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Construction and Characterization of an Isogenic Urease-Negative Mutant of *Helicobacter mustelae*

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Helicobacter mustelae **infects the ferret stomach and provides an opportunity to study pathogenic determinants of a *Helicobacter* species in its natural host. We constructed an isogenic urease-negative mutant of *H. mustelae* which produced no detectable urease and showed a reduced acid tolerance. This mutant provides an opportunity to further evaluate the role of urease in the pathogenesis of *Helicobacter* infection.**

Helicobacter pylori is a gram-negative spiral bacterium that is associated with peptic ulcer disease (4) and gastric malignancy (19, 20). *Helicobacter mustelae* is a closely related organism that naturally infects the stomach of ferrets, where it adheres to gastric mucosa and causes a chronic gastritis similar to that seen with *H. pylori* infection in humans (10). Experimental inoculation of ferrets with *H. mustelae* produces a stable colonization of the gastric mucosa and causes gastritis, a significant immune response, and a transient elevation in gastric pH (11). These features mimic the response to human infection with *H. pylori* (16–18) and suggest that *H. mustelae* infection in the ferret is a useful animal model.

Like all other known species of *Helicobacter* that infect the stomach, *H. mustelae* expresses a potent urease activity (5) that is presumed important in pathogenesis. Isogenic urease-negative mutants of *H. pylori* fail to colonize the stomach in the pig (6) and mouse (26) models. However, *H. pylori* does not naturally infect these animals. We sought to further examine the role of urease in *Helicobacter* pathogenesis by constructing and characterizing an isogenic urease-deficient mutant of *H. mustelae*. This mutant could then be used to study the role of urease in *H. mustelae* infection of the ferret, which is its natural host.

H. mustelae NCTC 12032 (National Collection of Type Cultures, London, England) was used to clone a partial fragment of the urease genes and served as the parent for construction of an isogenic urease-negative mutant. Cultures of *H. mustelae* were grown on 5% sheep blood plates at 37°C in a BBL GasPak jar containing an anaerobic gas pack envelope (BBL) without catalyst. *H. mustelae* chromosomal DNA was prepared with hexadecyltrimethyl ammonium bromide (1). Amplification reactions were carried out with a Thermal Cycler (Perkin-Elmer Cetus, Emeryville, Calif.) and standard amounts of Gene-Amp reagents (Perkin-Elmer Cetus) with 2.0 mM $MgCl_2$. Each primer (Table 1) was used at a concentration of 0.25 μM except the degenerate primers (2694F and 3379R), which were used at 10 μM. PCR was carried out for 30 to 35 cycles consisting of 94°C for 1 to 2 min, 40°C (for primer pairs whose target sequences were unknown) or 55°C for 1 min, and 72°C for 1 to 2 min (depending on the length of the amplified

TABLE 1. Oligonucleotides used for PCR

Name	Position[a]	Strand	Length (nucleotides)	Sequence (5′-3′)
2694F[b]	2671–2694	+	36	CAUCAUCAUCAUCCXAARGARYTXGAYAARYTXATG
3379R[b]	3379–3402	−	36	CUACUACUACUAYTCYTTXCGXSWDATYTTYTTCAT
3414F	3395–3414	+	20	GAAAAGAATATGTTTCTATG
4324R	4324–4346	−	23	TGGTGGCACACCATAAGCATGTC
2755F	73–97	+	39	GCATCCGCGGCCGCGAAAGAGGCGTAAAGCTAAATTATG[c]
4280R	1593–1616	−	35	CGTACCTGCAGTCTTTGTGAAAGGAATTGTAGGAT[c]
OLKm1	699–724	+	25	CTGCTAAGGTATATAAGCTGGTGGG
OLKm2	1305–1329	−	25	CATACTGTTCTTCCCCGATATCCTC

[a] Numbers refer to nucleotides in the sequences of the *H. pylori* urease genes (2694F, 3379R, 3414F, and 4324R [15]), sequences of the kanamycin resistance genes (OLKm1 and OLKm2 [25b]), and sequences of the *H. mustelae* urease genes (2755F and 4280R [see Fig. 2]).

[b] Degenerate oligonucleotides based on amino acids 5 to 12 of *H. pylori* UreA (2694F) and amino acids 1 to 8 of *H. pylori* UreB (3379R). The four triplet repeats (CAU for 2694F and CUA for 3379R) are added to the 5′ end for cloning using the CloneAmp system (Gibco-BRL). R = A or G; W = A or T; S = C or G; Y = C or T; D = A, G, or T; X = G, A, T, or C.

[c] Underlined nucleotides represent *Pst*I (4280R) and *Not*I (2755F) restriction endonuclease sites.

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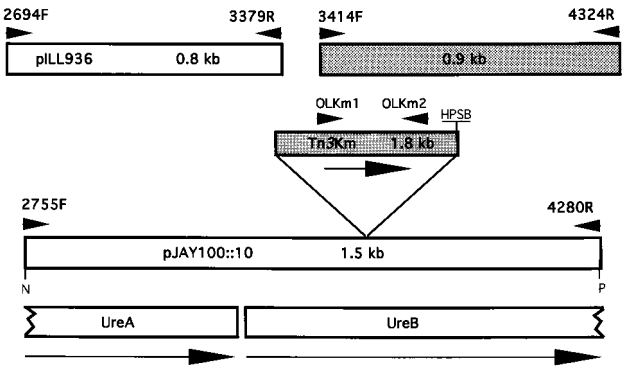

FIG. 1. Summary of the amplification of the *H. mustelae* urease genes and construction of the 1.5-kb urease gene fragment interrupted by the mini-Tn*3*Km transposon. Approximate fragment size (not drawn to scale) is as indicated. Arrowheads represent the designated oligonucleotide primers whose sequences are shown in Table 1. pILL936 is a derivative of pAMP1 containing a 0.8-kb insert of *H. mustelae* DNA that codes for all of UreA except the first 12 amino acids. The 0.9-kb fragment of *ureB* is represented by the broad shaded bar, and the mini-Tn*3*Km transposon is shown as a narrow shaded bar with its approximate position of insertion in pJAY100 (1.5 kb) to create pJAY100::10 (3.3 kb). Large arrows indicate the direction of transcription of the kanamycin gene and of *ureA* and *ureB* (shown as broken bars to indicate partial sequence). Restriction sites are indicated by H (*Hin*dIII), P (*Pst*I), S (*Sal*I), B (*Bam*HI), and N (*Not*I).

product). DNA sequencing was performed by the chain termination method (22), using a Sequenase kit (United States Biochemical Corp.). PCR products were sequenced directly with 1% dimethyl sulfoxide in the annealing mixture (28). Other DNA manipulations, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and immunoblotting were performed according to standard procedures (21) unless otherwise noted.

Figure 1 summarizes the amplification of the *H. mustelae* urease genes and construction of a 1.5-kb urease gene fragment interrupted by a mini-Tn*3*-Km transposon, which are described below. The *H. mustelae ureA* gene was amplified from chromosomal DNA by using degenerate primers that corresponded to amino acids 5 to 12 of UreA (2694F) and the first eight residues of UreB (3379R), regions that are highly conserved among *Helicobacter* ureases (27). This amplification reaction yielded a band at the expected size of approximately 0.8 kb. Uracil in the 5′ end of each primer permitted direct cloning of the PCR product into *Escherichia coli* MC1061 by using vector pAMP1 (CloneAmp system; Gibco-BRL, Gaithersburg, Md.), and this construct was designated pILL936. The insert from pILL936 was subcloned into M13mp18/19 and sequenced. The amplified DNA fragment coded for a predicted polypeptide of 225 residues (calculated molecular mass of 24,750 Da) which coded for all of UreA except the first 12 amino acids (Fig. 2).

To increase the likelihood of obtaining a transposon insertion, we next amplified a portion of the *ureB* gene of *H. mustelae*. Primers 3414F and 4324R were selected from the *H. pylori ureB* sequence in regions that are conserved among the known *Helicobacter* ureases (8, 14, 25). These primers amplified the expected 0.9-kb fragment from *H. pylori* and also amplified a fragment of the same size from *H. mustelae*. Direct sequencing of the *H. mustelae* PCR product verified that it was homologous to known urease sequences. A primer was selected from this sequence (4280R) and used in a PCR with a primer from the *H. mustelae ureA* sequence (2755F) in order to amplify an approximately 1.5-kb fragment of the *H. mustelae* urease genes. This 1.5-kb fragment was gel purified and asymmetrically cloned into pILL570 (15) that had been modified by the addition of a *Not*I cloning site, resulting in plasmid pJAY100.

Figure 2 shows the predicted amino acid sequence for the *H. mustelae* UreA and a portion of UreB, aligned with corresponding sequences from *H. pylori*, *H. felis*, and "*H. heilmannii*." The *H. mustelae* urease was 67 to 68% identical to other *Helicobacter* ureases for UreA and 79% identical for UreB (partial sequence). The presence of two structural subunits,

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UreA
H.m.                LHYAGELAKKRKERGVKLNYVEAVALISMEIMEHAREGKKTVADLMSLGRQVLK
H.h.  MKLTPKELDKLM.....Q..AK.I....T.......AHV..E..A...S.....QE..TL..
H.f.  ................R..EEALA.......T.......GRV..K..D.N.S.....QE..TW..
H.p.  .........................K.I............AH...E..A....A.E..QE..TL..

H.m.  AEDVMDGVAALVHEVQIEVCFPDGTLLVTVHNPIEN-NGKLHPGEFILKDEDIVLNAGKEAIEVKV
H.h.  .D...P...HMI...G..AG.....K...I.T.V.AGSD..A...V...N...T.....H.VQL..
H.f.  K.N......SMI...G..AN.....K...I.T.V.-D....A...VF..N...TI.......SL..
H.p.  PDD......SMI...G..AM.....K.....T...A.....V...LF..N...TI.E..K.VS...

H.m.  SNKGDRPIQVGSHFHFFETNKLLEFDREKRYGRRLDIASGTSVRFEPGESKTVRLIQFGGNQRIFG
H.h   K......V..........V....D.....A..K........A.......E...E..DI...K..Y.
H.f.  K......V..........V....D...A.SFCK........A.......E.S.E..DI...K..Y.
H.p.  K.V....V.I........V.RC.D.....TF.K........A.......E.S.E..DI...R..F.

H.m.  FNDLNNGQVNEDNKRKALAAAKAKGFIK
H.h.  ..A.VDR.ADH.G.KL..KR..E.H.GTINCGCDN-K              68%
H.f.  ..S.VDR.ADA.G.KLG.KR..E...GSV....EAT.DKQ          67%
H.p.  ..A.VDR.ADNES.KI..HR..ERG.HGAKSDDNYV.TIKE         67%

UreB
H.m.  MIKISRKEYVSMYGPTTGDKVRLGDTELIAEIEKDYTVYGEEIKFGGGKTIRDGMSQSVSPDVNEL
H.h.  .K.......................D..L.V.H.C.T.................G.TN..SSH..
H.f.  .K.................R......D..L.V.H.C.T.....................TN..SSY..
H.p.  .K.......................D....V.H...I....L.......L.E.....NN.SKE..
                                                        Δ
H.m.  DAVITNAMIIDYTGIYKADIGIKDGKIAGIGKAGNRDTQDGVGMDLVVGASTEAIAGEGLIVTAGG
H.h.  .L.....L.V.............N...H.......K.L....CNR.C..PA...L.A.........
H.f.  .L.L...L.V.............................K.M....DNN.C..PA...L.A.........
H.p.  .LI....L.V.....................G..K.M....KNN.S..PA...L.............

H.m.  IDIHIHFISPTQIPTALYSGVTTMIGGGTGPAAGTFATTISPGEWNIKQMIRAAEEYTMNLGFFGK
H.h.  ..........Q.....FA..I..........D..N....T..R..L.E.L..S...A....YL..
H.f.  ..........Q.....FA..............D..N....T..RA.L.S.L......A.....LA.
H.p.  ..........Q.....FA..............D..N....T..RR.L.W.L......S.....LA.

H.m.  GNTSNVKALEDQIKAGALGFKVHEDCGSTPAVINHSLDIAEKYDVQVAIHTDTLNEGGAVEDTLAA
H.h.  ..V.FEP..I..LE...I...I...W....SA...A.N..D.............A.C.....E.
H.f.  ..V.YEPS.R...E...I...I...W.....A.H.C.NV.DE.............A.C.....E.
H.p.  ..A..DAS.A...E...I...I...W.T..SA...A..V.D.............A.C....M..

H.m.  IGGRTIHTFHTEGAGGGHAPDIIKAAGEANILPASTNPTIPFTK
H.h.  .A.....................V..M...F.....................          79%
H.f.  .A.....................V..M...F.....................          79%
H.p.  .A...M.....................V...H.....................V        79%
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FIG. 2. Predicted partial amino acid sequences of UreA and UreB from *H. mustelae* (H.m.) aligned with the corresponding predicted sequences from "*H. heilmannii*" (H.h.), *H. felis* (H.f.), and *H. pylori* (H.p.). Sequence identity with *H. mustelae* is indicated by dots; gaps in sequence to optimize alignment are indicated by dashes. Percentages indicate percent amino acid identity compared with *H. mustelae*. The transposon in the urease-negative *H. mustelae* mutant was inserted immediately after residue 116 of UreB (alanine) that is indicated by a triangle.

TABLE 2. Matrix of percent amino acid identity (UreA and UreB) and percent nucleotide similarity (16S rRNA) for *H. mustelae* compared with other *Helicobacter* species[a]

Organism	% Identity											
	UreA				UreB				16S rRNA			
	Hm	Hf	Hp	Hh	Hm	Hf	Hp	Hh	Hm	Hf	Hp	Hh
Hm												
Hf	67				79				93.4			
Hp	67	74			79	88			93.8	95.3		
Hh	68	82	79		79	92	87		92.9	98.7	94.8	

[a] Hm, *H. mustelae*; Hf, *H. felis*; Hp, *H. pylori*; Hh, "*H. heilmannii*." The similarity matrix for 16S rRNA is derived from previously published results (25a). The amino acid identity matrices are based on published DNA sequences for the urease genes of *H. felis* (8), *H. pylori* (15), and "*H. heilmannii*" (25) and on the partial urease DNA sequences for *H. mustelae* reported here.

with greater interspecies homology in UreB, is characteristic of all other known *Helicobacter* ureases (8, 14, 25).

The ureases of *H. pylori*, *H. felis*, and "*H. heilmannii*" are more closely related to one another than they are to the urease of *H. mustelae* (Table 2). This finding is similar to the results of phylogenetic studies based on 16S rRNA sequences (Table 2), as well as G+C content (24, 25). It is also consistent with the ecological niche of these organisms. *H. pylori*, *H. felis*, and "*H. heilmannii*" have each been found to occur naturally in multiple overlapping species, while *H. mustelae* has so far been found only in ferrets and has no overlap in host range with other *Helicobacter* species.

Derivatives of pJAY100 harboring a mini-Tn*3*-Km transposable element were generated by random insertion of the transposon as described previously (14). *H. mustelae* NCTC 12032 was transformed with one of these derivatives (pJAY100::10) by electroporation, using a method previously described for *H. pylori* (7). Approximately 2,000 kanamycin-resistant colonies were obtained per μg of DNA. Of 180 colonies that were tested, 13 were resistant to kanamycin but sensitive to spectinomycin and therefore presumably contained a double crossover in the urease gene. All of these clones were shown to be qualitatively urease negative by suspending bacteria in urea-indole medium (Diagnostic Pasteur) as described previously (15). One of them, designated *H. mustelae* 10::Tn*3*Km, was selected for further characterization.

Chromosomal DNA from wild type *H. mustelae* and *H. mustelae* 10::Tn*3*Km was amplified with primers 2755F and 4280R, which had been used to clone the 1.5-kb urease fragment. The expected 1.5-kb band was obtained with the parent strain, but in 10::Tn*3*Km, these primers amplified an approximately 3.3-kb band, consistent with insertion of the 1.8-kb transposon (Fig. 3). Insertion of the transposon was further confirmed by amplification with primers OLKm1 and OLKm2, which amplified a 0.6-kb fragment of the kanamycin resistance gene from 10::Tn*3*Km but not from wild-type *H. mustelae* (Fig. 3).

The position of the transposon insertion was determined by DNA sequencing. Restriction mapping (data not shown) of pJAY100::10 showed that the mini-Tn*3*-Km was inserted approximately in the middle of the 1.5-kb urease fragment and that it was oriented with the multiple cloning site (which included *Pst*I) downstream (Fig. 1). Restriction of pJAY100::10 with *Pst*I yielded an approximately 0.8-kb band that was purified by electroelution and cloned into the *Pst*I site of pBluescript SK- (Gibco-BRL). DNA sequencing of this fragment showed that it contained 27 bp of the 38-bp repeat found at the end of the transposon (12), and this allowed us to place the transposon insertion between residues 116 and 117 of UreB.

H. mustelae 10::Tn*3*Km was microaerophilic, oxidase and catalase positive, and motile by microscopic examination. Comparison of growth curves for *H. mustelae* 10::Tn*3*Km and the wild-type *H. mustelae* showed that both strains reached the same cell density after 48 h growth in liquid culture (optical density at 600 nm of 1.06). Urease activity was quantitated by using an assay (9) based on the Berthelot reaction (3). Wild-type *H. mustelae* hydrolyzed 21.46 (standard deviation, 0.97) μmol of urea per min per mg of total protein, while there was no detectable urease activity in *H. mustelae* 10::Tn*3*Km (limit of detection, <12 nmol of urea per min per mg of total protein).

To provide direct evidence for loss of urease expression in *H. mustelae* 10::Tn*3*Km, whole-cell protein extracts from the mutant and wild type were electrophoresed and immunoblot-

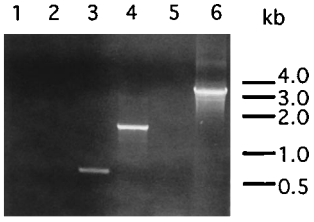

FIG. 3. Agarose gel electrophoresis of PCR products from amplification with primers OLKm1 and OLKm2 (lanes 1 to 3) and primers 2755F and 4280R (lanes 4 to 6). PCR template was no DNA (lanes 2 and 5), chromosomal DNA from wild-type *H. mustelae* (lanes 1 and 4), or chromosomal DNA from *H. mustelae* 10::Tn*3*Km (lanes 3 and 6). Primers OLKm1 and OLKm2 amplified a 0.6-kb fragment of the kanamycin gene from the urease mutant, *H. mustelae* 10::Tn*3*Km, but not from the wild-type *H. mustelae*. Amplification with primers 2755F and 4280R yielded a 1.5-kb band when the uninterrupted urease sequence served as template (*H. mustelae* wild type), and a 3.3-kb band when the urease was interrupted by insertion of the transposon (*H. mustelae* 10::Tn*3*Km).

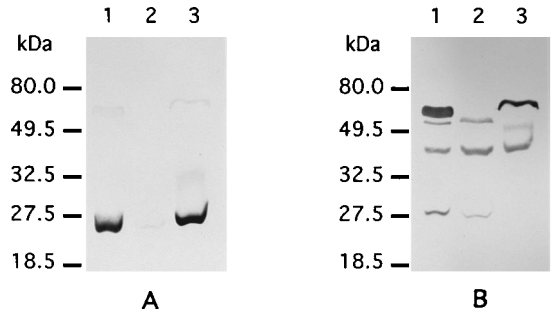

FIG. 4. Immunoblot of an SDS-polyacrylamide gel with antisera against the *H. pylori* UreA (A) and UreB (B) polypeptides. Samples are whole cell protein extracts from *H. mustelae* (lane 1), *H. mustelae* 10::Tn*3*Km (lane 2), and *H. pylori* 87A (lane 3) used as a positive control. The urease-negative mutant, *H. mustelae* 10::Tn*3*Km, expressed no detectable UreB and only a trace amount of UreA.

TABLE 3. Effect of pH on *H. mustelae* viability in vitro with and without urea

pH	Urea concn (mm)	Mean (SD) log viable *H. mustelae* cells/ml	
		Wild type, 8.44 (0.33)[a]	Urease mutant, 8.74 (0.37)
7.4	5	7.76 (0.17)	8.00 (0.27)
	0	7.79 (0.29)	7.88 (0.39)
3.0	5	7.55 (0.67)	3.11[b] (1.76)
	0	6.35 (0.90)	1.41[b] (1.33)
1.5	5	5.95 (0.70)	0.57[b] (0.98)
	0	0.0 (0.0)	0.0 (0.0)

[a] Mean (standard deviation) inoculum.
[b] Statistical significance (one-tailed Student's *t* test) compared with the wild type, $P < 0.001$.

ted with antiserum (a gift from H. Mobley) against the denatured UreA and UreB subunits from *H. pylori* (13). The results (Fig. 4) showed that wild-type *H. mustelae* expressed proteins of approximately 27 and 65 kDa that cross-reacted with antisera against UreA and UreB, respectively, and that corresponded in size to the *H. pylori* urease subunits. Transposon insertion into *ureB* abolished expression of the UreB polypeptide as expected, but it also nearly eliminated expression of UreA (Fig. 4). Similar results have been obtained with insertion of a transposon into the *ureB* gene of *H. pylori* (7). Since transposon insertion in *ureB* should not interfere with transcription and translation of the upstream *ureA* gene, this observation may suggest that UreA is unstable in vivo if not complexed with UreB to form the heterodimer.

Sensitivity of wild-type *H. mustelae* and of the urease-negative mutant to varying pH was assayed (23) by incubating approximately 5×10^8 bacteria in 1.0 ml of phosphate-buffered saline (pH 7.4, 3.0, or 1.5) with or without 5 mM urea for 15 min and then plating serial dilutions to determine viable counts (Table 3). Between three and five independent assays were performed for each strain under each condition. The wild-type and the mutant survived equally at pH 7.4, but survival of the mutant compared with the wild type was markedly reduced at pH 3.0. At pH 1.5, wild-type *H. mustelae* survived only if urea was present, while survival of the mutant was poor. Urea concentration did not significantly affect survival of the mutant at any pH. We did not observe a decreased viability of wild-type *H. mustelae* at neutral pH in the presence of urea, as has been described recently for *H. pylori* (2).

In summary, we have used transposon mutagenesis and allelic exchange to construct an isogenic urease-negative mutant of *H. mustelae* that now permits examination of the role of urease in the ferret model of *Helicobacter* infection.

Nucleotide sequence accession number. The DNA sequence of *H. mustelae ureA* and the partial sequence *ureB* from pJAY100 have been deposited with GenBank under accession number L33462.

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