

Urease Is Not Involved in the Virulence of *Yersinia pseudotuberculosis* in Mice

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A chromosomal locus (*ure*) involved in the production of urease activity in the bacterial pathogen *Yersinia pseudotuberculosis* was characterized. The genetic organization of the *Y. pseudotuberculosis ure* locus closely resembles that of the related ureolytic *Yersinia* species *Y. enterocolitica*. This locus encompasses seven open reading frames encoding polypeptides with predicted molecular weights of 10,894 (UreA), 15,820 (UreB), 61,001 (UreC), 25,801 (UreE), 24,551 (UreF), 20,330 (UreG), and 31,308 (UreD). The polypeptides have 85 to 96% identity with the corresponding Ure polypeptides of *Y. enterocolitica* serotype O:8. Restriction fragment length polymorphisms of the *ure* loci from 12 unrelated *Y. pseudotuberculosis* strains produced by *Hae*III and *Mbo*I indicate a low level of genetic variability of this locus in this species. The role of urease in the pathogenicity of *Y. pseudotuberculosis* was studied by constructing an isogenic urease-negative mutant obtained by disruption of structural gene *ureB* by *aphA-3'*, which encodes kanamycin resistance. Experimental infection of mice with this mutant demonstrates that urease is not essential for *Y. pseudotuberculosis* virulence. Urease might be required mostly during the saprophytic life of this pathogen.

Yersinia pseudotuberculosis is a ureolytic gram-negative bacterium widely spread in nature and responsible for sporadic infections in many animal species (4). Humans are occasionally infected with *Y. pseudotuberculosis* following ingestion of contaminated food or water, and pseudoappendicular syndrome is the main clinical form of illness (4). For patients in which the HLA-B27 class I major histocompatibility complex gene product is expressed, infection is frequently complicated by reactive arthritis (4). After crossing the acidic environment of the stomach, *Y. pseudotuberculosis* reaches the intestine and invades the lamina propria of the terminal ileum through M cells. The bacteria multiply in the Peyer's patches and drain into the mesenteric lymph nodes, causing acute mesenteric lymphadenitis. All pathogenic strains harbor a 70-kb plasmid called pYV that is essential for bacterial virulence and that encodes several Yops (*Yersinia* outer membrane proteins) and YadA (*Yersinia* adhesin) (for reviews, see references 3 and 41). However, none of these plasmid-encoded proteins have been proven to have a role in bacterial colonization of the intestinal mucosa. By contrast, the chromosomal outer membrane protein invasin, which is encoded by the *inv* gene, is necessary for efficient penetration of Peyer's patches by *Y. pseudotuberculosis* as well as by the other enteropathogenic *Yersinia* sp., *Yersinia enterocolitica* (19, 20, 32, 38). Additional virulence factors, remaining to be discovered, are most likely required for the infection of the digestive tract.

Among those factors, the urease of *Y. pseudotuberculosis* is an interesting candidate to consider. Ureases, which catalyze the hydrolysis of urea to carbamate and ammonia, are nickel metalloenzymes widely spread in the living world, being produced by bacteria, fungi, and plants (7, 27). They are heteromeric proteins composed, in most cases, of three structural subunits (α , β , and γ), but active urease requires accessory proteins involved in the assembly of the nickel metallocenter within the active site of the enzyme. Thus, the urease gene clusters contain from seven to nine genes depending upon the

species. The primary role of urease is to allow urease-producing microorganisms living in soil and water, such as *Y. pseudotuberculosis* (2), to use urea as a nitrogen source. However, urease can also play a significant role in the pathogenicity of several human pathogens, including *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Helicobacter pylori*, and *Y. enterocolitica* (9, 12, 15, 22). It has been shown that structural subunit β (UreB) of the urease catalytic site of the latter pathogen is present in the synovial fluid of patients with *Yersinia*-induced reactive arthritis (40) and is recognized by *Yersinia*-specific synovial fluid CD4⁺ T cells (33). Direct intra-articular injection of this polypeptide into preimmune rats induces arthritis (24), suggesting that it is an arthritogenic factor. UreB of *Y. enterocolitica* is, therefore, produced in vivo and plays a role in the pathogenesis of this bacterium.

In this work, we cloned and sequenced the complete urease locus of *Y. pseudotuberculosis* and constructed, by allelic replacement, an isogenic urease-negative *Y. pseudotuberculosis* mutant in which the structural *ureB* gene was disrupted. This mutant was found to be as virulent as the wild-type strain in a murine model of infection.

Cloning and sequencing the urease locus from *Y. pseudotuberculosis*. A genomic library from pYV-cured strain IP2777c (38) was constructed in the cosmid pHC79 (17). *Escherichia coli* HB101 transductants were screened for urease activity by replica plating on urea segregation agar which was prepared by combining component A (4 g of yeast extract, 4 g of peptone, 0.34 g of NaH₂PO₄, 1.03 g of Na₂HPO₄, 1 g of gelatin, 5 g of NaCl, 0.90 g of KH₂PO₄, 1.10 g of K₂HPO₄, and 15 g of agar in 900 ml of distilled H₂O [autoclave sterilized]) with component B (9 g of D-glucose, 6 g of urea, and 0.035 g of phenol red in 100 ml of distilled H₂O [filter sterilized]) and ampicillin (Ap; 100 μ g ml⁻¹) (18). The release of ammonia due to the urease activity raised the pH of the medium, inducing a change of phenol red from orange to red. Of the approximately 1,000 clones, a single clone expressed urease activity. This clone contained a 45-kb fragment inserted into cosmid pHC79. A 7.3-kb *Hind*III-*Pst*I fragment still retaining enzymatic activity was subcloned by previously described methods (35) into plasmid pBR325 (the recombinant plasmid was designated pBRU)

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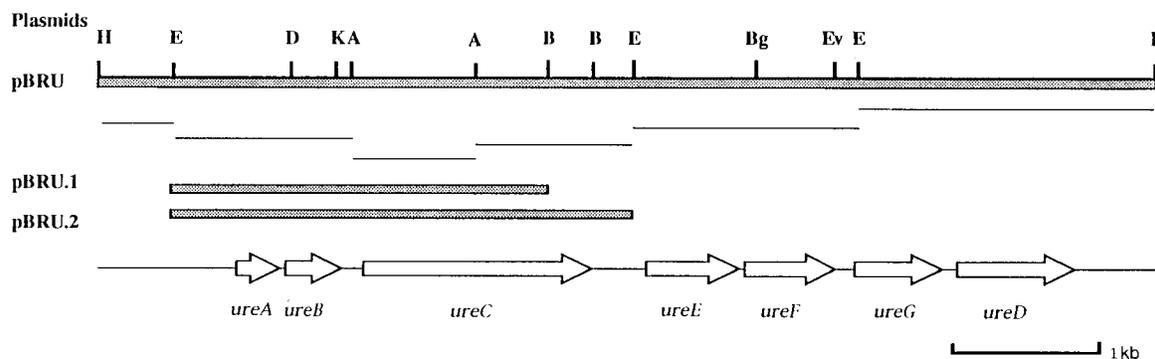


FIG. 1. Map of the *Y. pseudotuberculosis* urease locus. A restriction map is shown for pBRU, a pBR325 recombinant plasmid containing the urease genes of *Y. pseudotuberculosis*. Horizontal lines represent fragments of sequenced DNA. Only inserts of the recombinant plasmid pBRU and derivative plasmids pBRU.1 and pBRU.2 are shown. Open arrows indicate the positions of seven urease genes designated *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, *ureG*, and *ureD*. Abbreviations: A, *Afl*III; B, *Bam*HI; Bg, *Bgl*III; D, *Dra*III; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; K, *Kpn*I; P, *Pst*I.

(1). After restriction mapping of plasmid pBRU, smaller fragments were generated (Fig. 1) and inserted into the M13 mp18 and M13 mp19 sequencing vectors (25).

A 7,333-bp segment encompassing the urease locus was sequenced on both strands by the dideoxynucleotide chain termination method (36) with modified T7 DNA polymerase (Sequenase version 2.0), according to the manufacturer's instructions (Amersham France), and with α -³⁵S-dATP (Amersham France). Nucleotide sequence analysis revealed seven open reading frames, all in the same direction. With reference to the *ure* gene complex of *Y. enterocolitica* recently described (10), they were designated *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, *ureG*, and *ureD*. All the initiation codons of the *ure* genes were preceded by putative ribosome-binding sites. In comparison to the urease gene cluster of *Y. enterocolitica*, larger intergenic regions (between *ureC* and *ureE* and between *ureG* and *ureD*) were found in the *Y. pseudotuberculosis* gene cluster. The seven genes encoded putative polypeptides of 99, 144, 572, 231, 225, 187, and 281 amino acids with calculated molecular masses of 10,894, 15,820, 61,001, 25,801, 24,551, 20,330, and 31,308 Da, respectively. Structural subunits UreA (subunit γ ; pI = 4.3), UreB (subunit β ; pI = 9.3), and UreC (subunit α ; pI = 5.4) and accessory proteins UreE (pI = 5.9), UreF (pI = 5.9), UreG (pI = 5.3), and UreD (pI = 6.5) of *Y. pseudotuberculosis* shared 89.8, 90.2, 96.8, 93.9, 95.5, 83.4, and 85% amino acid identity, respectively, with the corresponding Ure polypeptides of *Y. enterocolitica* serotype O:8 (Fig. 2).

Urease is a nickel enzyme, and it has been demonstrated by crystallographic analysis of the *Klebsiella aerogenes* urease that four histidine residues, an aspartate, and a carbamylated lysine present in UreC are important in coordinating the two nickel ions at the active site (21). These amino acids are conserved in the UreC polypeptide of *Y. pseudotuberculosis* (His-139, -141, -251, and -277; Asp-364; and Lys-222). Other residues present in the UreC subunit of *K. aerogenes* and described as important in substrate binding and catalysis are conserved in the corresponding Ure polypeptide of *Y. pseudotuberculosis* (Gly-172, His-224, Cys-323, His-324, Ala-367, and Met-368) (Fig. 2). The UreE protein possesses a histidine-rich motif at the carboxy termini, as do UreE proteins of *K. aerogenes*, *Y. enterocolitica*, *P. mirabilis*, *Haemophilus influenzae*, and *H. pylori* (8, 10, 13, 23, 29). Finally, as in *Y. enterocolitica* and other ureolytic bacterial species (8, 10, 13, 23, 29), the UreG polypeptide of *Y. pseudotuberculosis* (Fig. 2) contains an ATP binding site (GXGG PVGXGKT) (37) which possibly provides the energy required for nickel activation.

Genetic homogeneity of the urease locus in wild-type isolates of *Y. pseudotuberculosis*. Restriction fragment length polymorphisms of the urease loci from a collection of 12 strains (IP1553, IP1554, IP1833, IP2515, IP2775, IP2777, IP2781, IP2783, IP2790, IP2821, IP2823, and IP2840), all urease positive and epidemiologically unrelated on the basis of molecular typing (30), were studied. Amplification of regions 1 to 4 (738 bp, 2,557 bp, 1,177 bp and 1,528 bp, respectively) encompassing the complete urease locus (Fig. 3) was carried out by PCR with primer sets defined from the nucleotide sequence of the *ure* locus of strain IP2777. PCR amplification was performed with a 100- μ l reaction volume and a model 480 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) as previously described (39). Amplified PCR products of the expected sizes were obtained from all tested strains and were digested by *Hae*III and *Mbo*I, two endonucleases cutting many sites within the *ure* locus. As shown in Fig. 3, the *ure* locus displays a remarkable homogeneity and a low level of genetic variability, thus contrasting with the considerable variation of the *H. pylori* urease locus (6, 14, 28).

Construction of a *Y. pseudotuberculosis* urease mutant and genetic complementation. To determine the role of urease in the pathogenicity of *Y. pseudotuberculosis*, a urease-deficient mutant was constructed from strain IP2777c by disrupting the *ureB* gene with a kanamycin (Km) resistance gene cassette as follows. From plasmid pBRU.1 (2.6-kb *Eco*RI-*Bam*HI fragment from pBRU cloned into pBR325 [Fig. 1]), a 309-bp *Dra*III-*Kpn*I fragment internal to *ureB* was deleted and replaced with a 1,240-bp *Pst*I fragment of plasmid pUC4K (44) encoding an aminoglycoside 3' phosphotransferase [APH (3')] type I hydrolyzing Km but not butirosin (42). The resulting insert was ligated to mobilizable suicide plasmid pGP704, which replicates only in strains containing a *pir* gene encoding the π protein (26). The recombinant plasmid (pBRU.3) was delivered, by electroporation (11), into pYV-cured strain IP2777c, and transformants were screened on Luria-Bertani (LB) agar with Km (25 μ g ml⁻¹) and Ap. Because the plasmid cannot replicate in this bacterium lacking the *pir* gene, Km^r and Ap^r *Yersinia* clones must have integrated the plasmid into the chromosome through recombination at homologous sites. A heterozygous merodiploid clone (Km^r and Ap^r), chosen randomly, was subcultured 12 times without Ap selection, and passaged bacteria were plated on LB agar plus Km and screened for Ap susceptibility. Clone MU2777, which was resistant to Km but susceptible to Ap, exhibited the same in vitro growth and biochemical characteristics as IP2777c except that

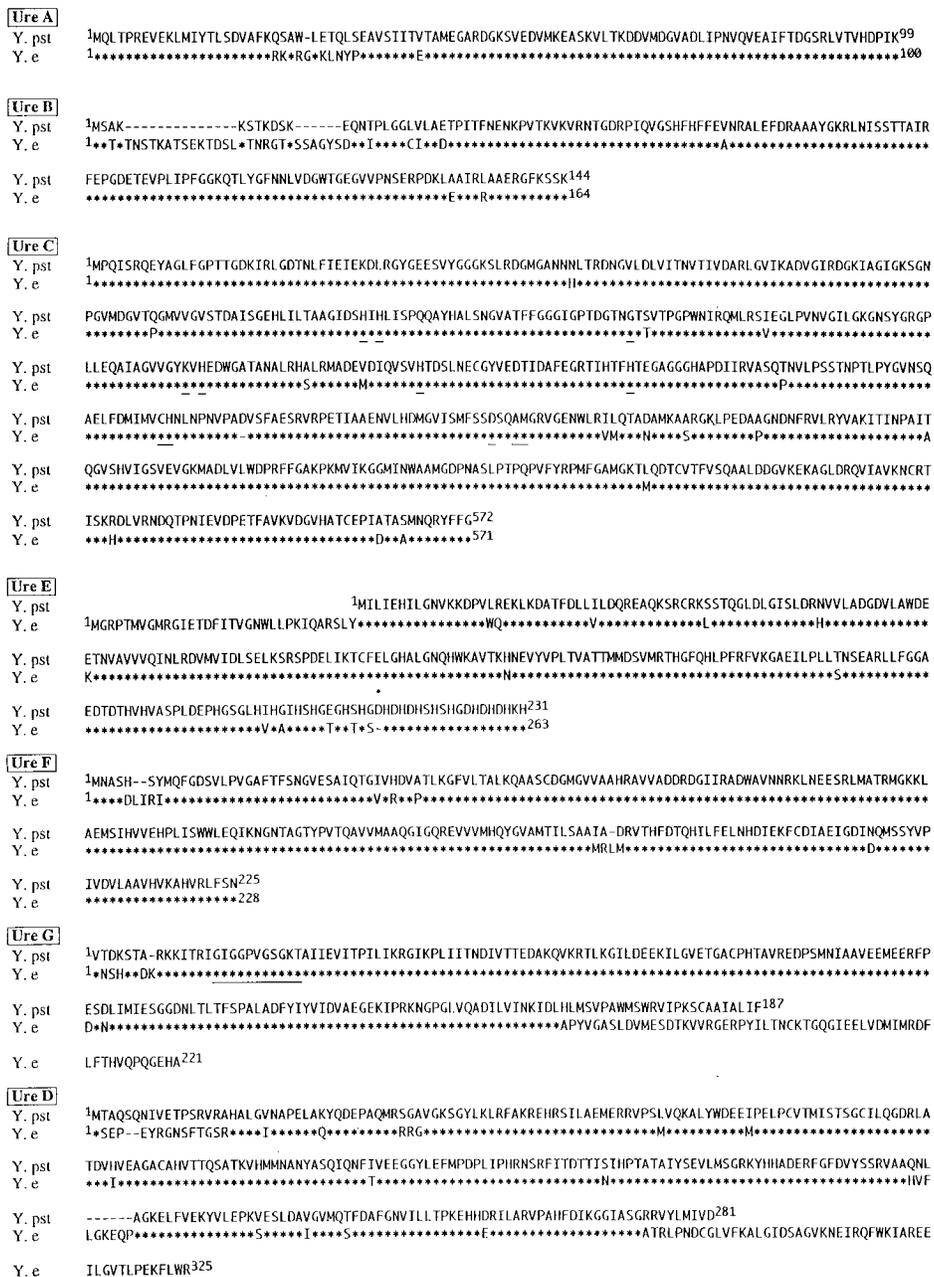


FIG. 2. Alignment of the amino acid sequences deduced from the nucleotide sequences of the *ure* gene complexes from *Y. pseudotuberculosis* (*Y. pst*) and *Y. enterocolitica* serotype O:8 (*Y. e*). Asterisks indicate identical amino acids. Dashes are gaps introduced to optimize homology between the sequences. Amino acids conserved in the enzymatic active sites of several ureolytic species are underlined.

it was devoid of ureolytic activity. Chromosomal DNA from the urease-deficient mutant was analyzed to confirm that only a single copy of *aphA-3'* was integrated into the chromosome, considering that the *aphA-3'* gene from Tn903 contains a single *HindIII* restriction site but no *EcoRI* restriction site (31). By Southern blot hybridization of digested DNA with an α -³²P-labeled probe internal to *aphA-3'* performed as previously described (30), a single band was found after restriction with *EcoRI* and two bands were found after restriction with *HindIII* (data not shown). In addition, from mutant MU2777, the DNA region flanking *aphA-3'* was amplified by PCR with primers

internal to *ureB*. Sequencing this PCR product showed that the *ureB* gene was disrupted at the expected site (position 1,359). The next step was to introduce the virulence plasmid pYV into urease-deficient mutant MU2777. We used pYV::Tn5, which expressed a fully virulent phenotype in *Y. pseudotuberculosis*. This recombinant plasmid was prepared as follows. A library of Km-resistant mutants was obtained from strain IP2777 (pYV⁺) after mating the recipient strain for 4 h at 30°C with the donor strain, *E. coli* SM10 (λ pir), harboring plasmid pMS90, a pJM703.1 derivative in which transposon Tn5 was inserted. This transposon encodes an APH (3') type II confer-

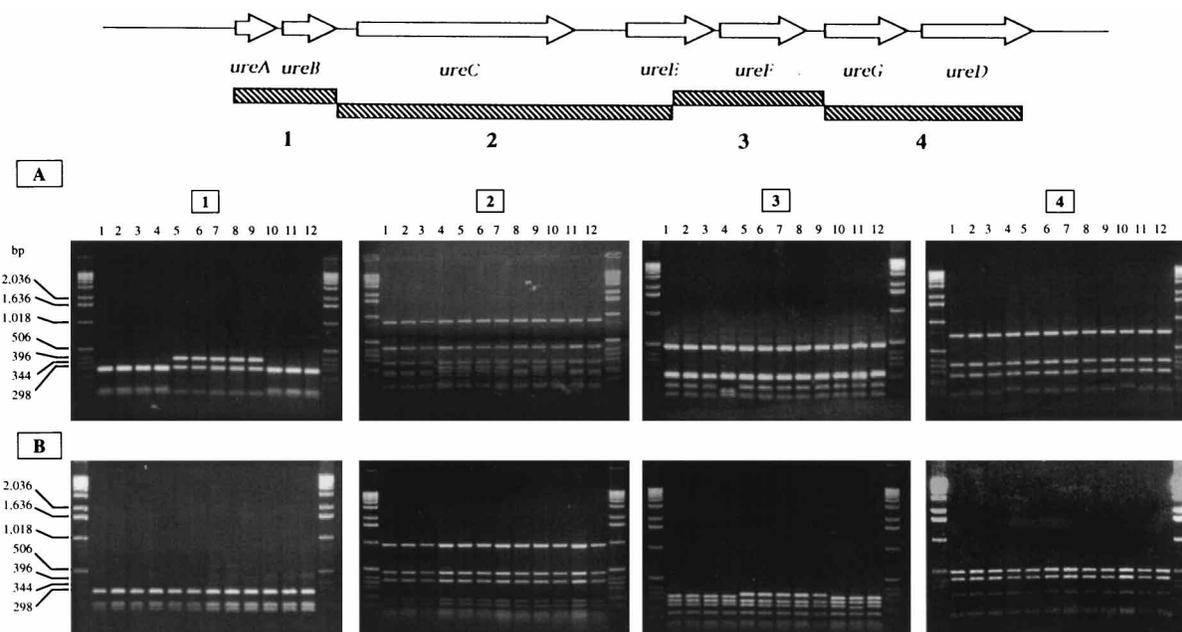


FIG. 3. PCR and restriction fragment length polymorphism of urease locus from *Y. pseudotuberculosis*. Four regions (1, 2, 3, and 4) of the urease loci of 12 *Y. pseudotuberculosis* strains were amplified by PCR with four primer sets (primer set 1, 5'-GATCTACACGCTGTCTGA-3' and 5'-TAAAACCACGCTCGGCGGCA A-3'; primer set 2, 5'-TTGCCCGCCGAGCGTGGTTTAA-3' and 5'-ACGGAGTCCATCATGGTG-3'; primer set 3, 5'-CACCATGATGGACTCCGT-3' and 5'-CC GGACCACCAATACCAAT-3'; primer set 4, 5'-ATTGGTATTGGTGGTCCGG-3' and 5'-GGGCTATCTTCCAAAATT-3'). PCR products were digested with *Hae*III (A) or *Mbo*I (B) according to the instructions provided by the manufacturer (New England Biolabs) and then were separated by electrophoresis in a 1.5% agarose gel in 1× Tris-borate-EDTA buffer. Lanes: 1, strain IP1553; 2, strain IP1554; 3, strain IP1833; 4, strain IP2515; 5, strain IP2775; 6, strain IP2777; 7, strain IP2781; 8, strain IP2783; 9, strain IP2790; 10, strain IP2821; 11, strain IP2823; 12, strain IP2840.

ring resistance to Km and butirosin (42). Like plasmid pGP704, this plasmid cannot replicate in *Yersinia* (Pir⁻). Transconjugants were selected on LB agar plates containing Km and colistin (25 µg ml⁻¹). The latter antibiotic inhibits the growth of *E. coli* but not that of *Y. pseudotuberculosis* (43). Plasmid pYV was extracted from pooled transconjugants and then introduced by electroporation into pYV-cured strain IP2777c. Transformants selected on LB agar with Km were pooled, and clones still virulent were picked by several passages in a mouse inoculated intravenously with a low-level bacterial challenge (10⁴ bacteria in 0.5 ml of phosphate-buffered saline [PBS]). One clone, designated IP2777.4, was found to be as virulent as wild-type strain IP2777 (pYV), and its in vitro growth was calcium dependent (Lcr⁺ phenotype), as assessed on Mox agar (16). In addition, it contained a single copy of Tn5 on plasmid pYV as determined by Southern blot hybridization of *Bam*HI-restricted pYV fragments (there is one *Bam*HI restriction site within Tn5) with a specific probe. Strain MU2777 was electrotransformed with plasmid pYV::Tn5, and transformants were selected on agar supplemented with butirosin (recombinant strain designated MU2777.4).

Finally, plasmid pBRU.2, a 3.1-kb *Eco*RI-*Eco*RI fragment encompassing the three structural genes (Fig. 1) inserted into plasmid pACYC184 (5), was constructed and introduced into strain MU2777.4. This *trans* complementation of the mutant resulted in the restoration of the ureolytic activity.

Virulence of the urease-deficient mutant of *Y. pseudotuberculosis*. The virulence of the urease-deficient mutant MU2777.4 and IP2777.4, both of which are strains harboring plasmid pYV::Tn5, was studied by infecting 6- to 8-week-old Swiss female mice (Janvier, Le Genest-Saint-Isle, France). The animals were given 10⁸ *Y. pseudotuberculosis* bacteria (in 0.2 ml of sterile water), grown at 30°C, by the intragastric route through

a gastric tube, a bacterial inoculum corresponding to about 0.3 50% lethal doses (LD₅₀) of control strain IP2777.4. Two hours after intragastric inoculation, the infected mice were sacrificed by cervical dislocation and bacteria were counted in ileum washings to assess the protective effect of urease against acidity, as described previously for *Y. enterocolitica* (9). A 10-cm-long portion of ileum was flushed three times with 1 ml of sterile PBS, and ileum washings were spread on agar with vancomycin (50 µg ml⁻¹) and Km and incubated for 48 h at 30°C. *Yersinia* colonies were identified on the basis of colony

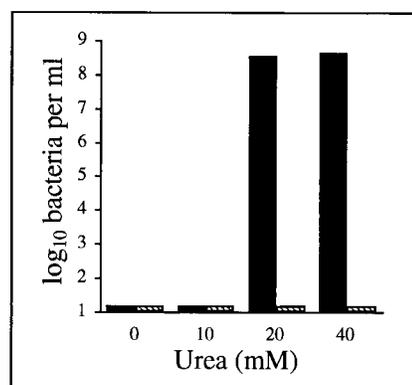


FIG. 4. Susceptibility to acidity of the urease-deficient mutant. Overnight bacterial cultures at 30°C were pelleted, and cells were resuspended (10^{8.85} to 10^{8.9} per ml) in PBS (pH 2) containing different concentrations of urea. The survival of *Y. pseudotuberculosis* after a 90-min contact at 37°C in the acidic environment is shown. The minimal detection limit was 10 CFU/ml. Symbols: solid column, wild type; hatched column, mutant.

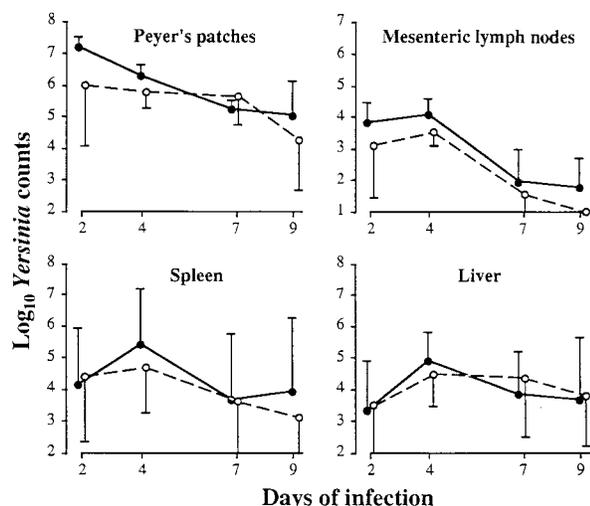


FIG. 5. Virulence of the urease-deficient mutant in mice. Swiss mice were given 10^8 *Yersinia* bacteria by the intragastric route. Bacterial counts in Peyer's patches, mesenteric lymph nodes, spleens, and livers were determined on days 2, 4, 7 and 9 after challenge. Minimal detection limits were 10 CFU for Peyer's patches and mesenteric lymph nodes, 30 CFU for ileum washings, and 10^2 CFU for spleens and livers. Each data point shows the mean value \pm the standard deviation for four animals. Symbols: ●, wild type; ○, mutant.

morphology and biochemical characteristics with API 20E strips (bioMérieux, La Balme-les-Grottes, France). No difference was detected between the two strains ($10^{3.85} \pm 10^{0.99}$ versus $10^{3.40} \pm 10^{0.44}$ CFU [means \pm standard deviations] in ileum washings for the urease-positive and -negative strains, respectively; $P > 0.10$). This result contrasts with a recent report showing, in a similar experimental model of infection, that a urease-negative mutant strain of *Y. enterocolitica* produced by *ureB* inactivation was recovered in ileum homogenates, 90 min after challenging mice, in smaller numbers (10-fold) than the wild-type strain, thus indicating a protective role against the host's natural defenses for urease (9). This discrepancy might be explained by the fact that *Y. pseudotuberculosis* tolerates acidity (pH 2) in vitro only when the concentration of urea reaches at least 20 mM (Fig. 4), a concentration 15-fold higher than that found in the gastric secretion of mammals (reference cited in reference 9). In contrast, *Y. enterocolitica* tolerates acidity at a concentration of urea of 0.3 mM (9). At days 2, 4, 7, and 9 following the intragastric challenge, Peyer's patches (four follicles per mouse), mesenteric lymph node chains, spleens, and livers were removed aseptically from infected animals and homogenized in PBS, and bacterial counts in organic homogenates were determined as described above. As shown in Fig. 5, bacterial counts for these tissues were found not to differ when mice were inoculated with either strain. In addition, the LD₅₀ for mice (34) of strain MU2777.4 introduced by the intravenous route, as determined on groups of five mice inoculated with progressive doses of bacteria ($10^{2.7}$ to $10^{4.7}$ bacteria), was similar to that of control strain IP2777.4 (LD₅₀ $< 10^2$ bacteria). Altogether, these results clearly indicate that urease is not a virulence factor in *Y. pseudotuberculosis* but rather might be required for the saprophytic life of this pathogen.

Nucleotide sequence accession number. The sequence obtained in this study was assigned GenBank accession number U40842.

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