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Isolation of *Helicobacter pylori* Genes That Modulate Urease Activity

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***Helicobacter pylori* urease, a nickel-requiring metalloenzyme, hydrolyzes urea to NH_3 and CO_2. We sought to identify *H. pylori* genes that modulate urease activity by constructing pHP8080, a plasmid which encodes both *H. pylori* urease and the NixA nickel transporter. *Escherichia coli* SE5000 and DH5α transformed with pHP8080 resulted in a high-level urease producer and a low-level urease producer, respectively. An *H. pylori* DNA library was cotransformed into SE5000 (pHP8080) and DH5α (pHP8080) and was screened for cotransformants expressing either lowered or heightened urease activity, respectively. Among the clones carrying urease-enhancing factors, 21 of 23 contained *hp0548*, a gene that potentially encodes a DNA helicase found within the *cag* pathogenicity island, and *hp0511*, a gene that potentially encodes a lipoprotein. Each of these genes, when subcloned, conferred a urease-enhancing activity in *E. coli* (pHP8080) compared with the vector control. Among clones carrying urease-decreasing factors, 11 of 13 clones contained the *flbA* (also known as *flhA*) flagellar biosynthesis/regulatory gene (*hp1041*), an *lcrD* homolog. The LcrD protein family is involved in type III secretion and flagellar secretion in pathogenic bacteria. Almost no urease activity was detected in *E. coli* (pHP8080) containing the subcloned *flbA* gene. Furthermore, there was significantly reduced synthesis of the urease structural subunits in *E. coli* (pHP8080) containing the *flbA* gene, as determined by Western blot analysis with UreA and UreB antiserum. Thus, flagellar biosynthesis and urease activity may be linked in *H. pylori*. These results suggest that *H. pylori* genes may modulate urease activity.**

Helicobacter pylori, a gram-negative, microaerophilic, motile, spiral-shaped bacterium, has been established as the etiologic agent of chronic gastritis (22, 37, 38, 60). Chronic infection with *H. pylori* results in gastric and duodenal ulcers (6, 22, 38) and is a risk factor for gastric adenocarcinoma (47). Isolates of *H. pylori* that contain the *cag* pathogenicity island may be involved in more severe disease (9).

Urease (urea amidohydrolase [EC 3.5.1.5]), produced in abundance by *H. pylori*, is central to the pathogenesis of *H. pylori* infection and disease, as evidenced by the failure of urease-negative mutants to colonize mice and gnotobiotic piglets (12, 13) (reviewed in references 38a and 42). The protein, comprised of six copies each of two structural subunits, UreA and UreB, is a nickel-requiring metalloenzyme that hydrolyzes urea to ammonia and carbon dioxide (reviewed in references 38a, 42, and 44). Urease-generated ammonia neutralizes gastric acid (22), causes damage to gastric epithelial cells (56), and is assimilated into proteins by synthesis of glutamine from ammonia and glutamate catalyzed by glutamine synthetase (19) or by synthesis of glutamate from ammonia and α-ketoglutarate catalyzed by glutamate dehydrogenase (16).

The nickel ions required for urease activity are transported into *H. pylori* by a high-affinity cytoplasmic membrane nickel transporter protein, NixA, encoded by the *nixA* gene (43). The nickel ions are incorporated into apourease, presumably by the urease accessory proteins (UreE, UreF, UreG, and UreH), to yield the catalytically active holoenzyme. A detailed structure-function analysis of *nixA* and NixA has been recently reported (17). The *nixA* gene was isolated by its ability to enhance urease activity in *Escherichia coli* carrying pHP808 (43), a plasmid that contains genes that encode the urease structural subunits and accessory proteins from *H. pylori* (28, 30). *nixA* mutants of *H. pylori* have reduced nickel transport and urease activity compared with the wild-type strain, thus confirming that *nixA* is a urease-enhancing factor (UEF) (5, 43). The *nixA* mutant of *H. pylori* still retained some urease activity (58% of that of the wild type) and nickel transport (30% of that of the wild type), suggesting that additional mechanisms of nickel transport may exist in *H. pylori*.

In contrast with other bacterial urease gene clusters (44), there do not appear to be any known regulatory signals for *H. pylori* urease, such as induction by urea for *Proteus mirabilis* urease (33) or induction by low nitrogen concentrations for *Klebsiella pneumoniae* urease (45). Thus, it has been hypothesized that *H. pylori* urease is constitutively expressed (16, 30). However, *H. pylori* urease can account for up to 10% of the total cellular protein (4, 29), a huge energy expenditure for this fastidious organism. Since the gastric mucosal lumen has a pH of 2 and the pH approaches neutrality at the gastric epithelial cell surface to which *H. pylori* adheres (51), it is conceivable that high levels of urease activity are not necessary during every stage of *H. pylori* infection (42). However, the regulatory signals for controlling urease levels have not yet been uncovered.

Previously it was observed that, when grown in minimal medium supplemented with 1 μM $NiCl_2$, *E. coli* containing the urease gene cluster on pHP808 failed to produce urease activity due to the inability to transport sufficient nickel ions for incorporation into apourease (43). Indeed, it has been very difficult to obtain high-level urease activity in *E. coli* (pHP808) under any growth condition. Urease activity was restored to *E. coli* (pHP808) only when it was cotransformed with the *nixA*-containing plasmid (43). Thus, we hypothesized that additional UEFs, as well as potential urease-decreasing factors (UDFs), could be isolated by screening an *H. pylori* DNA library in *E. coli* carrying pHP8080, a single plasmid that encodes both urease and NixA and is capable of generating

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urease activity in *E. coli*. To our knowledge, no one has reported a study designed to identify UDFs and UEFs from any urease-positive organism. To that end, we constructed pHP8080 and screened an *H. pylori* library for cotransformants containing potential UEFs or UDFs. Herein, we provide evidence that several *H. pylori* genes, in addition to *nixA*, modulate urease activity in *E. coli*. We isolated several candidate UEFs (*hp0511*, a gene that potentially encodes a lipoprotein, and *hp0548*, a gene that potentially encodes a DNA helicase found within the *cag* pathogenicity island) and a candidate UDF (*hp1041*, the *flbA* flagellar biosynthesis/regulatory gene [also known as *flhA*]).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *H. pylori* 26695 was kindly provided by Kate A. Eaton (Ohio State University, Columbus). *H. pylori* ATCC 43504 was obtained from the American Type Culture Collection (Rockville, Md.). *H. pylori* was grown on Brucella agar (Becton Dickinson, Cockeysville, Md.) with 10% (wt/vol) defibrinated pooled sheep blood (Waltz Farm, Smithsburg, Md.) in a microaerobic environment by using the CampyPak Plus system (Becton Dickinson). *E. coli* DH5α [F$^-$ *supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Clontech, Palo Alto, Calif.), SE5000 [F$^-$ *araD193* Δ(*argF lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA56*] (20), XL1-Blue MRF′ (*thi-1 gyrA96 relA1 recA1 endA1 supE44 hsdR17 lac* F′ [*proAB lacI*^{q}Z ΔM15 Tn*10*]) (Stratagene, La Jolla, Calif.), and SURE (*thi-1 gyrA96 relA1 recJ recB endA1 sbcC lac* Δ[*mcrCB-hsdMR-mrr*]*177 uvrC umu*::Tn*5* F′ [(*proAB lacI*^{q}Z ΔM15 Tn*10*]) (Clontech) were grown in Luria (L) broth and in Luria agar plus appropriate antibiotics (ampicillin [100 μg/ml], chloramphenicol [20 μg/ml], tetracycline [10 μg/ml], and/or kanamycin [50 μg/ml]) for maintenance of plasmids.

For preparation of urease extracts, strains were grown overnight in M9 minimal medium–1× M9 salts (per liter, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl), 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.68 μM thiamine-HCl, 0.5% Casamino Acids, plus 1% (vol/vol) L broth, 1 μM $NiCl_2$, and appropriate antibiotics.

Initial screening of UEFs and UDFs was achieved by using modified urea segregation agar, which contained the following per 900 ml: 4 g of yeast extract, 4 g of peptone, 0.34 g of NaH_2PO_4, 1.03 g of $Na_2HPO_4 \cdot 7H_2O$, 1 g of gelatin, 5 g of NaCl, 0.90 g of KH_2PO_4, 1.10 g of K_2HPO_4, and 15 g of agar. Following autoclaving, a 100-ml filter-sterilized solution of glucose (9 g), urea (6 g), phenol red (0.035 g), 10 μM $NiCl_2$, and appropriate antibiotics was added. Urea segregation agar medium was poured either into standard petri plates or into 96-well microtiter plates (200 μl per well) (Nunc, Roskilde, Denmark) covered with Parafilm. The pH of uninoculated medium was adjusted so that the color was yellow to light orange (~pH 6.9).

Molecular biology protocols. Standard molecular biology techniques were used as described (3, 52).

Construction of pHP8080. To construct pHP8080 (see Fig. 1), the *nixA* gene and flanking regions (corresponding to nucleotides 1,238 to 2,495 of the sequence with GenBank accession no. Z48742) were PCR amplified from pUEF201 (43), gel purified (Qiaquick Gel Extraction kit; Qiagen, Inc., Valencia, Calif.), and cloned into the *Nru*I and *Ava*I sites of pHP808, a pACYC184 derivative which carries the urease gene cluster from *H. pylori* (28, 30). The following primers were used: NixADM-F1 (5′-GCGTCGCGAGCCTTTTTACACCATTCTCC-3′; *Nru*I restriction endonuclease site is underlined), and NixADM-R1 (5′-GGCCTCGAG GCCAAGTTTTTCAAATCAAA-3′; *Ava*I site is underlined). The PCR conditions were as follows: 94°C for 5 min (first cycle only) and 94°C denaturation for 1 min, 50°C annealing for 90 s, and 72°C annealing for 2 min for a total of 30 cycles, followed by a 5-min extension at 72°C. PCR primers were designed by using a world wide web site (9a) and synthesized on an Applied Biosystems Oligonucleotide Synthesizer by the Biopolymer Core Facility at the University of Maryland, Baltimore. PCRs were carried out on a ThermoCycler (model PTC 150; MJ Research, Waterford, Mass.) by using *Pfu* DNA polymerase (Stratagene). This construct was confirmed by restriction analyses, by *nixA*-specific PCR (data not shown), and by qualitative and quantitative determination of the urease activity (see below) in comparison with that of the same *E. coli* strain carrying pHP808 alone.

Construction of *H. pylori* ATCC 43504 library. *Sau*3AI partially digested and blunt-ended chromosomal DNA (average size, 6 kb) from *H. pylori* ATCC 43504 was used for commercial preparation of a λ-ZAPII genomic library (*Eco*RV site used) (Stratagene) (43). The λ-ZAPII genomic library phage suspension (containing 10^7 phage particles) was incubated with *E. coli* XL1-Blue MRF′ (adjusted to optical density at 600 nm [OD_{600}] of 1.0) and 10^6 ExAssist helper phage particles for 15 min at 37°C. The suspension was then added to 20 ml of L broth and incubated at 37°C for 2 h and then heated at 65°C for 20 min. The suspension was centrifuged (2,500 × *g*; 10 min, 4°C), and the supernatant 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 was plated onto L agar plates containing ampicillin (200 μg/ml) and incubated for 18 h at 37°C (24). Colonies from 20 plates were pooled and used for a large-scale plasmid (pBluescript library) preparation by column chromatography (Qiagen Midi-Tip 100).

Urease activity determinations on urea segregation agar. Urease activities of *E. coli* cotransformants carrying pHP8080 plus the pBluescript library were determined by screening for colonies, grown overnight at 37°C, that changed the medium pH of modified urea segregation agar. As the pH of urea segregation agar rises, presumably due to urease-generated ammonia, the medium changes color from yellow or light orange to red. Cotransformants that were qualitatively confirmed to show enhanced or decreased urease activity were quantitatively assayed for urease activity (see below). *E. coli* SE5000 (pHP808/pBluescript) and SE5000 (pHP808/pUEF201) served as negative and positive controls, respectively (data not shown).

Urease extract preparations and protein determinations. *E. coli* (pHP8080/library) cotransformants were each grown from a single colony in 1.5 ml of M9 minimal medium overnight at 37°C. Bacteria were harvested by centrifugation (12,000 × *g*; 2 min, 4°C) and washed twice with 50 mM HEPES buffer (pH 7.5). Bacteria were bath sonicated (40% intensity, three pulses for 30 s each) (Cell Disruptor; Ultrasonics, Inc.) on ice to release cytosolic proteins, including urease; >99% of the bacteria were lysed by this method. Following centrifugation (12,000 × *g*, 2 min, 4°C), supernatants were placed on ice. The pellet fraction (cell debris) was retained for some experiments. The extracts retained the same urease activity after multiple freeze-thaw cycles. Protein determinations of the extracts were conducted by the bicinchoninic acid assay method (Pierce Chemical Company, Rockford, Ill.), according to the manufacturer's 30-min protocol. Bovine serum albumin was used as the standard.

Urease activity determinations by the phenol-hypochlorite urease assay. Urease activity of extracts was determined by measuring the amount of ammonia released from urea in the phenol-hypochlorite urease assay (61). Extracts were added to urease buffer (50 mM HEPES [pH 7.5] plus 25 mM urea) in a 1-ml final volume and were incubated at 37°C for 20 min. The reaction was stopped by removal of an aliquot and addition to a cuvette containing 1.5 ml of solution A (containing [per liter] 10 g of phenol and 50 mg of sodium nitroprusside). An equal volume (1.5 ml) of solution B (NaOH [5 mg/ml]–NaClO [0.044%, vol/vol]) was added, and the contents were mixed well. Following incubation at 37°C for 30 min, the absorbance at 625 nm was measured in a spectrophotometer (BioSpec 1601; Shimadzu Scientific Instruments, Inc., Columbia, Md.). A standard ammonium chloride concentration curve was determined to be linear over the range from 10 to 300 nmol of ammonia. A second standard curve, one for jack bean urease (Sigma Chemical Co., St. Louis, Mo.), was shown to be linear with respect to time and amount of urease added (data not shown). Absorbance values were converted to nanomoles of ammonia based on the ammonium chloride standard curve. Data are presented as urease specific activity, defined as nanomoles of NH_3/minute/milligram of protein. *E. coli* SE5000 (pHP808/pBluescript), which lacks *nixA*, served as the negative control, and the background activity (<10 nmol of NH_3/minute/milligram of protein) was subtracted from all values obtained to avoid measuring ammonia generated by urease-independent mechanisms. Statistical analysis of the data was conducted by the alternative Welch's *t* test by using InStat 2.03 software (GraphPad Software, San Diego, Calif.).

DNA sequencing and software analysis of the nucleotide sequence. Plasmids were sequenced by the dideoxy chain termination method (54) by using an Applied Biosystems 373A automated DNA sequencer with the Big Dye Terminator Cycle Sequencing Kit at the University of Maryland, Baltimore, Biopolymer Core Facility, with the T3 and T7 oligonucleotide primers and, when necessary, additional primers based on the nucleotide sequence of the insert. The following software programs were used to analyze and manipulate the sequence: DNASIS version 2.1 (Hitachi Software Engineering Company) and molecular biology world wide web sites (9b, 30a, 30b, 45a, 45b, 45c, 47a, 57a, 57b).

Subcloning of *flbA*. The *flbA* gene plus flanking DNA, corresponding to nucleotides 2,001 to 4,430 in pUDF104 (see the Results section), was subcloned by PCR amplification by using Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and the following primers: FlhA-F1, 5′-GCGCGGATCCGTG GCAAACGCCTTAATGAT-3′ (*Bam*HI restriction endonuclease site is underlined); and FlhA-R1, 5′-GCGCATCGATTGGTAAACTTGCATCATTCTCC-3′ (*Cla*I site is underlined). The following conditions were used: 94°C for 5 min (first cycle only) and 94°C for 90 s, 50°C for 3 min, and 72°C for 5 min for a total of 30 cycles, followed by a 5-min extension at 72°C. The product of the expected size (2,450 bp) was gel purified, simultaneously digested with *Bam*HI and *Cla*I, and directionally ligated into pBluescript to yield pBS-*flbA*. The constructs were confirmed by restriction analysis and by PCR.

Subclones of pUEF1004. Open reading frames (ORFs) *hp0511* (nucleotides 721 to 1,135) and *hp0548* (nucleotides 1,360 to 2,409) on pUEF1004 (see Results section) were subcloned by PCR amplification by using Vent DNA polymerase and the following primers: Hel-F1, 5′-GCGCGAATTCCCCTATGATTAGGG ACACAGAG-3′ (*Eco*RI site is underlined); Hel-R1, 5′-GCGCGGATCCTGC AATTTAGGAGCGTTTTG-3′ (*Bam*HI site is underlined); Orf511-F1, 5′-GC GCGGATCCTCTAATTCAAGGAGCCTAACTAAAA-3′ (*Bam*HI site is underlined); and Orf511-R1, 5′-GCGCGAATTCAACCCAATATCAGTTTGAT TGC-3′ (*Eco*RI site is underlined). The following conditions were used: 94°C for 5 min (first cycle only) and 94°C for 60 s, 63°C for 30 s, and 72°C for 2 min for a total of 30 cycles, followed by a 5-min extension at 72°C. The PCR products of the expected sizes (~415 bp for *hp0511* and ~1,050 bp for *hp0548*) were gel purified, simultaneously digested with *Eco*RI and *Bam*HI, and ligated into

pBluescript to yield pUEF1004-548 and pUEF1004-511, respectively. To construct pUEF1004-548ΔK, the 339-bp *Kpn*I fragment (including the ~295-bp 5′ end of *hp0548*) was removed from pUEF1004-548 and religated. Subclones were confirmed by PCR, restriction, and sequence analyses.

SDS-PAGE and Western blot analysis of urease extracts. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by the method of Laemmli (36), using a 12% resolving gel and 10 μg of protein loaded per lane. Proteins were transferred to Immobilin-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Mass.) by using a Bio-Rad Trans-Blot cell transfer apparatus (Bio-Rad, Hercules, Calif.). The membrane was blocked by using 5% nonfat dry milk and washed with Tris-buffered saline containing 0.05% Tween 20 (TBST) (3). Primary antibodies were anti-UreA or anti-UreB (1:100,000 to 1:200,000 dilution), obtained as described previously (30). The secondary antibody was goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Immunochemical Co.) (1:1,500 to 1:3,000 dilution). Blots were developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate according to the manufacturer's instructions (Life Technologies). Extracts from *E. coli* DH5α (pBluescript/pACYC184) and *E. coli* SE5000 (pBluescript/pACYC184) were used as negative controls. Blots were quantified by using Eagle Eye II software (Stratagene).

Nucleotide sequence accession number. The 4,582-bp nucleotide sequence of pUDF104 and the 2,693-bp nucleotide sequence of pUEF1004 have been deposited in GenBank under accession no. AF125197 and AF125214, respectively.

RESULTS

***E. coli* (pHP8080/pBluescript) has urease activity.** Previously, it was observed that *E. coli* (pHP808), which contains the *H. pylori* urease gene cluster, had no urease activity when the strain was grown in low nickel concentrations (1 μM); urease activity was obtained when *E. coli* (pHP808) was cotransformed with the *nixA*-containing plasmid, pUEF201, thus confirming that *nixA* is a UEF (5, 43). Further screening of an *H. pylori* library for other UEFs in *E. coli* (pHP808) failed to reproducibly yield additional cotransformants with altered urease activity (data not shown), suggesting that it is not possible to obtain additional UEFs unless a gene encoding a high-affinity nickel transporter is also present.

To determine whether we could obtain urease activity from *E. coli* carrying a single plasmid, we constructed pHP8080, a plasmid that encodes both *H. pylori* urease and the NixA nickel transporter (Fig. 1; see Materials and Methods section). We observed that *E. coli* SE5000 (pHP8080/pBluescript) was urease positive on urea segregation agar after overnight incubation (data not shown), as evidenced by a color change of urea segregation agar to red. In contrast, DH5α (pHP8080/pBluescript) failed to produce urease activity on urea segregation agar medium, even after 2 days of incubation at 37°C (data not shown). Urease assays by the phenol-hypochlorite method detected higher urease activity from SE5000 (pHP8080/pBluescript) than from DH5α (pHP8080/pBluescript) (Tables 1 and 2). As a negative control, SE5000 (pHP808/pBluescript), which lacks *nixA*, had no detectable urease activity (<10 nmol/min/mg). These findings indicate that pHP8080 is sufficient for urease production in *E. coli*. For comparison, note that the urease activity of *H. pylori* 43504 averages 30,000 ± 2,000 nmol of ammonia/min/mg of protein.

Urease activities of pUEF- and pUDF-containing cotransformants on urea segregation agar. To investigate genes that modulate urease activity in *E. coli* in addition to *nixA*, we transformed the low-level urease-producing *E. coli* strain DH5α (pHP8080) and the high-level urease-producing *E. coli* strain SE5000 (pHP8080) with a pBluescript library of *H. pylori*. Cotransformants were screened on urea segregation agar for enhanced urease activity or decreased urease activity, respectively. Among ~5,700 DH5α (pHP8080/library) cotransformants screened, 30 isolates (0.5%) turned urea segregation agar red after overnight growth (these are designated as pUEFs). Among ~1,400 SE5000 (pHP8080/library) cotransformants screened, 34 isolates (2%) showed delay in their ability to turn urea segregation agar red (these are designated as pUDFs), relative to that of the control, SE5000 (pHP8080/pBluescript) (data not shown).

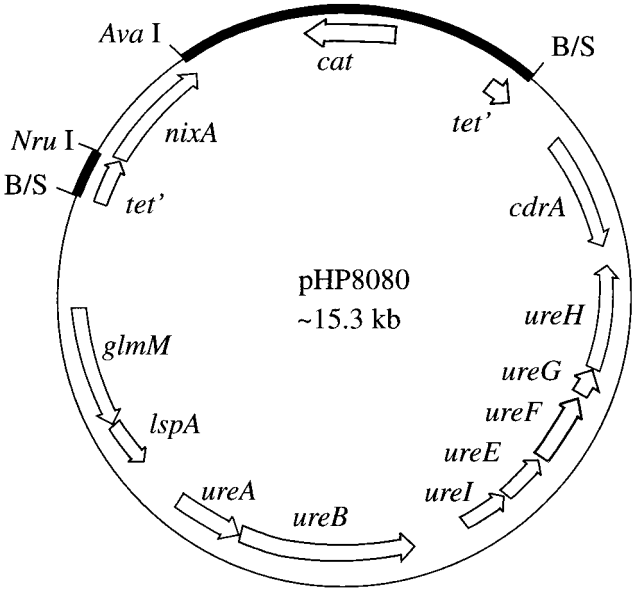

FIG. 1. Diagram of pHP8080. pHP8080 is a derivative of pHP808 (28, 30); pHP808, a pACYC184 derivative, contains the *H. pylori* urease gene cluster (*ureABIEFGH*) cloned into the *Bam*HI site through a *Sau*3AI partial digestion of *H. pylori* 43504 genomic DNA (restriction site designated as B/S). The *nixA* nickel transporter gene was PCR amplified from pUEF201 (43) and cloned into the *Nru*I and *Ava*I sites in pHP808, which removed the 3′ end of the tetracycline resistance marker. *glmM* (11) and *lspA* (58) refer to genes previously designated as *ureC* and *ureD*, respectively (10, 35) and are not required for urease activity (10, 44). Thick lines, pACYC184 vector sequences; *cat*, chloramphenicol resistance marker; *tet′*, truncated tetracycline resistance marker. Arrows show directions of transcription.

Urease activities of cotransformants containing pUEFs and pUDFs determined by the phenol-hypochlorite urease assay. Urease activity was quantified for all pUDF and pUEF *E. coli* (pHP8080) cotransformants. Of the 30 potential UEFs obtained, 23 DH5α (pHP8080/pUEF) library cotransformants (77%) had urease specific activities 3- to 13-fold higher than that of the control, DH5α (pHP8080/pBluescript) (Table 1; representative examples are shown). Indeed, most of the UEFs were so active that the extracts had to be diluted 1:10 to avoid saturating the assay. The majority of the pUEFs (21 of 23 isolates) had common restriction fragments (*Xba*I and *Hin*dIII double digest). There were also two unique restriction patterns observed (pUEF1014 and pUEF1023w) (Table 1).

Of the 34 potential UDFs obtained, 13 SE5000 (pHP8080/pUDF) library cotransformants (38%) had urease specific activities 3- to 750-fold lower than that of the control, SE5000 (pHP8080/pBluescript) (Table 2; representative examples are shown). The majority of the pUDFs (11 of 13 isolates) had common restriction patterns (*Xba*I and *Hin*dIII double digest). There were also two unique restriction patterns (pUDF123 and pUDF140). These results confirmed that *H. pylori* genes could modulate urease activity in *E. coli* (pHP8080). Furthermore, only a few genes are potentially involved in this modulation.

To minimize artifacts, we isolated plasmid DNA from confirmed DH5α (pHP8080/pUEFs) and SE5000 (pHP8080/pUDFs) and electroporated the plasmids into both DH5α and SE5000. Ampicillin-resistant, chloramphenicol-susceptible colonies (i.e., colonies of isolates lacking pHP8080) were retransformed with pHP8080 and reconfirmed by the phenol-hypochlorite urease assay. Thus, *E. coli* (pHP8080/pUEFs) or

TABLE 1. Summary of UEFs in DH5α (pHP8080)

Plasmid name	Insert size (kb)[a]	Candidate gene(s)	Mean urease activity ± SD[b]	n[c]	P value[d]	No. of similar clones
pBS	None	None	778 ± 628	33	NA[e]	NA
pUEF1004	2.7	*helAB, lpp*	6,518 ± 2,993	16	<0.0001	21[f]
pUEF1004-511	0.4	*lpp*	2,505 ± 428	8	0.0003	NA
pUEF1004-548	1.1	*helAB*	2,598 ± 838	9	<0.0001	NA
pUEF1004-548ΔK	0.7	*helB*	4,161 ± 816	7	0.0001	NA
pUEF1014	7.1	*hp0839–0846*	2,917 ± 2,000	7	0.0335	1
pUEF1023w	8.4	*hp1400–1403*	10,236 ± 4,449	10	<0.0001	1

[a] Size was determined based on the results of restriction analysis, PCR, and sequencing.
[b] Urease activity in DH5α (pHP8080) plus each of the plasmids listed. Data are expressed as specific activity (nanomoles of NH_3/minute/milligram of protein) (mean ± standard deviation).
[c] n, number of experiments.
[d] P value for urease activity as compared with urease activity from DH5α (pHP8080/pBluescript).
[e] NA, not applicable.
[f] Other clones determined to contain *helAB* (*hp0548*) and *lpp* (*hp0511*) based on the results of restriction analysis, PCR, and sequencing were as follows: pUEF1002, -1005, -1006, -1007, -1008, -1010, -1012, -1015, -1018, -1020, -1026, -1027, -1028, -1030, -1031, -1033, -1034, -1035, -1036, and -1038. Insert sizes varied from 2.7 to 12.3 kb.

E. coli (pHP8080/pUDFs) had a urease-enhancing or urease-decreasing phenotype, respectively, regardless of the strain of *E. coli* used (data not shown). The only exception to this was pUDF123, which had no urease activity due to an *E. coli* chromosomal mutation in the original isolate (data not shown). This clone was not analyzed further.

Sequence analysis of pUDFs. Of the 11 pUDFs that gave similar restriction enzyme patterns, we chose pUDF104 for further analysis. Sequence analysis of both DNA strands of the 4,582-bp insert of pUDF104 revealed three complete ORFs (Fig. 2, top), corresponding to *hp1039*, *hp1040*, and *hp1041* in sections 89 and 90 of the complete genome sequence of *H. pylori* (58) (corresponding to nucleotides 8,634 to 10,782 and 59 to 2,497 of the sequences with accession no. AE000611 and AE000612, respectively). The flanking regions of pUDF104 consisted of truncated *hp1038* at the left junction (T3 primer of pBluescript) and *hp1042* at the right junction (T7 primer of pBluescript). Since *hp1039* encodes a predicted hypothetical protein and *hp1040* encodes a predicted small ribosomal protein (RpsL5), we focused our attention on *hp1041*, which contains the *flbA* gene (2,202 bp). The FlbA protein is involved in secretion, assembly, and regulation of the expression of flagellar proteins in *H. pylori* (55). There is a predicted σ^{28} promoter (consensus sequence is $TAAAN_{15}GCCGAT(A/T)$; *flbA* sequence is $TAAGN_{14}ACCGAAAT$), upstream of the *flbA* gene.

The *flbA* gene encodes an 80.9-kDa cytoplasmic membrane protein (FlbA) that has seven predicted transmembrane domains within the amino-terminal half of the protein (55). The carboxy-terminal half of FlbA is hydrophilic and is likely located in the cytosol. There is no apparent signal sequence in FlbA. Comparison of the *H. pylori* FlbA predicted amino acid sequence with the FlbA and FlhA proteins of other strains of *H. pylori* (15, 55, 58) revealed a high degree of sequence conservation (>98% identity). The *H. pylori* FlbA was most similar with the FlbA protein from *Campylobacter jejuni* (55) (51.4% identity and 80.2% similarity).

The LcrD family is comprised of two subfamilies: FlhA and LcrD. *H. pylori* FlbA has a higher degree of amino acid homology with the FlhA subfamily (range, 34.6 to 51.4% amino acid sequence identity and 68.3 to 80.2% amino acid sequence similarity) than with the LcrD subfamily (range, 29.1 to 34.8% identity). At 733 amino acids, the *H. pylori* FlbA is the largest member of the FlhA subfamily.

The vector-insert junctions of four additional pUDFs were sequenced. pUDF109 was found to have sequence identical to that of pUDF104. Similarly, pUDF119 and pUDF141 were sequenced across the vector-insert junctions and found to have the same insert as pUDF104, except that both had the insert in the opposite orientation. Plasmid pUDF140, which has a unique restriction pattern, was sequenced across the junctions and was found to contain truncated genes *hp0554* and *hp0558*. Based on the size of the insert (4.0 kb) and the similar size of this region in the *H. pylori* genome, this clone probably con-

TABLE 2. Summary of UDFs in SE5000 (pHP8080)

Plasmid, extract	Insert size (kb)[a]	Candidate gene(s)	Mean urease activity ± SD[b]	n[c]	P value[d]	No. of similar clones[e]
pBS, cytosolic[f]	None	None	1,542 ± 786	41	NA[g]	NA
pBS, pellet	None	None	409 ± 203	5	NA	NA
pBS, supernatant	None	None	0 ± 0	3	NA	NA
pUDF104, cytosolic	4.7	*hp1038–1042*	450 ± 219	10	<0.0001	11
pBS-*flbA*, cytosolic	2.4	*flbA*	95 ± 73	11	<0.0001	NA
pBS-*flbA*, pellet	2.4	*flbA*	27 ± 19	5	<0.0001	NA
pBS-*flbA*, supernatant	2.4	*flbA*	0 ± 0	3	<0.0001	NA
pUDF140, cytosolic	4.0	*hp554–558*	0 ± 0	8	<0.0001	1

[a] Size was determined based on the results of restriction analysis, PCR, and sequencing.
[b] Urease activity in SE5000 (pHP8080) plus each of the plasmids listed. Data are expressed as specific activity (nanomoles of NH_3/minute/milligram of protein) (mean ± standard deviation).
[c] n, number of experiments.
[d] P value for urease activity as compared with urease activity from SE5000 (pHP8080/pBluescript).
[e] Other clones determined to contain *flbA* (*hp1041*) based on the results of restriction analysis, PCR, and sequencing were as follows: pUDF109, -119, -137, -138, -141, -145, -149, -153, -156, and -158.
[f] Cytosolic, pellet, and supernatant refer to crude extracts of the cytosol, the cell debris, and the extracellular supernatant, respectively.
[g] NA, not applicable.

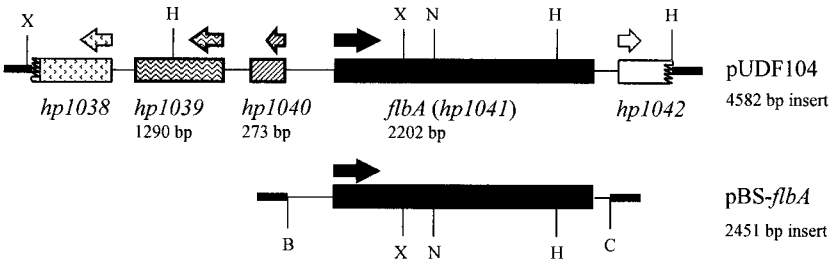

FIG. 2. Schematic maps of pUDF104 and pBS-*flbA*. The numbering of the ORFs of the genes in pUDF104 is based on the system developed by Tomb et al. (58). The size of each complete gene and the size of the total insert are shown. The *flbA* gene plus flanking DNA was subcloned into the *Bam*HI (B) and *Cla*I (C) sites in pBluescript by PCR as described in the Materials and Methods section to yield pBS-*flbA*. Truncated genes are denoted by jagged edges. H, *Hin*dIII; X, *Xba*I; N, *Nhe*I. Thick lines, pBluescript vector sequences. Arrows show directions of transcription.

tains genes *hp0554* to *hp0558*. This clone will not be discussed further in this study.

***flbA* is a UDF.** Since the *flbA* gene appeared to be the best candidate gene among the three complete predicted ORFs in pUDF104, we subcloned *flbA* (*hp1041*) by PCR (see Materials and Methods section) to yield pBS-*flbA* (Fig. 2, bottom). When pBS-*flbA* was cotransformed into SE5000 with pHP8080, urease specific activity was reduced by 15-fold in comparison with that of the control, SE5000 (pBluescript/pHP8080) (Table 2; see data for cytosolic fraction). These results confirm that *flbA* is a UDF in *E. coli* (pHP8080). Except for pUDF123 and pUDF140, all the other pUDFs were positive for the 2.4-kb *flbA* gene by PCR or by inference based on restriction analysis.

Given the potential role of FlbA as a component of the flagellar secretion apparatus (55), we investigated whether urease activity is directed to a different cellular compartment using crude extracts of the cytosol, the resulting pellet cell debris, or extracellular supernatants. We found that negligible urease activity was found in the pellets of *E. coli* (pBS-*flbA*/pHP8080) (Table 2), and no urease activity was found in extracellular supernatants of either *E. coli* (pBS-*flbA*/pHP8080) or *E. coli* (pBS/pHP8080). These results suggest that FlbA does not change the location of urease activity to another cellular compartment. Additionally, about 75% of the total urease activity is found in cytosolic extracts [compare data for cytosolic and pellet fractions of *E. coli* (pBS/pHP8080) given in Table 2]).

Sequence analysis of UEFs. One confirmed UEF-bearing plasmid, pUEF1004, was sequenced and contained the genes encoding a potential lipoprotein (*hp0511*; *lpp*) (58), a potential DNA helicase (*hp0548*), and a portion of *cagA*, as well as a fragment of IS*605* (Fig. 3). *hp0511* encodes a predicted 62-amino-acid polypeptide that is larger than the corresponding protein in *H. pylori* 26695, which is only 38 amino acids in length (58). *hp0511* or Hp0511 has no homology at the nucleotide or amino acid level with any other gene or protein in the database. The *cagA* gene and *hp0548* are found near the right junction of the *cag* pathogenicity island (1, 9, 58). In all *cag* pathogenicity islands investigated to date, the *hp0548* gene has a frameshift, presumably resulting in a nonfunctional protein (1, 9, 58). However, in the case of *H. pylori* 43504, used in this study, *hp0548* was not frameshifted but instead had a stop codon about one third of the way into the gene. Further analysis of *hp0548* suggested the potential for two ORFs, designated here as *helA* and *helB*.

Clones pUEF1010, pUEF1012, and pUEF1038 were sequenced and found to also contain *hp0548* and *hp0511* (Table 1). Except for clones pUEF1014 and pUEF1023w, all the remaining UEF-containing clones were positive for both *hp0548* and *hp0511* by PCR or by inference based on restriction analysis (Table 1).

Clones pUEF1014 and pUEF1023w were sequenced across the vector-insert junctions and were found to contain *H. pylori* genes *hp0839* through *hp0846* and *hp1400* through *hp1403*, respectively (Table 1), and will not be discussed further in this study.

***helB* and *lpp* are UEFs.** To determine which gene(s) in pUEF1004 was responsible for urease-enhancing activity, we subcloned the *hp0511* (*lpp*) and *hp0548* (*helAB*) genes (Fig. 3).

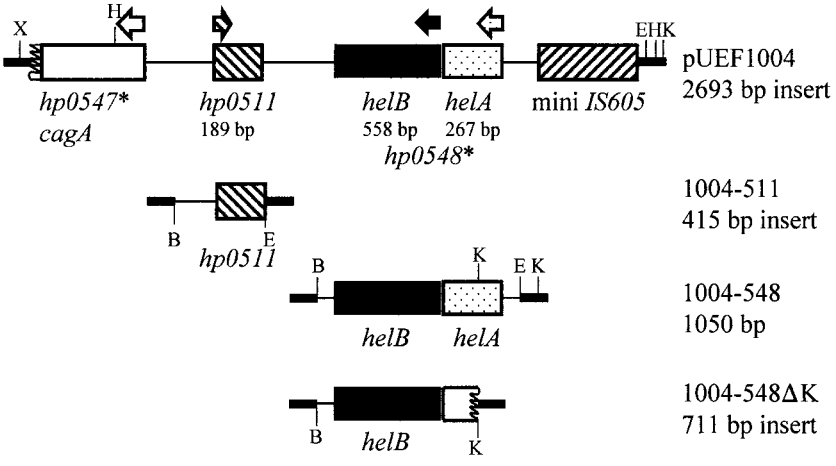

FIG. 3. Schematic maps of pUEF1004 and subclones. Subclones were constructed as described in the Materials and Methods section. E, *Eco*RI; K, *Kpn*I; B, *Bam*HI. *, genes in the *cag* pathogenicity island. Other abbreviations and notations are as described in the legend to Fig. 2.

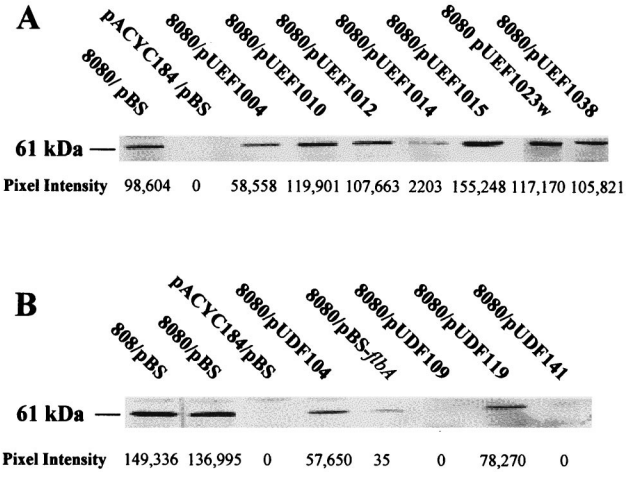

FIG. 4. Western blot analysis of urease extracts by using anti-UreB. Cytosolic protein extracts (10 μg) were electrophoresed through an SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-UreB as described in the Materials and Methods section. (A) Extracts from DH5α (pHP8080/pBluescript) and DH5α (pHP8080/pUEFs). (B) Extracts from SE5000 (pHP8080/pBluescript) and SE5000 (pHP8080/pUDFs). Density values are shown below individual blots. Results are representative of three experiments performed by using extracts prepared on 3 separate days. pBS, pBluescript; 808, pHP808; 8080, pHP8080.

Surprisingly, both genes separately conferred urease-enhancing activities (Table 1), although these activities were not as great as that of the clone that contains both genes (pUEF1004). When the 5′ end of the *helA* gene and the IS*605* fragment were deleted (construct pUEF1004-548ΔK in Fig. 3), urease activity still remained elevated (Table 1), suggesting that *helB* is the gene responsible for the urease-enhancing activity.

Urease protein levels in extracts from *E. coli* (pHP8080) carrying pUDFs or pUEFs. To determine whether genes on pUDFs or pUEFs affected the amounts of the urease structural proteins in *E. coli* (pHP8080), extracts from *E. coli* (pHP8080/pUDFs) or *E. coli* (pHP8080/pUEFs) were analyzed by SDS-PAGE and Western blot analysis by using anti-UreB antiserum. As expected, extracts from DH5α (pBluescript/pACYC184) and SE5000 (pBluescript/pACYC184), which have neither urease activity nor the urease genes, lacked the 61-kDa UreB polypeptide (large subunit of urease) as assayed by Western blotting (Fig. 4A and B). Also, SE5000 (pBluescript/pHP808), which has no detectable urease activity, was still able to synthesize UreB (Fig. 4A and B), as expected from previous studies in our laboratory (30). We found that extracts from DH5α (pHP8080/pUEFs) generally did not have an increase in the amount of UreB compared with the positive control, DH5α (pHP8080/pBluescript) (Fig. 4A). A similar finding was observed with extracts from subclones DH5α (pHP8080/pUEF1004-511) and DH5α (pHP8080/pUEF1004-548) (data not shown). In contrast, extracts from SE5000 (pHP8080/pUDFs) had a two- to threefold drop in the amount of UreB (for pUDF104 and pUDF119) or no detectable UreB (for pUDF109 and pUDF141) compared with the positive control, SE5000 (pHP8080/pBluescript) (Fig. 4B). Furthermore, extracts from SE5000 (pHP8080/pBS-*flbA*), which contains the subcloned *flbA* flagellar biosynthesis/regulatory gene and has greatly reduced urease specific activity (Table 1), had a dramatic drop in the amount of UreB (Fig. 4B). No UreB was detectable in pellets or culture supernatants of SE5000 (pHP8080/pBS-*flbA*) (data not shown). Comparable results for UEFs and UDFs were also obtained by using UreA (small subunit of urease)-specific antiserum (data not shown). These results suggest that *flbA* dramatically reduces the amounts of the urease structural subunits, UreA and UreB.

DISCUSSION

Our study suggests that *H. pylori* may potentially modulate urease levels in the cell, in contrast with previous reports (16, 30). This is supported by our discovery of both UEFs and UDFs that alter urease activity in *E. coli* strains expressing both *H. pylori* urease and the NixA nickel transporter on plasmid pHP8080. *E. coli* (pHP8080) is capable of generating urease activity, in contrast with *E. coli* (pHP808), which expresses the *H. pylori* urease structural and accessory genes but lacks *nixA* and thus has urease that is catalytically inactive due to lack of nickel ions in the urease active site. This observation indicates that expression of both the NixA nickel transporter and the urease structural and accessory proteins is necessary for high-level urease activity in *E. coli*. Most other bacterial ureases have been successfully expressed in *E. coli* in the absence of a nickel transporter-containing plasmid (reviewed in reference 44). As *E. coli* has a low-affinity nickel transporter, NikD (46), *H. pylori* apourease may have a lower affinity for nickel than other bacterial apoureases, which may be compensated by the high-affinity nickel transporter, NixA. At low nickel concentrations or under nickel-chelating conditions, little or no urease activity was observed in *E. coli* (pHP808) lacking *nixA* (28, 30, 43).

We used the high-level urease-producing strain, SE5000 (pHP8080/pBluescript), to obtain UDFs, and the low-level urease-producing strain, DH5α (pHP8080/pBluescript), to obtain UEFs. The urease activity difference between these *E. coli* strains is probably due to uncharacterized genetic differences in strain backgrounds. Our ability to uncover UEFs and UDFs was greatly facilitated by using urea segregation agar as a screening tool. Many cotransformants that exhibited UEF or UDF activity by the urea segregation agar method were subsequently confirmed by the phenol-hypochlorite urease assay (77% of UEFs and 38% of UDFs). Since the urea segregation agar screen relies on a pH indicator, it is possible that some UEFs or UDFs not subsequently confirmed by the phenol-hypochlorite urease assay could contain gene products that alter the medium pH rather than modulate urease activity. Nevertheless, our results suggest that the use of urea segregation agar as a screening tool is a highly effective method to obtain UEFs and UDFs.

UEFs and UDFs may act on urease activity in a direct or indirect fashion. For example, UEFs or UDFs may act upon the nickel transporter gene or the corresponding protein, which would affect the amount of urease activity observed by increasing or decreasing the amount of nickel ions available for incorporation into apourease. Also, UEFs or UDFs could affect the expression of the urease structural or accessory genes or the corresponding proteins.

Of the 13 UDFs obtained, 11 contained the *flbA* flagellar biosynthesis/regulatory gene. The *flbA* gene is a UDF, since SE5000 (pHP8080) containing the subcloned *flbA* gene (pBS-*flbA*) has almost no detectable urease activity (Table 1). Furthermore, SE5000 (pHP8080/pBS-*flbA*) had almost no UreB (Fig. 4B) or UreA (data not shown) by Western blot analysis. That the amounts of both structural subunits are reduced is not surprising since *ureA* and *ureB* are likely cotranscribed from the same promoter (35). As UreA and UreB are each essential for urease activity, loss of either one of these subunits would result in failure to produce catalytically active urease (44). FlbA does not redirect urease activity or an inactive urease protein to another cellular compartment, as little urease activ-

ity was observed in other compartments in crude extracts of *E. coli* (pBS-*flbA*/pHP8080) (Table 1) and no urease protein was detectable in other cellular compartments (data not shown).

We propose four possibilities whereby *flbA* may cause a reduction in the urease activity. First, FlbA may increase the turnover of either the urease structural subunits UreA and UreB or the mRNA for *ureA* and *ureB*. Second, FlbA may repress transcription of the urease genes. However, there are no DNA-binding motifs or motifs suggestive of a two-component regulatory system in FlbA or in any member of the LcrD family (40, 53, 57). Thus, the protein could be acting as a signal transducer through another protein (2, 8, 18, 48, 50). Third, FlbA could titrate σ^{28} away from activation of the urease gene cluster; it is unclear which sigma factor transcribes the *H. pylori* urease gene cluster. Finally, FlbA may decrease assembly of the very large (550 kDa) urease apoenzyme. Future experiments will center on determining the mechanism behind the decreased urease activity and decreased amounts of urease structural subunits produced by FlbA.

A mutation of *H. pylori* in the *flbA* gene has pleiotropic effects (55), as is the case for other members of the LcrD family (8, 23, 49). The *H. pylori flbA* mutant is nonmotile, has no flagella, and has reduced synthesis of the products of the *flaA*, *flaB*, and *flgE* flagellar biosynthesis genes. Notably, the *flbA* mutant has slightly increased levels of urease activity measured by a semiquantitative method (55), which supports our finding that *flbA* is a UDF in *E. coli*.

The FlbA protein is homologous with members of the LcrD family which are involved in secretion, assembly, and/or regulation of the expression of virulence-related proteins, and are structural constituents of a secretion apparatus (reviewed in references 26 and 27). The LcrD family is comprised of two distinct but evolutionarily related subfamilies: LcrD and FlhA. The LcrD subfamily is involved in type III secretion of virulence-related proteins (e.g., LcrD of *Yersinia* spp., MxiA of *Shigella flexneri*, InvA of *Salmonella* spp., and SepA/EscV of enteropathogenic *E. coli*) (2, 18, 31, 48, 49, 59). There does not appear to be a type III secretion system in *H. pylori* (58). The FlhA subfamily is involved in secretion and external localization of flagellar proteins (8, 19, 23, 40, 41, 50, 53, 55).

We also observed, based upon restriction, PCR, and sequence analyses, that 21 of the 23 UEFs have the *hp0511* and *hp0548* genes in common. These genes are adjacent to each other on all of our clones and thus do not appear to be chromosomal fragments ligated together from separate parts of the genome. Instead, it is likely that *H. pylori* 43504 has a different gene order than strain 26695, as has been widely found in *H. pylori* (7, 32). An intriguing finding is that the gene encoding the putative DNA helicase, *hp0548*, is found in the *cag* pathogenicity island and, instead of being frameshifted (i.e., inactive) as is found for other *H. pylori* strains (1, 9, 58), appears to be two ORFs, *helA* and *helB*. Our data suggest that the gene responsible for the urease-enhancing activity is *helB* (Table 1). Future experiments will center on understanding the mechanism of how *helB* causes urease-enhancing activity and whether the helicase produced can regulate urease gene transcription.

Other *H. pylori* genes have been shown to affect urease activity. Specifically, an *H. pylori* ATP-binding cassette (ABC) transporter gene cluster, *abcABCD*, enhances urease activity; this activity is reduced by 88% in an *H. pylori abcD* mutant (25). A *nixA abcC* double mutant of *H. pylori* also has almost no urease activity. AbcABCD may be involved in energy-driven transport of nickel ions into the cell (25). Other gene products that appear to affect urease activity are a P-type ATPase (39), a heat shock protein A (HspA) (34), and histidine-rich protein Hpn (21).

In summary, we have successfully obtained urease activity in *E. coli* carrying a single plasmid, pHP8080, that expresses both *H. pylori* urease and the NixA nickel transporter. Using this plasmid, we found candidate genes, UEFs and UDFs, from *H. pylori* that can modulate urease activity in *E. coli*. One confirmed UDF is the *flbA* flagellar biosynthesis/regulatory gene, which dramatically decreases urease activity and reduces the amounts of urease structural subunits detected on Western blot analysis for *E. coli* (pHP8080/pBS-*flbA*). Thus, flagellar biosynthesis and urease activity may be linked in *H. pylori*. We also identified two confirmed UEFs, *hp0511* and *helB*. *helB* is a potential component of a gene encoding a putative DNA helicase (*hp0548*) that is located in the *cag* pathogenicity island. Our findings should lead to a better understanding of the regulation of *H. pylori* urease. This may in turn lead to novel anti-urease therapies against *H. pylori* and other urease-producing bacteria.

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