INFECTION AND IMMUNITY, Mar. 1994, p. 793–798
0019-9567/94/$04.00+0

Vol. 62, No. 3

Immunological and Molecular Characterization of *Helicobacter felis* Urease

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Received 13 August 1993/Returned for modification 12 October 1993/Accepted 13 December 1993

Urease activity has recently been shown to be an important virulence determinant for *Helicobacter pylori*, allowing it to survive the low pH of the stomach during colonization. Experimental murine infection with *Helicobacter felis* is now being used as a model for *H. pylori* infection to study the effects of vaccines, antibiotics, and urease inhibitors on colonization. However, little information comparing the ureases of *H. felis* and *H. pylori* is available. Urease was partially purified from the cell surface of *H. felis* ATCC 49179 by A-5M agarose chromatography, resulting in an eightfold increase in specific activity over that of crude urease. The apparent K_m for urea for the partially purified urease was 0.4 mM, and the enzyme was inhibited in a competitive manner by flurofamide (50% inhibitory concentration = 0.12 μM). Antiserum to whole cells of *H. pylori* recognized both *H. pylori* and *H. felis* urease B subunits. Antiserum raised against *H. felis* whole cells recognized the large and small autologous urease subunits and the cpn60 heat shock molecule in both *H. felis* and *H. pylori*. However, this antiserum showed only a weak reaction with the B subunit of *H. pylori* urease. Two oligomeric DNA sequences were used as probes to evaluate the relatedness of *H. felis* and *H. pylori* urease gene sequences. One 30-mer from the *ureA* sequence, which had been shown previously to be specific for *H. pylori*, failed to hybridize to *H. felis* genomic DNA. A probe to the putative coding sequence for the active site of the *H. pylori ureB* subunit hybridized at low intensity to a 2.8-kb fragment of *Bam*HI-*Hin*dIII-digested *H. felis* DNA, suggesting that the sequences were homologous but not identical, a result confirmed from the recently published sequences of *ureA* and *ureB* from *H. felis*.

Helicobacter pylori is a microaerophilic, spiral, gram-negative rod that has been shown to be the etiological agent of active chronic gastritis and to be an important factor in the development of duodenal and gastric ulcers in humans (2, 17, 26, 28, 39, 46). Recent clinical studies have found an association between colonization with *H. pylori* and incidence of adenocarcinoma of the distal stomach (33, 35). Several properties of *H. pylori*, including motility, mucinase, adhesins, vacuolating cytotoxin, and urease, are believed to play roles in colonization of the gastric niche (7, 10, 13, 16, 23, 24, 27, 41). Of these, urease has been most extensively characterized; urease functions to protect *H. pylori* from the effects of low pH, by hydrolyzing urea to ammonia, which neutralizes the bactericidal effects of acid (18, 38). The K_m of *H. pylori* urease (0.3 mM) is lower than that determined for ureases of other organisms (18), which is appropriate for the relatively low concentrations of urea diffusing from the blood (1.7 to 3.4 mM) into the organism's niche in the mucous layer (18). Acid neutralization is important for colonization, since urease-negative mutants of *H. pylori* were unable to colonize germ-free piglets (9) and urease inhibition by acetohydroxamic acid prevented colonization of *Helicobacter felis* in mice (16). However, urease inhibitors apparently have no effect upon the elimination of *Helicobacter* spp. from animals already colonized (29). Such studies suggest that urease is critical for establishing colonization but may not be required after the organisms reside under the protective mucous layer in the stomach. Urease has been proposed to have other important properties that may contribute to gastric pathology. The high levels of ammonia generated via urease have toxic effects on cells and may break down gastric mucous (42), and urease may be a major proinflammatory component of *H. pylori* (1). Urease also elicits the production of antibody in infected individuals (4, 19, 34). Circulating immunoglobulin G to urease is not protective in colonized persons (36), and polyclonal mouse antibodies raised against purified urease can antagonize the ability of monoclonal antiurease antibodies to neutralize enzyme activity in vitro (30).

Investigation into the pathogenicity of *H. pylori* has been hampered by the lack of convenient animal models that closely mimic the chronic inflammatory disease seen in humans. Germ-free piglets, ferrets, and mice have been the most successful infection models, using *H. pylori*, *Helicobacter mustelae*, and *H. felis*, respectively, since *H. pylori* itself appears able to colonize naturally only humans and nonhuman primates (31). Because of the convenience of the mouse model, several studies have investigated the effects of vaccines, antibiotics, and urease inhibitors on colonization with *H. felis* (16). Despite the widespread use of *H. felis* in these studies, little has been reported about its urease activity. One examination of the properties of urease found in crude *H. felis* lysates showed that its K_m for urea was similar to that previously reported for urease purified from *H. pylori* (16). A recent study observed a high degree of homology among the first 12 N-terminal amino acids in the urease subunits of *H. pylori* and *H. felis* (44). While the urease genes from *H. felis* recently have been cloned (14, 15), there is relatively little information describing the biochemical and antigenic characteristics of this enzyme. The purpose of the present study was to determine the relatedness

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of the urease of *H. felis* to the corresponding enzyme from *H. pylori* by using biochemical, immunological, and DNA hybridization techniques. The results indicate that while *H. felis* urease shares biochemical properties with *H. pylori* urease, polyclonal antiserum raised against *H. felis* whole cells only weakly recognizes urease isolated from *H. pylori*. An oligonucleotide probe to the *H. pylori ureA* unique region failed to hybridize to DNA isolated from *H. felis*, demonstrating differences in the coding sequence of these enzymes.

MATERIALS AND METHODS

Growth of *H. felis* and *H. pylori*. *H. felis* ATCC 49179, originally isolated from the stomach of an adult cat (22), was kindly provided by James Fox at Massachusetts Institute of Technology. *H. pylori* UMAB41 and UMAB51 were recent clinical isolates from Harry L. T. Mobley at the University of Maryland School of Medicine. *H. pylori* 84-183 was obtained from the strain collection at Vanderbilt University Medical Center. Frozen stocks were prepared from overnight cultures of *H. felis* grown in brucella broth containing 10% fetal bovine serum (GIBCO, Grand Island, N.Y.), with shaking at 150 rpm in a 10% CO_2 atmosphere. *H. felis* was stored in liquid nitrogen from this overnight culture with 15% (vol/vol) glycerol added.

For isolation of urease from *H. felis*, a fresh culture was prepared by directly plating the frozen stock on tryptic soy agar containing 5% sheep blood, trimethoprim (5 μg/ml), polymyxin (2.5 U/ml), amphotericin B (2.5 μg/ml), and vancomycin (10 μg/ml) (Northeast Laboratories, Waterville, Mich.). Plates were incubated for 24 h at 40°C in a sealed jar with a microaerobic atmosphere (CampyPak system; BBL, Cockeysville, Md.). Growth from this plate was used to inoculate 60 fresh blood agar plates to produce sufficient cell growth after 24 h to enable isolation of urease.

Cultures of *H. pylori* and *H. felis* for DNA isolation were grown from frozen working stocks, prepared as described above, in tubes of brucella broth with 10% fetal bovine serum in a BBL GasPak container in a microaerobic atmosphere, as above. Such inocula were diluted 1:20 into the same medium in Erlenmeyer flasks and grown, again under reduced-oxygen conditions, with shaking for 18 to 24 h.

Preparation of partially purified urease. Urease was extracted from *H. felis* 49179 by a variation of the method reported by Evans et al. (12). Overnight growth was removed from the surface of the blood agar plates by washing with phosphate-buffered saline (PBS), and the resulting cells were pelleted by centrifugation (7,000 × *g* for 10 min). Urease was extracted from the surface of *H. felis* by resuspending 1.4 g (wet weight) of cells in 10 ml of 1% *n*-octyl glucopyranoside (Sigma, St. Louis, Mo.) for 20 min with gentle stirring at room temperature. The cells then were pelleted by high-speed centrifugation (30,330 × *g* for 30 min at 4°C), and the supernatant fluid (crude surface extract) was dialyzed overnight against 4 liters of PBS. The crude surface extract containing urease (53 mg) was fractionated by gel filtration on a Bio-Gel A-5M agarose column (Bio-Rad Laboratories, Richmond, Calif.) at room temperature with 0.05 M Tris-HCl (pH 8) elution buffer. The fractions with the highest urease specific activity were pooled and stored at 4°C.

Assay for urease activity. Urease activity in the pooled A-5M agarose-fractionated material was determined by an enzyme-coupled assay with glutamate dehydrogenase and NADH as previously described (20). Briefly, a dilution of sample was added to cuvettes (1-cm light path length) containing 3 ml of a reaction mixture of 31 mM Tris-HCl (pH 8), 810 μM oxoglutarate, 240 μM NADH, and 10 mM urea. The reaction was started by adding 96 U of glutamate dehydrogenase (Boehringer Mannheim, Indianapolis, Ind.), and the reduction of NADH was followed spectrophotometrically for 10 min at 37°C, with a standard wavelength of 340 nm in a dual beam spectrophotometer (Lambda 3B; Perkin-Elmer). One unit of urease activity was defined as the amount of enzyme that produced one micromole of NH_3 per minute, and the inhibitory effect of the urease inhibitor flurofamide (synthesized at Pfizer Central Research) was measured in the coupled assay.

Determination of apparent K_m and V_{max}. Urea concentrations from 0.156 to 50 mM were prepared in the standard coupled assay with the partially purified urease (0.78 μg per reaction), and calculations of specific activity were done as described above. Lineweaver-Burke plots of 1/S versus 1/V were derived with an Apple MacIntosh SE/30 using Cricket Graph. The apparent K_m and V_{max} were determined from a computer-generated regression line.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Column samples were analyzed by SDS-PAGE with a Bio-Rad Mini-Protean II gel system. Protein samples were denatured prior to electrophoresis for 6 min at 100°C in 5× sample buffer (0.31 M Tris-HCl [pH 6.8], 12.5% SDS, 25% dithiothreitol, 10% glycerol, and 0.01% bromophenol blue). Samples containing 5 to 10 μg of protein and molecular weight standard were loaded onto 4 to 15% gradient acrylamide gels (Bio-Rad), electrophoresed at 200 V for 30 to 45 min, and silver stained.

Immunoblot analysis of *H. felis* urease. Immunoblotting of *H. felis* urease and *H. pylori* preparations was done exactly as previously described (36, 37), by using hyperimmune rabbit serum and immunoglobulin G alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G. Preparations of *H. pylori* 84-183 purified urease and the purified heat shock protein (cpn60) obtained as previously described (5, 7) were also used in the immunoblot analysis. Antisera used in these experiments had been raised against *H. pylori* 84-183 whole cells as described previously (6). Preimmune and immune sera raised against *H. felis* whole cells was a generous gift from Richard Ferrero at the Institut Pasteur.

Preparation of genomic DNA for membrane hybridizations. Genomic DNA was prepared by a standard method (45). The concentration of DNA was measured spectrophotometrically at 260 nm; DNA was diluted to 100 μg/ml and purified on a cesium chloride gradient.

Southern blotting. DNA was digested for 24 h with the indicated restriction enzymes according to manufacturer's (New England Biolabs, Beverly, Mass.) directions. Additional restriction enzyme was added after 18 h to achieve complete digestion. After digestion, approximately equal amounts of DNA, as determined by ratios of optical density at 260 versus 280 nm and direct visualization of uncut DNA samples in agarose gels, were electrophoresed with *Hin*dIII-digested bacteriophage λ (Bethesda Research Laboratories, Inc., Bethesda, Md.) as the molecular weight standard in 0.8% agarose gels with TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8]. DNA samples were depurinated and capillary blotted to GeneScreen Plus (New England Nuclear, Boston, Mass.) positively charged nylon membranes according to the manufacturer's instructions. Oligonucleotides purified by PAGE were purchased from Genosys Biotechnologies, Inc. (The Woodlands, Tex.). End labelling of oligonucleotides was carried out with T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]-labelled ATP (Amersham) to achieve a specific activity of 5×10^6 dpm/mol (40).

All prehybridizations and hybridizations were carried out by standard protocols (40) in bottles in a Red Roller hybridization

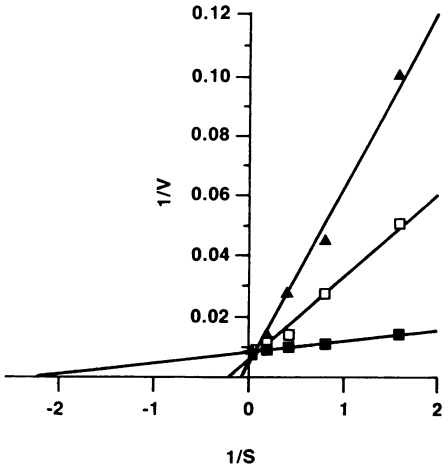

FIG. 1. Double reciprocal plot of A-5M agarose-fractionated urease enzymatic activity from *H. felis* ATCC 49179. ■, urease activity without flurofamide; □ and ▲, activities in the presence of 0.23 and 0.46 μM flurofamide, respectively.

oven (Hoefer, San Francisco, Calif.). Oligonucleotides were hybridized at 30°C (low stringency) and at 40°C (high stringency) in 6× SSPE (1.14 M sodium chloride; 1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]). Blots probed with oligonucleotides were washed four times with 100 ml of 0.1% SDS in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) for 15 min at 30°C (low stringency), 40°C (moderate stringency), or 50°C (high stringency). After being washed, the blots were immediately sealed in bags and exposed to X-Omat AR X-ray film (Kodak, Rochester, N.Y.).

RESULTS

Biochemical characteristics of *H. felis* urease. Partial purification of urease by A-5M agarose chromatography of crude surface extracts of *H. felis* 49179 resulted in recovery of 91% of total enzyme activity with an eightfold increase in specific activity (22 versus 174 U/mg of protein, respectively). Three major protein bands were observed in these fractions analyzed by SDS-PAGE: 66-, 54-, and 30-kDa species that correspond, respectively, to the UreB, heat shock, and UreA proteins that have been described previously for *H. pylori* (5, 11, 18). The apparent K_m for urea of the *H. felis* urease, determined from a double reciprocal plot (Fig. 1) was 0.4 mM, which is similar to a value determined previously by using crude cell extracts of *H. felis* 49179 (16). The apparent V_{max} from the reciprocal plot was 125 μmol/min/mg of protein. Figure 1 also demonstrates the competitive inhibition of urease catalytic activity observed with flurofamide. The apparent K_m of the enzyme increased in the presence of flurofamide at concentrations of 0.23 and 0.46 μM. The 50% inhibitory concentration for urease inhibition with flurofamide at a urea concentration of 0.4 mM was 0.12 μM.

SDS-PAGE and immunoblots. The *H. felis* urease preparation contained the two urease subunits plus a third band that showed similar migration to the *H. pylori* cpn60 molecule that copurifies (5) with urease (Fig. 2, lane a). The purified *H. pylori* cpn60 and urease molecules were included in the analysis (Fig. 2, lanes b and c, respectively). Antiserum raised against *H. pylori* whole cells strongly recognized the 66-kDa subunits of the purified *H. pylori* urease and *H. felis* urease molecules and

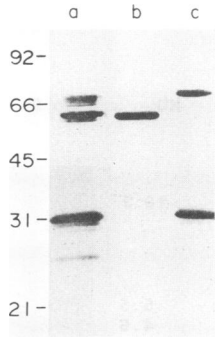

FIG. 2. SDS-PAGE (10% acrylamide) of *H. felis* and *H. pylori* preparations. Lanes: a, partially purified *H. felis*; b, purified *H. pylori* p54K (cpn60 homolog); c, purified *H. pylori* urease. After electrophoresis, bands were resolved by silver stain, as described in the text. The migration of molecular mass markers (in kilodaltons) is shown at the left.

showed weaker recognition of both small urease subunits (Fig. 3). There also was minimal recognition by this antiserum of the bands migrating at approximately 54 kDa from both species. Antiserum to whole *H. felis* cells recognized the large urease subunit from this organism as well as the putative 54-kDa heat shock protein. There was only weak recognition of the small urease subunit with an apparent molecular mass of 30 kDa. In addition, this antiserum only weakly recognized the purified large urease subunit from *H. pylori* (Fig. 3, lane c, α-*H. felis*) and the 54-kDa heat shock protein.

Southern hybridizations. Two oligomeric DNA sequences from the *H. pylori* urease operon were used as probes to evaluate the relatedness of the *H. felis* and *H. pylori* urease gene sequences. One 30-mer (5′-TTCGTTGTCTGCTTGTC TATCAACCAATGC-3′) was from a region of the gene for the small subunit (*ureA*, nucleotides 3256 to 3285) that previously had been shown to display no homology to the ureases of *Proteus mirabilis* or *Ureaplasma urealyticum* (21). A second 30-mer (5′-GCTTTTATCCAAGTGGTGGCACACCATAA G-3′) was putatively within the coding sequence (20) for the active site of the enzyme in *ureB* (nucleotides 4330 to 4359). *H. pylori* genomic DNA served as the positive control for the autologous *ureA* and *ureB* probes, and *Hin*dIII-digested phage λ was used as a negative control.

At high stringency (50°C hybridizations and washes) and at moderate stringency (40°C hybridizations and washes), with

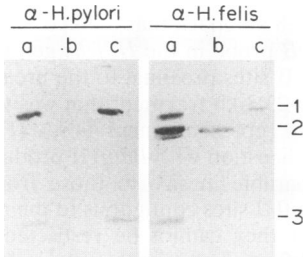

FIG. 3. Immunoblots of *H. felis* and *H. pylori* preparations with antisera to whole *H. pylori* cells (α-*H. pylori*) and to whole *H. felis* cells (α-*H. felis*). Antigens in lanes a, b, and c are exactly as described in the legend to Fig. 2. In each immunoblot, goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate was used to develop the reaction. The numbers on the right refer to the *ureB* product (1), cpn60 homolog (2), and *ureA* product (3).

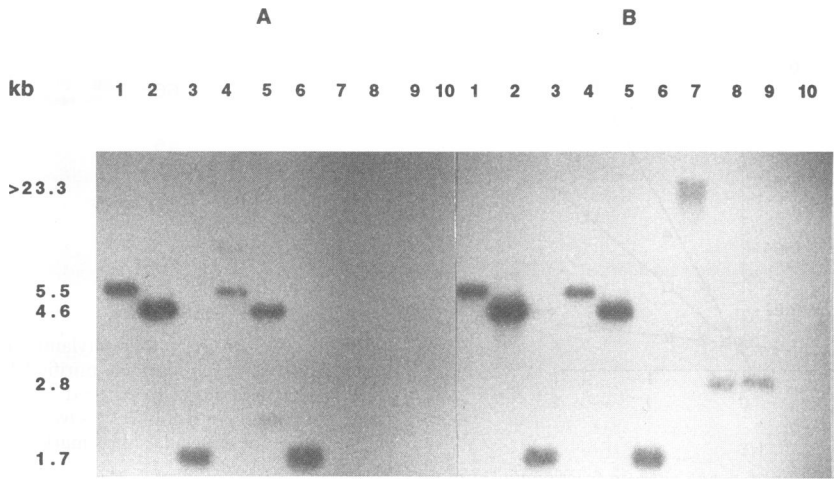

FIG. 4. Membrane hybridizations of duplicate genomic blots of *H. pylori* UMAB41 (lanes 1 to 3), UMAB51 (lanes 4 to 6), and *H. felis* 49179 (lanes 7 to 9) with oligonucleotide probes specific for the *ureA* (A) and *ureB* (B) genes. Hybridization and washes were carried out in 1.14 M NaCl at 40°C. Lanes 1, 4, and 7, *Bam*HI digests; lanes 2, 5, and 8, *Hin*dIII digests; lanes 3, 6, and 9, *Bam*HI-*Hin*dIII digests; lanes 10, bacteriophage lambda *Hin*dIII digest.

the oligonucleotide from the *ureA* subunit, single bands of approximately 5.5, 4.6, and 1.7 kb were visualized in the *Bam*HI, *Hin*dIII, and *Bam*HI-*Hin*dIII double digests, respectively, of genomic DNA from both *H. pylori* UMAB41 and UMAB51 (Fig. 4A, lanes 1 to 6). Under these conditions, no hybridization of this probe to *H. felis* DNA was detected (Fig. 4A, lanes 7 to 10). At lower stringency (30°C hybridization and wash), hybridization of the *H. felis* genomic DNA to the *ureA* 30-mer was detectable, but the probe also hybridized to λ DNA fragments, indicating that such results were nonspecific (data not shown).

In hybridization of the 30-mer from the *ureB* gene sequence to *Bam*HI, *Hin*dIII, and *Bam*HI-*Hin*dIII-digested genomic DNA from UMAB41 and UMAB51, at moderate and high stringency, bands of approximately 5.5, 4.6, and 1.7 kb, respectively, were again visualized (Fig. 4B, lanes 1 to 6). In addition, specific hybridization was now detected to *H. felis* DNA fragments of >23.3 kb with *Bam*HI-digested DNA, ~2.8 kb with *Hin*dIII-digested DNA, and ~2.8 kb with *Bam*HI-*Hin*dIII double digests (Fig. 4B; lanes 7 to 10). *H. felis* DNA contained gene sequences homologous to the *ureB* oligomer, but the lower intensity of the visualized bands compared with those from *H. pylori* suggested that such sequences were not identical. The relative sizes of the *H. felis*-hybridizing fragments suggested that there were no *Bam*HI sites between the two *Hin*dIII sites, ~2.8 kb apart, that flanked the fragment homologous to the *ureB* probe in the *H. felis* genome. In fact, there may be no *Bam*HI sites proximal to the urease gene sequence in *H. felis*. The >23.3-kb fragment that was visualized in these experiments may represent uncut DNA (Fig. 4B). Alternatively, although digestion with *Bam*HI produced *H. felis* DNA fragments comparable in size to those from *H. pylori*, it is possible that *Bam*HI sites contiguous to the urease operon are modified so that they cannot be restricted by this enzyme. Restriction-modification systems have been found to exist in *H. pylori* (25, 43).

DISCUSSION

Urease partially purified from *H. felis* ATCC 49179 shares several biochemical characteristics with urease isolated from *H. pylori*. The *H. felis* urease has a K_m for urea of 0.4 mM, which is similar to published values obtained with urease crude cell extracts from this organism and purified enzyme from *H. pylori* (5, 12, 16, 18); the urease recently isolated from *Helicobacter mustelae* has a similar K_m (8). Both the UreB and UreA subunits were detected in SDS-PAGE analysis of A-5M agarose-fractionated material from *H. felis*. The apparent molecular masses of the subunits (66 and 30 kDa, respectively) are similar to those reported for *H. pylori*. In addition, our results show that *H. felis* urease catalytic activity is competitively inhibited by flurofamide. Unlike ureases isolated from *Proteus vulgaris* and *Klebsiella pneumoniae*, the *Helicobacter* ureases retain activity at acidic pH and possess a relatively low K_m for urea, presumably reflecting the important function of urease in allowing *Helicobacter* spp. to colonize the gastric lumen, where urea is present in low concentrations (18).

Ferrero and Labigne have recently published the sequence of the *ureA* and *ureB* genes from *H. felis* (15). Comparison of the corresponding gene products derived from these sequences with those from sequences published on *H. pylori*, revealed a level of amino acid identity in the subunits between these species of 73.5% (UreA) and 88.2% (UreB), respectively. Despite this high degree of identity, the UreB polypeptide of *H. felis* was shown to have a lower mobility by SDS-PAGE than the corresponding subunit from *H. pylori*, suggesting that significant differences may exist between the UreB subunits from these organisms. This hypothesis is supported in our report by the immunoblotting studies performed with *H. pylori* and *H. felis* urease which revealed source differences in antigenic cross-reactivity. The antiserum raised to whole cells of *H. pylori* recognized autologous urease in the cell extracts prepared from this organism and common epitopes in the partially purified urease from *H. felis*. A stronger reaction of recognition with this antiserum was observed with the large urease B subunits from both organisms. Further analysis of the relatedness of these proteins was obtained with the antiserum to whole *H. felis* cells. This antiserum recognized the urease B subunits and 54-kDa proteins of both species, although a much weaker reaction was obtained against the *H. pylori* proteins. This result was obtained despite the addition of equal amounts of the various proteins to the gel. These observations suggest

that antigenic diversity exists between the urease proteins of *H. felis* and *H. pylori*. The nature of these differences is undoubtedly specific to both the urease molecules and to the antisera used in these studies. Cross-reactivity between the cpn60 heat shock proteins is not unexpected, since the chaperonins from phylogenetically diverse organisms are known to share common epitopes (7). However, differences observed with the anti-*H. felis* antiserum with respect to its weaker recognition of *H. pylori* UreB are somewhat surprising, considering that their ureases have previously shown similar biochemical properties and significant amino acid identity (32). In another study, Turbett et al. (44) compared the N-terminal amino acid sequences through the first 12 residues of the urease A and B subunits, using enzyme purified from several *Helicobacter* species. The UreB sequence from *H. felis* ATCC 49179 was identical to that of UreB from *H. pylori* NCTC 11637. Only one substitution was found in the corresponding region between the UreA molecules from these organisms. While these investigators found that the isoelectric points of the native ureases from these isolates were the same (pI 6.3), significant differences were found between the pIs of the corresponding UreA and UreB proteins of these species. While these results suggest that significant similarities between the ureases of *H. pylori* and *H. felis* exist, they also imply that certain differences exist as well. Furthermore, studies by Nagata et al. (30) have demonstrated the complex in vitro interactions of neutralizing antibody raised in mice against purified components of *H. pylori* urease. These investigators found that different antisera demonstrated marked differences in affinity for purified *H. pylori* urease as measured by ELISA.

The observation that these *ureA* and *ureB* probes visualized fragments of similar, if not identical, molecular size in the *H. pylori* genome is consistent with the known tandem arrangement of the *ureA* and *ureB* genes (3, 15, 21). Failure of the *ureA* probe to hybridize with genomic DNA from *H. felis* suggested that this 30-bp region is not present in the *H. felis ureA* sequence. This conclusion is confirmed by examining the recently published sequence of *ureA* (15). The less intense hybridization of the *ureB* probe to genomic DNA from *H. felis* indicates that similar but not identical sequences to those in *H. pylori* are present. Sequences related to the putative active site are most likely to be conserved.

Consideration of the known biochemical properties of the ureases from *H. pylori*, *H. mustelae*, and *H. felis* indicates that these enzymes play an important role in establishing colonization of these organisms in the acidic environment of the mammalian stomach. The current study indicates, however, that the ureases of *H. pylori* and *H. felis* are not identical in antigenicity and DNA sequence.

ACKNOWLEDGMENTS

We thank James Fox and Harry L. T. Mobley for providing bacterial isolates, Richard Ferrero for providing antisera, Candida Gower and Joan Petitpas for technical support, and Doreen Gale for skillful preparation of the manuscript.

This study was supported in part by the Medical Research Service of the Department of Veterans Affairs.

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