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Molecular Analysis of Urease Genes from a Newly Identified Uncultured Species of *Helicobacter*

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"*Gastrospirillum hominis*" is an uncultured gastric spiral bacterium that has recently been shown by 16S rDNA sequence analysis to be a newly recognized species of *Helicobacter* that infects humans, and it has been provisionally designated "*Helicobacter heilmannii*." We used PCR to directly amplify the urease structural genes of "*H. heilmannii*" from infected gastric tissue. DNA sequence analysis identified two open reading frames, *ureA* and *ureB*, which code for polypeptides with predicted molecular weights of 25,729 and 61,831, respectively. The urease subunit genes from "*H. heilmannii*" were cloned and expressed in *Escherichia coli*. Western blot (immunoblot) analysis showed that antiserum directed against the *ureA* and *ureB* gene products from *H. pylori* was cross-reactive with the corresponding polypeptides from "*H. heilmannii*." Analysis of the derived amino acid sequences of "*H. heilmannii*" UreA and UreB demonstrated that "*H. heilmannii*" urease is more highly related to the urease from *H. felis* (found in the stomachs of cats and dogs) than to the urease from *H. pylori*. These data are consistent with 16S rDNA sequence analysis and suggest that "*H. heilmannii*" is phylogenetically most closely related to *H. felis*.

Helicobacter pylori is a curved, microaerophilic, gram-negative rod that frequently infects human gastric mucosa. In the nearly 10 years since it was first cultivated (29), it has become clear that *H. pylori* is the most common cause of chronic gastritis and that it is involved in the pathogenesis of peptic ulcer disease and probably also gastric cancer (43). Because *H. pylori* is restricted in its host range, the search for an appropriate animal model has led to considerable interest in gastric spiral bacteria from other animals, particularly, *H. felis* in the cat and dog (35) and *H. mustelae* in the ferret (17).

Several reports have described an uncultivated gastric spiral bacterium that is ubiquitous in a wide range of animal hosts and that is occasionally found in human gastric mucosa (5, 9, 19, 26, 32). This organism (formerly "*Gastrospirillum hominis*") has recently been shown by 16S rDNA sequence analysis to be a newly identified *Helicobacter* species that is closely related to *H. felis* (42). It has been provisionally designated "*H. heilmannii*," although diversity in the 16S rRNA gene among three different isolates makes a definitive species designation premature (42). Human infection with "*H. heilmannii*" is associated with chronic gastritis similar to that seen with *H. pylori* but less severe and with a greater predominance of mononuclear cells (19). "*H. heilmannii*" is unique in the *Helicobacter* genus because its host range includes humans as well as a variety of other animals. For this reason, it might be particularly suitable for use in an animal model of pathogenesis if the appropriate conditions could be found for cultivation on artificial media.

"*H. heilmannii*" shares with *H. pylori* and other gastric helicobacters the expression of urease activity (19). Although it probably has other roles as well, one function of this enzyme may be protection of the organism from gastric acid upon initial colonization by the hydrolysis of urea to produce NH_4^+ ions. Initial evidence for this hypothesis comes from in vitro studies showing that wild-type *H. pylori* (28), but not a urease-deficient strain (39), are acid tolerant in the presence of physiological concentrations of urea. It has also been shown that pretreatment of ferrets with a urease inhibitor prevents colonization by *H. mustelae*, although it has no effect on an established infection (30). Furthermore, a urease-deficient *H. pylori* produced by nitrosoguanidine treatment was unable to colonize in the gnotobiotic piglet model (10), and preliminary evidence indicates that an isogenic urease-deficient mutant produced by allelic exchange (12) is also unable to colonize the gnotobiotic piglet (9a).

There is some functional heterogeneity among the known *Helicobacter* ureases, and there has been speculation that these differences may be important. For example, *H. muridarum* (27), whose natural habitat is the small and large bowel of rodents, has a urease that is significantly less acid stable than the urease from the gastric helicobacters (16). Presumably, the *H. muridarum* urease has evolved for some purpose other than buffering gastric acid, such as supplying the bacterium with nitrogen from urea. The urease from gastric helicobacters may also be involved in nutrient acquisition by the cytotoxic effects of ammonia or by the inflammatory effects of urease itself (2). Molecular analysis of the "*H. heilmannii*" urease will clarify the evolutionary relationship of this organism to other helicobacters and may help us to understand the role of urease in the ecological niche, host range specificity, and inflammatory response to *Helicobacter* infection. Furthermore, recent evidence suggests that immunization of mice with *H. pylori* urease confers protection against challenge with *H. felis* (3), which suggests that understanding the *Helicobacter* ureases may also be important in the development of a *Helicobacter* vaccine.

We used PCR to amplify, clone, and express the urease structural genes from "*H. heilmannii*." "*H. heilmannii*" cannot presently be cultivated on artificial media, but it can be maintained in vivo by inoculation of infected human gastric tissue into the stomach of a pathogen-free mouse (7). We therefore amplified the urease genes of a human isolate of "*H. heilmannii*" directly from infected mouse gastric tissue and

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TABLE 1. Oligonucleotide primers used for amplification of "*Helicobacter heilmannii*" urease genes[a]

Primer designation	Nucleotide sequence (5'-3')	Source of sequence
2794F	GCATCCGCGGCCGCTTTGATTAGTGCCCATATTATGGAAG	*H. pylori* 2769-2794[b]
4324R	GCATCCGCGGCCGCTGGTGGCACACCATAAGCATGTC	*H. pylori* 4346-4324[b]
1550F	AACCGCCGATGGCTTGGTGTGCGCT	*H. pylori* 1526-1550[b]
2943R	CTTCTACGGGAGTGTGGATG	"*H. heilmannii*" 514-495[c]
2365F	GCATCCGCGGCCGCACGTCAGTTGGTAGAGCACTACCT	*H. pylori* 2342-2365[b]
2860R	GCATCCGCGGCCGCGCGCCACGCCAGGCATCACATCATC	"*H. heilmannii*" 436-412[c]
4047F	GCTTTAAAATCCACGAAGAC	"*H. heilmannii*" 1579-1598[c]
5045R	GCCAAGCTCACTTTATTGGCTG	*H. pylori* 5066-5045[b]
4197F	GCATCCGCGGCCGCGACGCACCATCCACACCTTC	"*H. heilmannii*" 1729-1748[c]
5020R	GCATCCGCGGCCGCTTTAGAAGTTACTTCTTTGCCATC	*H. pylori* 5043-5020[b]
4F	AATGGTGTGCCACCACTT	*H. felis* 1719-1736[d]
9R	AAGCCCACTAACTCCGTTGC	*H. felis*[e]
2677F	GGAATTCAGGAGTTTAGGATGAAACTGACACCTAAAG	"*H. heilmannii*" 200-229[c]
5065R	CGGGATCCCACTAGAATAGGTTATAGAGTTGTG	"*H. heilmannii*" 2638-2614[c]

[a] Underlined nucleotides indicate restriction endonuclease sites used for cloning. *Not*I was used in all cases except primers 2677F (*Eco*RI) and 5065R (*Bam*HI). "F" and "R" in primer designations indicate forward and reverse primers, respectively.
[b] Sequence from the published *H. pylori* urease sequence (25) at the position indicated.
[c] Sequence from this paper (Fig. 4) at the position indicated.
[d] Sequence from the published *H. felis* urease sequence (14) at the position indicated.
[e] Sequence from an unpublished *H. felis* urease sequence (kindly provided by R. Ferrero and A. Labigne).

then cloned them into *Escherichia coli*. DNA sequence analysis showed that the urease of "*H. heilmannii*" contains two structural subunits whose predicted amino acid sequence is highly related to the ureases of *H. felis* (14) and, to a slightly lesser extent, *H. pylori* (25).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmid vectors. *E. coli* DH5α and MC1061 (37) were grown on Luria-Bertani agar or in Luria-Bertani broth without glucose at 37°C with shaking, unless otherwise described. Transformants were selected with media containing 100 μg of ampicillin per ml. Phagemid vector Bluescript II KS(−) (Stratagene, La Jolla, Calif.) was used to clone partial fragments of "*H. heilmannii*" urease. The complete urease structural genes were cloned into the expression vector pMAL-c2 (New England BioLabs, Beverly, Mass.).

DNA manipulations. All routine DNA manipulations were performed by standard methods (37), unless otherwise noted.

Extraction of "*H. heilmannii*" DNA. Biopsy of the gastric antrum from a patient with gastric ulcer revealed spiral-shaped bacteria that were distinct from *H. pylori* by light and electron microscopy. These bacteria could not be cultured in vitro but were maintained in vivo by oral inoculation of human gastric biopsy into pathogen-free mice, as previously described (7). Mice were sacrificed 1 month after inoculation, and about 25 mg of stomach tissue was placed in 200 μl of digestion buffer (50 mM Tris [pH 9], 1 mM EDTA) containing 1% Laureth 12 (PPG/Mazer Chemicals, Gurne, Ill.) and 0.2 mg of proteinase K (Sigma, St. Louis, Mo.) per ml. Samples were incubated at 37°C for 16 h, and the proteinase K was inactivated by heating to 95°C for 10 min. The cellular debris was sedimented (10,000 × *g* for 5 min at 4°C), and the supernatant was used as the DNA template for PCR reactions. DNA was extracted from one inoculated mouse and one uninoculated control mouse.

Oligonucleotide primers. Table 1 shows the nucleotide sequences and their source for all primers used to amplify the "*H. heilmannii*" urease structural genes.

PCR amplification. All PCR reactions were prepared in a dedicated hood, using aerosol-resistant pipette tips (Applied Scientific, San Francisco, Calif.). DNA extracts were thawed on ice, and 2 μl was added to a 100-μl reaction volume containing 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 25 pmol of each primer, and 1.5 mM $MgCl_2$ in GeneAmp (Perkin-Elmer Cetus) PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]). An overlay of sterile mineral oil was added to each tube, and amplification was done with a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Emeryville, Calif.). PCR conditions consisted of an initial 3 min of denaturation at 95°C, followed by 25 to 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 40 to 55°C, and 2 min of extension at 72°C (see Results for specific cycling conditions). After amplification, there was an additional 10 min of extension at 72°C. Negative controls (reaction mixture without DNA template and reaction mixture from an uninoculated mouse) were included with all PCR reactions. PCR product was detected by electrophoresing 10 μl of reaction solution in a 1% agarose gel containing 1 μg of ethidium bromide per ml. A 1-kb DNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the DNA size standard.

Cloning partial urease fragments. Three partial fragments (0.5, 1.5, and 0.8 kb) of the urease structural genes were amplified with primers containing *Not*I sites and were cloned (see Results and Fig. 1 for details). The PCR products from four to six 100-μl reaction mixtures were combined, extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 25 μl of sterile distilled water. The product was purified by low-melting-point agarose gel electrophoresis and cut with an excess of *Not*I (Bethesda Research Laboratories) for 16 h. Phagemid vector Bluescript II KS(−) (Stratagene) was cut with *Not*I, treated with alkaline phosphatase (Boehringer-Mannheim, Indianapolis, Ind.), and then ligated with the PCR product, using T4 DNA ligase (Bethesda Research Laboratories). *E. coli* DH5α (Bethesda Research Laboratories) was electrotransformed (8) with the recombinant plasmids, using a gene pulser (Bio-Rad, Richmond, Calif.), and then plated on Luria agar containing 100 μg of ampicillin per ml, isopropylthio-β-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). The appropriate recombinant plasmid from ampicillin-resistant transformants was purified by using a Qiagen midi-column (Qiagen, Chatsworth, Calif.).

DNA sequencing. Cloned partial urease fragments were sequenced on both strands with double-stranded template (Sequenase; United States Biochemicals, Cleveland, Ohio), using the dideoxy chain termination method (38). A fourth partial urease fragment (1.0 kb) was amplified and sequenced directly without cloning. The PCR products from four 100-μl reactions were combined, electroeluted by 1% agarose gel electrophoresis (24), and purified with an Elutip-d column (Schleicher & Schuell, Dassel, Germany) according to the manufacturer's instructions. The DNA was ethanol precipitated and sequenced as before with the following modifications: the DNA template and 25 pmol of primer were mixed with dimethyl sulfoxide to a final concentration of 1%, boiled for 3 min, and then plunged immediately into an ice water bath to allow annealing to occur. Labeling reactions were done in the presence of Mn^{2+} buffer, as described by the manufacturer (Sequenase).

DNA sequence analysis was performed with the University of Wisconsin Genetics Computer Group computer software package (6).

Cloning and expression of the complete urease structural genes. On the basis of sequences obtained from partial fragments of the urease genes, two new primers (2677F and 5065R) with *Eco*RI and *Bam*HI restriction endonuclease sites were designed to amplify the entire urease structural genes. PCR was carried out for 35 cycles with an annealing temperature of 55°C. The resulting approximately 2.4-kb fragment was cut with *Eco*RI and *Bam*HI and then electroeluted and ligated as described above into similarly digested pMAL-c2. The recombinant plasmid was transformed into *E. coli* MC1061, using the calcium chloride method, and ampicillin-resistant transformants were screened for the appropriate insert. A clone containing the insert and a control clone containing pMAL-c2 without insert were grown in the presence of ampicillin to an optical density (600 nm) of 0.4 to 0.6, and 1 ml of each culture was withdrawn for whole-cell protein extracts. The remaining culture was grown for an additional 4 h in the presence of 0.4 mM IPTG in order to induce expression of the putative urease subunits.

Urease activity detection. Detection of urease activity was performed with urea-indole diagnostic medium (Diagnostic Pasteur, Paris, France), as previously described (25).

DNA hybridization. DNA from PCR amplification was separated by agarose gel electrophoresis, transferred to a nylon membrane (PhotoGene; Bethesda Research Laboratories), and hybridized with a biotinylated DNA probe (BioNick; Bethesda Research Laboratories) according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Bacterial pellets were suspended in solubilization buffer (3% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8) and boiled for 5 min. Solubilized cell extracts were analyzed with a 4.5% acrylamide stacking gel and a 12.5% resolving gel at 200 V with a mini-slab gel apparatus (Bio-Rad).

Proteins were transferred to nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell) in a Mini Trans-Blot transfer cell (Bio-Rad) set at 100 V for 1 h with cooling. Membranes were blocked for 2 h with 5% (wt/vol) purified casein in phosphate-buffered saline (PBS; pH 7.4) at room temperature and rinsed with PBS. Antiserum (a gift from H. Mobley) against the denatured small and large urease subunits was prepared as previously described (21). Membranes were reacted at 4°C overnight with antisera diluted 1:1,000 in 1% (wt/vol) casein in PBS. The membrane was washed and then incubated in biotin-labeled secondary antibody (Amersham, Buckinghamshire, England) and then in peroxidase-labeled streptavidin (Amersham) as described in detail elsewhere (13). Proteins were visualized by chemiluminescence, using the ECL Western blotting (immunoblotting) protocol (Amersham) according to the manufacturer's instructions.

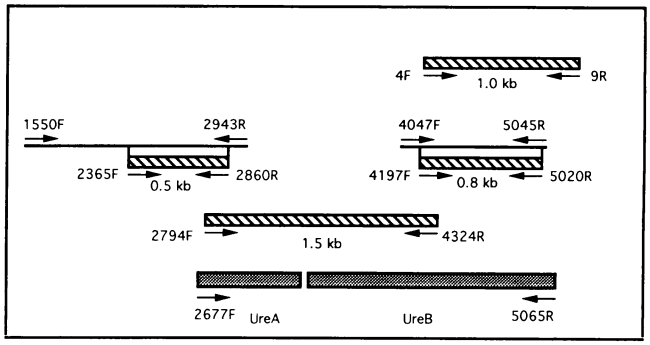

FIG. 1. Schematic diagram of the four overlapping partial urease fragments (hatched bars) in approximate relation to the urease structural subunits, *ureA* and *ureB* (shaded bars). Sizes of the partial fragments are shown below the hatched bars. For those fragments that required nested PCR (0.5 and 0.8 kb), the approximate position of the product from the first round of amplification (solid line) is shown above the hatched bars. Arrows represent the designated primers whose sequences are shown in Table 1.

Nucleotide sequence accession number. The DNA sequences reported here have been entered in the GenBank data base under accession number L25079.

RESULTS

Amplification and cloning partial urease fragments. Primers 2794F and 4324R were designed to amplify a 1.5-kb urease fragment (Fig. 1) based on conserved regions from the urease structural genes from *H. pylori* (25), *Proteus mirabilis* (22), *Ureaplasma urealyticum* (1), and *Klebsiella aerogenes* (33). PCR was carried out for 35 cycles with an annealing temperature of 55°C. Amplification of DNA extracts from mouse stomach inoculated with "*H. heilmannii*" and from the uninoculated control mouse showed several common bands but also a single, approximately 1.5-kb band that was present only in DNA extracted from the inoculated animal (Fig. 2A). With PCR product from the *H. pylori*-positive control as a probe, Southern blot showed hybridization only to the positive control and

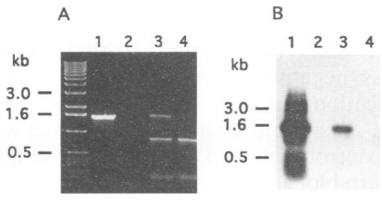

FIG. 2. (A) Agarose gel electrophoresis of PCR products from primers (2794F and 4324R) designed to amplify the 1.5-kb partial urease fragment of "*H. heilmannii*." DNA templates for amplification are as follows: lane 1, *H. pylori*; lane 2, distilled H_2O; lane 3, extract from mouse stomach inoculated with "*H. heilmannii*"; lane 4, extract from uninoculated mouse stomach. (B) Southern hybridization of PCR products from the gel shown in panel A probed with the 1.5-kb urease fragment from *H. pylori*. Lane designations are as for panel A.

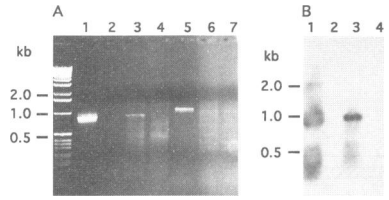

FIG. 3. (A) Agarose gel electrophoresis of PCR products from nested amplification of the 0.8-kb partial urease fragment of "*H. heilmannii*." Results from the first round of amplification (4047F and 5045R) are shown in lanes 5 to 7, whose DNA templates are as follows: lane 5, *H. pylori*; lane 6, extract from mouse stomach inoculated with "*H. heilmannii*"; lane 7, extract from uninoculated mouse stomach. Results from the second round of amplification (4197F and 5020R) are shown in lanes 1 to 4, whose DNA templates are as follows: lane 1, PCR product from lane 5 (*H. pylori*); lane 2, distilled H_2O; lane 3, PCR product from lane 6 (inoculated stomach); lane 4, PCR product from lane 7 (uninoculated stomach). (B) Southern hybridization of lanes 1 to 4 of the gel in panel A probed with the 0.8-kb urease fragment from *H. pylori*. Lane designations are as for panel A.

to the 1.5-kb band present in DNA extracted from the inoculated mouse stomach (Fig. 2B). The 1.5-kb fragment was digested with *Not*I, cloned into *Not*I-digested pBluescript II KS(−), and sequenced.

A second primer pair was selected to amplify a downstream 1.0-kb fragment. The forward primer (4047F) was chosen from a downstream portion of the 1.5-kb fragment of "*H. heilmannii*" urease sequence, and the reverse primer (5045R) was based on the *H. pylori* urease sequence. After 35 cycles at an annealing temperature of 55°C, these primers amplified an approximately 1.0-kb fragment from *H. pylori* DNA, but amplification of DNA extracted from inoculated and uninoculated mouse stomachs showed only a smear (Fig. 3A, lanes 5 to 7). Lowering the annealing temperature from 55 to 40°C failed to resolve a discrete band.

Another set of primers (4197F from "*H. heilmannii*" and 5020R from *H. pylori*) was then designed for use in a nested PCR reaction to amplify a 0.8-kb fragment (Fig. 1). After 25 cycles with primers 4047F and 5045R at an annealing temperature of 40°C, 2 μl of the reaction was used in a second PCR reaction (35 cycles; annealing temperature, 55°C) with primers 4197F and 5020R. Because of the greater potential for contamination with nested PCR, negative controls included both PCR reaction mix without DNA template and reaction mix with amplification product from an uninoculated mouse. The results showed the predicted 0.8-kb band with the positive control and a faint but discrete band at the same position with amplification from the inoculated animal (Fig. 3A, lanes 1 and 3). Direct amplification of infected gastric tissue with the nested primer pairs (4197F and 5020R) under the same conditions was negative. As expected, the negative control using amplification product from an uninoculated mouse showed only a smear, and no amplified product was present in the negative control without DNA template (Fig. 3A, lanes 2 and 4). Southern blot showed that the 0.8-kb product obtained from nested amplification of *H. pylori* DNA hybridized to the 0.8-kb band obtained from amplification of inoculated gastric tissue but not to the negative controls (Fig. 3B). The 0.8-kb fragment was then cloned into the *Not*I site of pBluescript II KS(−) and sequenced as before. The same strategy of nested PCR was then used to amplify, clone, and sequence a 0.5-kb upstream fragment (Fig. 1) that included the remainder of the *ureA* gene.

The sequence of the terminal portion of *ureB* was obtained by amplification of a 1-kb fragment with primers 4F and 9R (a gift from R. Ferrero and A. Labigne), which are based on the recently cloned *H. felis* urease (14). Primer 4F is in a region of *ureB* of *H. felis* that is identical at all but one position to the previously determined sequence from "*H. heilmannii*." Primer 9F is in a region downstream of the *H. felis ureB* gene that has an open reading frame encoding a polypeptide with 69% identity to the *H. pylori ureI* gene (25). PCR (35 cycles; annealing temperature, 55°C) demonstrated a band present in the inoculated mouse stomach that was not present in the uninoculated mouse (data not shown). This band was electroeluted, and the downstream portion was sequenced with primer 9F.

Sequence analysis of the "*H. heilmannii*" urease structural genes. Sequence analysis of the four overlapping partial urease fragments (Fig. 1) revealed two open reading frames, designated *ureA* and *ureB* (Fig. 4), that were transcribed in the same direction. No inverted repeats that might serve as transcription termination sequences were found. Both *ureA* and *ureB* began with an ATG start codon. Putative ribosome-binding sites similar to the *E. coli* consensus sequence (40) were found by inspection (Fig. 4). Upstream of the start of *ureA* was a region of noncoding DNA that showed no significant homology to other DNA sequences by FastA search of GenBank. A similar region has also been identified in *H. pylori* (25).

The intergenic space between *ureA* and *ureB* was 14 bp, longer than the 9 bp for *H. felis* and the 3 bp for *H. pylori*. It has been proposed (14, 25) that the intergenic spaces for *H. felis* and *H. pylori* make it theoretically possible for a single mutation in the stop codon to result in a fused single polypeptide, as is seen with jack bean urease. To be sure that the intergenic space we found was correct, we reamplified this region from the DNA extract and directly sequenced the PCR product. The resulting sequence was identical to the sequence obtained from the 1.5-kb cloned partial urease fragment.

The *ureA* and *ureB* genes from "*H. heilmannii*" code for polypeptides with predicted molecular weights of 25,729 and 61,831, respectively, very similar to the values previously obtained for *H. pylori* (25) and *H. felis* (14). A FastA search of the GenBank amino acid sequences found that these polypeptides were much more closely related to the *Helicobacter* ureases than to other bacterial or plant ureases. At the amino acid level, *ureA* from "*H. heilmannii*" was 79 and 82% identical to *ureA* from *H. pylori* and *H. felis*, respectively. *ureB*, which contains the putative active site (25) for the enzyme, showed 87 and 92% amino acid identity with *ureB* from *H. pylori* and *H. felis*, respectively (Fig. 5).

Expression of the "*H. heilmannii*" urease structural genes. A 2.4-kb DNA fragment was amplified (primers 2677F and 5065R) and confirmed by restriction fragment mapping to be the "*H. heilmannii*" urease genes (data not shown). This fragment, which contained the putative ribosome-binding sites for *ureA* and *ureB*, was asymmetrically cloned (*Eco*RI and *Bam*HI) into pMAL-c2 under control of the IPTG-inducible "tac" promoter. The primers were designed to be out of phase with the maltose-binding protein coded for by pMAL-c2 so as not to produce a fusion protein. Extracts of *E. coli* containing pMAL-c2 with the recombinant "*H. heilmannii*" urease and *E. coli* containing only pMAL-c2 were examined on SDS-PAGE gels under IPTG-induced and noninduced conditions. Neither clone expressed *ureA* or *ureB* under noninduced conditions, but the addition of IPTG resulted in the overexpression of polypeptides of approximately 27 and 61 kDa in the clone containing the urease genes (Fig. 6A). These polypeptides corresponded approximately in molecular weight to the mo-

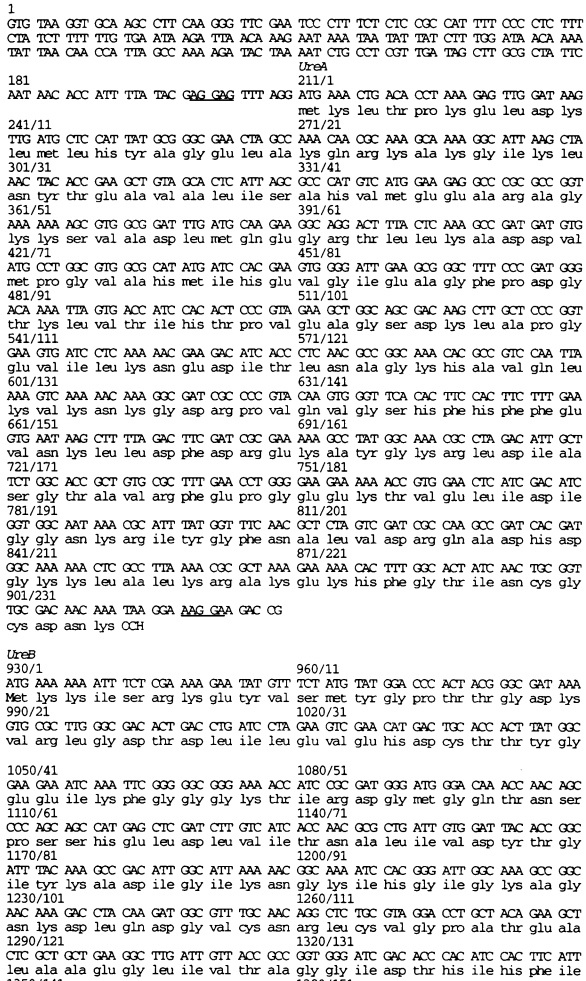

FIG. 4. Nucleotide sequences of the "*H. heilmannii*" *ureA* and *ureB* genes. The predicted amino acid sequence is shown below the nucleotide sequence. Numbers above the sequence indicate the nucleotide and amino acid positions. The putative ribosome-binding site (Shine-Dalgarno sequence) for each gene is underlined.

lecular weights reported for *ureA* and *ureB* from other *Helicobacter* species (44). Western blot analysis with polyclonal antiserum against *H. pylori ureA* (Fig. 6B) and *ureB* (Fig. 6C) showed cross-reactivity with the putative urease subunits from "*H. heilmannii*."

The clone containing the "*H. heilmannii*" urease was assayed for urease activity under induced and uninduced conditions and after growth on Luria agar as well as on minimal media, as previously described (4). No urease activity was detected under any conditions.

DISCUSSION

"*H. heilmannii*" is an uncultured gastric spiral bacterium that is ubiquitous in a wide range of animals and that occasionally infects humans, in whom it is associated with chronic gastritis (19). We previously used PCR to amplify directly from infected gastric tissue the 16S rRNA gene from this organism and identified it as a new species of *Helicobacter* (42). This is now one of several examples in which an uncultivated and previously uncharacterized organism has been identified by using the techniques of DNA amplification and molecular phylogeny based on 16S rRNA sequences (36). In this report, we use DNA amplification from infected gastric tissue to clone, sequence, and express the "*H. heilmannii*" structural genes for urease, a virulence determinant that is presumed important in all gastric helicobacters. This extends the molecular study of uncultivated microorganisms from phylogeny to analysis of pathogenic determinants.

The DNA sequence and Western blot analysis reported here support the classification of "*H. heilmannii*" as a new member of the *Helicobacter* genus. The presence of two structural subunits in "*H. heilmannii*" is characteristic of the *Helicobacter* genus (14, 25, 44) and is unique among bacterial ureases (31). The urease structural genes from "*H. heilmannii*" are highly homologous to *ureA* and *ureB* from *H. pylori* and *H. felis*, and both subunits are antigenically cross-reactive with antiserum directed against the corresponding subunits from *H. pylori*. The homology is greatest for *ureB*, which is consistent with the fact that this subunit is presumed to contain the urease catalytic site (25). The eight histidine residues and one cysteine residue that are believed to play an important role in nickel binding and enzymatic activity at the active site (25) are conserved in the "*H. heilmannii*" UreB polypeptide (Fig. 5).

The guanine-plus-cytosine (G+C) content of the "*H. heilmannii*" urease structural genes is 49.8%. Using the correlation between G+C content of structural genes and total genomic G+C content (34), we estimate that "*H. heilmannii*" has approximately 47% G+C content. Similar calculations

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UreA
H.h.  MKLTPKELDKLMLHYAGELAKQRKAKGIKLNYTEAVALISAHVMEEARAGKKSVADLMQEGRTLLK
H.f.  .................R..EE.L.R.V...........GR...K..D.N...........W..
H.p.  ....................K..E......V.........I.........TA.E.........

H.h.  ADDVMPGVAHMIHEVGIEAGFPDGTKLVTIHTPVEAGSDKLAPGEVILKNEDITLNAGKHAVQLKV
H.f.  KEN..D...S.........N...............-DNG.......F.......I....E.IS...
H.p.  PDD..D...S.........M.........V...I..-NG..V...LF.......I.E..K..SV..

H.h   KNKGDRPVQVGSHFHFFEVNKLLDFDREKAYGKRLDIASGTAVRFEPGEEKTVELIDIGGNKRIYG
H.f.  ..........................A.SFC...................S.............
H.p.  ..V......I.........RC.......TF....................S.........R..FG

H.h.  FNALVDRQADHDGKKLALKRAKEKHFGTINCGCDN-K
H.f.  ..S.......A.....G.......G..SV....EAT.DKQ        82% Identity
H.p.  ..........NES..I..H....RG.HGAKSDDNYV.TIKE        79% Identity


UreB
H.h.  MKKISRKEYVSMYGPTTGDKVRLGDTDLILEVEHDCTTYGEEIKFGGGKTIRDGMGQTNSPSSHEL
H.f.  ...................R.....................................S.......Y..
H.p.  ..............................A....Y.I....L.......L.E..S.S.N..KE..

H.h.  DLVITNALIVDYTGIYKADIGIKNGKIHGIGKAGNKDLQDGVCNRLCVGPATEALAAEGLIVTAGG
H.f.  ...L....................D...A.........M....D.N.....................
H.p.  ..I.....................D...A....G....M....K.N.S.........G.........

H.h.  IDTHIHFISPQQIPTAFASGITTMIGGGTGPADGTNATTITPGRWNLKEMLRASEEYAMNLGYLGK
H.f.  ...................V.............................A...S....A........F.A.
H.p.  ...................V.............................R...W....A...S....F.A.
                          •                                  •
H.h.  GNVSFEPALIDQLEAGAIGFKIHEDWGSTPSAINHALNIADKYDVQVAIHTDTLNEAGCVEDTLEA
H.f.  ....Y..S.R..I.................A..H.C..V..E.......................
H.p.  ..A.NDAS.A..I..............T.........DV.........................MA.
           •  •     •                                 •     •••
H.h.  IAGRTIHTFHTEGAGGGHAPDVIKMAGEFNILPASTNPTIPFTKNTEAEHMDMLM-CHHLDKNIKE
H.f.  ...................................................V......S...
H.p.  .....M...............I..V...H...............V...........V......S...

H.h.  DVEFADSRIRPQTIAAEDKLHDMGIFSITSSDSQAMGRVGEVITRTWQTADKNKKEFGRLPEEKGD
H.f.  ..Q..............Q......................................K.....
H.p.  ..Q..............T......................................K.....

H.h.  NDNFRIKRYISKYTINPAITHGISEYVGSVEVGKYADLVLWSPAFFGIKPNMIIKGGFIALSQMGD
H.f.  ..................G.A....D.........................................
H.p.  .........L.........A.............V...........V.....................

H.h.  ANASIPTPQPVYYREMFGHHGKAKFDTNITFVSQVAYENGIKHELGLQRVVLPVKNCRNITKKDLK
H.f.  ......................N...........A..KA...E....D.AAP.............
H.p.  ..................A......Y.A.......A..DK...E....E.Q..............MQ

H.h.  FNDVTAHIEVNPETYKVKVDGNEVTSHAADKLSLAQLYNLF
H.f.  ........D............K....K...E..........        92% Identity
H.p.  ...T............H.F...K....KP.N.V.....FSI.        87% Identity
```

FIG. 5. Predicted amino acid sequences of *ureA* and *ureB* from "*H. heilmannii*" (H.h.) aligned with the corresponding predicted sequences from *H. felis* (H.f.) and *H. pylori* (H.p.). Sequence identity with "*H. heilmannii*" is indicated by small dots; gaps in sequence to optimize alignment are indicated by dashes. Percentages indicate percent amino acid identity compared with "*H. heilmannii*." Heavy black dots (•) are shown above the conserved histidine and cysteine residues in UreB that are believed to play an important role in nickel binding and enzymatic activity.

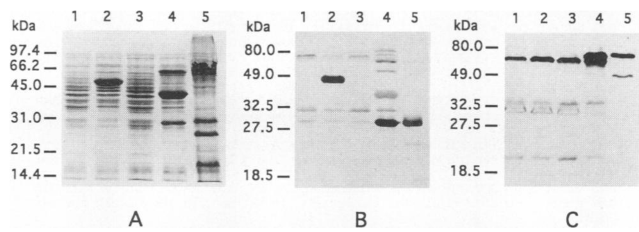

FIG. 6. (A) SDS-PAGE of whole-cell protein from *E. coli* MC1061 harboring the expression vector pMAL-c2 under noninduced conditions (lane 1) and induced with IPTG (lane 2). The same strain with the "*H. heilmannii*" *ureA* and *ureB* genes cloned into pMAL-c2 is shown uninduced (lane 3) and induced with IPTG (lane 4), compared with *H. pylori* (lane 5). The prominent bands migrating at approximately 48 (lane 2) and 40 (lane 4) kDa represent maltose-binding protein (MBP)–β-galactosidase fusion and MBP alone, respectively. (B) Western blot of a duplicate of the gel shown in panel A blotted with monospecific polyclonal antiserum against the *H. pylori ureA* polypeptide. Lane designations are as in panel A. The reaction of antiserum against UreA with MBP–β-galactosidase fusion protein (lane 2) and, to a lesser degree, the MBP alone (lane 4) likely reflects preimmune exposure to *E. coli* MBP. (C) Western blot of a duplicate of the gel shown in panel A blotted with monospecific polyclonal antiserum against the *H. pylori ureA* polypeptide. Lane designations are as in panel A.

based on the G+C content of the urease structural genes from *H. pylori* (43.2%) and *H. felis* (47.1%) lead to predicted genomic G+C contents of 39.8 and 44.1%, respectively. These values are slightly higher than the measured G+C contents of 37% for *H. pylori* (18) and 42.5% for *H. felis* (35), and so our estimate may be somewhat high. Nevertheless, this analysis is consistent with the placement of "*H. heilmannii*" among the more recently evolved *Helicobacter* species whose genomic G+C content is known to vary widely from 24% (*H. nemestrinae*) to 42.5% (*H. felis*), compared with the phylogenetically older members of the genus (*H. muridarum*, *H. mustelae*, *H. cinaedi* and *H. fenelliae*) that show a narrower range of 35 to 38% (41).

"*H. heilmannii*" and *H. felis* are very similar in 16S rRNA sequences and morphology, which differs only by the presence of periplasmic fibers in *H. felis*. Some investigators have wondered whether "*H. heilmannii*" is a phase variant of *H. felis* that has lost its periplasmic fibers. The urease sequence data presented here are consistent with the interpretation that these two organisms are different species. The overall DNA identity in the urease structural genes between "*H. heilmannii*" and *H. felis* is 80.1%. Although there are no data on the extent of heterogeneity in urease DNA sequence among strains of *H. felis*, it was recently reported that partial DNA sequences of *ureC* from 15 strains of *H. pylori* were between 95.3 and 99.2% identical (15). Thus, the urease sequence data are consistent with 16S rDNA data and suggest that "*H. heilmannii*" is closely related to, but different from, *H. felis*.

There are probably other genes in "*H. heilmannii*" that are associated with *ureA* and *ureB*, as has been described for *H. pylori* (4). Immediately downstream of the *H. pylori ureB* gene is *ureI* (4), which has no known homolog in other bacterial ureases and whose function is unknown. The presence of *ureI* in "*H. heilmannii*" is implied by our success in using a primer in this region to amplify the downstream portion of *ureB*. The failure to obtain urease activity with expression of only the structural genes is consistent with the finding in *H. pylori* (4, 20, 25) and other bacteria (22, 33) that expression of a catalytically active urease requires several accessory genes. Therefore, it may be possible to obtain functional expression of the "*H. heilmannii*" urease by overexpression of the structural genes in *trans* to the accessory genes from *H. pylori*.

The availability now of urease DNA sequences from three species of *Helicobacter* (*H. pylori*, *H. felis*, and "*H. heilmannii*"), which differ in host range specificity and histopathology, will allow further study of whether the urease contributes to these differences. Although the molecular techniques for exchanging genes among *Helicobacter* species have not yet been developed, we can expect that shuttle vectors derived from *Helicobacter* plasmids (23) will make this possible in the near future. This will permit the construction of *Helicobacter* strains that produce proteins from different *Helicobacter* species or that produce chimeric proteins, which will allow a more precise evaluation of the role of the urease subunits in pathogenesis.

The DNA and predicted amino acid sequences reported here are subject to the fidelity constraints of *Taq* DNA polymerase. The measured error rate of *Taq* polymerase has ranged from 2×10^{-4} to less than 10^{-5} errors per nucleotide per cycle (11). We can therefore estimate that after 35 cycles the error frequency will range from approximately 1 per 150 to 1 per 3,000 bp (11). For the 2,450 bp of the urease structural genes, we expect <1 to 17 errors per molecule. Since some of these errors will not lead to a change in amino acid, we expect between 0 and 6 errors among the 802 amino acids making up

the "*H. heilmannii*" urease structural genes. Of the 373 bp that were sequenced in duplicate from separately amplified products, no differences were found.

In summary, we report the cloning, sequencing, and expression of urease structural genes from "*H. heilmannii*," a newly identified uncultured species of *Helicobacter* in humans. The *ureA* and *ureB* genes from "*H. heilmannii*" code for polypeptides that are most closely related to the corresponding gene products from *H. felis*, the *Helicobacter* species commonly found in the stomach of cats and dogs. These data support 16S rDNA sequence analysis and suggest that "*H. heilmannii*" is phylogenetically most closely related to *H. felis*. The marked conservation among the known *Helicobacter* UreA and UreB polypeptide sequences is consistent with an essential role of urease in the pathogenesis of all gastric *Helicobacter* species.

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