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Expression of *Helicobacter pylori* Urease Genes in *Escherichia coli* Grown under Nitrogen-Limiting Conditions

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***Helicobacter pylori* produces a potent urease that is believed to play a role in the pathogenesis of gastroduodenal diseases. Four genes (*ureA*, *ureB*, *ureC*, and *ureD*) were previously shown to be able to achieve a urease-positive phenotype when introduced into *Campylobacter jejuni*, whereas *Escherichia coli* cells harboring these genes did not express urease activity (A. Labigne, V. Cussac, and P. Courcoux, J. Bacteriol. 173:1920–1931, 1991). Results that demonstrate that *H. pylori* urease genes could be expressed in *E. coli* are presented in this article. This expression was found to be dependent on the presence of accessory urease genes hitherto undescribed. Subcloning of the recombinant cosmid pILL585, followed by restriction analyses, resulted in the cloning of an 11.2-kb fragment (pILL753) which allowed the detection of urease activity (0.83 ± 0.39 μmol of urea hydrolyzed per min/mg of protein) in *E. coli* cells grown under nitrogen-limiting conditions. Transposon mutagenesis of pILL753 with mini-Tn*3*-Km permitted the identification of a 3.3-kb DNA region that, in addition to the 4.2-kb region previously identified, was essential for urease activity in *E. coli*. Sequencing of the 3.3-kb DNA fragment revealed the presence of five open reading frames encoding polypeptides with predicted molecular weights of 20,701 (UreE), 28,530 (UreF), 21,744 (UreG), 29,650 (UreH), and 19,819 (UreI). Of the nine urease genes identified, *ureA*, *ureB*, *ureF*, *ureG*, and *ureH* were shown to be required for urease expression in *E. coli*, as mutations in each of these genes led to negative phenotypes. The *ureC*, *ureD*, and *ureI* genes are not essential for urease expression in *E. coli*, although they belong to the urease gene cluster. The predicted UreE and UreG polypeptides exhibit some degree of similarity with the respective polypeptides encoded by the accessory genes of the *Klebsiella aerogenes* urease operon (33 and 92% similarity, respectively, taking into account conservative amino acid changes), whereas this homology was restricted to a domain of the UreF polypeptide (44% similarity for the last 73 amino acids of the *K. aerogenes* UreF polypeptide). With the exception of the two UreA and UreB structural polypeptides of the enzyme, no role can as yet be assigned to the nine proteins encoded by the *H. pylori* urease gene cluster.**

In 1983, Warren and Marshall (35) first described a spiral, gram-negative, microaerophilic bacterium they called *Campylobacter pyloridis* (20). Recently renamed *Helicobacter pylori* (12), this bacterium has a natural predilection for the gastric epithelium of humans. *H. pylori* is now recognized as the etiologic agent of antral chronic gastritis (2) and is a probable contributor to the pathogenesis of peptic ulcer disease (2). This bacterium is characterized by the production of a potent urease (7, 22) which has been described as a common trait of all bacteria belonging to the *Helicobacter* genus.

Like most bacteria, *H. pylori* is sensitive to acid pH; however, it can tolerate acidity when physiological levels of urea are present (20). By hydrolyzing urea to carbon dioxide and ammonia, which are released into the microenvironment of the bacterium, *H. pylori* urease is thought to allow survival of the bacterium in the acidic environment of the stomach. Recently, animal model studies have provided evidence that suggests that urease is an important factor in the colonization of the gastric mucosa (5, 8). In addition to this role, urease is also suspected to either directly or indirectly cause damage to the gastric mucosa. It has been hypothesized that the accumulation of high concentrations of ammonia at the tissue surface is responsible for the back diffusion of hydrogen ions, thus increasing acidity at the tissue level (13). Alternatively, ammonia could be directly toxic to intercellular tight junctions and might alter mucosal integrity. A recent study demonstrated that the cytotoxic effects of *H. pylori* on cultured human gastric epithelial cells could be potentiated by the presence of ammonia (4, 33).

To ultimately clarify the role of urease in the pathogenic process, we cloned the genes responsible for urease activity in *H. pylori* (16). The use of a shuttle cosmid cloning approach allowed replication and movement of a cloned portion of *H. pylori* chromosomal DNA. Whereas the presence of this fragment was not associated with urease activity when introduced into *Escherichia coli*, it permitted biosynthesis of urease when transferred by conjugation to *Campylobacter jejuni*. By using this approach, the urease genes were localized to a 34-kb portion of the *H. pylori* chromosome (the pILL585 recombinant cosmid) and were mapped to a 4.2-kb region. On the basis of sequencing data, four open reading frames (ORFs) were found, of which two (*ureA* and *ureB*) encoded the two structural subunits of the urease enzyme. Sequences highly homologous with the consensus sequence of the nitrogen regulation site (binding site for factor σ54) were found 310 bp upstream of the *ureA*, *ureB*, and *ureD* genes (16). This sigma factor has been found to be required for the expression of a variety of genes, in particular those whose products function in the assimilation of nitrogen (24).

Growth conditions that resulted in detectable urease activity in *E. coli* host strains harboring the recombinant cosmid pILL585 were subsequently defined. After subcloning and mutagenesis studies, five additional *H. pylori* urease

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TABLE 1. Vectors and hybrid plasmids used in this study

Plasmid	Vector	Phenotypic characteristics[a]	Size (kb)	Origin of the insert	Source or reference
None	pILL550	RepEc RepCj Mob Km	8.3		17
None	pILL570	RepEc Mob Sp	5.3		16
None	pILL575	RepEc RepCj Mob Km Cos	10		16
pILL585	pILL575	RepEc RepCj Mob Km Cos	44	*Sau*3A partial digest of 85P	16
pILL590	pILL550	RepEc RepCj Mob Km	16.4	*Sau*3A partial digest of pILL585	16
pILL753	pILL570	RepEc Mob Sp	16.5	*Sau*3A partial digest of pILL585	This study
pILL763	pILL570	RepEc Mob Sp	14.75	*Cla*I-*Pst*I fragment of pILL753::1	This study
pILL768	pILL570	RepEc Mob Sp	15.35	*Eco*RI fragment of pILL753::16	This study

[a] Abbreviations: RepEc and RepCj, plasmid capable of replicating in *E. coli* and *C. jejuni* cells, respectively; Mob, conjugative plasmid due to the presence of OriT; Km and Sp, resistance to kanamycin and spectinomycin, respectively; Cos, presence of lambda cos site.

genes were identified and sequenced. The results presented here demonstrate, for the first time, the functional expression of *H. pylori* genes in *E. coli*. The findings provide new information relevant to the understanding of the expression and regulation of the *H. pylori* urease genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *H. pylori* 85P was isolated from a patient with gastritis (16). *E. coli* MC1061 (18) was used for the cloning procedure, whereas *E. coli* HB101 (1) was used as a host for quantitative analyses of urease expression. Vectors and hybrids used in this study are listed in Table 1. *E. coli* strains were grown in L broth without glucose (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl [each per liter], pH 7.0) or on L agar plates (containing 1.5% agar) at 37°C. Antibiotic concentrations for the selection of transformants were as follows (in milligrams per liter): kanamycin, 20; tetracycline, 8; ampicillin, 100; spectinomycin, 100; carbenicillin, 100. Nitrogen-limiting medium consisted of ammonium-free M9 minimal agar medium (pH 7.4) containing 0.4% D-glucose as the carbon source and 0.2% freshly prepared filter-sterilized L-glutamine (29). For the optimization of urease activity in *E. coli*, the M9 minimal medium was supplemented with various nitrogen sources, each at a final concentration of 10 mM.

Molecular cloning and DNA analyses. Restriction endonuclease digestions and other common DNA manipulations were performed by standard procedures according to the methods of Maniatis et al. (18) or as previously described (16).

Urease activity. Qualitative detection of urease activity was achieved as previously described (16). Urease activity was quantitated by the Berthelot reaction by using a modification of the procedure already described (8). Briefly, bacteria were harvested from agar plates in 2.0 ml of sterile 0.85% NaCl and centrifuged at 11,000 × *g* for 10 min at 4°C. The pellets were washed twice in 0.85% NaCl and resuspended in PEB (100 mM sodium phosphate buffer [pH 7.4] containing 10 mM EDTA). To prepare sonicated extracts, cells were disrupted by four 30-s bursts by using a Branson Sonifier model 450 set at 30 W for a 50% cycle. Cell debris was removed prior to urease determinations. Freshly prepared samples (10 to 50 μl) were added to 200 μl of urea substrate solution (50 mM urea prepared in PEB) and reacted at room temperature for up to 30 min. The reactions were terminated by the addition of 400 μl of phenol-nitroprusside reagent and 400 μl of alkaline hypochlorite reagent. The reaction mixture was incubated at 50°C. Reaction mixture blanks, in which urease activity was inactivated by boiling for 5 min prior to addition of substrate, were treated in a similar manner. The quantity of ammonia liberated was determined from a standard curve correlating the A_{625} to the ammonium concentration (from NH_4Cl). The release of 2 μmol of ammonia was considered equivalent to the hydrolysis of 1 μmol of urea. Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of bacterial protein.

Protein determination. Protein concentrations were estimated with a commercial version of the Bradford assay (Sigma Chemicals).

Transposon mutagenesis. The mini-Tn*3*-Km delivery system, as previously described (15), was used to generate random insertional mutations within the DNA fragment cloned into pILL570. This procedure involved (i) the transformation of *E. coli* HB101, containing the transposase-encoding plasmid (pTCA, Tc^r), with the pILL570 derivative plasmid to be mutagenized; (ii) transfer of the pOX38::mini-Tn*3*-Km plasmid into that strain; and then (iii) the transfer of cointegrates into a lambda *cre* recipient strain (Rif^r), with selection of resolved structures by high concentrations of kanamycin (500 mg/liter) and spectinomycin (300 mg/liter) (15).

DNA sequencing. Sequencing of single-stranded DNA (21, 30) was performed by the dideoxynucleotide chain termination method (31), by using the Sequenase kit (U.S. Biochemical Corp.) as previously described (16). To sequence double-stranded DNA, samples (3 μg) of plasmid DNA purified on a CsCl gradient (36) were first denatured with a solution of 1 M NaOH (total volume, 20 μl) and neutralized with 2 μl of 2 M ammonium acetate (pH 4.6). Sixty microliters of 100% ice-cold ethanol was then added to the samples, and the DNA was precipitated at −70°C for 10 min and recovered by centrifugation. The pellet was washed with 60 μl of 80% ice-cold ethanol, resuspended in 10 μl of sequencing buffer containing 0.5 pmol of the primer, and incubated for 3 min at 65°C. Samples were incubated at room temperature for 30 min prior to being sequenced.

Nucleotide sequence accession number. The nucleotide sequence accession number for the sequence shown in Fig. 3 is M84338.

RESULTS

Detection of urease activity in *E. coli* harboring the pILL585 recombinant cosmid. *E. coli* transformants harboring the pILL585 cosmid were plated on either glucose-containing M9 minimal medium supplemented with 0.2% L-glutamine (as the sole nitrogen source) or L medium and were incu-

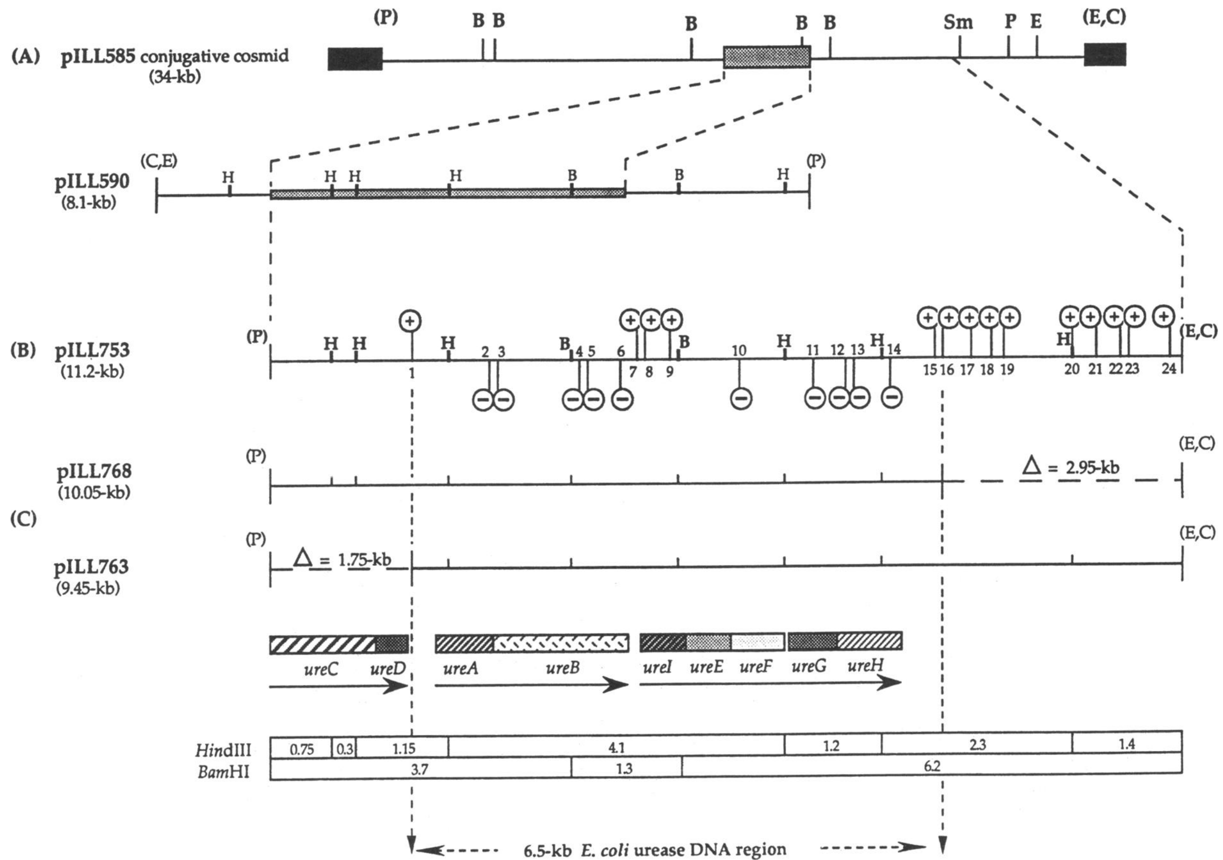

FIG. 1. Subcloning and transposon mutagenesis of pILL753. (A) Linear restriction maps of the pILL585 hybrid cosmid and the pILL590 plasmid (16). The shaded boxes represent the DNA fragment required for urease expression in *C. jejuni*. (B) Random insertion of mini-Tn*3*-Km transposon (15). The numbers (1 to 24) as well as circles correspond to the sites of insertion of the transposon into pILL753; plus signs indicate that the transposon did not inactivate urease expression, whereas negative signs indicate that urease expression was abolished. (C) Linear restriction maps of the pILL763 and pILL768 hybrid plasmids generated by deletions (Δ) within pILL753. The locations of the genes (*ureA* to *ureH*) are indicated by bars. The lengths of the bars correspond to the lengths of DNA required to encode the polypeptides. The arrows refer to the orientation of transcription. The numbers in the boxes at the bottom of the figure indicate the sizes (in kilobases) of the restriction fragments. Numbers in parentheses correspond to the sizes of the *H. pylori* DNA fragments inserted into one of the cloning vectors (pILL575 [16], pILL550 [17], or pILL570 [16]). Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; C, *Cla*I; Sm, *Sma*I. Letters in parentheses indicate that the restriction sites belong to the vector.

bated at 37°C for 48 h. The transformants were then screened for urease activity by using a qualitative colorimetric assay, which was carried out with urea-indole medium. Activity was observed only for *E. coli* HB101 transformants subcultured several times (more than five passages) on minimal medium at 37°C under aerobic conditions. Henceforth, these were the conditions used to screen for urease expression in *E. coli* clones. No urease activity was detected for transformants grown on the nitrogen-rich medium.

Transformation of *E. coli* HB101 with the pILL590 plasmid, containing a 4.2-kb fragment identified as the minimal region necessary for urease expression in *C. jejuni* (16), did not result in a urease-positive phenotype in *E. coli* cells, even after subculturing the bacteria on nitrogen-limiting media. This indicated that genes present on the cosmid, but absent from the pILL590 plasmid, were required for urease expression in this host.

Subcloning of the DNA fragment required for urease activity in *E. coli*. In the absence of detectable urease activity in the *E. coli* strain harboring the pILL590 recombinant plasmid, the 34-kb insert of the pILL585 cosmid was partially digested with the endonuclease *Sau*3A so as to generate fragments ranging from 7 to 12 kb. These were treated with alkaline phosphatase to prevent any rearrangement of the initial genome and ligated with the *Bam*HI-linearized pILL570 plasmid. After transformation in *E. coli* HB101, each spectinomycin-resistant transformant was subsequently tested for the capacity to hydrolyze urea. A clone that exhibited a urease-positive phenotype was found to harbor a recombinant plasmid containing an 11.2-kb insert. This plasmid was designated pILL753. The *Bam*HI and *Hin*dIII recognition sites were mapped with respect to the unique *Eco*RI and *Pst*I restriction sites of the pILL570 vector (Fig. 1). Comparison of the pILL753 plasmid restriction map with that of the previously described recombinant plasmid pILL590 demonstrated that the insert in pILL753 consisted of an additional 4.6-kb DNA fragment located downstream of the four urease genes *ureC*, *ureD*, *ureA*, and *ureB*.

Optimization of urease activity in *E. coli*. To define the culture conditions giving optimal expression of *H. pylori* urease genes in *E. coli*, the activity of cells harboring

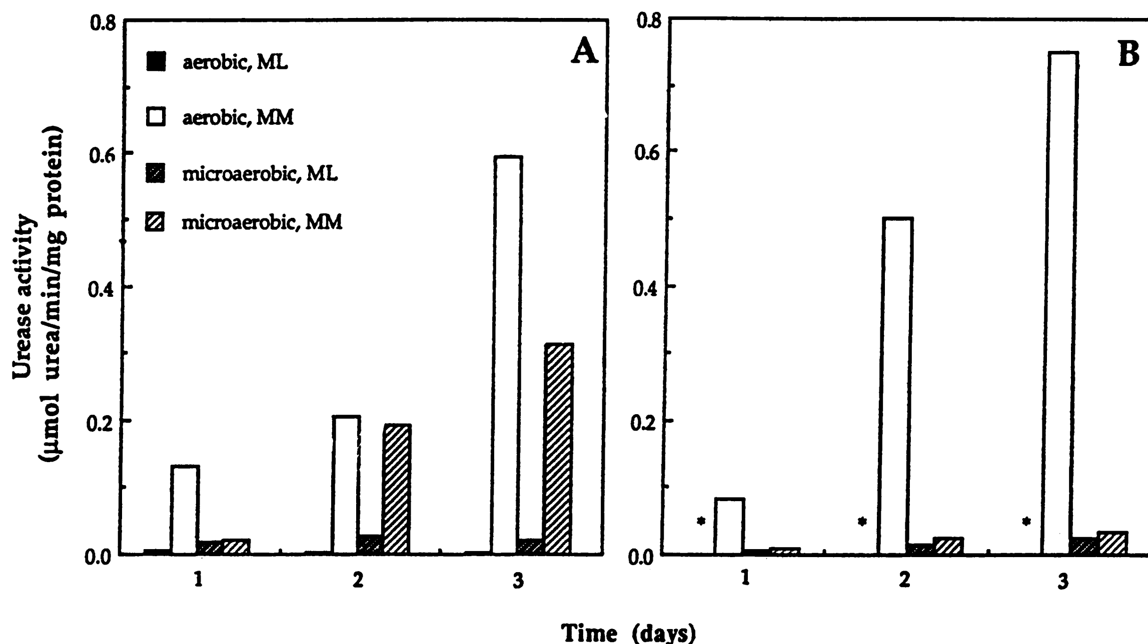

FIG. 2. Urease activity expressed over time by *E. coli* HB101 cells harboring pILL753. Plates prepared from either L agar medium (ML) or M9 minimal medium supplemented with 10 mM L-arginine (MM) were each inoculated with a 100-μl aliquot of culture suspended (10^8 bacteria per ml) in sterile 0.85% NaCl. The plates were incubated, aerobically or microaerobically, at 30°C (A) or 37°C (B), and activity was measured at the appropriate times. Asterisks indicate that no urease activity was detected.

pILL753 was quantitated after growth on solid nitrogen-limiting medium supplemented with various nitrogen sources. The relative activities of cultures on medium supplemented with L-arginine, NH_4Cl, L-glutamine, urea, and L-glutamate were 100, 46, 36, 20, and 2.7%, respectively. The finding of optimal activity for cultures grown on L-arginine as the source of nitrogen appears to be consistent with urease expression in *Klebsiella aerogenes* (9, 28). Urease activity was not detected in cultures grown on nitrogen-rich medium.

Time course analysis of urease expression in *E. coli* cells harboring pILL753, grown under various culture conditions, indicated that maximal urease activity occurred after 3 days of aerobic growth at 37°C on solid minimal medium supplemented with L-arginine (Fig. 2). The urease activity of cultures grown on nitrogen-rich medium was greatest when microaerobic conditions were employed. Conversely, microaerobic conditions had a repressive effect on the activities of nitrogen-limited cultures.

The urease activity of *E. coli* cells harboring pILL753, grown aerobically for 3 days at 37°C on minimal medium supplemented with arginine, was 0.83 ± 0.39 μmol of urea hydrolyzed min^{-1} mg of $protein^{-1}$. By comparison, the *H. pylori* isolate used to clone the urease genes hydrolyzed urea at a rate of 23.2 ± 2.3 μmol min^{-1} mg of $protein^{-1}$.

Identification and location of the genes required for urease activity in *E. coli*. To determine the DNA region required for the expression of a urease-positive phenotype, pILL753 derivatives carrying the mini-Tn*3*-Km transposable element were first isolated and *E. coli* HB101 transformants bearing the transposons were then screened for urease activity. These were designated pILL753::x, where x refers to the site of mini-Tn*3*-Km insertion as mapped on Fig. 1. Of 24 insertions selected for analysis, 10 derivatives (2 to 6 and 10 to 14) had lost the capacity to hydrolyze urea. In contrast, the remaining 14 still expressed the urease-positive phenotype. These results confirmed that insertional mutations mapping to the *ureA* or *ureB* gene (i.e., mutants 2 to 6) were deleterious to the urease activity. It was also demonstrated that a 2.6-kb DNA fragment located further downstream of the *ureB* gene was necessary for the expression of a urease-positive phenotype in *E. coli* cells grown under nitrogen-limiting conditions. A 600-bp DNA fragment located immediately downstream of the *ureB* gene did not appear to be essential for the expression of urease activity in *E. coli*.

Additional analyses, including the generation of deletions

TABLE 2. Mutagenesis of *E. coli* clones and effect on urease activity

Plasmid	Urease activity[a] (μmol of urea min^{-1} mg^{-1})	Location of disruption or deletion
pILL753	0.83 ± 0.39	
pILL753::3	Neg[b]	*ureA*
pILL753::6	Neg	*ureB*
pILL753::8	1.1 ± 0.23	*ureI*
pILL753::10	Neg	*ureF*
pILL753::11	Neg	*ureG*
pILL753::13	Neg	*ureH*
pILL753::16	0.66 ± 0.11	Downstream of *ureH*
pILL763	2.14 ± 0.16	*ureC* and *ureD*
pILL768	0.57 ± 0.28	3' end of 11.2-kb insert

[a] Activities of bacteria grown aerobically for 3 days at 37°C on solid M9 minimal medium supplemented with 10 mM L-arginine. The values represent the means ± standard deviations calculated from at least three determinations.

[b] No activity detected (limit of detection was <1 nmol of urea min^{-1} mg of $protein^{-1}$.

1 31
A CTC TTT AGC ATT TTC TAG GA TTT TTT AGG AGC AAC GCT CTT AGA TCC TTA GTT TTT AGC
—leu phe ser ile phe AMB

61 91
TCT CTG ATT TTT TGT TTA TCA AAA AAT TGG GGG CTT TTT TTG TTT TTA TTT TTT GTC AAT

121 151
TTA CTA TTT TTC TTT ATG ATT AGC TCA AGC AAC AAA AGT TAT TCG TAA GGT GCG TTT GTT

181 SD 211
GTA AAA ATT TTT GTT TGG AAG GAA AAG GCA ATG CTA GGA CTT GTA TTG TTA TAT GTT GGG
ureI Met leu gly leu val leu leu tyr val gly

241 271
ATT GTT TTA ATC AGC AAT GGG ATT TGC GGG TTA ACC AAA GTC GAT CCT AAA AGC ACT GCG
ile val leu ile ser asn gly ile cys gly leu thr lys val asp pro lys ser thr ala

301 331
GTG ATG AAC TTT TTT GTG GGT GGG CTC TCC ATT ATT TGT AAT GTG GTT GTC ATC ACT TAT
val met asn phe phe val gly gly leu ser ile ile cys asn val val val ile thr tyr

361 391
TCC GCT CTC AAC CCT ACA GCC CCT GTA GAA GGT GCT GAA GAT ATT GCT CAA GTA TCA CAC
ser ala leu asn pro thr ala pro val glu gly ala glu asp ile ala gln val ser his

421 451
CAT TTG ACT AAT TTC TAT GGG CCA GCG ACT GGG TTA TTG TTT GGT TTC ACC TAC TTG TAT
his leu thr asn phe tyr gly pro ala thr gly leu leu phe gly phe thr tyr leu tyr

481 511
GCG GCT ATC AAC CAC ACT TTT GGT TTG GAT TGG AGG CCC TAC TCT TGG TAT AGC TTA TTC
ala ala ile asn his thr phe gly leu asp trp arg pro tyr ser trp tyr ser leu phe

541 571
GTA GCG ATC AAC ACG ATT CCT GCT GCG ATT TTA TCC CAC TAT AGC GAT ATG CTT GAT GAC
val ala ile asn thr ile pro ala ala ile leu ser his tyr ser asp met leu asp asp

601 631
CAC AAA GTG TTA GGC ATC ACT GAA GGC GAT TGG TGG GCG ATC ATT TGG TTG GCT TGG GGT
his lys val leu gly ile thr glu gly asp trp trp ala ile ile trp leu ala trp gly

661 691
GTT TTG TGG CTT ACC GCT TTC ATT GAA AAC ATC TTG AAA ATC CCT TTA GGG AAA TTC ACT
val leu trp leu thr ala phe ile glu asn ile leu lys ile pro leu gly lys phe thr

721 751
CCA TGG CTT GCT ATC ATT GAG GGC ATT TTA ACC GCT TGG ATC CCT GCT TGG TTA CTC TTT
pro trp leu ala ile ile glu gly ile leu thr ala trp ile pro ala trp leu leu phe

781 811
ATC CAA CAC TGG GTG TGA GAT GAT CAT
ile gln his trp val OPA

782 812
TCC AAC ACT GGG TGT GAG ATG ATC ATA GAG CGT TTA ATA GGC AAT CTA AGG GAT TTA AAC
ureE Met ile ile glu arg leu ile gly asn leu arg asp leu asn

842 871
CCC TTG GAT TTC AGC GTG GAT TAT GTG GAT TTG GAA TGG TTT GAA ACG AGG AAA AAA ATC
pro leu asp phe ser val asp tyr val asp leu glu trp phe glu thr arg lys lys ile

902 932
GCT CGC TTT AAA ACC AGG CAA GGC AAA GAC ATA GCC GTA CGC CTT AAA GAC GCT CCC AAG
ala arg phe lys thr arg gln gly lys asp ile ala val arg leu lys asp ala pro lys

962 992
TTG GGT TTC TCT CAA GGA GAT ATT TTA TTT AAA GAA GAG AAG GAA ATT ATC GCC GTT AAT
leu gly phe ser gln gly asp ile leu phe lys glu glu lys glu ile ile ala val asn

1022 1052
ATC TTG GAT TCT GAA GTC ATT CAC ATC CAA GCT AAG AGC GTG GCA GAA GTA GCG AAA ATA
ile leu asp ser glu val ile his ile gln ala lys ser val ala glu val ala lys ile

1082 1112
TGC TAT GAA ATA GGA AAC CGC CAT GCG GCT TTA TAC TAT GGC GAG TCT CAA TTT GAA TTT
cys tyr glu ile gly asn arg his ala ala leu tyr tyr gly glu ser gln phe glu phe

1142 1172
AAA ACA CCA TTT GAA AAG CCC ACG CTA GCG TTA CTA GAA AAG CTA GGG GTT CAA AAT CGT
lys thr pro phe glu lys pro thr leu ala leu leu glu lys leu gly val gln asn arg

1202 1232
GTT TTA AGT TCA AAA TTG GAT TCC AAA GAA CGC TTA ACC GTG AGC ATG CCC CAT AGT GAG
val leu ser ser lys leu asp ser lys glu arg leu thr val ser met pro his ser glu

1262 1292 SD
CCT AAT TTT AAG GTC TCA CTG GCG AGC GAT TTT AAA GTG GTC ATG AAA TAG AAA AAC AA
pro asn phe lys val ser leu ala ser asp phe lys val val met lys AMB

1321 1351
CAA ATG GAT AAA GGA AAA AGC GTG AAA AGC ATT GAA AAA AGC GTG GGT ATG CTC CCA AAA
F Met asp lys gly lys ser val lys ser ile glu lys ser val gly met leu pro lys

1381 1411
ACT CCA AAG ACA GAC AGC AAT GCT CAT GTG GAT AAT GAA TTT CTG ATT CTG CAA GTC AAT
thr pro lys thr asp ser asn ala his val asp asn glu phe leu ile leu gln val asn

1441 1471
GAT GCG GTG TTC CCC ATT GGA TCT TAC ACG CAT TCT TTT GGG CTT TTG GCT AGA AAC TTA
asp ala val phe pro ile gly ser tyr thr his ser phe gly leu leu ala arg asn leu

1501 1531
CAT CCA GCA AAA AAG GTT ACT AAT AAA GAA AGC GCT TTA AAA TAT TTA AAA GCC AAT CTC
his pro ala lys lys val thr asn lys glu ser ala leu lys tyr leu lys ala asn leu

1561 1591
TCT AGC CAG TTC CTT TAC ACG GAA ATG CTG AGC TTG AAA CTC ACC TAT GAA AGC GCT CTC
ser ser gln phe leu tyr thr glu met leu ser leu lys leu thr tyr glu ser ala leu

1621 1651
CAA CAA GAT TTA AAA AGG ATC TTA GGG GTT GAA GAA ATC ATT ACG CTA TCC ACA AGC CCC
gln gln asp leu lys arg ile leu gly val glu glu ile ile thr leu ser thr ser pro

1681 1711
ATG GAA TTG CGA TTA GCC AAT CAA AAG CTA GGC AAT CGT TTC ATT AAA ACC TTA CAA GCC
met glu leu arg leu ala asn gln lys leu gly asn arg phe ile lys thr leu gln ala

1741 1771
ATG AAC GAA TTA GAC ATT GGC GCA TTT TTT AAC GCT TAC GCT CAA CAA ACC GAA GAC CCC
met asn glu leu asp ile gly ala phe phe asn ala tyr ala gln gln thr glu asp pro

1801 1831
ACC CAT GCC ACT AGC TAT GGC GTT TTT GCG GCG AGT TTG GGG ATT GAA TTG AAA AAG GCT
thr his ala thr ser tyr gly val phe ala ala ser leu gly ile glu leu lys lys ala

1861 1891
TTA AGG CAT TAT CTT TAT GCA CAA ACT TCT AAC ATG GTA ATT AAC TGC GTT AAA AGC GTC
leu arg his tyr leu tyr ala gln thr ser asn met val ile asn cys val lys ser val

1921 1951
CCA CTA TCT CAA AAC GAT GGG CAA AAA ATC TTA TTG AGC TTG CAA AGC CCT TTT AAC CAG
pro leu ser gln asn asp gly gln lys ile leu leu ser leu gln ser pro phe asn gln

1981 2011
CTC ATA GAA AAA ACC CTA GAA CTA GAC GAA AGC CAC TTG TGC GCG GCA AGC GTT CAA AAC
leu ile glu lys thr leu glu leu asp glu ser his leu cys ala ala ser val gln asn

2041 2071
GAC ATT AAG GCG ATG CAG CAT GAG AGT TTA TAC TCG CGC CTT TAT ATG TCT TGA ATT TTA
asp ile lys ala met gln his glu ser leu tyr ser arg leu tyr met ser OPA

2102 SD 2132
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ureG Met val lys ile gly val cys gly pro val gly ser gly

2162 2192
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lys thr ala leu ile glu ala leu thr arg his met ser lys asp tyr asp met ala val

2222 2252
ATC ACT AAT GAT ATT TAC ACG AAA GAA GAC GCA GAA TTT ATG TGT AAA AAT TCG GTG ATG
ile thr asn asp ile tyr thr lys glu asp ala glu phe met cys lys asn ser val met

2282 2312
CCA CGA GAG AGG ATC ATT GGC GTA GAA ACA GGA GGC TGT CCG CAC ACG GCT ATT AGA GAA
pro arg glu arg ile ile gly val glu thr gly gly cys pro his thr ala ile arg glu

2342 2372
GAC GCT TCT ATG AAT TTA GAA GCC GTA GAA GAA ATG CAT GGC CGT TTC CCT AAT TTG GAA
asp ala ser met asn leu glu ala val glu glu met his gly arg phe pro asn leu glu

2402 2432
TTG CTT TTG ATT GAA AGC GGA GGC AGT AAC CTT TCA GCG ACT TTC AAC CCA GAG CTA GCG
leu leu leu ile glu ser gly gly ser asn leu ser ala thr phe asn pro glu leu ala

2462 2492
GAC TTT ACG ATC TTT GTG ATT GAT GTG GCT GAG GGC GAT AAA ATC CCC AGA AAA GGC GGG
asp phe thr ile phe val ile asp val ala glu gly asp lys ile pro arg lys gly gly

2522 2552
CCA GGA ATC ACG CGT TCA GAC TTG CTT GTC ATC AAT AAG ATT GAT TTA GCC CCC TAT GTG
pro gly ile thr arg ser asp leu leu val ile asn lys ile asp leu ala pro tyr val

2582 2612
GGA GCC GAC TTG AAA GTC ATG GAA AGG GAT TCT AAA AAA ATC GCG GCG AAA AGC CCT TTA
gly ala asp leu lys val met glu arg asp ser lys lys ile ala ala lys ser pro leu

2642 2672
TTT TTA CCG AAT ATC CGC GCT AAA GAA GGT TTA GAC GAT GTG ATC GCT TGG ATC AAG CGC
phe leu pro asn ile arg ala lys glu gly leu asp asp val ile ala trp ile lys arg

2702
AAC GCT TTA TTG GAA GAT TGA TGA ACA CTT
asn ala leu leu glu asp OPA

2701 SD 2731
CAA CGC TTT ATT GGA AGA TTG ATG AAC ACT TAC GCT CAA GAA TCC AAG CTC AGG TTA AAA
ureH Met asn thr tyr ala gln glu ser lys leu arg leu lys

2761 2791
ACC AAA ATA GGG GCT GAC GGG CGG TGC GTG ATT GAA GAC AAT TTT TTC ACG CCC CCC TTT
thr lys ile gly ala asp gly arg cys val ile glu asp asn phe phe thr pro pro phe

2821 2851
AAG CTC ATG GCG CCC TTT TAC CCT AAA GAC GAT TTA GCG GAA ATC ATG CTT TTA GCG GTA
lys leu met ala pro phe tyr pro lys asp asp leu ala glu ile met leu leu ala val

2881 2911
AGC CCT GGC TTA ATG AAA GGC GAT GCA CAA GAT GTG CAA TTG AAC ATC GGT CCA AAT TGC
ser pro gly leu met lys gly asp ala gln asp val gln leu asn ile gly pro asn cys

2941 2971
AAG TTA AGG ATC ACT TCG CAA TCC TTT GAA AAA ATC CAT AAC ACT GAA GAC GGG TTT GCT
lys leu arg ile thr ser gln ser phe glu lys ile his asn thr glu asp gly phe ala

3001 3031
AGC AGA GAC ATG CAT ATC GTT GTG GGG GAA AAC GCT TTT TTA GAC TTC GCG CCC TTC CCG
ser arg asp met his ile val val gly glu asn ala phe leu asp phe ala pro phe pro

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3061                                    3091
TTA ATC CCC TTT GAA AAC GCG CAT TTT AAG GGC AAT ACC ACG ATT TCT TTG CGC TCT AGC
leu ile pro phe glu asn ala his phe lys gly asn thr thr ile ser leu arg ser ser

3121                                    3151
TCC CAA TTG CTC TAT AGT GAA ATC ATT GTC GCA GGG CGA GTG GCG CGC AAT GAG TTG TTT
ser gln leu leu tyr ser glu ile ile val ala gly arg val ala arg asn glu leu phe

3181                                    3211
AAA TTC AAC CGC TTG CAC ACC AAA ATC TCT ATT TTA CAA GAT GAG AAA CCC ATC TAT TAT
lys phe asn arg leu his thr lys ile ser ile leu gln asp glu lys pro ile tyr tyr

3241                                    3271
GAC AAC ACG ATT TTA GAT CCC AAA ACC ACC GAC TTA AAT AAC ATG TGC ATG TTT GAT GGC
asp asn thr ile leu asp pro lys thr thr asp leu asn asn met cys met phe asp gly

3301                                    3331
TAT ACG CAT TAT TTG AAT TTG GTG CTG GTC AAT TGC CCC ATA GAG CTG TCT GGC GTG CGA
tyr thr his tyr leu asn leu val leu val asn cys pro ile glu leu ser gly val arg

3361                                    3391
GGA TTG ATT GAA GAG AGC GAA GGA GTG GAT GGA GCC GTG AGT GAA ATC GCT AGT TCT CAT
gly leu ile glu glu ser glu gly val asp gly ala val ser glu ile ala ser ser his

3421                                    3451
TTA TGC CTG AAA GCT TTA GCG AAA GGC TCA GAA CCC TTG TTG CAT TTA AGA GAA AAA ATC
leu cys leu lys ala leu ala lys gly ser glu pro leu leu his leu arg glu lys ile

3481                                    3511
GCT CGC TTT ATC ACG CAA ACG ATT ACG CCA AAG GTT TAA AAA ACA CTT TAA AAA AGA TTA
ala arg phe ile thr gln thr ile thr pro lys val OCH

3541
TAC CCT TTA GTC TTT TTT AA
```

FIG. 3. Nucleotide sequence of the *H. pylori* accessory urease genes. Numbers above the sequence indicate the nucleotide positions. Predicted amino acid sequences, in sequential order, for UreI (bp 211 to 795), UreE (bp 800 to 1309), UreF (bp 1324 to 2091), UreG (bp 2123 to 2719), and UreH (bp 2722 to 3516) are shown below the sequence. The putative ribosome-binding sequences (Shine-Dalgarno [SD] sites) are underlined.

within the pILL753 insert, were conducted to better understand the conditions required for the expression of an active urease in *E. coli* cells. *E. coli* subclones bearing the derivative plasmids were quantitated for urease activity under the nitrogen-limiting conditions defined above. The results are summarized in Table 2. All subclones were derivatives of the same vector (pILL570) so that the results could be compared. One of these (pILL768) was achieved by self-religating the large *Eco*RI fragment generated from pILL753::16 (Fig. 1). This construction led to a 2.95-kb deletion at the 3′ end of the pILL753 insert. Cells harboring this plasmid expressed a comparatively low urease activity (Table 2). Plasmid pILL763 was obtained by cloning the *Cla*I-*Pst*I restriction fragment of the pILL753::1 plasmid into the linearized pILL570 vector. This construction, in which a 1.75-kb DNA fragment containing the *ureC* and *ureD* genes was deleted, had a urease activity that was approximately two times greater than that of cells harboring pILL753. In no case did deletions (or insertions) lead to constitutive urease activity.

Sequence analyses of the region required for urease expression in *E. coli*. The nucleotide sequence of the 3.2-kb DNA fragment located immediately downstream of the *ureB* gene, within the 11.2-kb fragment required for urease expression in *E. coli*, was determined. Briefly, restriction fragments originating from the pILL753 plasmid or from the pILL753::10, pILL753::11, pILL753::12, and pILL753::13 derivative plasmids were cloned in both orientations in M13mp18 and/or M13mp19. In addition, 12 oligonucleotide primers were synthesized to confirm the reading and/or to generate sequences overlapping the independently sequenced fragments; these were used as primers in double-stranded-DNA sequencing analyses.

The analysis of the sequence revealed five ORFs designated *ureI*, *ureE*, *ureF*, *ureG*, and *ureH*. These genes are all transcribed in the same direction and were predicted to encode peptides of 195, 170, 256, 199, and 265 amino acids, respectively. No ORF of any significant length was found on the strand complementary to the sequence shown in Fig. 3. The five ORFs begin with the characteristic ATG start codon. Four of the five ORFs were preceded by sites similar to the *E. coli* consensus ribosome-binding (Shine-Dalgarno) sequence (32).

DISCUSSION

Presented here is the first case of functional expression of genes originating from *H. pylori* in *E. coli*. This was made possible by growing *E. coli* cells harboring the urease recombinant cosmid pILL585 (16) on minimal medium containing a nitrogen-limiting source. The results obtained in this work suggest that urease activity in *E. coli* cells was dependent on the presence of a set of genes hitherto undescribed. The region encoding this set of genes was found to be located immediately downstream of the four genes, *ureC*, *ureD*, *ureA*, and *ureB*, previously described (16). It consisted of a 3.2-kb fragment having five ORFs which were designated (according to the nomenclature used for *K. aerogenes* [26] and *Proteus mirabilis* [14]) *ureI*, *ureE*, *ureF*, *ureG*, and *ureH*.

Generation of insertional mutations, as well as deletions, over the 11.2-kb DNA fragment (pILL753) subcloned from the original cosmid demonstrated that the *ureA*, *ureB*, *ureF*, *ureG*, and *ureH* genes were required for expression of urease activity in *E. coli*. Conversely, insertional mutations within the *ureI* gene did not markedly affect the urease activity in *E. coli* cells. However, recent work demonstrated that the disruption of *ureI* in *H. pylori* chromosomal DNA led to a urease-negative phenotype (6). Deletion of the *ureC* and *ureD* genes (as in pILL763) resulted in activities that were consistently greater than those in cells carrying plasmids with these loci intact, suggesting a regulatory role for this region of the *H. pylori* urease gene cluster. No conclusions regarding the requirement of *ureE* for enzyme expression could be drawn, as mutations were not obtained within this gene locus.

It seems likely that pILL753 does not carry the full complement of genes necessary for complete urease expression. The main evidence for this is that *E. coli* cells harboring pILL753 had a urease activity approximately 27 times lower than that of the *H. pylori* isolate used for the original cloning. It is interesting that *C. jejuni* required fewer foreign genes for enzyme expression than did *E. coli* and therefore must provide some of the functions of the *H. pylori* genes cloned in pILL753; however, no obvious DNA homology was found when a probe consisting of the *H. pylori* urease accessory genes was used in Southern hybridization experiments, which might confirm this hypothesis.

The requirement for accessory genes has been demonstrated for numerous microbial ureases, including *K. aerogenes* (26), *Klebsiella pneumoniae* (11), *Proteus vulgaris* (25), *Proteus mirabilis* (14, 34), *Providencia stuartii* (27), a urease-positive *E. coli* (3), and *Staphylococcus saprophyticus* (10). The genetic organization of the urease regions of some of these is now well documented. Figure 4 displays the comparison of three urease-encoding regions of various

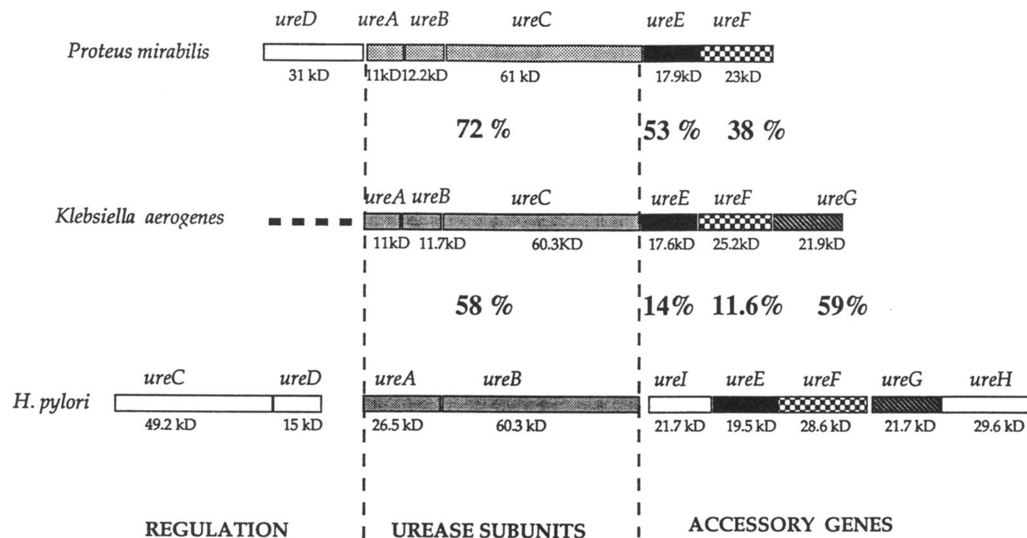

FIG. 4. Genetic organization of the urease operon. Shown are the relative positions of genes encoding polypeptides associated with the urease operons of *P. mirabilis* (14), *K. aerogenes* (26), and *H. pylori* (16). Percentages refer to the proportions of identical amino acids between two related genes. Open boxes represent genes which are unique to a specific operon.

bacterial species and stresses the similarities as well as singularities of each. The degree of relatedness, in terms of the genetic organization and the polypeptides encoded, was greatest between *P. mirabilis* and *K. aerogenes* vis-à-vis *H. pylori*. This has been the subject of a previous communication (16). While the UreG polypeptide of *H. pylori* was found to be highly similar to that of *K. aerogenes* (92% conservation and 59% identity), the degrees of similarity and identity between the UreE and UreF polypeptides of the bacteria were 33 and 14, and 44 and 11.6%, respectively.

Mulrooney and Hausinger (26) have shown that in *K. aerogenes* the genes encoding the UreE, UreF, and UreG accessory proteins are involved in the activation of the apoenzyme via the incorporation of nickel into the urease subunits (23). Because of the presence of a large string of histidine residues at the carboxy termini of the *Klebsiella* and *Proteus ureE* gene products, it has been proposed that the UreE polypeptide interacts through this metal-binding site with nickel ions which subsequently are transferred to apourease (26). No such string of histidine residues or related metal-binding sites was found in the corresponding gene product of the *H. pylori* urease gene cluster.

The urease region of *H. pylori* exhibits some unique features which are worthy of mention. First, the genes designated *ureI* and *ureH* are unique to *H. pylori*. Second, the urease region consists of three blocks of genes that are transcribed in the same direction and have an intergenic region of 420 bp between *ureD* and *ureA* (16) and 200 bp between *ureB* and *ureI*; this compares with the *Klebsiella* and *Proteus* gene clusters that behave as single gene blocks (the largest intergenic regions being 11 bp [26] and 26 bp [14], respectively). These findings suggest a genetic organization peculiar to *H. pylori*, in which the three gene blocks might be regulated independently.

It is generally thought that the *H. pylori* urease is constitutively expressed. Nevertheless, it has not been possible to confirm this because of the lack of a minimal medium capable of sustaining this fastidious bacterium. Evidence which suggests that *H. pylori* urease gene expression may in fact be regulated is provided here. Experiments are currently being undertaken to better understand the regulation of urease gene expression in *H. pylori*, which may eventually be of relevance to the successful cloning and expression of other *H. pylori* genes of interest.

REFERENCES

1. **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.
2. **Buck, G. E.** 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3**:1–12.
3. **Collins, C. M., and S. Falkow.** 1988. Genetic analysis of an *Escherichia coli* urease locus: evidence of DNA rearrangement. J. Bacteriol. **170**:1041–1045.
4. **Cover, T. L., W. Puryear, G. J. Perez-Perez, and M. Blaser.** 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59**:1264–1270.
5. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59**:2470–2475.
6. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1991. Construction of isogenic mutants of *Helicobacter pylori* deficient in urease activity. (Abstract.) Ital. J. Gastroenterol. **23**(Suppl. 2):O3.
7. **Ferrero, R. L., S. L. Hazell, and A. Lee.** 1988. The urease enzymes of *Campylobacter pylori* and a related bacterium. J. Med. Microbiol. **27**:33–40.
8. **Ferrero, R. L., and A. Lee.** 1991. The importance of urease in acid protection for the gastric-colonising bacteria *Helicobacter pylori* and *Helicobacter felis* sp. nov. Microb. Ecol. Health Dis. **4**:121–134.
9. **Friedrich, B., and B. Magasanik.** 1977. Urease of *Klebsiella aerogenes*: control of its synthesis by glutamine synthetase. J. Bacteriol. **131**:446–452.
10. **Gatermann, S., and R. Marre.** 1989. Cloning and expression of *Staphylococcus saprophyticus* urease gene sequences in *Staphylococcus carnosus* and contribution of the enzyme to virulence. Infect. Immun. **57**:2998–3002.
11. **Gerlach, G.-F., S. Clegg, and W. A. Nichols.** 1988. Characterisation of the genes encoding urease activity of *Klebsiella pneumoniae*. FEMS Microbiol. Lett. **50**:131–135.
12. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. McConnell, and W. E. S. Harper.** 1989.

Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. Int. J. Syst. Bacteriol. **39**:397–405.

13. **Hazell, S., and A. Lee.** 1986. *Campylobacter pyloridis*, urease, hydrogen ion back diffusion, and gastric ulcers. Lancet **ii**:15–17.
14. **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.
15. **Labigne, A., P. Courcoux, and L. Tompkins.** 1992. Cloning of *Campylobacter jejuni* genes required for leucine biosynthesis, and construction of *leu* negative mutant of *C. jejuni* by shuttle transposon mutagenesis. Res. Microbiol. **143**:15–26.
16. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173**:1920–1931.
17. **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.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Marshall, B. J., L. J. Barrett, C. Prakash, R. W. McCallum, and R. L. Guerrant.** 1990. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. Gastroenterology **99**:697–702.
20. **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.
21. **Messing, 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.
22. **Mobley, H. L. T., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol. **26**:831–836.
23. **Mobley, H. L. T., and R. P. Hausinger.** 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. **53**:85–108.
24. **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.
25. **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.
26. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J. Bacteriol. **172**:5837–5843.
27. **Mulrooney, S. B., M. J. Lynch, H. L. T. Mobley, and R. P. Hausinger.** 1988. Purification, characterization, and genetic organization of recombinant *Providencia stuartii* urease expressed by *Escherichia coli*. J. Bacteriol. **170**:2202–2207.
28. **Mulrooney, S. B., H. S. Pankratz, and R. P. Hausinger.** 1989. Regulation of gene expression and cellular localisation of cloned *Klebsiella aerogenes* (*K. pneumoniae*) urease. J. Gen. Microbiol. **135**:1769–1776.
29. **Pahel, G., D. M. Rothstein, and B. Magasanik.** 1982. Complex *glnA-glnL-glnG* operon of *Escherichia coli*. J. Bacteriol. **150**:202–213.
30. **Sanger, F., 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.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **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.
33. **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.
34. **Walz, S. E., S. K. Wray, S. I. Hull, and R. A. Hull.** 1988. Multiple proteins encoded within the urease gene complex of *Proteus mirabilis*. J. Bacteriol. **170**:1027–1033.
35. **Warren, J., and B. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i**:1273–1275.
36. **Zhang, H., R. Scholl, J. Browse, and C. Somerville.** 1988. Double stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. **16**:1220.