JOURNAL OF BACTERIOLOGY, Mar. 1991, p. 1920–1931
0021-9193/91/061920-12$02.00/0

Vol. 173, No. 6

# 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.

(Parts of this work were presented at the First and the Second International Meetings of the European *Campylobacter pylori* Study Group Workshop on Gastroduodenal Pathology and *Campylobacter pylori*, 1988, Bordeaux, France, and 1989, Ulm, Germany; and at the Annual Meeting of the American Society for Microbiology, 1990.)

## 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::Tn*7* 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* $\lambda^-$ *rpsL minA minB*) (1) was used for preparation of minicells. 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 $10^9$ 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 *Sau*3A, 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*II 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 *Sau*3A or *Hin*dIII 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 | *Sau*3A partial digest of 85P |
| pILL587 | pILL550 | ++[a] | 7.1 | *Sau*3A partial digest of pILL585 |
| pILL588 | pILL550 | +/−[a] | 8.4 | *Sau*3A partial digest of pILL585 |
| pILL589 | pILL550 | +/−[a] | 9 | *Sau*3A partial digest of pILL585 |
| pILL590 | pILL550 | ++[a] | 8.1 | *Sau*3A partial digest of pILL585 |
| pILL594 | pILL570 | −[b] | 5.1 | *Bam*HI fragment of pILL590 |
| pILL599 | pILL570 | −[b] | 4.1 | *Hin*dIII fragment of pILL590 |
| pILL615 | pILL533 | ND[c] | 9 | *Pst*I-*Eco*RI fragment of pILL589 |
| pILL720 | pILL570 | +/−[b] | 6.8 | *Bal*31 digest of *Cla*I-treated pILL740 |
| pILL721 | pILL570 | −[b] | 5.5 | *Bal*31 digest of *Cla*I-treated pILL740 |
| pILL722 | pILL570 | −[b] | 4.5 | *Bal*31 digest of *Cla*I-treated pILL740 |
| pILL723 | pILL550 | −[b] | 5.3 | *Hin*dIII partial digest of pILL590 + self-religation |
| pILL724 | pILL550 | −[b] | 6.6 | *Hin*dIII partial digest of pILL590 + self-religation |
| pILL725 | pILL550 | −[b] | 2.6 | *Hin*dIII partial digest of pILL590 + self-religation |
| pILL740 | pILL570 | ND | 8.1 | *Pst*I-*Eco*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] *Cla*I-*Bgl*II 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 μl, 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-μm pore size; Schleicher & Schuell, Inc.) by the Southern technique (46) and hybridized at 68°C with $^{32}$P-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 simultanously 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 $10^8$ 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 ($10^8$ bacteria per ml) was centrifuged, washed in sterile water, pelleted again, and suspended in 100 μl of donor *E. coli* cells. Each mating mix (100 μl) 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-μl volume of heart infusion broth, and 100 μl of broth containing bacteria was plated on Columbia medium containing vancomycin, polymixin, 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 [$^{35}$S]methionine (50 μCi/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 En$^3$Hance (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 *Hin*dIII 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 *Bam*HI, *Eco*RI, *Pst*I, and *Sma*I 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.** *Bam*HI-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 *Bam*HI-generated fragments led to a urease$^+$ phenotype in *C. jejuni*, indicating that the genes involved in the expression of urease contain at least one *Bam*HI 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 μg) was partially digested with endonuclease *Sau*3A to generate fragments ranging from 6 to 12 kb. The fragments were treated with alkaline phosphatase to prevent any rearrangement of the initial

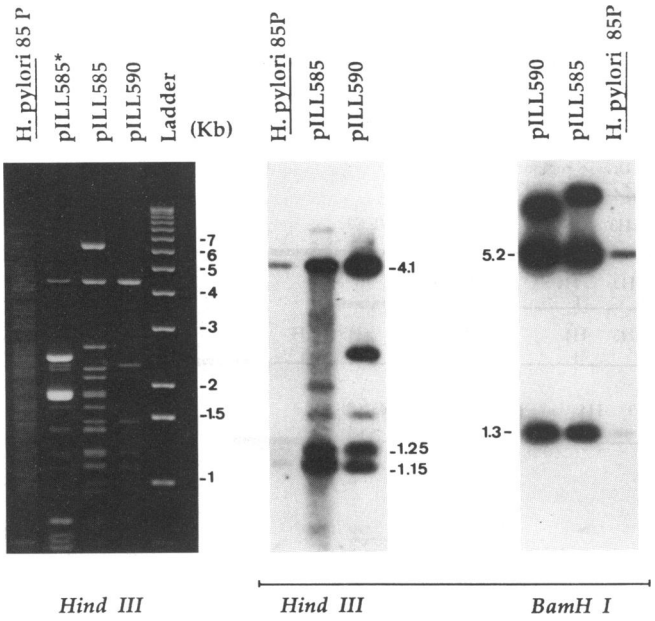


FIG. 1. Comparison of the urease region when cloned in shuttle cosmid pILL575 (pILL585) or in shuttle plasmid pILL550 (pILL590) following their propagation in *E. coli* or *C. jejuni* C31 (pILL585*). Plasmid DNA (0.5 μg) or *H. pylori* 85P total DNA (3 μg) was digested with *Hin*dIII or *Bam*HI restriction 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 extend 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, *Cla*I, *Eco*RI, *Hin*dIII, *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 *Hin*dIII 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 *Hin*dIII-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 *Hin*dIII. 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 *Hin*dIII 0.3-, 1.15-, and 1.25-kb fragments as well as the 1.5-kb *Hin*dIII-*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 distincts ORFs.

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

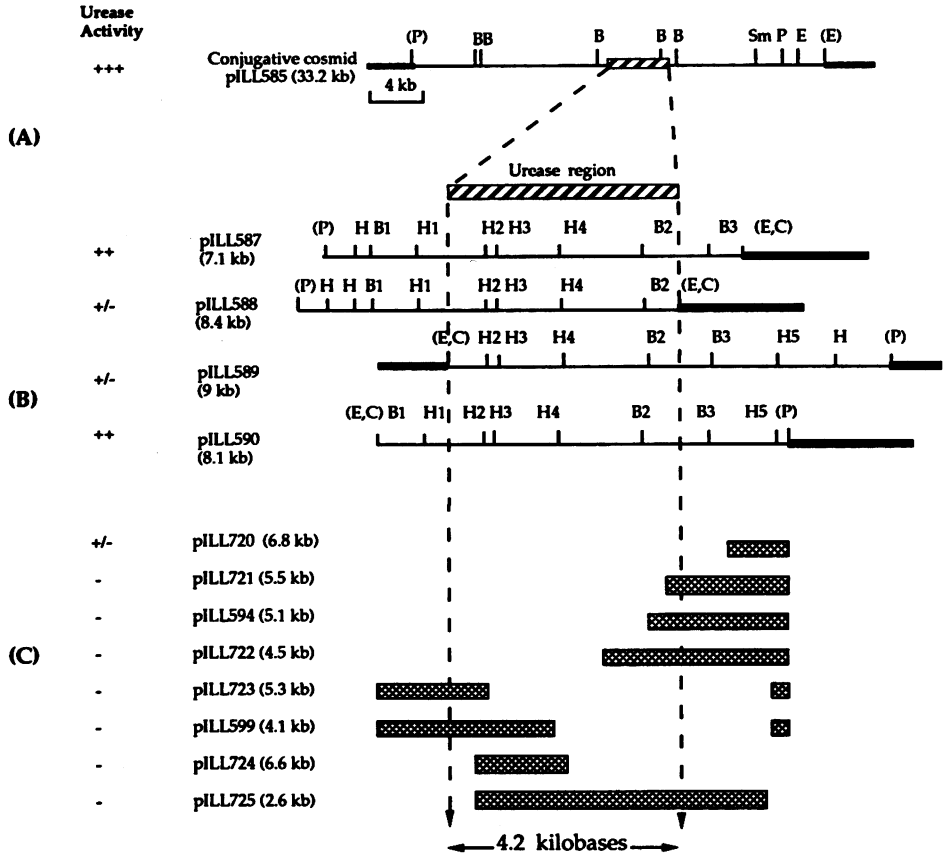


FIG. 2. Linear restriction maps of hybrid cosmid pILL585 (A) and (B) hybrid plasmids pILL587, pILL588, pILL589, and pILL590 resulting from *Sau*3A partial digestion of pILL585 and the subsequent cloning of the generated fragments into vector pILL550 (heavy line). (C) Boxes represent the extent of the deletions generated with nuclease *Bal* 31 or restriction endonuclease *Hin*dIII 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, *Hin*dIII; 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 - - - - YYGCZ, 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 synthetized in minicells harboring

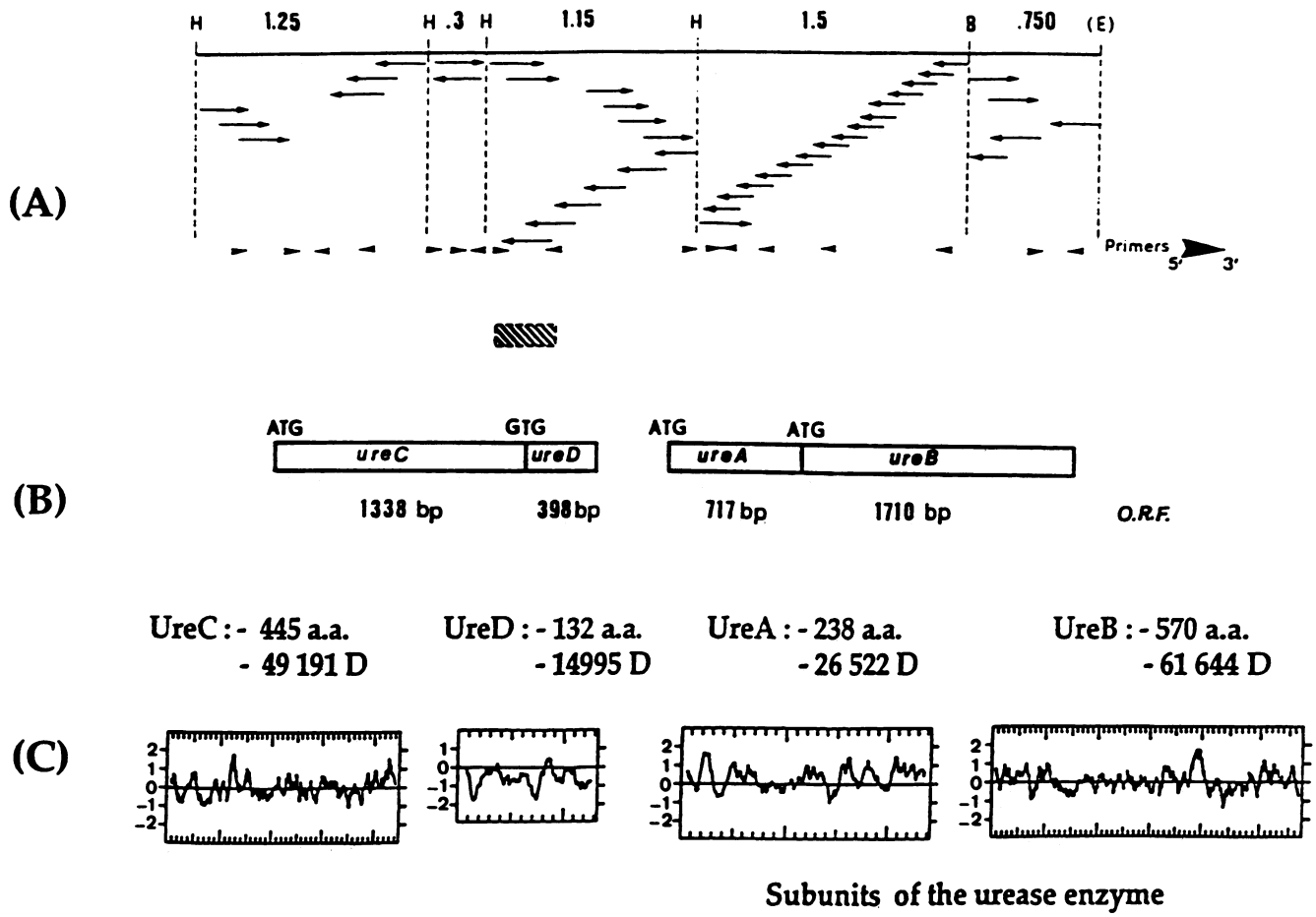


FIG. 3. DNA sequence of urease region. (A) Strategy for sequencing the urease region from pILL588 hybrid plasmid as described in the text. Arrows correspond to the size of the DNA fragment sequenced. Arrowheads represent the oligonucleotides used to achieve and confirm the nucleotide determination. The 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

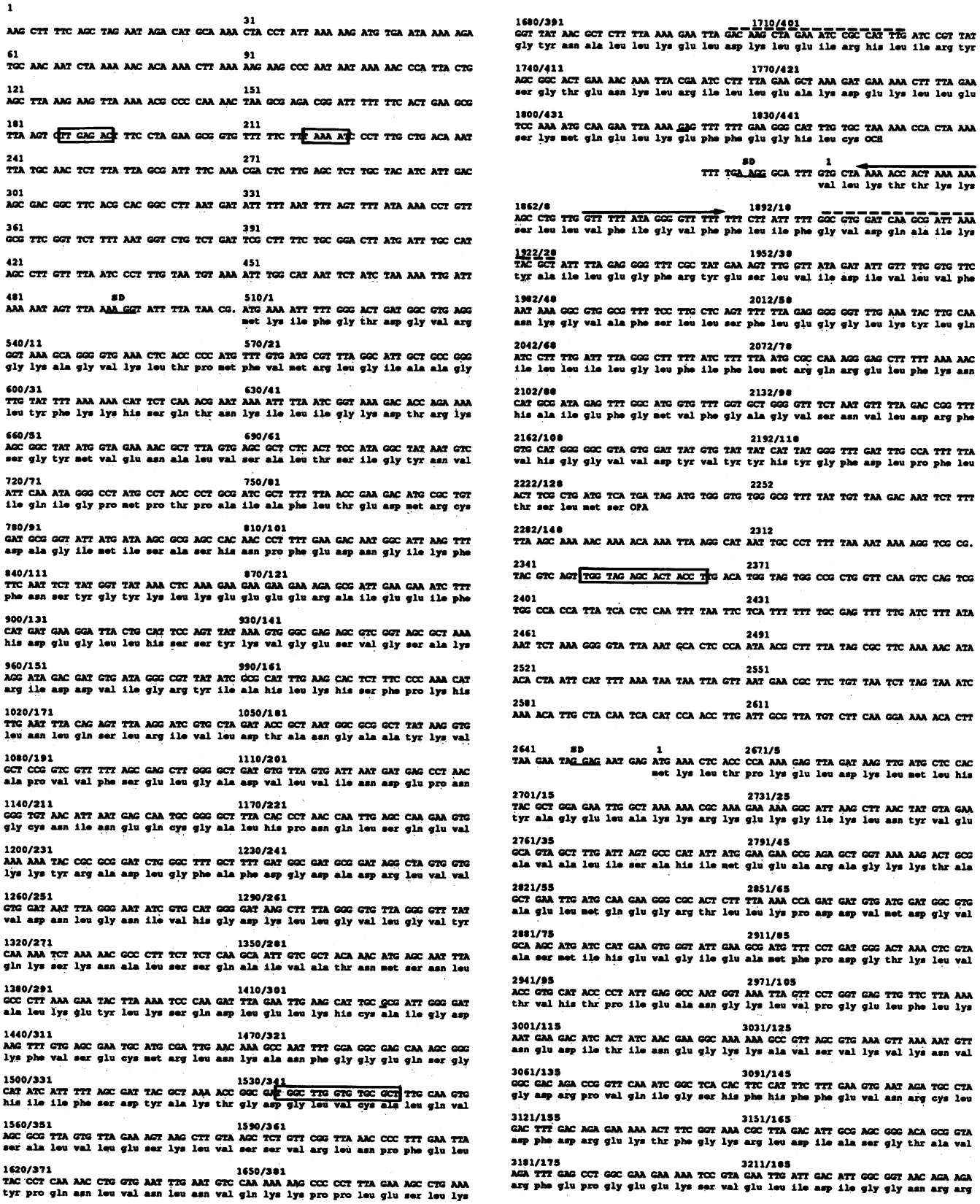

FIG. 4. Nucleotide sequence of the *H. pylori* urease genes. Numbers above the sequence indicate the nucleotide position. Predicted amino acid sequences, in sequential order, for UreC (bp 510 to 1844), UreD (bp 1841 to 2235), UreA (bp 2659 to 3372), and UreB (bp 3379 to 5085) are shown below the DNA 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.

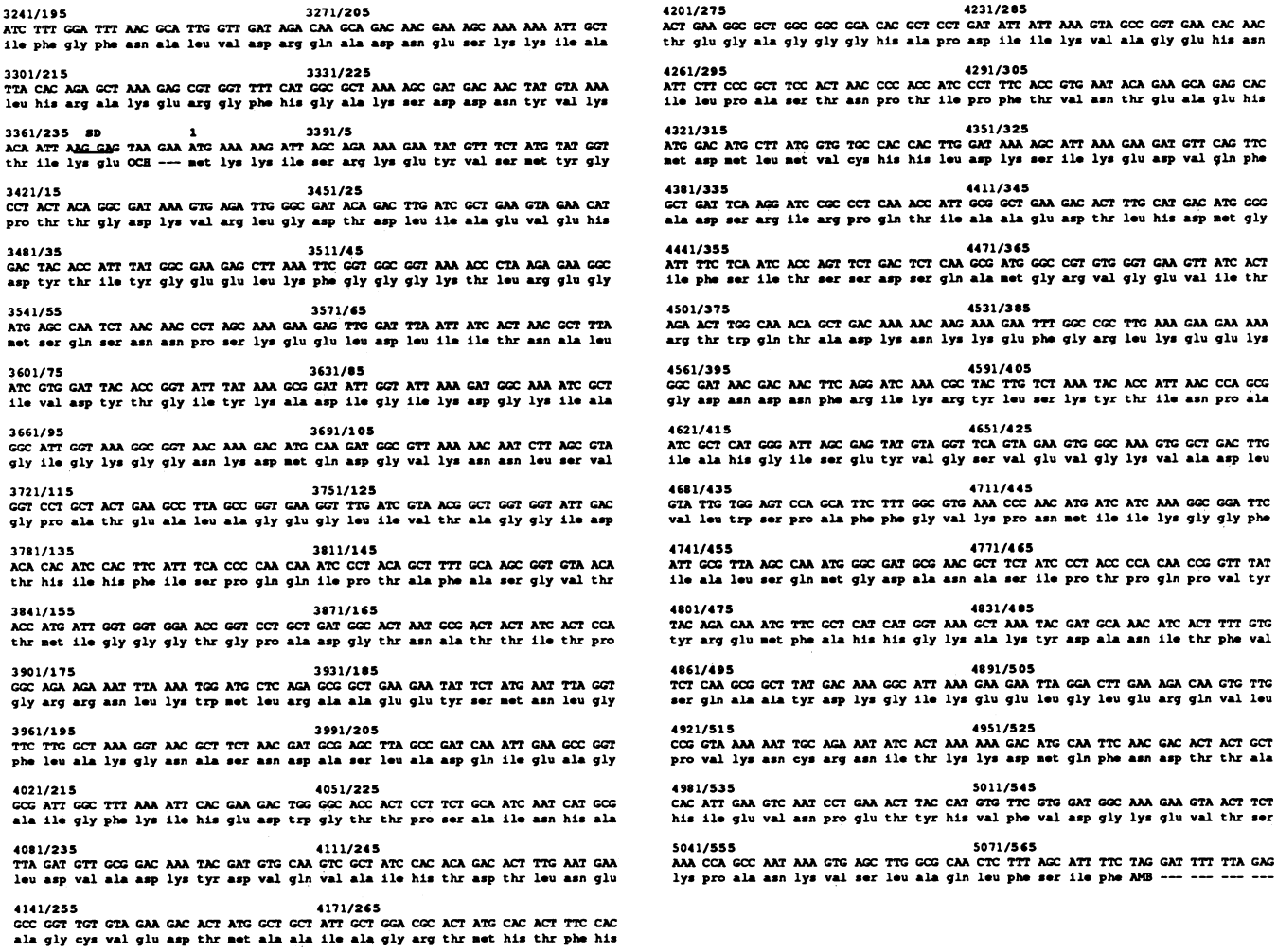


FIG. 4—*Continued.*

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-

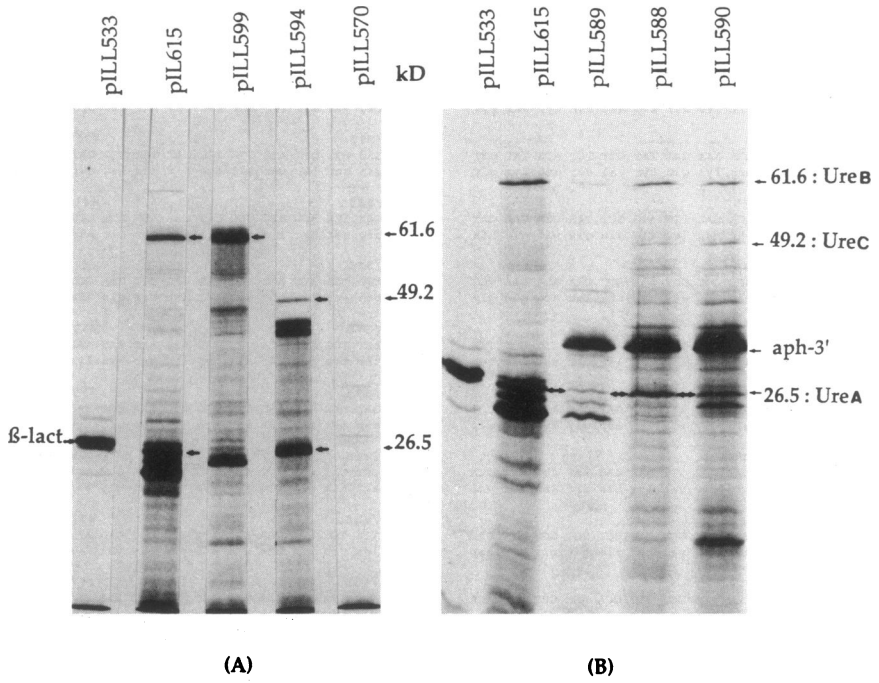


FIG. 5. Fluorographs of plasmid-mediated polypeptides expressed in minicells labeled with [$^{35}$S]methionine analyzed on 10% (A) or 12.5% (B) sodium dodecyl sulfate-polyacrylamide gels. (A) Polypeptides encoded by the pILL533 vector, by the pILL615 plasmid consisting of the *Eco*RI-*Pst*I insert of 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 *Hin*dIII-1 restriction site and the *Eco*RI 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 relevent 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 σ54), 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

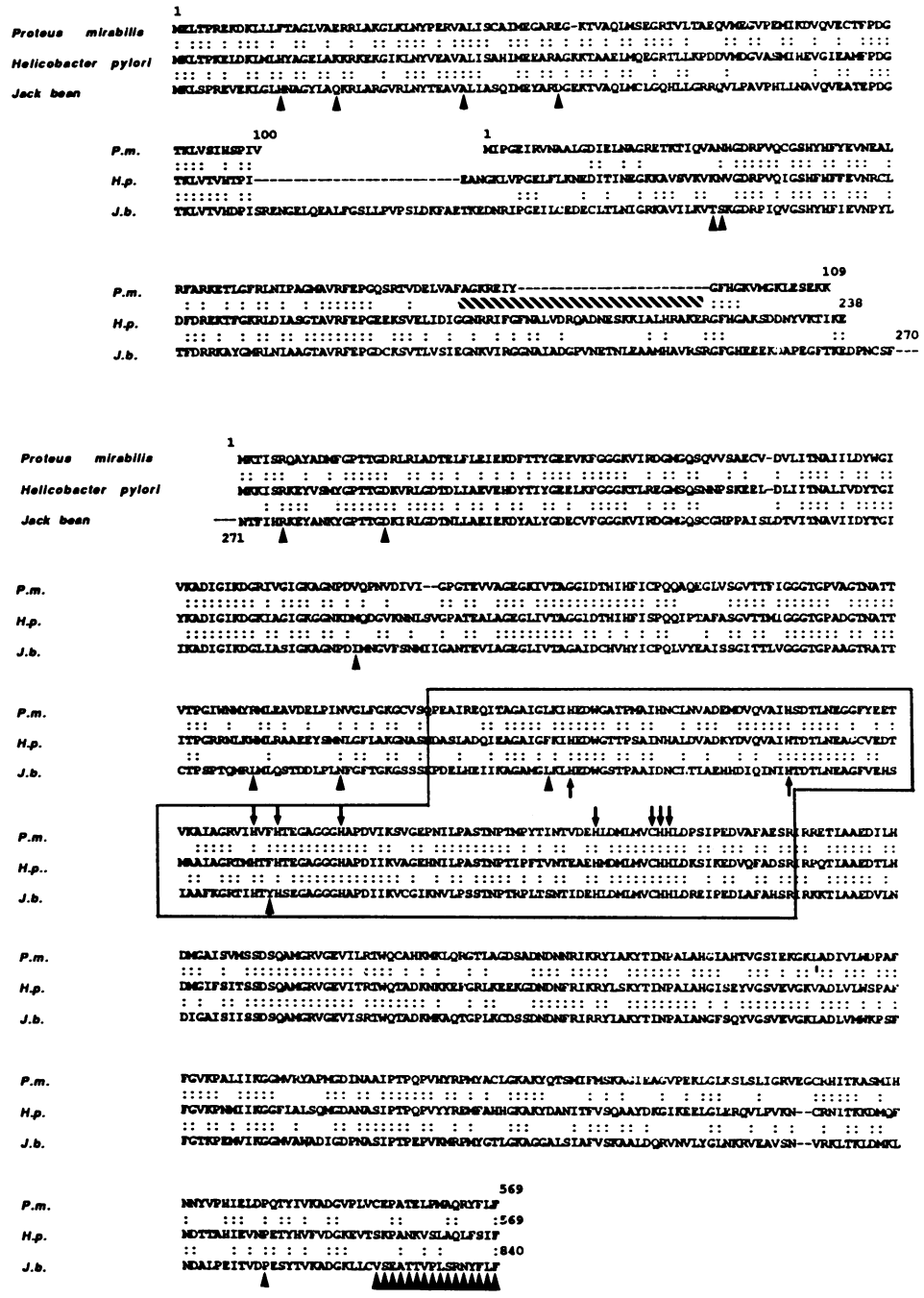


FIG. 6. Alignment of the first 270 amino acids of the jack bean urease (bottom line) with that of the two small subunits of *Proteus mirabilis* and the product of the *ureA* gene (top) and alignment of the last 550 amino acids of the jack bean urease with that of the large subunit of *P. mirabilis* and 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 shuttling 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.

## ACKNOWLEDGMENTS

We thank J. L. Fauchère for providing *H. pylori* 85P and M. Blaser for help with the writing as well as for critically reading the manuscript.

## REFERENCES

1. **Alder, H. I., D. Fisher, A. Cohen, and A. A. Hardlget.** 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. USA **57**:321–326.
2. **Blanchard, A.** 1990. *Ureaplasma urealyticum* urease genes; use of a UGA tryptophan codon. Mol. Microbiol. **4**:669–676.
3. **Boehm, D., R. Welch, and I. S. Snyder.** 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect. Immun. **58**:1959–1964.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
5. **Buck, G. E.** 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3**:1–12.
6. **Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali.** 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res. **18**:362.
7. **Clayton, C. L., B. W. Wren, P. Mullany, A. Topping, and S. Tabaqchali.** 1989. Molecular cloning and expression of *Campylobacter pylori* species-specific antigens in *Escherichia coli* K-12. Infect. Immun. **57**:623–629.
8. **Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser.** 1990. Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265**:9464–6469.
9. **Dunn, B. E., G. I. Perez-Perez, and M. Blaser.** 1989. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter pylori* proteins. Infect. Immun. **57**:1825–1833.
10. **Evans, D. G., D. J. Evans, Jr., and D. Y. Graham.** 1989. Receptor-mediated adherence of *Campylobacter pylori* to mouse Y-1 adrenal cell monolayers. Infect. Immun. **57**:2272–2278.
11. **Evans, D. J., D. G. Evans, K. E. Smith, and D. Y. Graham.** 1989. Serum antibody responses to the *N*-acetylneuraminyllactose-binding hemagglutinin of *Campylobacter pylori*. Infect. Immun. **57**:664–667.
12. **Evans, R. M., and S. M. Hollenberg.** 1988. Zinc fingers: gilt by association. Cell **52**:1–3.
13. **Feinberg, A., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
14. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
15. **Gill, R. E., F. Heffron, and S. Falkow.** 1979. Identification of the protein encoded by the transposable element Tn3 which is required for its transposition. Nature (London) **282**:797–801.

15a. **Guerrant, R. L., R. A. Pennie, L. J. Barrett, and A. O'Brien.** 1985. 3rd International Workshop on *Campylobacter* Infections, abstr. 093.

16. **Hazell, S., and A. Lee.** 1986. *Campylobacter pyloridis*, urease, hydrogen ion back diffusion, and gastric ulcers. Lancet **ii**:15–17.
17. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. **153**:658–663.
18. **Higgins, C. F. I. D. Hiles, K. Whalley, and D. J. Jamieson.** Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent trnasport systems. EMBO J. **4**:1033–1040.
19. **Hopp, T., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–382.
20. **Hu, L., and H. L. T. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. **58**:992–998.
21. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
22. **Hupertz, V., and S. Czinn.** 1988. Demonstration of a cytotoxin from *Campylobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. **7**:576–578.
23. **Jones, B. D., and H. L. T. Mobley.** 1989. *Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. J. Bacteriol. **171**:6414–6422.
24. **Labigne-Roussel, A., P. Courcoux, and L. Tompkins.** 1988. Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. J. Bacteriol. **170**:1704–1708.
25. **Labigne-Roussel, A., J. Harel, and L. Tompkins.** 1987. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. **169**:5320–5323.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
27. **Levi, S., K. Beardshall, G. Haddad, R. Flayford, P. Ghosh, and J. Calam.** 1989. *Campylobacter pylori* and duodenal ulcers: the gastrin link. Lancet **i**:1167–1168.
28. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1983. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Marshall, B. J., L. Barret, C. Prakesh, R. Mc Callum, and R. Guerrant.** 1988. Protection of *Campylobacter pyloridis* but not *Campylobacter jejuni* against acid susceptibility by urea, p. 402–403. *In* B. Kaijser and E. Falsen (ed.), Campylobacter IV. University of Göteborg, Göteborg, Sweden.
30. **Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren, and J. A. Armstrong.** 1984. Original isolation of *Campylobacter pyloridis* from human gastric mucosa. Microbios Lett. **25**:83–88.
31. **Meissing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19**:269–276.
32. **Mobley, H. L. T., and R. P. Hausinger.** 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. **53**:85–108.
33. **Morett, E., and M. Buck.** 1989. In vivo studies on the interaction of RNA polymerase-54 with the *Klebsiella pneumoniae* and *Rhizobium meliloti nifH* promoters. J. Mol. Biol. **210**:65–77.
34. **Mörsdorf, G., and H. Kaltwasser.** 1990. Cloning of the genes encoding urease from *Proteus vulgaris* and sequencing of the structural genes. FEMS Microbiol. Lett. **66**:67–74.
35. **Nakazawa, T., M. Ishibashi, H. Konishi, T. Takemoto, M. Shigeeda, and T. Kochiyama.** 1989. Hemagglutination activity of *Campylobacter pylori*. Infect. Immun. **57**:989–991.
36. **Neman-Simha, V., and F. Megraud.** 1988. In vitro model for *Campylobacter pylori* adherence properties. Infect. Immun. **56**:3329–3333.
37. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
38. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–359.
39. **Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik.**

1982. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**:1–22.

40. **Sanger, F., S., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
42. **Sarosiek, J., A. Slomiany, and B. L. Slomiany.** 1988. Evidence for weakening of gastric mucus integrity by *Campylobacter pylori*. Scand. J. Gastroenterol. **23**:585–590.
43. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
44. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**:784–791.
45. **Smoot, D. T., H. L. T. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. **58**:1992–1994.
46. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
47. **Takishima, K., T. Suga, and G. Mamiya.** 1988. The structure of jack bean urease. The complete amino acid sequence, limited proteolysis and reactive cysteine residues. Eur. J. Biochem. **175**:151–165.
48. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. The ATP operon nucleotide sequence of the genes for the gamma sub-unit, beta-subunit, epsilon-subunit of *Escherichia coli* ATP synthetase. EMBO J. **1**:945–951.
49. **Warren, J., and B. Marshal.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i**:1273–1275.
50. **Winship, P. R.** 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res. **17**:1266.