PURINE SALVAGE IN *HELICOBACTER PYLORI*

by

ERICA FRANCESCA MILLER

(Under the Direction of Robert J. Maier)

ABSTRACT

Purines are essential for all living cells. This fact is reflected in the high degree of pathway conservation for purine metabolism across all domains of life. The availability of purines within a mammalian host is thought to be a limiting factor for infection, as demonstrated by the importance of purine synthesis and salvage genes among many bacterial pathogens. *Helicobacter pylori,* a primary causative agent of peptic ulcers and gastric cancers, colonizes a niche that is otherwise uninhabited by bacteria: the surface of the human gastric epithelium. Despite many studies over the past 30 years that have addressed virulence mechanisms such as acid resistance, little knowledge exists regarding this organism's purine metabolism. To fill this gap in knowledge, we asked whether *H. pylori* can carry out *de novo* purine biosynthesis, and whether its purine salvage network is complete. Based on genomic data from the fully sequenced *H. pylori* genomes, we combined mutant analysis with physiological studies to determine that *H. pylori*, by necessity, must acquire purines from its human host. Furthermore, we found the purine salvage network to be complete, allowing this organism to use any single purine nucleobase or nucleoside for growth. In the process of elucidating these pathways, we discovered a nucleoside transporter in *H. pylori* that, in contrast to the biochemically-characterized homolog NupC, aids in uptake of purine rather than pyrimidine nucleosides into

the cell. Lastly, we investigated an apparent pathway gap in the genome annotation—that of adenine degradation—and in doing so uncovered a new family of adenosine deaminase that lacks sequence homology with all other adenosine deaminases studied to date. These newly characterized *H. pylori* proteins exemplify evolution in action, and underscore the need for functional annotations that do not rely solely on sequence homology with biochemically characterized orthologs. The results of our studies expand the database that is needed for future genome annotations and for prediction of metabolic capabilities in other prokaryotes.

INDEX WORDS: Purine, purine salvage, adenosine deaminase, Add, PurB, PurA, GuaB, GuaA, GuaC, NupC, purine uptake, adenine, *Helicobacter*, *Helicobacter pylori*, epsilon-proteobacteria, nucleoside, nucleotide, nucleobase

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DEDICATION

This work is dedicated to those who suffer, whether from disease of the stomach or, metaphorically, of the heart. May they be brought to realize their true nature: expansive blissful, and connected.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

Helicobacter pylori Bacteriology 1

Epidemiology and Disease 3

Purine Metabolism in Bacteria 5

De Novo IMP Biosynthesis 6

Overview of Purine Salvage 8

Purine Uptake 8

Conversion Between Nucleobases, Nucleosides, and Nucleotides 12

Synthesis of AMP and GMP 14

Conversions Between Adenine and Guanine 18

Adenosine Deaminase 20

Purines: Relevance to Virulence in *H. pylori* 23

Scope of this Study 24

References 26

2 EFFICIENCY OF PURINE UTILIZATION IN *HELICOBACTER PYLORI*: ROLES FOR ADENOSINE DEAMINASE AND A NUPC HOMOLOG 46

Abstract 47

Introduction 48

Results 50

Discussion 55

Materials and Methods 58

Acknowledgements 61

References 62

3 IDENTIFICATION OF A NEW CLASS OF ADENOSINE DEAMINASE FROM *HELICOBACTER PYLORI* WITH HOMOLOGS AMONG DIVERSE TAXA 78

Abstract 79

Introduction 80

Materials and Methods 81

Results/Discussion....87

Conclusions 93

Acknowledgements 94

References 95

4 DISSERTATION SUMMARY AND FUTURE DIRECTIONS 105

LIST OF TABLES

Page

Table 1.1: Genes for *De novo* IMP biosynthesis among the ε-proteobacteria..............................42

Table 1.2: Catalytically important residues for adenosine deaminase..43

Table 2.1: Growth rates and end-point yields of H. pylori grown in EMF12 medium with various purine sources 67

Table 2.2: Growth of EM207 (**Δ***nupC*) in EMF12 supplemented with a single purine source 68

Table 2.3: Comparison of radiolabeled nucleoside uptake by *H. pylori* 26695 versus EM207 69

Table 2.4: Components present in the defined chemical medium EMF12...................................70

Table 2.5: *H. pylori* strains used in this study...71

Table 2.6: Oligonucleotide primers used in this study 72

Table 3.1: Purification summary of native *H. pylori* adenosine deaminase 100

Table 3.2: Protein bands identified using MALDI-TOF 100

Table 3.3: Substrate preference and estimated kinetic constants of Hp0267 100

Table 3.4: Metals analysis of purified Hp0267...101

LIST OF FIGURES

Page

Figure 1.1: Overview of *de novo* IMP biosynthesis 44

Figure 1.2: GMP and AMP biosynthesis and degradation 45

Figure 2.1: Overview of the current model for purine conversions in *H. pylori* 73

Figure 2.2: Growth of *H. pylori 26695* in the chemically defined medium EMF12 74

Figure 2.3: Growth of gua and pur mutants in EMF12 supplemented with individual Purines……………………………………………………………………………… 75

Figure 2.4: Sequence comparison between *H. pylori* NupC homolog (HP1180) and three *E. coli* CNT paralogs 76

Figure 3.1: Denaturing SDS-PAGE of native Hp0267 purification 101

Figure 3.2: pH and temperature optima of Hp0267 102

Figure 3.3: Hp0267 ADD activity upon incubating with divalent metals 103

Figure 3.4: Sequence alignment of Hp0267, Tm0936, and orthologs 104

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

***Helicobacter pylori* Bacteriology**

The gastric epithelia of over 50% of the adult human population is currently colonized with *H. pylori*, a Gram-type negative, helical-shaped ε-proteobacterium that resists the acidity of the stomach and causes chronic infection (1, 2). From a phylogenetic standpoint, *H. pylori* is a fascinating example of how closely related genera can be metabolically diverse. The best-studied members of the ε-proteobacteria are the genera *Campylobacter* and *Helicobacter,* which inhabit the gastrointestinal tract or liver of various animals, while lesser-known genera such as *Arcobacter* are halophiles that thrive in seawater (3) or exist as nitrogen-fixing symbionts within the roots of salt marsh plants (4). Even within the genus *Helicobacter*, a division exists between the gastric species, which colonize the stomach, and the enterohepatic species, which colonize the liver and colon (5).

Most *Helicobacter* species (including *H. pylori*) possess high levels of catalase activity to combat oxidative stress (6) and urease activity to combat the acidity of the gastric lumen (7). Nevertheless, the terms "microaerophile" and "acidophile" are incorrect descriptors for *H. pylori*. *H. pylori* is not a true acidophile, growing optimally at neutral pH and mitigating acid exposure so long as urea is available. It is the rapid and sustained intracellular hydrolysis of this substrate that allows the bacterium to maintain its cytoplasm at pH 7.4 (8). Neither is *H. pylori* a true

microaerophile; it has recently been shown that under high cell density or under high partial pressure of CO_2 *H. pylori* can grow in the presence of atmospheric oxygen (9, 10).

Definitions aside, it is clear that despite possessing a relatively small genome (1.6 Mbp) and a scant regulatory network for gene expression (11, 12), *H. pylori* has devised solutions to a very real problem: how to live and thrive in the human stomach. In the thirty years since the discovery of *H. pylori*, we are beginning to get a clearer picture of the conditions encountered by the bacterium during colonization. The bacteria are found close to the surface of the gastric epithelium—generally within 25 μm of this surface (13). Attachment to the host cells is common, and perhaps most of the bacterium's nutrients are derived from the host cell products (14, 15). During colonization, *H. pylori* is continually dislodged from the host cell surface, either due to peristalsis of the stomach or due to the rapid turnover of the host epithelial layer. The bacteria solve this problem by exploiting their high motility, which depends upon several polar flagella and good sense of direction (chemotaxis) (16-18). The *H. pylori* proteome constitutively maintains its urease pools at approximately 5-10% of the total proteome (by mass), suggesting that the issue of low pH is constant burden to the cell (19). Nevertheless, the precise pH near the host epithelia, where the majority of bacteria spend most of their time, remains controversial (20, 21). It is clear, however, that urease is important (22), both for motility through the mucus lining of the stomach (23) and to combat acidic pH (8, 24).

Arguably, *H. pylori* is an interesting pathogen simply from the perspective of its unique physiology and colonization strategies. Regarding this organism's pertinence to public health, however, more important questions arise. What has this bacterium done to threaten public health? What aspects of its survival strategies in the host are important to study and why? These

questions will be addressed in the following sections of this chapter, along with a literature review pertaining to the scope of this dissertation work.

Epidemiology and Disease

What has *H. pylori* done to threaten public health? There are two main factors that influence any decision to study a bacterial pathogen. First, is the frequency of infection high? Second, what is the degree of morbidity and/or mortality associated with the infection? The discovery of *H. pylori* as a causative agent for gastric ulcers was a noteworthy event in medical history, partly due to the controversial methodologies (namely, drinking the organism) used by Warren & Marshall to challenge conventional wisdom regarding ulcers (2), and also due to the discovery of a previously overlooked site for prokaryotic colonization. Indeed, the human gastric lumen and mucus frequently fall below pH 2 (25), and such acidic conditions pose a formidable barrier against the survival of pathogens capable of inhabiting this niche. Interestingly, it appears that *H. pylori* has been associated with humans for at least the past 50,000 years (26), a fact that has enabled the use of *H. pylori* genotyping as a marker to unveil migration patterns of ancient humans (27).

Worldwide infection rates vary between 20 – 80% of the adult population (28). These percentages vary by geographic region and by demographic factors. For example, higher infection rates correlate with low socioeconomic status (29, 30). Infection by *H. pylori* is chronic unless treated with a combination of proton-pump inhibitors and antibiotics (31). Although the mode by which *H. pylori* is transmitted remains unknown, populations exhibiting poor hygiene and sanitation possess infection rates on the upper end of this spectrum (32, 33). *H. pylori* is typically acquired during childhood, and the risk of infection is highest in individuals

who live with infected family members (34). These observations lend likelihood to the possibility of person-to-person transmission, but it has also been suggested that contaminated water supplies could be the culprit (35). *H. pylori* is notoriously fastidious outside the human host, however, and it remains to be determined whether the organism can remain infectious for an extended period of time in an aquatic environment.

While the majority of infected individuals remain asymptomatic, between 1 and 10% of individuals develop chronic gastritis, gastric-esophogeal reflux disease (GERD), and/or ulcers of the stomach and the duodenum (29, 31). Another potential consequence of *H. pylori* infection is anemia, especially in females, and is thought to be due to poor iron absorption in infected individuals (36). Classified as a Group 1 carcinogen by the World Health Organization, *H. pylori* has been shown to induce chronic inflammation leading to gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma (37). Significant advances have been made in recent years to define the mechanisms behind *H.pylori*-induced carcinogenesis. These studies are of major relevance to public health, as gastric cancer is the second leading cause of cancer-related death worldwide (38). Lack of symptoms does not necessarily preclude the development of *H. pylori* associated pathologies; indeed, a chronic inflammatory response is common even among asymptomatic *H. pylori* carriers (39).

Therapies to eradicate *H. pylori* can prevent certain cancers that are associated with its infection (1, 37, 40), however due to increased antibiotic resistance and the high cost of these regimens, there is some uncertainty as to whether current antibiotic therapies will continue to be a viable treatment against *H. pylori* infection (41). Several potential vaccines targeting *H. pylori* are safe and effective in humans, but have not yet been capitalized upon (42). Interestingly, if a vaccine were deployed against *H. pylori* as a public health intervention, morbidity and mortality

could be attenuated worldwide, the cost for which would be substantially lower than the current economic burden associated its with its high infection rates (43).

Purine Metabolism in Bacteria

Purines are a group of nitrogen- and carbon-rich molecules that are essential for all living cells. These molecules are perhaps most recognized for their function within DNA and RNA as carriers of the genetic code. Equally important, however, is the essential role of purines in cellular chemistry. Adenine moieties form part of the redox cofactors NAD(P) and FAD, which participate in electron transfer and are abundant inside the cell; for *Escherichia coli* grown in a minimal medium their respective concentrations are approximately 1 mM and 0.05 mM (44). The purine nucleotides ATP and GTP play an important role as energy currency for the cell, and are maintained at approximately 3 mM and 0.9 mM, respectively (44). For these nucleotides to serve as energy donors the concentrations of the tri-phosphate forms must be kept higher than those of the di- and mono-phosphates, providing a reservoir of potential energy that can then be released via hydrolysis of these phosphodiester bonds. It is primarily the hydrolysis of purine nucleotide phosphodiester bonds which provides the force to drive otherwise non-spontaneous cellular chemistry. Furthermore, although ATP is the primary nucleotide used for energy-coupling, the role of GTP cannot be underestimated; growing bacterial cells direct half of their energy budget toward synthesis of protein, for which two ATPs and two GTPs are used to attach each amino acid monomer to the growing peptide chain (45). A further role for purines are as signaling molecules, for example cAMP and (p)ppGpp, involved in regulation of catabolism and the stringent response, respectively.

Considering that purines are both essential and relatively concentrated within the cell, it is not surprising that organisms possess robust mechanisms to acquire these molecules exogenously, and/or to synthesize them using amino acid precursors. In addition, because *de novo* purine synthesis is expensive, many organisms possess a network of reactions that can salvage pre-formed purines and convert these to either adenine or guanine nucleotides depending on the needs of the cell.

***De Novo* IMP Biosynthesis**

The steps for purines biosynthesis are nearly identical across all three domains of life. Two major steps are required to convert amino acid precursors into purine nucleotides. In the first major transition, Inosine monophosphate (IMP) is formed (Fig 1.1). Second, either AMP or GMP can be synthesized from the IMP precursor, each in two distinct steps (Fig 1.2). The best-studied microbial pathways for IMP biosynthesis are found in the enterobacteria *E. coli* and *Salmonella enterica*, and in *Bacillus subtilis.* All three organisms employ the same set of enzyme-catalyzed reactions (46). The first committed step in *de novo* IMP synthesis begins with the transfer of an amide group from glutamine onto the activated ribose sugar 5-phosphoribosyl 1-α-diphosphate (PRPP). This reaction is catalyzed by glutamine-PRPP amidotransferase (*E. coli* PurF) (47, 48). The product of this reaction is phosphoribosylamine (PAR), which contains the amide group, linked by an N-β-glycosidic bond, onto which the purine ring is sequentially synthesized. Ten further catalytic steps are required to form the desired product IMP, and the total cost of synthesis is five ATP. This value rises to seven ATP equivalents if we include the two ATP equivalents needed to synthesize PRPP, and even more if we include the cost of synthesizing the initial amino acid precursors. The point here is that *de novo* purine biosynthesis

requires a huge energy investment for the cell, and when this pathway is not necessary for survival, which is the case for certain symbionts, then a strong selective pressure exists for its elimination from the genome (49-51).

Most ε-proteobacteria for which whole genome sequences are available possess all ten gene homologs required for IMP biosynthesis. The exceptions are *H. pylori* and *H. acinonychis*, which lack all but two of these genes (Table 1). The absence of this suite of genes for IMP synthesis is true for all strains of *H. pylori* that have been sequenced to date. Since two close cousins (*H. hepaticus* and *H. mustelae*) have both retained this pathway, we can surmise that the set of genes for purine biosynthesis were lost at some point after the *Helicobacter* genus split from the other Campylobacteriaceae but before speciation occurred between *H. pylori* and *H. acinonychis.*

Interestingly, although the genomic data point strongly toward an inability of *H. pylori* to synthesize purines, a study by Mendz *et al* (1996) reported growth of *H. pylori* in a minimal medium without an added purine source, and from this it was concluded that *H. pylori* can synthesize purines *de novo*. One year later, the first complete *H. pylori* genome was sequenced (11). In hindsight, we can now compare genomic evidence with physiology and conclude that a discrepancy exists between these two approaches. The question of whether *H. pylori* can indeed carry out *de novo* synthesis of IMP must be re-examined.

Historically, it has been challenging to study the specific nutrient requirements of *H. pylori* due to its fastidious nature. Most minimal media were supplemented with undefined, complex supplements such as bovine serum albumin (BSA) or fetal bovine serum (FBS) (52-54). Nevertheless, with the recent optimization of a chemically defined growth medium for *H. pylori* (55, 56), the potential for contaminating purines can be eliminated. Thus, one aim in this work

was to re-examine the possibility of *de novo* purine synthesis by *H. pylori* using a fully defined chemical medium.

Overview of Purine Salvage

Organisms can exploit the presence of pre-formed purines in the environment, thereby avoiding *de novo* synthesis and/or allowing the use of purine breakdown products as a source of carbon, nitrogen and energy. Cells have evolved a network of "salvage" reactions that include both the uptake and the conversion of purines. Conversion pathways serve two major roles. The first is to exchange between purine forms, one instance being the assembly of a nucleotide from a free purine base. The second set of pathways serves to change the purine moiety itself, an example being the conversion of AMP to GMP. Polymerization of purine nucleotides and deoxynucleotides into RNA or DNA is not considered among the purine salvage pathways. An organism is said to possess a "complete" salvage pathway if it can grow and replicate using any single purine nucleoside or base as its sole source of purine. In the sections that follow, the major roles of purine salvage will be detailed.

Purine Uptake

Bacteria acquire pre-formed purines from the environment through uptake mediated by transporters. Nucleosides and nucleobases can enter passively through porins in the outer membrane, and in *E. coli,* via the nucleoside-specific transporter Tsx, whose crystal structure has been solved (57, 58). To subsequently cross the inner membrane, nucleosides and nucleobases are taken up separately by way of energy-requiring transport systems. Phosphorylated

nucleosides are not substrates for bacterial transporters with the exception of certain intracellular pathogens that can take up host ATP in exchange for their own "spent" ADP (59, 60).

If purines are not synthesized by *H. pylori de novo*, then we can assume transporter systems must exist to allow entry of pre-formed purines across the inner membrane. It was shown previously that *H. pyori* can take up a variety of radiolabeled purines, namely adenine, guanine, hypoxanthine, and the nucleosides adenosine and guanosine (54). Radiolabeled deoxyadenosine, but not deoxyguanosine, were also taken up by whole cells. The remaining nucleosides (inosine, xanthosine) and deoxynucleosides (deoxyinosine, deoxyxanthosine) were not tested (54). Thus, we know that certain purines are taken up by *H. pylori*, but the mechanisms of uptake remain to be determined. Two genes in the fully sequenced *H. pylori* genomes feasibly encode nucleoside and/or nucleobase transporters, and these homologs will be further discussed.

1. Uptake of Nucleosides

In prokaryotes, nucleoside transporters come in several forms. These include the Concentrative Nucleoside Transporters (CNT), the nucleoside-specific Major Facilitator Symporters (MFS), and ABC-transporters. While *H. pylori* does not possess obvious homologs to either MFS or ABC-type nucleoside transporters, a CNT homolog is present and will be discussed.

CNTs are widely distributed among both prokaryotes and eukaryotes, and are characterized by their use of an electrochemical ion gradient to power nucleoside symport across the lipid bilayer. In general, bacterial CNTs are H^+-coupled nucleoside symporters, while

eukaryotic CNTs use sodium as the companion ion for symport (61). Exceptions to this rule are found in the fungi (62).

All CNTs share a relatively high degree of sequence conservation across the three domains of life. For example, *E. coli* NupC is a CNT-type transporter that shares 26% identity (37% similarity) to the functional region of human hCNT1 protein (61). Nevertheless, although homology-based approaches can predict the presence of a CNT transporter, the nucleoside substrate preference for this family of transporters can vary. In humans, three nucleoside transporters exist: one is specific for pyrimidine nucleosides (hCNT1) (63), a second for purine nucleosides (hCNT2) (64), and a third accepts both pyrimidine and purine nucleosides (hCNT3) (65). Despite having different substrate capabilities, the sequence identity between these three CNT paralogs is greater than 70%, underscoring the notion that high homology can inform but cannot necessarily predict substrate preference. Indeed, recombinant hCNT1 (pyrimidine-specific) can be altered via mutation at just two residues in order to confer a complete switch to hCNT2-like purine-specific transport (66). Therefore, although this family of transporters shares a conserved 3D architecture and general function, precise substrate preference cannot be determined from sequence homology alone.

To date, the only well-studied bacterial CNT is the *E. coli* protein NupC. Initial characterization of this transporter used mating/recombination experiments and mutant analysis to map the location of NupC in the genome, and to determine its substrate specificity (67). Further studies using prepared *E. coli* membrane vesicles showed that transport by NupC depends upon the proton-motive force (68). It was not until fifteen years later that *E. coli* NupC was shown to be encoded by a single gene (69, 70). More recently, studies of *E. coli* NupC have identified key residues involved in transport, and have precisely determined the substrate

specificity (61, 71). Studies using recombinant *E. coli* NupC expressed in *Xenopus* oocytes showed that, similar to human hCNT1, this protein exhibits a strong preference for pyrimidine rather than purine nucleosides (61). In *Bacillus subtilis*, the role of NupC *in vivo* was studied using mutant analysis (72), and was postulated to prefer pyrimidine substrates. However, no studies have pinpointed the substrate specificity for this protein.

H. pylori possesses a CNT-family protein (HP1180), annotated as NupC (11, 73, 74). This annotation was likely based on the sequence similarity between HP1180 and *E. coli* NupC (61, 70), since *E. coli* NupC is the only bacterial CNT whose substrate specificity has been determined. HP1180 has not been studied, and therefore we cannot assume that its substrate preference is the same as for *E. coli*. The gene encoding HP1180 is located in a three-gene operon along with purine nucleoside phosphorylase (PNP) (*hp1178*) and phosphopentomutase (*hp1179*). Mutants lacking *hp1178* are deficient for growth on purine nucleosides, suggesting that the assignment of PNP is correct (75). Thus, if co-expression is indicative of related function, we can hypothesize that HP1180 is involved in purine rather than pyrimidine transport, in contrast to the function identified for *E. coli* NupC.

2. Uptake of Nucleobases

Nucleobase uptake systems in prokaryotes comprise a subset of the Major Facilitator Superfamily (MFS) of transporters, and fall into two families: the Nucleobase Cation Symporter Family 1 (NCS1) and the Nucleobase-Ascorbate Transporter family (NAT or NCS2) (76). The first family of transporters (NCS1) contains only one experimentally-studied bacterial example: CodB from *E. coli* (77). Many homologs to CodB exist in diverse members within the domain bacteria. *H. pylori* does not possess a homolog to a NCS1 family transporter.

Despite the use of “ascorbate” to describe the NAT family, only mammalian NAT members can transport ascorbate (78). Despite the diverse substrate recognition among the NAT family, all members use a cation for symport (Na^+ in mammals, H^+ in bacteria, fungi, and plants) (78). Members of the NCS2 family in bacteria include PurP from *E. coli* which transports adenine (79), and YgfO and YicE, which transport xanthine (80). *B. subtilis* also possesses several members of this family including a xanthine-specific permease (PbuX) (81) and a guanine-hypoxanthine permease (PbuG) (82). Recently, two purine-specific nucleobase transporters of the NCS2 family were found in *Borrelia burgdorferi,* denoted BB022 and BB023 (83). A simple BLAST search against the *H. pylori* 26695 proteome using *B. subtilis* PbuG and *B. burgdorferi* BB022 as queries revealed a homolog to these transporters (30% identical to each) encoded by *hp1175.*

<u>Conversions Between Nucleobases, Nucleosides and Nucleotides</u>

Free purine bases can be attached to the C1 carbon of PRPP through an N-glycosyl bond. This reaction is catalyzed by phosphoribosyltransferase (PRTase), and occurs as follows:

$$\text{nucleobase} + \text{PRPP} \rightarrow \text{nucleoside monophosphate} + PP_i$$

E. coli possesses three purine PRTase enzymes, one specific for adenine (APRTase)(84), another for guanine and xanthine (XGPRTase)(85), and a third for hypoxanthine (HPRTase)(86). *H. pylori* possesses only two phosphoribosyl-transferases: APRTase (encoded by *hp0572*) and XGPRTase (encoded by *hp0735*) (11). APRTase activity was detected in *H. pylori* cell lysates (54), but has not been studied in detail. *H. pylori* XGPRTase was purified from recombinant *E.*

coli and was shown to utilize all 6-oxopurine substrates: guanine, hypoxanthine, and xanthine (87).

Purine nucleoside phosphorylase (PNP) is a second enzyme class that catalyzes the condensation between a purine base and a ribose or deoxyribose sugar. In this case, the product is a nucleoside as opposed to a nucleoside monophosphate:

nucleobase + (deoxy)ribose-1-P ←→ (deoxy)ribonucleoside + P_i

At equilibrium this reversible reaction favors nucleoside formation, however the observed direction *in vivo* is primarily toward nucleoside cleavage, partly due to limiting concentration of intracellular (deoxy)ribose-1-P, but also due to the rapidity at which PRTase enzymes quickly consume free purine bases (86, 88). There are two PNPs in *E. coli*. The first to be described (PNP-I) accepts all purine nucleosides and deoxynucleosides as substrates except xanthosine (89). A second PNP (PNP-II) was later discovered in *E. coli* (90). This second PNP shares less than 10% identity with PNP-I, but it does share some overlap in substrate specificity, accepting all purine nucleosides and deoxynucleosides except adenosine (91).

H. pylori possesses two putative PNPs, encoded by *hp1178* and *hp1530* (11). While the gene product HP1178 has not been studied biochemically, an *H. pylori* mutant lacking *hp1178* was attenuated for growth on adenosine and guanosine as its sole purine source (75). This mutant was not tested for growth on the remaining two purine nucleosides (xanthosine and inosine), and therefore it cannot yet be determined whether HP1178 shares the same substrate specificity as *E. coli* PNP-I. Interestingly, although HP1178 is a strong homolog (54% identical) to *E. coli* PNP-I, HP1530 shares low overall sequence similarity to other known PNPs, including

E. coli PNP-II. It appears that the assignment for HP1530 was instead based on the presence of conserved domains indicating that it is a member of the phosphorylase superfamily (92, 93). Due to high sequence variability among members of this superfamily, some members are undetectable using simple sequence similarity-based approaches such as BLAST (93). Until HP1530 is studied experimentally, the assignment of "purine nucleoside phosphorylase" to describe this hypothetical enzyme is too specific; among the other possible functions of this enzyme are 5'-methyladenosine phosphorylase or uridine phosphorylase, which are the two other members of the phosphorylase superfamily (93).

An alternative, direct strategy to convert a nucleoside into a nucleotide monophosphate occurs through phosphorylation of a nucleoside by a nucleoside kinase (NK). Interestingly, while most eukaryotes possess NKs for all nucleoside substrates, nearly all bacteria possess only guanosine-inosine kinase but lack adenosine kinase (94). An exception to this rule can be found in members of the genus *Mycobacterium*, which possesses an adenosine kinase that apparently evolved from another enzyme of the ribokinase family via convergent evolution (95). *H. pylori* possesses no homologs for adenosine kinase, or guanosine-inosine kinase.

Synthesis of AMP and GMP

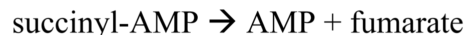
IMP is the product of *de novo* purine biosynthesis, and from this molecule either AMP or GMP can be synthesized. The pathway for AMP and GMP biosynthesis is the same for all organisms, regardless of the ability of that organism to synthesize IMP *de novo*. *H. pylori* possesses strong homologs to the canonical enzymes which catalyze the synthesis of these two nucleotides (Fig 1.2).

The first step in the synthesis of AMP from IMP is catalyzed by adenylosuccinate synthase (EC 6.3.4.4), which is highly conserved in bacteria and is denoted PurA. *H. pylori* possesses a strong homolog (55% identical) to *E. coli* PurA (encoded by *hp0255*). The overall reaction catalyzed is:

IMP + L-aspartate + GTP → succinyl-AMP + GDP + P_i

The crystal structure of *E. coli* PurA has been solved (96), and the enzyme has been studied in-detail with regard to ligand binding, kinetics, allosteric regulation, and important active site residues (44, 97, 98). Notably, GTP is used for energy coupling in this reaction (as opposed to ATP). This is one of several mechanisms used to regulate the ratio of ATP to GTP inside the cell.

Following its synthesis, succinyl-AMP is immediately cleaved by adenylosuccinate lyase (EC 4.3.2.2), releasing AMP and fumarate. Denoted PurB in bacteria, adenylosuccinate lyase also catalyzes the ninth step in the pathway toward IMP biosynthesis, a similar reaction that likewise liberates a molecule of fumarate. Therefore, the two reactions catalyzed by PurB are:

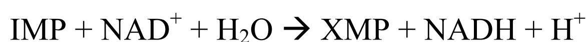

succinyl-AMP → AMP + fumarate

SAICAR → AICAR + fumarate

In these reactions, SAICAR stands for succinyl-aminoimidazole carboxamide ribotide, and AICAR stands for aminoimidazole carboxamide ribotide. PurB is a member of the argininosuccinate lyase (ASL)/fumarase C superfamily (99). The homolog to PurB in *H. pylori*

(encoded by *hp1112*) shares overall low sequence homology with *E. coli* PurB, but nevertheless is 49% identical to *B. subtilis* PurB, for which the structure and catalytic mechanism has been partially detailed (100, 101), aided by the crystal structure of the close homolog PurB from *Thermotoga maritima* (102).

Similar to AMP synthesis, the synthesis of GMP from IMP is catalyzed in two steps. In the first step, IMP is oxidized to XMP by the enzyme IMP dehydrogenase (GuaB, EC 1.1.1.205), which uses NAD^+ as the oxidant.



$$IMP + NAD^+ + H_2O \rightarrow XMP + NADH + H^+$$

In *H. pylori*, the homolog for this enzyme is encoded by *hp0829*, and is 55% identical IMP dehydrogenase from *E. coli*. Of the purine conversion enzymes in *H. pylori*, only IMP dehydrogenase (denoted here *Hp*IMPDH) has been studied empirically using purified enzyme in order to study potential inhibitors of catalytic activity. The K_m of *Hp*IMPDH for IMP and NAD^+, respectively, were 18 μM and 73 mM, which is similar to values seen for other bacterial IMP dehydrogenases (103). The authors were particularly interested in the potential for this enzyme as a drug target. Two key residues, Ala165 and Tyr358, confer susceptibility of IMP dehydrogenase to a certain set of inhibitors (103). *Hp*IMPDH possesses these key residues, and indeed the purified enzyme was potently inhibited *in vitro* by these compounds. Growth of *H. pylori* in rich media was also affected by these same compounds (103), however their efficacy has yet to be studied using an *in vivo* infection model. The gene encoding *Hp*IMPDH (*guaB*) has not been studied using mutant analysis.

To convert XMP to GMP requires transfer of an amido group from L-glutamine onto XMP by the enzyme GMP synthase:

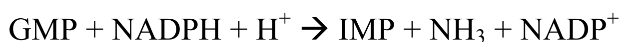
This reaction requires the hydrolysis of the opposite purine nucleotide, ATP, as a source of energy. As previously noted, the cell's strategy of using the opposite purine nucleotide as an energy source for synthesis of the former can serve to balance the ratio of ATP to GTP within the cell (44). The crystal structure of *E. coli* GMP synthase has been solved, revealing a bifunctional enzyme possessing two catalytic domains: a glutaminase domain and an ATP pyrophosphatase domain (104). This enzyme belongs to the Class I glutamine amidotransferase family, which exhibit channeling of ammonia from one catalytic domain to the other in order to prevent diffusion of this intermediate (105). *H. pylori* possesses a homolog for GMP synthetase (encoded by *hp0409*) that shares over 50% identity with its homologs in *E. coli* and *Thermus thermophiles*, enzymes for which crystal structures have been solved.

In summary, strong homologs exist in *H. pylori* for enzymes required for synthesis of both AMP and GMP. One of these enzymes (*Hp*IMPDH) has been studied biochemically. None have been studied *in vivo* using mutant analysis. One of my aims in this current work was to provide *in vivo* evidence for these purine conversion enzymes using mutant analysis.

Conversion Between Adenine and Guanine

Guanine and its derivatives can be converted into adenine derivatives (and visa versa) in order to satisfy cellular needs. In both cases, the purine must first be converted to the common intermediate IMP, from which either AMP or GMP can be made (Fig 1.2).

In the case of guanine derivatives, the conversion occurs through GMP. This molecule is reduced to IMP via reductive deamination by GMP reductase (GuaC) (106):



$$GMP + NADPH + H^{+} \rightarrow IMP + NH_3 + NADP^{+}$$

A close relative of IMP dehydrogenase, GMP reductase is highly conserved among many organisms including bacteria and mammals (107-109). In *E. coli,* GuaC is the sole means by which guanine and its derivatives are converted to adenine (110). In *E. coli*, GuaC is inhibited by ATP and activated by GTP, in accordance with its role in balancing adenine and guanine pools (106, 108). A homolog to GuaC is present in *H. pylori* (32% identity with *E. coli* GuaC, encoded by *hp0854*), but it has not been studied.

Adenine and its derivatives are likewise degraded to IMP first, then to GMP (Fig 1.2). Generally, the first step in this process relies on a hydrolytic deamination reaction on C-6 of the adenine moiety, a reaction that is irreversible and requires no ATP. In bacteria, this deamination is catalyzed by either adenosine deaminase (ADD) or by adenine deaminase (ADE). Some bacteria possess both ADE and ADD (111, 112), while others possess only one of the two (111, 113). In certain prokaryotes, there exists a roundabout mechanism for adenine degradation that relies on the histidine biosynthesis pathway. Through this pathway, ATP is converted into AICAR, which can be channeled into the final steps of the *de novo* IMP biosynthetic pathway

(114). *E. coli* Δ*add* mutants that lack histidine in the growth medium use this alternative route. When hisidine is added to the medium, the genes required for histidine biosynthesis are no longer expressed and at this point the Δ*add* mutation causes auxotrophy for guanine or guanine derivatives (115). *H. pylori* lacks homologs to both ADD and ADE, and furthermore lacks a histidine biosynthetic pathway. Also absent from *H. pylori*, and indeed from all ε-proteobacteria, is a homolog to the *E. coli* tRN-specific adenosine deaminase (TadA). TadA forms inosine from adenosine at the wobble position of tRNAs (116), and in theory could be responsible for adenosine degradation in the cell, however TadA cannot substitute for ADD in *E. coli* (115). Therefore, even if *H. pylori* did possess a homolog for TadA, the chances are slight that this enzyme could serve as the primary deamination pathway for adenine derivatives. In summary, the mechanism of adenine or adenosine deamination in *H. pylori* remains unsolved.

The question of a missing pathway for adenine/adenosine degradation in *H. pylori* was noted upon publication of the first full genome sequence (117), and has likewise been found missing from subsequent *H. pylori* strains (118). Early studies of purine salvage in *H. pylori* reported the presence of adenine deaminase (ADE) activity in cell extracts (54), however this conclusion was not fully supported. The authors observed a spectral peak corresponding to adenine decrease over time, concomitant with an increase in hypoxanthine. Either ADE or ADD activity could explain this substrate-product shift, so the presence of these enzymes in *H. pylori* remains unclear. Reynolds & Penn (1994) showed that *H. pylori* can grow using adenine as a sole purine source (53). Their observation must first be corroborated using a fully (rather than partially) defined growth medium, however if we assume this phenotype to be true, then *H. pylori* must possess an enzyme (ADE or ADD) that can degrade adenine for use in the synthesis of guanine nucleotides.

Adenosine Deaminase

ADD is a member of the amidohydrolase superfamily of proteins, whose members are diverse and include urease, cytosine deaminase, adenosine and adenine deaminase, dihydroorotase, N-acyl-D-amino acid amidohydrolase, chorohydrolase, and others. This superfamily is widely distributed among eukaryotes and prokaryotes. The common ancestral enzyme of this superfamily is so deeply rooted that, among its members, the amino acid sequences often share no homology whatsoever (119). Members of this superfamily are therefore recognized by their three-dimensional structure and by the presence of certain highly conserved residues (119). All members share a common $(\beta/\alpha)_8$-barrel 3-D structure along with three conserved histidine residues and one aspartic acid residue that together coordinate a divalent metal ion (119). The preferred divalent metal ligand varies depending on the enzyme. Examples include nickel (urease) (120, 121), zinc (adenosine deaminase, dihydroorotase, guanine deaminase (122-124), iron(II) (cytosine deaminase, chlorohydrolase) (125, 126), and cadmium/zinc (phosphotriesterase) (127, 128). Also variable are the number of metal ions in the catalytic core. Urease, for example, holds two Ni^{+2} ions per β-subunit (121, 129), while adenosine deaminase holds only one Zn^{+2} ion (123, 130). The presence of two rather than one metal atom requires, in addition to the conserved histidines, a carbamylated or carboxylated lysine to serve as a bridging ligand (122, 131).

Interestingly, several amidohydrolases have recently been recognized as having evolved from existing paralogs over the past forty years in certain soil-dwelling bacteria for the purpose of degrading S-triazine herbicides. One such example is the enzyme atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. ADP. This iron(II)-containing enzyme dechlorinates atrazine in the first step of atrazine metabolism (126, 132, 133). Due to the recent appearance of this

enzyme, presumably it has evolved from an ancestral protein whose substrate remains to be determined (119).

Adenosine deaminase is a highly efficient biocatalyst that significantly lowers the activation energy required for the hydrolytic deamination of adenosine at position C-6 of the purine ring. This efficiency is demonstrated by *E. coli* ADD, which provides a rate enhancement of 2×10^{12} (134). Adenosine deaminase has been purified and studied from numerous organisms, both prokaryotic and eukaryotic (112, 135-138), however the majority of structural and kinetic studies have been performed using *Mus musculus* (murine) ADD. Adenosine deamination occurs via an SN2 mechanism in which a water molecule is polarized, through the aid of Zn^{+2} and Asp295 (Asp277 in *E. coli*), to form the hydroxide nucleophile for attack (139-141). An unstable tetrahedral intermediate is formed (123), after which the leaving group is protonated by a residue that has yet to be confirmed (140). The binding pocket for Zn^{+2} and adenosine is located deep within the β-barrel, inaccessible to the surrounding solvent (141). Many of the important residues for substrate binding and catalysis have been elucidated for murine ADD (Table 1.2), and are based on bioinformatics analyses, site-directed mutagenesis and crystal structures of ADD bound to various adenosine and transition-state analogs (123, 139-144). *E. coli* ADD is 33% identical (50% similar) to murine ADD, and therefore it is not surprising that these same critical residues are conserved in *E. coli* ADD (145).

The K_{cat}/K_m value for murine ADD is 1.4×10^7 $M^{-1}s^{-1}$, indicating that the catalyzed reaction is nearly diffusion-limited (134). Substrates for bacterial and mammalian ADDs include both adenosine and 2'-deoxyadenosine as substrates; K_m values for adenosine range between 20 and 70 μM, while values for 2'-deoxyadenosine range between 20-400 μM (112, 135, 136, 146, 147). The molecular weight for a single monomer of ADD typically falls between 25-55 kDa,

and, depending upon the bacterial species, exist as a monomer, homodimer, or homotetramer (112, 135, 147, 148). Aside from these differences in quaternary structure, certain striking commonalities exist among all ADDs studied to date; for instance, the presence of a single Zn^{+2} ion as the physiologically relevant metal ligand. In addition, all ADDs studied biochemically to date are orthologs that descended from a common ancestor and cluster unambiguously in a phylogenetic tree (119, 144). Their close relatedness is illustrated by their representation in a single cluster of orthologous groups (cog1816) within the COG database (149).

One of the most powerful aspects of the COG database is the ability to examine the phylogenic representation of a particular COG among the prokaryotic phyla. For example, adenosine deaminase (cog1816) does not appear to be universally represented among prokaryotes. Members of cog1816 are absent from the archaea, and appear to have spotty representation among both the proteobacteria and the firmicutes. An important caveat to remember, however, is that only 66 genomes are represented in the COG database, and some phyla are represented by only a single genome, therefore generalizations cannot be made regarding a COG's true phylogenetic representation. Nevertheless, it is curious that many organisms which lack a cog1816 homolog also contain a "gap" in their purine conversion pathway—a gap that can only be filled by the presence of ADD or ADE. Examples of such organisms include *Methanococcus maripaludis* S2, *Termotoga maritima*, *Synechocystis* sp. PCC 6803, *Pasteurella multocida,* as well as most epsilon- and delta-proteobacteria including *H. pylori.*

For the above reasons, we predict the existence of an enzyme in *H. pylori* that can degrade adenosine (or adenine), and that is not a member of cog1816. Efforts to elucidate this enzyme from the proteome of *H. pylori* are described herein.

Purines: Relevance to Virulence in *H. pylori*

Purine salvage is important for numerous human bacterial pathogens. Of particular importance are genes involved in converting between guanine and adenine. For example, uropathogenic *E. coli* requires genes for the synthesis and degradation of GMP for full virulence (150). *S. enterica* is likewise attenuated when lacking *guaA* and *guaB* (151). Other pathogens for which purine interconversion pathways are important include *Yersinia pestis, B. burgdorferi, and Vibrio cholerae* (152-154). Notably, these five pathogens encounter distinct host environments ranging from intestinal mucosa to the urothelium, from intracellular replicative niches to transitory passage through the bloodstream. The reliance on purine interconversion pathways by many pathogens suggests a general limitation for purines in diverse niches within the human host (155), and could at least partially explain why most bacterial pathogens possess an expensive *de novo* pathway for IMP biosynthesis. Despite this trend, some pathogens (for example *B. burgdorferi* (50) and *Chlamydia trachomatis* (51)) rely entirely on host purines in combination with partial salvage pathways. These two pathogens provide evidence that, in certain host environments, sufficient purines are available for bacterial replication. Which of these situations is true for *H. pylori*? Does it possess machinery for *de novo* purine synthesis, salvage, or both?

Similar to *B. burgdorferi* and *C. thrachomatis*, *H. pylori* possesses a reduced genome due to its extended evolution within the relatively stable niche of the gastric mucosa. On the other hand, *H. pylori* is not an intracellular pathogen, and in that respect is more similar to *E. coli* and *V. cholerae*. Purines are more highly concentrated inside the host cell cytosol (155, 156), arguing perhaps in favor of the preservation of *de novo* IMP biosynthesis in *H. pylori*. Several questions are therefore of medical relevance, and are central to this current work. Does *H. pylori* rely on host purines for survival? Does successful host infection depend upon a purine salvage

network, and if so, which components of this network are important? In the interest of providing a foundation for rational drug engineering, it is important to piece together this salvage network, especially the component of this network that appears to be missing from the genome: adenosine deaminase. *H. pylori* ADD will likely be a novel isoform that is dissimilar to human ADD isoforms I and II, and thus may represent an attractive candidates for the design of new antibiotics targeting this organism.

Scope of this Study

The work presented herein serves to characterize the poorly understood *H. pylori* purine metabolic network, in particular the conversions between guanine and adenine derivatives. The following questions will be addressed:

1. What are the purine requirements for H. pylori? [1]

To address this question I use a fully defined chemical growth medium lacking BSA or other common supplements, and in doing so unambiguously determine whether *de novo* purine biosynthesis is possible in this organism. I furthermore address whether *H. pylori*'s salvage network is complete by asking if a particular purine is necessary for growth, or whether any purine source can suffice. Lastly, I examine whether the growth efficiency of *H. pylori* depends upon the purine source present in the medium. These studies build a baseline understanding of the purine requirements in this organism, and therefore serve as valuable information from which hypotheses can be drawn in subsequent sections of this dissertation work.

2. What genes are responsible for H. pylori's purine conversion network? [1]

To determine the associated genes I use mutant analysis in combination with growth studies in a fully defined medium. Determining which specific purines can support growth of these *H. pylori* mutant strains define the importance of these genes within the purine metabolic network, and substantiate the current gene annotations with experimental evidence for gene function.

3. How are purines taken up by H. pylori?

I address one facet of this question by examining the *hp1180* locus, annotated as encoding a "pyrimidine nucleoside transporter, NupC." I hypothesize that this gene actually encodes a purine nucleoside transporter, and test this using a *nupC* mutant by way of phenotype characterization and radiolabeled nucleoside uptake studies.

4. How does H. pylori degrade adenine?

There is no homolog in the sequenced *H. pylori* genomes for adenosine or adenine deaminase. Therefore, an adenine degradation pathway is not included among the hypotheses tested in the mutant analyses described above. Addressing this question must therefore begin by identifying the gene encoding this elusive adenine-degrading enzyme. After doing this, I study this enzyme biochemically, as it possesses no strong homologs in the current NCBI database and therefore warrants additional enzymological studies toward understanding purine metabolism in bacteria.

[1] Prior to publishing our findings, a study was published that addressed these questions (75).

References

1. **Malaty, H., P. Sutton, and H. Mitchell.** 2010. Epidemiology of *Helicobacter pylori* infection. Helicobacter **8:**8-12.

2. **Marshall, B., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. The Lancet **323:**1311-1315.

3. **Donachie, S. P., J. P. Bowman, S. L. On, and M. Alam.** 2005. *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. International Journal of Systematic and Evolutionary Microbiology **55:**1271-1277.

4. **McClung, C., D. Patriquin, and R. Davis.** 1983. *Campylobacter nitrofigilis* sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel. International Journal of Systematic Bacteriology **33:**605-612.

5. **Solnick, J. V., and D. B. Schauer.** 2001. Emergence of Diverse *Helicobacter* Species in the Pathogenesis of Gastric and Enterohepatic Diseases. Clinical Microbiology Reviews **14:**59-97.

6. **Hazell, S. L., D. J. Evans, and D. Y. Graham.** 1991. *Helicobacter pylori* catalase. Journal of General Microbiology **137:**57-61.

7. **Hu, L., and H. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infection and Immunity **58:**992-998.

8. **Stingl, K., and H. De Reuse.** 2005. Staying alive overdosed: How does *Helicobacter pylori* control urease activity? International Journal of Medical Microbiology **295:**307-315.

9. **Bury-Moné, S., N. O. Kaakoush, C. Asencio, F. Mégraud, M. Thibonnier, H. De Reuse, and G. L. Mendz.** 2006. Is *Helicobacter pylori* a true microaerophile? Helicobacter **11:**296-303.

10. **Park, S. A., A. Ko, and N. G. Lee.** 2011. Stimulation of growth of the human gastric pathogen *Helicobacter pylori* by atmospheric level of oxygen under high carbon dioxide tension. BMC Microbiology **11:**96.

11. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, and B. A. Dougherty.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

12. **Danielli, A., G. Amore, and V. Scarlato.** 2010. Built shallow to maintain homeostasis and persistent infection: insight into the transcriptional regulatory network of the gastric human pathogen *Helicobacter pylori*. PLoS Pathogens **6:**e1000938.

13. **Schreiber, S., M. Konradt, C. Groll, P. Scheid, G. Hanauer, H.-O. Werling, C. Josenhans, and S. Suerbaum.** 2004. The spatial orientation of *Helicobacter pylori* in the gastric mucus. Proceedings of the National Academy of Sciences **101:**5024-5029.

14. **Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow.** 2003. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. Science **300:**1430-1434.

15. **Tan, S., L. S. Tompkins, and M. R. Amieva.** 2009. *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. PLoS Pathogens **5:**e1000407.

16. **Mizote, T., H. Yoshiyama, and T. Nakazawa.** 1997. Urease-independent chemotactic responses of *Helicobacter pylori* to urea, urease inhibitors, and sodium bicarbonate. Infection and Immunity **65:**1519-1521.

17. **Croxen, M. A., G. Sisson, R. Melano, and P. S. Hoffman.** 2006. The *Helicobacter pylori* chemotaxis receptor TlpB (HP0103) is required for pH taxis and for colonization of the gastric mucosa. Journal of Bacteriology **188:**2656-2665.

18. **Schreiber, S., M. Konradt, C. Groll, P. Scheid, G. Hanauer, H. O. Werling, C. Josenhans, and S. Suerbaum.** 2004. The spatial orientation of *Helicobacter pylori* in the gastric mucus. Proceedings of the National Academy of Sciences **101:**5024-5029.

19. **Mobley, H., M. J. Cortesia, L. Rosenthal, and B. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. Journal of Clinical Microbiology **26:**831-836.

20. **Scott, D. R., E. A. Marcus, Y. Wen, J. Oh, and G. Sachs.** 2007. Gene expression in vivo shows that *Helicobacter pylori* colonizes an acidic niche on the gastric surface. Proceedings of the National Academy of Sciences **104:**7235-7240.

21. **Allen, A., and G. Flemström.** 2005. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. American Journal of Physiology-Cell Physiology **288:**C1-C19.

22. **Eaton, K. A., C. Brooks, D. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infection and Immunity **59:**2470-2475.

23. **Celli, J. P., B. S. Turner, N. H. Afdhal, S. Keates, I. Ghiran, C. P. Kelly, R. H. Ewoldt, G. H. McKinley, P. So, and S. Erramilli.** 2009. *Helicobacter pylori* moves through mucus by reducing mucin viscoelasticity. Proceedings of the National Academy of Sciences **106:**14321-14326.

24. **Megraud, F., F. Bonnet, M. Garnier, and H. Lamouliatte.** 1985. Characterization of *Campylobacter pyloridis* by culture, enzymatic profile, and protein content. Journal of Clinical Microbiology **22:**1007-1010.

25. **Schade, C., G. Flemström, and L. Holm.** 1994. Hydrogen ion concentration in the mucus layer on top of acid-stimulated and-inhibited rat gastric mucosa. Gastroenterology **107:**180.

26. **Linz, B., F. Balloux, Y. Moodley, A. Manica, H. Liu, P. Roumagnac, D. Falush, C. Stamer, F. Prugnolle, and S. W. van der Merwe.** 2007. An African origin for the intimate association between humans and *Helicobacter pylori*. Nature **445:**915-918.

27. **Dominguez-Bello, M. G., and M. J. Blaser.** 2011. The Human Microbiota as a Marker for Migrations of Individuals and Populations. Annual Review of Anthropology **40:**451-474.

28. **Makola, D., D. A. Peura, and S. E. Crowe.** 2007. *Helicobacter pylori* infection and related gastrointestinal diseases. Journal of Clinical Gastroenterology **41:**548-558.

29. **Malaty, H. M.** 2007. Epidemiology of *Helicobacter pylori* infection. Best Practice & Research Clinical Gastroenterology **21:**205-214.

30. **Staat, M. A., D. Kruszon-Moran, G. M. McQuillan, and R. A. Kaslow.** 1996. A population-based serologic survey of *Helicobacter pylori* infection in children and adolescents in the United States. Journal of Infectious Diseases **174:**1120-1123.

31. **Fischer, W., S. Prassl, and R. Haas.** 2009. Virulence mechanisms and persistence strategies of the human gastric pathogen *Helicobacter pylori*, p. 129-171, Molecular Mechanisms of Bacterial Infection via the Gut. Springer.

32. **Malaty, H. M., and O. Nyren.** 2003. Epidemiology of *Helicobacter pylori* infection. Helicobacter **8:**8-12.

33. **Bardhan, P. K.** 1997. Epidemiological features of *Helicobacter pylori* infection in developing countries. Clinical Infectious Diseases **25:**973-978.

34. **Kivi, M., and Y. Tindberg.** 2006. *Helicobacter pylori* occurrence and transmission: A family affair? Scandinavian Journal of Infectious Diseases **38:**407-417.

35. **Bellack, N., M. Koehoorn, Y. MacNab, and M. Morshed.** 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. Epidemiology and Infection **134:**439-449.

36. **Ciacci, C., F. Sabbatini, R. Cavallaro, F. Castiglione, S. Di Bella, P. Iovino, A. Palumbo, R. Tortora, D. Amoruso, and G. Mazzacca.** 2004. *Helicobacter pylori* impairs iron absorption in infected individuals. Digestive and Liver Disease **36:**455-460.

37. **Fuccio, L., R. Zagari, M. Minardi, and F. Bazzoli.** 2007. Systematic review: *Helicobacter pylori* eradication for the prevention of gastric cancer. Alimentary Pharmacology & Therapeutics **25:**133-141.

38. **Parkin, D. M., F. Bray, J. Ferlay, and P. Pisani.** 2005. Global cancer statistics, 2002. CA: A Cancer Journal for Clinicians **55:**74-108.

39. **Dooley, C. P., H. Cohen, P. L. Fitzgibbons, M. Bauer, M. D. Appleman, G. I. Perez-Perez, and M. J. Blaser.** 1989. Prevalence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. The New England Journal of Medicine **321:**1562.

40. **Zullo, A., C. Hassan, F. Cristofari, A. Andriani, V. De Francesco, E. Ierardi, S. Tomao, M. Stolte, S. Morini, and D. Vaira.** 2010. Effects of *Helicobacter pylori* Eradication on Early Stage Gastric Mucosa–Associated Lymphoid Tissue Lymphoma. Clinical Gastroenterology and Hepatology **8:**105-110.

41. **Selgrad, M., and P. Malfertheiner.** 2012. Commentary: is *Helicobacter pylori* antibiotic resistance surveillance needed and how can it be delivered? Alimentary Pharmacology & Therapeutics **36:**197-198.

42. **Velin, D., and P. Michetti.** 2010. Advances in vaccination against *Helicobacter pylori*. Expert Review of Gastroenterology & Hepatology **4:**157-166.

43. **Rupnow, M. F., A. H. Chang, R. D. Shachter, D. K. Owens, and J. Parsonnet.** 2009. Cost-effectiveness of a potential prophylactic *Helicobacter pylori* vaccine in the United States. Journal of Infectious Diseases **200:**1311-1317.

44. **Jensen, K. F., G. Dandanell, B. Hove-Jensen, and M. Willenoes.** 2008. Nucleotides, nucleosides, and nucleobases. *In* J. Ingraham (ed.), *EcoSal* - *Escherichia coli* and *Salmonella*, Web edition ed. ASM Press, Washington, DC.

45. **Stouthamer, A.** 1973. A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie van Leeuwenhoek **39:**545-565.

46. **Ebbole, D., and H. Zalkin.** 1987. Cloning and characterization of a 12-gene cluster from *Bacillus subtilis* encoding nine enzymes for de novo purine nucleotide synthesis. Journal of Biological Chemistry **262:**8274-8287.

47. **Smith, J. L.** 1998. Glutamine PRPP amidotransferase: snapshots of an enzyme in action. Current Opinion in Structural Biology **8:**686-694.

48. **Messenger, L. J., and H. Zalkin.** 1979. Glutamine phosphoribosylpyrophosphate amidotransferase from *Escherichia coli*. Purification and properties. Journal of Biological Chemistry **254:**3382-3392.

49. **Hammond, D. J., and W. E. Gutteridge.** 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Molecular and Biochemical Parasitology **13:**243-261.

50. **Pettersson, J., M. E. Schrumpf, S. J. Raffel, S. F. Porcella, C. Guyard, K. Lawrence, F. C. Gherardini, and T. G. Schwan.** 2007. Purine salvage pathways among *Borreli*a species. Infection and Immunity **75:**3877-3884.

51. **Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, and Q. Zhao.** 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science **282:**754-759.

52. **Nedenskov, P.** 1994. Nutritional requirements for growth of *Helicobacter pylori*. Applied and Environmental Microbiology **60:**3450-3453.

53. **Reynolds, D. J., and C. W. Penn.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140:**2649-2656

54. **Mendz, G., B. Jimenez, S. Hazell, A. Gero, and W. O'Sullivan.** 1994. Salvage synthesis of purine nucleotides by *Helicobacter pylori*. Journal of Applied Microbiology **77:**674-681.

55. **Testerman, T. L., D. J. McGee, and H. L. T. Mobley.** 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. Journal of Clinical Microbiology **39:**3842-3850.

56. **Testerman, T. L., P. B. Conn, H. L. T. Mobley, and D. J. McGee.** 2006. Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. Journal of Clinical Microbiology **44:**1650-1658

57. **Ye, J., and B. Van Den Berg.** 2004. Crystal structure of the bacterial nucleoside transporter Tsx. The EMBO Journal **23:**3187-3195.

58. **Maier, C., E. Bremer, A. Schmid, and R. Benz.** 1988. Pore-forming activity of the Tsx protein from the outer membrane of *Escherichia coli*. Demonstration of a nucleoside-specific binding site. Journal of Biological Chemistry **263:**2493-2499.

59. **Tjaden, J., H. Winkler, C. Schwöppe, M. Van Der Laan, T. Möhlmann, and H. Neuhaus.** 1999. Two nucleotide transport proteins in *Chlamydia trachomatis*, one for net nucleoside triphosphate uptake and the other for transport of energy. Journal of Bacteriology **181:**1196-1202.

60. **Williamson, L. R., G. V. Piano, H. H. Winkler, D. C. Krause, and D. O. Wood.** 1989. Nucleotide sequence of the *Rickettsia prowazekii* ATP/ADP translocase-encoding gene. Gene **80:**269-278.

61. **Loewen, S. K., S. Y. M. Yao, M. D. Slugoski, N. N. Mohabir, R. J. Turner, J. R. Mackey, J. H. Weiner, M. P. Gallagher, P. J. F. Henderson, and S. A. Baldwin.** 2004. Transport of physiological nucleosides and anti-viral and anti-neoplastic nucleoside drugs by recombinant *Escherichia coli* nucleoside-H+ cotransporter (NupC) produced in *Xenopus laevis* oocytes. Molecular Membrane Biology **21:**1-10.

62. **Loewen, S. K., A. M. L. Ng, N. N. Mohabir, S. A. Baldwin, C. E. Cass, and J. D. Young.** 2003. Functional characterization of a H+/nucleoside co-transporter (CaCNT) from *Candida albicans*, a fungal member of the concentrative nucleoside transporter (CNT) family of membrane proteins. Yeast **20:**661-675.

63. **Ritzel, M., S. Yao, M. Huang, J. Elliott, C. Cass, and J. Young.** 1997. Molecular cloning and functional expression of cDNAs encoding a human Na+-nucleoside cotransporter (hCNT1). American Journal of Physiology-Cell Physiology **272:**C707-C714.

64. **Rlteel, M. W. L., S. Y. M. Yaof, A. M. L. Ng, J. R. Mackeyt, C. E. Cass, and J. D. Young.** 1998. Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Molecular Membrane Biology **15:**203-211.

65. **Ritzel, M. W. L., A. M. L. Ng, S. Y. M. Yao, K. Graham, S. K. Loewen, K. M. Smith, R. G. Ritzel, D. A. Mowles, P. Carpenter, and X. Z. Chen.** 2001. Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system *cib*). Journal of Biological Chemistry **276:**2914-2927.

66. **Loewen, S. K., A. M. L. Ng, S. Y. M. Yao, C. E. Cass, S. A. Baldwin, and J. D. Young.** 1999. Identification of amino acid residues responsible for the pyrimidine and purine nucleoside specificities of human concentrative Na+ nucleoside cotransporters hCNT1 and hCNT2. Journal of Biological Chemistry **274:**24475-24484.

67. **Munch‐Petersen, A., and B. Mygind.** 1976. Nucleoside transport systems in *Escherichia coli* K12: Specificty and regulation. Journal of Cellular Physiology **89:**551-559.

68. **Munch-Petersen, A., B. Mygind, A. Nicolaisen, and N. J. Pihl.** 1979. Nucleoside transport in cells and membrane vesicles from *Escherichia coli* K12. Journal of Biological Chemistry **254:**3730-3737.

69. **Zhang, Y., J. E. Craig, and M. P. Gallagher.** 1992. Location of the nupC gene on the physical map of *Escherichia coli* K-12. Journal of Bacteriology **174:**5758-5759.

70. **Craig, J. E., Y. Zhang, and M. P. Gallagher.** 1994. Cloning of the nupC gene of *Escherichia coli* encoding a nucleoside transport system, and identification of an adjacent insertion element, IS 186. Molecular Microbiology **11:**1159-1168.

71. **Patching, S. G., S. A. Baldwin, A. D. Baldwin, J. D. Young, M. P. Gallagher, P. J. F. Henderson, and R. B. Herbert.** 2005. The nucleoside transport proteins, NupC and NupG, from *Escherichia coli*: specific structural motifs necessary for the binding of ligands. Org. Biomol. Chem. **3:**462-470.

72. **Saxild, H. H., L. N. Andersen, and K. Hammer.** 1996. *dra-nupC-pdp* operon of *Bacillus subtilis*: nucleotide sequence, induction by deoxyribonucleosides, and transcriptional regulation by the deoR-encoded DeoR repressor protein. Journal of Bacteriology **178:**424-434

73. **Baltrus, D. A., M. R. Amieva, A. Covacci, T. M. Lowe, D. S. Merrell, K. M. Ottemann, M. Stein, N. R. Salama, and K. Guillemin.** 2009. The complete genome sequence of *Helicobacter pylori* strain G27. Journal of Bacteriology **191:**447-448.

74. **Suerbaum, S., C. Josenhans, T. Sterzenbach, B. Drescher, P. Brandt, M. Bell, M. Dröge, B. Fartmann, H. P. Fischer, and Z. Ge.** 2003. The complete genome sequence of the carcinogenic bacterium *Helicobacter hepaticus*. Proceedings of the National Academy of Sciences **100:**7901-7906.

75. **Liechti, G., and J. B. Goldberg.** 2012. *Helicobacter pylor*i relies primarily on the purine salvage pathway for purine nucleotide biosynthesis. Journal of Bacteriology **194:**839-854.

76. **de Koning, H., and G. Diallinas.** 2000. Nucleobase transporters. Molecular Membrane Biology **17:**75-94.

77. **Danielsen, S., M. Kilstrup, K. Barilla, B. Jochimsen, and J. Neuhard.** 1992. Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. Molecular Microbiology **6:**1335-1344.

78. **Diallinas, G., and C. Gournas.** 2008. The ubiquitous Nucleobase-Ascorbate Transporter (NAT) family: Lessons from model microbial genetic systems. Channels **2:**363-372.

79. **Burton, K.** 1983. Transport of nucleic acid bases into *Escherichia coli*. Journal of General Microbiology **129:**3505-3513.

80. **Karatza, P., and S. Frillingos.** 2005. Cloning and functional characterization of two bacterial members of the NAT/NCS2 family in *Escherichia coli*. Molecular Membrane Biology **22:**251-261.

81. **Christiansen, L. C., S. Schou, P. Nygaard, and H. H. Saxild.** 1997. Xanthine metabolism in *Bacillus subtilis*: characterization of the xpt-pbuX operon and evidence for purine-and nitrogen-controlled expression of genes involved in xanthine salvage and catabolism. Journal of Bacteriology **179:**2540-2550.

82. **Saxild, H. H., K. Brunstedt, K. I. Nielsen, H. Jarmer, and P. Nygaard.** 2001. Definition of the *Bacillus subtilis* PurR Operator Using Genetic and Bioinformatic Tools and Expansion of the PurR Regulon with glyA, guaC, pbuG, xpt-pbuX, yqhZ-folD, and pbuO. Journal of Bacteriology **183:**6175-6183.

83. **Jain, S., S. Sutchu, P. A. Rosa, R. Byram, and M. W. Jewett.** 2012. *Borrelia burgdorferi* Harbors a Transport System Essential for Purine Salvage and Mammalian Infection. Infection and Immunity **80:**3086-3093.

84. **Hochstadt-Ozer, J., and E. Stadtman.** 1971. The regulation of purine utilization in bacteria. III. The involvement of purine phosphoribosyltransferases in the uptake of adenine and other nucleic acid precursors by intact resting cells. The Journal of Biological Chemistry **246:**5312.

85. **Liu, S., and G. Milman.** 1983. Purification and characterization of Escherichia coli guanine-xanthine phosphoribosyltransferase produced by a high efficiency expression plasmid utilizing a lambda PL promoter and CI857 temperature-sensitive repressor. Journal of Biological Chemistry **258:**7469-7475.

86. **Jochimsen, B., P. Nygaard, and T. Vestergaard.** 1975. Location on the chromosome of *Escherichia coli* of genes governing purine metabolism. Molecular and General Genetics MGG **143:**85-91.

87. **Duckworth, M., A. Ménard, F. Megraud, and G. L. Mendz.** 2006. Bioinformatic analysis of *Helicobacter pylori* XGPRTase: a potential therapeutic target. Helicobacter **11:**287-295.

88. **Jensen, K. F., and P. Nygaard.** 1975. Purine nucleoside phosphorylase from *Escherichia coli* and *Salmonella typhimurium*. European Journal of Biochemistry **51:**253-265.

89. **Robertson, B. C., and P. A. Hoffee.** 1973. Purification and properties of purine nucleoside phosphorylase from *Salmonella typhimurium*. Journal of Biological Chemistry **248:**2040-2043.

90. **Hammer-Jespersen, K., R. Buxton, and T. Hansen.** 1980. A second purine nucleoside phosphorylase in *Escherichia coli* K-12. Molecular and General Genetics MGG **179:**341-348.

91. **Dandanell, G., R. H. Szczepanowski, B. Kierdaszuk, D. Shugar, and M. Bochtler.** 2005. *Escherichia coli* purine nucleoside phosphorylase II, the product of the xapA gene. Journal of Molecular Biology **348:**113.

92. **Marchler-Bauer, A., S. Lu, J. B. Anderson, F. Chitsaz, M. K. Derbyshire, C. DeWeese-Scott, J. H. Fong, L. Y. Geer, R. C. Geer, and N. R. Gonzales.** 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Research **39:**D225-D229.

93. **Pugmire, M. J., and S. E. Ealick.** 2002. Structural analyses reveal two distinct families of nucleoside phosphorylases. Biochemical Journal **361:**1.

94. **Park, J., and R. Gupta.** 2008. Adenosine kinase and ribokinase–the RK family of proteins. Cellular and Molecular Life Sciences **65:**2875-2896.

95. **Long, M. C., V. Escuyer, and W. B. Parker.** 2003. Identification and characterization of a unique adenosine kinase from *Mycobacterium tuberculosis*. Journal of Bacteriology **185:**6548-6555.

96. **Poland, B. W., H. J. Fromm, and R. B. Honzatko.** 1996. Crystal Structures of Adenylosuccinate Synthetase from *Escherichia coli* Complexed with GDP, IMP Hadacidin, NO3, and Mg 2+. Journal of Molecular Biology **264:**1013-1027.

97. **Bass, M. B., H. J. Fromm, and M. M. Stayton.** 1987. Overproduction, purification, and characterization of adenylosuccinate synthetase from *Escherichia coli*. Archives of Biochemistry and Biophysics **256:**335-342.

98. **Kang, C., N. Sun, B. W. Poland, A. Gorrell, R. B. Honzatko, and H. J. Fromm.** 1997. Residues essential for catalysis and stability of the active site of *Escherichia coli* adenylosuccinate synthetase as revealed by directed mutation and kinetics. Journal of Biological Chemistry **272:**11881-11885.

99. **Tsai, M., J. Koo, P. Yip, R. F. Colman, M. L. Segall, and P. L. Howell.** 2007. Substrate and Product Complexes of *Escherichia coli* Adenylosuccinate Lyase Provide New Insights into the Enzymatic Mechanism. Journal of Molecular Biology **370:**541-554.

100. **Lee, T. T., C. Worby, Z. Q. Bao, J. E. Dixon, and R. F. Colman.** 1999. His68 and His141 are critical contributors to the intersubunit catalytic site of adenylosuccinate lyase of *Bacillus subtilis*. Biochemistry **38:**22-32.

101. **Palenchar, J. B., J. M. Crocco, and R. F. Colman.** 2009. The characterization of mutant *Bacillus subtilis* adenylosuccinate lyases corresponding to severe human adenylosuccinate lyase deficiencies. Protein Science **12:**1694-1705.

102. **Toth, E. A., and T. O. Yeates.** 2000. The structure of adenylosuccinate lyase, an enzyme with dual activity in the *de novo* purine biosynthetic pathway. Structure **8:**163-174.

103. **Gollapalli, D. R., I. S. MacPherson, G. Liechti, S. K. Gorla, J. B. Goldberg, and L. Hedstrom.** 2010. Structural determinants of inhibitor selectivity in prokaryotic IMP dehydrogenases. Chemistry & Biology **17:**1084-1091.

104. **Tesmer, J. J. G., T. J. Klem, M. L. Deras, V. J. Davisson, and J. L. Smith.** 1996. The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. Nature Structural & Molecular Biology **3:**74-86.

105. **Raushel, F. M., J. B. Thoden, and H. M. Holden.** 2003. Enzymes with molecular tunnels. Accounts of Chemical Research **36:**539-548.

106. **Mager, J., and B. Magasanik.** 1960. Guanosine 5'-phosphate reductase and its role in the interconversion of purine nucleotides. Journal of Biological Chemistry **235:**1474-1478.

107. **Martinelli, L. K. B., R. G. Ducati, L. A. Rosado, A. Breda, B. P. Selbach, D. S. Santos, and L. A. Basso.** 2011. Recombinant *Escherichia coli* GMP reductase: kinetic, catalytic and chemical mechanisms, and thermodynamics of enzyme–ligand binary complex formation. Molecular BioSystems **7:**1289-1305.

108. **Andrews, S. C., and J. Guest.** 1988. Nucleotide sequence of the gene encoding the GMP reductase of *Escherichia coli* K12. Biochemical Journal **255:**35.

109. **Li, J., Z. Wei, M. Zheng, X. Gu, Y. Deng, R. Qiu, F. Chen, C. Ji, W. Gong, and Y. Xie.** 2006. Crystal structure of human guanosine monophosphate reductase 2 (GMPR2) in complex with GMP. Journal of Molecular Biology **355:**980-988.

110. **Kessler, A. I., and J. S. Gots.** 1985. Regulation of guaC expression in *Escherichia coli*. Journal of Bacteriology **164:**1288-1293.

111. **Nygaard, P., P. Duckert, and H. H. Saxild.** 1996. Role of adenine deaminase in purine salvage and nitrogen metabolism and characterization of the ade gene in *Bacillus subtilis*. Journal of Bacteriology **178:**846-853.

112. **Pornbanlualap, S., and P. Chalopagorn.** 2011. Adenosine deaminase from *Streptomyces coelicolor*: Recombinant expression, purification and characterization. Protein Expression and Purification **78:**167-173.

113. **G Ducati, R., A. Breda, L. A Basso, and D. S Santos.** 2011. Purine Salvage Pathway in *Mycobacterium tuberculosis*. Current Medicinal Chemistry **18:**1258-1275.

114. **White, R. H.** 1997. Occurrence and biosynthesis of 5-aminoimidazole-4-carboxamide ribonucleotide and N-(beta-D-ribofuranosyl) formamide 5'-phosphate in *Methanobacterium thermoautotrophicum* delta (H). Journal of Bacteriology **179:**563-566.

115. **Yeung, C.-Y., D. Ingolia, D. Roth, C. Shoemaker, M. Al-Ubaidi, J.-Y. Yen, C. Ching, C. Bobonis, R. Kaufman, and R. Kellems.** 1985. Identification of functional murine adenosine deaminase cDNA clones by complementation in *Escherichia coli*. Journal of Biological Chemistry **260:**10299-10307.

116. **Wolf, J., A. P. Gerber, and W. Keller.** 2002. tadA, an essential tRNA-specific adenosine deaminase from *Escherichia coli*. The EMBO Journal **21:**3841-3851.

117. **Marais, A., G. L. Mendz, S. L. Hazell, and F. Mégraud.** 1999. Metabolism and genetics of *Helicobacter pylori*: the genome era. Microbiology and Molecular Biology Reviews **63:**642-674.

118. **Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, and T. L. Madden.** 2008. NCBI BLAST: a better web interface. Nucleic Acids Research **36:**W5-W9.

119. **Holm, L., and C. Sander.** 1998. An evolutionary treasure: unification of a broad set of amidohydrolases related to urease. Proteins: Structure, Function, and Bioinformatics **28:**72-82.

120. **Dunn, B. E., G. P. Campbell, G. Perez-Perez, and M. Blaser.** 1990. Purification and characterization of urease from *Helicobacter pylori*. Journal of Biological Chemistry **265:**9464-9469.

121. **Dixon, N. E., C. Gazzola, R. L. Blakeley, and B. Zerner.** 1975. Jack bean urease (EC 3.5. 1.5). Metalloenzyme. Simple biological role for nickel. Journal of the American Chemical Society **97:**4131-4133.

122. **Thoden, J. B., G. N. Phillips Jr, T. M. Neal, F. M. Raushel, and H. M. Holden.** 2001. Molecular structure of dihydroorotase: a paradigm for catalysis through the use of a binuclear metal center. Biochemistry **40:**6989-6997.

123. **Wilson, D. K., F. B. Rudolph, and F. A. Quiocho.** 1991. Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. Science **252:**1278-1284.

124. **Maynes, J. T., R. G. Yuan, and F. F. Snyder.** 2000. Identification, expression, and characterization of *Escherichia coli* guanine deaminase. Journal of Bacteriology **182:**4658-4660.

125. **Ireton, G. C., G. McDermott, M. E. Black, and B. L. Stoddard.** 2002. The structure of *Escherichia coli* cytosine deaminase. Journal of Molecular Biology **315:**687-697.

126. **Seffernick, J. L., H. McTavish, J. P. Osborne, M. L. de Souza, M. J. Sadowsky, and L. P. Wackett.** 2002. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a metalloenzyme. Biochemistry **41:**14430-14437.

127. **Omburo, G. A., J. M. Kuo, L. S. Mullins, and F. Raushel.** 1992. Characterization of the zinc binding site of bacterial phosphotriesterase. Journal of Biological Chemistry **267:**13278-13283.

128. **Vanhooke, J. L., M. M. Benning, F. M. Raushel, and H. M. Holden.** 1996. Three-dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate. Biochemistry **35:**6020-6025.

129. **Jabri, E., and P. A. Karplus.** 1996. Structures of the *Klebsiella aerogenes* urease apoenzyme and two active-site mutants. Biochemistry **35:**10616-10626.

130. **Cooper, B. F., V. Sideraki, D. K. Wilson, D. Y. Dominguez, S. W. Clark, F. A. Quiocho, and F. B. Rudolph.** 1997. The role of divalent cations in structure and function of murine adenosine deaminase. Protein Science **6:**1031-1037.

131. **Benning, M. M., J. M. Kuo, F. M. Raushel, and H. M. Holden.** 1995. Three-dimensional structure of the binuclear metal center of phosphotriesterase. Biochemistry **34:**7973-7978.

132. **Seffernick, J. L., and L. P. Wackett.** 2001. Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. Biochemistry **40:**12747-12753.

133. **De Souza, M. L., M. J. Sadowsky, and L. P. Wackett.** 1996. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. Journal of Bacteriology **178:**4894-4900.

134. **Frick, L., J. P. Mac Neela, and R. Wolfenden.** 1987. Transition state stabilization by deaminases: rates of nonenzymatic hydrolysis of adenosine and cytidine. Bioorganic Chemistry **15:**100-108.

135. **Gabellieri, E., S. Bernini, L. Piras, P. Cioni, E. Balestreri, G. Cercignani, and R. Felicioli.** 1986. Purification, stability and kinetic properties of highly purified adenosine deaminase from *Bacillus cereus* NCIB 8122. Biochimica et Biophysica Acta **884:**490-496.

136. **Daddona, P. E., and W. N. Kelley.** 1977. Human adenosine deaminase. Purification and subunit structure. Journal of Biological Chemistry **252:**110-115.

137. **Ma, P. F., and J. R. Fisher.** 1969. Comparative studies of mammalian adenosine deaminases—some distinctive properties in higher mammals. Comparative biochemistry and physiology **31:**771-781.

138. **Ling, F., Y. Inoue, and A. Kimura.** 1991. Purification and characterization of adenosine deaminase from *Klebsiella* sp. LF 1202. Journal of Fermentation and Bioengineering **71:**89-92.

139. **Sideraki, V., K. A. Mohamedali, D. K. Wilson, Z. Chang, R. E. Kellems, F. A. Quiocho, and F. B. Rudolph.** 1996. Probing the functional role of two conserved active site aspartates in mouse adenosine deaminase. Biochemistry **35:**7862-7872.

140. **Sideraki, V., D. K. Wilson, L. C. Kurz, F. A. Quiocho, and F. B. Rudolph.** 1996. Site-directed mutagenesis of histidine 238 in mouse adenosine deaminase: substitution of histidine 238 does not impede hydroxylate formation. Biochemistry **35:**15019-15028.

141. **Wilson, D. K., and F. A. Quiocho.** 1993. A pre-transition-state mimic of an enzyme: X-ray structure of adenosine deaminase with bound 1-deazaadenosine and zinc-activated water. Biochemistry **32:**1689-1694.

142. **Maier, S. A., J. R. Galellis, and H. E. McDermid.** 2005. Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. Journal of Molecular Evolution **61:**776-794.

143. **Mohamedali, K. A., L. C. Kurz, and F. B. Rudolph.** 1996. Site-directed mutagenesis of active site glutamate-217 in mouse adenosine deaminase. Biochemistry **35:**1672-1680.

144. **Ribard, C., M. Rochet, B. Labedan, B. Daignan-Fornier, P. Alzari, C. Scazzocchio, and N. Oestreicher.** 2003. Sub-families of α/β barrel enzymes: a new adenine deaminase family. Journal of Molecular Biology **334:**1117-1131.

145. **Chang, Z., P. Nygaard, A. C. Chinault, and R. E. Kellems.** 1991. Deduced amino acid sequence of *Escherichia coli* adenosine deaminase reveals evolutionarily conserved amino acid residues: implications for catalytic function. Biochemistry **30:**2273-2280.

146. **Kurz, L. C., L. Moix, M. C. Riley, and C. Frieden.** 1992. The rate of formation of transition-state analogs in the active site of adenosine deaminase is encounter-controlled: implications for the mechanism. Biochemistry **31:**39-48.

147. **Nygaard, P.** 1978. Adenosine deaminase from *Escherichia coli*. Methods in Enzymology **51:**508-512.

148. **Jun, H.-k., T.-S. Kim, and T. Sakai.** 1991. Purification and characterization of extracellular adenosine deaminase from a *Streptomyces* sp. Journal of Fermentation and Bioengineering **71:**6-11.

149. **Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin.** 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic acids research **29:**22-28.

150. **Russo, T. A., S. T. Jodush, J. J. Brown, and J. R. Johnson.** 1996. Identification of two previously unrecognized genes (*guaA* and *argC*) important for uropathogenesis. Molecular Microbiology **22:**217-229.

151. **Wang, J. Y., M. F. Pasetti, F. R. Noriega, R. J. Anderson, S. S. Wasserman, J. E. Galen, M. B. Sztein, and M. M. Levine.** 2001. Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated delta-*guaBA Salmonella enterica* serovar Typhi strain CVD 915. Infection and Immunity **69:**4734-4741

152. **Ivanovics, G., E. Marjai, and A. Dobozy.** 1968. The growth of purine mutants of *Bacillus anthracis* in the body of the mouse. Journal of General Microbiology **53:**147-162.

153. **Jewett, M. W., K. A. Lawrence, A. Bestor, R. Byram, F. Gherardini, and P. A. Rosa.** 2009. GuaA and GuaB are essential for *Borrelia burgdorferi* survival in the tick-mouse infection cycle. Journal of Bacteriology **191:**6231-6241.

154. **Straley, S. C., and P. A. Harmon.** 1984. Growth in mouse peritoneal macrophages of *Yersinia pestis* lacking established virulence determinants. Infection and Immunity **45:**649-654.

155. **Samant, S., H. Lee, M. Ghassemi, J. Chen, J. L. Cook, A. S. Mankin, and A. A. Neyfakh.** 2008. Nucleotide biosynthesis is critical for growth of bacteria in human blood. PLoS Pathogens **4:**e37.

156. **Traut, T. W.** 1994. Physiological concentrations of purines and pyrimidines. Molecular and Cellular Biochemistry **140:**1-22.

Table 1.1 Homologs for *de novo* IMP biosynthesis enzymes among members of the ε-proteobacteria.

	Homolog[a]									
Species	*purF*	*purD*	*purN*	*purL*	*purM*	*purK*	*purE*	*purC*	*purB*	*purH*
Helicobacter pylori	-	+	-	-	-	-	-	-	+	-
Helicobacter achinonychis	-	+	-	-	-	-	-	-	+	-
Helicobacter mustelae	+	+	+	+	+	+	+	+	+	+
Helicobacter hepaticus	+	+	+	+	+	+	+	+	+	+
Campylobacter jejuni	+	+	+	+	+	+	+	+	+	+
Acrobacter butzleri	+	+	+	+	+	+	+	+	+	+
Geobacter sulfurreducens	+	+	+	+	+	+	+	+	+	+
Wolinella succinogenes	+	+	+	+	+	+	+	+	+	+
Sulfurospirillum barnesii	+	+	+	+	+	+	+	+	+	+
Sulfurovum sp. NBC37N1	+	+	+	+	+	+	+	+	+	+

[a] Gene names are based on the reference pathway in *Escherichia coli*. The presence or absence of homologs in these organisms is based upon current annotations in the NCBI GenBank database.

Table 1.2 Catalytically important residues for adenosine deaminase.

Residue (*Mus musclus*)	**Residue (*E. coli*)**	**Role**	**Reference**
His15	His12	Coordinates Zn^{+2} ion	Wilson *et al*. (1991)
His17	His14	Coordinates Zn^{+2} ion	Wilson *et al*. (1991)
His214	His196	Coordinates Zn^{+2} ion	Wilson *et al*. (1991)
Asp295	Asp277	Coordinates Zn^{+2} ion	Wilson *et al*. (1991) Sideraki *et al*. (1996)
Asp296	Asp278	H-bonds with substrate	Wilson *et al*. (1991) Sideraki *et al*. (1996)
His238	His220	Stabilizes hydroxide nucleophile	Wilson & Quiocho (1993)
Asp19	Asp17	H-bonds with substrate Conserved among ADDs[a]	Wilson *et al*. (1991) Ribard *et al*. (2003)
Glu217	Glu199	Involved in catalysis	Wilson *et al*. (1991) Mohamedali *et al*. (1996)
Ala183	Ala168	Active site involvement	Ribard *et al*. (2003)
Gly184	Gly169	H-bonds with N-3 of purine ring	Wilson *et al*. (1991)
Ser103	Ser100	Possible ribose binding	Ribard *et al*. (2003)
Arg101	Arg98	Conserved in ADDs but not ADEs[b]	Maier *et al*. (2005)
Phe10	Phe7	Conserved among ADDs and ADEs	Ribard *et al*. (2003)

[a] ADD = adenosine deaminase
[b] ADE = adenine deaminase

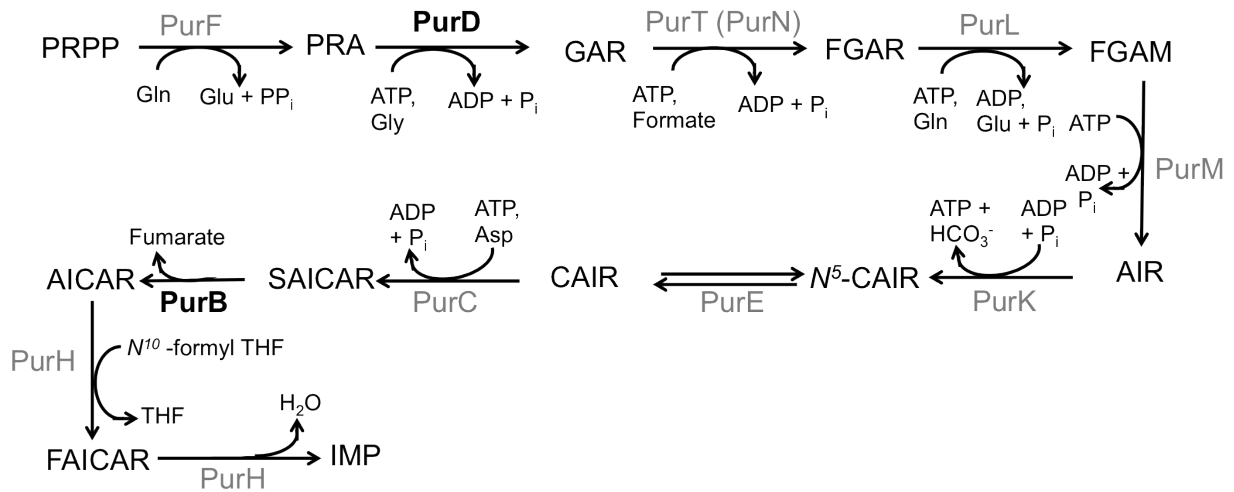


Figure 1.1 Overview of *de novo* IMP biosynthesis. The canonical, highly conserved eleven-step pathway is used to synthesize purines using only amino acid precursors. Enzymes are named in accordance with their designations in *E. coli*. Bolded enzymes indicate homologs are present in one or more *H. pylori* strains.

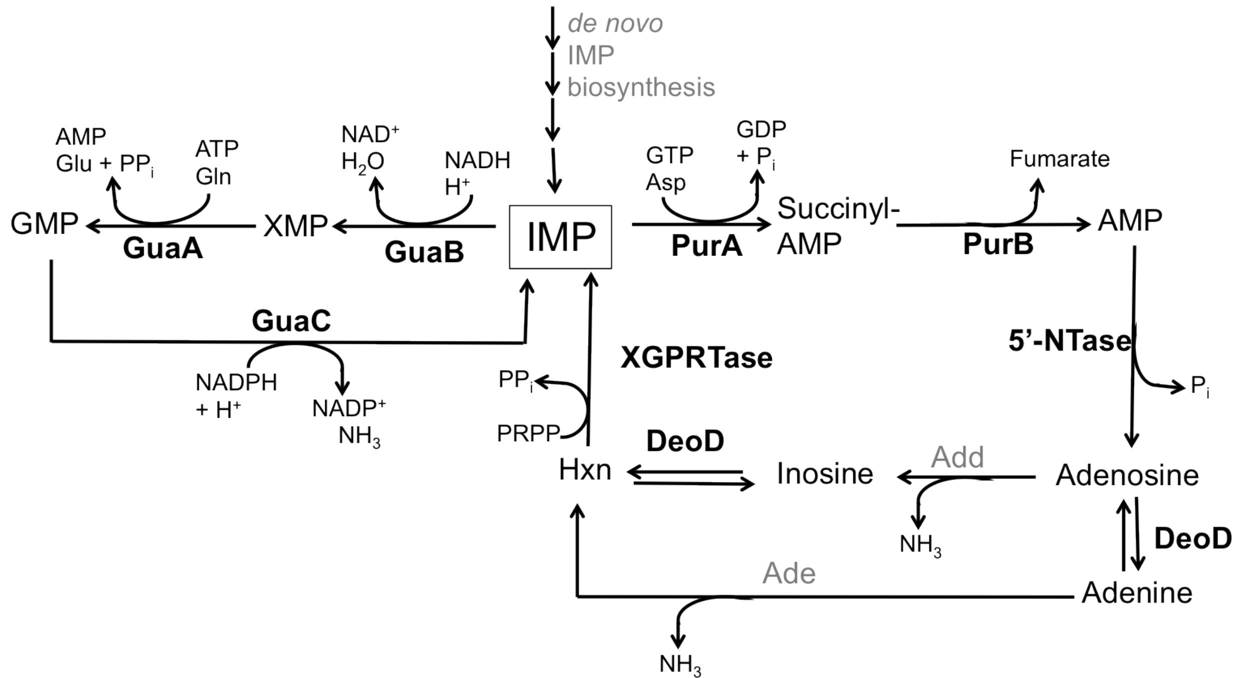


Figure 1.2 GMP and AMP biosynthesis and degradation. Degradation of adenine into hypoxanthine can occur either through Add or Ade. Bolded enzymes indicate that a homolog to this protein exists that is conserved in all *H. pylori* strains. Enzymes are named according to the convention in *E. coli*.

CHAPTER 2

EFFICIENCY OF PURINE UTILIZATION BY *HELICOBACTER PYLORI*: ROLES FOR ADENOSINE DEAMINASE AND A NUPC HOMOLOG[1]

[1] Miller, E. F., S. Vaish, and R. J. Maier. 2012. PloS One 7:e38727. Reprinted here with permission of publisher.

Abstract

The ability to synthesize and salvage purines is crucial for colonization by a variety of human bacterial pathogens. *Helicobacter pylori* colonizes the gastric epithelium of humans, yet its specific purine requirements are poorly understood, and the transport mechanisms underlying purine uptake remain unknown. Using a fully defined synthetic growth medium, we determined that *H. pylori* 26695 possesses a complete salvage pathway that allows for growth on any biological purine nucleobase or nucleoside with the exception of xanthosine. Doubling times in this medium varied between 7 and 14 hours depending on the purine source, with hypoxanthine, inosine and adenosine representing the purines utilized most efficiently for growth. The ability to grow on adenine or adenosine was studied using enzyme assays, revealing deamination of adenosine but not adenine by *H. pylori* 26695 cell lysates. Using mutant analysis we show that a strain lacking the gene encoding a NupC homolog (HP1180) was growth-retarded in a defined medium supplemented with certain purines. This strain was attenuated for uptake of radiolabeled adenosine, guanosine, and inosine, showing a role for this transporter in uptake of purine nucleosides. Deletion of the GMP biosynthesis gene *guaA* had no discernible effect on mouse stomach colonization, in contrast to findings in numerous bacterial pathogens. In this study we define a more comprehensive model for purine acquisition and salvage in *H. pylori* that includes purine uptake by a NupC homolog and catabolism of adenosine via adenosine deaminase.

Introduction

The bacterial pathogen *Helicobacter pylori* is known for its ability to colonize and persist in the human stomach, a niche that is largely uninhabited by other bacteria. Infection by *H. pylori* greatly increases the risk of duodenal and gastric ulcers, gastric cancers, and MALT lymphoma (1). As it infects between 20%-80% of the adult population worldwide, *H. pylori* is regarded as one of the most successful human pathogens (1). One reason for this success is that it has evolved for millennia in close association with humans (2) and is well-adapted to acquire nutrients, including purines, from the host gastric epithelium and from the mucus environment (3, 4). Purines are critical for cellular growth and replication, therefore it is important to examine the mechanisms by which *H. pylori* acquires and salvages purines, and to determine the roles of these pathways in host colonization.

It was recently shown that, in contrast to previous reports, purines are absolutely required for growth of *H. pylori* 26695 (Abstract no. 2273, Miller, E. F. & Maier, R. J., Annual Meeting of the American Society for Microbiology, May 2011), a result confirmed for three other strains of *H. pylori* (5). These observations corroborate predictions made from the RAST-annotated *H. pylori* genomes, which all lack the pathway for *de novo* IMP synthesis (6). *H. pylori* therefore relies on a purine salvage pathway (Figure 1), which has been partially characterized already (5, 7, 8). Although several different strains were used for these prior studies, the gene homologs for purine salvage are well-conserved among the sequenced strains of *H. pylori,* making it likely that purine utilization is similar across strains (6).

Here we study the purine requirements of *H. pylori* strain 26695, for which, if a complete salvage pathway is present, any biological purine base or nucleoside would suffice to support growth. It was recently shown that all four biological purine bases (adenine, guanine,

hypoxanthine, or xanthine), as well as two nucleosides (adenosine and guanosine) can individually serve as the sole purine source for *H. pylori* strain G27 (5). Nevertheless, purine requirements for *H. pylori* have not been studied in a completely defined medium, and it is also unclear which purines *H. pylori* uses most efficiently. Historically, it has been challenging to study specific nutrient requirements in this organism because most minimal growth media include undefined biological supplements such as bovine serum albumin (BSA) or foetal bovine serum (FBS) (7, 9, 10). However, with the recent optimization of a chemically defined growth medium for *H. pylori* (11, 12), the potential for contaminating purines is eliminated and we can ask not only which purines are sufficient for growth, but which are used most efficiently.

If *H. pylori* can utilize any biological purine source including adenine or adenosine, then it is surprising that *H. pylori* lacks homologs to enzymes that can deaminate adenine or adenosine. *H. pylori* cell extracts can convert radiolabeled adenine into hypoxanthine, suggesting that adenine deaminase is the key enzyme responsible for catabolizing AMP to IMP (13). However, subsequent studies showed that a Δ*deoD* mutant was unable to grow on adenine as a sole purine source, implying instead that adenosine deaminase is the responsible enzyme (5).

Another gap in our knowledge of *H. pylori* purine salvage is the mechanism by which purines are transported into the cell. The *H. pylori* protein HP1180 is homologous to the bacterial concentrative nucleoside transporter (CNT) NupC, a H^+/nucleoside symporter. The best-studied bacterial NupC proteins (from *Escherichia coli* and *Bacillus subtilis*) transport pyrimidine rather than purine nucleosides (14-16). In *H. pylori* nothing is known about the substrate specificity of HP1180, however its corresponding gene is co-transcribed with phosphopentomutase (*hp1179*), and purine nucleoside phosphorylase (*hp1178*) both of which are

involved in purine metabolism. We therefore predicted that this *H. pylori* NupC homolog might play a role in purine uptake.

The overall aim of this study was to create a more precise understanding of how *H. pylori* 26695 transports and metabolizes purines, the efficiency to which each biological purine is used, and the importance of purine conversions in host colonization.

Results

Growth of *H. pylori* in a synthetic medium supplemented with various purine sources. *H. pylori* 26695 was grown in a modified version of Ham's F12 (designated EMF12, Table 2.4). This medium does not represent the minimum requirements for *H. pylori* growth, which are known (12), but instead contains a wide array of vitamins and amino acids in order to reduce the number of limiting components. In addition, the salt and iron concentrations were modified as described (12).

When grown in EMF12 supplemented with hypoxanthine, *H. pylori* 26695 reached an average final OD_{600} of 0.13 ± 0.02 (approx. 8.1 x 10^7 cfu/ml), which corresponded to three generations of growth (Figure 2). *H. pylori* grown in F12 generally achieves a maximum growth yield of between 10^7 – 10^8 cfu/ml (12). In the absence of a purine source the optical density of the cell culture decreased, confirming reports that *H. pylori* requires a purine source for growth (5). Serial dilutions were plated at 0 and 18 hours post-inoculation to verify that the number of cells in the presence of hypoxanthine increased during log phase of growth (data not shown). To further assess whether purine auxotrophy is strain-specific, we grew two other wild-type strains, 43504 and X47, under these same conditions and likewise observed growth that was dependent on the presence of hypoxanthine (data not shown).

To examine the ability of *H. pylori* 26695 to utilize a variety of purine sources, we grew cells in EMF12 supplemented individually with each of the following purines: hypoxanthine, adenine, guanine, xanthine, and the four corresponding nucleosides inosine, adenosine, guanosine, and xanthosine. With the exception of xanthosine, all purines tested were capable of supporting growth. The doubling time (T_d) varied among the purine sources (Table 2.1), and were longest (greater than 12 h) in media supplemented with guanine, xanthine, or guanosine. Inoculum size affected growth in the presence of certain purines. Hypoxanthine, adenine, inosine and adenosine supported growth at a starting OD_{600} of 0.01 (10^6 cfu/ml), while growth on guanine, xanthine, or guanosine required a minimum starting OD_{600} of 0.025 (2.6×10^6 cfu/). Xanthosine as a sole purine source did not support exponential growth; despite observing a slight increase in OD_{600} during the first twelve hours after inoculation, viable cells were undetectable after 12 hours. Attempts to enhance growth by increasing the concentration of xanthosine to 1 mM or by increasing the initial inoculum to 5×10^6 cfu/ml were of no added benefit for supporting growth (data not shown).

Growth of *H. pylori* 26695 *gua* and *pur* mutants in a minimal medium. In most organisms, GMP is synthesized from IMP by the enzymes IMP dehydrogenase (GuaB) and GMP synthetase (GuaA), while adenylosuccinate synthetase (PurA) and adenylosuccinate lyase (PurB) catalyze the formation of AMP from IMP (Figure 1). The conversion of GMP back to IMP is carried out in one step by GMP dehydrogenase (GuaC). *H. pylori* possesses homologs for *purA*, *purB*, *guaA*, *guaB*, and *guaC* (17, 18). Recent reports using *H. pylori* G27 showed that the *gua* and *pur* genes perform similar roles in purine salvage as do their homologs in other bacteria (5). In order to confirm these results in *H. pylori* 26695, we constructed gene deletions in *guaA*, *guaB*, *guaC*,

purA and *purB* (Table 2.5, Materials and Methods). To study the phenotypes of each mutant, strains were grown for 20 h in the chemically defined medium EMF12 supplemented with one of seven different purine sources (Figure 3). As predicted based on the canonical purine conversion pathway outlined in Figure 1, strain EM202k (*guaA*) required guanine or guanosine for significant growth (see Figure 3 legend). EM203k (*guaB*) was able to utilize xanthine in addition to guanine and guanosine as a sole purine source. EM204 (*guaC*) lacked the ability to degrade GMP back into IMP and therefore grew in the presence of all purines except guanine, guanosine, and xanthine. Similarly, strains EM205 (*purA*) and EM206 (*purB*) grew only in the presence of exogenous adenine or adenosine. These results support the conclusion that the *gua* and *pur* genes are responsible for inter-conversion between GMP and AMP in *H. pylori*.

***H. pylori* X47 Δ*guaA* is proficient for mouse colonization.** Several bacterial pathogens rely on purine biosynthesis and salvage genes for full virulence, in particular the *gua* genes (19-21), and for this reason a strain lacking *guaA* was chosen to assess whether the same is true for *H. pylori*. Strain EMX02k (Δ*guaA*) was engineered using the mouse-adapted parent strain *H. pylori* X47 (22). Prelimary growth studies were carried out in brain-heart infusion (BHI), which, compared to EMF12, more closely resembles conditions encountered in the host. The growth rate of EMX02k in BHI was slower ($T_d = 12.9 \pm 0.5$ h) than the wild-type ($T_d = 5.5 \pm 0.2$ h) ($P < 0.01$, student's t-test). The growth rate of EMX02k was restored ($T_d = 4.8 \pm 0.3$ h) upon addition of 1 mM guanosine to the medium.

Mice were inoculated with *H. pylori* X47 or EMX02k via oral gavage, and infection was allowed to persist for three weeks before sacrifice and enumeration of viable *H. pylori* from the homogenized stomachs. Strain EMX02k was unattenuated for colonization as compared to wild-

type ($p = 0.45$, Wilcoxon rank-sum test, $n = 8$ mice per condition, H_o: no difference in colonization between parent and mutant strain), indicating that the pathway for GMP biosynthesis is not important for colonization by *H. pylori*.

Adenosine deaminase activity enables growth of *H. pylori* 26695 with adenine or adenosine as the sole purine source. To better understand how *H. pylori* metabolizes adenine and adenosine to satisfy its purine requirements, we measured ammonium production by cell lysates in the presence of either adenine or adenosine. While no ammonium was generated upon incubating cell extracts (8 mg protein/ml) with adenine, incubation with adenosine caused ammonium to increase linearly over a period of 40 minutes. Reactions containing heat-killed cell lysates in the presence of adenosine produced no increase in ammonium over time, confirming that adenosine-dependent ammonium production was indeed enzymatic. The observed specific activity of adenosine deaminase in *H. pylori* cell lysates was 0.074 (± 0.03) μmoles NH_4^+ min^{-1} mg^{-1} at pH 8.6, as determined from the initial rate of ammonium production over time from three independent experiments.

We further sought to test whether the adenosine deaminase activity can vary depending upon the presence of adenosine in the defined growth medium. There was no significant difference in adenosine deaminase activity between cells grown in either adenosine or hypoxanthine (data not shown), indicating that under these conditions *H. pylori* does not regulate the production and/or activity of this enzyme in response to changes in adenosine availability.

***H. pylori* HP1180 aids in uptake of purine nucleosides.** HP1180 from *H. pylori* 26695 is a member of the CNT family of nucleoside transporters, and is present in all sequenced *H. pylori*

strains. An amino acid sequence alignment compares HP1180 against *E. coli* NupC (Figure 4). The latter protein transports pyrimidine nucleosides and adenosine, but does not transport guanosine or nucleobases, and transports inosine inefficiently (16). Two other *E. coli* CNT transporters of unknown substrate specificity YeiJ (NupX) and YeiM and are included in the alignment.

HP1180 possesses conserved motifs (outlined in black boxes) found in both prokaryotic and eukaryotic CNT transporters, which supports its annotation as a NupC homolog. However, HP1180 is actually more similar to the *E. coli* NupC paralogs YeiJ (44% identical, 65% similar) and YeiM (45% identical, 64% similar) than it is to NupC (28% identical, 52% similar). Furthermore, we suspected that the co-expression of *hp1180* with purine-related genes point to a role for this NupC homolog in purine uptake. We measured growth rates of a *nupC* deletion mutant (EM207) in a defined medium containing individual biological purine nucleobases and/or nucleosides. Strain EM207 exhibited a significantly longer doubling time compared to wild-type in all purine supplements except adenine or adenosine (Table 2.2). No growth was observed in media supplemented with guanine, xanthine, or guanosine. These results indicate a role for this NupC homolog in purine uptake.

We then directly measured purine nucleoside uptake using radiolabeled substrates. Transport of [^{14}C]-adenosine, [^{3}H]-inosine and [^{3}H]-guanosine was slower for strain EM207 compared to wild-type (Table 2.3). Furthermore, nucleoside uptake by EM207 remained the same between the 5- and 20-minute time points ($P > 0.25$) in contrast to the wild-type, which after 20 minutes had taken up significantly higher levels of nucleoside. These results support a role for this NupC homolog in purine uptake, and suggest that HP1180 may be a non-redundant transporter of purines in *H. pylori*.

Discussion

Similar to other pathogens that have evolved in close association with their hosts (23-25), *H. pylori* does not have the ability to synthesize purines *de novo* (5), a conclusion that our study confirmed for *H. pylori* 26695 using a fully defined medium that obviates the need for biological supplements typically added at high concentrations (5% BSA and/or 10% FBS, for example). We showed all individual biological purines except xanthosine allow for growth, but certain nucleosides and nucleobases support faster growth.

Guanosine supported the slowest growth among the purines tested, while xanthosine failed to support growth at all. Similarly, a strain of *E. coli* that cannot synthesize IMP *de novo* was severely attenuated for growth in media containing xanthosine as a sole purine source (26). It is likely that the inability of *H. pylori* to grow using xanthosine is attributed to either a deficiency for transport, or a rate-limiting step in xanthosine utilization that cannot be overcome.

In this study we confirmed recent reports identifying the genetic basis for synthesis of GMP and AMP from the common intermediate IMP (5). Deletions in *guaA, guaB, guaC, purA* and *purB* resulted in growth phenotypes that would be expected based on the predicted functional roles for these genes. Surprisingly, certain mutant strains (for example Δ*purA*) achieved a higher growth yield than wild-type in the presence of certain purines. It is possible that certain gene deletions impart a growth advantage for the organism, as was shown for a *Lactobacillus lactis* purine auxotroph supplemented with inosine (27). Alternatively, a compensatory mutation may have occurred that enhanced the ability of this strain to utilize one purine over another. Taken together these data show that the genetic basis for conversion between GMP and AMP in *H. pylori* 26695 is likely identical to the conserved pathway used by most organisms.

We know that *H. pylori* can use adenine or adenosine to satisfy its purine requirements, and that this phenotype relies upon deamination of the adenine moiety into a hypoxanthine moiety. Some prokaryotes (e.g. members of the phylum Firmicutes) deaminate adenine directly via adenine deaminase (28), while other bacteria possess adenosine deaminase. A third strategy is to convert AMP into IMP by exploiting the histidine biosynthetic pathway (29), however *H. pylori* lacks the necessary genes for histidine biosynthesis and thus a histidine-purine connection does not explain its ability to use adenine.

In contrast to previous reports of adenine deaminase activity in *H. pylori*, (7), we detected hydrolysis of adenosine but not adenine by *H. pylori* cell extracts. A recent study showed that the gene encoding purine nucleoside phosphorylase (*deoD*) is required for adenine utilization in *H. pylori* (5). Other bacteria that rely on adenosine deaminase to metabolize adenine also require by necessity a nucleoside phosphorylase (see Figure 1) (30, 31), thus this phenotype is congruent with the presence of an *H. pylori* adenosine deaminase. Although no homologs for adenosine deaminase exist in *H. pylori*, it is possible that one of several aminohydrolases—an enzyme family that is known to rapidly evolve to accommodate novel substrates (32, 33)—may serve as adenosine deaminase in this organism.

H. pylori was shown previously to take up adenosine and guanosine, as well as the nucleobases adenine, guanine and hypoxanthine (13). Our study is the first to examine a mechanism for purine uptake by showing that a *nupC* mutant is deficient for transport of radiolabeled inosine, guanosine or adenosine. Although previously studied bacterial NupC transporters are pyrimidine-selective (15, 16), these proteins represent one of two broad phylogenetic clusters for bacterial CNTs: the second distinct cluster contains *E. coli* YeiJ, YeiM, as well as HP1180 (34). Furthermore, certain eukaryotic CNT transporters can switch from

being pyrimidine-selective to purine-selective due to a single amino acid substitution (35), highlighting the potential for CNT transporters to evolve altered substrate preference.

To our surprise, the Δ*nupC* strain was growth-attenuated in the presence of several nucleobases, indicating a role for HP1180 in uptake of nucleobases. *E. coli* NupC does not transport nucleobases (16), and therefore further studies are needed to resolve this association between this *H. pylori* NupC homolog and nucleobase utilization.

Many known pathogens require either *guaA/guaB* or *purA/purB* for full virulence (19-21, 36-38). Surprisingly, our results suggest that *guaA* has no effect on *H. pylori* colonization. It is possible that guanosine and guanine are therefore not limiting for this pathogen *in vivo*. Guanosine is the least abundant purine nucleoside in human serum (39, 40), however to our knowledge no studies have measured purine concentrations in gastric mucus. It is noteworthy that a certain proportion of *H. pylori* cells attach to gastric epithelial cells during infection and can thus access nutrients from within host cells (4). Because purine concentrations are higher for intracellular than for extracellular fluids (41, 42), these epithelium-associated bacteria may experience purine concentrations different from that of bacteria inhabiting the mucus. Overall, these colonization data suggest that guanine/guanosine are not limiting for *H. pylori* growth *in vivo*. We now have a better understanding of *H. pylori*'s ability to transport and salvage purines from the environment, and the importance of purine salvage for virulence. It would be relevant in the future to identify the genetic basis for adenosine deaminase, as well as to characterize the *H. pylori* NupC homolog for its precise transport function.

Materials and Methods

Strains and growth conditions. *Helicobacter pylori* strain ATCC 26695 was used as the parental strain for physiology experiments. *H. pylori* strain X47 was the parental strain for *in vivo* colonization studies. *H. pylori* were routinely grown on *Brucella* Agar (Oxoid Ltd., Hampshire, England) supplemented with 10% defibrinated sheep blood (QuadFive, Ryegate, MT) (BA plates), and incubated in 37°C incubator with gas concentrations maintained at 5% CO_2, 2% O_2 and balanced N_2. Plates were supplemented with 25 µg/ml kanamycin or 30 µg/ml chloramphenicol as required. Liquid cultures were grown in glass bottles with rubber or silicone stoppers to minimize gas exchange. Cultures were grown at 37°C and were aerated by shaking at 200 rpm. For growth in a rich medium, Brain-Heart Infusion (Becton, Dickinson and Co., Sparks, MD, pH 7.4) supplemented with 0.4% β-cyclodextrin (BHI) was used, and the initial gas concentrations (total pressure of 101 kPa) were: O_2 (7%), CO_2 (5%), H_2 (10%) and N_2 (78%). EMF12 (pH 7.0) was used for studies of purine requirements (see below for preparation). Initial gas concentrations for cells grown in EMF12 were: O_2 (20%), CO_2 (10%), H_2 (10%), and N_2 (60%).

Preparation of EMF12 defined medium. The backbone recipe for EMF12 was previously described by Ham (1965), and detailed instructions for its preparation can be found therein (43). Modifications to this original medium were based on previously described optimization (12), and are specified in Table 2.4. All components were dissolved in double-distilled H_2O. An exception to the original protocol outlined by Ham *et al.* (1965) was the final pH adjustment, which was performed after the addition of $NaHCO_3$ rather than prior to. The purine source in

EMF12 varied according to the experiment, and was at a concentration of 0.06 mM unless otherwise noted.

Construction of *H. pylori* deletion mutants. Overlapping PCR and allele-exchange mutagenesis was used to generate deletion mutants. *H. pylori* 26695 genomic DNA was used as a template to amplify an approximately 400 bp DNA fragment both upstream and downstream of the target locus (Table 2.6). Primers designated 1 and 2 (for example, guaA1 and guaA2) were used to amplify the region upstream of the target locus, while primers designated 3 and 4 (for example guaA3 and guaA4) were used to amplify the region downstream of the target locus. The *aphA3* gene (encoding for kanamycin resistance) or the *cat* cassette (encoding for chloramphenicol resistance) was amplified using primers Aph5 & Aph6 or cat5 & cat6. Both antibiotic resistance cassettes contain an upstream promoter and lack a transcription termination sequences in order to avoid polar effects on downstream genes. Primers 2 and 3 contain 5' end regions that anneal to either end of *aphA3* or *cat*, depending upon which antibiotic marker is to be used in allelic exchange. Final overlapping PCR reactions resulted in a sandwich fusion in which the antibiotic resistance cassette is flanked by upstream and downstream regions surrounding the gene locus. Following excision and purification from an agarose gel, this PCR product was introduced into *H. pylori* by natural transformation. Mutants were then selected on BA plates containing kanamycin or chloramphenicol as appropriate. Successful disruption of the target allele was confirmed by PCR/gel electrophoresis and by direct sequencing of the PCR fragment (Georgia Genomics Facility).

Adenosine deaminase assay. The deamination of adenosine and/or adenine by *H. pylori* cell-free extract was monitored by measuring the increase in ammonium over time in the presence of either adenosine or adenine. The reaction was initiated by the addition of 10 µg cell-free extract to a reaction buffer containing 40 mM HEPES, 100 mM NaCl, 0.27 mM KCl, and 10 mM adenosine or adenine (pH 8.6) at a final volume of 250 µl. The concentration of NH_4^+ was measured at each time point using the phenol-hypochlorite method (44), which monitors the absorbance at 625nm as compared to a standard curve of known ammonium concentrations.

Nucleoside uptake assay. Radiolabeled [8-^{14}C]-adenosine, [8-^{3}H]-guanosine, and [2,8-^{3}H]-inosine (Moravek Biochemicals Inc., Brea, CA), were used in uptake assays. *H. pylori* 26695 cells were grown in BHI to an OD_{600} of 0.2. Radiolabeled nucleosides were injected into the bottles at a final concentration of 0.5 µCi/ml (for tritium-labeled nucleosides) or 0.2 µCi/ml (for ^{14}C-labeled adenosine) along with 20 µM unlabeled nucleoside. Bottles were shaken at 37°C, and nucleoside uptake was measured at 5 min and 20 min using a previously described method (45). BHI containing radiolabeled nucleosides but lacking cells was filtered and subtracted from all experimental cpm values to account for substrate adhering to the filter.

Mouse Colonization assay. *H. pylori* X47 or the isogenic mutant strain EMX02k were grown for 24 hours on BA plates. 5-6 week-old female C57BL/6NCr mice (NCI, Frederick, MD) were infected via oral gavage with 0.2 ml of twice-washed bacterial cells suspended in PBS (5 x 10^7 *H. pylori* cells/mouse). Mice were sacrificed by CO_2 asphyxiation and cervical dislocation three weeks after inoculation, and stomachs were removed, weighed, and homogenized in PBS. Samples of 100 µl from serial dilutions of the stomach homogenate were spread onto BA plates

containing amphotericin B (10 μg/ml), vancomycin (10 μg/ml), and bacitracin (100 μg/ml). After incubation at 37°C and 2% oxygen for 5-7 days, *H. pylori* colonies were enumerated and colonization was expressed as cfu per gram stomach tissue.

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References

1. **Makola, D., D. A. Peura, and S. E. Crowe.** 2007. *Helicobacter pylori* infection and related gastrointestinal diseases. Journal of Clinical Gastroenterology **41:**548-558.

2. **Linz, B., F. Balloux, Y. Moodley, A. Manica, H. Liu, P. Roumagnac, D. Falush, C. Stamer, F. Prugnolle, and S. W. Van Der Merwe.** 2007. An African origin for the intimate association between humans and *Helicobacter pylori*. Nature **445:**915-918.

3. **Schreiber, S., M. Konradt, C. Groll, P. Scheid, G. Hanauer, H. O. Werling, C. Josenhans, and S. Suerbaum.** 2004. The spatial orientation of *Helicobacter pylori* in the gastric mucus. Proceedings of the National Academy of Sciences **101:**5024-5029.

4. **Tan, S., L. S. Tompkins, and M. R. Amieva.** 2009. *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. PLoS Pathogens **5:**e1000407.

5. **Liechti, G., and J. B. Goldberg.** 2012. *Helicobacter pylor*i relies primarily on the purine salvage pathway for purine nucleotide biosynthesis. Journal of Bacteriology **194:**839-854.

6. **Overbeek, R., T. Begley, R. M. Butler, J. V. Choudhuri, H. Y. Chuang, M. Cohoon, V. de Crécy-Lagard, N. Diaz, T. Disz, and R. Edwards.** 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Research **33:**5691-5702.

7. **Mendz, G., B. Jimenez, S. Hazell, A. Gero, and W. O'Sullivan.** 1994. Salvage synthesis of purine nucleotides by *Helicobacter pylori*. Journal of Applied Microbiology **77:**674-681.

8. **Duckworth, M., A. Ménard, F. Megraud, and G. L. Mendz.** 2006. Bioinformatic analysis of *Helicobacter pylori* XGPRTase: a potential therapeutic target. Helicobacter **11:**287-295.

9. **Nedenskov, P.** 1994. Nutritional requirements for growth of *Helicobacter pylori*. Applied and Environmental Microbiology **60:**3450-3453.

10. **Reynolds, D. J., and C. W. Penn.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140:**2649-2656

11. **Testerman, T. L., D. J. McGee, and H. L. T. Mobley.** 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. Journal of Clinical Microbiology **39:**3842-3850.

12. **Testerman, T. L., P. B. Conn, H. L. T. Mobley, and D. J. McGee.** 2006. Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. Journal of Clinical Microbiology **44:**1650-1658

13. **Mendz, G. L., A. J. Shepley, S. L. Hazell, and M. A. Smith.** 1997. Purine metabolism and the microaerophily of *Helicobacter pylori*. Archives of Microbiology **168:**448-456.

14. **Patching, S. G., S. A. Baldwin, A. D. Baldwin, J. D. Young, M. P. Gallagher, P. J. F. Henderson, and R. B. Herbert.** 2005. The nucleoside transport proteins, NupC and NupG, from *Escherichia coli*: specific structural motifs necessary for the binding of ligands. Org. Biomol. Chem. **3:**462-470.

15. **Saxild, H. H., L. N. Andersen, and K. Hammer.** 1996. *dra-nupC-pdp* operon of *Bacillus subtilis*: nucleotide sequence, induction by deoxyribonucleosides, and transcriptional regulation by the deoR-encoded DeoR repressor protein. Journal of Bacteriology **178:**424-434

16. **Loewen, S. K., S. Y. M. Yao, M. D. Slugoski, N. N. Mohabir, R. J. Turner, J. R. Mackey, J. H. Weiner, M. P. Gallagher, P. J. F. Henderson, and S. A. Baldwin.** 2004. Transport of physiological nucleosides and anti-viral and anti-neoplastic nucleoside drugs by recombinant *Escherichia coli* nucleoside-H+ cotransporter (NupC) produced in *Xenopus laevis* oocytes. Molecular Membrane Biology **21:**1-10.

17. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, and B. A. Dougherty.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

18. **Baltrus, D. A., M. R. Amieva, A. Covacci, T. M. Lowe, D. S. Merrell, K. M. Ottemann, M. Stein, N. R. Salama, and K. Guillemin.** 2009. The complete genome sequence of *Helicobacter pylori* strain G27. Journal of Bacteriology **191:**447-448.

19. **Jewett, M. W., K. A. Lawrence, A. Bestor, R. Byram, F. Gherardini, and P. A. Rosa.** 2009. GuaA and GuaB are essential for *Borrelia burgdorferi* survival in the tick-mouse infection cycle. Journal of Bacteriology **191:**6231-6241.

20. **Russo, T. A., S. T. Jodush, J. J. Brown, and J. R. Johnson.** 1996. Identification of two previously unrecognized genes (*guaA* and *argC*) important for uropathogenesis. Molecular Microbiology **22:**217-229.

21. **Wang, J. Y., M. F. Pasetti, F. R. Noriega, R. J. Anderson, S. S. Wasserman, J. E. Galen, M. B. Sztein, and M. M. Levine.** 2001. Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated delta-*guaBA Salmonella enterica* serovar Typhi strain CVD 915. Infection and Immunity **69:**4734-4741

22. **Kleanthous, H., T. Tibbitts, T. Bakios, K. Georgokopoulos, G. Myers, T. Ermak, J. Fox, and T. Monath.** 1995. In vivo selection of a highly adapted *H. pylori* isolate and the development of an *H. pylori* mouse model for studying vaccine efficacy and attenuating lesions. Gut **37:**A94.

23. **Pettersson, J., M. E. Schrumpf, S. J. Raffel, S. F. Porcella, C. Guyard, K. Lawrence, F. C. Gherardini, and T. G. Schwan.** 2007. Purine salvage pathways among *Borreli*a species. Infection and Immunity **75:**3877-3884.

24. **McClarty, G., and B. Qin.** 1993. Pyrimidine metabolism by intracellular *Chlamydia psittaci*. Journal of Bacteriology **175:**4652-4661.

25. **Hammond, D. J., and W. E. Gutteridge.** 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Molecular and Biochemical Parasitology **13:**243-261.

26. **Zalkin, H., and P. Nygaard.** 1996. Biosynthesis of Purine Nucleotides *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd ed. ASM Press, Washington, DC.

27. **Martinussen, J., S. L. L. Wadskov-Hansen, and K. Hammer.** 2003. Two nucleoside uptake systems in *Lactococcus lactis:* competition between purine nucleosides and cytidine allows for modulation of intracellular nucleotide pools. Journal of Bacteriology **185:**1503-1508.

28. **Nygaard, P., P. Duckert, and H. H. Saxild.** 1996. Role of adenine deaminase in purine salvage and nitrogen metabolism and characterization of the ade gene in *Bacillus subtilis*. Journal of Bacteriology **178:**846-853.

29. **White, R. H.** 1997. Occurrence and biosynthesis of 5-aminoimidazole-4-carboxamide ribonucleotide and N-(beta-D-ribofuranosyl) formamide 5'-phosphate in *Methanobacterium thermoautotrophicum* delta (H). Journal of Bacteriology **179:**563-566.

30. **Hoffmeyer, J., and J. Neuhard.** 1971. Metabolism of exogenous purine bases and nucleosides by *Salmonella typhimurium*. Journal of Bacteriology **106:**14-24

31. **Kilstrup, M., K. Hammer, P. Ruhdal Jensen, and J. Martinussen.** 2005. Nucleotide metabolism and its control in lactic acid bacteria. FEMS Microbiology Reviews **29:**555-590.

32. **Seffernick, J. L., and L. P. Wackett.** 2001. Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. Biochemistry **40:**12747-12753.

33. **Roodveldt, C., and D. S. Tawfik.** 2005. Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. Biochemistry **44:**12728-12736.

34. **Ritzel, M. W. L., A. M. L. Ng, S. Y. M. Yao, K. Graham, S. K. Loewen, K. M. Smith, R. G. Ritzel, D. A. Mowles, P. Carpenter, and X. Z. Chen.** 2001. Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system *cib*). Journal of Biological Chemistry **276:**2914-2927.

35. **Loewen, S. K., A. M. L. Ng, S. Y. M. Yao, C. E. Cass, S. A. Baldwin, and J. D. Young.** 1999. Identification of amino acid residues responsible for the pyrimidine and purine nucleoside specificities of human concentrative Na+ nucleoside cotransporters hCNT1 and hCNT2. Journal of Biological Chemistry **274:**24475-24484.

36. **Chiang, S. L., and J. J. Mekalanos.** 1998. Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. Molecular Microbiology **27:**797-805.

37. **Straley, S. C., and P. A. Harmon.** 1984. Growth in mouse peritoneal macrophages of *Yersinia pestis* lacking established virulence determinants. Infection and Immunity **45:**649-654.

38. **Ivanovics, G., E. Marjai, and A. Dobozy.** 1968. The growth of purine mutants of *Bacillus anthracis* in the body of the mouse. Journal of General Microbiology **53:**147-162.

39. **Colonna, A., T. Russo, F. Esposito, F. Salvatore, and F. Cimino.** 1983. Determination of pseudouridine and other nucleosides in human blood serum by high-performance liquid chromatography. Analytical Biochemistry **130:**19-26.

40. **Hartwick, R. A., A. M. Krstulovic, and P. R. Brown.** 1979. Identification and quantitation of nucleosides, bases and other UV-absorbing compounds in serum, using reversed-phase high-performance liquid chromatography: II. Evaluation of human sera. Journal of Chromatography A **186:**659-676.

41. **Traut, T. W.** 1994. Physiological concentrations of purines and pyrimidines. Molecular and Cellular Biochemistry **140:**1-22.

42. **Samant, S., H. Lee, M. Ghassemi, J. Chen, J. L. Cook, A. S. Mankin, and A. A. Neyfakh.** 2008. Nucleotide biosynthesis is critical for growth of bacteria in human blood. PLoS Pathogens **4:**e37.

43. **Ham, R. G.** 1965. Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proceedings of the National Academy of Sciences **53:**288–293.

44. **Weatherburn, M.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry **39:**971-974.

45. **Mehta, N. S., S. Benoit, J. V. Mysore, R. S. Sousa, and R. J. Maier.** 2005. *Helicobacter hepaticus* hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and in causing liver lesions in A/J mice. Infection and Immunity **73:**5311-5318.

46. **Krogh, A., B. È. Larsson, G. Von Heijne, and E. L. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. Journal of Molecular Biology **305:**567-580.

Tables

Table 2.1 Growth rates and end-point yields of *H. pylori* grown in EMF12 medium with various purine sources.

Purine source	Initial OD_{600}	Final OD_{600} [a]	T_d (h) [a]	Generations achieved
hypoxanthine	0.02	0.129 ± 0.01	7.3 ± 1.6	2.6
adenine	0.02	0.128 ± 0.02	9.8 ± 1.0	2.6
guanine	0.025	0.106 ± 0.02	12.8 ± 2.8	2.0
xanthine	0.025	0.104 ± 0.04	12.4 ± 2.8	2.0
inosine	0.02	0.132 ± 0.02	7.6 ± 2.2	2.7
adenosine	0.02	0.129 ± 0.02	7.6 ± 1.6	2.6
guanosine	0.025	0.108 ± 0.02	14.1 ± 3.8	2.1
xanthosine	0.025	0.039 ± 0.01	NG[b]	<1

[a] Doubling times were calculated using at least five data points taken during exponential growth. Values are the mean ± SD of three or more independent experiments. Doubling times in guanine, xanthine and guanosine were significantly longer than for hypoxanthine, inosine, or adenosine (student's t-test, $P < 0.05$). Final OD_{600} values were not significantly different from one another.
[b] NG = No growth observed after 36 hours

Table 2.2 Growth of EM207 (Δ*nupC*) in EMF12 supplemented with a single purine source.

Purine source	**T_d (h)** [a]	
	26695 (wild-type)	EM207 (Δ*nupC*)
hypoxanthine	7.3 ± 1.6	13.6 ± 2.3 *
adenine	9.8 ± 1.0	9.7 ± 0.1
guanine	12.8 ± 2.8	NG
xanthine	12.4 ± 2.8	NG
inosine	7.6 ± 2.2	14.3 ± 3.3 *
adenosine	7.6 ± 1.6	9.1 ± 1.0
guanosine	14.1 ± 3.8	NG
xanthosine	NG [b]	NG

[a] The initial OD_{600} was standardized to 0.025. Doubling times were calculated using at least four data points taken during exponential growth. Results are the mean ± SD of three independent growth cultures from two independent experiments.
* Significantly longer doubling times compared to wild-type (student's t-test $P < 0.05$).
[b] NG = No growth observed after 36 hours

Table 2.3 Comparison of radiolabeled nucleoside uptake by *H. pylori* 26695 versus EM207 (Δ*nupC*).

	[^{14}C] adenosine uptake (cpm/10^8 cells) [a]	
	5 min	20 min
26695 (wild-type)	137 ± 23	343 ± 42 $^{\Psi}$
EM207 (Δ*nupC*)	18 ± 11 $^{\phi}$	45 ± 9 *
	[^{3}H] inosine uptake (cpm/10^8 cells)	
26695 (wild-type)	80 ± 16	210 ± 11 $^{\Psi}$
EM207 (Δ*nupC*)	20 ± 6 $^{\phi}$	25 ± 7 *
	[^{3}H] guanosine uptake (cpm/10^8 cells)	
26695 (wild-type)	139 ± 26	449 ± 34 $^{\Psi}$
EM207 (Δ*nupC*)	39 ± 15 $^{\phi}$	68 ± 30 *

[a] Values are the mean ± SEM of four independent growth cultures. Trends were similar among three independent experiments.
* Significantly lower uptake compared to wild-type (student's t-test, $P < 0.01$)
$^{\phi}$ Significantly lower uptake compared to wild-type (student's t-test, $P < 0.05$)
$^{\Psi}$ Significant increase in nucleoside uptake for 20 min versus 5 min time point (student's t-test, $P < 0.05$)

Table 2.4 Components present in the defined chemical medium EMF12.

Amino acids		Vitamins/Cofactors	
Component	**Concentration (mM)**	**Component**	**Concentration (mM)**
L-arginine	1	Choline	10^{-1}
L-histidine	10^{-1}	Biotin	3×10^{-5}
L-Isoleucine	3×10^{-2}	Pantothenic acid	10^{-3}
L-leucine	10^{-1}	Niacinamide	3×10^{-4}
L-lysine	2×10^{-2}	Pyridoxine	3×10^{-4}
L-methionine	3×10^{-2}	Thiamine	10^{-3}
L-phenylalanine	3×10^{-2}	Folic acid	3×10^{-3}
L-serine	10^{-1}	Vitamin B_{12}	10^{-3}
L-threonine	10^{-1}	*Myo*-inositol	10^{-1}
L-tryptophan	10^{-2}		
L-tyrosine	3×10^{-2}		
L-valine	10^{-1}	**Metals**	
L-glutamine	1	**Component**	**Concentration (mM)**
L-cysteine	2×10^{-1}	$FeCl_2 \cdot 6H_2O$	7.5×10^{-2}
L-asparagine	10^{-1}	$MgCl_2 \cdot 6H_2O$	6×10^{-1}
L-proline	3×10^{-1}	$CaCl_2 \cdot 2H_2O$	3×10^{-1}
L-alanine	10^{-1}	$CuSO_4 \cdot 5H_2O$	10^{-5}
L-aspartic acid	10^{-1}	$ZnSO_4 \cdot 7H_2O$	3×10^{-3}
L-glutamic acid	10^{-1}	$FeCl_2 \cdot 6H_2O$	7.5×10^{-2}
L-glycine	10^{-1}		

Salts		Other	
Component	**Concentration (mM)**	**Component**	**Concentration (mM)**
KCl	3	Hypoxanthine [a]	6×10^{-2}
NaCl	104	Thymidine	3×10^{-3}
Na_2HPO_4	1	Pyruvate	1
$NaHCO_3$	14	β-cyclodextrin	3.26
		Phenol red	3×10^{-3}

Table 2.5 *H. pylori* strains used in this study.

Strain	Characteristics[a]	Source or reference
H. pylori 26695	Wild-type strain	ATCC
EM202k	Δ*guaA::aphA3,* Kan^r	This study
EM203k	Δ*guaB::aphA3,* Kan^r	This study
EM204	Δ*guaC::aphA3,* Kan^r	This study
EM205	Δ*purA::aphA3,* Kan^r	This study
EM206	Δ*purB::aphA3,* Kan^r	This study
EM207	Δ*nupC:aphA3,* Kan^r	This study
H. pylori X47	Mouse-adapted wild-type strain	(22)
EMX02k	Δ*guaA::aphA3,* Kan^r	This study

Table 2.6 Oligonucleotide primers used in this study.

Primer name	Sequence (5' → 3')
AphA5	CAAGACGATAAATGCGTC
AphA6	CTAGGTACTAAAACAATTC
Cat-5	GATATAGATTGAAAAGTGGAT
Cat-6	TTATCAGTGCGACAAACTGGG
guaA1	GAAGACTTAGAAGGTATG
guaA2	GTATAACATAGTATCGACGCAATATTGAGAATCAATC
guaA3	GAATTGTTTTAGTACCTAGAATAGCACTAAAAGTGGG
guaA4	GCGCATCGCTCTCTTCTAC
guaB1	GAAGCGAGTTCGAATCTGG
guaB2	GTATAACATAGTATCGACGGGTGAATTGTAAAAGAA
guaB3	GAATTGTTTTAGTACCTAGCCTTAATCTAATTTTAAATC
guaB4	GGGAAGTATTTGCGGGC
guaC1	GCTCAAATCGCCTTCGGTG
guaC2	GTATAACATAGTATCGACCTTTCAATGACATGATCC
guaC3	GAATTGTTTTAGTACCTAGCAATGGAGACGCAATC
guaC4	CATTAGCGATCAAGCCC
purA1	ATGGAATATCTTTAGAAG
purA2	GTATAACATAGTATCGACAAAGCTTGTTAGAATACC
purA3	GAATTGTTTTAGTACCTAGGAAAGAGAAGACACGATT
purA4	TCTAATTCCTGGTTAGC
purB1	TTAACGCTCTTAGCCCATG
purB2	GTATAACATAGTATCGACATAGCGTTCTAACACCGAC
purB3	GAATTGTTTTAGTACCTAGGTGTTTGAATAAGGCGC
purB4	CCATAAAGAGAGGCGCTC
nupC1	TTACGGCCTATCTTATCGC
nupC2	GTATAACATAGTATCGACCGTCAAATCCCTAATGCA
nupC3	GAATTGTTTTAGTACCTAGGCTAAACGCTCATTAAAAGG
nupC4	CTGATGCGTGGCAATTCC

Figures

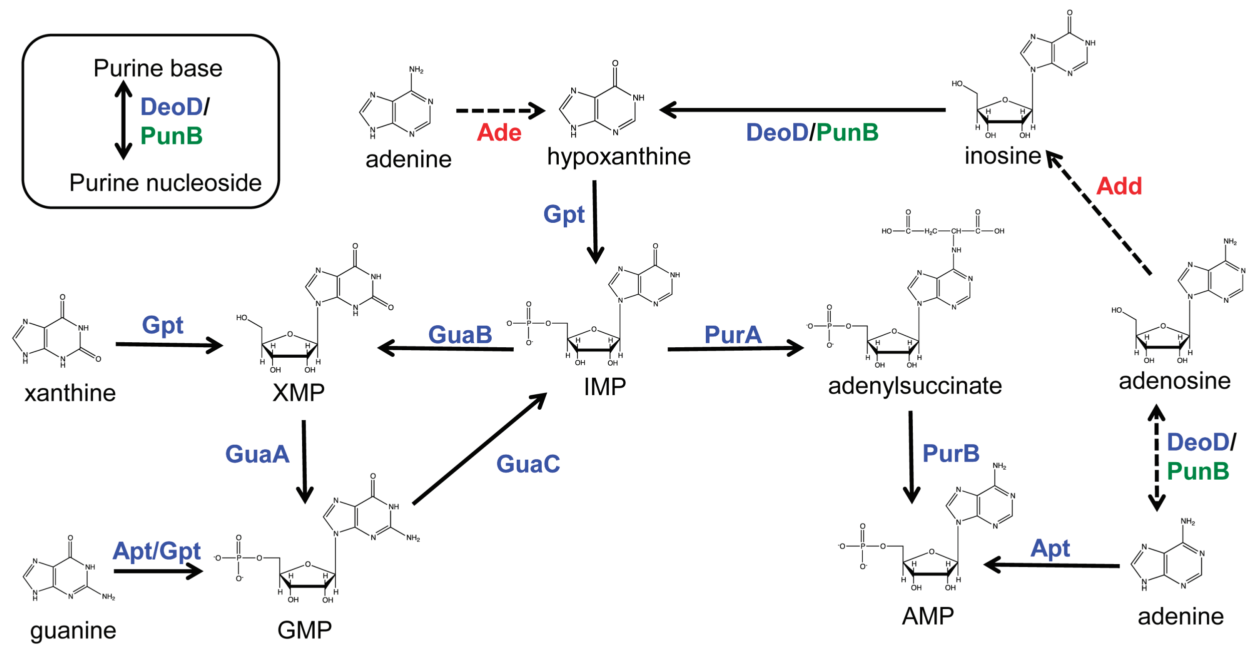

Figure 2.1 Overview of the current model for purine conversions in *H. pylori*. This network allows for salvage of purine nucleobases and nucleosides, as well as inter-conversion between GMP and AMP. Color code: blue; enzymes that have been studied in *H. pylori* by mutant analysis and/or biochemistry, green; enzymes for which genes have been identified, but whose role has not yet been confirmed, red; putative functional roles whose genetic basis has not yet been identified. Abbreviations: GuaB, IMP dehydrogenase; GuaA, GMP synthetase; GuaC, GMP reductase; PurA, adenylosuccinate synthetase; PurB, adenylosuccinate lyase; Gpt, hypoxanthine-guanine phosphoribosyl-transferase; Apt, adenine phosphoribosyltransferase; DeoD, purine nucleoside phosphorylase; PunB, purine nucleoside phosphorylase; Ade, adenine deaminase; Add, adenosine deaminase; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; AMP, adenosine monophosphate.

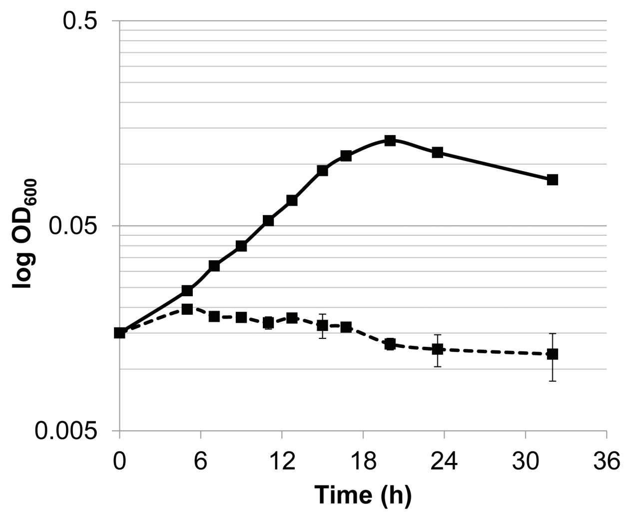

Figure 2.2 Growth of *H. pylori* 26695 in the chemically defined medium EMF12. Liquid growth medium EMF12 was supplemented with 60 μM hypoxanthine (solid line) or contained no purines (dashed line). *H. pylori* cells were inoculated at an initial OD_{600} of 0.015 (approx. 1.3 x 10^7 cfu/ml). Growth was monitored over time by measuring the absorbance at 600 nm. Results are the mean ± SD of three independent cultures.

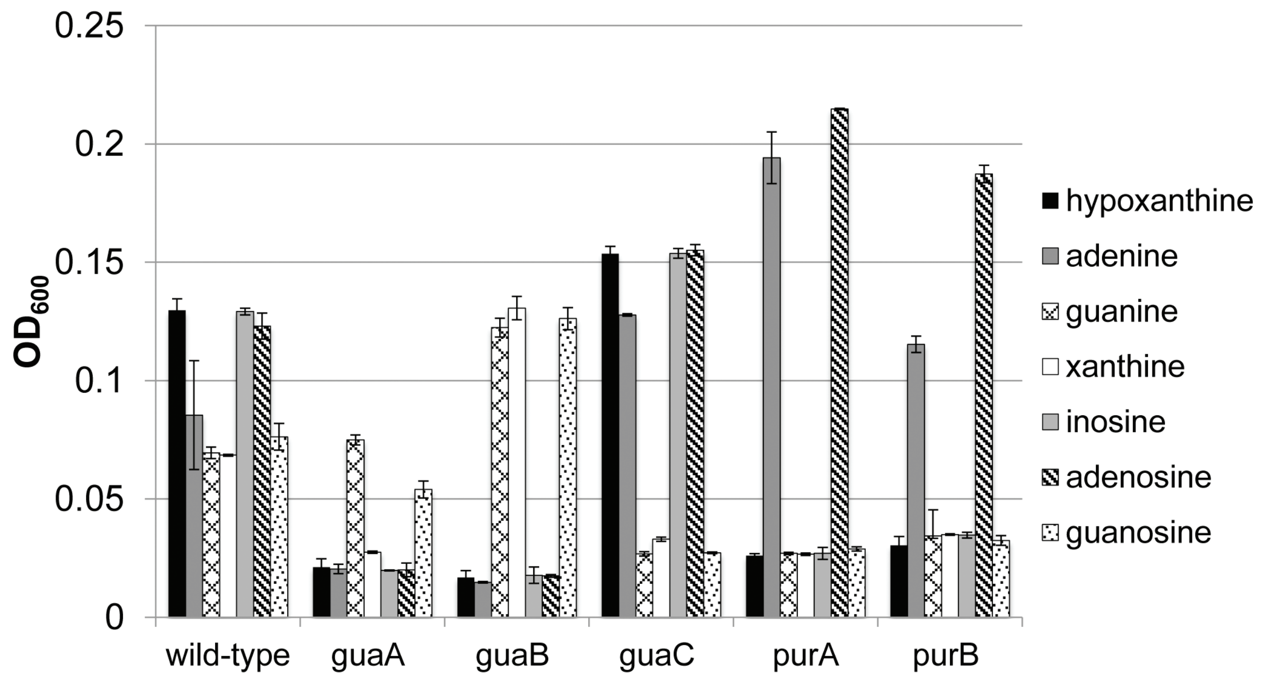

Figure 2.3 Growth of *gua* and *pur* mutants in EMF12 supplemented with individual purines. *H. pylori* strains were grown in EMF12 supplemented with one of seven purine sources. After 20 hours, the optical density was measured. Positive growth was defined as a statistically significant increase in OD_{600} relative to the baseline OD_{600} of 0.025 (student's t-test, $P < 0.05$). Results are the mean ± SD of three independent growth cultures.

```
YeiJ      --MDVMRSVLGMVVLLTIAFLLSVNKKKISLRTVGAALVLQVVIGGIMLWLPPGRWVAEK 58
YeiM      --MDIMRSVVGMVVLLAIAFLLSVNKKSISLRTVGAALLLQIAIGGIMLYFPPGKWAVEQ 58
HP1178    MIFSSLFSVVGMAVLFLIAWVFSSNKRAINYRTIVSAFVIQVALGALALYVPLGREMLQG 60
NupC      -MDRVLHFVLALAVVAILALLVSSDRKKIRIRYVIQLLVIEVLLAWFFLNSDVGLGFVKG 59
               :  *:.:.*:  :* :.* ::: *  * :   ::::: :. : *    *    :

YeiJ      VAFGVHKVMAYSDAGSAFIFGSLVGPKMDTLFDGAGFIFGFRVLPAIIFVTALVSILYYI 118
YeiM      AALGVHKVMSYSDAGSAFIFGSLVGPKMDVLFDGAGFIFAFRVLPAIIFVTALISLLYYI 118
HP1178    LASGIQSVISYGYEGVRFLFGNLA-PNAKGDQGIGGFVFAINVLAIIIFFASLISLLYYL 119
NupC      FSEMFEKLLGFANEGTNFVFGSND--------QGLAEFFFLKVLCPIVFISALIGILQHI 111
           :  ...::.:.  *  *:**.            . .* :.**  *:*.::*:.:* ::

YeiJ      GVMGILIRILGGIFQKALNISKIESFVAVTTIFLGQNEIPAIVKPFIDRLNRNELFTAIC 178
YeiM      GVMGLLIRILGSIFQKALNISKIESFVAVTTIFLGQNEIPAIVKPFIDRMNRNELFTAIC 178
HP1178    KIMPLFINLIGGALQKCLGTSRAESMSAAANIFVAHTEAPLVIKPYLKSMSDSEIFAVMC 179
NupC      RVLPVIIRAIGFLLSKVNGMGKLESFNAVSSLILGQSENFIAYKDILGKISRNRMYTMAA 171
           :: ::*. :*  :.*  . .: **: *.:.:::.:.*     *  :  :. ..:::  .

YeiJ      SGMASIAGSTMIGYAALGVPVEYLLAASLMAIPGGILFARLLSPATESSQVSFNNLSFTE 238
YeiM      SGMASIAGSMMIGYAGMGVPIDYLLAASLMAIPGGILFARILSPATEPSQVTFENLSFSE 238
HP1178    VGMASVAGPVLAGYASMGIPLPYLIAASFMSAPGGLLFAKIIYPQNE-TISSHADVSIEK 238
NupC      TAMSTVSMSIVGAYMTMLEP-KYVVAALVLNMFSTFIVLSLINPYRVDASEENIQMSNLH 230
           .*:::: . : .*  :  *  *::** .:   . ::.  :: *    :     ::*  .

YeiJ      TPPKSIIEAAATGAMTGLKIAAGVATVVMAFVAIIALINGIIGGVGGWFGFEHASLESIL 298
YeiM      TPPKSFIEAAASGAMTGLKIAAGVATVVMAFVAIIALINGIIGGIGGWFGFANASLESIF 298
HP1178    HVN--AIEAIANGASTGLNLALHVGAMLLAFVGMLALINGLLGVVGGFLGMEHLSLGLIL 296
NupC      EGQ-SFFEMLGEYILAGFKVAIIVAAMLIGFIALIAALNALFATVTGWFGYS-ISFQGIL 288
               :*  .    :*:::*  *.::::.*:.::* :*.::. : *::*    *:  *:

YeiJ      GYLLAPLAWVMGVDWSDANLAGSLIGQKLAINEFVAYLNFSPYLQTAG--TLDAKTVAII 356
YeiM      GYVLAPLAWIMGVDWSDANLAGSLIGQKLAINEFVAYLSFSPYLQTGG--TLEVKTIAII 356
HP1178    GTLLKPLAFMLGIPWSQAGIAGEIIGIKIALNEFVGYMQLLPYLGDNPPLILSEKTKAII 356
NupC      GYIFYPIAWVMGVPSSEALQVGSIMATKLVSNEFVAMMDLQKIASTLS-----PRAEGII 343
          * :: *:*:::*:  *:*  .*.::. *:. ****. :.:              :: .**

YeiJ      SFALCGFANFGSIGVVVGAFSAVAPHRAPEIAQLGLRALAAATLSNLMSATIAGFFIGLA 416
YeiM      SFALCGFANFGSIGVVVGAFSAISPKRAPEIAQLGLRALAAATLSNLMSATIAGFFIGLA 416
HP1178    TFALCGFANLSSVAMLIGGLGSLVPKKKDLIVRLALKAVLVGTLSNFMSATIAGLFIGLNAH418
NupC      SVFLVSFANFSSIGIIAGAVKGLNEEQGNVVSRFGLKLVYGSTLVSVLSASIAALVL--- 400
          :. * .***:.*:.:: *.. .:  .:   : ::.*: :  .** ..:**:**.:.:
```

Figure 2.4 Sequence comparison between *H. pylori* NupC homolog (HP1180) and three *E. coli* CNT paralogs. Sequences for *E. coli* YeiJ (GenBank™ accession number AAA60513.1), *E. coli* YeiM (GenBank™ accession number AAA60518.1), HP1180 (GenBank™ accession number AAD08224.1), and *E. coli* NupC (GenBank™ accession number CAA52821.1) were

aligned using ClustalW. Membrane-spanning helices were predicted using the TMHMM program (46). Conserved regions typical of CNT transporters are boxed in black (34).

CHAPTER 3

IDENTIFICATION OF A NEW CLASS OF ADENOSINE DEAMINASE FROM *HELICOBACTER PYLORI* WITH HOMOLOGS AMONG DIVERSE TAXA[1]

[1] Miller, E. F., and R. J. Maier. 2013. The Journal of Bacteriology (in press). Reprinted here with permission of publisher.

Abstract

Early studies of *H. 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 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 studies 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 phyletic distribution among prokaryotes is broad.

Introduction

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 ε-proteobacteria, colonizes the gastric epithelium of humans. Infection is chronic, and can lead to peptic ulcers, gastric cancer, and/or MALT lymphoma (6-8). The bacterium's reduced genome size and specialized machinery for acid resistance are consequences of its over 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, adenosine deaminase (ADD) activity was detected in *H. pylori* strain 26695 cell-free 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) for protein expression. *H. pylori* cells were grown on blood agar (BA) plates or in EMF12 liquid media 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 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 psi), and cell-free extract was obtained by centrifugation (20,000 x g, 20 min). The supernatant was stirred slowly at 4°C while finely ground ammonium sulfate was added to 45% saturation. After 30 minutes, the sample was centrifuged (20,000 x 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 NP Buffer, and dialyzed twice for 2 hours in 0.8 L 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 flow-through 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 flow-through from this step was similarly concentrated to 0.4 ml and applied to a Hi-Load 16mm/60cm 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 & 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). Protein concentration was determined using the 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 *Hp*0267 using 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 minutes, followed by incubation in a 1:1 solution of acetonitrile (ACN):water for three rounds of 15 minutes each. The gel slices were then incubated successively for 15 minutes 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 at room temperature for 60 min in the dark. Gel slices were washed once with 100 mM ammonium bicarbonate, then

incubated overnight at 37°C in 25 mM ammonium bicarbonate containing 0.1 μg trypsin. The trypsin solution was removed and saved, while the gel slices were briefly incubated in 50% ACN containing 0.1% formic acid. Both 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 2,5-Dihydroxybenzoic acid and 0.1% trifluoroacetic acid. The samples were run using a Bruker Daltonics Autoflex MALDI-TOF instrument using 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 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 (BioRad, Cat# 161-363). The subunit composition of Hp0267 was determined using gel filtration chromatography by comparing the V_e/V_o 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'-AGCACTCGAGATCAAGAAAT CATAGGAGCG-3' and 5'-AGCAGAATTCTTAGATCACCCTTTTCCCC-3'. The 1.23 kbp amplicon was digested using *Xho*I and *Eco*RI and ligated into pTrcHisC (Invitrogen), allowing

for expression of Hp0267 with a hexahistidine fusion peptide at the N-terminus. Recombinant *E. coli* BL21 Rosetta PLysS (Novagen) containing the expression vector were grown in 0.3 L Luria Bertani broth, and expression was stimulated for 12 hoursat 37°C using 0.1 mM 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 cOmplete EDTA-free protease inhibitor cocktail (Roche Applied Science). Cells were passed twice through a French pressure cell (20,000 psi), and soluble cell extract was prepared by centrifugation (20,000 x g, 20 min). Soluble Hp0267 was purified via gravity chromatography using 1 ml Ni-NTA agarose resin (Quiagen) 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 L lysis buffer. Elution fractions 2-4 were pooled together. Enterokinase (0.02 μg) (New England Biolabs, Cat# P8070S) was added, and digestion proceeded for 16 hours 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 BSA.

Enzyme assays. Substrates for enzyme assays were obtained from the following sources: adenosine (Sigma, A4036), guanine (Sigma, G11950), cytosine (Sigma, C3506), adenine (Sigma, A9126), atrazine (Chem Service, P5380, West Chester, PA), 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, end-point 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 10 mM of the desired substrate (2 mM for guanine, 2.5 mM for S-adenosylhomocysteine). 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 5-6), MES (pH 6-7), HEPES (pH 7-8), Bicine (pH 8-9), carbonate/bicarbonate (pH 9-10.5). The carbonate/bicarbonate buffer was prepared as previously described (19). Reactions were initiated by adding 150 ng of enzyme, allowed to proceed for 5 minutes at 37°C, and the ammonium concentration was determined (17). Each condition (pH and buffer) required its own standard curve of known NH_4Cl concentrations.

The temperature optimum of Hp0267 was determined by measuring the initial rate of ADD activity over a range of 25°C - 70°C in Bicine (pH 8.5) containing 10 mM adenosine.

Prior to adding enzyme, the reaction mix was pre-heated for 1 minute. A thermocycler (MyCycler, BioRad) was used to incubate the reactions.

Metals 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.

Steady-state kinetics analysis. The data used for calculating kinetic constants were obtained using substrate concentrations ranging from 0.5 - 10 mM (adenosine) and 0.1 – 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 weight 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 e-20. Only sequences containing the

signature metal-binding motif were considered. Based on the conserved motif found within this alignment (P[A/G]XXNXHXH) 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-free 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 3.1, Fig 3.1). 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 3.2). 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 the ADD activity in cell lysates by approximately 10-fold. These data suggested that *hp0267* encoded an adenosine deaminase.

Attempts to generate a *hp0267* deletion mutant in *H. pylori* through allelic replacement of the gene with an antibiotic resistance cassette (*cat* or *AphA*3) produced no viable clones despite including 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-base pair sequence overlap with 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 weight of between 45-60 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 & Methods). There was no difference in ADD activity between His_6-Hp0267 and the final enzyme lacking the hexahistidine tag, indicating that the enzyme's integrity had been maintained during enterokinase treatment. 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 hours at 4°C) was increased 3-fold by storing the enzyme at -20°C with 1 mg/ml BSA and 40% glycerol. The pH optimum of purified Hp0267 was shown to be approximately 8.5, and the optimum temperature is between 33-40°C (Fig 3.2).

Hp0267 exhibited slight but significant deaminase activity on 2'-deoxyadenosine, and no detectable activity on guanine, cytosine or adenine (Table 3.3). No dechlorination of atrazine was measured even after a 3-hour 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 indicates a low catalytic efficiency and specificity when compared to previously characterized ADDs, which typically achieve nearly

diffusion-limited reaction rates ($K_{cat}/K_m \approx 10^7\ M^{-1}s^{-1}$), and exhibit K_m values for adenosine in the micromolar range (27-32).

We obtained a model of the 3D structure of Hp0267 using the program Phyre (33), 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 found that not only can Hp0267 catalyze the deamination of SAH, but 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 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 (34, 35). 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, 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 (36). To determine the metal contained within Hp0267, samples of recombinant, purified enzyme were analyzed using Inductively Coupled Plasma Optical Emission Spectrometery (ICP-OES) (Table 3.4). These results suggest that Fe, Ni, or Zn can be coordinated by 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 possibility 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 minutes, and then measuring ADD activity (Fig 3.3). 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, 37). 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, 32, 38-40). 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) (41). Biochemically studied members of cog0402 include atrazine chlorohydrolase (18, 42), cytosine deaminase (37, 43), guanine deaminase (44), 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 3.4). The hallmark metal-binding residues that define all AHS members are His65, His67, His207, and Asp372 (numbering of residues corresponds to *H. pylori* Hp0267) (36, 45). Residues that are both (a) conserved within this alignment, and (b) 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 Figure 4, it appears that Gln can substitute Glu210. In addition to these residues for which structural data has 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 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/epsilon-proteobacteria, for which comparatively strong homologs to Hp0267 (30-50% identity) are found. With only three exceptions, all epsilon/delta-proteobacteria 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 *Aciduliporofundum 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 (42, 46), 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 as substrates (42). 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 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 (35). Our comparative genomic data reveals 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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References

1. **Wilson, D. K., F. B. Rudolph, and F. A. Quiocho.** 1991. Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. Science (New York, NY) **252:**1278.

2. **Akedo, H., H. Nishihara, K. Shinkai, K. Komatsu, and S. Ishikawa.** 1972. Multiple forms of human adenosine deaminase: I. Purification and characterization of two molecular species. Biochimica et Biophysica Acta (BBA)-Enzymology **276:**257-271.

3. **Gabellieri, E., S. Bernini, L. Piras, P. Cioni, E. Balestreri, G. Cercignani, and R. Felicioli.** 1986. Purification, stability and kinetic properties of highly purified adenosine deaminase from *Bacillus cereus* NCIB 8122. Biochimica et Biophysica Acta (BBA)-General Subjects **884:**490-496.

4. **Ling, F., Y. Inoue, and A. Kimura.** 1991. Purification and characterization of adenosine deaminase from *Klebsiella* sp. LF 1202. Journal of Fermentation and Bioengineering **71:**89-92.

5. **Chang, Z., P. Nygaard, A. C. Chinault, and R. E. Kellems.** 1991. Deduced amino acid sequence of *Escherichia coli* adenosine deaminase reveals evolutionarily conserved amino acid residues: implications for catalytic function. Biochemistry **30:**2273-2280.

6. **Bayerdörffer, E., B. Rudolph, A. Neubauer, C. Thiede, N. Lehn, S. Eidt, and M. Stolte.** 1995. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of *Helicobacter pylori* infection. The Lancet **345:**1591-1594.

7. **Forman, D., D. Newell, F. Fullerton, J. Yarnell, A. Stacey, N. Wald, and F. Sitas.** 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. BMJ: British Medical Journal **302:**1302.

8. **Polk, D. B., and R. M. Peek.** 2010. *Helicobacter pylori*: gastric cancer and beyond. Nature Reviews Cancer **10:**403-414.

9. **Linz, B., F. Balloux, Y. Moodley, A. Manica, H. Liu, P. Roumagnac, D. Falush, C. Stamer, F. Prugnolle, and S. W. van der Merwe.** 2007. An African origin for the intimate association between humans and *Helicobacter pylori*. Nature **445:**915-918.

10. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, and B. A. Dougherty.** 1997. The

complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

11. **Mobley, H., M. J. Cortesia, L. Rosenthal, and B. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. Journal of Clinical Microbiology **26:**831-836.

12. **Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs.** 2000. A H+-gated urea channel: the link between *Helicobacter pylor*i urease and gastric colonization. Science **287:**482-485.

13. **Liechti, G., and J. B. Goldberg.** 2012. *Helicobacter pylor*i relies primarily on the purine salvage pathway for purine nucleotide biosynthesis. Journal of Bacteriology **194:**839-854.

14. **Miller, E. F., S. Vaish, and R. J. Maier.** 2012. Efficiency of Purine Utilization by *Helicobacter pylori:* Roles for Adenosine Deaminase and a NupC Homolog. PloS one **7:**e38727.

15. **White, R. H.** 1997. Occurrence and biosynthesis of 5-aminoimidazole-4-carboxamide ribonucleotide and N-(beta-D-ribofuranosyl) formamide 5'-phosphate in *Methanobacterium thermoautotrophicum* delta (H). Journal of Bacteriology **179:**563-566.

16. **Shah, P., G. Gutierrez‐Sanchez, R. Orlando, and C. Bergmann.** 2009. A proteomic study of pectin-degrading enzymes secreted by *Botrytis cinerea* grown in liquid culture. Proteomics **9:**3126-3135.

17. **Weatherburn, M.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry **39:**971-974.

18. **Seffernick, J. L., H. McTavish, J. P. Osborne, M. L. de Souza, M. J. Sadowsky, and L. P. Wackett.** 2002. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a metalloenzyme. Biochemistry **41:**14430-14437.

19. **Delory, G., and E. King.** 1945. A sodium carbonate-bicarbonate buffer for alkaline phosphatases. Biochemical Journal **39:**245.

20. **Zhang, Z., W. Miller, A. A. Schäffer, T. L. Madden, D. J. Lipman, E. V. Koonin, and S. F. Altschul.** 1998. Protein sequence similarity searches using patterns as seeds. Nucleic Acids Research **26:**3986-3990.

21. **Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, and T. L. Madden.** 2008. NCBI BLAST: a better web interface. Nucleic Acids Research **36:**W5-W9.

22. **Salama, N. R., B. Shepherd, and S. Falkow.** 2004. Global transposon mutagenesis and essential gene analysis of *Helicobacter pylori*. Journal of Bacteriology **186:**7926-7935.

23. **Allaby, R. G., and M. Woodwark.** 2004. Phylogenetics in the bioinformatics culture of understanding. Comparative and Functional Genomics **5:**128-146.

24. **Seffernick, J. L., M. L. de Souza, M. J. Sadowsky, and L. P. Wackett.** 2001. Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different. Journal of Bacteriology **183:**2405-2410.

25. **Hermann, J. C., R. Marti-Arbona, A. A. Fedorov, E. Fedorov, S. C. Almo, B. K. Shoichet, and F. M. Raushel.** 2007. Structure-based activity prediction for an enzyme of unknown function. Nature **448:**775-779.

26. **Shao, Z., W. Seffens, W. Mulbry, and R. Behki.** 1995. Cloning and expression of the s-triazine hydrolase gene (*trzA*) from *Rhodococcus corallinus* and development of *Rhodococcus* recombinant strains capable of dealkylating and dechlorinating the herbicide atrazine. Journal of Bacteriology **177:**5748-5755.

27. **Schomburg, I., A. Chang, and D. Schomburg.** 2002. BRENDA, enzyme data and metabolic information. Nucleic Acids Research **30:**47-49.

28. **Kurz, L. C., L. Moix, M. C. Riley, and C. Frieden.** 1992. The rate of formation of transition-state analogs in the active site of adenosine deaminase is encounter-controlled: implications for the mechanism. Biochemistry **31:**39-48.

29. **Pornbanlualap, S., and P. Chalopagorn.** 2011. Adenosine deaminase from *Streptomyces coelicolor*: Recombinant expression, purification and characterization. Protein Expression and Purification **78:**167-173.

30. **Daddona, P. E., and W. N. Kelley.** 1977. Human adenosine deaminase. Purification and subunit structure. Journal of Biological Chemistry **252:**110-115.

31. **Gabellieri, E., S. Bernini, L. Piras, P. Cioni, E. Balestreri, G. Cercignani, and R. Felicioli.** 1986. Purification, stability and kinetic properties of highly purified adenosine deaminase from *Bacillus cereus* NCIB 8122. Biochimica et Biophysica Acta **884:**490-496.

32. **Nygaard, P.** 1978. Adenosine deaminase from *Escherichia coli*. Methods in Enzymology **51:**508-512.

33. **Bennett‐Lovsey, R. M., A. D. Herbert, M. J. Sternberg, and L. A. Kelley.** 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins: Structure, Function, and Bioinformatics **70:**611-625.

34. **Zulty, J. J., and M. K. Speedie.** 1989. Purification and characterization of S-adenosylhomocysteine deaminase from streptonigrin-producing *Streptomyces flocculus*. Journal of Bacteriology **171:**6840-6844.

35. **Speedie, M., J. Zulty, and P. Brothers.** 1988. S-adenosylhomocysteine metabolism in *Streptomyces flocculus*. Journal of Bacteriology **170:**4376-4378.

36. **Holm, L., and C. Sander.** 1998. An evolutionary treasure: unification of a broad set of amidohydrolases related to urease. Proteins: Structure, Function, and Bioinformatics **28:**72-82.

37. **Ireton, G. C., G. McDermott, M. E. Black, and B. L. Stoddard.** 2002. The structure of *Escherichia coli* cytosine deaminase. Journal of Molecular Biology **315:**687-697.

38. **Cooper, B. F., V. Sideraki, D. K. Wilson, D. Y. Dominguez, S. W. Clark, F. A. Quiocho, and F. B. Rudolph.** 1997. The role of divalent cations in structure and function of murine adenosine deaminase. Protein Science **6:**1031-1037.

39. **Jun, H.-k., T.-S. Kim, and T. Sakai.** 1991. Purification and characterization of extracellular adenosine deaminase from a *Streptomyces* sp. Journal of Fermentation and Bioengineering **71:**6-11.

40. **Seibert, C. M., and F. M. Raushel.** 2005. Structural and catalytic diversity within the amidohydrolase superfamily. Biochemistry **44:**6383-6391.

41. **Tatusov, R. L., N. D. Fedorova, J. D. Jackson, A. R. Jacobs, B. Kiryutin, E. V. Koonin, D. M. Krylov, R. Mazumder, S. L. Mekhedov, and A. N. Nikolskaya.** 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics **4:**41.

42. **De Souza, M. L., M. J. Sadowsky, and L. P. Wackett.** 1996. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. Journal of Bacteriology **178:**4894-4900.

43. **Porter, D. J.** 2000. *Escherichia coli* cytosine deaminase: the kinetics and thermodynamics for binding of cytosine to the apoenzyme and the Zn2+ holoenzyme are similar. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology **1476:**239-252.

44. **Maynes, J. T., R. G. Yuan, and F. F. Snyder.** 2000. Identification, expression, and characterization of *Escherichia coli* guanine deaminase. Journal of Bacteriology **182:**4658-4660.

45. **Wilson, D. K., F. B. Rudolph, and F. A. Quiocho.** 1991. Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. Science **252:**1278-1284.

46. **Seffernick, J. L., and L. P. Wackett.** 2001. Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. Biochemistry **40:**12747-12753.

Tables

Table 3.1 Purification summary of native *H. pylori* adenosine deaminase.

Sample	Activity (μmol min^{-1} ml^{-1})	Protein (mg)	Specific Activity (μmol min^{-1} mg^{-1})	Yield (%)	Fold purification
Cell lysate	1110	331	0.03	100	1
20K supernatant	1074	233	0.05	97	1.4
45% - 55% AS 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

Table 3.2 Protein bands identified using MALDI-TOF.

Band number	HP identifier	Protein annotation	Sequence coverage (%)	E-value
1	HP0891	Conserved hypothetical protein	35	6.3e-009
2	HP1563	Alkyl hydroperoxide reductase (AhpC)	52	4e-011
3	HP1104	Cinnamyl-alcohol dehydrogenase	34	2e-011
4	HP0267	Chlorohydrolase	39	2e-012
5	HP0974	Phosphoglycerate mutase	47	6.3e-013

Table 3.3 Substrate preference and estimated kinetic constants of Hp0267

Substrate	SA[a] (μmol min^{-1} mg^{-1})	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
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, 2.5 mM for S-adenosylhomocysteine).
[b] NC = Not able to calculate due to either low or non-detectable activity
[c] BDL = Activity below the limit of detection for this substrate

Table 3.4 Metals analysis of purified Hp0267

Metal	Concentration (μM)	Molar ratio (metal:enzyme)
Fe	48.6 ± 0.9	0.49
Zn	21.4 ± 1.7	0.21
Ni	14.6 ± 0.2	0.15
Mn	1.9 ± 0.1	0.02
Mg	BDL[a]	-
Mo	BDL	-
Co	BDL	-
Cd	BDL	-

[a] BDL = below detection limit

Figures

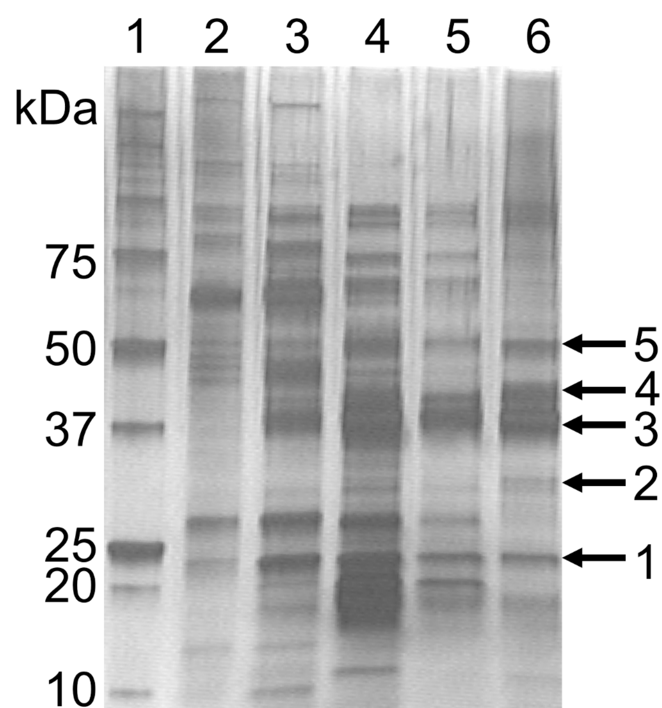

Figure 3.1 Denaturing SDS-PAGE of native *Hp0267* purification. Bands were stained using silver nitrate. Lane 1: Molecular weight marker, Lane 2: 20K supernatant, Lane 3: Ammonium sulfate precipitation, Lane 4: Anion exchange FT, Lane 5: Cation exchange FT, Lane 6: Size-exclusion chromatography. Arrows indicate bands that were excised from a Coomassie-stained gel for MALDI-TOF analysis.

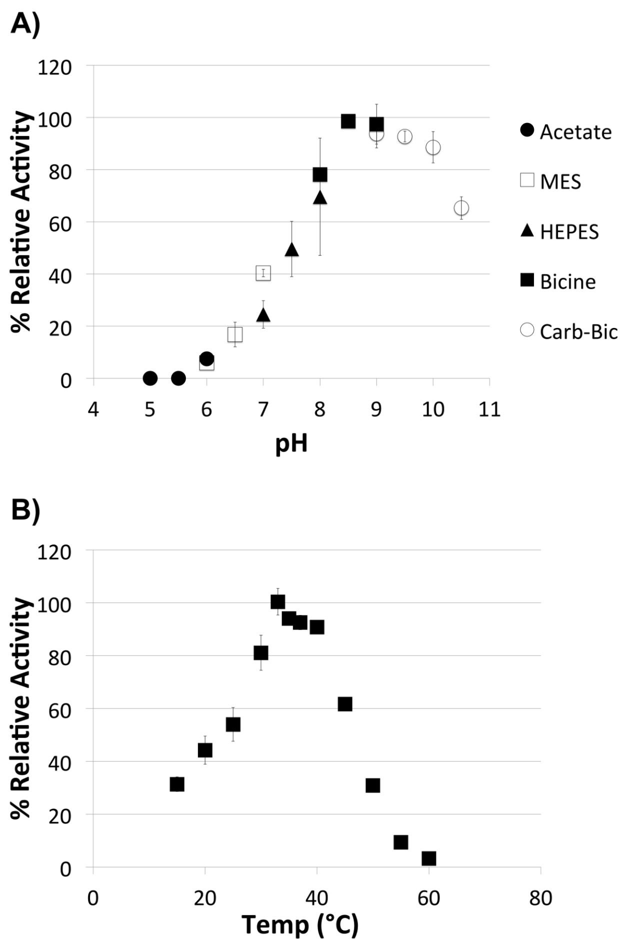

Figure 3.2 pH and temperature optima of Hp0267. (A) A range of pH-appropriate buffers were used as indicated in the figure legend. All reactions were incubated at 37°C. **(B)** Reactions were carried out in Bicine buffer at pH 8.5, and the temperature of incubation was varied. Reaction rates are reported as a percentage of the maximum specific activity observed on that day (90 μmol min^{-1} mg^{-1}). Results are the mean ± SD of at least three independent determinations.

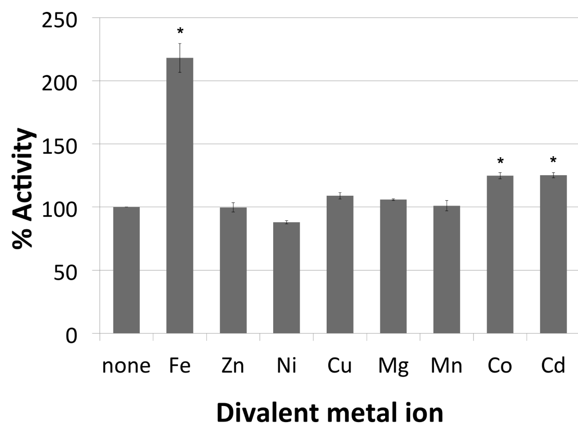

Figure 3.3 Hp0267-ADD activity upon incubating with divalent metals. The ADD activity of purified Hp0267 was measured following incubation with various divalent metals. Results shown are the mean ± SD of three independent replicates, and are expressed as the percentage of activity relative to a control incubation lacking added metals. Asterisks indicate a significant increase in activity relative to the control ($P < 0.01$, paired Student's t-test)

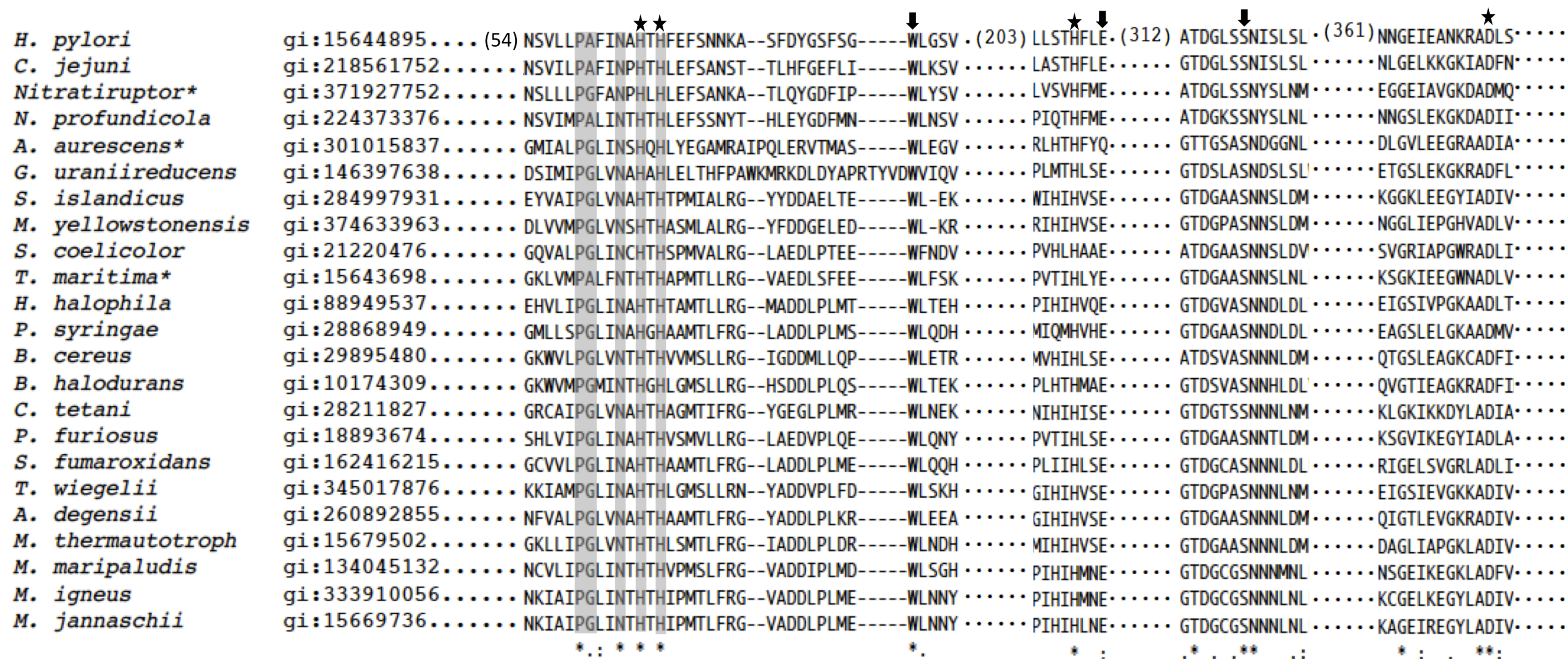

Figure 3.4 Sequence alignment of Hp0267, Tm0936, and orthologs. Proteins for which crystal structures are available are indicated by an asterisk adjacent to the species' name, and can be accessed from the Protein Data Bank: *Nitratiruptor* Sp. Sb155-2 (PDB: 3V7P), *Arthrobacter aurescens* Tc1 (PDB: 3LSC), *Thermotoga maritima* (PDB: 2PLM). Stars above the sequence alignment indicate residues that coordinate the metal ligand. Downward arrows indicate residues that are both (a) conserved and (b) 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.

CHAPTER 4

DISSERTATION SUMMARY AND FUTURE DIRECTIONS

This dissertation has expanded our knowledge of *H. pylori* purine metabolism by offering a more complete picture of the organism's purine requirements and its purine conversion network. Previous studies suggesting that *de novo* purine biosynthesis is possible in *H. pylori* were not supported by my data nor by recent studies (1). Furthermore, I studied the growth of this fastidious organism in a defined medium and showed that any purine nucleoside or nucleobase can serve as the sole source of purine for *H. pylori*. From the studies described in Chapter 2, I conclude that the salvage pathway for purines in this organism is complete, and that a *de novo* pathway for their synthesis is absent. By characterizing *H. pylori gua* and *pur* mutants, I defined the non-redundant roles of these key players in the building and breaking down of guanine and adenine, two processes that are critically important for a complete purine salvage network. These data, along with data from quantitative growth studies that identified the relative efficacies of various purines in supporting *H. pylori* growth, provided a strong foundation for the remainder of this dissertation and will be a useful framework for future studies of this organism's metabolism.

To my surprise, unlike many other bacterial pathogens that are attenuated for virulence upon disruption of their purine salvage network, an *H. pylori* mutant lacking *guaA* colonized a murine host with efficiency similar to wild-type. I had previously shown that *guaA* is essential for growth in the absence of guanine or guanine derivatives. Likewise, it is important that a

balance of GMP and AMP be maintained in the cell, a balance that depends upon GuaA (2). The question, therefore, is if the host can fulfill the pathogen's need for guanine and can also provide an appropriate balance of adenine and guanine, then why would *H. pylori* maintain this costly biosynthetic pathway? Could the presence of GuaA be important only during later stages of chronic *H. pylori* infection? A caveat to recognize here is that a murine infection model is not an optimal system for studying a primate-specific pathogen such as *H. pylori*. The availability of purines likely varies depending on the host, and the establishment of chronic infection by *H. pylori* is not thought to be replicable in a murine model (3). Conditions such as gastric pH, epithelial cell turnover rate, and host cell surface proteins almost certainly vary between mice and humans. Nonetheless, if we do assume that GMP biosynthesis is not important for colonization (in other words, that all purines are readily obtainable in its gastric niche), then the new class of inhibitors currently being developed against *H. pylori* GuaA should not be pursued further (4). Perhaps purine transport would be a better target for drug design against this organism.

To this end, my dissertation also focused on identifying possible purine transporters in *H. pylori* strain 26695. I identified a mechanism by which *H. pylori* takes up purine nucleosides—an ortholog of the pyrimidine nucleoside transporter NupC. Using radiolabeled purine nucleoside uptake experiments, I showed that Hp1180 aids in the uptake of the three main biological purine nucleosides adenosine, guanosine, and inosine. Curiously, my results show that not only does the substrate specificity of Hp1180 differ from that of its homologs in *E. coli* and *B. subtilis*, but also that this transporter is important for growth on several purine nucleobases. All CNT-type transporters studied to date recognize nucleosides exclusively and not nucleobases, therefore I hypothesize that Hp1180 does not actually transport nucleobases but

that it instead facilitates their use in an indirect way. For example, perhaps the purine nucleoside phosphorylase DeoD, which is found in the periplasm of certain proteobacteria (5, 6), could function in tandem with Hp1180 by converting free bases into nucleosides that can subsequently be taken up by Hp1180.

Due to the importance of Hp1180 for growth in several purine nucleosides and nucleobases, I sought to use an *H. pylori nupC* mutant for animal infection studies. This data, unfortunately, varied greatly between experiments and was therefore inconclusive. Further studies will be needed to determine the importance of *nupC* in colonization and/or persistent infection by *H. pylori*. Also informative would be transport studies using recombinant Hp1180 expressed in *Xenopus* oocytes, a method that avoids using bacterial membrane preparations and would therefore be useful for unambiguous determination of the substrates carried by Hp1180 (7).

A final discovery in this dissertation was the identification of a new class of adenosine deaminase. In my studies of the *gua* and *pur* genes, I had relied on the strong homology of their gene products to the canonical and highly conserved pathway in other well-studied bacteria. This same luxury was not afforded in my attempt to identify a mechanism for breaking down AMP to IMP. Apart from understanding that this pathway relies on the deamination of the adenine moiety, the actual enzymes involved varies among bacterial species, and no homologs to these were identified in *H. pylori*. I approached this problem by identifying the enzyme activity in *H. pylori* cell-free extracts that catalyzes the deamination of an adenine moiety. I detected high adenosine deaminase activity, and then used this sensitive activity assay to track the enzyme's fractionation through sequential purification steps, at which point I could identify the enzyme using MALDI-TOF. This adenosine deaminase, which also exhibits S-adenosyl-

homocysteine (SAH) deaminase activity, is encoded by the locus *hp0267,* previously mis-annotated as encoding a "chlorohydrolase" (8, 9).

Hp0267 is a member of cog0402 and is, therefore, more closely related to cytosine and guanine deaminases than to previously characterized adenosine deaminases, which are all members of cog1816. I examined the phyletic distribution of Hp0267 orthologs and found that the delta/epsilon-proteobacteria contained the most similar homologs. Among the *Campylobactereaceae*, for example, the percentage of identity is between 30-50%, while among other genera such as *Sulfurovum* and *Nitratiruptor* the identities are lower, between 30-40%. Surprisingly, the second-highest ranking group of Hp0267 orthologs I found were among the archaea, with identities ranging between 20-30%. Although these percentages fall within the "twilight zone" of homology (10), I mitigated this somewhat by using PHI-BLAST programmed to find a conserved motif that I had described for Tm0936 and Hp0267 orthologs (Chapter 3) (11). Although I cannot generalize to say that this group of Tm0936 and Hp0267 orthologs consists entirely of adenosine deaminases, I also noted that the presence of an Hp0267 ortholog within a genome correlates with the absence of a cog1816 ADD in that genome. Only three species from the delta/epsilon-proteobacteria possess both a cog1816 ADD and an Hp0267 ortholog (*Campylobacter lari*, *Wolinella succinogenes*, and *Sulfurospirillum deleyianum*), whereas the majority of members possess only an ortholog to Hp0267. Likewise, among the archaea that possess an ortholog to Hp0267, only one species possesses a cog1816 adenosine deaminase homolog. Although the evidence is strong that Hp0267 represents a second class of prokaryotic adenosine deaminase, I was unable to prove the functional role of this enzyme *in vivo* using mutant analysis. Further studies using mutant analysis, ADD inhibitors, or

conditional mutants are needed to prove whether Hp0267, Tm0936 and their close orthologs do indeed carry out the physiological role of adenosine deamination *in vivo*.

I concluded the studies of Hp0267 with an analysis of the metal contained within its active site. Like its cousin cytosine deaminase, Hp0267 coordinates an Fe^{+2} ion as its physiologically relevant metal ligand. This is in contrast to cog1816 adenosine deaminases, which coordinate Zn^{+2} as the relevant metal ligand.

In summary, this dissertation has served to characterize a complete purine salvage pathway in this important human pathogen. In addition, by studying a NupC homolog that transports purine rather than pyrimidine nucleosides (Hp1180), and a chlorohydrolase homolog that degrades adenosine and SAH (Hp0267), my work has uncovered two proteins that exemplify how the current methods used for whole-genome annotation lag behind our increasing knowledge of how many enzymes and transporters evolve to accommodate new substrates. Namely, that only a few key mutations within an enzyme's active site can imbue it with an entirely new functional role (12, 13). Until more robust computational methods are developed to annotate genomes—methods that rely on more than just thresholds of percentages of shared identity—a certain amount of prudence is needed when predicting the function of a gene product. In turn, it is equally important that new enzyme families be biochemically characterized in order to populate the existing databases with accurate information. As biochemists we must also remain mindful that bioinformatic methods are only as accurate as the biochemical data on which they are based.

References

1. **Liechti, G., and J. B. Goldberg.** 2012. *Helicobacter pylor*i relies primarily on the purine salvage pathway for purine nucleotide biosynthesis. Journal of Bacteriology **194:**839-854.

2. **Jensen, K. F., G. Dandanell, B. Hove-Jensen, and M. Willenoes.** 2008. Nucleotides, nucleosides, and nucleobases. *In* J. Ingraham (ed.), *EcoSal* - *Escherichia coli* and *Salmonella*, Web edition ed. ASM Press, Washington, DC.

3. **Kusters, J. G., A. H. van Vliet, and E. J. Kuipers.** 2006. Pathogenesis of *Helicobacter pylori* infection. Clinical Microbiology Reviews **19:**449-490.

4. **Hedstrom, L., G. Liechti, J. B Goldberg, and D. R Gollapalli.** 2011. The antibiotic potential of prokaryotic IMP dehydrogenase inhibitors. Current Medicinal Chemistry **18:**1909-1918.

5. **Link, A. J., E. Carmack, and J. R. Yates III.** 1997. A strategy for the identification of proteins localized to subcellular spaces: Application to *E. coli* periplasmic proteins. International Journal of Mass Spectrometry and Ion Processes **160:**303-316.

6. **Coldham, N. G., and M. J. Woodward.** 2004. Characterization of the *Salmonella typhimurium* proteome by semi-automated two dimensional HPLC-mass spectrometry: detection of proteins implicated in multiple antibiotic resistance. Journal of Proteome Research **3:**595-603.

7. **Loewen, S. K., S. Y. M. Yao, M. D. Slugoski, N. N. Mohabir, R. J. Turner, J. R. Mackey, J. H. Weiner, M. P. Gallagher, P. J. F. Henderson, and S. A. Baldwin.** 2004. Transport of physiological nucleosides and anti-viral and anti-neoplastic nucleoside drugs by recombinant *Escherichia coli* nucleoside-H+ cotransporter (NupC) produced in *Xenopus laevis* oocytes. Molecular Membrane Biology **21:**1-10.

8. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, and B. A. Dougherty.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

9. **Baltrus, D. A., M. R. Amieva, A. Covacci, T. M. Lowe, D. S. Merrell, K. M. Ottemann, M. Stein, N. R. Salama, and K. Guillemin.** 2009. The complete genome sequence of *Helicobacter pylori* strain G27. Journal of Bacteriology **191:**447-448.

10. **Rost, B.** 1999. Twilight zone of protein sequence alignments. Protein Engineering **12:**85-94.

11. **Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, and T. L. Madden.** 2008. NCBI BLAST: a better web interface. Nucleic Acids Research **36:**W5-W9.

12. **Seffernick, J. L., M. L. de Souza, M. J. Sadowsky, and L. P. Wackett.** 2001. Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different. Journal of Bacteriology **183:**2405-2410.

13. **Ritzel, M. W. L., A. M. L. Ng, S. Y. M. Yao, K. Graham, S. K. Loewen, K. M. Smith, R. G. Ritzel, D. A. Mowles, P. Carpenter, and X. Z. Chen.** 2001. Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system *cib*). Journal of Biological Chemistry **276:**2914-2927.