Identification of a New Class of Adenosine Deaminase from *Helicobacter pylori* with Homologs among Diverse Taxa

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Early studies of *Helicobacter pylori*'s nutritional requirements alluded to a complete purine salvage network in this organism. Recently, this hypothesis was confirmed in two strains of *H. pylori*, whose purine requirements were satisfied by any single purine base or nucleoside. Most of the purine conversion enzymes in *H. pylori* have been studied using mutant analysis; however, the gene encoding adenosine deaminase (ADD) in *H. pylori* remained unidentified. Through stepwise protein purification followed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF), we discovered that *H. pylori* ADD is encoded by *hp0267*, an apparently essential gene. *Hp0267* shares no sequence homology with previously characterized ADDs, yet both are members of the amidohydrolase superfamily. *Hp0267* is grouped within *cog0402*, while other ADDs studied to date are found in *cog1816*. The *hp0267* locus was previously misannotated as encoding a chlorohydrolase. Using purified recombinant *Hp0267*, we determined the enzyme's pH optimum, temperature optimum, substrate specificity, and estimated kinetic constants. In contrast to other known ADDs, *Hp0267* contains Fe(II) as the relevant metal ligand. Furthermore, *Hp0267* exhibits very low deaminase activity on 2'-deoxyadenosine, a substrate that is readily hydrolyzed by *cog1816* ADDs. Our preliminary comparative genomic analysis suggests that *Hp0267* represents a second enzyme class of adenosine deaminase whose phylogenetic distribution among prokaryotes is broad.

Adenosine deaminase (ADD) is a primary enzyme of the purine salvage network in many organisms. Following the first in-depth studies of mammalian adenosine deaminases (1, 2), homologs to these ADDs were studied in a variety of bacteria (3–5). All ADDs studied to date, both bacterial and mammalian, are zinc metalloenzymes of the amidohydrolase superfamily (AHS) and share significant sequence similarity to one another. Collectively, ADDs are grouped within *cog1816*, a feature indicative of their common orthology.

Helicobacter pylori, a member of the epsilonproteobacteria, colonizes the gastric epithelium of humans. Infection is chronic and can lead to peptic ulcers, gastric cancer, and/or mucosa-associated lymphoid tissue (MALT) lymphoma (6–8). The bacterium's reduced genome size and specialized machinery for acid resistance are consequences of its >50,000-year evolution within the niche of the human gastric environment (9–12). One example of *H. pylori*'s niche specialization is the absence of a pathway for *de novo* purine biosynthesis, which renders the organism reliant on exogenous purines for growth (13, 14). Nevertheless, its purine salvage network is complete, and thus, any single purine source can satisfy *H. pylori*'s purine requirement (13, 14). While adenine and adenosine can both serve as sole purine sources for this organism, there are no apparent homologs in the sequenced *H. pylori* genomes for enzymes known to catalyze the deamination of an adenine moiety (examples of such enzymes include adenine deaminase, adenosine deaminase, and AMP deaminase). Also absent is an alternative route for adenine degradation that relies on histidine biosynthesis (10, 15). Recently, ADD activity was detected in *H. pylori* strain 26695 cell extracts (14); however, the gene encoding this ADD in *H. pylori* has not been identified.

MATERIALS AND METHODS

Strains and growth conditions. *Helicobacter pylori* 26695 was the parent strain for all experiments unless otherwise specified and was the source of genomic DNA for genetic manipulations. *Escherichia coli* strain Top10

(Invitrogen) was used for DNA cloning, and strain BL21 Rosetta pLysS (Novagen) was used for protein expression. *H. pylori* cells were grown on blood agar (BA) plates or in EMF12 liquid medium as described previously (14). When appropriate, plates were supplemented with chloramphenicol (30 µg/ml), kanamycin (25 µg/ml), hypoxanthine (1 mM), or guanine (1 mM).

Partial purification of native *H. pylori* ADD. *Helicobacter pylori* 26695 cells were grown overnight as lawns on BA plates, harvested into phosphate-buffered saline (PBS), and washed twice with PBS. Cells were stored as a pellet at –80°C prior to use. The following steps were carried out at 4°C. The cell pellet was resuspended in 10 ml of native purification (NP) buffer, which contained 50 mM HEPES, 50 mM NaCl, 0.5 mM EDTA, and 0.5 mM dithiothreitol (DTT) (pH 8.3 at 4°C). For initial cell lysis, one dissolved tablet of cOmplete EDTA-free protease inhibitor cocktail (Roche Applied Science) was included. Lysis was achieved by two passages through a French pressure cell (20,000 lb/in²), and cell extract was obtained by centrifugation (20,000 × g, 20 min). The supernatant was stirred slowly at 4°C while finely ground ammonium sulfate was added to 45% saturation. After 30 min, the sample was centrifuged (20,000 × g, 15 min), and the supernatant was brought to 55% saturation with ammonium sulfate. This sample was centrifuged likewise, and the precipitated protein was washed once with NP buffer containing ammonium sulfate at 55% saturation. The protein was dissolved in 1 ml of NP buffer and dialyzed twice for 2 h in 0.8 liter NP buffer to remove residual ammonium sulfate. The dialyzed sample was applied to a 5-ml HiTrap Q column (GE Healthcare), and unbound protein was eluted using 35 ml of NP buffer at a rate of 4 ml/min. This flowthrough was concentrated to 0.5 ml by ultrafiltration (Amicon Ultra-4 centrifugal filter, 10-kDa cutoff; EMD Millipore) and was applied to a 5-ml HiTrap SP column (GE Healthcare). The

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flowthrough from this step was similarly concentrated to 0.4 ml and applied to a Hi-Load 16-mm/60-cm Superdex 75 column (GE Healthcare; prep grade). The column was eluted with NP buffer at a flow rate of 0.4 ml/min, and 5-ml fractions were collected. Fractions containing significant ADD activity (fractions 11 and 12) were pooled and concentrated to 0.25 ml.

The presence of native Hp0267 was monitored during purification by measuring ADD activity (see "Enzyme assays" below). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Samples were visualized by denaturing SDS-PAGE stained with either silver nitrate or Coomassie brilliant blue G-250.

Identification of Hp0267 using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF). Following denaturing SDS-PAGE of the partially purified sample, five prominent bands from the Coomassie-stained gel were excised and treated separately with trypsin using a variation of a published protocol (16). Gel slices were equilibrated in water for 10 min, followed by incubation in a 1:1 solution of acetonitrile (ACN)–water for three rounds of 15 min each. The gel slices were then incubated successively for 15 min each in 100 mM ammonium bicarbonate, then in ammonium bicarbonate and ACN (1:1), and then in ACN alone. Following a 60-min incubation at 65°C in 25 mM ammonium bicarbonate (with 10 mM DTT), the samples were alkylated by adding 55 mM iodoacetamide in 20 mM ammonium bicarbonate and incubating the samples at room temperature for 60 min in the dark. Gel slices were washed once with 100 mM ammonium bicarbonate and then incubated overnight at 37°C in 25 mM ammonium bicarbonate containing 0.1 µg of trypsin. The trypsin solution was removed and saved, while the gel slices were briefly incubated in 50% ACN containing 0.1% formic acid. The two solutions were combined, the gel slices discarded, and the liquid samples dried using a SPD111V SpeedVac concentrator (Savant).

For MALDI-TOF analysis, samples were dissolved in 1:1 ACN–water containing 15 mg/ml of 2,5-dihydroxybenzoic acid and 0.1% trifluoroacetic acid. The samples were run using a Bruker Daltonics Autoflex MALDI-TOF instrument using the reflectron mode. The peptide fingerprints were analyzed using the Mascot Server (Matrix Sciences) against the NCBI nr database. The MALDI-TOF and search were performed by the Proteomic and Mass Spectrometry (PAMS) facility at the University of Georgia.

Molecular mass determinations. To determine the enzyme's single subunit mass, 10 µg of purified enzyme was heated to 95°C in the presence of 1% β-mercaptoethanol and 3% SDS. Following gel electrophoresis (10% polyacrylamide), the migration of Hp0267 was compared to known molecular weight standards (Bio-Rad; catalog number 161-363). The subunit composition of Hp0267 was determined using gel filtration chromatography by comparing the elution volume to void volume (V_e/V_0) ratios of the native, partially purified Hp0267 and of the recombinant, purified Hp0267 to a standard curve of known molecular weights (Sigma; MWGF70).

Expression and purification of recombinant Hp0267. The gene locus *hp0267* was amplified from *H. pylori* genomic DNA using the following primers: 5'-AGCACTCGAGATCAAGAAATCATAGGAGCG-3' and 5'-AGCAGAATTCTTAGATCACCTTTTCCCC-3'. The 1.23-kbp amplicon was digested using XhoI and EcoRI and ligated into pTrcHisC (Invitrogen), allowing for expression of Hp0267 with a hexahistidine fusion peptide at the N terminus. Recombinant *E. coli* BL21 Rosetta PLYS (Novagen) containing the expression vector was grown in 0.3 liter of Luria-Bertani broth, and expression was stimulated for 12 hours at 37°C using 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction, cells were washed once with PBS and were resuspended in 10 ml of lysis buffer (20 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl, 10 mM imidazole) containing 1 dissolved tablet of cComplete EDTA-free protease inhibitor cocktail (Roche Applied Science). Cells were passed twice through a French pressure cell (20,000 lb/in²), and soluble cell extract was prepared by centrifugation (20,000 × g, 20 min). Soluble Hp0267 was purified via gravity chromatography using 1 ml of nickel-

nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) as outlined by the manufacturer. The wash and elution buffers contained 40 mM and 200 mM imidazole, respectively, but were otherwise identical to the initial lysis buffer. Hp0267 was eluted in 1-ml increments, and each was dialyzed for 120 min in 1 liter of lysis buffer. Elution fractions 2 to 4 were pooled. Enterokinase (0.02 µg) (New England BioLabs; catalog number P8070S) was added, and digestion proceeded for 16 h at 18°C. The sample was again passed through Ni-NTA agarose resin to remove the cleaved hexahistidine tags as well as any uncleaved enzyme. Enterokinase was removed using size exclusion chromatography. The final buffer used for this chromatography and for subsequent storage contained 20 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, and 10 mM imidazole. Assays used for kinetic analyses were performed immediately following purification. Hp0267 was stored long-term at –20°C in 40% glycerol containing 1 mg/ml of bovine serum albumin (BSA).

Enzyme assays. The following substrates were used for enzyme assays: adenosine (Sigma; A4036), guanine (Sigma; G11950), cytosine (Sigma; C3506), adenine (Sigma; A9126), atrazine (P5380; Chem Service, West Chester, PA), and 5'-S-adenosyl-L-homocysteine (Sigma; A9384). Adenosine deaminase assays were carried out at 37°C in 40 mM bicine (pH 8.5 at 25°C) containing 100 mM NaCl and 10 mM adenosine. Reactions were performed in a final volume of 125 µl and were initiated by the addition of enzyme. The initial (linear) rate of adenosine deamination was determined by measuring the formation of ammonium over time using the phenol-hypochlorite method (17). This colorimetric endpoint assay is commonly used to determine *H. pylori* urease activity and specifically measures ammonium with a detection limit of <40 µM. This same method was used to measure the hydrolytic deamination of alternative substrates by replacing adenosine with a 10 mM concentration of the desired substrate (2 mM for guanine and 2.5 mM for S-adenosylhomocysteine) (SAH). Atrazine chlorohydrolase activity was assayed as described previously (18).

Temperature and pH optima. To determine the optimum pH for catalysis, ADD assays using purified Hp0267 were carried out over a range of pH values in buffers appropriate for each pH range tested. All assays were carried out in 50 mM buffer, 50 mM NaCl, and 10 mM adenosine in a reaction volume of 125 µl. The buffers used were acetate (pH 5 to 6), morpholineethanesulfonic acid (MES) (pH 6 to 7), HEPES (pH 7 to 8), bicine (pH 8 to 9), and carbonate-bicarbonate (pH 9 to 10.5). The carbonate-bicarbonate buffer was prepared as previously described (19). Reactions were initiated by adding 150 ng of enzyme and allowed to proceed for 5 min at 37°C, and the ammonium concentration was determined (17). Each condition (pH and buffer) required its own standard curve of known NH₄Cl concentrations.

The temperature optimum of Hp0267 was determined by measuring the initial rate of ADD activity over a range of 25°C to 70°C in bicine (pH 8.5) containing 10 mM adenosine. Prior to addition of enzyme, the reaction mixture was preheated for 1 min. A thermocycler (MyCycler; Bio-Rad) was used to incubate the reactions.

Metal analysis. Recombinant, purified Hp0267 was analyzed using a Thermo Jarrell-Ash Enviro 36 inductively coupled plasma optical emission spectrometer (ICP-OES) to screen for and quantify the concentration of bound metals. A buffer blank was obtained by ultrafiltration of the purified enzyme to separate buffer from protein. The final protein concentration used for analysis was 4.56 mg/ml (0.1 mM). Both the sample and the blank were analyzed in triplicate, and the values for the blank were subtracted from those of the original sample. Samples were processed according to EPA guidelines using commercially available standards (SPEX).

To measure the activation of ADD by various metals, purified Hp0267 was incubated for 30 min at room temperature in the presence of 0.2 mM divalent metal salt. Following this incubation, the ADD activity was determined as described in "Enzyme assays" above.

Steady-state kinetics analysis. The data used for calculating kinetic constants were obtained using substrate concentrations ranging from 0.5

TABLE 1 Purification summary of native *H. pylori* adenosine deaminase

Sample	Activity ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Protein (mg)	Sp act ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Yield (%)	Fold purification
Cell lysate	1,110	331	0.03	100	1
20,000 $\times$ g supernatant	1,074	233	0.05	97	1.4
45%–55% AS ^a fraction	168	3.4	0.28	15	8.3
Anion exchange	115	0.99	0.30	10	9.2
Cation exchange	68	0.19	0.68	6.1	20.5
Size exclusion	85	0.04	1.88	7.7	56.6

^a AS, ammonium sulfate precipitation.

to 10 mM (adenosine) and 0.1 to 2.5 mM (SAH). The initial rate of ammonium formation at each substrate concentration was determined, and these values were plotted and analyzed by nonlinear regression using the SigmaPlot Enzyme Kinetics Module to estimate the K_m and $V_{\max}$ for each substrate. To calculate k_{cat} , we used the theoretical molecular mass of Hp0267 (45,540 Da).

Bioinformatics methods. Hp0267 and Tm0936 orthologs used for sequence alignments were selected from BLAST hits exhibiting E values greater than 10^{-20} . Only sequences containing the signature metal-binding motif were considered. Based on the conserved motif found within this alignment (P[A/G]XXNXHXXH) and on the presence of other key conserved residues, PHI-BLAST was then used to detect the presence of Hp0267 orthologs within various taxonomic groups (20, 21).

RESULTS AND DISCUSSION

hp0267 encodes an adenosine deaminase. We previously showed that *H. pylori* cell extracts contain adenosine deaminase activity (14). To identify the responsible enzyme, ADD activity was monitored over the course of several protein purification steps such that the enzyme was eventually visible as one of several bands on a denaturing SDS-PAGE gel (Table 1 and Fig. 1). During each purification step, we observed that only a single fraction possessed ADD activity, suggesting that *H. pylori* possesses a single adenosine deaminase. Five bands were excised from the gel, and these proteins were identified using MALDI-TOF (Table 2). Band 4 was identified as Hp0267, which is a member of the amidohydrolase (AHS) superfamily and therefore noted as a possible adenosine deaminase. Heterologous expression of Hp0267 in *E. coli* elevated

TABLE 2 Protein bands identified using MALDI-TOF

Band no.	HP identifier	Protein annotation	Sequence coverage (%)	E value
1	HP0891	Conserved hypothetical protein	35	6.3×10^{-9}
2	HP1563	Alkyl hydroperoxide reductase (AhpC)	52	4×10^{-11}
3	HP1104	Cinnamyl-alcohol dehydrogenase	34	2×10^{-11}
4	HP0267	Chlorohydrolase	39	2×10^{-12}
5	HP0974	Phosphoglycerate mutase	47	6.3×10^{-13}

the ADD activity in cell lysates approximately 10-fold. These data suggested that *hp0267* encoded an adenosine deaminase.

Attempts to generate an *hp0267* deletion mutant in *H. pylori* through allelic replacement of the gene with an antibiotic resistance cassette (*cat* or *aphA3*) produced no viable clones despite the inclusion of hypoxanthine and guanosine (1 mM each) in the selective medium. We considered the possibility that deleting *hp0267* could affect the upstream locus (encoding dihydroorotase), which shares a 13-bp sequence overlap the 5' end of *hp0267*. To address this, we attempted an insertion into (rather than a complete allelic replacement of) *hp0267* that avoided the 5' region of the gene. This method also failed to produce clones. We then attempted to transform *H. pylori* with a plasmid (pGEM-T) containing the allelic replacement cassette as opposed to a linear DNA fragment. Clones were recovered following transformation; however, PCR analysis showed that these clones still possessed an intact *hp0267* locus, and we surmised that a single-crossover event had occurred between plasmid and chromosomal DNA in the region flanking *hp0207*. An attempt at mutagenesis of *hp0267* was performed using *H. pylori* strain 43504 and, similarly, only single-crossover mutants were recovered. This selection for a single-rather than a double-crossover event provides further evidence that the *hp0267* locus may be essential. Interestingly, a global transposon mutagenesis of the *H. pylori* G27 genome resulted in three insertions within the *hp0267* locus (22). It is possible that the essential nature of this gene is strain dependent.

Molecular mass of native Hp0267. The band corresponding to Hp0267 as visualized by SDS-PAGE is approximately 46 kDa (Fig. 1). This correlates with the predicted subunit mass as determined from the translated gene sequence. The enzyme's subunit composition was estimated by gel filtration chromatography using a standard curve of proteins of known mass. From this, both native (partially purified) and recombinant (purified) Hp0267 were estimated to have a molecular mass of between 45 and 60 kDa. These data suggest that Hp0267 functions as a monomer *in vivo*.

Characterization of purified Hp0267: pH optimum, temperature optimum, substrate specificity, and estimated kinetic constants. To obtain sufficient quantities of purified, active enzyme, Hp0267 from *H. pylori* strain 26695 was expressed heterologously in *E. coli* such that a hexahistidine tag and an enterokinase cleavage domain were fused to the enzyme's N terminus. Following Ni affinity purification of this fusion protein, the hexahistidine tag was removed with enterokinase, and the tag-free enzyme was obtained chromatographically (see Materials and Methods). There was no difference in ADD activity between His₆-Hp0267 and the final enzyme lacking the hexahistidine tag, indicating that the enzyme's integrity had been maintained during enterokinase treat-

FIG 1 Denaturing SDS-PAGE of native *Hp0267* purification. Bands were stained using silver nitrate. Lane 1, molecular mass marker; lane 2, 20,000 $\times$ g supernatant; lane 3, ammonium sulfate precipitation; lane 4, anion exchange flowthrough; lane 5, cation exchange flowthrough; lane 6, size exclusion chromatography. Arrows indicate bands that were excised from a Coomassie-stained gel for MALDI-TOF analysis.

FIG 2 pH and temperature optima of Hp0267. (A) A range of pH-appropriate buffers were used as indicated in the symbol key (Carb-Bic, carbonate-bicarbonate). All reaction mixtures were incubated at 37°C. (B) Reactions were carried out in bicine buffer at pH 8.5, and the temperature of incubation was varied. Reaction rates are reported as a percentage of the maximum specific activity observed on that day ($90 \mu\text{mol min}^{-1} \text{mg}^{-1}$). Results are the means $\pm$ SDs of at least three independent determinations.

ment. Purified Hp0267 was most stable in 20 mM phosphate buffer (pH 8.0) containing 100 mM NaCl and 10 mM imidazole. Reducing agents (DTT or β -mercaptoethanol), EDTA, and K^+ had no significant effect on enzyme stability. The half-life of Hp0267 (24 h at 4°C) was increased 3-fold by storing the enzyme at -20°C with 1 mg/ml of BSA and 40% glycerol. The pH optimum of purified Hp0267 was shown to be approximately 8.5, and the optimum temperature is between 33 and 40°C (Fig. 2).

Hp0267 exhibited slight but significant deaminase activity on 2'-deoxyadenosine and no detectable activity on guanine, cyto-

sine, or adenine (Table 3). No dechlorination of atrazine was measured even after a 3-h incubation with Hp0267. This result contradicts the previous annotation of Hp0267, which was perhaps based on its approximately 30% sequence identity with atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. strain ADP. This result highlights the danger of explicit functional predictions based solely on sequence identities with other characterized enzymes. This may be especially true of AHS enzymes belonging to cog0402, which are known to be functionally diverse and incredibly adaptable toward evolving new substrate preferences (23–26). The low activity of Hp0267 on 2'-deoxyadenosine is noteworthy because other ADDs studied to date use this substrate efficiently or nearly as efficiently as adenosine (27). The kinetic constants for adenosine indicate a low catalytic efficiency and specificity compared to those of previously characterized ADDs, which typically achieve nearly diffusion-limited reaction rates ($k_{\text{cat}}/K_m \approx 10^7 \text{ M}^{-1}\text{s}^{-1}$), and exhibit K_m values for adenosine in the micromolar range (3, 27–31).

We obtained a model of the three-dimensional (3D) structure of Hp0267 using the program Phyre (32), which revealed the enzyme's tertiary structure to be similar to that of a characterized S-adenosylhomocysteine (SAH) deaminase from *Thermotoga maritima* (Tm0936) (25). There is currently a group of enzymes in the NCBI database annotated as "SAH deaminases," likely because of their orthology with Tm0936. We therefore included SAH among the substrates tested, and we found not only that Hp0267 can catalyze the deamination of SAH but also that it has a lower K_m for this substrate than for adenosine. This high affinity for SAH was nevertheless tempered by a 30-fold-lower V_{max} compared with that of adenosine as a substrate. One explanation for the observed activity on SAH could be that the physiological role for Hp0267 is as an SAH deaminase; however, there is only one such enzyme described in the literature (from *Streptomyces flocculus*), and the specifics of this degradation pathway are uncertain (33, 34). In addition, it was shown that Tm0936 catalyzes the deamination of adenosine nearly as efficiently as for SAH, and furthermore, the *T. maritima* genome lacks a homolog to adenosine deaminase (25). We therefore hypothesize that the biological roles of Tm0936 and Hp0267 are as adenosine deaminases.

Hp0267 binds Fe(II). Enzymes of the AHS superfamily coordinate either one or two divalent metal ions at the active site (35). To determine the metal contained within Hp0267, samples of recombinant, purified enzyme were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) (Table 4). These results suggest that Fe, Ni, or Zn can be coordinated by

TABLE 3 Substrate preference and estimated kinetic constants of Hp0267

Substrate	SA ^a ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Adenosine	227 ± 12	295 ± 30	4.0 ± 1.0	226	5.7×10^4
2'-Deoxyadenosine	0.3 ± 0.1	NC ^b	NC	NC	NC
S-Adenosylhomocysteine	6.9 ± 0.2	7.3 ± 0.9	0.9 ± 0.3	5.6	6.4×10^3
Atrazine	BDL ^c	NC	NC	NC	NC
Guanine	BDL	NC	NC	NC	NC
Cytosine	BDL	NC	NC	NC	NC
Adenine	BDL	NC	NC	NC	NC

^a SA, specific activity of purified Hp0267 in the presence of 10 mM substrate (1.0 mM for guanine and 2.5 mM for S-adenosylhomocysteine).

^b NC, not able to be calculated due to either low or nondetectable activity.

^c BDL, activity below the detection limit.

TABLE 4 Metal analysis of purified Hp0267

Metal	Concn (μ M)	Molar ratio (metal/enzyme)
Fe	48.6 $\pm$ 0.9	0.49
Zn	21.4 $\pm$ 1.7	0.21
Ni	14.6 $\pm$ 0.2	0.15
Mn	1.9 $\pm$ 0.1	0.02
Mg	BDL ^a	
Mo	BDL	
Co	BDL	
Cd	BDL	

^a BDL, below detection limit.

Hp0267. All metals were present at a molar ratio of less than 1:1 metal to enzyme, likely due to the absence of added metals in the growth medium used to express the recombinant enzyme, or possibly due to the loss of bound metals during purification. We sought to test the effect of incubating purified Hp0267 with various divalent metals for 30 min, by measuring ADD activity (Fig. 3). Incubation with divalent Fe, Co, or Cd elevated ADD activity, with the highest activation induced by Fe. Neither Ni nor Zn affected ADD activity of Hp0267. These data suggest that Fe(II) is the preferred ligand used for catalysis by Hp0267. This would make sense, as other characterized members of cog0402 (atrazine chlorohydrolase and cytosine deaminase) likewise coordinate iron (18, 36). Nevertheless, the ability of Hp0267 to function with iron is unique among all other known ADDs, which coordinate zinc as the relevant active-site metal (29, 31, 37–39). The stoichiometry of the bound metal is likely 1:1 (based on the Phyre-predicted structure for Hp0267); however, more studies are necessary to prove this.

Phylogenetic and sequence analysis of Hp0267. The *hp0267* locus is conserved among all *H. pylori* genomes currently in the NCBI database, and the presence of this gene correlates with an absence of a homolog to adenosine deaminase. Hp0267 shares no significant sequence identity (<15%) with other characterized ADDs, all of which are members of cog1816. Nevertheless, Hp0267 is a member of the AHS and is therefore a distant ortholog of ADD, falling within cog0402 (cytosine deaminase and related metal-dependent hydrolases) (40). Biochemically studied members of cog0402 include atrazine chlorohydrolase (18, 41), cytosine deaminase (36, 42), guanine deaminase (43), and Tm0936 (SAH deaminase) from *T. maritima* (25).

Hp0267 and Tm0936 share relatively low sequence identity (23%) and apparently do not share the same divalent cation preference; however, these differences belie the significant structural and biochemical similarities between the two. Because Hp0267 and Tm0936 are biochemically more similar to one another than to other characterized members of this COG, we constructed a sequence alignment that includes Hp0267, Tm0936, and representative orthologs with the intention of identifying conserved residues that could provide a signature motif for predicting adenosine deaminase activity of cog0402 members (Fig. 4). The hallmark metal-binding residues that define all AHS members are His65, His67, His207, and Asp372 (numbering of residues corresponds to *H. pylori* Hp0267) (35, 44). Residues that both are conserved within this alignment and were shown in Tm0936 to be involved in substrate binding or catalysis are Trp85 and Ser318 and Glu210. While Trp85 and Ser318 are strictly conserved among

the group of orthologs in Fig. 4, it appears that Gln can replace Glu210. In addition to these residues for which structural data have been provided, there are other notable conserved residues whose function is unknown. In particular, the region just upstream and including His65 and His67 appears to be a signature unique to this group of enzymes, and it reads P(A/G)XXNXHXH.

We further sought to determine whether the presence of Hp0267 homologs within a genome correlates inversely with the presence of a cog1816 ADD homolog. If so, this pattern would indicate that Hp0267 performs the same functional role as the cog1816 ADDs. We began with members of the delta/epsilonproteobacteria, for which comparatively strong homologs to Hp0267 (30 to 50% identity) are found. With only three exceptions, all epsilon/deltaproteobacterial genomes that contain a Hp0267 homolog have no homolog for *E. coli* ADD (E-value cutoff < 1e–20) (21). We then extended our query to include members of the Archaea, the second-highest scoring taxonomic group with respect to their Hp0267 homologs. All archaeal genomes (with the exception of *Aciduliprofundum boonei*) possessing a Hp0267 homolog contain no homolog to *E. coli* ADD. This inverse correlation between Hp0267 homologs and cog1816 ADDs strongly suggests that Hp0267 represents a separate class of ADDs in prokaryotes.

Conclusions. We described herein a member of cog0402 in *H. pylori* that catalyzes the deamination of both adenosine and SAH. Based on our native purification data, Hp0267 appears to be the sole adenosine deaminase in this organism. The existence of this enzyme explains *H. pylori*'s ability to grow using only adenine and/or adenosine as a sole purine source. Hp0267 and its close homologs from diverse taxonomic groups are currently annotated as “chlorohydrolases.” This is very likely a misannotation, as there are few chlorinated molecules in nature that would be physiologically relevant substrates for these enzymes. It is tempting to propose that atrazine chlorohydrolase (AtzA), which evolved over a period of 40 years or less (41, 45), was derived from an ADD that exhibited promiscuous activity on chlorinated molecules resembling adenosine. Alternatively, AtzA could have evolved from another cog0402 member such as cytosine deaminase; however, AtzA was shown not to use cytosine or other related pyrimidines

FIG 3 Hp0267 ADD activity upon incubation with divalent metals. The ADD activity of purified Hp0267 was measured following incubation with various divalent metals. Results shown are the means $\pm$ SDs of three independent replicates and are expressed as the percentage of activity relative to a control incubation lacking added metals. Asterisks indicate a significant increase in activity relative to the control ($P < 0.01$, paired Student's *t* test).

FIG 4 Sequence alignment of Hp0267, Tm0936, and orthologs. Proteins for which crystal structures are available are indicated by an asterisk adjacent to the species' name and can be accessed from the Protein Data Bank (PDB): *Nitratiruptor* sp. strain Sb155-2 (PDB: 3V7P), *Arthrobacter aurescens* Tc1 (PDB: 3LSC), and *Thermotoga maritima* (PDB: 2PLM). Stars above the sequence alignment indicate residues that coordinate the metal ligand. Downward arrows indicate residues that both (i) are conserved and (ii) are in close proximity to the substrate in the crystal structure of Tm0936. Highlighted in gray is the proposed signature motif for this group of orthologs.

as the substrates (41). It is unknown whether adenosine is a substrate for AtzA.

Hp0267 acts on both adenosine and SAH, with k_{cat}/K_m values that suggest that either of these reactions could be the enzyme's primary physiological role. Nevertheless, if SAH were indeed a major substrate for Hp0267 or for its ortholog Tm0930, this would imply that a degradation pathway is present for SAH, of which only one is known to exist in bacteria (34). Our comparative genomic data reveal that the presence of an Hp0267 homolog correlates with the absence of a cog1816 ADD. This is true for diverse prokaryotic taxa, supporting a hypothesis that Hp0267 represents a previously unknown form of adenosine deaminase whose phyletic representation is broad.

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