

The *Helicobacter pylori* UreI Protein Is Not Involved in Urease Activity but Is Essential for Bacterial Survival In Vivo

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We produced defined isogenic *Helicobacter pylori* ureI mutants to investigate the function of UreI, the product of one of the genes of the urease cluster. The insertion of a cat cassette had a strong polar effect on the expression of the downstream urease genes, resulting in very weak urease activity. Urease activity, measured in vitro, was normal in a strain in which ureI was almost completely deleted and replaced with a nonpolar cassette. In contrast to previous reports, we thus found that the product of ureI was not necessary for the synthesis of active urease. Experiments with the mouse-adapted *H. pylori* SS1 strain carrying the nonpolar ureI deletion showed that UreI is essential for *H. pylori* survival in vivo and/or colonization of the mouse stomach. The replacement of ureI with the nonpolar cassette strongly reduced *H. pylori* survival in acidic conditions (1-h incubation in phosphate-buffered saline solution at pH 2.2) in the presence of 10 mM urea. UreI is predicted to be an integral membrane protein and may therefore be involved in a transport process essential for *H. pylori* survival in vivo.

Helicobacter pylori is a microaerophilic gram-negative bacterium which colonizes the gastric mucosa of humans (9). *H. pylori* is associated with gastritis and peptic ulcer disease and has been shown to increase the risk of gastric cancers. Urease is a major virulence factor of *H. pylori*. It is involved in neutralizing the acidic microenvironment of the bacterium and also plays a role in *H. pylori* metabolism (10, 24).

The urease-encoding region of the *H. pylori* genome is composed of two gene clusters common to all strains (8) (Fig. 1), one comprising the *ureAB* genes encoding the structural urease subunits and the other containing the *ureEFGH* genes encoding the accessory proteins required for nickel incorporation into the urease active site. There is a gene of unknown function, *ureI*, immediately upstream from the latter gene cluster and transcribed in the same direction (Fig. 1). The distances separating *ureI* from *ureE* (1 bp) and *ureE* from *ureF* (11 bp) suggest that *ureI-ureE-ureF* constitutes an operon. Cotranscription of *ureI* and *ureE* has been demonstrated by Northern blot analysis (1). An *H. pylori* N6 mutant with the *ureI* gene disrupted by a MiniTn3-Km transposon was previously obtained (12). This strain (N6-*ureI*::TnKm-8) presented a urease-negative phenotype, so it was concluded that *ureI* was an accessory gene required for full urease activity.

The sequence of UreI from *H. pylori* and those of the AmiS proteins, encoded by the aliphatic amidase operons of *Pseudomonas aeruginosa* and *Rhodococcus* sp. strain R312, are similar (4, 25). Aliphatic amidases catalyze the intracellular hydrolysis of short-chain aliphatic amides to produce the corresponding organic acid and ammonia. We have shown that *H. pylori* also has such an aliphatic amidase, which hydrolyzes acetamide and propionamide in vitro (21).

The sequence similarity between UreI and AmiS together with the very similar structures of the urease and amidase substrates (urea: NH₂-CO-NH₂; acetamide: CH₃-CO-NH₂) and the fact that ammonia is produced by both enzymes

opened new perspectives for an investigation of the function of the *H. pylori* UreI protein.

Construction of defined mutations of the *H. pylori* ureI gene. *H. pylori* strains with defined mutations in *ureI* were generated by allelic exchange to determine whether the UreI protein was necessary for full urease activity. For this purpose, two plasmids (pILL823 and pILL834) with cassettes carrying antibiotic resistance genes inserted in *ureI* were constructed in *Escherichia coli*.

In one plasmid, pILL823 (Fig. 2), the *ureI* gene was inactivated by the insertion of a promoterless *cat* gene, conferring resistance to chloramphenicol (CM). A 780-bp blunt-ended *Bam*HI restriction fragment containing the “*cat* cartridge” from pCM4 (Pharmacia, Uppsala, Sweden) was introduced into a unique *Hpa*I site, between codons 21 and 22 of *ureI*, in pILL753 (8).

The second plasmid, pILL834, carried a *ureI* gene in which all but the first 21 codons were deleted and replaced with a nonpolar cassette (subcloned from pUC18K2 [18]) composed of the *aphA-3* kanamycin (KM) resistance gene (23) with its promoter and terminator regions deleted. In *Shigella flexneri* (18) and other organisms (such as *Yersinia enterocolitica* [2]), this cassette has been shown not to affect the transcription of the genes downstream within an operon as long as these distal genes have intact translation signals. There is only 1 bp separating *ureI* from *ureE* (Fig. 1), and *ureE* does not have a ribosome binding site (RBS) of its own; so the expression of *ureI* and *ureE* is transcriptionally and translationally coupled. Therefore, the *ureI* deletion was accompanied by the addition of an RBS immediately upstream from *ureE*. As shown in Fig. 2, three intermediates, pILL824, pILL825, and pILL833, were constructed in order to produce the final plasmid, pILL834.

Introduction of *ureI* mutations into *H. pylori*. *H. pylori* *ureI* mutants were produced by allelic exchange following electroporation with a concentrated preparation of pILL823 and pILL834 (as previously described [21]) of *H. pylori* N6 (11) and of mouse-adapted *H. pylori* SS1 (Sydney strain) (16). Bacteria showing chromosomal allelic exchange with pILL823 were selected on CM (4 µg/ml), and those with chromosomal allelic exchange with pILL834 were selected on KM (20 µg/ml). We

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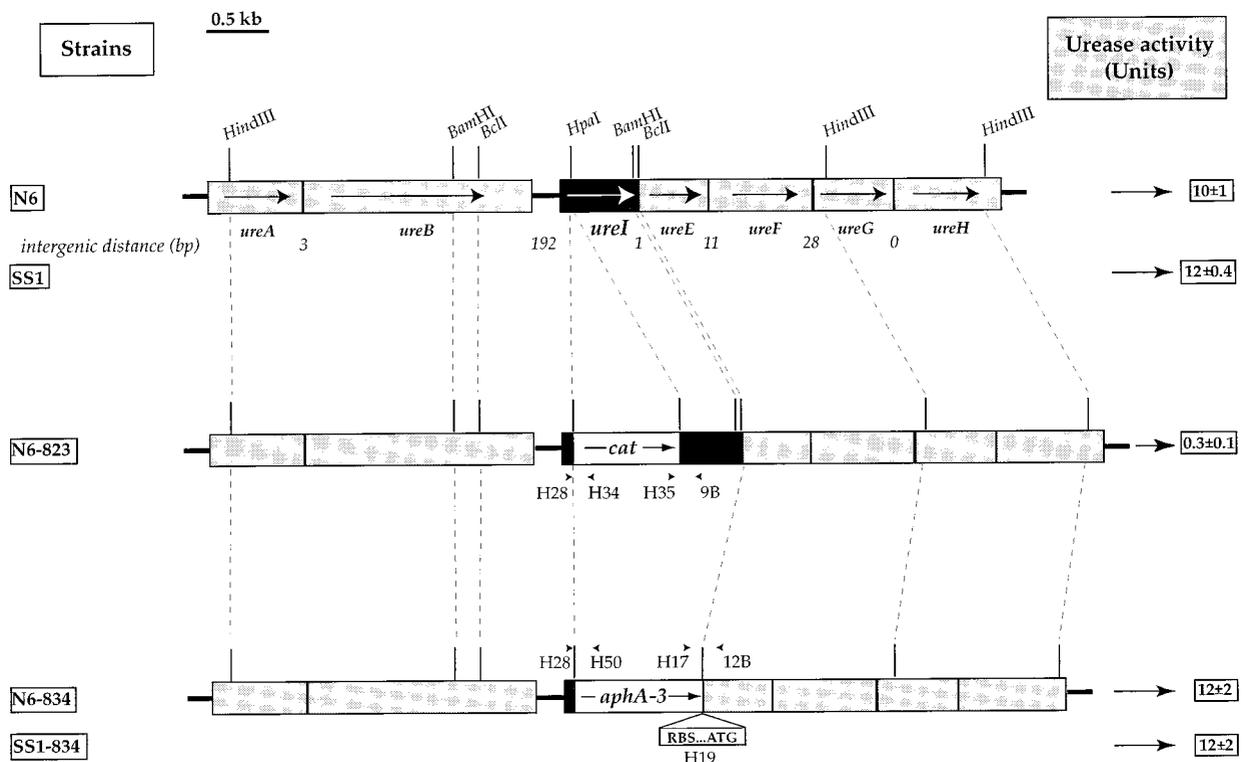


FIG. 1. The urease gene cluster of *H. pylori* parental strains N6 and SS1 and of the derived mutants deficient in UreI, strains N6-823, N6-834, and SS1-834. The genes are indicated by boxes with arrows showing the direction of their transcription. The distances between the *ure* genes are given in base pairs. The sites hybridizing to the primers used to confirm correct allelic exchange in strains N6-823, N6-834, and SS1-834 are shown. Blank boxes represent the cassettes containing the genes conferring resistance to CM (*cat*) or to KM (*aphA-3*). The urease activities of these strains are given on the right side of the figure. Urease activity was measured at pH7 as the release of ammonia in crude extracts of bacteria grown for 48 h on blood agar plates as described previously (8). One unit corresponds to the amount of enzyme required to hydrolyze 1 μ mol of urea/min/mg of total protein. The data are means \pm standard deviations calculated from three to five determinations.

checked that the desired allelic exchange had taken place in strains N6-823, N6-834, and SS1-834 (Fig. 1) by performing PCR with the appropriate oligonucleotides (Table 1). The PCR products obtained with genomic DNA of these strains were as expected: for strain N6-823, 140 bp with primers H28 and H34, 220 bp with primers H35 and 9B, and 1.2 kilobase pairs (kb) with primers H28 and 9B; for strains N6-834 and SS1-834, 150 bp with primers H28 and H50, 180 bp with primers H17 and 12B, and 1 kb with primers H28 and 12B.

The growth rate of strain N6-834 carrying mutant *ureI* with a nonpolar cassette was compared to that of the parental strain, N6. No difference in the colony size was observed on blood agar medium plates. Identical doubling times and stationary phase optical densities were measured for both strains grown in brain heart infusion (Oxoid) liquid medium containing 0.2% β -cyclodextrin (Sigma). UreI is thus not essential for *H. pylori* growth in vitro.

Urease activities of *H. pylori ureI* mutants. The urease activities of strains N6-823, N6-834, and SS1-834 were measured in vitro on crude extracts as described previously (8) and compared to the activities of the parental strains, N6 and SS1 (Fig. 1). Urease activity was almost completely abolished in strain N6-823 (0.3 ± 0.1 U). Strains N6-834 and SS1-834, with nonpolar *ureI* mutations, had wild-type levels of activity (N6-834 and SS1-834: 12 ± 2 U; N6: 10 ± 1 U; SS1: 12 ± 0.4 U).

These results strongly suggested that the urease-negative phenotype of the N6-*ureI*::TnKm-8 strain (12) and the very weak urease activity of the N6-823 strain were due to a polar effect of the inserted cassettes on the expression of the downstream genes *ureE* and *ureF* (Fig. 1). This hypothesis was tested

by measuring the urease activity of strain N6-823 complemented in *trans* with an *E. coli/H. pylori* shuttle plasmid expressing the *ureEF* genes. This plasmid, pILL845 (Fig. 2), was obtained by insertion of a 2.8-kb *ClaI*-*Bam*HI fragment of pILL834 (comprising the 3' end of *ureB*, *ureI* with the nonpolar cassette replacing deleted codons, and intact *ureE* and *ureF* genes) into the corresponding sites of the pHel2 shuttle vector (14). Strain N6-823 was electroporated with a DNA preparation of pILL845 (as described in reference 21), and transformants were selected on KM (20 μ g/ml) and CM (4 μ g/ml). In strain N6-823 harboring pILL845, a high level of urease activity was restored (25 U), confirming that the very low level of urease activity of strain N6-823 was due to a polar effect on the expression of accessory genes *ureEF*.

Colonization test for the *H. pylori* SS1-834 mutant in the mouse animal model. The mouse model for infection by the *H. pylori* SS1 strain (Sydney strain) (16), validated in our laboratory (6, 13), was used to test the function of UreI in vivo. Mice were infected either with the nonpolar *ureI* mutant, SS1-834, or with the parental strain, SS1 (which had gone through an equivalent number of in vitro subcultures) as a positive control. This experiment was repeated three times and produced identical results. Two independently constructed SS1-834 mutants were used. The first mutant strain had gone through 30 in vitro subcultures; the second had gone through only 20. R. Ferrero (10a) showed that, under the same experimental conditions, strain SS1 can undergo up to 80 in vitro subcultures without losing its colonization capacity.

In each experiment, aliquots (100 μ l) containing 10^6 *H. pylori* SS1 or SS1-834 bacteria prepared in peptone broth were

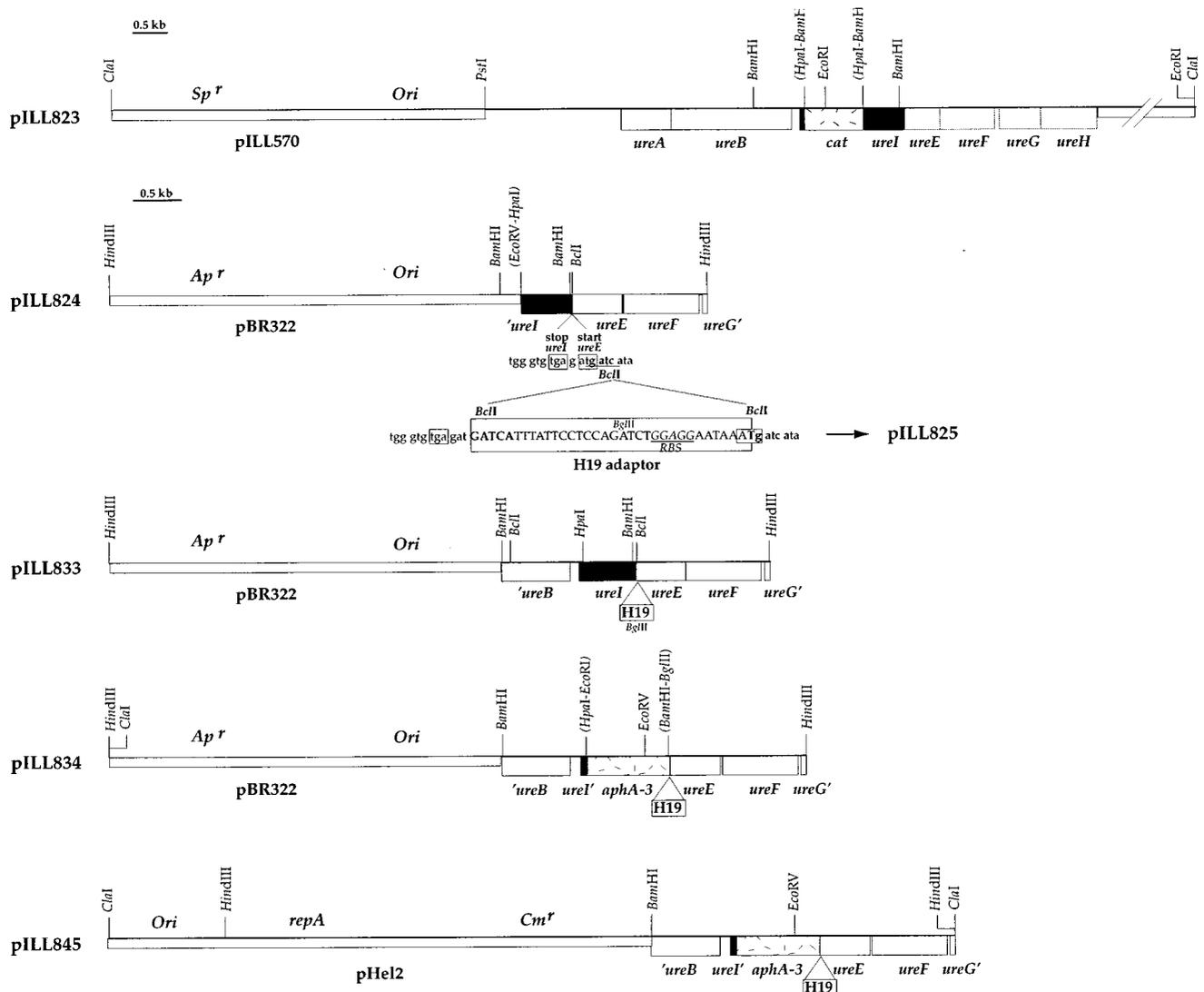


FIG. 2. Restriction map of pILL823, pILL824, pILL833, pILL834, and pILL845. Small boxes mark the vector of each plasmid; large boxes correspond to genes. Ori indicates the position of the ColE1 origin of replication. repA is the gene coding for the RepA protein, which is necessary for autonomous replication of pHel2 in *H. pylori*. Sp, Ap, and Cm indicate the genes conferring resistance to spectinomycin, ampicillin, and CM, respectively. The sequence of the DNA region comprising the ureI stop codon and the ureE start codon, including the BclI site where adapter H19 was inserted, is given below pILL824, which was obtained by the insertion of a 1.8-kb HpaI-HindIII fragment from pILL753 (8) into pBR322. Plasmid pILL825 was produced by the insertion of the H19 adaptor (carrying an RBS and ATG in frame with ureE) (Table 1) into the BclI site of pILL824; the resulting ureI-ureE intergenic sequence is also shown. The stop codon of ureI and the start codon of ureE are boxed, and the RBS is underlined. In pILL833, the BamHI fragment of pILL825 was replaced by a 1.3-kb blunt-ended PvuII-BamHI fragment of pILL753. Plasmid pILL834 was obtained by replacement of the HpaI-BglII fragment of pILL833 with an 850-bp blunt-ended EcoRI-BamHI fragment of pUC18K2 containing the nonpolar KM cassette (18). Parentheses indicate the position of restriction sites removed by ligation.

administered orogastrically to 10 mice each (6- to 8-week-old Swiss specific-pathogen-free mice) as described by Ferrero et al. (13). Mice were killed 4 weeks after inoculation. We tested for the presence of *H. pylori* with a direct urease test on biopsies performed on half the stomach (13). The remaining gastric tissues were used for quantitative culture of *H. pylori* as described by Ferrero et al. (13). In every experiment, the stomachs of the 10 SS1-infected mice all tested positive for urease. The bacterial load was between 5×10^4 and 5×10^5 CFU per g of stomach tissue. None of the stomachs of the mice infected with strain SS1-834 tested positive for urease, and no *H. pylori* cells were cultured from them. Thus, the UreI protein is essential for *H. pylori* in vivo survival and/or colonization of the mouse stomach.

TABLE 1. Names and nucleotide sequences of oligonucleotides used in this study

Primer ^a	Oligodeoxynucleotide sequence (5' to 3')
H17	TTTGACTTACTGGGGATCAAGCCTG
H19	GATCATTTATTCTCCAGATCTGGAGGAATAAAT
H28	GAAGATCTCTAGGACTTGTATTGTTATAT
H34	TATCAACGGTGGTATATCCAGTG
H35	GCAGTTATTGGTGCCCTTAAACG
H50	CCGGTGATATTCTCATTTTAGCC
8A	GCGAGTATGTAGGTTTCAGTA
9B	GTGATACTTGAGCAATATCTTCAGC
12B	CAAATCCACATAATCCACGCTGAAATC

^a H19 was used as the adaptor, and the others were used as primers for PCR amplification.

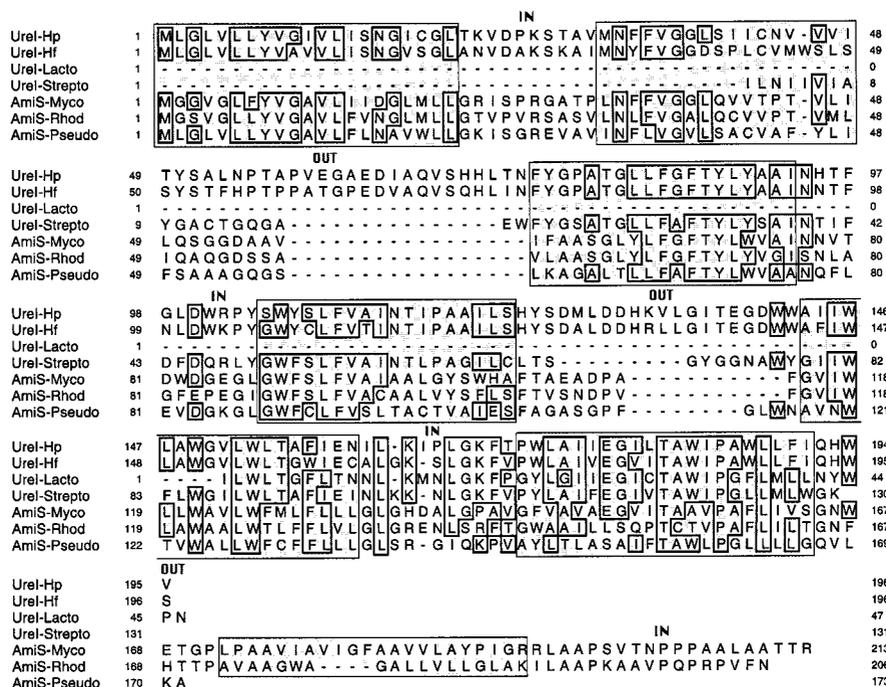


FIG. 3. Alignment of the amino acid sequence of UreI from *H. pylori* with those of similar proteins and prediction of the two-dimensional structure of members of the protein family comprising UreI and AmiS. Residues identical at one position in at least four sequences are boxed; dashes indicate gaps inserted to optimize alignment. The organisms from which the sequences originated and the degree of identity of each with the *H. pylori* UreI protein are as follows: Urel-Hp, *H. pylori* (195 residues; accession no., M84338); Urel-Hf, *H. felis*; 74% identity over 196 residues (accession no., A41012); Urel-Lacto, *L. fermentum*; 55% identity over the 46-residue partial sequence (accession no., D10605); Urel-Strepto, *S. salivarius*; 54% identity over the 129-residue partial sequence (accession no., U35248); AmiS-Myco, *M. smegmatis*; 39% identity over 172 residues (accession no., X57175); AmiS-Rhod, *Rhodococcus* sp. strain R312; 37% identity over 172 residues (accession no., Z46523); and AmiS-Pseudo, *P. aeruginosa*; 37% identity over 171 residues (accession no., X77161). Predicted transmembrane α -helices are shown as shaded boxes. The regions separating these boxes are hydrophilic loops labeled IN when they are predicted to be intracellular and OUT when they are predicted to be extracellular.

Survival of the *H. pylori* N6-834 mutant in acidic conditions.

Survival under acidic conditions in the presence of 10 mM urea or in the absence of urea was tested with strains N6 and N6-834. The experimental procedures were those described by Clyne et al. (7). Exponentially grown bacteria were harvested and washed in phosphate-buffered saline (PBS; Boehringer, Mannheim, Germany), and 2×10^8 CFU of bacteria per ml were resuspended in PBS at pH 2.2 or 7 in the presence of 10 mM urea or in the absence of urea and incubated for 1 h at 37°C. To evaluate bacterial survival, quantitative cultures (5 days of growth) of the *H. pylori* strains were performed. At pH 7, in the absence of urea, both strains survived similarly (10^8 CFU/ml). In agreement with the results of Clyne et al. (7), none of the strains survived at pH 7 in the presence of urea because the final pH rose to 9. As expected (7), both strains were killed at pH 2.2 in the absence of urea, and significant survival at pH 2.2 in the presence of urea was observed with strain N6 (5×10^6 CFU/ml; final pH 6.5). In contrast, when the nonpolar *ureI* mutant strain N6-834 was incubated at pH 2.2 in the presence of urea, a low level of survival was observed (10^3 CFU/ml) and the pH was unchanged (pH 2.3) after 1 h of incubation.

Alignment of the UreI and AmiS protein sequences and two-dimensional structure prediction. A systematic search for UreI homologs in the protein data banks was carried out. We found that *H. pylori* is not the only ureolytic bacterium with a *ureI* gene. Two phylogenetically related gram-positive organisms, *Streptococcus salivarius*, a dental plaque bacterium (5), and *Lactobacillus fermentum*, a lactic acid bacterium (15), carry genes (the available sequences are only partial) coding for UreI homologs (Fig. 3) located immediately upstream from the ure-

ase structural genes. The *ureI* gene has also been detected in various *Helicobacter* species; the *Helicobacter felis ureI* gene has been entirely sequenced (10b) (Fig. 3). PCR experiments have suggested that there is a *ureI* gene in *Helicobacter heilmannii* (22) and in *Helicobacter mustelae* (unpublished data).

Sequence similarities between the UreI protein of *H. pylori* (21.7 kDa) and the AmiS proteins expressed by the aliphatic amidase operons from *P. aeruginosa* (25) and *Rhodococcus* sp. strain R312 (4) have been reported. In *Mycobacterium smegmatis*, there is an additional AmiS homolog encoded by a gene, open reading frame P3, located immediately upstream from an amidase gene (17).

Alignment of these UreI and AmiS proteins [with the Clustal W(1.60) program] defined strongly conserved stretches of amino acids (Fig. 3). All but one of these conserved blocks are in highly hydrophobic segments. These regions, each 17 to 22 residues long, are probably folded into transmembrane α -helices (Fig. 3). Six transmembrane regions were predicted for the proteins from *H. pylori*, *H. felis*, and *P. aeruginosa*, and seven were predicted for those from *Rhodococcus* sp. strain R312 and *M. smegmatis* (these are highly reliable predictions, performed with pHD, a profile-fed neural network system [20]). The orientations of the UreI and AmiS proteins in the membrane were deduced (20) from the charges of the intercalated hydrophilic regions, which are short in these proteins (Fig. 3). These results strongly suggest that the members of the family comprising UreI and AmiS, found in both gram-positive and -negative bacteria, are integral membrane proteins. These proteins have no signal sequence and should therefore be inserted into the cytoplasmic membrane in gram-negative bacteria.

Conclusions. The urease cluster of *H. pylori* is unique among the many urease operons of gram-negative bacteria that have been sequenced (19) in that it has an extra gene, *ureI*. The function of UreI has therefore been the subject of much speculation. It has mostly been assigned the function of an accessory protein required for nickel incorporation at the urease active site or of a nickel transporter. We have demonstrated that UreI is not required for full activation of *H. pylori* urease during *in vitro* growth. UreI is thus not a nickel transporter since such a protein, NixA (3), already identified in *H. pylori*, is necessary for full urease activity. We showed herein that replacing *ureI* with a nonpolar cassette has no effect on urease activity measured *in vitro*. This is the first time that a nonpolar cassette (18) has been shown to be functional in *H. pylori*. This will certainly be a valuable tool for genetic analysis of complex *H. pylori* operons.

We observed that UreI was essential for survival *in vivo* and/or for colonization of the mouse stomach. This could be due to the reduced resistance to acidity of the *ureI* mutant, as suggested by the results of tests of *in vitro* survival in acidic conditions with 10 mM urea. UreI has a sequence similar to those of the AmiS proteins, proposed to be involved in the transport of short-chain amides (25), molecules structurally similar to urea. The UreI and AmiS proteins have the characteristics of integral membrane proteins, probably of the cytoplasmic membrane. Different roles for UreI can tentatively be proposed. UreI might be involved in (i) transport of urea or short-chain amides, (ii) an uptake system for maintaining appropriate intracellular ammonia concentrations, or (iii) the export of excess intracellular ammonium. An essential role for UreI as an amide transporter seems unlikely because in mouse colonization experiments (performed as described above) an SS1 mutant deficient in aliphatic amidase (carrying the mutation described in reference 21) colonized mice as efficiently as the parental strain, SS1. In addition, amidase activity was not significantly modified by the deletion of *ureI* in strain N6-834. Our results concerning acidity survival are most compatible with UreI being involved in ammonium export.

Finally, UreI, as a membrane protein essential for the survival of *H. pylori* *in vivo*, is an interesting potential target for new antibacterial drugs.

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