

Shuttle Cloning and Nucleotide Sequences of *Helicobacter pylori* Genes Responsible for Urease Activity

AGNÈS LABIGNE,* VALÉRIE CUSSAC, AND PASCALE COURCOUX

Unité des Entérobactéries, INSERM U199, Institut Pasteur, 75724 Paris Cedex 15, France

Received 17 September 1990/Accepted 9 January 1991

Production of a potent urease has been described as a trait common to all *Helicobacter pylori* so far isolated from humans with gastritis as well as peptic ulceration. The detection of urease activity from genes cloned from *H. pylori* was made possible by use of a shuttle cosmid vector, allowing replication and movement of cloned DNA sequences in either *Escherichia coli* or *Campylobacter jejuni*. With this approach, we cloned a 44-kb portion of *H. pylori* chromosomal DNA which did not lead to urease activity when introduced into *E. coli* but permitted, although temporarily, biosynthesis of the urease when transferred by conjugation to *C. jejuni*. The recombinant cosmid (pILL585) expressing the urease phenotype was mapped and used to subclone an 8.1-kb fragment (pILL590) able to confer the same property to *C. jejuni* recipient strains. By a series of deletions and subclonings, the urease genes were localized to a 4.2-kb region of DNA and were sequenced by the dideoxy method. Four open reading frames were found, encoding polypeptides with predicted molecular weights of 26,500 (*ureA*), 61,600 (*ureB*), 49,200 (*ureC*), and 15,000 (*ureD*). The predicted UreA and UreB polypeptides correspond to the two structural subunits of the urease enzyme; they exhibit a high degree of homology with the three structural subunits of *Proteus mirabilis* (56% exact matches) as well as with the unique structural subunit of jack bean urease (55.5% exact matches). Although the UreD-predicted polypeptide has domains relevant to transmembrane proteins, no precise role could be attributed to this polypeptide or to the UreC polypeptide, which both mapped to a DNA sequence shown to be required to confer urease activity to a *C. jejuni* recipient strain.

Helicobacter pylori (previously designated *Campylobacter pylori*) is a small, curved, gram-negative bacillus found in the stomach of patients with active chronic gastritis and duodenal ulcers. Since its discovery by Warren and Marshall (49) and successful isolation by Marshall et al. in 1984 (30), clinical, histological, and bacteriological investigations have been conducted worldwide in an attempt to determine the role of the bacteria as a causative agent in gastroduodenal diseases. *H. pylori* is now recognized as the etiological agent of active chronic gastritis (5), and there is accumulating evidence that the organism contributes to peptic ulceration.

Several properties commonly associated with *H. pylori* are suspected to play a role in the pathogenic process of gastritis as well as ulcer formation. These include adhesion to the gastric epithelium layer (17), a property which correlates with the expression of hemagglutinins (11, 35), and adhesion to cell lines (10, 36); the production of proteases capable of degrading mucus glycoproteins (42); and production of cytotoxins (22). Whether or not the genes expressing these traits are harbored by all *H. pylori* isolates is still unknown. In contrast, the expression of very high urease activity responsible for hydrolysis of urea to ammonia and carbon dioxide has been described as a common trait to all *H. pylori* so far isolated (5). Although it is not yet clear how the urease enzyme acts, it is suspected to play a major role in the ability of the bacteria to colonize and cause damage to the gastric mucosa. It has been proposed that the urease enzyme might (i) allow the survival of the bacteria in an acidic medium (29), a prerequisite for colonization; and (ii) be responsible for the enhancement of the back-diffusion of hydrogen ions (16) or stimulate gastrin production (27), resulting in increased acidity leading to gastric mucosal

damage. (iii) More recently, Smoot et al. (45) have demonstrated that *H. pylori* was cytotoxic to cultured human gastric epithelial cells and that this toxicity was due in part to ammonia produced by hydrolysis of urea.

To clarify the role of urease in the pathogenic process, we attempted to identify the genes responsible for urease activity in *H. pylori*. Because direct cloning into *Escherichia coli* did not result in expression of urease activity, we developed a shuttle approach for the cloning and expression of *H. pylori* genes. A genomic library was prepared from the total DNA of an *H. pylori* strain in an *E. coli* host strain, using a cosmid cloning vector derived from the shuttle vector pILL550 that we designed previously for investigation of the genetics of *Campylobacter* species (25). Each recombinant cosmid was then shuttled from *E. coli* to a *Campylobacter jejuni* recipient strain to examine for expression of the *H. pylori* genes in a *Campylobacter* background. By using this shuttle approach and constructing a series of plasmid derivatives of a hybrid cosmid harboring the *H. pylori* genes responsible for the urease activity in *C. jejuni*, we were able to identify and sequence the two genes encoding the polypeptides of the urease enzyme as well as additional sequences required for the urease expression.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *H. pylori* 85P, isolated from a patient with gastritis, and *C. jejuni* C31 were kindly provided by J. L. Fauchere (Hôpital Necker-Enfants

* Corresponding author.

TABLE 1. Vectors used in this study

Vector	Phenotypic characteristics ^a	Size (kb)	Reference
pILL533	RepEc mob Ap	4.7	This paper
pILL550	RepEc RepCj mob Km	8.3	25
pILL560	RepEc mob Ap	4.5	24
pILL570	RepEc mob Sp	5.3	This paper
pILL575	RepEc RepCj mob Km Cos	10	This paper

^a RepEc and RepCj, Plasmid capable of replicating in *E. coli* and *C. jejuni* cells, respectively; mob, conjugative plasmid due to the presence of OriT; Ap, Km, and Sp, resistance to ampicillin, kanamycin, and spectinomycin, respectively; cos, presence of lambda cos site.

Malades, Paris, France) and R. L. Guerrant (15a), respectively. *E. coli* HB101 (4) (*hsdR hsdM recA supE44 lacZ4 leuB6 proA2 thi-1 Sm*) and *E. coli* S17-1 (44) (RP4-2-Tc::Mu-Km::Tn7 Tmp Sm) were used as hosts in transformation experiments and for plasmid mobilization, respectively. *E. coli* P678-54 (F⁻ *thr-1 leu-6 thi-1 lacY1 malA1 xyl-7 ara-13 mtl-2 ton-A2 gal-6 λ⁻ rpsL minA minB*) (1) was used for preparation of micells. Vectors and hybrid plasmids used in this study are listed in Tables 1 and 2.

Culture conditions. *E. coli* strains were grown in L broth without glucose (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter [pH 7.0]) or on L-agar plates (1.5% agar) at 37°C. *C. jejuni* strains were grown on Columbia agar base (Difco Laboratories) or heart infusion broth (Difco) both supplemented with vancomycin (10 mg/liter), cephalothin (15 mg/liter), polymixin B (2,500 U/liter), trimethoprim (5 mg/liter), and amphotericin B (4 mg/liter). *H. pylori* strains were grown on blood agar plates (tryptic soy agar plus 5% sheep blood) or brucella broth supplemented with 10% fetal calf serum and nalidixic acid (50 mg/liter), vancomycin, trimethoprim, and amphotericin B all in the same concentrations as for *C. jejuni*. Both *C. jejuni* and *H. pylori* were grown under microaerobic conditions in an anaerobic jar with a carbon dioxide generator envelope (BBL 70304). Antibiotic concentrations for the selection of transformants or transconjugants were as follows (in milligrams per liter): kanamycin, 20; tetracycline, 8; ampicillin, 100; spectinomycin, 20.

Urease activity detection. Detection of urease activity was

achieved by resuspending 10⁹ bacteria into 1 ml of urea-indole medium (Diagnostic Pasteur) and incubating for 24 h at 37°C. Release of ammonia due to urease activity raised pH, inducing a color change from orange to red.

Preparation of DNA. Whole-cell DNA from *C. jejuni* was prepared as described previously (24). Whole-cell DNA from *H. pylori* was prepared following the same protocol except that the initial volume of liquid growth was 150 ml (instead of 10 ml); volumes were centrifuged and directly resuspended in 0.2 ml of the lysozyme solution without a washing step. Plasmid DNA was isolated by an alkaline lysis procedure (28).

Cosmid cloning. Chromosomal DNA from *H. pylori* 85P was partially cleaved with restriction endonuclease *Sau3A*, sized on a sucrose gradient (10 to 40%), and ligated into the *Bam*HI-digested and alkaline phosphatase-treated shuttle cosmid vector pILL575 DNA. This cosmid vector was constructed by inserting the 1.7-kb *Bgl*III DNA polymerase I large fragment-treated restriction fragment of pERG153, which contains the "cos site" of phage lambda, into the unique *Pvu*II restriction site of the shuttle cloning vector pILL550 (25). Cosmids were packaged into phage lambda particles (21) and used to infect *E. coli* HB101 harboring helper plasmid pRK212.1 (14).

DNA analysis and cloning methodologies. Restriction endonucleases were purchased from Amersham Corp. Enzymatic reaction conditions were as recommended by the manufacturers except when partial digestions with *Sau3A* or *Hind*III were conducted; then digestions were performed at 20°C to slow down the enzyme activity. DNA fragments were separated by electrophoresis in horizontal slab gels containing 0.7, 1, or 1.4% agarose and run in Tris-acetate buffer (28). The 1-kb ladder from Bethesda Research Laboratories was used as a molecular weight standard. All of the hybrid plasmids generated either by cloning or by deleting cloned material by the action of exonuclease *Bal* 31 (Bethesda Research Laboratories) were constructed following the protocols described by Maniatis et al. (28). Electroelution of DNA fragments from agarose gels containing ethidium bromide (0.4 µg/ml) was performed by punching a well in front of the DNA band of interest and migrating the DNA into the running buffer present in the well: DNA was recovered by extracting the eluate once with phenol-chloroform (vol/vol),

TABLE 2. Hybrid plasmids and their properties

Plasmid	Vector	Urease activity	Insert size (kb)	Origin of insert
pILL585	pILL575	+++ ^a	33.2	<i>Sau3A</i> partial digest of 85P
pILL587	pILL550	++ ^a	7.1	<i>Sau3A</i> partial digest of pILL585
pILL588	pILL550	+/- ^a	8.4	<i>Sau3A</i> partial digest of pILL585
pILL589	pILL550	+/- ^a	9	<i>Sau3A</i> partial digest of pILL585
pILL590	pILL550	++ ^a	8.1	<i>Sau3A</i> partial digest of pILL585
pILL594	pILL570	- ^b	5.1	<i>Bam</i> HI fragment of pILL590
pILL599	pILL570	- ^b	4.1	<i>Hind</i> III fragment of pILL590
pILL615	pILL533	ND ^c	9	<i>Pst</i> I- <i>Eco</i> RI fragment of pILL589
pILL720	pILL570	+/- ^b	6.8	<i>Bal</i> 31 digest of <i>Clal</i> -treated pILL740
pILL721	pILL570	- ^b	5.5	<i>Bal</i> 31 digest of <i>Clal</i> -treated pILL740
pILL722	pILL570	- ^b	4.5	<i>Bal</i> 31 digest of <i>Clal</i> -treated pILL740
pILL723	pILL550	- ^b	5.3	<i>Hind</i> III partial digest of pILL590 + self-religation
pILL724	pILL550	- ^b	6.6	<i>Hind</i> III partial digest of pILL590 + self-religation
pILL725	pILL550	- ^b	2.6	<i>Hind</i> III partial digest of pILL590 + self-religation
pILL740	pILL570	ND	8.1	<i>Pst</i> I- <i>Eco</i> RI fragment of pILL590

^a Urease activity was detected in *C. jejuni* following mobilization of the hybrid plasmid from *E. coli* to *C. jejuni*.

^b *Clal*-*Bgl*III fragment of the indicated plasmid was subcloned into the vector pILL550 and then mobilized into *C. jejuni* cells in which the urease activity was detected.

^c ND, Not determined.

then several times with butanol to reduce the eluate volume to 100 μ l, and finally with diethyl ether. DNA was then precipitated with cold ethanol before recovering it by centrifugation. DNA polymerase I large fragment, T4 DNA polymerase (used to make blunt-end fragments) and T4 DNA ligase were purchased from Amersham, and calf intestine phosphatase was purchased from Pharmacia.

Hybridization. DNA restriction fragments fractionated by agarose gel electrophoresis were transferred to nitrocellulose sheets (0.45- μ m pore size; Schleicher & Schuell, Inc.) by the Southern technique (46) and hybridized at 68°C with ³²P-labeled deoxyribonucleotide probes (Amersham) labeled by random priming (13), using as primers the random hexamers from Pharmacia. Hybridization was revealed by autoradiography with Kodak XAR-Omat film.

Mating between donor *E. coli* cells and *C. jejuni* C31 recipients. Conditions to run series of 24 mating experiments simultaneously were adapted from the previously described protocol (25) as follows. *E. coli* cells harboring the IncP helper plasmid plus the hybrid plasmid to mobilize were grown in L broth without antibiotic with gentle shaking to a density of 10⁸ bacteria per ml. *Campylobacter* recipient cells were grown overnight in heart infusion broth (100 ml) supplemented as described above at 37°C with vigorous shaking under microaerobic conditions. A 4-ml portion of the overnight recipient cultures (10⁸ bacteria per ml) was centrifuged, washed in sterile water, pelleted again, and suspended in 100 μ l of donor *E. coli* cells. Each mating mix (100 μ l) was spread on the surface of Mueller-Hinton medium freshly poured into a 12-well tissue culture plate (each well containing 3 ml of Muller-Hinton) and incubated in microaerobic conditions for 5 h at 37°C. The bacteria were harvested by shaking three glass beads per well with a 100- μ l volume of heart infusion broth, and 100 μ l of broth containing bacteria was plated on Columbia medium containing vancomycin, polymyxin, cephalothin, trimethoprim, amphotericin B, and kanamycin. Plates were incubated at 37°C in microaerobic conditions for 48 to 60 h.

Analysis of proteins expressed in minicells. Minicells harboring the appropriate hybrid plasmid were isolated and labeled with [³⁵S]methionine (50 μ Ci/ml) (15). Approximately 100,000 cpm of acetone-precipitable material was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% gel (26). Standard proteins with molecular weights ranging from 94,000 to 14,000 (low-molecular-weight kit from Bio-Rad Laboratories) were run in parallel. The gel was stained and examined by fluorography, using En³Hance (New England Nuclear).

DNA sequencing. Appropriate DNA fragments were cloned into M13mp19 (31). Plaques containing inserts were identified by using X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and isopropyl- β -D-thiogalactopyranoside. Sequential series of overlapping clones were produced by using the Cyclone I Biosystem (I.B.I.). Single-stranded DNA templates were prepared by the polyethylene glycol method (41), and the sequence was determined by dideoxynucleotide chain termination (40), using a Sequenase kit (United States Biochemical Corp.). Sequence analyses performed directly on the product of amplification (polymerase chain reaction) were performed by using dimethyl sulfoxide (1% final concentration) in the annealing mixture (50). The amplification was carried out from total DNA extracted from *H. pylori* 85P for 25 cycles in a DNA Thermal Cycler (Perkin Elmer-Cetus), using the two oligonucleotides shown in Fig. 4 and a GeneAmp kit (Perkin Elmer-Cetus). The DNA was

denatured at 94°C for 2 min, annealed at 55°C for 2 min, and extended at 75°C for 2 min.

Nucleotide sequence accession number. The nucleotide sequence accession number is X57132.

RESULTS

Cosmid cloning of a DNA sequence responsible for urease expression from *H. pylori* 85P. Due to unsuccessful attempts at cloning and expressing the *H. pylori* genes of interest into *E. coli* recipient strains, we designed a shuttle approach for identifying the genes involved in urease expression in *H. pylori* strains, using *C. jejuni* as a final recipient strain. *C. jejuni* is a *Campylobacter* species naturally devoid of urease activity and was thought to be a more closely related host for the expression of *H. pylori* genes than *E. coli*. We modified the *E. coli*-*C. jejuni* shuttle vector pILL550 that we described previously (25) by introducing a DNA fragment containing a cos site (see Materials and Methods), so that the shuttle vector could be used as a cosmid vector. Bacteriophage lambda-transducing particles carrying recombinant cosmid molecules with segments (35 to 48 kb) of the chromosomal DNA of *H. pylori* 85P total DNA were prepared with pILL575 as a vector and were transduced into *E. coli* K-12 strain HB101 harboring the IncP helper plasmid pRK212.1. A total of 400 independent tetracycline- and kanamycin-resistant *E. coli* transductants harboring recombinant cosmids were frozen as a gene bank. None of the 400 *E. coli* transductants exhibited urease activity. Each recombinant cosmid was then mobilized from *E. coli* to *C. jejuni* C31, and kanamycin transconjugants were tested for their capacity to hydrolyze urea, a phenotype designated urease⁺. Of 106 *C. jejuni* kanamycin-resistant transconjugants tested, 1 exhibited urease activity; the enzymatic activity of the transconjugant was considerably lower than that observed with the wild-type *H. pylori* strain (85P). Whereas with *H. pylori* the reaction was immediate, 4 h of incubation were required to detect the activity from the transconjugants. The positive colony harbored a recombinant plasmid designated pILL585, 54 kb in size, which was purified from *E. coli* transductants as well as from *Campylobacter* transconjugants. Comparison of the HindIII restriction profiles of pILL585 isolated from either *E. coli* or *C. jejuni* (Fig. 1) clearly showed that the recombinant cosmid once in *C. jejuni* cells was totally unstable and gave rise to DNA rearrangements associated with deletions. As a consequence, in the subsequent steps, the DNA material used was solely that prepared from *E. coli* strains. The BamHI, EcoRI, PstI, and SmaI restriction sites of pILL585 were located by single and double digestion, and the resulting restriction map of the cosmid was established as shown in Fig. 2.

Defining the smallest urease-expressing DNA region of cosmid pILL585. BamHI-generated DNA fragments from the 44-kb insert were cloned into shuttle vector pILL550, transformed in the mobilizing *E. coli* S17-1, and mobilized into *C. jejuni*, and the resulting transconjugants harboring the hybrid plasmids were assayed for urease activity. None of the BamHI-generated fragments led to a urease⁺ phenotype in *C. jejuni*, indicating that the genes involved in the expression of urease contain at least one BamHI site. In the absence of suitable restriction sites and to define the smallest DNA fragment able to confer the urease⁺ phenotype on *C. jejuni* C31, pILL585 cosmid DNA (20 μ g) was partially digested with endonuclease Sau3A to generate fragments ranging from 6 to 12 kb. The fragments were treated with alkaline phosphatase to prevent any rearrangement of the initial

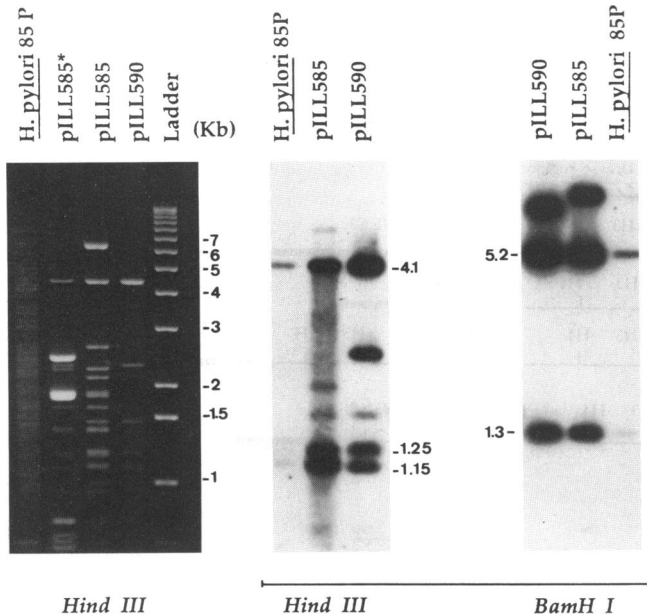


FIG. 1. Comparison of the urease region when cloned in shuttle cosmid pILL575 (pILL585) or in shuttle plasmid pILL550 (pILL590) following their propagation in *E. coli* or *C. jejuni* C31 (pILL585*). Plasmid DNA (0.5 μ g) or *H. pylori* 85P total DNA (3 μ g) was digested with *Hind*III or *Bam*HI restriction enzymes. The resulting fragments were separated by electrophoresis through a 1% agarose gel for 17 h at 2.5 V/cm, transferred to nitrocellulose, and hybridized with *in vitro* 32 P-labeled *Eco*RI-*Pst*I 8.1-kb insert from pILL590. Values on the left correspond to the sizes (in kilobases) of the 1-kb ladder used as the standard; those on the right indicate the size of the restriction fragments present in chromosomal DNA, hybrid cosmid pILL585, and the derivative plasmid (pILL590).

genome and were ligated with *Bam*HI-treated pILL550 (75 ng). After transformation into *E. coli* S17-1, kanamycin-resistant transformants were mated with *C. jejuni* C31 and kanamycin transconjugants were assayed for urease activity. Of 60 transconjugants tested, 4 were urease positive; they contained plasmids designated pILL587, pILL588, pILL589, and pILL590 with inserts of 7.8, 8.6, 9, and 8.1 kb, respectively. The plasmids exhibited instability in *C. jejuni* cells, however, to a lesser extent than cosmid pILL585. Based on the rapidity of the reaction relative to that of recombinant cosmid pILL585 (urease⁺⁺⁺), pILL587 and pILL590 exhibited a phenotype which was designated urease⁺⁺, whereas pILL588 and pILL589 were urease^{+/-}. The *Bam*HI, *Clal*, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II, and *Sma*I recognition sites were mapped in the four plasmids, and the orientation of the insert relative to the vector was determined as illustrated in Fig. 2: comparison of the restriction endonuclease-generated maps showed that expression of the urease activity was independent of the orientation of the insert relative to the vector and that they shared a common DNA sequence of 4.2 kb, designated the urease region. Plasmid pILL590 with relatively high urease expression was chosen as the prototype plasmid for further characterization of the urease region. Using the 8.1-kb *Eco*RI-*Pst*I restriction fragment of pILL590 as a probe, we confirmed by Southern hybridization that the sequences cloned did not suffer any rearrangements through the cloning process, i.e., that the *Hind*III and *Bam*HI restriction fragments of subclones and cosmid were present at the same size as in the original strain

of *H. pylori* (Fig. 1). This hybridization allowed us to conclude that a single copy of the urease region was present in the *H. pylori* strain. Moreover, hybridizing the 8.1-kb insert of pILL590 against nondigested total DNA extracted from *H. pylori* 85P did not allow us to visualize a band which would migrate as a supercoiled form of plasmid DNA, suggesting that the genetic information that we cloned originated from chromosomal DNA (data not shown).

To generate deletions, unique restriction sites were added on each side of the insert by cloning the 8.1-kb fragment of pILL590 into vector pILL570 (*Sp*^r, 5.3 kb in size). The pILL570 vector was constructed from pILL560, described previously (24), following the removal of the DNA fragment containing the ampicillin resistance marker (located between the *Dra*I site [position 3232 of pBR322] and the *Eco*RI site [position 1 of pBR322]) and ligation with the filled ends of the *Hind*III-generated interposon Ω (37). A series of deletions starting at either end of the 8.1-kb insert of the resulting plasmid pILL740 and extending up to 3.6 kb in the insert were performed by (i) using *Bal*31, (ii) subcloning restriction fragments, or (iii) partially digesting the plasmid with *Hind*III. Following these steps, each deleted insert was cloned again into pILL550, introduced into a mobilizing *E. coli* strain (S17.1) and shuttled into *C. jejuni* to determine urease activity. The results are summarized in Fig. 2. Any deletion extending into the previously defined urease region (4.2 kb in length) led to a negative phenotype, suggesting that this region was the smallest DNA fragment absolutely required for the urease expression in a *C. jejuni* host.

DNA sequence of the urease region of *H. pylori* 85P. The 5,100 bp depicted in Fig. 3A were sequenced with the following strategy; the *Hind*III 0.3-, 1.15-, and 1.25-kb fragments as well as the 1.5-kb *Hind*III-*Bam*HI and 0.7-kb *Bam*HI-*Eco*RI fragments originating from pILL588 were independently sequenced by creating overlapping deletions on the restriction fragments cloned into M13mp19 DNA phage. In addition, 16 synthetic oligonucleotide primers were synthesized to generate sequences overlapping the five independently sequenced fragments and to sequence the complementary strand when required.

The 5,100 bp spanning the urease region were analyzed for open reading frames (ORFs): four ORFs of >132 codons were found encoded by the same strand (Fig. 3B), whereas no ORF of any significant length was found on the reverse complement of the sequence shown in Fig. 4. These four ORFs were designated *ureA*, *ureB*, *ureC*, and *ureD*; three of them begin with the characteristic ATG start codon, and one begins with the less frequent GTG start codon (Fig. 3). The four ORFs were each preceded by sites similar to the *E. coli* consensus ribosome-binding (Shine-Dalgarno) sequence (43): GGAG preceding the *ureA* and *ureB* genes and AAGG preceding the *ureC* and *ureD* genes. The precise positions are indicated in Fig. 4. The atypical GTG start codon of *ureD* is localized a few nucleotides upstream of the *ureC* stop codon, and a unique frameshift is responsible for the separation of the left end side of the urease region into two ORFs. To be sure that these two ORFs really did exist in *H. pylori*, we used the polymerase chain reaction to amplify a 450-bp DNA fragment spanning the 3' end of the putative *ureC* gene and the 5' end of the *ureD* gene from total DNA extracted from *H. pylori* 85P. The amplified DNA was then directly sequenced and the same nucleotide sequence as the one determined from DNA cloned and propagated in *E. coli* was found in the genome of *H. pylori*, indicating that *ureC* and *ureD* actually represented two distinct ORFs.

From the 5' to the 3' end of the sequenced region, several

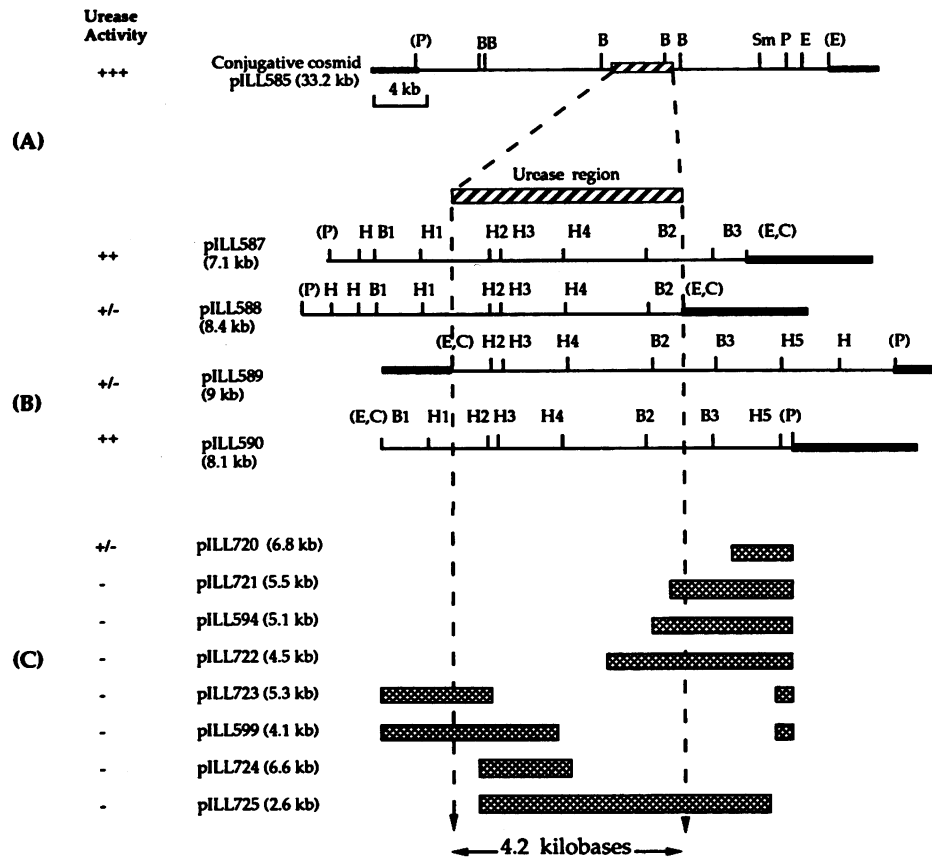
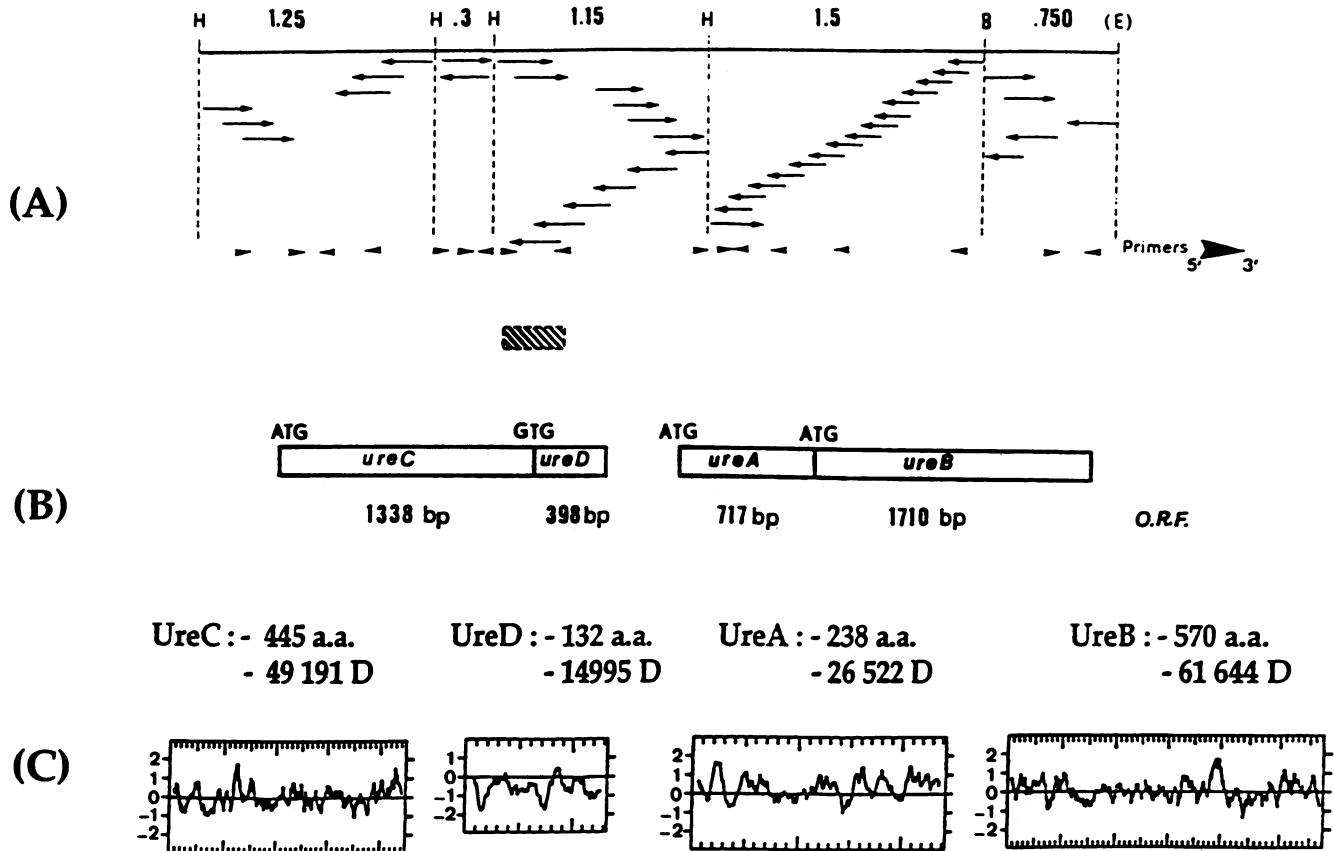


FIG. 2. Linear restriction maps of hybrid cosmid pILL585 (A) and (B) hybrid plasmids pILL587, pILL588, pILL589, and pILL590 resulting from *Sau3A* partial digestion of pILL585 and the subsequent cloning of the generated fragments into vector pILL550 (heavy line). (C) Boxes represent the extent of the deletions generated with nuclease *Bal 31* or restriction endonuclease *HindIII* performed on plasmid pILL590. Numbers in parentheses correspond to the size of the *H. pylori* DNA fragment inserted into the cloning vector (pILL575 or pILL550). B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sm, *Sma*I restriction sites; these letters within parentheses indicated that the restriction sites originated from the cloning vector. +++, ++, or +/- refer to the urease activity detected in the *C. jejuni* kanamycin-resistant transconjugants harboring the indicated recombinant plasmid.

other remarkable features were found at the DNA level (Fig. 3B). (i) Upstream of the *ureC* gene an *E. coli* consensus promoterlike sequence ($\sigma 70$) was found between nucleotides 188 and 225, where 10 nucleotides matched the 12 required nucleotides (TTGACA, -35 and TATAAT, -10) (38). (ii) A stem-loop structure with the features of a typical rho-independent transcriptional stop signal (38) was found downstream of the *ureC* gene (Fig. 4). (iii) Exactly 310 bp upstream of the *ureD* and *ureA* genes, sequences were found which exhibit a high degree of homology with the nitrogen regulation site specifying the $\sigma 54$ recognition sequence (*nif* promoter) (33): 13 and 14 of the 16 nucleotides of the consensus *nif* promoter sequence TGGYAYR ---- YYG CZ, where Y = T or C, R = G or A, and Z = A or T (i.e., TGGTAGA ---- TacCT for *ureA* and TGGCtTG ---- gCGCT for *ureD*), were conserved and aligned in such a way that the critical spacing between the GG and the GC doublets was 10 bp. No stem-loop structure was identified downstream of the *ureD* gene which might lead to the termination of the putative transcript initiated at the first *nif* promoterlike sequence or downstream of the second *nif* promoterlike sequence mapping within the 480-bp noncoding region flanked by the *ureD* and *ureA* genes.

Analysis of polypeptides expressed in minicells. pILL615, pILL589, pILL588, pILL590, pILL594, and pILL599 hybrid

plasmids as well as the corresponding cloning vectors were introduced by transformation into *E. coli* P678-54, a minicell-producing strain. Minicells were isolated and the polypeptides encoded by the plasmids synthesized in *E. coli* were labeled with [35 S]methionine and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The two polypeptides expressed from DNA fragments corresponding to *ureA* and *ureB* were clearly detected from these experiments (Fig. 5); they migrated with polypeptides having apparent molecular weights of 66,000 and 30,000 and were shown to disappear when deletions mapped to the *ureA* and *ureB* loci encoding the 61.6- and 26.5-kDa predicted polypeptides: *UreA* was expressed by pILL615, pILL589, pILL588, and pILL590 as well as by the subcloned pILL599, but not by pILL594; reciprocally, *UreB* was expressed by pILL594 but not by pILL599, as expected. The intensity of the bands corresponding to the expressed polypeptide relative to that of type III 3'-aminoglycoside phosphotransferase conferring kanamycin resistance (35,000 in molecular weight) was independent of the orientation of the insert in the cloning vector, suggesting that the *ureA* and *ureB* genes were expressed from promoter sequences located in the cloned sequences; a polypeptide with an apparent electrophoretic mobility corresponding to the *ureC* gene predicted product (49.2 kDa) was synthesized in minicells harboring



Subunits of the urease enzyme

FIG. 3. DNA sequence of urease region. (A) Strategy for sequencing the urease region from pILL588 hybrid plasmid as described in the text. Arrows correspond to the size of the DNA fragment sequenced. Arrowheads represent the oligonucleotides used to achieve and confirm the nucleotide determination. The hatched box represents the DNA fragment sequenced directly from the chromosomal DNA of *H. pylori* following the enzymatic amplification of this DNA region with two flanking oligonucleotides. (B) Schematic representation of the four ORFs deduced from the nucleotide sequence analysis and their size in nucleotides. ATG and GTG correspond to the initiator codons relative to each putative gene. (C) Predicted hydropathy profiles (39) for each of the four urease polypeptides with their size and calculated molecular weight. The left vertical axis indicates the relative hydrophilicity (positive ordinate) or hydrophobicity (negative ordinate).

pILL594, pILL588, and pILL590, but was absent in those containing pILL615 and pILL589, which both share the same insert cloned into different vectors (pILL533 and pILL550, respectively). In addition, due to the absence of a precise deletion or insertional mutation in the *ureD* gene, it was impossible to draw conclusions about the effective expression of the *ureD* gene in the *E. coli* host strain.

DISCUSSION

In this work, we report for the first time the cloning and functional expression of genes originating from *H. pylori*. This was made possible by virtue of a shuttle approach which allowed us to move randomly cloned segments of DNA from *E. coli* to *C. jejuni*, a *Campylobacter* species more closely related to *H. pylori* than bacteria belonging to the *Enterobacteriaceae* family. Using this approach, we cloned the urease gene cluster and were able to demonstrate urease activity in *C. jejuni* cells, whereas in *E. coli* the same DNA sequences, whether introduced by cosmid or by derivative subcloned plasmids, did not result in expression of enzyme activity. We have shown that a critical 4.2-kb DNA

fragment, designated the urease region, originated from the chromosomal DNA and was present as a single copy. This phenomenon, present in all other *H. pylori* isolates tested (data not shown), indicates that the high-level expression of enzyme activity in *H. pylori* is not due to the presence of multiple gene copies of the urease-encoding sequences, as has been suggested (20).

The 4.2-kb DNA fragment required for urease activity encompasses two genes encoding polypeptides with calculated molecular weights of 61,000 and 26,500 which unambiguously appeared to be the structural subunits of the urease enzyme as they aligned perfectly with the unique polypeptide chain of 840 amino acid residues of jack bean urease (47) and the three polypeptide chains of 11, 12, and 61 kDa of *P. mirabilis* (23) and *P. vulgaris* (34) (data not shown). This perfect alignment allowed us to confirm the results recently published by Hu and Mobley (20) as well as by Dunn et al. (8) based on the purified enzyme and to conclude that *H. pylori* urease consists solely of two polypeptide chains and not three as was first reported (9), a situation unique among the bacterial ureases (20, 32). That the *ureA* and *ureB* genes are arranged such that the stop

1

31
 AAG CTT TTC AGC TAG AAT AGA CAT GCA AAA CTA CCT ATT AAA AAG ATG TGA ATA AAA AGA

61
 TGC AAG AAT CTA AAA AAC ACA AAA CTT AAA AAG AAG CCC AAT AAT AAA AAC CCA TTA CTG

121
 AGC TTA AAG AAG TTA AAA ACG CCC CAA AAC TAA GCG AGA GCG ATT TTT TTC ACT GAA GCG

181
 TTA AGT CTT GAG AGC TTC CTA GAA GCG GTC TTT TTC TTT AAA ATC CCT TTG CTG ACA AAT

241
 TTA TGC AAG TCT TTA TTA GCG ATT TTC AAA CGA CTC TTG AGC TCT TGC TAC ATC ATT GAC

301
 AGC GAC GGC TTC ACG CAC GGC CTT AAT GAT ATT TTT AAT TTT AGT TTT ATA AAA CCT GTT

361
 GCG TTC GGT TCT TTT AAT GGT CTG TCT GAT TCG CTT TTC TGC GGA CTT ATG ATT TGC CAT

421
 AGC CTT GTT TTA ATC CCT TTG TAA TGT AAA AAT TGG CAT AAT TCT ATC TAA AAA TTG ATT

481
 AAA AAT AGT TTA AAA GGT ATT TTA TAA CG. ATG AAA ATT TTT GCG ACT GAT GCG GTG AGG
 met lys ile phe gly thr asp gly val ary

540/11
 GGT AAA GCA GCG GTG AAA CTC ACC CCC ATG TTT GTG ATG CGT TTA GGC ATT GCT GCG GCG
 gly lys ala gly val lys leu thr pro met

600/31
 TGC TAT TTT AAA AAA CAT TCT CAA ACG AAT AAA ATT TTA ATC GGT AAA GAC ACC AGA AAA
 leu tyr phe lys lys his ser gln thr asn lys ile leu gly lys asp thr ary lys

660/51
 AGC GCG TAT ATG GTA GAA AAC GCT TTA GTG AGC GCT CTC ACT TCC ATA GGC TAT AAT GTC
 ser gly tyr met val glu asn ala leu val ser ala leu thr ser ile gly tyr asn val

720/71
 ATT CAA ATA GCG CCT ATG CCT ACC CCT GCG ATC GCT TTT TTA ACC GAA GAC ATG GCG TGT
 ile gln ile gly pro met pro thr pro ala ile ala phe leu thr glu asp met ary cys

780/91
 GAT GCG GGT ATT ATG ATA ACC GCG AGC CAC AAC CCT TTT GAA GAC AAT GCG ATT AAG TTT
 asp ala gly ile met ile ser ala ser his asn pro phe glu asp asn gly ile lys phe

840/111
 TTC AAT TCT TAT GGT TAT AAA CTC AAA GAA GAA GAA AGA GCG ATT GAA GAA ATC TTT
 phe asn ser tyr gly tyr lys leu lys glu glu glu arg ala ile glu glu ile phe

900/131
 CAT GAT GAA GGA TTA CTG CAT TCC AGT TAT AAA GTG GCG GAG AGC GTC GGT AGC GCT AAA
 his asp glu gly leu leu his ser ser tyr lys val gly glu ser val gly ser ala lys

960/151
 AGG ATA GAC GAT GTG ATA GCG COT TAT ATC GCG CAT TTG AAG CAC TCT TTC CCC AAA CAT
 ary ile asp asp val ile gly arg tyr ile ala his leu lys his ser phe pro lys his

1020/171
 TTG AAT TTA CAG AGT TTA AGC ATC GTG CTA GAT ACC GCT AAT GCG GCG GCT TAT AAG GTG
 leu asn leu gln ser leu arg ile val leu asp thr ala asn gly ala ala tyr lys val

1080/191
 GCT CCG GTC GTT TTT AGC GAG CTT GCG GCT GAT GTG TTA GTG ATT AAT GAT GAG CCT AAC
 ala pro val val phe ser leu leu gly ala asp val leu val ile asn asp glu pro asn

1140/211
 GCG TGT AAT ATT AAT GAG CAA TCG GCG GCT TTA CAC CCT AAC CAA TTG AGC CAA GAA GTG
 gly cys asn ile asn glu cys gly ala leu his pro asn gln leu ser gln glu val

1200/231
 AAA AAA TAC CAC CCG GAT CTG GCG TTT GCT TTT GAT GCG GAT GCG GAT AGC CTA GTG GTG
 lys lys tyr ary ala asp leu gly phe ala phe asp gly asp ala asp ary leu val val

1260/251
 GTG GAT AAT TTA GCG AAT ATC GTG CAT GCG GAT AAG CTT TTA GCG GTG TTA GCG GTT TAT
 val asp asn leu gly asn ile val his gly asp lys leu leu gly val leu gly val tyr

1320/271
 CAA AAA TCT AAA AAC GCG CTT TCT TCT CAA GCA ATT GTC GCT ACA AAC ATG AGC AAT TTA
 gln lys ser lys asn ala leu ser ser gln ala ile val ala thr asn met ser asn leu

1380/291
 GCG CTT AAA GAA TAC TTA AAA TCC CAA GAT TTA GAA TTG AAG CAT TGC GCG ATT GCG GAT
 ala leu lys glu tyr leu lys ser gln asp leu glu leu lys his cys ala ile gly asp

1440/311
 AAG TTT GTG AGC GAA TGC ATG CGA TTG AAC AAA GCG AAT TTT CGA GCG GAC CAA AGC GCG
 lys phe val ser glu cys met ary leu asn lys ala asn phe gly gly glu gln ser gly

1500/331
 CAT AAT ATT TTT AGC GAT TAC GCT AAA ACC GCG GAT GCG TTG GTG TGC GCT TTG CAA GTG
 his ile ile phe ser asp tyr ala lys thr gly asp gly leu val cys ala leu gln val

1560/351
 AGC GCG TTA GTG TTA GAA AGT AAG CTT GTA ACC TCT GTT CCG TTA AAC CCC TTT GAA TTA
 ser ala leu val leu glu ser lys leu val ser ser val ary leu asn pro phe glu leu

1620/371
 TAC CTT CAA AAC CTG GTG AAT TTG AAT GTC CAA AAA AAG CCC CCT TTA GAA AGC CTG AAA
 tyr pro gln asn leu val asn leu asn val gln lys lys pro pro leu glu ser leu lys

1680/391
 GGT TAT AAC GCT CTT TTA AAA GAA TTA GAC AAG CTA GAA ATC CCG CAT TTG ATC CGT TAT
 gly tyr asn ala leu leu lys glu leu asp lys leu glu ile arg his leu ile arg tyr

1740/411
 AGC GCG ACT GAA AAC AAA TTA CGA ATC CTT TTA GAA GCT AAA GAT GAA AAA CTT TTA GAA
 ser gly thr glu asn lys leu arg ile leu leu glu ala lys asp glu lys leu leu glu

1800/431
 TCC AAA ATC CAA GAA TTA AAA GCG TTT TTT GAA GCG CAT TTG TGC TAA AAA CCA CTA AAA
 ser lys met gln glu leu lys glu phe phe gly his leu cys OCH

1710/491
 SD
 TTT TGA AGC GCA TTT GTC CTA AAA ACC ACT AAA AAA
 val leu lys thr thr lys lys

1862/8
 AGC CTG TTG GGT TTT AEA GCG GGT TTT CTT ATT TTT GCG CTG GAT CAA GCG ATT AAA
 ser leu leu val phe ile gly val phe phe leu ile phe gly val asp gln ala ile lys

1922/29
 TAC GCT ATT TTA GAG GCG TTT CCG TAT GAA AGT TTG GTT AEA GAT ATT GTT TTG CTG TTC
 tyr ala ile leu glu gly phe ary tyr glu ser leu val ile asp ile val leu val phe

1982/48
 AAT AAA GCG GTG GCG TTT TCC TTG CTC AGT TTT TTA GAG GCG GGT TTG AAA TAC TTG CAA
 asn lys gly val ala phe ser leu leu ser phe leu glu gly gly leu lys tyr leu gln

2042/68
 ATC CTT TTG ATT TTA GCG CTT TTT ATC TTT TTA ATC CCG CAA AGG GAG CTT TTT AAA AAC
 ile leu leu ile leu gly leu phe ile phe leu met ary gln ary glu leu phe lys asn

2102/88
 CAT GCG ATA GAG TTT GCG ATG GTG TTT GGT GCT GCG GGT TCT AAT GTT TTA GAC CCG TTT
 his ala ile glu phe gly met val phe gly ala gly val ser asn val leu asp ary phe

2162/108
 GTG CAT GCG GCG GTA GTG GAT TAT GTG TAT TAT CAT TAT GCG TTT GAT TTG CCA TTT TTA
 val his gly gly val val asp tyr val tyr tyr his tyr gly phe asp leu pro phe leu

2222/128
 ACT TCG CTG ATG TCA TGA TAG ATG TCG GTG TCG GCG TTT TAT TGT TAA GAC AAT TCT TTT
 thr ser leu met ser OPA

2282/148
 TTA AGC AAA AAC AAA ACA AAA TTA AGC CAT AAT TGC CCT TTT TAA AAT AAA AGG TCG CG.

2341
 TAC GTC AGT TGG TAG AGC ACT ACC TTG ACA TGG TAG TGG CCG CTG GTT CAA GTC CAG TCG

2401
 TGG CCA CCA TTA TCA CTC CAA TTT TAA TTC TCA TTT TTT TGC GAG TTT TTG ATC TTT ATA

2461
 AAT TCT AAA GCG GTA TTA AAT GCA CTC CCA ATA ACG CTT TTA TAG CCG TTC AAA AAC ATA

2521
 ACA CTA ATT CAT TTT AAA TAA TAA TTA GTT AAT GAA GCG TTC TGT TAA TCT TAG TAA ATC

2581
 AAA ACA TTG CTA CAA TCA CAT CCA ACC TTG AAT GCG TTA TGT CTT CAA GGA AAA ACA CTT

2641
 SD
 TAA GAA TAG GAG AAT GAG ATG AAA CTC ACC CCA AAA GAG TTA GAT AAG TTG ATG CTC CAC
 met lys leu thr pro lys glu leu asp lys leu met leu his

2701/15
 TAC GCT GCA GAA TTG CCT AAA AAA CCG AAA GAA GCG ATT AAG CTT AAC TAT GTA GAA
 tyr ala gly glu leu ala lys lys arg lys glu lys gly ile lys leu asn tyr val glu

2761/35
 GCA GTA GCT TTG ATT AGT GCG CAT ATT ATG GAA GAA GCG AGA GCT GGT AAA AAG ACT GCG
 ala val ala leu ile ser ala his ile met glu glu ala arg ala gly lys lys thr ala

2821/55
 GCT GAA TTG ATG CAA GAA GCG CCG ACT CTT TTA AAA CCA GAT GAT GTG ATG GAT GCG GTG
 ala glu leu met gln glu gly arg thr leu leu lys pro asp asp val met asp gly val

2881/75
 GCG AGC ATG ATC CAT GAA GTG GGT ATT GAA GCG ATG TTT CCT GAT GCG ACT AAA CTC GTA
 ala ser met ile his glu val gly ile glu ala met phe pro asp gly thr lys leu val

2941/95
 AGC GTG CAT ACC CCT ATT GAG GCG AAT GGT AAA TTA GTT CCT GGT GAG TTG TTC TTA AAA
 thr val his thr pro ile glu ala asn gly lys leu val pro gly glu leu phe leu lys

3001/115
 AAT GAA GAC ATC ACT ATC AAC GAA GCG AAA AAA GCG GTT AGC GTG AAA GTT AAA AAT GTT
 asn glu asp ile thr ile asn glu gly lys lys ala val ser val lys val lys asn val

3061/135
 GCG GAC AGA CCG GTT CAA ATC GCG TCA CAC TTC CAT TTC TTT GAA GTG AAT AGA TGC CTA
 gly asp ary pro val gln ile gly ser his phe his phe phe glu val asn ary cys leu

3121/155
 GAC TTT GAC AGA CAA AAA ACT TTC GGT AAA CCG TTA GAC ATT GCG AGC GCG ACA GCG GTA
 asp phe asp ary glu lys thr phe gly lys arg leu asp ile ala ser gly thr ala val

3181/175
 AGA TTT GAG CCT GCG GAA GAA AAA TCC GTA CAA TTG ATT GAC ATT GCG GGT AAC AGA AGA
 ary phe glu pro gly glu glu lys ser val glu leu ile asp ile gly gly asn ary ary

FIG. 4. Nucleotide sequence of the *H. pylori* urease genes. Numbers above the sequence indicate the nucleotide position. Predicted amino acid sequences, in sequential order, for UreC (bp 510 to 1844), UreD (bp 1841 to 2235), UreA (bp 2659 to 3372), and UreB (bp 3379 to 5085) are shown below the DNA sequence. Putative ribosome-binding sequences (Shine-Dalgarno [SD] sites) are underlined; boxed sequences correspond to the promoterlike sequences ($\sigma 70$ as well as $\sigma 54$), and arrows above the sequence indicate stem-loop structures with the features of a rho-independent transcriptional stop signal.

3241/195
ATC TTT GGA TTT AAC GCA TTG GTT GAT AGA CAA GCA GAC AAC GAA AGC AAA AAA ATT GCT
ile phe gly phe asn ala leu val asp arg gln ala asp asn glu ser lys lys ile ala

3301/215
TCA CAC AGA GCT AAA GAG CGT GGT TTT CAT GGC GCT AAA AGC GAT GAC AAC TAT GTA AAA
leu his arg ala lys glu arg gly phe his gly ala lys ser asp asp asn tyr val lys

3361/235 SD 1
ACA ATT AAG GAG TAA GAA ATC AAA AAG ATT AGC AGA AAA GAA TAT GTT TCT ATG TAT GGT
thr ile lys glu OCH --- met lys lys ile ser arg lys glu tyr val ser met tyr gly

3421/15
CCT ACT ACA GGC GAT AAA GTG AGA TTG GCG GAT ACA GAC TTG ATC GCT GAA GTA GAA CAT
pro thr thr gly asp lys val arg leu gly asp thr asp leu ile ala glu val glu his

3481/35
GAC TAC ACC ATT TAT GGC GAA GAG CTT AAA TTC GGC GGT AAA ACC CTA AGA GAA GGC
asp tyr thr ile tyr gly glu glu leu lys phe gly gly lys thr leu arg glu gly

3541/55
ATG AGC CAA TCT AAC AAC CCT AGC AAA GAA GAG TTG GAT TTA ATT ATC ACT AAC GCT TTA
met ser gln ser asn asn pro ser lys glu glu leu asp leu ile ile thr asn ala leu

3601/75
ATC GTG GAT TAC ACC GGT ATT TAT AAA GCG GAT ATT GGT ATT AAA GAT GGC AAA ATC GCT
ile val asp tyr thr gly ile tyr lys ala asp ile gly ile lys asp gly lys ile ala

3661/95
GCC ATT GGT AAA GGC GGT AAC AAA GAC ATC CAA GAT GGC GTT AAA AAC AAT CTT AGC GTA
gly ile gly lys gly gly asn lys asp met gln asp gly val lys asn asn leu ser val

3721/115
GGT CCT GCT ACT GAA GGC TTA GGC GGT GAA GGT TTG ATC GTA ACC GCT GGT GGT ATT GAC
gly pro ala thr glu ala leu ala gly glu gly leu ile val thr ala gly gly ile asp

3781/135
ACA CAC ATC CAC TTC ATT TCA CCC CAA CAA ATC GCT ACA GCT TTT GCA AGC GGT GTA ACA
thr his ile his phe ile ser pro gln gln ile pro thr ala phe ala ser gly val thr

3841/155
ACC ATC ATT GGT GGT GGA ACC GGT CCT GCT GAT GGC ACT AAT GCG ACT ACT ATC ACT CCA
thr met ile gly gly gly thr gly pro ala asp gly thr asn ala thr thr ile thr pro

3901/175
GGC AGA AGA AAT TTA AAA TGG ATC CTC AGA GCG GCT GAA GAA TAT TCT ATG AAT TTA GGT
gly arg arg asn leu lys trp met leu arg ala ala glu glu tyr ser met asn leu gly

3961/195
TTC TTG GCT AAA GGT AAC GCT TCT AAC GAT GCG AGC TTA GCG GAT CAA ATT GAA GCG GGT
phe leu ala lys gly asn ala ser asn asp ala ser leu ala asp gln ile glu ala gly

4021/215
GGC ATT GGC TTT AAA ATT CAC GAA GAC TGG GCG ACC ACT CCT TCT GCA ATC AAT CAT GCG
ala ile gly phe lys ile his glu asp trp gly thr thr pro ser ala ile asn his ala

4081/235
TTA GAT GTT GCG GAC AAA TAC GAT GTG CAA GTC GCT ATC CAC ACA GAC ACT TTG AAT GAA
leu asp val ala asp lys tyr asp val gln val ala ile his thr asp thr leu asn glu

4141/255
GCC GGT TGT GTA GAA GAC ACT ATG GCT GCT ATT GCT GGA GCG ACT ATG CAC ACT TTC CAC
ala gly cys val glu asp thr met ala ala ile ala gly arg thr met his thr phe his

3271/205
TCA CAC AGA GCT AAA GAG CGT GGT TTT CAT GGC GCT AAA AGC GAT GAC AAC TAT GTA AAA
leu his arg ala lys glu arg gly phe his gly ala lys ser asp asp asn tyr val lys

3331/225
TCA CAC AGA GCT AAA GAG CGT GGT TTT CAT GGC GCT AAA AGC GAT GAC AAC TAT GTA AAA
leu his arg ala lys glu arg gly phe his gly ala lys ser asp asp asn tyr val lys

3391/5
ACA ATT AAG GAG TAA GAA ATC AAA AAG ATT AGC AGA AAA GAA TAT GTT TCT ATG TAT GGT
thr ile lys glu OCH --- met lys lys ile ser arg lys glu tyr val ser met tyr gly

3451/25
CCT ACT ACA GGC GAT AAA GTG AGA TTG GCG GAT ACA GAC TTG ATC GCT GAA GTA GAA CAT
pro thr thr gly asp lys val arg leu gly asp thr asp leu ile ala glu val glu his

3511/45
GAC TAC ACC ATT TAT GGC GAA GAG CTT AAA TTC GGC GGT AAA ACC CTA AGA GAA GGC
asp tyr thr ile tyr gly glu glu leu lys phe gly gly lys thr leu arg glu gly

3571/65
ATG AGC CAA TCT AAC AAC CCT AGC AAA GAA GAG TTG GAT TTA ATT ATC ACT AAC GCT TTA
met ser gln ser asn asn pro ser lys glu glu leu asp leu ile ile thr asn ala leu

3631/85
ATC GTG GAT TAC ACC GGT ATT TAT AAA GCG GAT ATT GGT ATT AAA GAT GGC AAA ATC GCT
ile val asp tyr thr gly ile tyr lys ala asp ile gly ile lys asp gly lys ile ala

3691/105
GCC ATT GGT AAA GGC GGT AAC AAA GAC ATC CAA GAT GGC GTT AAA AAC AAT CTT AGC GTA
gly ile gly lys gly gly asn lys asp met gln asp gly val lys asn asn leu ser val

3751/125
GGT CCT GCT ACT GAA GGC TTA GGC GGT GAA GGT TTG ATC GTA ACC GCT GGT GGT ATT GAC
gly pro ala thr glu ala leu ala gly glu gly leu ile val thr ala gly gly ile asp

3811/145
ACA CAC ATC CAC TTC ATT TCA CCC CAA CAA ATC GCT ACA GCT TTT GCA AGC GGT GTA ACA
thr his ile his phe ile ser pro gln gln ile pro thr ala phe ala ser gly val thr

3871/165
ACC ATC ATT GGT GGT GGA ACC GGT CCT GCT GAT GGC ACT AAT GCG ACT ACT ATC ACT CCA
thr met ile gly gly gly thr gly pro ala asp gly thr asn ala thr thr ile thr pro

3931/185
GGC AGA AGA AAT TTA AAA TGG ATC CTC AGA GCG GCT GAA GAA TAT TCT ATG AAT TTA GGT
gly arg arg asn leu lys trp met leu arg ala ala glu glu tyr ser met asn leu gly

3991/205
TTC TTG GCT AAA GGT AAC GCT TCT AAC GAT GCG AGC TTA GCG GAT CAA ATT GAA GCG GGT
phe leu ala lys gly asn ala ser asn asp ala ser leu ala asp gln ile glu ala gly

4051/225
GGC ATT GGC TTT AAA ATT CAC GAA GAC TGG GCG ACC ACT CCT TCT GCA ATC AAT CAT GCG
ala ile gly phe lys ile his glu asp trp gly thr thr pro ser ala ile asn his ala

4111/245
TTA GAT GTT GCG GAC AAA TAC GAT GTG CAA GTC GCT ATC CAC ACA GAC ACT TTG AAT GAA
leu asp val ala asp lys tyr asp val gln val ala ile his thr asp thr leu asn glu

4171/265
GCC GGT TGT GTA GAA GAC ACT ATG GCT GCT ATT GCT GGA GCG ACT ATG CAC ACT TTC CAC
ala gly cys val glu asp thr met ala ala ile ala gly arg thr met his thr phe his

4201/275
ACT GAA GGC GCT GGC GCG GGA CAC GCT CCT GAT ATT ATT AAA GTA GCC GGT GAA CAC AAC
thr glu gly ala gly gly gly his ala pro asp ile ile lys val ala gly glu his asn

4231/285
ATT CTT CCC GCT TCC ACT AAC CCC ACC ATC CCT TTC ACC GTG AAT ACA GAA GCA GAC CAC
ile leu pro ala ser thr asn pro thr ile pro phe thr val asn thr glu ala glu his

4261/295
ATG GAC ATG CTT ATG GTG TGC CAC CAC TTG GAT AAA AGC ATT AAA GAA GAT GTT CAG TTC
met asp met leu met val cys his his leu asp lys ser ile lys glu asp val gln phe

4321/315
GCT GAT TCA AGG ATC GCG CCT CAA ACC ATT GCG GCT GAA GAC ACT TTG CAT GAC ATG GCG
ala asp ser arg ile arg pro gln thr ile ala ala glu asp thr leu his asp met gly

4351/325
GCT GAT TCA AGG ATC GCG CCT CAA ACC ATT GCG GCT GAA GAC ACT TTG CAT GAC ATG GCG
ala asp ser arg ile arg pro gln thr ile ala ala glu asp thr leu his asp met gly

4411/345
GCT GAT TCA AGG ATC GCG CCT CAA ACC ATT GCG GCT GAA GAC ACT TTG CAT GAC ATG GCG
ala asp ser arg ile arg pro gln thr ile ala ala glu asp thr leu his asp met gly

4441/355
ATT CTT TCA ATC ACC AGT TCT GAC TCT CAA GCG ATG GCG GGT GTG GGT GAA GTT ATC ACT
ile phe ser ile thr ser ser asp ser gln ala met gly arg val gly glu val ile thr

4501/375
AGA ACT TGG CAA ACA GCT GAC AAA AAC AAG AAA GAA TTT GCG CCG TTG AAA GAA GAA AAA
arg thr trp gln thr ala asp lys asn lys lys glu phe gly arg leu lys glu glu lys

4591/405
GCC GAT AAC GAC AAC TTC AGG ATC AAA CCG TAC TTG TCT AAA TAC ACC ATT AAC CCA GCG
gly asp asn asp asn phe arg ile lys arg tyr leu ser lys tyr thr ile asn pro ala

4621/415
GCT GCT CAT GCG ATT AGC GAG TAT GTA GGT TCA GTA GAA GTG GCG AAA GTG GCT GAC TTG
ile ala his gly ile ser glu tyr val gly ser val glu val gly lys val ala asp leu

4681/435
GTA TTG TGG AGT CCA GCA TTC TTT GCG GTG AAA CCC AAC ATG ATC ATC AAA GCG GGA TTC
val leu trp ser pro ala phe phe gly val lys pro asn met ile ile lys gly gly phe

4741/455
ATT GCG TTA AGC CAA ATG GCG GAT GCG AAC GCT TCT ATC CCT ACC CCA CAA CCG GTT TAT
ile ala leu ser gln met gly asp ala asn ala ser ile pro thr pro gln pro val tyr

4801/475
TAC AGA GAA ATG TCT GCT CAT CAT GGT AAA GCT AAA TAC GAT GCA AAC ATC ACT TTT GTG
tyr arg glu met phe ala his his gly lys ala lys tyr asp ala asn ile thr phe val

4861/495
TCT CAA GCG GCT TAT GAC AAA GCG ATT AAA GAA GAA TTA GCA CTT GAA AGA CAA GTG TTG
ser gln ala ala tyr asp lys gly ile lys glu glu leu gly leu glu arg gln val leu

4921/515
CGG GTA AAA AAT TGC AGA AAT ATC ACT AAA AAA GAC ATG CAA TTC AAC GAC ACT ACT GCT
pro val lys asn cys arg asn ile thr lys lys asp met gln phe asn asp thr thr ala

4981/535
CAC ATT GAA GTC AAT CCT GAA ACT TAC CAT GAT GTC TTC GTG GAT GCG AAA GAA GTA ACT TCT
his ile glu val asn pro glu thr tyr his val phe val asp gly lys glu val thr ser

5041/555
AAA CCA GCG AAT AAA GTG AGC TTG GCG CAA CTC TTT AGC ATT TTC TAG GAT TTT TTA GAG
lys pro ala asn lys val ser leu ala gln leu phe ser ile phe AMB --- --- ---

FIG. 4—Continued.

codon of the *ureA* gene is separated from the methionine initiator codon of the *ureB* gene by a single codon suggests that a single mutation in the stop codon of *ureA* could lead to the fusion of the two polypeptides encoded by *ureA* and *ureB* and therefore generate a single polypeptide. Based on this, the *H. pylori* urease appears phylogenetically more closely related to the jack bean urease than to the three-subunit bacterial ureases; this statement is also documented by the higher degree of conservation observed between the 26.5-kDa subunit of *H. pylori* with the amino-terminal sequence of jack bean urease (48%) compared with that of the homologous urease subunits of *P. mirabilis* (42%).

Although the urease of *H. pylori* has been described as an extracellular enzyme (8), i.e., has to be transported to the external membrane, no leader peptide sequence was found for either of the two polypeptides. In addition, there is an excellent agreement between the amino acid sequence deduced from the DNA analyses and that of the N-terminal amino acid of the *H. pylori* subunits purified and sequenced (8, 20), indicating that no maturation of the N-terminal ends of the urease subunits is required either for the export of the subunits or to generate enzymatic activity.

Clayton et al. (7) were the first to report the cloning of specific *H. pylori* antigens reacting with antisera raised

against the purified *H. pylori* urease. Subsequently, they demonstrated by DNA sequence analyses (6) that the antigens expressed in *E. coli* by the cloned DNA fragment correspond to two polypeptides of the urease enzyme (i.e., UreA and UreB); however, there was no expression of urease activity. The *ureA* and *ureB* nucleotide sequences presented in this work and the sequences reported by Clayton et al. (6) for a different *H. pylori* isolate indicate that the urease subunits of *H. pylori* are highly conserved polypeptides as >98% of the amino acids of the urease enzyme were conserved, if one excludes from the comparison the carboxy-terminal sequence on which there is substantial disagreement. However, the high degree of conservation in primary structure observed between the urease sequences originating from bacteria (*P. mirabilis*) or from a plant (jack bean) suggests that the whole urease enzyme cannot be used as a specific antigen for serological tests since it is likely that common epitopes will be present in the different bacterial urease proteins which might lead to false-positive serological tests. Nevertheless, alignment of the three sequences allowed us to identify a domain (shown in Fig. 6) of the *ureA* gene product which is unique to *H. pylori*; no equivalent sequence exists in the urease subunits of *P. mirabilis* or *Ureaplasma urealyticum* (2) (data not shown). We are pres-

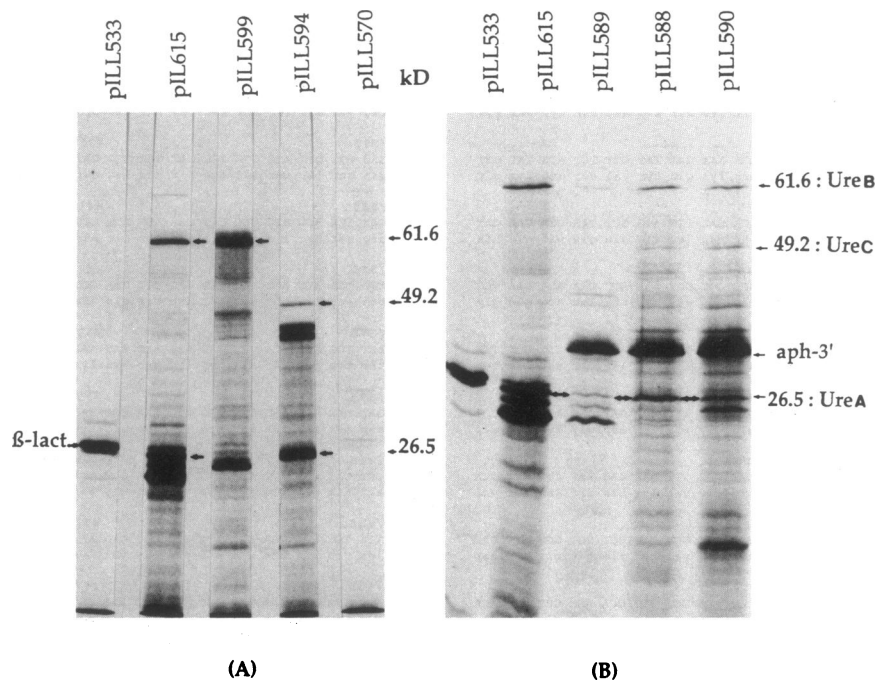


FIG. 5. Fluorographs of plasmid-mediated polypeptides expressed in minicells labeled with [35 S]methionine analyzed on 10% (A) or 12.5% (B) sodium dodecyl sulfate-polyacrylamide gels. (A) Polypeptides encoded by the pILL533 vector, by the pILL615 plasmid consisting of the *EcoRI-PstI* insert of plasmid pILL589 (urease-positive plasmid) cloned into vector pILL533, and by pILL594 and pILL599 urease-negative plasmids in comparison with the polypeptides expressed by the corresponding vector, pILL570. (B) Polypeptides expressed by three urease-positive plasmids, pILL589, pILL588, and pILL590. Numbers in kilodaltons refer to the calculated molecular weights of the predicted polypeptides UreA, UreB, and UreC. Aph-3', Migration of type III 3'-aminoglycoside phosphotransferase (35 kDa); β -lact, migration of β -lactamase (30 kDa), both encoded by the vectors.

ently investigating the degree of conservation of this peptide region among *H. pylori* isolates as well as testing its immunogenic properties to determine whether it can be used as a specific epitope for diagnostic purposes or as a potentially protective epitope. Alignment of sequences also permits localization of the active site of the *H. pylori* urease; this site is indicated in Fig. 6 and contains the eight histidine residues and one cysteine residue which, respectively, are believed to play a major role in the binding of nickel ions and in enzymatic activity in jack bean urease. That these residues are conserved and perfectly aligned between the three sequences of urease suggests a similar role in *H. pylori*, a hypothesis which can now be assessed by directed mutagenesis of the *ureB* gene.

In addition to the *ureA* and *ureB* genes, we have shown that DNA sequences located upstream of the *ureA* and *ureB* genes were absolutely required for urease expression in *C. jejuni*. The nucleotide sequence determination of the DNA fragment extending from the *HindIII*-1 restriction site and the *EcoRI* site of pILL588 permitted identification of two ORFs designated *ureC* and *ureD* which potentially encode polypeptides with molecular weights of 49,200 and 14,900, respectively. The search for amino acids relevant to sequences involved in metal (12)-, calcium (3)-, or ATP-binding sites (18, 48) was negative, as was that of similarities between the deduced amino acid sequence of the *ureC* and *ureD* genes and protein sequences from the NBRF data base.

No precise role can be attributed to the UreC-predicted polypeptide in the absence of similarities between its amino acid sequence and that of the identified UreD, UreE, or

UreF polypeptide of the *P. mirabilis* urease operon (23, 32). These polypeptides are believed to be involved in the processing of nickel ions and/or transport of the urea from the extracellular to the intracellular compartment (32). From the present results, we cannot definitively conclude that the *ureC* gene product per se is required for urease expression; one might suspect, due to the identification of nitrogen binding sites, that solely the DNA sequences corresponding to this ORF are important as *cis*-acting sites for urease expression. Additional experiments will be required to clarify this point.

ureD encodes a possible polypeptide of 14.9 kDa with features (Fig. 3C) typical of membrane-spanning domains of transmembrane proteins (39), which suggests that this polypeptide could serve a transport or anchoring function. In addition, features at the DNA level suggest that the *ureD* gene and its product are related to urease function. The same sequence localized 310 bp upstream of both the *ureD* and *ureA* genes is highly homologous with the consensus sequence of the nitrogen regulation site (binding site of factor σ_{54}), suggesting that the expressions of the *ureA-ureB* genes and the *ureD* gene are under the same transcriptional control. Northern (RNA) hybridization as well as primer extension experiments are now in progress to determine whether these promoterlike sequences are used for the initiation of the transcripts corresponding to the *ureA+ureB* and *ureD* genes in *H. pylori* as well as in *E. coli*, or whether they are a remnant of a promoter which allowed genes to be expressed and regulated in an ancestral genome.

Differences in the rapidity and intensity of urease activity in *C. jejuni* cells harboring plasmids with DNA inserts larger

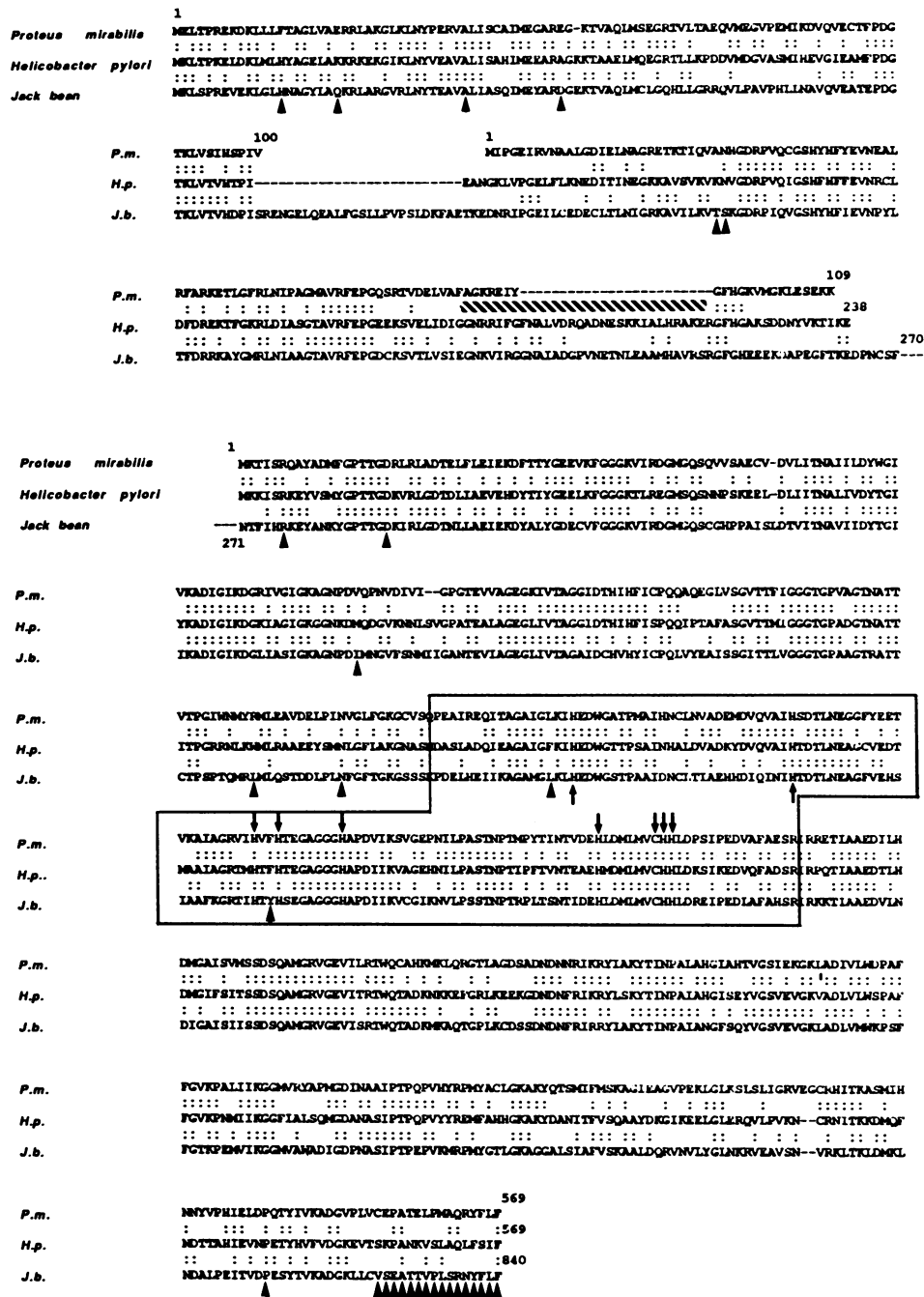


FIG. 6. Alignment of the first 270 amino acids of the jack bean urease (bottom line) with that of the two small subunits of *Proteus mirabilis* and the product of the *ureA* gene (top) and alignment of the last 550 amino acids of the jack bean urease with that of the large subunit of *P. mirabilis* and of the product of the *ureB* gene (bottom). Colons between two lines indicate the presence of the identical amino acid in two polypeptide chains. Numbers above the letters represent amino acid positions for each subunit. Dashed lines represent gaps introduced to optimize the alignments. The box in which arrows point out eight histidine residues and one cysteine residue correspond to the jack bean urease active site (47). Triangles point out the discrepancies with the *H. pylori* urease sequence as published by Clayton et al. (6). Hatch lines correspond to a peptide sequence unique to *H. pylori*.

than the 4.2-kb obligately required fragment indicates that, in addition to the four identified genes or loci, other genes or DNA sequences located upstream as well as downstream appear to be involved in high-level activity.

The absence of detectable urease activity in *E. coli* cells is not yet understood: in vivo transcription and translation

experiments performed in a minicell-producing strain indicated functional transcription as well as translation of at least the *ureA*, *ureB*, and *ureC* genes. All of our attempts to induce urease activity in recombinant *E. coli* cells by changing growth conditions (temperature, media, or addition of urea) or to detect activity from sonicated bacterial cells in

the presence or absence of nickel ions were unsuccessful. That by shutting the DNA fragment cloned in *E. coli* to *C. jejuni* cells we were capable to detect urease activity indicates that *C. jejuni* cells are capable of complementing a function that *E. coli* cells cannot accomplish; this function should be investigated. The cloning and expression of the urease genes of *H. pylori* allow us to think that the shuttle approach described in this paper might be generalized as a possible route for the successful cloning and expression of other *H. pylori* genes of interest.

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