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Helicobacter pylori Glutamine Synthetase Lacks Features Associated with Transcriptional and Posttranslational Regulation

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***Helicobacter pylori* urease, produced in abundance, is indispensable for the survival of *H. pylori* in animal hosts. Urea is hydrolyzed by the enzyme, resulting in the liberation of excess ammonia, some of which neutralizes gastric acid. The remaining ammonia is assimilated into protein by glutamine synthetase (EC 6.3.1.2), which catalyzes the reaction: NH_3 + glutamate + ATP→glutamine + ADP + P_i. We hypothesized that glutamine synthetase plays an unusually critical role in nitrogen assimilation by *H. pylori*. We developed a phenotypic screen to isolate genes that contribute to the synthesis of a catalytically active urease. *Escherichia coli* SE5000 transformed with plasmid pHP808 containing the entire *H. pylori* urease gene cluster was cotransformed with a pBluescript plasmid library of the *H. pylori* ATCC 43504 genome. A weakly urease-positive 9.4-kb clone, pUEF728, was subjected to nucleotide sequencing. Among other genes, the gene for glutamine synthetase was identified. The complete 1,443-bp *glnA* gene predicts a polypeptide of 481 amino acid residues with a molecular weight of 54,317; this was supported by maxicell analysis of cloned *glnA* expressed in *E. coli*. The top 10 homologs were all bacterial glutamine synthetases, including *Salmonella typhimurium glnA*. The ATP-binding motif GDNGSG (residues 272 to 277) of *H. pylori* GlnA exactly matched and aligned with the sequence in 8 of the 10 homologs. The adenylation site found in the top 10 homologs (consensus sequence, NLYDLP) is replaced in *H. pylori* by NLFKLT (residues 405 to 410). Since the Tyr (Y) residue is the target of adenylation and since the *H. pylori* glutamine synthetase lacks that residue in four strains examined, we conclude that no adenylation occurs within this motif. Cloned *H. pylori glnA* complemented a *glnA* mutation in *E. coli*, and GlnA enzyme activity could be measured spectrophotometrically. In an attempt to produce a GlnA-deficient mutant of *H. pylori*, a kanamycin resistance cassette was cloned into the *Tth*111I site of *H. pylori glnA*. By using the standard technique of allelic exchange mutagenesis, no verifiable glutamine synthetase double-crossover mutant of strain UMAB41 could be isolated, suggesting that the mutation is lethal. We conclude that glutamine synthetase is critical for nitrogen assimilation in *H. pylori* and is active under all physiologic conditions.**

Helicobacter pylori, the etiologic agent of gastritis and peptic ulcer disease in humans, produces urease as one of its most abundant protein components (19). This species produces an anomalously high urease activity compared to the many other bacterial species that synthesize this enzyme (27). Urease is clearly indispensable for *H. pylori*, which cannot survive in an animal model of infection in its absence (5). Urea, the nitrogenous waste product of mammals, is hydrolyzed by the enzyme, resulting in the liberation of an excess of ammonia, some of which is neutralized by gastric acid. The remaining ammonia must also be dealt with.

Ammonia, a preferred nitrogen source for bacteria, is assimilated into protein and other nitrogenous compounds in bacteria by a single pathway (29). Glutamine synthetase (EC 6.3.1.2) catalyzes the reaction NH_3 + glutamate + ATP→glutamine + ADP + P_i (29). Glutamine, in turn, serves as the nitrogen donor for other nitrogenous compounds including alanine, glycine, serine, histidine, tryptophan, CTP, AMP, carbamoyl-phosphate, and glucosamine 6-phosphate (29). Because of the central role of glutamine synthetase in nitrogen metabolism, most species have developed complex regulatory schemes that modulate the expression and activity of the enzyme by the use of multiple promoters (σ^{70} and σ^{54}), positive activators (NR_I and NR_{II}), posttranslational adenylation, and allosteric inhibition by nitrogenous compounds (21).

During *H. pylori* infection of the gastric mucosa, the potential for the generation of ammonia is high. Plentiful urea is provided by the host, and plentiful urease is synthesized by the bacterium (15, 22, 24). Because of the high concentration of urease-generated ammonia produced, we hypothesized that glutamine synthetase plays an unusually critical role in nitrogen assimilation by *H. pylori*. The recent release of the complete nucleotide sequence of the *H. pylori* genome revealed a notable absence of many regulatory pathways found in *Escherichia coli* (34). Our work confirms this general trend for *H. pylori glnA* and its protein product. We describe the isolation and sequencing of the *glnA* gene of *H. pylori* and characterization of the gene product when expressed in *E. coli.*

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *H. pylori* strains were passaged on blood agar (brucella agar supplemented with 10% [vol/vol] sheep blood) at 37°C in an anaerobic jar with palladium catalyst and an activated Campypak (Becton-Dickinson). Isolates were stored at −70°C in Trypticase soy broth (BBL) supplemented with 15% (vol/vol) glycerol. For liquid culture, *H. pylori* strains were inoculated from fresh blood agar plates into 250 ml of Mueller-Hinton broth supplemented with 4% (vol/vol) fetal calf serum in a 500-ml flask. Cultures were incubated for 48 h with shaking (200 rpm) at 37°C in an anaerobic jar containing an activated Campypak.

Gene bank preparation. Chromosomal DNA isolated from *H. pylori* ATCC 43504 was used for preparation of a λ ZAPII genomic library (Stratagene) as described previously (25). Chromosomal DNA isolated from *H. pylori* UMAB41

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype and comments	Source or reference
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*; used as the recipient for electroporation	31
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^r)]	Stratagene
SURE	*recB recF sbcC201 uvrC umuC*::Tn*5* (Kan^r) *lac*Δ(*hsdRMS*) *endA1 gyrA96 thi relA1 supE44* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^r)]	Stratagene
SE5000	*araD139*Δ(*argF-lac*)*U169 rpsL150 relA1 flb-3501 deoC1 ptsF25 rbsR recA56*	F. C. Gherardini (10)
M5004	M5004 [λ$^-$ *trpA9825*(Oc), IN(*rrnD-rrnE*)*1 glnA3*], a glutamine synthetase-deficient mutant	M. Berlyn, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. (23)
W3110	Glutamine synthetase-sufficient parent strain of M5004 [λ$^-$ IN(*rrnD-rrnE*)*1 rph1*]	M. Berlyn, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. (23)
H. pylori		
UMAB41	Isolated from a gastric biopsy specimen taken by endoscopy from a patient with complaints of abdominal pain and a history of peptic ulcer disease	27
Leung	Gastric biopsy isolate	J. Gilbert (11)
ATCC 43504	Human gastric antrum, Australia	American Type Culture Collection (12)
HUH1	Gastric biopsy isolate	D. Smoot, Howard University School of Medicine
HUH40	Gastric biopsy isolate	D. Smoot, Howard University School of Medicine
X47ZAL	Gastric biopsy isolate	H. Kleanthous, Oravax
Plasmids		
pHP1	1,489-bp fragment conferring Kan^r originating from streptococcal plasmid pJH1, cloned into pUC	H. Kleanthous (35)
pSKCAT4	1.5-kb *Eco*RI fragment derived from *H. pylori* NTCC 11639 plasmid pUOA26 cloned into the *Eco*RI site of pBluescript SK$^-$, chloramphenicol cassette	J. Gilbert (38)
pHC79	Cosmid vector, 6.43-kb Amp^r Tet^r derived from pBR322	17 (GenBank accession no. L08873)
pUEF728	λZAPII genomic clone of *H. pylori* ATCC 43504 converted to a pBluescript-based library clone; carries entire *glnA* gene	This study
pHP808	pHP9D11 cosmid clone derivative encoding the entire urease gene cluster	18
pUEF16-7A	Cosmid clone of *H. pylori* UMAB41	This study
pUEF730	1.5-kb *Kpn*I-*Spe*I *glnA*-containing fragment cloned into the *Kpn*I-*Spe*I site of pBluescript SK$^+$	This study

was used for preparation of a pHC79-based cosmid gene bank with *Sau*3A partials as described previously (18).

pBluescript plasmid library. The λ ZAPII genomic library phage suspension (10^7 phage) was incubated with *E. coli* XL1-Blue (adjusted to an optical density at 600 nm [OD_{600}] = 1.0) along with 10^6 Exassist helper phage for 15 min at 37°C. The suspension was then added to 20 ml of Luria broth and incubated at 37°C for 2 h and then at 65°C for 20 min. The suspension was centrifuged (4,500 rpm [2,400 × *g*] for 10 min at 4°C), and the supernatant was collected. Supernatant (1 μl) was added to a suspension (OD_{600} = 1.0) of *E. coli* SURE and incubated at 37°C for 15 min. After incubation, 100 μl of the suspension was plated onto Luria agar plates containing ampicillin (200 μg/ml) and incubated for 18 h at 37°C (14). Colonies from 20 plates were pooled and used for a large-scale plasmid preparation, which represented the pooled pBluescript plasmid library.

Plasmid isolation. Plasmid DNA was isolated by alkaline sodium dodecyl sulfate (SDS) extraction (2). DNA was purified on plasmid purification columns (Qiagen tip-100) as directed by the manufacturer.

Recombinant DNA methods. Recombinant DNA techniques, including restriction endonuclease digestion, ligation, and transformation, were done by standard techniques (2, 31).

DNA hybridization. DNA restriction fragments isolated from a λZAPII gene bank clone were used as gene probes to identify pHC79-based cosmid clones containing complete *glnA* sequences. Fragments were labeled with [γ-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham) and used to probe dot blots of the cosmid gene library. Southern blots of cosmid clones digested with various restriction enzymes were prepared by standard methods (31) and developed with the enhanced chemiluminescence system (Amersham) as specified by the manufacturer.

Nucleotide sequencing. Double-stranded DNA was used as a template for sequencing by the dideoxy chain termination method (32). The reactions were performed with reagents from the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems) and *Taq* polymerase. A model 373A DNA Sequencer (Applied Biosystems) was used, and the sequence was determined in each direction. DNAsis software (Hitachi, version 2.1) was used for analysis of the DNA sequence, base composition, identification of open reading frames (ORFs), restriction sites, and other basic analyses. Apparent homologies between ORFs were sought in GenBank and SwissProt data bases with Wisconsin Package programs (version 8.1; Genetics Computer Group, Inc.).

Electroporation. Plasmid DNA was introduced into *H. pylori* with a Gene Pulser electroporator (Bio-Rad) in 15% glycerol–9% sucrose at 12.5 kV/cm, 25-μF capacitance, and 200 Ω by the method of Ferrero et al. (7).

PCR and oligonucleotide primer design. PCR was performed as previously described for *H. pylori* (9, 37) with primers designed from *aphA* (35), *glnA* (determined in this report), and vector pBluescript SK (GenBank accession no. X52330) sequences. Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems automated DNA synthesizer model 380B.

Protein labeling in maxicells. Plasmid-encoded polypeptides were labeled with [^{35}S]methionine (specific activity, 800 to 1,000 Ci/mmol; Amersham Corp.) by using UV-treated *E. coli* SE5000 transformed with pBluescript or pUEF730 and the method of Gherardini et al. (10). Labeled polypeptides were solubilized in SDS-gel sample buffer, electrophoresed on an SDS–12% polyacrylamide gel, and visualized by autoradiography.

Glutamine synthetase assay. Glutamine synthetase activity was quantitated by an assay that measures the synthesis of γ-glutamylhydroxamate from glutamine and hydroxylamine. A spectrophotometric assay that measures γ-glutamylhydroxamate formation as a gauge of the total amount of glutamine synthetase present has been described by Bender et al. (4). Soluble protein (300 μg) derived from French pressure cell lysates (bacterial suspensions passaged at 20,000 lb/in^2) was assayed in a reaction volume of 8 ml. Aliquots (500 μl) were taken at 30-min intervals and placed in 1 ml of stop mix (55 g of $FeCl_3 \cdot 6H_2O$ per liter, 20 g of trichloroacetic acid per liter, 21 ml of concentrated HCl per liter). The precipitate was removed by centrifugation, and the absorbance was measured at 540 nm.

Urease assays. *H. pylori* was harvested from liquid culture by centrifugation (10,000 × *g* for 10 min at 4°C), washed twice with 20 mM sodium phosphate (pH

6.8), suspended in 5 ml of 20 mM sodium phosphate (pH 6.8)–5 mM dithiothreitol–1 mM EDTA, and ruptured in a precooled French pressure cell at 20,000 lb/in^2. Lysates were centrifuged (27,000 $\times$ *g* for 30 min at 4°C), and the supernatants were removed with a Pasteur pipette and used directly for the assay. The protein concentration was determined by the bicinchoninic acid method as specified by the manufacturer (Pierce) with bovine serum albumin as a standard. Rates of urea hydrolysis were measured by the spectrophotometric assay of Hamilton-Miller and Gargan (13) calibrated for the measurement of ammonia as described previously (28).

Allelic exchange mutagenesis. A kanamycin resistance cassette (35), isolated on a 1.5-kb *Eco*RV fragment from pHP1 (kindly provided by H. Kleanthous, Oravax), was cloned into the *Tth*111I site of plasmid pUEF730 which had been rendered blunt. This construct was electroporated into *H. pylori* ATCC 43504 and Leung. Electrotransformants were obtained on brucella agar plates containing 4% sheep blood, kanamycin (50 μg/ml), and L-glutamine supplementation at 50 μg/ml or 1 mg/ml. Initially, growth was more substantial at the higher glutamine concentration but was not as robust as that of the wild type. A putative glutamine synthetase mutant was designated HP-GS1. With subsequent passage, the growth of HP-GS1 became hardy on brucella agar containing 4% sheep blood and kanamycin (50 μg/ml) with and without 1 mg of L-glutamine per ml. These results were more consistent with the presence of a cointegrate in *H. pylori* rather than the presence of a double-crossover mutation in *glnA*. PCR was used to evaluate the construct.

Nucleotide sequence accession numbers. The sequences of the 1,443- and 523-bp ORFs have been deposited in GenBank under accession no. AF053357 and AF053715, respectively.

RESULTS

Urease-enhancing factor assay. As a part of a strategy to investigate genes that contribute to the synthesis of a catalytically active urease, we developed a phenotypic screen to isolate such genes. *E. coli* SE5000 transformed with plasmid pHP808 containing the entire *H. pylori* urease gene cluster was cotransformed with a pBluescript plasmid library of the *H. pylori* ATCC 43504 genome. Cam^r Amp^r colonies were plated on urea segregation agar, a medium designed to identify urease-positive colonies [*E. coli* SE5000(pHP808) does not produce sufficient urease on this medium to give a positive reaction]. One strongly positive clone has been identified previously as encoding NixA, a high-affinity nickel transporter (25). An additional clone was identified that turned positive for urease after 3 days of incubation. The urease activity was qualitatively positive but was not quantifiable by a spectrophotometric assay.

Nucleotide sequencing and plasmid pUEF728. We recovered a 9.4-kb plasmid, pUEF728, in addition to pHP808 from the weakly urease-positive clone and subjected the plasmid to restriction mapping. The *H. pylori* chromosomal DNA insert, released by digestion with *Eco*RI, was estimated to be 6.5 kb. By using universal pBluescript T3 and T7 primers and additional primers based on the acquired sequence, the nucleotide sequence of approximately 1.8 kb was determined for each end of the insert. Two significant ORFs of 523 and 1,284 bp were identified, one at each end of the insert. The first ORF predicted a truncated polypeptide of 174 amino acids and a molecular weight of 20,487. The most closely related polypeptide, identified by WORDSEARCH in GenBank, was that of a GTP-binding protein of *E. coli* (1). The second ORF, based on very strong amino acid sequence identity to other gene bank entries, appeared to represent the *H. pylori glnA* gene encoding glutamine synthetase. After inspection of amino acid sequence alignments, the 3′ end of the gene did not, however, appear to be complete and was found to be fused to vector sequences.

To obtain the remaining portion of *glnA*, a 1,418-kb *Hph*I-*Pvu*II fragment of *glnA* (bp 527 to 1945) (Fig. 1) was isolated from pUEF728 and used as a probe to identify cosmid clones also containing these sequences. Of 2,304 pHC79-based cosmid clones screened, two hybridized strongly with the probe. One of these, designated pUEF16-7A, was isolated. A Southern blot of this cosmid DNA, digested with *Hin*dIII, was probed independently with two probes: a 1-kb *Afl*II-*Tth*111I fragment (upstream sequences and the 5′ end of *glnA*) and a 0.4-kb *Tth*111I-*Eag*I fragment (3′ end of *glnA*). Both probes hybridized to a 3.8-kb *Hin*dIII. This fragment was subcloned into pBluescript and subjected to nucleotide sequencing with a primer designed from sequences near the 3′ end of the truncated *glnA* on the previous clone. Overlapping sequences representing the end of *glnA* were identified and used to compile the complete *glnA* ORF (Fig. 1).

***H. pylori glnA*.** The complete 1,443-bp *glnA* gene predicts a polypeptide of 481 amino acid residues with a molecular weight of 54,317. The G+C content of the ORF is 42.4%, slightly higher than the 39% for total genomic DNA (12, 34). An apparent Shine-Dalgarno site of AGG is located 7 bp upstream of the translational start site. No σ^{70} or σ^{54} promoter sequence is readily identifiable, nor is a *rho*-independent transcriptional terminator located downstream of the 3′ end of the gene. Analysis of sequences upstream (573 bp) and downstream (451 bp) of *glnA* did not reveal any homologs related to ammonia assimilation or nitrogen regulation. However, the sequence of the entire insert of pUEF728 was not determined. Examination of this region in the complete genome sequence of *H. pylori* ATCC 26695 (34) confirmed the lack of additional nitrogen regulatory homologs immediately upstream and downstream of *glnA*. A short ORF upstream of *glnA* predicts a 38-amino-acid peptide with no homologs. An ORF downstream of *glnA* predicts a polypeptide of 78 kDa with two homologs elsewhere in the *H. pylori* genome, also with no known functions. The upstream and downstream ORFs are both transcribed in the direction opposite to that of *glnA*, suggesting that they are not part of an operon.

***H. pylori* GlnA.** The predicted GlnA amino acid sequence was used to search the SwissProt data base for similar proteins by using WORDSEARCH. The top 10 matches were all bacterial glutamine synthetases from (most to least similar) *Salmonella typhimurium*, *Azotobacter vinelandii*, *Methylococcus capsulatus*, *E. coli*, *Azospirillum brasilense*, *Synechocystis* sp., *Proteus vulgaris*, *Fremyella diplosiphon*, *Neisseria gonorrhoeae*, and *Vibrio alginolyticus*. When aligned, the most similar homolog, *S. typhimurium* GlnA, was found to have 48.3% amino acid sequence identity and 65.3% amino acid sequence similarity (identical residues and conservative replacements) (Fig. 2). The least similar of the top 10 homologs, *V. alginolyticus* GlnA, has 38.7% identity and 57.4% similarity.

The ATP-binding motif GDNGSG (residues 272 to 277) exactly matched and aligned with the sequence in 8 of the 10 homologs and was similar to sequences in the other two matches (five of six for *Synechocystis* sp. and four of six for *M. capsulatus*).

Lack of adenylation site. The adenylation site found in all of the 10 most similar homologs (consensus sequence, NL<u>Y</u>DLP) (33) is replaced in *H. pylori* by NL<u>F</u>KLT (residues 405 to 410). Since the Tyr407 residue is the well-conserved target of adenylation (33) and since the *H. pylori* glutamine synthetase apparently lacks that residue, we concluded that no adenylation occurs for this enzyme within this motif. Another tyrosine-containing sequence, NPYLAF (residues 376 to 381), is found upstream of the consensus adenylation site and is well conserved among the homologs but has not been demonstrated as an adenylation site.

Since the change from Tyr407Phe could have resulted from a single-base-pair change (T1219A), we determined the sequence of this region of *glnA* from other strains. Using primers directed against sequences centered 31 bp upstream of the start codon of *glnA* and 21 bp downstream of *glnA*, we amplified a 1,522-bp sequence from two additional strains (HUH1

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CT TGA TCA ACT TGC ACC CAT GTG GAT GCA AGA GCG GTT TCC TAC C          45
TT TCA TTG AAA TCT AGG CTA GGA AAG TTA AGA TTT AAA GCA GGA A          90
GA ACA CTT CCC AAA ATA TTC TCT AGA TGC TGT ATC TTT TTA CCT T         135
GT TCG CGA TTT AGA GAA AGT GAA TAA TAA AGT GCT TCA TTA AGT C         180
CC TTG TTT TCA TCT TTA AGT CTT TCA TTC TGA TCT AAG GTC TGC T         225
TA AGA CCG CCA TTC AAA TAT GCG GCT TCA GCA TTA TAC AGA GCC G         270
CA CCT TTT AAA GGT TTT CCG TAC CTA TCG CAG AAA ACT CCA TTT A         315
TA AGT AAG AAG TTT TGT TTC ATA ATT TTT CCT TAA ATT TAA AAT A         360
AC ACC CCA TTT CTA GGT AGA GAT AGG GCG GCA ATT ATA CTT ATA A         405
CT AGC AAA CGA ATA ATA CCA CAA TAA AAG AAA CAA AAT GCC AAA A         450
TT AAA AAA GGT GGG TTT TAG TCT CTA TAA AAT TTA GAG CAT CAA T         495
TC TAC TTT TTT TAC CCC ATA AAA TGC TAT AAT CAC CCC TAT CAA T         540
                               S.D.
CA AAC TCA ATT CAT AAC AAT TAA AGG TGG TTA ATG ATA GTA AGA A         585
                               ---
                                            M   I   V   R  T   5
CT CAA AAT AGT GAA AGC AAG ATC AAA GAA TTT TTT GAA TTT TGC A         630
    Q   N   S   E   S   K   I   K   E   F   F   E   F   C  K   20
AA GAA AAT GAA GTG GAA TTT GTG GAT TTT AGA TTC AGC GAT ATT A         675
    E   N   E   V   E   F   V   D   F   R   F   S   D   I  K   35
AA GGC ACT TGG AAT CAC ATC GCT TAT TCT TTT GGG GCT TTA ACG C         720
    G   T   W   N   H   I   A   Y   S   F   G   A   L   T  H   50
AT GGC ATG TTT AAA GAG GGG ATT CCT TTT GAT GCG AGT TGT TTT A         765
    G   M   F   K   E   G   I   P   F   D   A   S   C   F  K   65
AG GGC TGG CAA GGC ATT GAA CAC TCC GAT ATG ATT TTA ACC CCC G         810
    G   W   Q   G   I   E   H   S   D   M   I   L   T   P  D   80
AT TTG GTG CGT TAT TTC ATT GAT CCT TTT AGC GCA GAT GTG AGC G         855
    L   V   R   Y   F   I   D   P   F   S   A   D   V   S  V   95
TG GTC GTG TTT TGC GAT GTG TAT GAT GTG TAT AAA AAC CAG CCT T         900
    V   V   F   C   D   V   Y   D   V   Y   K   N   Q   P  Y   110
AT GAA AAA TGC CCC AGA AGT ATC GCT AAA AAA GCC TTA CAG CAT T         945
    E   K   C   P   R   S   I   A   K   K   A   L   Q   H  L   125
TA AAA GAT TCA GGT TTG GGC GAT GTG GCT TAT TTT GGT GCG GAG A         990
    K   D   S   G   L   G   D   V   A   Y   F   G   A   E  S   140
GC GAA TTT TTC ATC TTT GAT TCC ATT AAA ATT AAA GAC GCT TCC A        1035
    E   F   F   I   F   D   S   I   K   I   K   D   A   S  N   155
AT TCC CAA TAC TAC GAA GTG GAT AGC GAA GAA GGC GAA TGG AAT C        1080
    S   Q   Y   Y   E   V   D   S   E   E   G   E   W   N  R   170
GG GAT AGG AGC TTT GAA AAT GGC GTG AAT TTT GGG CAT AGA CCG G        1125
    D   R   S   F   E   N   G   V   N   F   G   H   R   P  G   185
GC AAG CAA GGG GGC TAT ATG CCT GTG CCG CCA ACG GAT ACG ATG A        1170
    K   Q   G   G   Y   M   P   V   P   P   T   D   T   M  M   200
TG GAT ATT CGC ACT GAA ATT GTG AAA GTC TTA AAC CAA GTG GGG T        1215
    D   I   R   T   E   I   V   K   V   L   N   Q   V   G  L   215
TA GAA ACT TTT GTC GCC CAT CAT GAA GTC GCG CAA GCG CAA GGC G        1260
    E   T   F   V   A   H   H   E   V   A   Q   A   Q   G  E   230
AA GTG GGC GTG AAA TTT GGG GAT TTA GTG GAA GCC GCT GAC AAT G        1305
    V   G   V   K   F   G   D   L   V   E   A   A   D   N  V   245
TC CAA AAA CTC AAA TAT GTG GTT AAA ATG GTC GCT CAT TTA AAC G        1350
    Q   K   L   K   Y   V   V   K   M   V   A   H   L   N  G   260
GC AAA ACC GCC ACT TTC ATG CCA AAA CCT TTA TAC GGG GAT AAC G        1395
    K   T   A   T   F   M   P   K   P   L   Y   G   D   N  G   275
GG AGC GGG ATG CAC ACC CAT GTG AGC GTT TGG AAA AAC AAC GAA A        1440
    S   G   M   H   T   H   V   S   V   W   K   N   N   E  N   290
AC CTT TTT AGC GGC GAA ACT TAT AAG GGC TTG AGT GGG TTA GCG T        1485
    L   F   S   G   E   T   Y   K   G   L   S   G   L   A  L   305
TG CAT TTT TTA GGG GGT GTG TTG CGT CAC GCT AGA GGG TTA GCC G        1530
    H   F   L   G   G   V   L   R   H   A   R   G   L   A  A   320
CT TTC ACT AAC GCT TCC ACT AAT TCT TAC AAA CGC CTA ATT CCA G        1575
    F   T   N   A   S   T   N   S   Y   K   R   L   I   P  G   335
GC TAT GAA GCC CCA TCT ATT TTA ACT TAT TCA GCT AAC AAC AGG A        1620
    Y   E   A   P   S   I   L   T   Y   S   A   N   N   R  S   350
GC GCT AGC GTG CGT ATC CCT TAT GGG ATT TCT AAA AAT AGC GCG A        1665
    A   S   V   R   I   P   Y   G   I   S   K   N   S   A  R   365
GG TTT GAA TTC AGG TTT CCT GAC AGC TCA TCA AAC CCC TAC CTG G        1710
    F   E   F   R   F   P   D   S   S   S   N   P   Y   L  A   380
CT TTT GGG GCT ATT TTA ATG GCG GGC ATT GAT GGC ATT AAA AAT A        1755
    F   G   A   I   L   M   A   G   I   D   G   I   K   N  K   395
AA ATG GAT CCC GGC GAA GCG ATG GAC ATT AAC CTT TTC AAG CTA A        1800
    M   D   P   G   E   A   M   D   I   N   L   F   K   L  T   410
CT TTA GAC GAA ATT ATA GAA CAG GGT ATC ATA CAA ATG CCC CAC A        1845
    L   D   E   I   I   E   Q   G   I   I   Q   M   P   H  T   425
CT TTA AGG AGA TCA TTA GAA GAA ATG CTA GCC GAT AAG CAG TAT C        1890
    L   R   R   S   L   E   E   M   L   A   D   K   Q   Y  L   440
TA AAA CAG GGT CAG GTC TTT AGC GAA CAA TTT ATC CAC GCC TAT C        1935
    K   Q   G   Q   V   F   S   E   Q   F   I   H   A   Y  Q   455
AG TCT CTT AAA TTC CAT TCT GAA GTG TTC CCA TGG GAG AGC AAA C        1980
    S   L   K   F   H   S   E   V   F   P   W   E   S   K  P   470
CC CAT CCT TTT GAA TTT ATC ACC ACT TAT TCA TGC TAA AAC AAT G        2025
    H   P   F   E   F   I   T   T   Y   S   C   *              481
AG TGG GTT CAT AAA TCC CAC TCT AAA AAT CTA GAA TAA GGC AAA A        2070
AT ACC TTA TTT TTA AAA AGA GTG GTT GAA AGA ATT GAT TTT CTT T        2115
GT TTG TTG GTT TTA TTT TAA TAG GGT ATT GAT CAT AAA ATT TAA A        2160
```

FIG. 1. Nucleotide sequence of *H. pylori glnA*. The nucleotide sequence for *glnA* and the predicted amino acid sequence are shown. A Shine-Dalgarno (S.D.) sequence is underlined. *, stop codon.

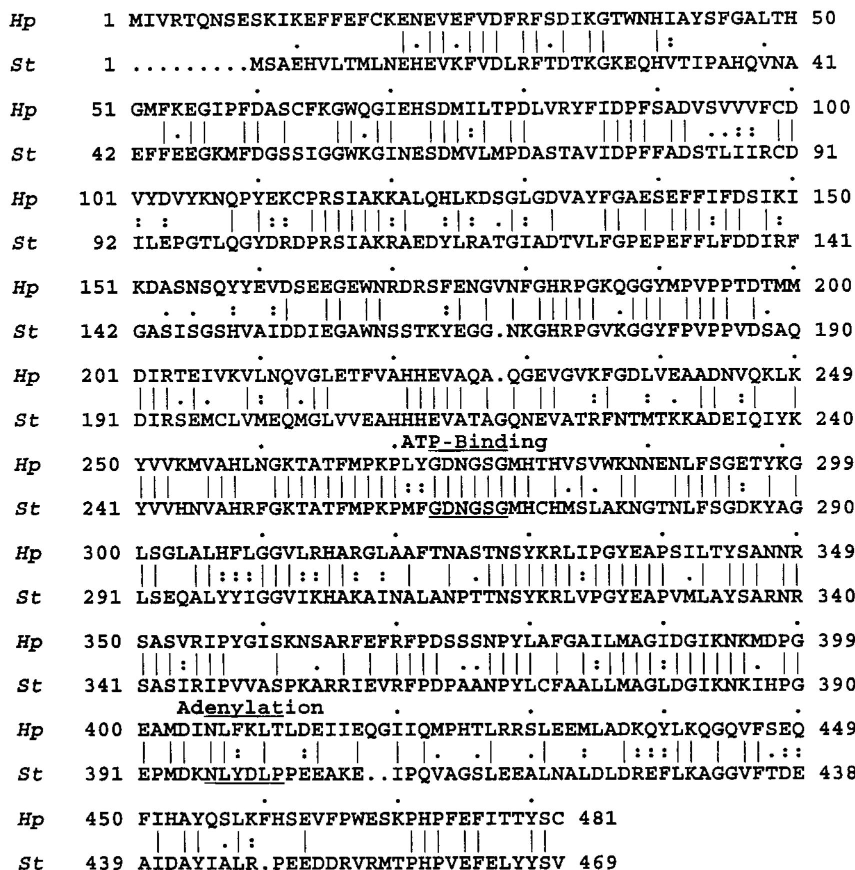

FIG. 2. Alignment of *H. pylori* GlnA and *S. typhimurium* GlnA. The predicted amino acid sequences of the glutamine synthetases of *H. pylori* (*Hp*) and *S. typhimurium* (*St*) were aligned. Identical residues are denoted by a vertical line; conservative replacements are shown by a colon (:). An ATP-binding motif and the adenylation site (Tyr residue within the motif) are identified by lines drawn above the sequences.

and HUH40) and determined the nucleotide sequence. The Tyr407Phe change is conserved in these two additional strains (Fig. 3) and also in strain 26695, for which the complete genome sequence was determined (34). This suggests that the activity of *H. pylori* glutamine synthetase is indeed not modulated by adenylation and thus is not regulated by posttranslational modification.

Protein labeling in maxicells. To demonstrate whether a GlnA product of the predicted size was produced by plasmid pUEF730, plasmid-encoded gene products were labeled with [^{35}S]methionine in maxicells. Proteins encoded by this plasmid were analyzed on autoradiographs of an SDS–12% polyacrylamide gel; proteins synthesized by the pBluescript vector were included as a control (Fig. 4). In addition to vector-encoded polypeptides, pUEF730 encoded a polypeptide estimated to be 56 kDa (Fig. 4, arrow), consistent with the molecular weight of 54,317 predicted by the nucleotide sequence.

Complementation of an *E. coli glnA* mutant. To determine whether cloned *H. pylori glnA* can complement a *glnA* mutation in *E. coli*, plasmid pUEF730 carrying *glnA* on a 1,522-bp *Spe*I-*Kpn*I fragment cloned into the *Spe*I-*Kpn*I site of pBluescript was constructed. Plasmid pUEF730 or vector pBluescript SK+ was transformed into *E. coli* W3110 and its *glnA*-deficient mutant M5004. These strains were plated onto minimal salts agar supplemented with tryptophan (20 μg/ml) and L-glutamine (0, 50, or 1,000 μg/ml). All strains grew within 48 h in the presence of glutamine (Fig. 5). *E. coli* M5004(pBluescript), however, was unable to grow in the absence of glutamine, whereas *E. coli* M5004(pUEF730) and W3110 grew as individual colonies after 48 h. Thus, expression of cloned *H. pylori glnA* is able to complement a *glnA* mutation of *E. coli*. The 48 h needed for growth of the complemented mutant is consistent with the observation that the cloned expression of *H. pylori* GlnA activity is weak compared to that of native *E. coli*.

Glutamine synthetase activity. To determine whether the catalytic activity of glutamine synthetase could be detected in *H. pylori* and *E. coli* transformed with cloned *H. pylori glnA*, a spectrophotometric assay for glutamine synthetase was used. Use of a standard spectrophotometric assay (4) that measures total glutamine synthetase activity without regard to the ad-

ADENYLATION SITE

UMAB41	399	G E A M D I N L **F** K L T L D E I R E	416
HUH1	399	G E A M D I N L **F** K L T L D E I R E	416
HUH40	399	A K A M D I N L **F** K L T L D E I R E	416
ATCC26695	399	G E A M D I N L **F** K L T L D E I R E	416
St	390	G E P M D K N L **Y** D L P P E E A K E	406

FIG. 3. Adenylation site of *H. pylori* GlnA. The predicted amino acid sequences of the conserved adenylation site of GlnA from four *H. pylori* strains (three determined in this study and ATCC 26695 [34]) and *S. typhimurium* (accession no. P06201) (St) are aligned. The Tyr residue in *S. typhimurium* is the site of adenylation in this and most species but is replaced with Phe in *H. pylori.*

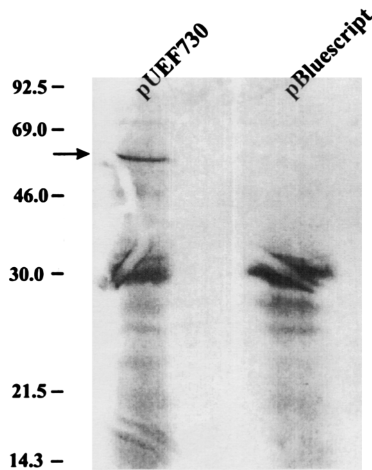

FIG. 4. Autoradiograph of plasmid-encoded polypeptides. *E. coli* SE5000(pBluescript) and SE5000(pUEF730) were UV-irradiated and plasmid-encoded polypeptides were labeled with [^{35}S]methionine and electrophoresed on an SDS–15% polyacrylamide gel, which was dried and autoradiographed. The mobilities of molecular mass markers are shown in kilodaltons at the left. The arrow points to a polypeptide synthesized by pUEF730 but not by pBluescript.

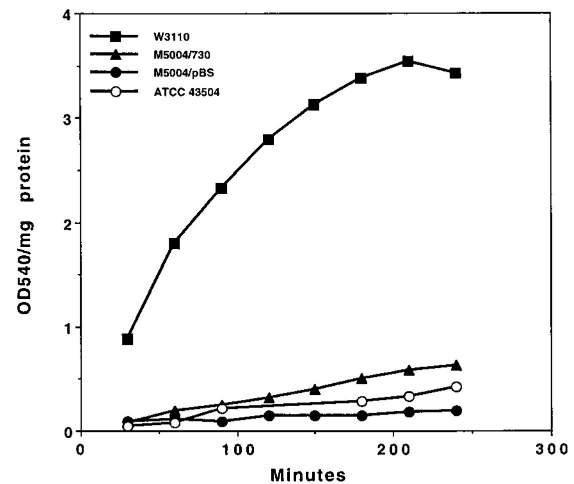

FIG. 6. Glutamine synthetase activity of *E. coli* transformed with plasmids. Glutamine synthetase activity was measured in *E. coli* W3110 (wild type for glutamine synthetase activity) and *E. coli* M5004 (glutamine synthetase mutant) transformed with either vector pBluescript (pBS) or pUEF730 (730) carrying *H. pylori glnA*. For reference, the activity in *H. pylori* ATCC 43504 is also included. Values are the averages of five independent determinations. For determinations made with the *E. coli* strain, each curve is significantly different from the other two ($P < 0.001$).

enylation status of the enzyme revealed significant activity in *H. pylori* and *E. coli* M5004(pUEF730) (Fig. 6). *E. coli* M5004, the *glnA*-deficient mutant, transformed with pUEF730 encoding *H. pylori* GlnA showed significantly higher rates of activity at 0.0029 OD_{540}/min/mg of protein ($P < 0.0001$) than did the same strain transformed with pBluescript, which had no significant activity (0.0001 OD_{540}/min/mg of protein). The glutamine synthetase activity of *E. coli* M5004(pUEF730), however, was significantly lower ($P < 0.0001$) than that of *E. coli* W3110 (0.0241 OD_{540}/min/mg of protein), the parent strain containing the wild-type *E. coli glnA* gene. The weaker GlnA activity of the complemented *E. coli* mutant [*E. coli* M5004(pUEF730)] was consistent with the slower growth of this strain than of the GlnA-sufficient parent strain, *E. coli* W3110.

Mutation of glutamine synthetase may be lethal to *H. pylori*. To determine the physiological consequences of mutation of *glnA*, we attempted to construct a GlnA-deficient mutant of *H. pylori* (see Materials and Methods) (Fig. 7A); four strains (UMAB41, ATCC 43504, Leung, and X47ZAL) were electroporated on approximately 30 different occasions with pUEF760. For UMAB41, no Kan^r colonies were ever isolated. For the other strains, pinpoint Kan^r colonies were observed but could not be successfully passaged. For example, one isolate, a putative glutamine synthetase mutant designated HP-GS1, was observed on blood agar containing L-glutamine (50 μg/ml). This mutant could not be successfully propagated, and because of the inability to obtain a sufficient number of cells, the presence of a double-crossover mutation could not be verified.

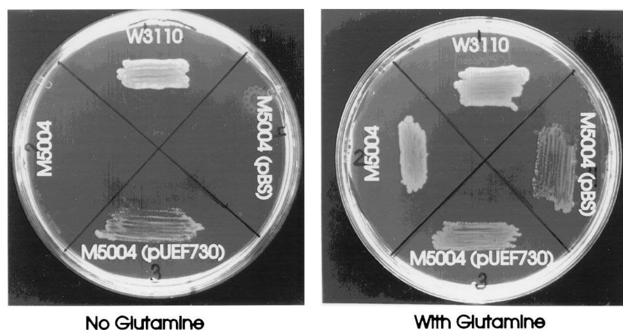

FIG. 5. Complementation of an *E. coli glnA* mutant with cloned *H. pylori glnA*. A glutamine synthetase mutant of *E. coli*, strain M5004, transformed or not with plasmid pUEF730 (contains *H. pylori glnA*) or pBluescript vector, and the parent strain, W3110 (produces glutamine synthetase), were plated onto minimal medium supplemented or not with glutamine (50 μg/ml). The plates were photographed after 48 h of growth at 37°C.

In subsequent experiments, some of the Kan^r isolates, obtained on blood agar supplemented with L-glutamine, were successfully passaged but regained the vigorous growth properties of the wild-type strains. These colonies retained glutamine synthetase activity. PCR analysis (Fig. 7B) of one of these strains derived from ATCC 43504 demonstrated that this isolate represented a cointegrate of the plasmid into the chromosome. When primers specific for the *glnA* ORF (GlnA2$^+$ and GlnA2$^-$) were used for PCR amplification, a 0.83-kb fragment was amplified from the parent strain; in the cointegrate, both a 0.83-kb fragment (wild-type size) and a 2.17-kb fragment (representing *glnA*::*aphA*) were amplified, indicating that both the wild-type gene and the insertionally inactivated gene were present. When one *glnA* (GlnA2$^+$) and one *aphA* (KanF$^-$) primer were used, a product was not amplified from the parent strain but the expected 1.48-kb product was amplified from the cointegrate. When primers specific for the pBluescript vector (pBS$^+$ and pBS$^-$) were used, vector sequences (1.34-kb) were amplified from the cointegrate and plasmid constructs but not from the parent strain. These results are all consistent with the cointegration of pUEF760 (the insertional inactivation construct) into the chromosome of *H. pylori.*

DISCUSSION

We have described how the glutamine synthetase gene of *H. pylori* has been cloned, sequenced, and expressed in *E. coli*. The amino acid sequence of the polypeptide predicted from the nucleotide sequence closely aligned with the amino acid sequences of previously described glutamine synthetases from

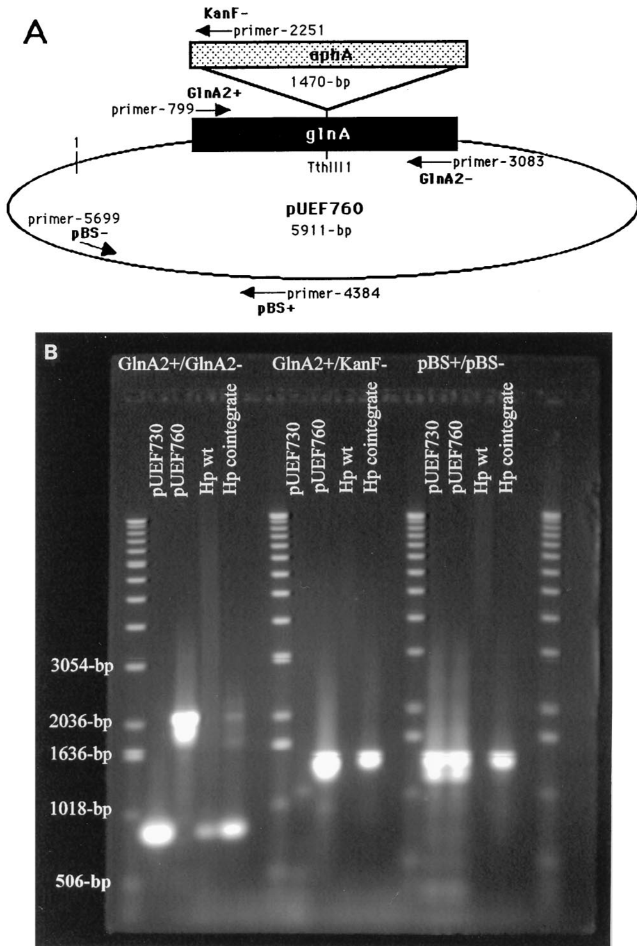

FIG. 7. Constructs used in allelic exchange mutagenesis. (A) The *H. pylori glnA* gene, cloned into pBluescript, was insertionally inactivated by cloning a kanamycin resistance cassette into the *Tth*111I site (see Materials and Methods). The construct was electroporated into *H. pylori* strains and plated on blood agar containing kanamycin (50 μg/ml) supplemented or not with L-glutamine (50 μg/ml or 1 mg/ml). The location of primers used for the PCR analysis are shown along with their coordinates (given in base pairs with reference to the "1" on pBluescript vector). (B) PCR analysis of the parental strain and putative *glnA* cointegrate. Purified plasmid or chromosomal DNA was used as the template along with primer pairs (top) identified in panel A for PCR amplification of fragments specific for *glnA*, *glnA*::*aphA*, or pBluescript sequences. Plasmid pUEF730 contains intact *glnA*; plasmid pUEF760 carries insertionally inactivated *glnA*. Hp wt is wild-type strain ATCC 43504, and Hp cointegrate is the putative cointegrate. Sizes of relevant standards are given in base pairs.

other bacterial species (nearly 50% amino acid sequence identity to GlnA of *S. typhimurium*). The cloned *H. pylori* gene complemented a *glnA* mutation in *E. coli*, produced measurable glutamine synthetase activity, and encoded a polypeptide of the expected size (~54 kDa) when expressed in *E. coli* maxicells.

The ATP-binding motif (33), required for activity, is well conserved in the *H. pylori* enzyme. However, the residue (Tyr407) within the adenylation motif, necessary for regulation by posttranslational modification, is absent in the *H. pylori* GlnA in all strains tested, as well as in strain ATCC 26695. Since adenylation is the key mechanism used to turn off enzymatic activity following translation of the enzyme, the glutamine synthetase may be catalytically active under all environmental conditions. This may be necessary because of the constant release of ammonia due to urease-mediated urea hydrolysis. The lack of such control is an exception rather than the rule among bacterial glutamine synthetases, since the enzymes from most species have rigorously conserved the Tyr-containing adenylation site (conserved in all 10 of the most closely related GlnA homologs). When the consensus adenylation site itself was used to search protein databases, 31 alignments from bacterial species were identified. Of these, only three lack the conserved Tyr residue, replacing it with Phe: *Clostridium acetobutylicum* (36), *Lactobacillus delbrueckii* (20), and *Methanococcus voltae* (accession no. P21154). Biochemical evidence is also provided, without the nucleotide sequence, that the GlnA of the cyanobacterium *Anabaena* does not undergo adenylation (8).

In most procaryotes, the ammonia assimilation pathway mediated by glutamine synthetase is regulated at every level including transcriptional regulation (σ^{70} and σ^{54} promoters, positive transcriptional activator NR_I or NtrC, and catabolite activator protein-binding site), translational regulation (strength of Shine-Dalgarno sequence), posttranslational regulation (adenylation of the conserved Tyr residue), and allosteric regulation by the binding of certain nitrogenous compounds (29). For *H. pylori*, which has a relatively modest-sized genome of 1.7 Mb (34), one could speculate that this level of complexity of regulation may be absent or reduced. Evidence for this is the absence of NtrC or NtrB homologs encoded by sequences downstream of *glnA*, their usual location in procaryotes (21).

H. pylori appears to lack proteins required for transcriptional and posttranslational regulation of glutamine synthetase. Consistent with the observation that none of the *H. pylori* strains tested possessed the conserved Tyr407 residue, which is the target of adenylation in glutamine synthetase, homologs of proteins which are required to carry out this adenylation in other gram-negative enteric bacterial species do not appear to be present in the *H. pylori* genome. In *E. coli*, the P_{II} protein (GlnB), uridylyltransferase/uridyl-removing enzyme (GlnD), and adenylyltransferase (GlnE) are all required for posttranslational modification of glutamine synthetase (30). Since *E. coli* GlnA is highly homologous (47% amino acid sequence identity) to *H. pylori* GlnA, *E. coli* GlnB, GlnD, and GlnE sequences were used to search for homologs in *H. pylori*. Homologs of these proteins were not identified in the recently published genome of *H. pylori* (no amino acid sequence identity of more than 12%) (34).

While the *H. pylori* genome does contain possible homologs of the transcriptional regulators NtrB (GlnL) (25% amino acid sequence identity to the *E. coli* protein) and NtrC (GlnG) (34% amino acid sequence identity to the *E. coli* protein), it is unclear whether *glnA* is under the transcriptional control of these proteins. First, both homologs are located distally to GlnA in the published *H. pylori* genome (34). Second, in other studied systems (30), a deuridylylated P_{II} protein is required for the NtrC protein to dephosphorylate NtrB and halt transcription of glutamine synthetase. Similarly, uridylylation of P_{II} is required for covalent modification of NtrB, leading to transcription of *glnA* (30). Since these modifying enzymes are absent, it is likely that transcription of *glnA* is not controlled in this manner.

Glutamine synthetase is intimately linked to urease, the most thoroughly investigated virulence factor of *H. pylori* (26). Two important roles have been proposed for the urease of *H. pylori*. First, hydrolysis of urea present in the host liberates ammonia, which can neutralize gastric acid, allowing the bacterium to survive its initial plunge into the gastric juice while it traverses the mucus layer to the safety of the epithelium surface. Second, ammonia that is not externalized by diffusion can serve as a preferred nitrogen source for incorporation into

amino acids. This is accomplished by the action of glutamine synthetase on ammonia, glutamate, and ATP to form glutamine. Mutants of *H. pylori* deficient in urease cannot colonize the gastric mucosa of any animal model thus far tested (5, 6). Apparently, this inability to colonize is not solely due to acid protection mediated by urease. Gnotobiotic piglets, treated with omeprazole, a proton pump inhibitor, to raise the gastric pH to neutrality, remain resistant to colonization with a urease-negative mutant of *H. pylori* (6). This suggests that an additional role of urease, perhaps that of ammonia assimilation, is critical for successful colonization of the gastric mucosa. Thus, glutamine synthetase may play an unusually critical role in nitrogen metabolism in *H. pylori* and may be required to process the ammonia liberated by urea hydrolysis.

That glutamine synthetase is required for *H. pylori* viability and colonization is supported by our inability to introduce and document a double-crossover allelic exchange mutation in which a kanamycin resistance cassette was used to insertionally inactivate *glnA*. Mutations of nonessential *H. pylori* genes are carried out routinely in this manner and result in direct isolation of double-crossover mutants (3, 16). The formation of cointegrates, in which the electroporated plasmids carrying the insertionally inactivated gene is integrated into the chromosome, is in our experience a rare event unless the mutation is lethal. In this study, we observed and documented such a cointegrate formation, suggesting that *glnA* is an essential gene.

The isolation of *glnA* by the urease-enhancing assay appears to have been fortuitous. It is unlikely that synthesis of active glutamine synthetase contributed to the isolation of the clone. Indeed, upon sequence analysis and alignment with other GlnA sequences, we determined that the 3′ end of the gene was not present and thus was probably not producing active enzyme. Furthermore, if the enzyme was active, it would have reduced, not increased, the ammonia concentration and thus would not have raised the local pH, allowing the detection of an alkali-induced color change of phenol red in the urea segregation agar. This pH-elevating effect was most probably mediated by another gene product encoded on the clone, less than half of which was sequenced.

Given what we know, it is logical that GlnA remains unregulated following translation of the enzyme. *H. pylori* appears to be restricted to the gastric mucosa of humans. Because there are no alternate niches that would include radical changes in temperature, pH, or nitrogen availability, there would be no need for sophisticated levels of regulation for the enzyme. Also because urease does not appear to be regulated, there would be a constant supply of ammonia as long as there is a supply of urea. This pathway of nitrogen assimilation appears to be required for viability, and thus glutamine synthetase appears to represent an unusually critical enzyme in *H. pylori.*

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