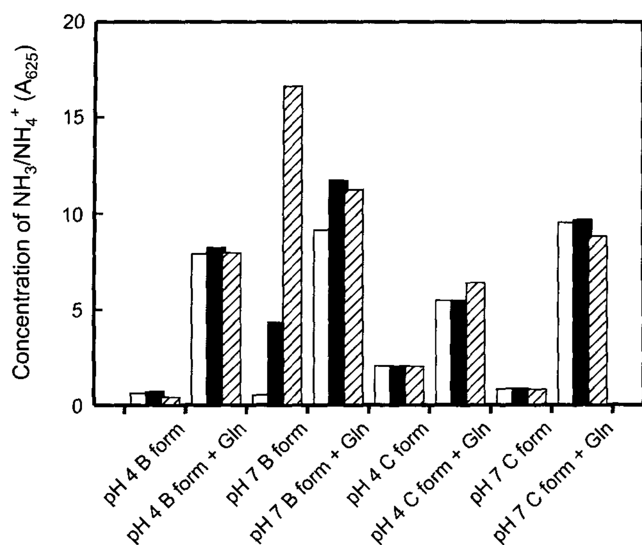

Fig. 3. Effect of glutamine and urea on *H. pylori*'s ammonium production.

Bacillary or coccoidal *H. pylori* cells were suspended in 20 mM citrate-phosphate buffer containing urea and incubated for 10 min at 37°C with or without 10 mM glutamine. After incubation, supernatants were taken by centrifugation for 10 min at 9,000 xg, and directly used for ammonium analysis. Symbols used: □, no urea; ■, 0.1 mM urea; ▨, 1 mM urea. (cf.) Bacillary (B form) and coccoidal cells (C form) were grown on agar plates for 2 days and 2 weeks, respectively.

our preliminary studies employing a RT-PCR technique suggested that *H. pylori* possesses highly versatile potential to express γ-GTP [Ki *et al.*, 2002; *Abstr. 9th Int. Sym. GIM-2002*, Gyongju, Korea. p112. P5- 16].

To determine the subcellular location of this enzyme, subcellular fractionation of *H. pyrori* was undertaken. As a result, the γ-GTP pool was found to be exclusively in the periplasmic and outer membrane fraction (Table 1). Therefore, the enzyme's action would be restricted immediate to the cell's external compartment, thereby releasing a component of bleb with outer membrane vesicles [17]. It should also be mentioned that the *H. pylori* coccoids, lacking urease activity, possessed a large amount of external γ-GTP with a

Table 2. Effect of various compounds on extracellular pH of *H. pylori* suspension.

Additive (5 mM)	pH
γ-Glu-$NHNH_2$	- 0.05
γ-Glu-OEt	- 0.09
Glutamine	- 0.05
GSH (red)[a]	- 2.81
Acrylamide	+ 0.08
Acetamide	+ 0.09
Urea	+ 2.23
None	+ 0.06

Intact *H. pylori* cells were suspended into a 0.8% NaCl solution to give a turbidity of 0.3 at 660 nm. Initial pH of the cell suspension without the compounds was adjusted to be 6.0 ± 0.1 by 0.1 HCl or NaOH. Following the addition of each compound, change of the medium pH was monitored using a digital pH-meter for 5 min at room temperature.

[a]pH was suddenly dropped at the beginning of GSH addition, and then did not change further. Abbreviation: γ-Glu-$NHNH_2$, γ-glutamylhydrazide; γ-Glu-OEt, γ-glutamylethyl ester; GSH (red), glutathione, reduced.

maximal specific activity at about pH 4.0, compatible to the urease with optimum pH near 5.0 [12]. These findings suggested that *H. pylori* γ-GTP functions under an alternative to urease, especially after being attacked by acid.

Cooperative Ammonium Production by *H. pylori* γ-GTP and Urease

As expected, *H. pylori* did not immediately utilize urea, but it was capable of producing ammonia with glutamine at pH 4.0 or coccoids. Furthermore, at pH 7.0, the urea-originated ammonium production seemed to be rather interrupted by adding glutamine, giving a near constant ammonium concentration - an estimated average value of $A_{625} = 8.0 \pm 2.0$ per 1×10^3 cells per minute, throughout the conditions tested (Fig. 3). From this finding, it was highly likely that *H. pylori in vivo* would adequately be covered by a certain concentration of ammonium ions, except in defect of the relative enzymes. We also found that, unlike the case of urease, hydrolysis of substrate by γ-GTP or amidase in the whole-cell suspension did not result in the

Table 3. Effect of medium composition on *H. pylori*'s membrane ATPase.

Condition	Final pH	Protein (μg)	Control	ATPase inhibition (%)	
				Azide	Vanadate
None	3.05	0.53	0.02	0	0
5 mM urea	6.43	0.79	0.22	0	95
5 mM urea +1% Glc +1 mM Mg^{2+}	6.52	2.26	0.23	2	86
5 mM urea +1 mg casamino acids/ml +1 mM Mg^{2+}	6.60	2.10	0.20	1	77
5 mM urea + monovalent metals (Na, K, Li, Rb)	6.60	1.25	0.34	14	92
5 mM urea + divalent metals (Zn, Ni, Mn, Mg, Cd)	6.66	1.24	0.29	8	80

H. pylori cell suspension ($A_{660} = 0.2$), containing 0.1 M NaCl, 0.25 M sucrose, and 50 mM citrate, was incubated in the presence or absence of the above additives for 30 min at 37°C in a CO_2 incubator. After incubation, cells were harvested by centrifugation (9,000 xg, 10 min), disrupted to isolate cell membrane (see Materials and Methods for isolation). Membrane vesicles suspended were placed on an ice-bath for 10 min with or without 0.1 mM ATPase inhibitor, before adding 1 mM ATP. The inhibitory extent was presented as percent ratio relative to the control values. Data are means of a triplicate assay.

Table 4. Determination of ammonium transport by cation-motive ATPase.

Vesicular metal cations	ATPase activity (O.D. at 320 nm)	Ammonium import (O.D. at 625 nm)
None	0.113 ± 0.009	0.142
Zn^{2+}	0.231 ± 0.016	0.265
Cd^{2+}	0.074 ± 0.004	0.087
Ni^{2+}	0.081 ± 0.006	0.094
Mn^{2+}	0.245 ± 0.019	0.321
Ca^{2+}	0.159 ± 0.011	0.191
Mg^{2+}	0.244 ± 0.017	0.282

Membrane vesicles (ca. 10 mg proteins) containing metal salt were suspended into a solution containing 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM NH_4Cl, and 20 mM HEPES (pH 6.0). To this was added 1 mM ATP and the mixture incubated for 30 min at 37°C. After incubation, the vesicles were harvested by ultracentrifugation (19,000 xg, 1 h) and washed by resuspending into the above HEPES buffer (pH 6.0). To determine ammonium concentration, the washed vesicles were treated with 1% n-octyl glucoside and reacted with an assay kit as described in Materials and Methods.

pH-change (Table 2), suggesting some impact of ammonium ions *per se* on bacterial physiology, other than the generally considered roles, such as on pH-neutralization or as a nitrogen source.

Effect of Ammonium Ions on Cation-Motive ATPase of *H. pylori* Membrane

Under low pH condition (pH≤4), the presence of urea or ammonium chloride is necessary for providing bacterial maintenance of the basal membrane ATPase activity [36]. To this, additional presence of energy sources (e.g., D-glucose or a mixture of 20-amino acids) did not result in any further increase of the enzyme pool, but it was obviously increased by adding mono- or divalent metal cations (Table 3). It was interesting that, under these situations, enzyme sensitivities against Na-azide, which is specific for F-type ATPase, were increased. These observations suggested a possible role of ammonium ions in keeping the membrane energetic.

To confirm this suggestion, we determined ammonium dependence of cation-motive ATPase, by using metal-saturated membrane vesicles. The result showed that all of the monovalent cations appeared to accelerate ATPase activity except NH_4^+, which in turn revealed that ammonium ions could possibly surrogate these cations [36]. In case of divalent cations, the ammonium-involved ATPase activity varied greatly with metal types, showing a close correlation with vesicular uptake of ammonium ions (Table 4). To study the effect of ammonium ions on pH dependence of the membrane ATPase, an experiment was carried out by using membrane vesicles saturated with a mixture of divalent metal cations. As shown in Fig. 4, the extravesicular ammonium ions seemed to affect ATPase strictly in a pH-dependent manner, where apparent enzyme inhibition by ammonium chloride at pH above 6.0 was quite remarkable. More importantly, such ammonium-mediated enzyme activity did not appear to be affected by a protonophore over the pH regions examined (Table 5). These data demonstrate

Table 5. Effect of NH_4^+ on Ni^{2+}-saturated vesicle's ATPase.

pH	H^+		NH_4^+	
	Ni^{2+}	Ni^{2+}+I*	Ni^{2+}	Ni^{2+}+I
5.0	0.113 ± 0.027	0.107 ± 0.026	0.123 ± 0.021	0.104 ± 0.006
6.0	0.118 ± 0.056	0.126 ± 0.051	0.152 ± 0.005	0.143 ± 0.009
7.0	0.067 ± 0.007	0.058 ± 0.011	0.072 ± 0.006	0.078 ± 0.004
8.0	0.026 ± 0.004	0.023 ± 0.001	0.017 ± 0.008	0.031 ± 0.012

*Hydrogen ionophore I (tridodecylamine; Sigma) - 10 μM was used.

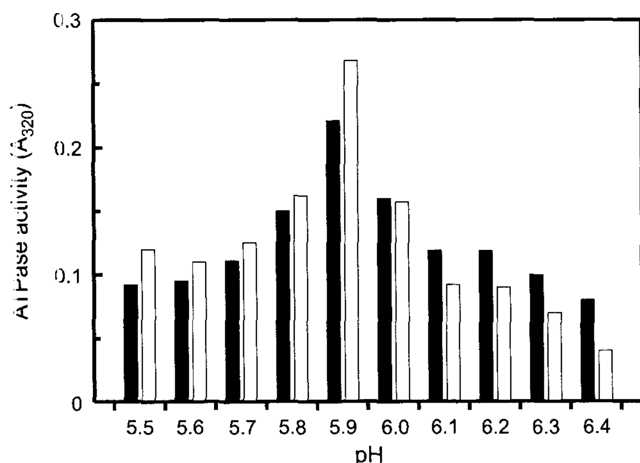

Fig. 4. Change of pH dependence of *H. pylori*'s cation-motive ATPase by ammonium chloride.

Membrane vesicles saturated with a mixture of divalent cations presented in Table 4 were used to determine ATPase in the presence (□) or absence (■) of 10 mM NH_4Cl.

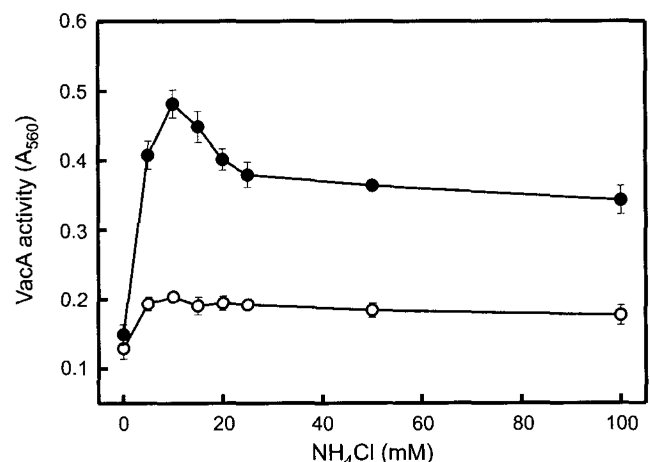

Fig. 5. Effect of ammonium chloride on vacuolating activity of *H. pylori* VacA.

For each test, about 0.1 mg of the purified VacA sample (~ 0.5 U) was employed. Symbols used: ○, control; ●, with VacA protein.

that proton itself would not be pumped out but, instead, via ammonium extrusion by a putative transport system of *H. pylori* membrane.

Role of Ammonium Ions on Vacuolating Function of *H. pylori* VacA

H. pylori VacA is a cytotoxin that vacuolates various eukaryotic cells [5]. However, studies *in vitro* show that VacA by itself cannot induce intracellular vacuoles. As presented in Fig. 5, vacuolation by VacA in HeLa cells was strictly dependent upon ammonium chloride, being maximal by initial addition of 10 mM NH_4Cl.

Discussion

H. pylori is unique in producing a large quantity of γ-GTP and urease, and their most suitable *in vivo* substrates, glutamine and urea, are major ammonium carriers in human plasma. Therefore, the incidence of abnormal accumulation of ammonium ions near *H. pylori* habitats should not be ignored. In this regard, the present study was undertaken to examine *H. pylori* potential for ammonium production and also to elucidate its significance on the bacterial physiology. The results showed two remarkable phenomena; the role of γ-GTP in maintenance of the bacterial ammonium pool in conjunction with depressed urease activity, and apparently active γ-GTP in whole cells defective of urease, especially at low pH or coccoids. Based on these findings, we suggest that γ-GTP, as an alternative to urease, may be important in keeping ammonium sink, most likely beneath the outer membrane of *H. pylori*.

In literature, glutamine synthetase was reported to be essential for *H. pylori* [13], implying that glutamine should not be transported without enzymatic deamination to glutamate. The bacterial expression of multiple transporters of this amino acid was also reported recently. Also, glutamate is suggested to play a key role for normal metabolism of both reductive and oxidative TCA cycles [8]. These notions are highly compatible with the findings of the bacterial property of external γ-GTP accumulation, especially in response to acid shock. Thus, creating ammonium cloud by *H. pylori* seems to be fundamental. Undoubtedly, we cannot rule out a large production of ammonia by urease that is suggested to be essential for acid survival. But its diffusible extrusion is likely to be constricted, which consequently exert adverse effect on the cell's metabolism, unless there is an elevated ammonium leakage due to cell lysis. In fact, at neutral pH, urea shows toxicity against this bacterium due to uncontrollable ammonium production. It is therefore important to correlate the bacterial ammonium production with its essential necessity in this bacterium. The existence of an unprecedented electrogenic ammonium transporter has recently been proposed, but it raised a great deal of controversy [23, 31]. In this study, two putative ammonium-motive ATPases, specific towards either mono- or divalent cations, are suggested to participate in ammonium extrusion, perhaps by keeping the transmembrane potential with relevant cation-flux. On this supposition, ammonium ions are thought to be indispensable for the bacterium.

However, it should not be ignored that undesirable toxicity toward host tissue near the *H. pylori* infection can happen due to progressive accumulation of the ammonium ions; Besides disturbance of the TCA cycle, some fatal ammonium effects that can be seen pathologically are as follows; Histologically, degenerative vacuolation is a characteristic *H. pylori*-associated pathogenesis that is accelerated by an acid-inducible VacA from the bacterium. Without ammonium ions, however, VacA cannot function easily (see Fig. 5). Furthermore, due to the ammonium's potassium-surrogating property on mammalian gastric H^+,K^+-ATPase [32], increased ammonium concentration may cause uncontrolled proton secretion from the gastric parietal cells, and the resulting acidity would synergistically accelerate the rate of vacuolar degeneration. This sequential event is thought to be the major risk factor for developing mucosal impairment.

In conclusion, implicit in this paper is the assumption that *H. pylori* may steadily maintain an ammonium cloud *in vivo* through two enzyme systems, i.e., γ-GTP and urease, which are likely to be prerequisite for regulating cation flux, metals or proton concentration across bacterial membrane. Because of such a unique *H. pylori* potential, we strongly point out a number of serious problems regarding the milieu host tissue. It seems that the putative ammonium-motive cation antiporter, a P-type ATPase, should be targeted.

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