INFECTION AND IMMUNITY, Sept. 2001, p. 5914–5920 Vol. 69, No. 9
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.9.5914–5920.2001


# Cloning, Expression, and Catalytic Activity of *Helicobacter hepaticus* Urease

CATHERINE S. BECKWITH,[1]* DAVID J. McGEE,[2]† HARRY L. T. MOBLEY,[2] AND LELA K. RILEY[1]

*Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri 65211,[1] and Department of Microbiology and Immunology, University of Maryland, Baltimore, Maryland 21201[2]*

Received 2 February 2001/Returned for modification 18 April 2001/Accepted 15 June 2001

***Helicobacter hepaticus*** **causes disease in the liver and lower intestinal tract of mice. It is strongly urease positive, although it does not live in an acidic environment. The *H. hepaticus* urease gene cluster was expressed in *Escherichia coli* with and without coexpression of the *Helicobacter pylori* nickel transporter NixA. As for *H. pylori*, it was difficult to obtain enzymatic activity from recombinant *H. hepaticus* urease; special conditions including $NiCl_2$ supplementation were required. The *H. hepaticus* urease cluster contains a homolog of each gene in the *H. pylori* urease cluster, including the urea transporter gene *ureI*. Downstream genes were homologs of the *nik* nickel transport operon of *E. coli*. Nongastric *H. hepaticus* produces urease similar to that of *H. pylori*.**

*Helicobacter hepaticus* is a gram-negative, microaerophilic, urease-positive spiral rod (13). It is a pathogen of mice and causes chronic active hepatitis, hepatic tumors, and proliferative typhlocolitis (40, 53). Although it was first identified in the liver, the primary site of *H. hepaticus* colonization is the intestinal tract; it has not been found in the stomach. After the initial identification of *H. hepaticus* in 1992, this bacterium was found to infect large numbers of rodents used in biomedical research (39, 44). Since that time, additional *Helicobacter* species, including *H. bilis, H. cholecystus, H. rodentium*, and "*H. typhlonicus*" have been identified in laboratory rodents with disease of the hepatobiliary or intestinal tracts (14–18, 21).

The best-known and most-studied member of the genus *Helicobacter* is *H. pylori*, which causes peptic ulcer and gastric cancers in humans (8). Nongastric helicobacters also cause human illness. *H. pullorum, H. canis, H. fennelliae*, and *H. cinaedi* are associated with enteritis; in addition, proctocolitis and bacteremia have been reported for some *Helicobacter* species (11, 49).

Urease is an important virulence factor in *H. pylori* and in *H. mustelae*, the gastric pathogen of ferrets. In those species, urease is required to colonize the gastric mucosa of laboratory animals (3, 10, 51). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide (33). Ammonium ion causes a pH increase that allows *Helicobacter* cells to survive and grow in a highly acidic niche (43). Urease contributes to disease by both direct and indirect mechanisms. Urease itself activates phagocytes, induces cytokine production, and enhances gastric inflammation (22). Ammonia can be used as a nitrogen source for protein synthesis (19), and ammonium ion is toxic to gastric epithelial cells (47).

Urease is a heteromultimer nickel-containing metalloenzyme (33). *H. pylori* urease contains 12 copies each of structural subunits, UreA and UreB, encoded by the genes *ureA* and *ureB* (20). Production of enzymatically active urease requires these structural genes and four accessory genes, *ureE, ureF, ureG*, and *ureH*, which are essential for assimilation of nickel ions into the apoenzyme. An additional gene, *ureI*, encodes an integral membrane protein that transports urea to the cytoplasm under acidic conditions (42, 55).

Urease is enzymatically active only when nickel ions are incorporated during assembly of the mature enzyme (33). *Escherichia coli* carrying the *H. pylori* urease gene cluster is only weakly active except under specific culture conditions (24). The *E. coli* host must be grown in medium supplemented with $NiCl_2$ and devoid of amino acids which chelate nickel ions, thus making them unavailable for intracellular transport. *H. pylori* possesses redundant mechanisms for nickel acquisition, so that active urease is produced even in amino acid-rich medium. One method of transport is via the high-affinity nickel transport protein, NixA (32). Providing a copy of *nixA* in *E. coli* carrying *H. pylori* urease genes leads to greatly enhanced urease activity by improving nickel transport into the cell (30, 32). An additional method of nickel transport in *H. pylori* may be via an ATP-binding cassette (ABC) transporter composed of the genes *abcABCD* (23).

While urease is an important virulence factor for gastric helicobacters which inhabit a highly acidic environment, the function of urease in the nongastric helicobacters, whose environment is not acidic, is unclear. Recently, a partial sequence of the *H. hepaticus* urease structural genes was published (45). We have extended that information by sequencing, cloning, and expressing the entire *H. hepaticus* urease gene cluster. This knowledge will be useful for understanding comparative aspects of the role of urease in the pathogenesis of gastric versus nongastric helicobacters.

**Bacterial strains, plasmids, and media.** *H. hepaticus* strain MU94-1 was isolated from the liver of a naturally infected mouse and grown on chocolate agar as previously described (13). *H. pylori* ATCC 49503 was purchased from the American Type Culture Collection (Rockville, Md.) and grown on 10% sheep blood agar. *Escherichia coli* DH5α (Gibco BRL Life Technologies, Gaithersburg, Md.) was grown on Luria-Bertani (LB) agar or in LB broth (41). Kanamycin (50 μg/ml) and/or

* Corresponding author. Present address: Department of Comparative Medicine, Stanford University School of Medicine, Stanford, CA 94305-5410. Phone: (650) 723-5305. Fax: (650) 725-0940. E-mail: CBeckwith@Stanford.edu.

† Present address: Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile, AL 36688.

TABLE 1. Plasmids used in this study

| Plasmid | Vector | Insert size (kb) | Source of insert | Relevance |
|---|---|---|---|---|
| pHHuc1 | pCR-XL-TOPO | 6.1 | *H. hepaticus* | Urease cluster genes |
| pC2 | pCR-XL-TOPO | 7 | Irrelevant fragment | Negative control for pHHuc1 |
| pACYC184-*nixA* | pACYC184 | 1.2 | *H. pylori* | Nickel transporter gene *nixA* |
| pACYC184 | pACYC184 | None | None | Vector control for pACYC184-*nixA* |
| p2:5A | pUC18 | 4.3 | *H. hepaticus* | Library clone |

chloramphenicol (20 μg/ml) were added to media when needed to maintain plasmids.

The plasmid pHP8080 (30) was digested with the *Nru*I and *Ava*I restriction endonucleases. A 1.2-kb fragment containing *nixA* was ligated into corresponding sites in pACYC184 and designated pACYC184-*nixA* (Table 1).

**Construction of an *H. pylori ureAB* probe.** A 1.6-kb PCR fragment containing the *H. pylori* urease genes *ureA* and *ureB* was amplified from *H. pylori* genomic DNA with the PCR primers Hp2794f and Hp4324r (Table 2). The PCR product was labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, Ind.) by PCR according to the manufacturer's guidelines (The DIG System User's Guide for Filter Hybridization, 2000; Roche).

**Hybridizations.** *H. hepaticus* genomic DNA was digested with the restriction endonuclease *Hin*dIII, electrophoresed, and transferred to nylon membranes according to standard techniques (4, 41). Membranes were hybridized with the *H. pylori ureAB* probe under stringent conditions (65°C), washed, incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche), and detected using the chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl) phenyl phosphate; Roche) using the manufacturer's protocol. A single band of 2.8 kb was identified, indicating that a single copy of the structural urease genes is present in *H. hepaticus* (data not shown).

An *H. hepaticus* plasmid library (29) harbored in *E. coli* strain DH5αMCR was screened for clones containing urease genes by colony hybridization with the *H. pylori ureAB* probe by standard techniques (4, 41). Membranes containing plasmid DNA were hybridized and washed under stringent conditions (65°C), incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche), and detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Roche). An *E. coli* clone that hybridized with the *ureAB* probe was selected; the corresponding plasmid was designated p2:5A.

**DNA sequencing and analysis.** Vector-insert junctions of the plasmid p2:5A were sequenced by the dideoxy chain termination method using the ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, Calif.) and M13/pUC sequencing primers. The obtained sequence had strong homology with *Helicobacter* urease genes (BLAST; National Center for Biotechnology Information, National Library of Medicine, NIH [http://www.ncbi.nlm.nih.gov/BLAST]), so the entire 4.3-kb insert of plasmid p2:5A (Table 1) was sequenced. The insert contained one open reading frame (ORF) with homology to *ureA* and a partial ORF with homology to *ureB* (GCG software package, Wisconsin Package Version 10.1; Genetics Computer Group, Inc., Madison, Wisc.; and Omiga Version 2.0; Oxford Molecular, Ltd., Madison, Wisc.).

Since the entire *H. hepaticus* urease cluster was not present in a single clone, the 3′ end of *ure*B and downstream genes were amplified by cassette-mediated PCR using the TaKaRa LA PCR in vitro cloning kit (PanVera Corporation, Madison, Wisc.). Specific primers HhureB1 and HhureB2 (Table 1) were designed near the 3′ end of the *H. hepaticus ureB* gene. *H. hepaticus* genomic DNA, digested with the restriction enzyme *Pst*I and ligated to cassettes, was amplified with cassette primer C1 and HhureB1, 400 μM (each) dNTP, 10× LA BufferII, and 2.5 mM $MgCl_2$. Samples were denatured at 94°C for 9 min before 2.0 U of TaKaRa LA Taq DNA polymerase was added. Thirty cycles of 15 s at 94°C, 2 s at 58.7°C, and 6.25 min at 72°C were completed followed by a final extension at 72°C for 10 min. The nested primers C2 and HhureB2 reamplified products from the first PCR. The approximately 6.2-kb product was directly sequenced to reduce the chance of incorporating PCR-generated mistakes commonly fixed in individual strands when the PCR product is cloned before sequencing.

Analysis of the combined sequences of the library clone and the cassette-mediated PCR product revealed seven ORFs homologous to the *H. pylori* urease structural genes *ureA* and *ureB*, the urea transporter gene *ureI*, and the accessory genes *ureE, ureF, ureG*, and *ureH* (Fig. 1). The deduced amino acid

TABLE 2. Oligonucleotide primers

| Primer | Nucleotide sequence | Organism | Reference | Product |
|---|---|---|---|---|
| Hp2769f | 5′-TTTGATTAGTGCCCATATTATGGAAG | *H. pylori* | 48 | *ureAB* probe |
| Hp4346r | 5′-TGGTGGCACACCATAAGCATGTC | *H. pylori* | 48 | *ureAB* probe |
| Hh653f | 5′-CCGGAATTCGGCTTTGCATACCCTATTGACAAAC[a] | *H. hepaticus* | This work | Urease cluster |
| Hh6778r | 5′-CCCGAGCTCTGCGTGGTGGAACATATAAGGATAG[a] | *H. hepaticus* | This work | Urease cluster |
| HhureB1 | 5′-ATGGAATGATTGTAGCAGCAAAAATAGGGG | *H. hepaticus* | This work | Cassette-mediated PCR |
| HhureB2 | 5′-GGATTCTAATGCTTCTATTCCTACTCCTGAACCTG | *H. hepaticus* | This work | Cassette-mediated PCR |

[a] Underlined nucleotides indicate restriction endonuclease sites.

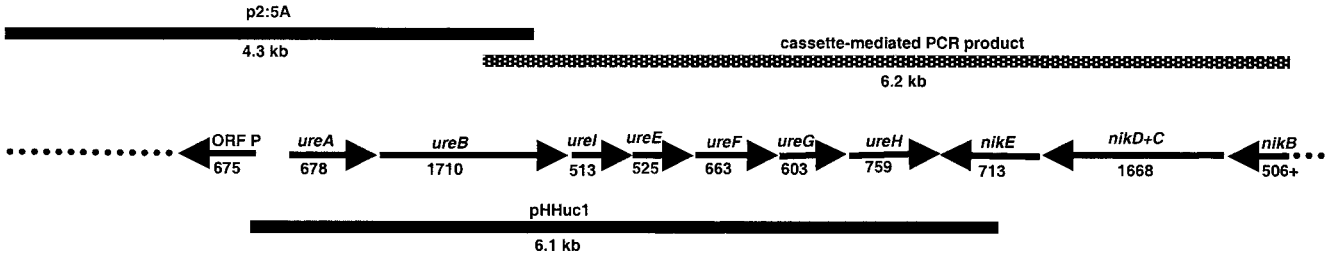


FIG. 1. Map of *H. hepaticus* genes included on plasmids and PCR products. Arrows represent ORFs and directions of transcription. Solid bars represent plasmid inserts, and the patterned bar represents the cassette-mediated PCR product. Size is shown in base pairs or kilobases for genes and larger fragments, respectively. Dotted lines represent incompletely sequenced fragments.

sequences were highly homologous to those corresponding to urease genes of other *Helicobacter* species (Table 3). *H. hepaticus* UreB had 100% identity to two urease signature consensus patterns (Motifs program, GCG package).

ATG codons initiated each ORF of the *H. hepaticus* urease cluster except *ureF*, which began with a TTG codon. A putative ribosome-binding site preceded each ORF except the accessory gene *ureE*. Two intergenic regions, *ureI–ureE* and *ureE-ureF*, had overlapping start and stop codons. The intergenic region between *H. hepaticus ureB* and *ureI* contained only 9 bp in contrast to approximately 200 bp between these genes in *H. pylori*. The G+C content of the *H. hepaticus* urease cluster genes was 36%.

The *H. hepaticus* urease cluster contained a homolog of the *H. pylori* urea transporter gene, *ureI*. Kyte Doolittle hydropathy analysis of the encoded amino acid sequence predicted six hydrophobic regions separated by hydrophilic regions, very similar to *H. pylori* UreI. Alignment of the amino acid sequences of the two UreI proteins showed two gaps of 15 and 9 amino acids in the *H. hepaticus* product within extracellular loops of *H. pylori* UreI (55) (Fig. 2).

DNA in the downstream region flanking the *H. hepaticus* urease cluster had homology with bacterial ABC transporters, which function as dipeptide, oligopeptide, and nickel transporters. These ORFs were on the complementary strand of the cassette-mediated PCR product with opposite polarity to genes of the urease cluster (Fig. 1) and aligned closely with the *E. coli nik* operon, a nickel transport system (28, 34). A partial *nikB* ORF was identified in *H. hepaticus* followed by a long ORF that encompassed domains homologous to *nikC* and *nikD*, and finally, a *nikE* homolog. Both the *nikD* and *nikE* homologs contained consensus patterns for the ABC transporter family signature and ATP/GTP-binding site motifs A and B (28, 31, 34, 52). A predicted ORF, ORF P, upstream of the *ureA* gene in the *H. hepaticus* clone p2:5A contained three sets of direct repeats with 51, 43, and 43 bases each and had homology to putative periplasmic proteins of *Campylobacter jejuni* and *Neisseria meningitidis* (36, 37).

TABLE 3. Amino acid sequence identity of predicted products of the *H. hepaticus* urease gene cluster

| Comparison species | % Identity of: | | | | | | |
|---|---|---|---|---|---|---|---|
| | UreA | UreB | UreI | UreE | UreF | UreG | UreH |
| *H. pylori* | 66 | 77 | 62 | 37 | 46 | 82 | 43 |
| *H. felis* | 66 | 76 | 58 | | | | |
| *H. mustelae* | 71 | 84 | | | | | |
| *"H. heilmannii"* | 66 | 76 | | | | | |

**Cloning the *H. hepaticus* urease cluster.** To express recombinant *H. hepaticus* urease, the entire cluster of genes was cloned as a single fragment amplified by long PCR. Primers Hh653f and Hh6778r (Table 2) amplified the urease structural genes, accessory genes, and approximately 300 bases of flanking sequence from *H. hepaticus* DNA. Long PCR reactions contained 100 to 200 ng of *H. hepaticus* genomic DNA, 0.3 μM (each) primer, 300 μM (each) dNTP, 1 mM $MgSO_4$, 2.5 U of Platinum *Pfx* polymerase, and *Pfx* Amplification Buffer (Gibco BRL Life Technologies) in a 50-μl volume. Reactions were denatured for 3 min followed by 20 cycles of 94°C for 15 s, 58.2°C for 2 s, and 68°C for 6 min 20 s with a final 7-min extension at 68°C. A 6.1-kb product was obtained.

Terminal 3′ deoxyadenosine overhangs were added to the PCR product by incubation with 200 μM dNTPs, 1 U of *Taq* DNA polymerase, and 10× PCR buffer (Roche) at 72°C for 15 min. The fragment was ligated to pCR-XL-TOPO (TOPO XL PCR cloning kit; Invitrogen, Carlsbad, Calif.) according to the manufacturer's recommendations and was designated pHHuc1 (Table 1). The ligation reaction was electroporated into DH5α that had been previously transformed with pACYC184-*nixA* to facilitate identification of urease-positive clones. After cloning, the nucleotide sequence of the insert in pHHuc1 was redetermined and compared to sequence of the cassette-mediated PCR product and plasmid p2:5A to ensure that no base errors were introduced during PCR amplification.

A 7-kb control DNA fragment supplied with the TOPO XL PCR cloning kit was amplified, ligated to the vector, and designated pC2. The plasmid pC2 was cotransformed into DH5α carrying pACYC184-*nixA* as a urease-negative control strain. A second DH5α control strain was prepared with pHHuc1 and insert-free pACYC184 (Table 1). DH5α was also transformed with pHHuc1 and pC2 as single plasmids.

**Qualitative tests for urease activity.** *E. coli* DH5α cotransformants carrying pHHuc1 plus pACYC184-*nixA* and control strains were screened for enzymatic activity on modified urea segregation agar (30). Only cotransformants carrying pHHuc1 and pACYC184-*nixA* turned the medium pink as the pH rose because of urea hydrolysis.

**Antigenic cross-reactivity of *H. hepaticus* urease structural subunits.** Cultured bacteria were centrifuged (10,000 × *g*, 10 min, 4°C), washed twice in 50 mM HEPES, pH 7.5, and frozen at −20°C until used. Pellets were resuspended in 50 mM

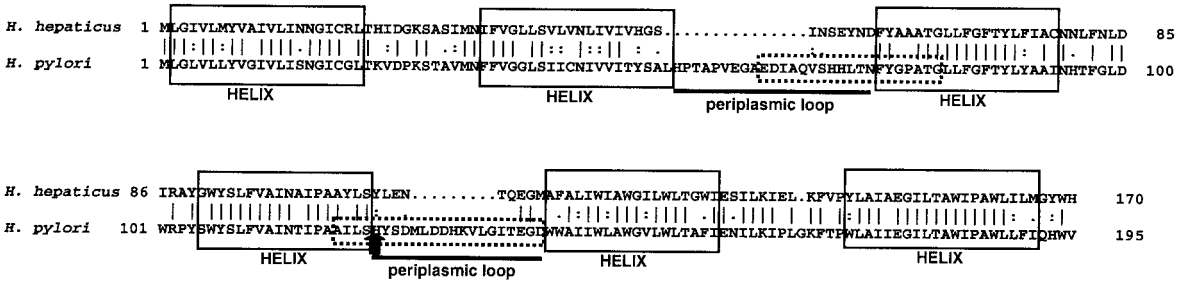


FIG. 2. Alignment of UreI amino acid sequences from *H. hepaticus* MU94-1 and *H. pylori*. Identical residues are depicted by "|." Similar residues are depicted with ":" or "." Transmembrane helices and periplasmic loops are shown as determined by Weeks et al. (55) for *H. pylori* UreI. Helices are enclosed in solid boxes. Solid bars indicate periplasmic loops. Peptides used by Scott et al. (42) to develop anti-UreI antibodies are enclosed in dashed boxes. The arrowhead points to histidine 123 of *H. pylori* UreI.

HEPES, sonicated 3 times for 30 s, and centrifuged at 12,000 × *g*, and the supernatant was retained. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (27). Proteins were electrophoresed, transferred to an ImmobilonP membrane (Millipore, Bedford, Mass.), and detected using the Lumi-Light$^{PLUS}$ Western Blotting Kit (Roche) using the manufacturer's protocol. Antibodies to *H. pylori* UreA and UreB (24) identified immunoreactive bands at approximately 27 and 60 kDa, respectively, in *H. pylori, H. hepaticus*, and DH5α containing plasmid pHHuc1 (data not shown). No bands were identified for DH5α transformed with pC2.

**Enzymatic activity of recombinant *H. hepaticus* urease.** Growth media for DH5α strains varied based on which plasmids were carried. DH5α cotransformed with pHHuc1 and pACYC184-*nixA*, or corresponding control constructs (Table 1), was grown in M9 minimal medium (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 CaC $l_2$, and 1.68 μM thiamine-HCl) supplemented with 0.5% casamino acids, 1% LB, 0.1 μM $NiCl_2$, 50 μg of kanamycin/ml, and 20 μg of chloramphenicol/ml (30). Bacteria were centrifuged, washed, and sonicated as described for sodium dodecyl sulfate-polyacrylamide gel electrophoresis Urease activities were determined by the phenol-hypochlorite assay, a spectrophotometric assay that measures ammonia production, as previously described (30, 54). Data were statistically analyzed using Sigmastat for Windows (Version 2.03; SPSS, Inc., San Rafael, Calif.). High levels of urease activity were measured only when the *H. hepaticus* urease cluster and the nickel transporter gene were both present in the same strain (pHHuc1 and pACYC184-*nixA*) (Fig. 3A). When either of these plasmids was present in combination with its negative control, only negligible ammonia was produced.

Starter cultures of DH5α singly transformed with pHHuc1 or pC2 were grown as for cotransformed DH5α. Cultures were then diluted 1:500 into M9 minimal medium with 3 μM $NiCl_2$ and kanamycin (no casamino acids or LB). These cultures were incubated at 37°C with shaking until optical density measurements were stable, approximately 44 h. Casamino acids and LB were not used to prevent amino acid chelation of nickel ions (24). DH5α(pHHuc1) had consistent urease activity (Fig. 3B), while DH5α(pC2) had negligible activity. Sonicated wild-type *H. hepaticus* MU94-1 had an average urease activity similar to that for *H. pylori* (Fig. 3C).

**Nucleotide sequence accession numbers.** The sequence of pHHuc1 containing the urease gene cluster of *H. hepaticus* MU94-1 was deposited in GenBank under accession number AF3322656. Sequence of the 5′ and the 3′ flanking regions was deposited under accession numbers AF332654 and AF332655, respectively. The latter two sequences were determined in one direction only.

**Discussion.** This is the first report of the cloning and sequencing of a complete urease gene cluster for a *Helicobacter* species other than *H. pylori*. The urease gene cluster of *H. hepaticus* is similar to the urease cluster of *H. pylori* in many ways. As in other helicobacters, two structural subunit genes are present (Fig. 1), in contrast to the more common bacterial pattern of a three-subunit urease (33). Both structural subunits of *H. hepaticus* cross-react with immune sera directed against *H. pylori* urease subunits, indicating that recombinant *H. hepaticus* urease is stable and important antigenic epitopes are conserved. Indeed, protein sequence alignment of the UreA and UreB structural subunits of *H. pylori* and *H. hepaticus* confirm this high degree of relatedness (Table 3).

Despite overall similarities of the *H. hepaticus* and *H. pylori* urease gene clusters, there are notable differences. In *H. hepaticus*, the *ureB–ureI* intergenic distance is 9 bp, compared to approximately 200 bp (strain dependent) in *H. pylori*; in *H. pylori*, the sequence contains a promoter for *ureI* and downstream accessory genes (1, 26). This sequence difference suggests that the two species differ in regulation of *ureI* and the accessory genes. Although the overall sequence of UreI in *H. hepaticus* and *H. pylori* is well conserved, alignment shows gaps in the *H. hepaticus* product (Fig. 2). This may explain why antibodies to *H. pylori* UreI failed to detect products in Western blots of *H. hepaticus* and other nongastric species (42). The antibodies used by Scott et al. (42) were directed against peptides within extracellular loops of *H. pylori* UreI which are truncated in *H. hepaticus* UreI. *H. hepaticus* UreI also lacks the critical histidine 123 residue, important for acid activation of urea transport in *H. pylori* (55). A similar histidine residue is also present in UreI of gastric *H. felis* (GenBank accession no. A41012) (46). The presence of such a histidine residue in gastric helicobacters and its absence in nongastric *H. hepaticus* may represent specific adaptations of these organisms to acidic versus nonacidic environments.

The significant urease activity of DH5α(pHHuc1) proves that all of the *H. hepaticus* genes essential for urease activity

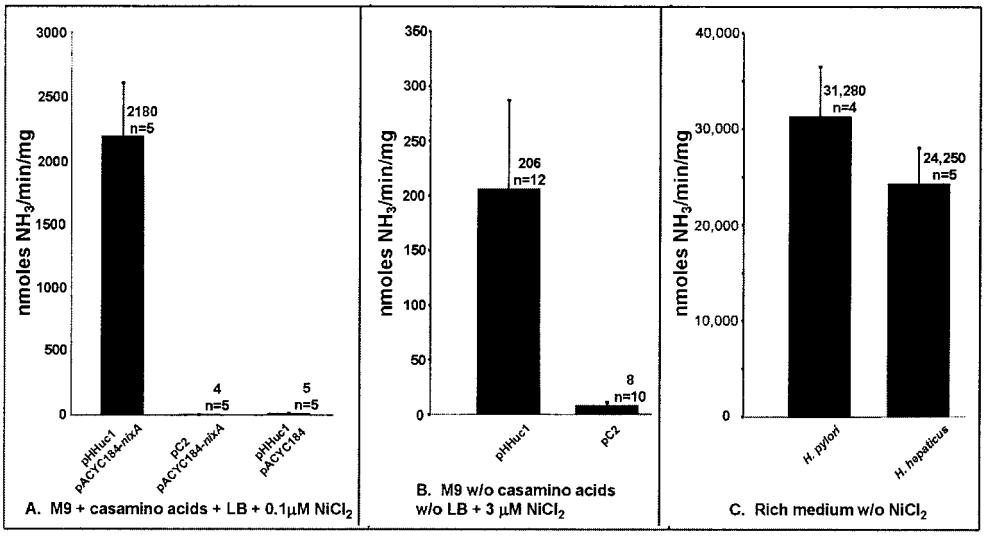


FIG. 3. Urease activities of sonicated bacterial proteins in the phenol-hypochlorite assay. Scales differ for the three panels. (A) *E. coli* DH5α cotransformed with *H. hepaticus* urease genes (pHHuc1) and the *H. pylori* nickel transporter (pACYC184-*nixA*), or control plasmids ($P = 0.008$; Kruskal-Wallis one-way analysis of variance on ranks). (B) *E. coli* DH5α singly transformed with *H. hepaticus* urease genes (pHHuc1) or control plasmid (pC2) ($P = <0.001$; Mann-Whitney rank sum test). (C) Wild-type *H. pylori* and *H. hepaticus*. Differences are not statistically significant (*t* test).

are present on this plasmid (Fig. 3B). Without a specific nickel transporter, urease activity was obtained only when *E. coli* cells were grown in medium devoid of amino acids which chelate nickel ions and prevent their assimilation into the apoenzyme (24, 32). When the NixA nickel transporter was coexpressed with the *H. hepaticus* urease gene cluster in DH5α, much higher levels of urease activity were obtained (Fig. 3A). These levels were similar to urease activities of cloned *H. pylori* urease genes and emphasize the importance of nickel acquisition to urease activity. In the presence of a nickel transporter, urease activity was high even when host cells were grown in medium supplemented with amino acids. Urease activity of DH5α cotransformed with pHHuc1 and pACYC184-*nixA* remained about 10-fold lower than that of wild-type *H. hepaticus* (Fig. 3C). This is similar to findings with comparable clones carrying *H. pylori* genes and suggests that other factors in addition to urease genes and nickel transport may be necessary for full wild-type levels of urease activity.

It is likely that wild-type *H. hepaticus* possesses a specialized system for nickel transport, but it is not known whether that system is a *nixA* homolog, another transporter gene, or perhaps multiple redundant means of nickel transport as is found in *H. pylori*. ORFs flanking the downstream end of the *H. hepaticus* urease gene cluster are most closely related to the *nik* operons of *E. coli* (34) and *Brucella suis* (25). Both of these operons were documented to mediate nickel transport, and mutation of *B. suis nikA* led to decreased urease activity. The closest *H. pylori* match to the *H. hepaticus nik* homologs is the *dpp* operon, a putative dipeptide ABC transporter (2, 50). A role for *dpp* genes in nickel transport has not yet been tested. Another set of *H. pylori* ABC transporter genes, *abcABCD*, appears to be necessary for full urease activity, since mutation of *abcD* led to decreased urease activity (23). That system, however, has not been proven to be specific for nickel.

Identification of a putative nickel transporter flanking the urease gene cluster in *H. hepaticus* points to a difference in genome organization between *H. hepaticus* and *H. pylori*. Documented nickel transporter genes in *H. pylori* are located at separate sites on the chromosome distant from the urease gene cluster (2, 50). In contrast, the *H. pylori* urease cluster is flanked by *lspA* upstream and *cdrA* downstream, respectively (2, 50).

*H. hepaticus* inhabits a biological niche where the pH is nearly neutral, yet it produces an amount of urease activity similar to that of gastric *H. pylori* (Fig. 3C). It is not apparent why such high levels of urease activity would be necessary in the lower bowel and liver. Possible roles for urease in *H. hepaticus* include improving survival during passage through the stomach, as for *Yersinia enterocolitica* (7), and producing ammonia as a source of nitrogen for protein biosynthesis (6, 19). Urease activity could significantly contribute to pathology, since ammonia damages host cells (47) and urease itself stimulates phagocyte chemotaxis, activates immune cells, and induces cytokine production (9).

Among the nongastric helicobacters, no clear pattern can be discerned correlating urease activity with virulence or site of colonization. Both urease-positive and urease-negative *Helicobacter* species have been identified in the liver and/or biliary tracts of various animal species in association with disease (14–18, 49). Some reports link helicobacters with human diseases of the liver and biliary tract (5, 12, 35, 38). Ultimately, understanding the role of urease in the pathogenesis of the enterohepatic helicobacters may contribute to a better understanding of some human hepatobiliary tract diseases. Future studies will clarify properties of the specific gene products and their roles in colonization and pathogenesis of *H. hepaticus*.

This work was supported in part by NIH PHS grant AI10098 (D.J.M.) and AI25567 (H.L.T.M.).

We thank Robert S. Livingston for the use of the *H. hepaticus* library and Howard Wilson for computer graphics assistance.

## REFERENCES


1. **Akada, J. K., M. Shirai, H. Takeuchi, M. Tsuda, and T. Nakazawa.** 2000. Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. Mol. Microbiol. **36:**1071–1084.
2. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176–180.
3. **Andrutis, K. A., J. G. Fox, D. B. Schauer, R. P. Marini, J. C. Murphy, L. Yan, and J. V. Solnick.** 1995. Inability of an isogenic urease-negative mutant stain of *Helicobacter mustelae* to colonize the ferret stomach. Infect. Immun. **63:**3722–3725.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, New York, N.Y.
5. **Avenaud, P., A. Marais, L. Monteiro, B. Le Bail, P. Bioulac Sage, C. Balabaud, and F. Megraud.** 2000. Detection of *Helicobacter* species in the liver of patients with and without primary liver carcinoma. Cancer **89:**1431–1439.
6. **Burne, R. A., and Y. Y. Chen.** 2000. Bacterial ureases in infectious diseases. Microbes Infect. **2:**533–542.
7. **De Koning-Ward, T. F., and R. M. Robins-Browne.** 1995. Contribution of urease to acid tolerance in *Yersinia enterocolitica*. Infect. Immun. **63:**3790–3795.
8. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
9. **Dunn, B. E., and S. H. Phadnis.** 1998. Structure, function and localization of *Helicobacter pylori* urease. Yale J. Biol. Med. **71:**63–73.
10. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect. Immun. **62:**3604–3607.
11. **Fox, J.** 1998. Enterohepatic helicobacters: natural and experimental models. Ital. J. Gastroenterol. Hepatol. **30:**S264–S269.
12. **Fox, J. G., F. E. Dewhirst, Z. Shen, Y. Feng, N. S. Taylor, B. J. Paster, R. L. Ericson, C. N. Lau, P. Correa, J. C. Araya, and I. Roa.** 1998. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology **114:**755–763.
13. **Fox, J. G., F. E. Dewhirst, J. G. Tully, B. J. Paster, L. Yan, N. S. Taylor, M. J. Collins, Jr., P. L. Gorelick, and J. M. Ward.** 1994. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J. Clin. Microbiol. **32:**1238–1245.
14. **Fox, J. G., P. L. Gorelick, M. C. Kullberg, Z. Ge, F. E. Dewhirst, and J. M. Ward.** 1999. A novel urease-negative *Helicobacter* species associated with colitis and typhlitis in IL-10-deficient mice. Infect. Immun. **67:**1757–1762.
15. **Fox, J. G., L. L. Yan, F. E. Dewhirst, B. J. Paster, B. Shames, J. C. Murphy, A. Hayward, J. C. Belcher, and E. N. Mendes.** 1995. *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated from bile, livers, and intestines of aged, inbred mice. J. Clin. Microbiol. **33:**445–454.
16. **Franklin, C. L., C. S. Beckwith, R. S. Livingston, L. K. Riley, S. V. Gibson, C. L. Besch-Williford, and R. R. Hook, Jr.** 1996. Isolation of a novel *Helicobacter* species, *Helicobacter cholecystus* sp. nov., from the gallbladders of Syrian hamsters with cholangiofibrosis and centrilobular pancreatitis. J. Clin. Microbiol. **34:**2952–2958.
17. **Franklin, C. L., L. K. Riley, R. S. Livingston, C. S. Beckwith, C. L. Besch-Williford, and R. R. Hook, Jr.** 1998. Enterohepatic lesions in SCID mice infected with *Helicobacter bilis*. Lab. Anim. Sci. **48:**334–339.
18. **Franklin, C. L., L. K. Riley, R. S. Livingston, C. S. Beckwith, R. R. Hook, Jr., C. L. Besch-Williford, R. Hunziker, and P. L. Gorelick.** 1999. Enteric lesions in SCID mice infected with "*Helicobacter typhlonicus*," a novel urease-negative *Helicobacter* species. Lab. Anim. Sci. **49:**496–505.
19. **Garner, R. M., J. Fulkerson, Jr., and H. L. Mobley.** 1998. *Helicobacter pylori* glutamine synthetase lacks features associated with transcriptional and post-translational regulation. Infect. Immun. **66:**1839–1847.
20. **Ha, N. C., S. T. Oh, J. Y. Sung, K. A. Cha, M. H. Lee, and B. H. Oh.** 2001. Supramolecular assembly and acid resistance of Helicobacter pylori urease. Nat. Struct. Biol. **8:**505–509.
21. **Haines, D. C., P. L. Gorelick, J. K. Battles, K. M. Pike, R. J. Anderson, J. G. Fox, N. S. Taylor, Z. Shen, F. E. Dewhirst, M. R. Anver, and J. M. Ward.** 1998. Inflammatory large bowel disease in immunodeficient rats naturally and experimentally infected with *Helicobacter bilis*. Vet. Pathol. **35:**202–208.
22. **Harris, P. R., H. L. Mobley, G. I. Perez-Perez, M. J. Blaser, and P. D. Smith.** 1996. *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology **111:**419–425.
23. **Hendricks, J. K., and H. L. Mobley.** 1997. *Helicobacter pylori* ABC transporter: effect of allelic exchange mutagenesis on urease activity. J. Bacteriol. **179:**5892–5902.
24. **Hu, L. T., and H. L. Mobley.** 1993. Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. Infect. Immun. **61:**2563–2569.
25. **Jubier-Maurin, V., A. Rodrigue, S. Ouahrani-Bettache, M. Layssac, M. A. Mandrand-Berthelot, S. Kohler, and J. P. Liautard.** 2001. Identification of the *nik* gene cluster of *Brucella suis*: regulation and contribution to urease activity. J. Bacteriol. **183:**426–434.
26. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173:**1920–1931.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
28. **Linton, K. J., and C. F. Higgins.** 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. **28:**5–13.
29. **Livingston, R. S., L. K. Riley, R. R. Hook, Jr., C. L. Besch-Williford, and C. L. Franklin.** 1999. Cloning and expression of an immunogenic membrane-associated protein of *Helicobacter hepaticus* for use in an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **6:**745–750.
30. **McGee, D. J., C. A. May, R. M. Garner, J. M. Himpsl, and H. L. Mobley.** 1999. Isolation of *Helicobacter pylori* genes that modulate urease activity. J. Bacteriol. **181:**2477–2484.
31. **Mimura, C. S., S. R. Holbrook, and G. F. Ames.** 1991. Structural model of the nucleotide-binding conserved component of periplasmic permeases. Proc. Natl. Acad. Sci. USA **88:**84–88.
32. **Mobley, H. L., R. M. Garner, and P. Bauerfeind.** 1995. *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. Mol. Microbiol. **16:**97–109.
33. **Mobley, H. L., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol. Rev. **59:**451–480.
34. **Navarro, C., L. F. Wu, and M. A. Mandrand-Berthelot.** 1993. The *nik* operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. Mol. Microbiol. **9:**1181–1191.
35. **Nilsson, I., S. Lindgren, S. Eriksson, and T. Wadstrom.** 2000. Serum antibodies to *Helicobacter hepaticus* and *Helicobacter pylori* in patients with chronic liver disease. Gut **46:**410–414.
36. **Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R. M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, M. A. Rajandream, K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt, and B. G. Barrell.** 2000. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. Nature **404:**502–506.
37. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665–668.
38. **Ponzetto, A., R. Pellicano, N. Leone, M. A. Cutufia, F. Turrini, W. F. Grigioni, A. D'Errico, P. Mortimer, M. Rizzetto, and L. Silengo.** 2000. *Helicobacter* infection and cirrhosis in hepatitis C virus carriage: is it an innocent bystander or a troublemaker? Med. Hypotheses **54:**275–277.
39. **Riley, L. K., C. L. Franklin, R. R. Hook, Jr., and C. Besch-Williford.** 1996. Identification of murine helicobacters by PCR and restriction enzyme analyses. J. Clin. Microbiol. **34:**942–946.
40. **Russell, R. J., D. C. Haines, M. R. Anver, J. K. Battles, P. L. Gorelick, L. L. Blumenauer, M. A. Gonda, and J. M. Ward.** 1995. Use of antibiotics to prevent hepatitis and typhlitis in male scid mice spontaneously infected with *Helicobacter hepaticus*. Lab. Anim. Sci. **45:**373–378.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Scott, D. R., E. A. Marcus, D. L. Weeks, A. Lee, K. Melchers, and G. Sachs.** 2000. Expression of the *Helicobacter pylori ureI* gene is required for acidic pH activation of cytoplasmic urease. Infect. Immun. **68:**470–477.
43. **Scott, D. R., D. Weeks, C. Hong, S. Postius, K. Melchers, and G. Sachs.** 1998. The role of internal urease in acid resistance of *Helicobacter pylori*. Gastroenterology **114:**58–70.
44. **Shames, B., J. G. Fox, F. Dewhirst, L. Yan, Z. Shen, and N. S. Taylor.** 1995. Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. J. Clin. Microbiol. **33:**2968–2972.
45. **Shen, Z., D. B. Schauer, H. L. Mobley, and J. G. Fox.** 1998. Development of a PCR-restriction fragment length polymorphism assay using the nucleotide sequence of the *Helicobacter hepaticus* urease structural genes *ureAB*. J. Clin. Microbiol. **36:**2447–2453.
46. **Skouloubris, S., J. M. Thiberge, A. Labigne, and H. De Reuse.** 1998. The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival in vivo. Infect. Immun. **66:**4517–4521.
47. **Smoot, D. T., H. L. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. **58:**1992–1994.
48. **Solnick, J. V., J. O'Rourke, A. Lee, and L. S. Tompkins.** 1994. Molecular

analysis of urease genes from a newly identified uncultured species of *Helicobacter*. Infect. Immun. **62:**1631–1638.
49. **Stanley, J., D. Linton, A. P. Burnens, F. E. Dewhirst, S. L. On, A. Porter, R. J. Owen, and M. Costas.** 1994. *Helicobacter pullorum* sp. nov.—genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology **140:**3441–3449.
50. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:** 539–547.
51. **Tsuda, M., M. Karita, M. G. Morshed, K. Okita, and T. Nakazawa.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect. Immun. **62:**3586–3589.
52. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
53. **Ward, J. M., M. R. Anver, D. C. Haines, J. M. Melhorn, P. Gorelick, L. Yan, and J. G. Fox.** 1996. Inflammatory large bowel disease in immunodeficient mice naturally infected with *Helicobacter hepaticus*. Lab. Anim. Sci. **46:**15–20.
54. **Weatherburn, M.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:**971–974.
55. **Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs.** 2000. A H+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Science **287:**482–485.

*Editor:* D. L. Burns