

Identification and Characterization of the Acid Phosphatase HppA in *Helicobacter pylori*

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An acid phosphatase (HppA) activated by NH_4Cl was purified 192- and 34-fold from the periplasmic and membrane fractions of *Helicobacter pylori*, respectively. SDS–polyacrylamide gel electrophoresis revealed that HppA from the latter appears to be several kilodaltons larger in molecular mass than from the former by about 24 kDa. Under acidic conditions ($\text{pH} \leq 4.5$), the enzyme activity was entirely dependent on the presence of certain mono- and/or divalent metal cations (e.g., K^+ , NH_4^+ , and/or Ni^{2+}). In particular, Ni^{2+} appeared to lower the enzyme's K_m for the substrates, without changing V_{max} . The purified enzyme showed differential specificity against nucleotide substrates with pH; for example, the enzyme hydrolyzed adenosine nucleotides more rapidly at pH 5.5 than at pH 6.0, and vice versa for CTP or TTP. Analyses of the enzyme's N-terminal sequence and of an HppA⁻ *H. pylori* mutant revealed that the purified enzyme is identical to rHppA, a cloned *H. pylori* class C acid phosphatase, and shown to be the sole bacterial 5'-nucleotidase uniquely activated by NH_4Cl . In contrast to wild type, HppA⁻ *H. pylori* cells grew more slowly. Strikingly, they imported Mg^{2+} at a markedly lowered rate, but assimilated urea rapidly, with a subsequent increase in extracellular pH. Moreover, mutant cells were much more sensitive to extracellular potassium ions, as well as to metronidazole, omeprazole, or thiophenol, with considerably lowered MIC values, than wild-type cells. From these data, we suggest that the role of the acid phosphatase HppA in *H. pylori* may extend beyond 5'-nucleotidase function to include cation-flux as well as pH regulation on the cell envelope.

Keywords: *Helicobacter pylori*, acid phosphatase, HppA, ammonium ions

Helicobacter pylori is a Gram-negative bacterium that colonizes the gastric mucosa of approximately half the world's population and is believed to be the main etiological agent of gastric diseases and/or carcinomas [19, 20]. *H. pylori* apparently favors neutral pH for growth, but it resides in extremely low pH conditions, which results from the presence of gastric acid or the occasional acid shocks that occur when the gastric mucus layer is damaged [31, 32]. It is still largely unknown why this bacterium has chosen to adopt such a hostile environment as its ecological niche.

A widely accepted mechanism for the resistance of *H. pylori* against acid shocks is the bacterial production of vast amounts of urease [8, 32, 38]. In fact, *H. pylori* has a unique property of urea intake via an acid-activated channel, UreI [36]. Interestingly, urease itself is remarkably susceptible to acid and is immediately inactivated at pH 4.0 [8]. Moreover, the excessive amount of intracellular ammonia or ammonium ions produced from urea breakdown should be rapidly removed from the bacterial cell to avoid lethal toxicity. We therefore suspect that there is an underlying mechanism by which *H. pylori* effectively controls ammonium flux across the bacterial membrane, perhaps in conjunction with metal cation transport, which is thought to be essential for the survival of acidophiles and their adaptation to an acidic environment [1, 4, 16].

Interestingly, ATPases have been suggested to be involved in the regulation of pH and cation concentrations across cell membranes, but the role of ATPases in *H. pylori* has not yet been fully understood [3, 22]. Previously, Yun *et al.* [47, 48] reported that vanadate (Na_3VO_4)-sensitive ATPase was most prominent in this bacterium. This finding was initially unexpected, since bacterial membranes such as that of *Escherichia coli* were considered to contain mainly azide-sensitive ATPase [12, 14]. Surprisingly, the *H. pylori* genome contains genes that encode for three subunits of the F_0F_1 -ATPase, which are known to be downregulated

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under acidic conditions [21, 39]. Moreover, the membrane-residing ATPase pool of intact *H. pylori* cells is completely inactivated at pH 4.0, unless a sufficient amount of ammonium ions is present in the medium [13]. Ammonium ions may therefore be paradoxically important for the physiology of *H. pylori*, which supports the existence of an ammonium-motive factor [48]. Unraveling the dynamics of ATPase and ammonium ions in acidic conditions will undoubtedly further the understanding of the cryptic working of *H. pylori*.

We have thus sought to identify a putative ATPase or phosphomonoesterase, activated by NH_4Cl , from *H. pylori*. In this study, we purified and characterized an acid phosphatase specifically activated by NH_4Cl , which was determined to belong to the class C acid phosphatase family. A related *H. pylori* enzyme, HP1285-HppA, belonging to the acid phosphatase family was recently cloned and characterized [9, 27]. As a 5'-nucleotidase, the cloned enzyme activity was known to be enhanced by some divalent metal cations. In addition, this enzyme has been suggested to be able to hydrolyze the free as well as the bound forms of flavin mononucleotide (FMN) to flavodoxin (FldA) in *H. pylori* [33]: FldA is known to serve as an electron acceptor for pyruvate:oxidoreductase in the biosynthesis of acetylcoenzyme A [11]. Using an HppA-defective *H. pylori* mutant, the purified enzyme was identified as the HppA, based on the fact that there was no acid phosphatase activated by NH_4Cl . To determine the enzyme's role in *H. pylori*, we examined and compared the functional properties of the mutant and wild type. Strikingly, the mutant featured accelerated urea assimilation, with a lowered rate of Mg^{2+} uptake. We also observed that the mutant was highly sensitive to potassium ions, and to various agents such as metronidazole, omeprazole, and thiophenol.

MATERIALS AND METHODS

H. pylori Strain and Culture Conditions

H. pylori ATCC strain 26695 was obtained from the American Type Culture Collection (Rockville, MD, USA). The *H. pylori* cells were grown under a fixed air condition [13]: In brief, *H. pylori* colonies grown on agar plate were scraped out, and then inoculated onto 1-L Erlenmeyer flasks containing brain heart infusion medium (Difco, USA; pH 6.8) supplemented with 5% virus-free horse serum (GibcoBRL; Life Technologies, USA). The cells were then incubated for 1–2 days at 37°C in an atmosphere of 5% O_2 , 10% CO_2 , and 85% N_2 in a chamber, using a rotary shaker (90 rpm). Cells in the logarithmic growth phase were harvested by centrifugation for 10 min at 9,000 $\times g$, and the resulting cell pellet was washed twice by suspension in 20 mM Tris-HCl buffer (pH 8.0). The washed cells were directly used as intact *H. pylori* cells.

Subcellular Fractionations

Fresh *H. pylori* cells were incubated in the above same chamber for 30 min at 37°C in a buffered solution containing 20 mM Tris-HCl,

pH 8.0. After incubation, the cell suspension was centrifuged for 10 min, and the resultant cell pellet was thoroughly washed by repeated suspension and centrifugation, using a hand homogenizer with the above buffer. The washed cells were then swirled by thermal change; that is, freezing/thawing in 20 mM Tris-HCl containing 10% sucrose and 10 mM EDTA. Lysozyme was added to the suspension to give a final concentration of 0.5 mg/ml and incubated for 30 min at room temperature. After incubation, the reaction mixture was centrifuged (9,000 $\times g$, 10 min) to harvest spheroplasts and the supernatant was ultracentrifuged (190,000 $\times g$, 1 h) to isolate the outer membrane and the aqueous layer periplasmic compartment. The spheroplasts were exposed to ultrasonic waves (Sonic & Materials Inc., USA; VCX 400 frq., 20 KHz) and centrifuged for 10 min at 9,000 $\times g$ to remove any cell debris. The resulting supernatant was ultracentrifuged again to isolate the inner membrane from the aqueous cytoplasmic compartment. The sediments were carefully washed twice using a glass-teflon homogenizer with 20 mM HEPES containing 0.25 M sucrose (HS buffer; pH 6.0), and concentrated by ultrafiltration (polysulfone: 10,000 NMWC) [13].

Purification of Acid Phosphatase from the *H. pylori* Membrane

All purification steps were carried out at 4°C unless stated otherwise. Buffers: A, 100 mM HEPES-Tris (pH 8.0) supplemented with 100 mM KCl, 10% glycerol, 1% *n*-octyl β -D-glucopyranoside (OGP), 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM MgCl_2 , and 0.5 mM phenylmethanesulfonyl fluoride (PMSF); B, 20 mM HEPES-Tris (pH 8.0) containing 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, and 0.1% OGP. *H. pylori* membrane fractions containing both inner and outer membranes were dissolved in buffer A. After incubating for 2 h with gentle stirring, the insoluble fractions were removed by centrifugation (10,000 $\times g$, 10 min). The supernatant was then collected as the solubilized membrane fractions. The OGP extract was concentrated and then chromatographed on a DEAE-Sepharose CL-6B column that was previously equilibrated with buffer B. After washing thoroughly with buffer B, the bound proteins were eluted with a linear KCl gradient (0–1 M) and fractionated. Fractions containing ATPase activity that was increased in the presence 100 mM NH_4Cl were pooled and dialyzed against 1 M ammonium sulfate with buffer B. The resulting sample was loaded onto a Phenyl-Sepharose column that was pre-equilibrated with buffer B containing 1 M ammonium sulfate and thoroughly washed with the same buffer. The bound proteins were eluted using an ammonium-sulfated gradient (a linear 1 M to 0 M) and fractionated. The active fractions were concentrated using a Centricon-YM 10 (Amicon).

Purification of Soluble Acid Phosphatase

All purification steps were carried out at 4°C if not otherwise stated. Buffers: C, 20 mM HEPES-Tris (pH 7.4) containing 100 mM NaCl and 1 mM EDTA; D, 20 mM HEPES-Tris buffer (pH 8.4); E, 20 mM HEPES-Tris buffer (pH 9.0). Ammonium sulfate was added to saturation to the soluble periplasmic fraction and stored at 4°C overnight. The resulting precipitate was collected by centrifugation (10,000 $\times g$ for 20 min) and dissolved in buffer C, followed by dialysis against buffer C. The dialyzed sample was chromatographed on a SP-Sepharose column equilibrated with buffer C (pH 7.4). The active fraction purified through the first iteration of chromatography was again loaded onto a SP-Sepharose column equilibrated with buffer D (pH 8.4). The eluted active fraction was further chromatographed using Phe-Sepharose and Q-Sepharose resins (buffer E; pH 9.0).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli [15] using 15% polyacrylamide gel in a vertical mini-slab gel apparatus (Bio-Rad). The electrophoresis was performed at a constant voltage of 200 V for 45 min. The gel was then stained using Coomassie Brilliant Blue R-250.

Analysis of the N-Terminal Sequence of Purified Enzyme

About 10 µg of the purified enzyme from the *H. pylori* periplasm was electrophoresed on a 15% Tris-glycine polyacrylamide gel and electroblotted onto PVDF in 25 mM Tris–192 mM glycine–20% methanol for 2 h at 100 V. The membrane was stained with Ponceous S, and the band was excised. The amino-terminal 15 residues were sequenced using the Edman reaction, at Korea University Basic Science Institute.

Construction of HppA-Defective *H. pylori* Mutant

Gene replacement was used to construct the HppA-defective mutant with the plasmid kindly provided by Dr. Renata Godlewska [9]. The plasmid was constructed by insertion of a ClaI-ended kanamycin-resistant cassette carrying the *Campylobacter jejuni aph* (3') III gene (1.4 kb) into unique ClaI sites in the cloned PCR fragments of the gene for HP1285. The plasmid was introduced into *H. pylori* by natural transformation as described by Chevalier *et al.* [5] (see Fig. 5A). In brief, natural transformation was achieved by dropping 10 µl of the plasmid preparation (0.52 µg) onto 5 h-grown *H. pylori* culture on nonselective plates. After incubation for 20 h under the preceding culture conditions, cells on the plates were transferred onto new media containing 25 µg kanamycin/ml and then incubated for 3 to 5 days. Using this procedure, we were able to construct the HppA-deficient mutant strain from the *H. pylori* ATCC 26695.

Enzyme Assays

ATPase. The enzyme sample was added to a reaction mixture containing 1 mM ATP, 10 mM MgCl₂, and 20 mM HEPES (pH 6.0) to give a final volume of 1 ml. The reaction was then incubated for 30 min at 37°C. After incubation, the reaction mixture was transferred to an ice bath. The liberated inorganic phosphate was quantitated according to the method of Yoda and Hokins [46]; briefly, 1 ml of prechilled 12% perchloric acid containing 3.6% ammonium molybdate was added to the reaction mixture and the molybdophosphate adduct was extracted with 3 ml of *n*-butylacetate. After centrifugation at 1,000 rpm for 5 min, the absorbance of the colored upper phase was measured at 320 nm. If necessary, ATPase activity was also assessed using a microtiter reader, as described previously [14].

pNPPase. Typically, the enzyme was reacted with 0.1 mM para-nitrophenylphosphate (pNPP) in 1 ml of 20 mM sodium acetate/HEPES buffer supplemented with 1 mM MgCl₂ (pH 5.5 or 6.0), and the amount of liberated *p*-nitrophenol was measured at 405 nm.

Urease. The liberated ammonium ions upon urea hydrolysis were reacted with an indophenol blue-forming kit containing phenol-nitroprusside and alkaline hypochlorite (Sigma), and the resulting blue color was quantitated at 625 nm [45]. One unit (U) of enzyme activity was defined as the amount capable of hydrolyzing 1 µmol of substrate per minute. Enzyme specific activity was calculated as µmol of substrate hydrolyzed per minute per milligram of protein. The magnesium ion level was determined using a flame atomic absorption spectrophotometer (AA-670; Simadzu Co. Ltd., Japan).

Protein Determination

The protein concentrations were determined using a Bradford assay kit (Bio-Rad) with bovine serum albumin as a standard.

RESULTS

Purification of Acid Phosphatase from *H. pylori*

Fresh *H. pylori* cells were disrupted and the cell lysates were fractionated into components to determine the component enriched with ATPase activated by NH₄Cl. Strikingly, much of the enzyme activity (over 80% of the total) was shown to be concentrated in the cell envelope fraction of either the soluble or membrane-bound forms. We therefore isolated them apiece. Any fraction that

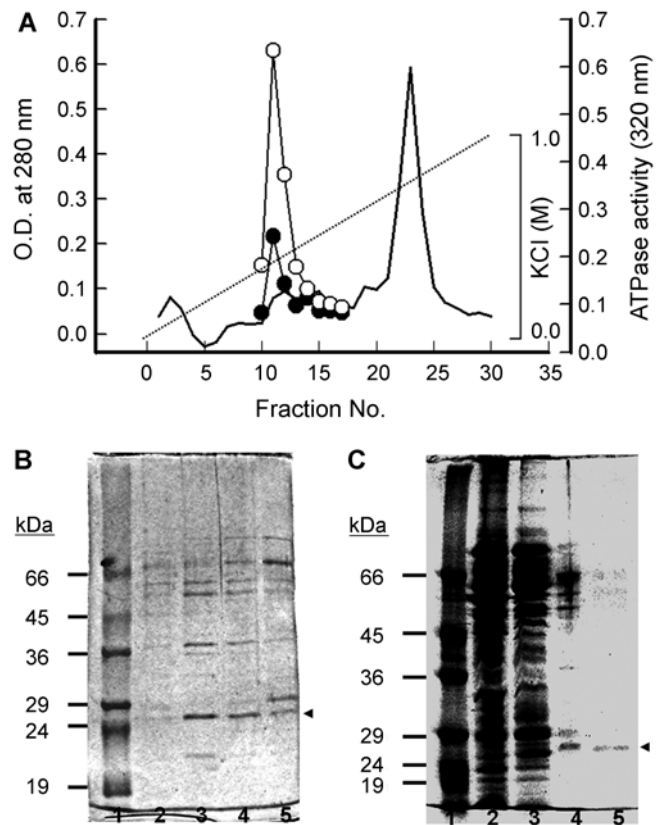


Fig. 1. Purification and SDS-PAGE analysis of ammonium-activated phosphatase from the *H. pylori* membrane.

A. DEAE-Sepharose chromatography: after KCl-gradient elution (0–1 M; 20 mM HEPES-Tris and 0.1% *n*-octylglucoside, at pH 8.0), ATP hydrolytic activity was measured in the presence (○) and absence (●) of 100 mM NH₄Cl. The dashed line indicates absorbance at 280 nm. **B.** SDS-PAGE analysis of the pooled ammonium-activated enzyme in **A**; lanes 1 to 5; the positions and molecular mass of marker proteins, and eluted fractions 10 to 13 from DEAE-Sepharose column chromatography; notice that the ammonium-activated peak is at fraction 11. **C.** SDS-PAGE analysis of purification steps: lane 1, molecular weight standards; lane 2, crude membrane; lane 3, solubilized membrane; lane 4, active fraction from DEAE-Sepharose chromatography; lane 5, active fraction from Phenyl-Sepharose chromatography. Arrowheads point to bands with a molecular mass of ca. 27,000 Da.

displayed an increase in ATP hydrolysis with NH_4Cl was regarded as containing the ammonium-motive acid phosphatase. The DEAE-Sepharose column chromatography of the OGP-treated *H. pylori* membrane sample resulted in a sharp peak, which eluted at approximately 0.4 M KCl, that was active in the presence of NH_4Cl (Fig. 1A). To isolate the same one from the periplasmic fraction, a SP-Sepharose resin was used instead of the DEAE-Sepharose. In this chromatography, the enzyme also eluted at 0.4 M KCl. The former was then further purified up to 34-fold with a final yield of 7%, and the latter was further purified up to 192-fold, with the same final yield (Table 1). SDS-PAGE revealed that their molecular mass was approximately 27 kDa (Fig. 1C) and 24 kDa (Fig. 2), respectively, assuming that the membrane-bound form may contain an extra unknown domain. As will be described shortly, the 24 kDa band of Fig. 2 corresponds to the subunits of trimeric native HppA of 72 kDa [27]. It was intriguing that, unlike the membranous enzyme, the soluble enzyme exhibited increased activity only if certain heavy metal salts such as NiCl_2 was additionally present together with NH_4Cl in the reaction mixture.

Properties of the Purified Enzyme

The rate of nucleotide hydrolysis by the purified enzyme from the bacterial periplasmic compartment was shown to be dependent on pH. As shown in Fig. 3, the enzyme hydrolyzed adenosine nucleotides more rapidly at pH 5.5 than at pH 6.0. However, the opposite trend was observed for CTP and TTP in that hydrolysis was more rapid at pH 6.0 than at pH 5.5. The enzyme was virtually inactive at $\text{pH} \leq 4.5$ but displayed substantial activity in the presence

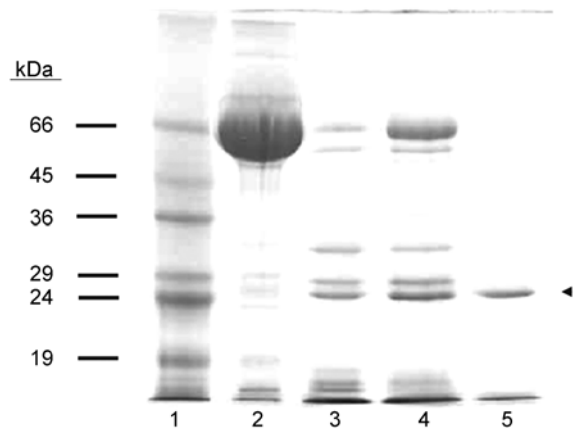


Fig. 2. SDS-PAGE analysis of the purification of soluble acid phosphatase from *H. pylori* periplasm.

Lane 1, the positions and molecular mass of marker proteins; lane 2, ammonium sulfate precipitate; lane 3, active fraction from the 1st SP-Sepharose chromatography (pH 7.4); lane 4, active fraction from the 2nd SP-Sepharose chromatography (pH 8.4); lane 5, active fraction from Q-Sepharose chromatography. The arrowhead points to the single band with a molecular mass of 24,000 Da.

of NH_4Cl and NiCl_2 (Fig. 4). This enhanced activity, however, gradually diminished when the medium pH was increased, resulting in nearly no effect at pH values greater than 7. Various metal ions had a wide range of differential effects on enzyme activity (Table 2). Enzyme activity was most effectively increased in the presence of Ni^{2+} and, to a lesser degree by Cu^{2+} or Mg^{2+} , whereas Mn^{2+} , Zn^{2+} or Ca^{2+} markedly inhibited enzyme activity; note that Cu^{2+} instead of Ni^{2+} was previously shown to be the most potent activator in cloned rHppA [27]. Strikingly, K^+ and NH_4^+ considerably

Table 1. Summary of the purification of acid phosphatase from *H. pylori*.

(A) Membrane enzyme

Purification step	Total enzyme ^a (U)	Total protein (mg)	Sp. act. (U/mg of protein)	Purification fold	Yield (%)	NH_4^+ -induced fold ^b
Crude membrane	2.4	40	0.06	1	100	1.5
Solubilization	2.2	12	0.18	3	92	2.3
DEAE-Sepharose	0.52	1	0.52	9	22	4.3
Phe-Sepharose	0.16	0.08	2.05	34	7	6.4

^aEnzyme units in cell membrane obtained from 1 liter of culture broth; ca. 3 g of cells (wet wt). Substrate used; 1 mM ATP with 10 mM MgCl_2 .

^b100 mM NH_4Cl was used; data values are activity ratio relative to the control without NH_4Cl .

(B) Periplasmic enzyme

Purification step	Total activity ^a (U)	Total protein (mg)	Sp. act. (U/mg of protein)	Purification fold	Yield (%)
$(\text{NH}_4)_2\text{SO}_4$ ppt.	2.78	35	0.08	1	100
SP-Sepharose 1st	1.5	0.68	2.21	28	54
SP-Sepharose 2nd	1.13	0.25	4.52	57	41
Phe-Sepharose	0.6	0.07	8.59	107	21
Q-Sepharose	0.2	0.013	15.38	192	7

^aEnzyme units in bacterial periplasm obtained from the same culture broth as (A).

Substrate used; pNPP.

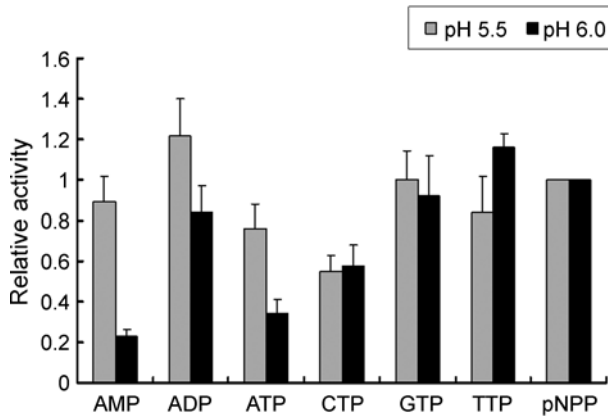


Fig. 3. Substrate specificity of the purified soluble acid phosphatase. The enzyme was incubated with 1 mM of each indicated nucleotide substrate in 20 mM sodium acetate buffer (pH 5.5 and 6) containing 10 mM $MgCl_2$. The data represent the ratio of nucleotide hydrolysis at given pH conditions relative to the control pNPP. Error bars indicate standard deviations of results from triplicate assays.

increased the rate of pNPP hydrolysis, but did not significantly affect the enzyme's ATP hydrolysis activity.

In kinetic studies of the purified enzyme from *H. pylori* membrane, similar kinetic parameters for pNPP and ATP were observed: a K_m of 0.76 ± 0.06 mM and V_{max} of $1.9 \pm 0.08 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for pNPP, and a K_m of 0.79 ± 0.11 mM and V_{max} of $2.1 \pm 0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for ATP. Ni^{2+} did not appear to affect the enzyme activity and was not examined here. In contrast, the periplasmic enzyme activity was markedly enhanced in the presence of Ni^{2+} , with considerably

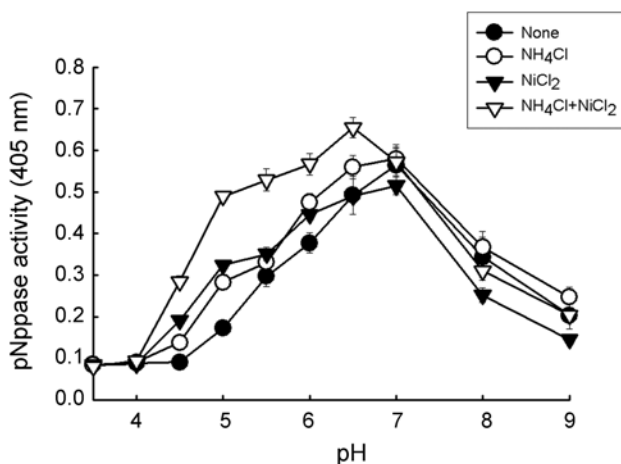


Fig. 4. Effects of cations at varying pHs on pNPP hydrolysis by the purified soluble acid phosphatase.

The buffer system was composed of 20 mM acetate, MES, HEPES, and Tris, and pH was adjusted using 0.1 N HCl or NaOH. The enzyme reaction with 0.1 mM pNPP was carried out at varying pH values in the presence or absence of additives (20 mM NH_4Cl and/or 0.1 mM $NiCl_2$). After incubation for 10 min at 37°C , the amount of *p*-nitrophenol was measured at 405 nm. Symbols used: closed circle, no cation; open circle, plus NH_4Cl ; closed inverse triangle, plus $NiCl_2$; open inverse triangle, plus both.

Table 2. Effects of metal cations on purified acid phosphatase from the *H. pylori* periplasm.

Metal cation	Conc. (mM)	Relative activity (%) ^a	
		pNPP	ATP
None	-	100	100
Mg^{2+}	10	189 ± 15	391 ± 20
Ni^{2+}	0.1	296 ± 21	643 ± 52
Cu^{2+}	0.01	290 ± 18	253 ± 13
Fe^{2+}	0.1	116 ± 10	79 ± 6
Ca^{2+}	1	86 ± 6	ND
Co^{2+}	0.1	115 ± 10	ND
Mn^{2+}	0.1	27 ± 3	ND
Zn^{2+}	0.01	63 ± 4	ND
Na^+	20	108 ± 8	73 ± 5
K^+	20	200 ± 13	81 ± 5
NH_4^+	20	157 ± 11	102 ± 8

^aData represent percentage change in the rate of hydrolysis of the given substrates in the presence of metal cations; values are the means \pm SE of three independent assays.

ND, not determined.

lowering the K_m values for pNPP and ATP (Table 3): The K_m for pNPP was 0.43 ± 0.03 mM, which decreased to 0.12 ± 0.01 mM after the addition of Ni^{2+} . Meanwhile, the K_m for ATP was significantly higher (4.14 ± 0.5 mM) than the K_m for pNPP but was also lowered, over 6-fold, in the presence of Ni^{2+} to 0.67 ± 0.1 mM. Interestingly, Ni^{2+} did not significantly affect the V_{max} for both pNPP and ATP: V_{max} values for pNPP and ATP were 15.4 ± 1.2 and $12.3 \pm 0.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively, compared with those determined in the presence of Ni^{2+} (18.9 ± 1.4 and $14.5 \pm 1.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$).

Identification of the N-Terminal Sequence

The N-terminal 15 residues of the purified enzyme from *H. pylori* periplasm were determined to be K1-E2-X3-V4-S5-

Table 3. Kinetic parameters of the purified acid phosphatase from *H. pylori*.

Enzyme source ^a	Substrate	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
Membrane	pNPP	0.76 ± 0.06	1.9 ± 0.08
	ATP	0.79 ± 0.11	2.1 ± 0.1
Periplasm	pNPP	0.43 ± 0.03	15.4 ± 1.2
	pNPP + Ni^{2+}	0.12 ± 0.01	18.9 ± 1.4
	ATP	4.14 ± 0.5	12.3 ± 0.9
	ATP + Ni^{2+}	0.67 ± 0.1	14.5 ± 1.1

^aFor membrane, active fraction from the Phe-Sepharose chromatography; for periplasm, active fraction from the Q-Sepharose chromatography. All enzyme reactions were carried out at 37°C for 10 min (20 mM HEPES/citrate, pH 6) in the presence of 20 mM NH_4Cl ; $NiCl_2$ conc. used, 0.1 mM. Initial velocities were extrapolated from Δ O.D. values/min, and the kinetic parameters were determined from the Lineweaver-Burk plot. Data represent the means \pm SE in a triplicate assay.

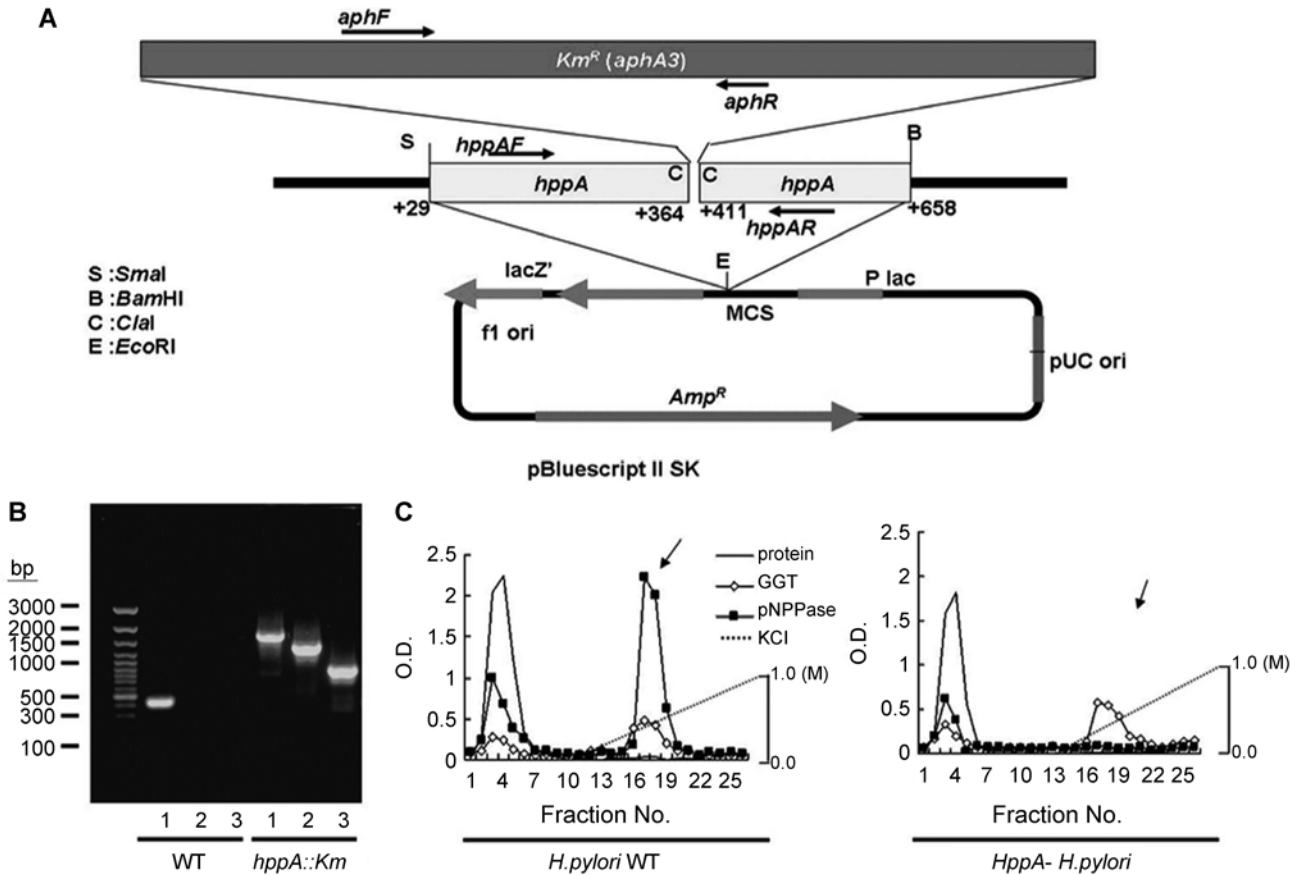


Fig. 5. Construction and identification of the HppA⁻ *H. pylori* mutant.

A. Schematic diagram of the construction of *hppA*⁻ (*hp1285-isogenic hppA::Km*). Arrows indicate the location of the primers for PCR amplification, and numbers from left to right point to restriction sites for *Sma*I, *Cl*aI, *Cl*aI, and *B*aMHI, respectively. **B.** Agarose gel electrophoresis. For diagnostic PCRs of genomic DNA from wild type and the mutant *H. pylori*, three pairs of primers were used; that is, *hppAF*/*hppAR*, *hppAF*/*aphR*, and *aphF*/*hppAR* for lanes 1 to 3, respectively. A 1 kb bp ladder marker was run in the left-most lane. **C.** Identification of HppA⁻ mutant. A single active peak, eluted at approximately 0.4 M KCl (dotted lines, KCl gradient) from SP-Sepharose chromatography of the wild type (WT; left), was absent in the mutant (see arrows). For control, a constitutive enzyme γ -glutamyltranspeptidase (GGT) was also assayed.

P6-L7-T8-R9-S10-V11-K12-Y13-X14-Q15, homologous to that of rHppA, a cloned *H. pylori* acid phosphatase [9, 27]. Strikingly, the N-terminal residues, except for the undeterminable X-marked amino acids, were identical to the 15 residues – from K22 to Q36 – of the corresponding protein present in the *H. pylori* strains ATCC 49503 and CZD 115. A stretch of 21 hydrophobic residues near the precursor’s amino terminus might be cleaved and trimmed to be activated. Alternatively, a computational analysis using the Signal-IP program suggests that this domain may be a signal sequence yet ill-defined [26]. Although arbitrary, a finishing touch, most likely with a fatty acid, could take place in the enzyme protein to direct its localization.

Construction and Properties of the HppA⁻ *H. pylori* Mutant

Kanamycin-resistant transformants (Fig. 5A) were confirmed with PCR analysis by employing suitable *hppA* and

aph(3')-III gene primers: *hppAF*, 5'-TGC ATC AAA TAC ACC CCA GA-3'; *hppAR*, 5'-TCA TCT TCC CAT GTG CCA TA-3'; *aphF*, 5'-GAA AGC TGC CTG TTC CAA AG-3'; *aphR*, 5'-CAG GCT TGA TCC CCA GTA AG-3' (Fig. 5B). The elution profiles of the *H. pylori* lysate by SP-Sepharose column chromatography showed two active peaks; one in the unbound elution and the other in the 0.4 M KCl elution, and the latter one, which was responsible for acid phosphatase activated by NH₄Cl and/or NiCl₂, did not appear in the HppA⁻ mutant cells (Fig. 5C). This indicates that our purified enzyme is the HppA. Incidentally, Reilly and Calcutt [27] reported that the cloned acid phosphatase rHppA encoded by *hppA* of *H. pylori* has a native mass of about 72 kDa, 3-fold the mass of rHppA under denatured conditions. Recall that in Fig. 2, the mass of the single band is 24 kDa, confirming that the purified enzyme here is the native form of the rHppA. After all, we were able to successfully construct acid phosphatase-

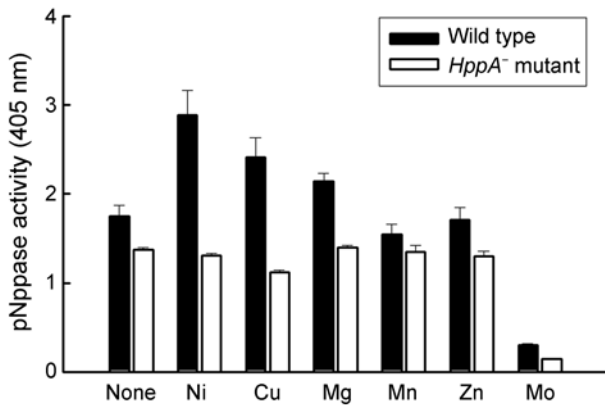


Fig. 6. Effects of metal cations on pNPP hydrolysis by cell lysates of the wild type and HppA⁻ *H. pylori* mutant. Cell lysates (0.9 ml; from cell suspension of A₆₆₀, ca. 0.5 in 50 mM HEPES, pH 5.5), with or without each heavy metal (0.1 mM), were dispensed into cuvettes (1-cm path-length) and placed for 5 min at room temp. Then 0.1 ml of 10 mM pNPP was added to the cuvettes and incubated for 10 min at 37°C. The HppA⁻ *H. pylori* cells exhibit pNPPase activity, which was not at all enhanced by the presence of heavy metal cations. Error bars indicate standard deviations from three replicate experiments.

deficient mutants of the *H. pylori* 26695 and 43504 strains. In this study, *H. pylori* 26695 and its acid phosphatase isogenic mutant were used in comparative experiments.

We first examined the effects of divalent cations on pNPP hydrolysis. In these experiments, an enhanced pNPPase activity by divalent cations was observed only in the wild-type *H. pylori* cells (Fig. 6), showing consistent metal specificity with what was observed with the purified soluble enzyme as indicated in Table 2. These data illustrate that the enzyme encoded by *hppA* is the sole acid phosphatase activated by NH₄Cl and/or heavy metals in *H. pylori*. The effects of ATPase inhibitors on the pNPP hydrolytic activity of the wild type and mutant were also determined and the data are presented in Table 4. The total pNPPase activity of the mutant was 30–40% lower than that of wild type. Whereas 1 mM ammonium molybdate [(NH₄)₆Mo₇O₂₄·4H₂O] almost completely inhibited pNPPase activity in both the wild type and mutant, no discernible effects were observed with 1 mM NaN₃ and 1 mM Na-tartrate. Moreover, NaF, omeprazole, and Na₃VO₄ (sodium orthovanadate) exerted different inhibitory effects on pNPPase activity in the wild type and mutant; that is, pNPPase activity of the mutant was more sensitive to NaF, omeprazole, and Na₃VO₄ than that of the wild type. In other words, the remaining residual pNPPase activities observed in the wild type after the addition of the above inhibitors were completely absent in the mutant. Ni²⁺ plus K⁺ or NH₄⁺ maximized the phosphatase activity in wild-type cells, whereas no changes were observed in the mutant cells.

A comparison of ammonia/ammonium accumulation and extracellular pH changes in the wild type and mutant revealed that the latter renders them to accumulate more

Table 4. Differential effects of additives on phosphatase activity of wild type vs. HppA⁻ mutant cells.

Additive	Conc. (mM)	Activity in whole cells (A ₄₀₅) ^a	
		Wild type	Mutant
None	-	1.67 ± 0.1	1.08 ± 0.06
Azide	1	1.82 ± 0.1	1.19 ± 0.08
AmMo	1	0.11 ± 0.02	0
NaF	1	1.01 ± 0.08	0.05 ± 0.02
OMP	1	0.39 ± 0.03	0
NaT	1	1.48 ± 0.1	1.11 ± 0.1
Na ₃ VO ₄	1	0.42 ± 0.05	0
Na ⁺ + Ni ²⁺	20/0.1	1.61 ± 0.1	1.12 ± 0.1
K ⁺ + Ni ²⁺	20/0.1	2.57 ± 0.15	1.09 ± 0.1
NH ₄ ⁺ + Ni ²⁺	20/0.1	2.25 ± 0.12	1.06 ± 0.1

^aActivities are expressed as optical densities at 405 nm of *p*-nitrophenol produced from pNPP in a whole-cell suspension (A₆₆₀ = 0.5) in the presence or absence of additives, after incubation for 20 min at 37°C. Abbreviations used: AmMo, ammonium molybdate; NaF, sodium fluoride; OMP, omeprazole; NaT, sodium tartrate; Na₃VO₄, acid-activated sodium orthovanadate. The data represent the means ± SE from three independent assays.

rapidly outside the bacterium, with a subsequent steeper rise in extracellular pH (Fig. 7A, 7B, and 7C); note that magnesium ions appeared to accelerate urea assimilation in wild-type cells but not in mutant cells. Strikingly, the extracellular [Mg²⁺] in the mutant strain remained relatively constant with only a mild decrease, whereas that of the wild type decreased precipitously, 20 min after administration of MgCl₂ (Fig. 7D). It was interesting that, in our preliminary studies, urea significantly diminished the deadly effect of acid (pH 3–4) by adding metal salts such as MgCl₂ not on the mutant but on the wild-type cells (data not shown). To clarify whether the accelerated urea assimilation by the mutant cells was attributable rather to higher cellular urease activity than that of wild type, the above same experiment

Table 5. Summary of the data obtained from the disc agar diffusion assays.

Compound	Conc. (μmol/disc)	Disc zone diameter (mm)	
		Wild Type	Mutant
K-PO ₄	50	0	11 ± 1 (BS)
	75	0	15 ± 1 (BS/BC)
	100	11 ± 2 (BS)	20 ± 2 (BS/BC)
Na-PO ₄	75	14 ± 1.5 (BS)	0
	100	16 ± 3 (BS)	0
Metronidazole	10	12 ± 1 (BS)	14 ± 1 (BS)
Omeprazole	0.2	15 ± 1 (BS)	16 ± 1 (BS)
Thiophenol	1	9 ± 0.5 (BC)	14 ± 1 (BC)

To *H. pylori*-seeded agar plate with culture medium, 6-mm filter paper discs containing additives were placed, and then incubated for 1 day in a 10% CO₂ incubator. Abbreviations: BS, bacteriostatic; BC, bactericidal; BS/BC, hazy zone with inner transparent zone. The data represent the means from three separate experiments.

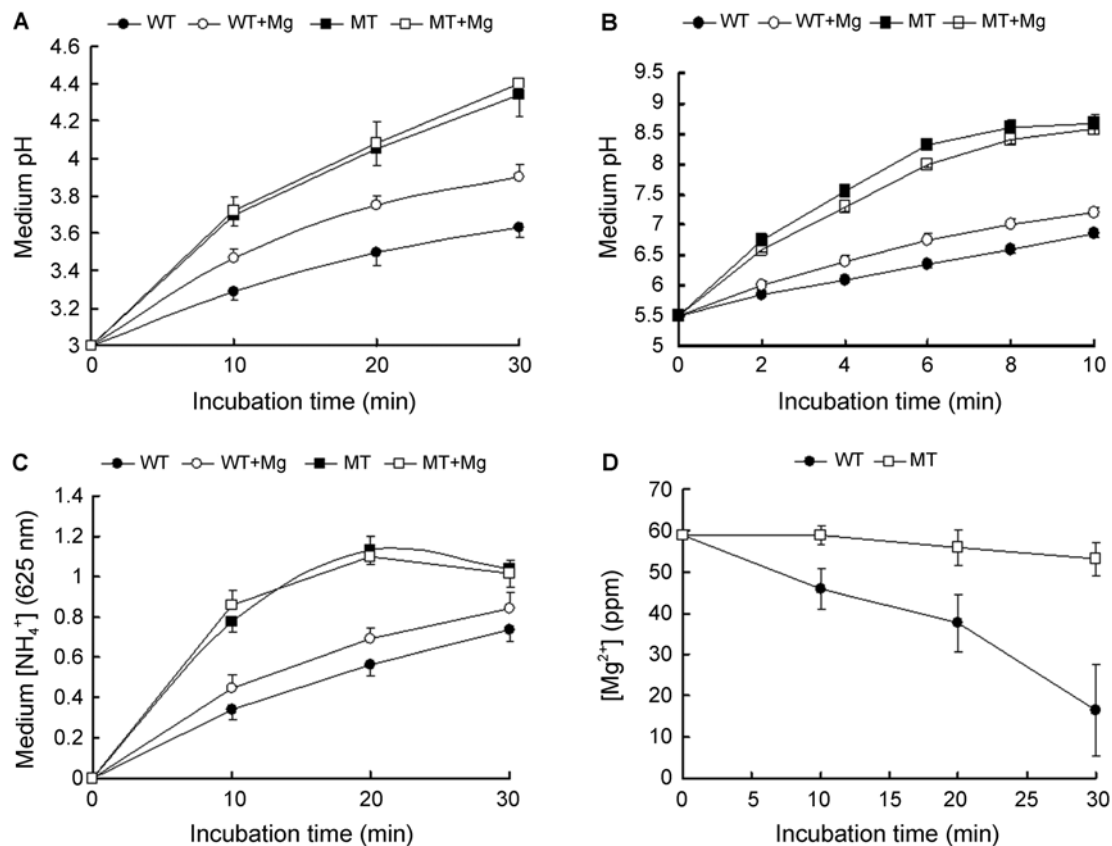


Fig. 7. Time-course profiles of changes in the extracellular pH and ammonium production after the addition of urea and/or MgCl₂ to the whole-cell suspensions of the wild type and HppA⁻ *H. pylori*.

A. Change in extracellular pH at 37°C, after urea (5 mM, final conc.) was added to the cell suspensions of pH 3, with (open symbols) or without 10 mM MgCl₂ (closed symbols): the 1-day cultured wild type (WT; circle) and mutant cells (MT; rectangle) were suspended in 5 mM of acetate–KH₂PO₄–boric acid (*A*₆₆₀, ca. 0.25). Note that MgCl₂ apparently enhanced the rate at which the pH increased in wild-type cells relative to the mutant cells. **B.** The same condition as (A), except that the initial pH of the cell suspensions was adjusted to 5.5: A rapid alkalization of the mutant cell suspension can be seen. **C.** Time course of ammonium production in (A); notice the exponential production of ammonium ions from urea by HppA⁻ cells, regardless of MgCl₂. **D.** Uptake of MgCl₂ by the wild-type and mutant cells: Cell suspensions (pH 3) initially contained 5 mM MgCl₂, and the decrease of MgCl₂ in the medium was determined by a flame atomic absorption spectrophotometer.

was done with cell lysates. Results revealed that wild-type and mutant cell lysates exhibited nearly the same urea hydrolysis activity (data not shown).

In the disc agar diffusion assay, we found that the mutant was unusually sensitive to extracellular potassium ions, which was lethal at a concentration higher than 50 μmol per paper disc (\approx 50 mM KPO₄), and yet resistant against sodium concentrations at which wild-type cells are deadly (Table 5). The mutant cells were also much more vulnerable to exposure to thiophenol and, to a lesser extent, to metronidazole or omeprazole, than wild-type cells, with varying degrees of lowered MIC values.

DISCUSSION

The results of this study provide evidence for a novel role of the acid phosphatase HppA in *H. pylori*'s regulation of

ammonium and potassium flux, with the aid of heavy metals. Drug sensitivity testing also revealed that this enzyme displays protective effects against agents known to block other bacterial ATPases as well as against the clinically relevant proton pump inhibitor omeprazole. To the best of our knowledge, this is the first study in which the *H. pylori*'s native HppA was purified and characterized.

HppA belongs to a family of bacterial nonspecific acid phosphohydrolases (NSAPs) that have an optimal dephosphorylating activity at acid to neutral pH [9]. HppA is an acid phosphatase from *H. pylori* 49503 and is homologous to HP1285 and JHP1205 from the *H. pylori* 26695 and J99 strains, respectively. The *hppA* gene is located at position 1362349–1361660 of the *H. pylori* chromosome [39]. NSAPs belong to a family of enzymes called DDDD phosphohydrolases [28] and to a larger superfamily of HD hydrolases, including P-type ATPases and haloacid dehalogenases, and are classified into class

A, B, and C on the basis of amino acid sequence clustering [2]. As noted earlier, HppA of *H. pylori* has been cloned and classified as a class C acid phosphatase and found to function as a 5'-nucleotidase [9, 27]. Recently, HppA in *H. pylori* was suggested to catalyze the hydrolysis of flavin-mononucleotide (FMN), a cofactor of flavodoxin (FldA), to riboflavin [33]. However, its precise cellular location and physiological role in the bacterium has remained largely unknown.

We have previously shown that a P-type ATPase may contribute to ammonium flux across the *H. pylori* membrane [13, 47, 48]. Consistent with our previous findings, the membrane-bound HppA exhibited an ammonium-induced enhancement of ATP hydrolysis in DEAE-Sepharose column chromatography and was further purified as a 27 kDa protein (Fig. 1), which likely contains an extra fatty acyl moiety attached as a final modification after cleavage of the N-terminal domain of the HppA precursor protein [27]. More confirmatory data were obtained with *H. pylori*'s membrane vesicles. Ammonium extrusion in these vesicles saturated with ammonium ions was most enhanced after the addition of ATP as well as divalent ions; Ni, Mg, and Cu, in decreasing order (data not shown). This suggests that this enzyme may function in the cell membrane to transport NH_4^+ , with Ni^{2+} promoting maximal ATP hydrolysis. In fact, ammonium production from urea was augmented considerably by Mg^{2+} in the wild type, but not in HppA-defective *H. pylori* cells (Fig. 7). These combined results indicate that NH_4^+ transport, or flux, may be correlative to proton-coupled heavy metal transport [10] in which HppA would be necessary. Indeed, the absence of HppA may cause uncontrolled ammonium flux and pH during urea assimilation, which is perhaps lethal to bacterial cells. Of note, there is no reported sequence homology to known NH_4^+ transporters in the genome of *H. pylori* [6, 39], despite the widespread belief that a protective mechanism must exist to prevent the toxic accumulation of urea by-products [24]. It has been postulated that *H. pylori*'s urease transporter UreI is involved in NH_3 or NH_4^+ transport in regard to acid tolerance [36]. However, this hypothesis has never been experimentally verified, and the results obtained in this study indicate that the ammonium-activated acid phosphatase HppA may play a pivotal role in the ammonium flux across the *H. pylori* membrane.

Reilly *et al.* [27] have previously shown that the cloned HppA most optimally hydrolyzed pyrimidine 5' monophosphates at pH 5.2 and purine 5' monophosphates at pH 4.6 in the presence of Cu^{2+} . Interestingly, native *H. pylori* HppA was inactive at $\text{pH} \leq 4.5$ unless ammonium and/or nickel cations were added (Fig. 4). Moreover, the hydrolysis of adenosine nucleotides, in particular, was found to be highly pH-dependent, with an optimum pH of less than pH 6 (Fig. 3). Also consistent with previous findings is the enzyme's affinity for metal cations [17]. Of note, a significant portion of the

acid-controlled genes are involved in metal cation metabolism, mainly nickel or iron [4]. Within the Class C acid phosphatase family, lipoprotein e (P4) was reported to be involved in heme transport [28], and uteroferrin (Uf), which is known as porcine purple acid phosphatase, is believed to take part in the maternal/fetal iron transport [25]. It has also been shown that the post-transcriptional modification of urease and the enzyme activity are dependent on the nickel cofactor [40], and its possible role in metal uptake has been suggested [16, 23, 32]. However, whether metal affinity signifies the enzyme's alternate function as a metal transporter is still unclear.

An interesting finding was encountered while examining the effects of some salts on bacterial growth; the disc-zone of growth inhibition test revealed that a high potassium level could be deadly to the HppA-defective *H. pylori* cells, which were nearly 10-fold as much sensitive to KPO_4 as the wild type, when compared at 100 μmol of potassium per paper disc (Table 5). From this, we inferred that HppA would be involved in potassium transport or potassium flux across the *H. pylori* membrane. In regard to sodium sensitivity, the viability of the mutant strain was unaffected, whereas that of the wild type was significantly decreased at sodium concentrations higher than 75 μmol . Based on these data, HppA may also be involved in regulating sodium flux. In fact, previous studies on the medullary collecting duct of rats have shown that NH_4^+ uptake occurs through the Na^+/K^+ -ATPase [41–44]. NH_4^+ may substitute for K^+ as a counter-ion for Na^+ transport and may be actively transported by the vertebrate Na^+/K^+ -ATPase [7, 29, 43]. In addition, it is generally accepted that NH_4^+ can replace K^+ in maintaining ATP hydrolysis by the vertebrate enzyme [18, 28, 35, 41]. This competitive binding between NH_4^+ and K^+ is physiologically insignificant, given the low NH_4^+ concentration found in the extracellular fluid under normal conditions. However, this may be of value in the extracellular environment of *H. pylori* where excess ammonium ions are produced by urease. In plants, membrane-bound inorganic pyrophosphatases (H^+ -PPases) are known to provide the membrane for energy through PPi hydrolysis, making the proton movement possible across membranes with a Na^+/H^+ exchanger [34]. Whether the enriched presence of HppA in the *H. pylori* membrane serves an important function as do H^+ -PPases in plants is an interesting question.

Furthermore, our data on the differential drug sensitivity of whole cells and enzyme activity suggest that it may contribute to drug resistance in *H. pylori*. With lowered MIC values, all drugs tested exerted more inhibitory effects on the acid phosphatase-deficient mutant, suggesting a predominant drug protective effect of the enzyme (Table 5). Remarkably, ammonium molybdate, OMP, and Na_3VO_4 completely abolished the pNPPase activity in the mutant, whereas significant activity was retained in the wild type. These data hint at the possibility that there may be a close

relationship between HppA and bacterial drug resistance. This is of vital clinical relevance, since the recent rise of resistance to drugs in *H. pylori* strains has posed challenges in treating GI tract diseases associated with *H. pylori* infection. The current treatment of *H. pylori* involves expensive combinations of various medicines, including antibiotics and proton pump inhibitors, and is not always effective [30].

Several limitations in this study should be noted. Because of the low purification yield, enzyme from the *H. pylori* membrane was not characterized to the same extent as the soluble counterpart. Obtaining clear inner and outer membrane fractions was also difficult. Furthermore, there may be a chance that the cytoplasmic protein leaked out and became associated with the membrane surface [37]. Nonetheless, this study revealed some important features of HppA in *H. pylori* as follows: (1) This enzyme is the sole bacterial acid phosphatase uniquely activated by NH₄Cl; (2) It is concentrated in the cell envelope and present in soluble and in membrane-bound forms; (3) Of the metal cations, nickel exerts the most potent effect on HppA by lowering the enzyme's K_m for substrates; (4) Enzyme hydrolysis activity on nucleotides varies with pH; and (5) Both the soluble and membrane-bound forms have different requirements for mono- and/or divalent cations on different substrates. In addition, comparison of the HppA-defective mutant and the wild type revealed that the enzyme action on the substrates does not occur haphazardly but is inextricably tied to cation-flux and pH regulation on *H. pylori*'s envelope. Finally, HppA not only has potential implications in bacterial resistance but also may serve as a promising target in the eradication of *H. pylori*.

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