

Yersinia pseudotuberculosis Harbors a Type IV Pilus Gene Cluster That Contributes to Pathogenicity

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Received 13 May 2002/Returned for modification 25 June 2002/Accepted 29 July 2002

Fimbriae have been shown to play an essential role in the adhesion of pathogenic gram-negative bacteria to host cells. In the enteroinvasive bacterium *Yersinia pseudotuberculosis*, we characterized a previously unknown 11-kb chromosomal locus involved in the synthesis of type IV pili. The locus consists of 11 open reading frames forming a polycistronic unit and encoding putative Pil proteins, PilLMNOPQRSUVW. When introduced into *Escherichia coli*, the *Y. pseudotuberculosis* operon reconstituted bundles of filaments at a pole on the bacterial surface, demonstrating that the *pil* locus was functional in a heterogenous genetic background. Environmental factors regulated transcription of the *Y. pseudotuberculosis* operon; in particular, temperature, osmolarity, and oxygen tension were critical cues. Deletion of the type IV pilus gene cluster was associated with a reduction of *Y. pseudotuberculosis* pathogenicity for mice infected orally. Forty-one percent of *Y. pseudotuberculosis* strains isolated from human or animal sources harbored the type IV pilus locus. Therefore, the *pil* locus of *Y. pseudotuberculosis* might constitute an “adaptation island,” permitting the microorganism to colonize a vast reservoir.

Type IV pili are appendages emanating from the surfaces of several gram-negative bacteria, including species pathogenic for animals and plants (*Actinobacillus pleuropneumoniae*, *Aeromonas veronii* biovar *sobria*, *Aeromonas caviae*, *Dichelobacter nodosus*, *Eikenella corrodens*, enteropathogenic *Escherichia coli* [EPEC] and enterotoxigenic *E. coli* [ETEC], *Legionella pneumophila*, *Moraxella bovis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Vibrio cholerae*) (9, 11, 12, 15, 16, 18, 19, 20, 21, 25, 27, 40, 45, 49). These structures, which may be peritrichous or polar on the cell surface, sometimes form bundles and have been implicated in a variety of bacterial functions, including cell adhesion (24), bacteriophage adsorption (17, 19), plasmid transfer (19), and twitching motility, a form of flagellum-independent locomotion (26, 44). Pili are composed of pilin subunits, the primary structures of which are conserved (24). All pilins are produced from prepilin molecules through cleavage of the leader sequence by a prepilin peptidase. There is a long hydrophobic segment (20 to 30 amino acid residues [aa]) at the N-terminal region of the mature pilin protein. Type IV pili are classified into two subclasses, IVA and IVB, on the basis of similarities in the deduced amino acid sequences of prepilins. Type IVA prepilins have a very short leader sequence (5 to 6 aa), and the N-terminal amino acid of the mature pilin is a methylated phenylalanine. Type IVB prepilins tend to have longer signal sequences (13 to 30 aa), and the N-terminal amino acid of the mature protein may be a methionine (methylated in some cases), leucine, tryptophan, or serine. The assembly machinery involved in the formation of fimbriae con-

sists of a set of proteins encoded by genes either scattered throughout the bacterial genome (IVA subclass) or organized into operons consisting of 11 to 14 genes (IVB subclass). Many of the proteins involved in type IV pilus biogenesis are homologous to proteins involved in type II secretion and natural transformation (DNA uptake) systems in various bacteria (6, 7, 10, 14, 18, 28, 30, 34, 41).

Fimbriae of the IVB subclass have been described exclusively in enteric pathogens such as *V. cholerae*, *E. coli* (EPEC and ETEC), and *S. enterica* (1, 11, 12, 43, 48). The gram-negative bacterium *Yersinia pseudotuberculosis* is responsible for animal and human infections of the digestive tract (ileitis and mesenteric lymphadenitis) following ingestion of contaminated food or water (3). Here, we provide evidence that the enteropathogenic species *Y. pseudotuberculosis* also harbors a functional IVB subclass fimbrial gene cluster. We report the genetic organization of this cluster and its distribution among strains of this species. We also show that the type IV pilus gene cluster contributes to the pathogenicity of *Y. pseudotuberculosis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Ninety-two human and animal strains of *Y. pseudotuberculosis* were used: 47 were of serotype I (367/89, 376/89, 892/97, 102/88, 442/93, 310/89, YPT2, YPT3, YPT4, YPT6, YPT7, YPT8, YPT9, YPT10, YPT11, YPT12, 347/89, 72/91, 2/91, 2775, 32777, 32953, 2783, 2790, 2853, WS53/94, WS37/95, WS27/93, WS31/96, WS34/94, 33036, 33034, 33052, 32845, 32849, 33053, 32982, 417/94, 40/92, 736/91, 3625/92, 1924/95, 9314/74, 58/87, 1937/88, 2063/88, and 145/89), 11 were of serotype II (165/89, 297/89, 300/89, MA, 1432/94, 2515, 2892, 2926, 2929, WS25/91, and 33054), 22 were of serotype III (304/89, 201/90, 97/88, 32829, 32842, 32984, 32826, 32861, 32945, 32977, 32975, 32992, YPIII, 2666, 2887, 2889, YPT1, YPT5, 32607, 33001, 766/95, and 1216/93), 5 were of serotype IV (ST, AH, NT, 3255/96, and 1830), 4 were of serotype V (199/90, 2821, 2843, and 99/91), and 3 were of serotype VI (682/90, 1553, and 1554). They were kindly provided by S. Aleksić (Hygiene Institut Hamburg, Hamburg, Germany), E. Falsen (University of Göteborg, Göteborg, Sweden), D. Caugant

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics ^a	Reference or origin
pUC18	Cloning vector; Ap	Appligene
pACYC184	Cloning vector; Tet	New England BioLabs
pHC79	Cloning vector; Ap	Stratagene
pCVD442	Suicide vector containing the counterselectable marker <i>sacB</i> ; Ap	5
pUC4K	Km; source of aminoglycoside phosphotransferase <i>aphA-Ia</i> gene	Amersham Pharmacia
pLS92.1	pUC18 Ω ; 3.4-kb <i>EcoRV</i> -digested DNA from <i>Y. pseudotuberculosis</i> 32777	This work
pMM2.1	pHC79 Ω ; 30- to 35-kb partially <i>Sau3A</i> -digested DNA from <i>Y. pseudotuberculosis</i> 32777	This work
pMM2.A6	pHC79 Ω ; 30- to 35-kb partially <i>Sau3A</i> -digested DNA from <i>Y. pseudotuberculosis</i> 32777	This work
pMM3.D6	pHC79 Ω ; 30- to 35-kb partially <i>Sau3A</i> -digested DNA from <i>Y. pseudotuberculosis</i> 32777	This work
pMM5	pACYC184 Ω ; 10.8-kb <i>NcoI</i> -digested fragment from pMM2.1	This work
pMM1	pUC18 Ω ; 1,693-bp <i>SstI/BamHI</i> PCR-generated fragment with primers 1 and 2 encompassing the upstream region of <i>pilL</i> from <i>Y. pseudotuberculosis</i> 32777	This work
pMM2	pMM1 Ω ; 1,162-bp <i>BamHI/PstI</i> PCR-generated fragment with primers 3 and 4 encompassing part of <i>pilV</i> and its 3' flanking region from <i>Y. pseudotuberculosis</i> 32777	This work
pMM3	pMM2 Ω ; 1,264-bp <i>BamHI</i> -digested fragment from pUC4K containing <i>aphA-Ia</i>	This work
pMM4	pCVD442 Ω ; 4,524-bp <i>SstI</i> -digested fragment from pMM3	This work

^a Ap, Km, and Tet, resistance to ampicillin, kanamycin, and tetracycline, respectively; Ω , in vitro insertion.

(National Institute of Public Health, Oslo, Norway), E. Carniel (Institut Pasteur, Paris, France), R. Van Noyen (Imelda Hospital, Bonheiden, Belgium), and N. Takeda (Kurashiki Central Hospital, Okayama, Japan). Strain MIV is a mutant engineered from the parent strain 32777 (serotype 1). The *E. coli* DH5 α , NM554 (32), and MC1061 strains (46) were used as hosts for cloning. *E. coli* SY327 λ pir and SM10 λ pir were recipients for replication of the suicide vector pCVD442 and its derivatives. The cloning vectors used in this work and the recombinant plasmids constructed in this study are listed in Table 1.

Bacterial growth conditions. *Y. pseudotuberculosis* and *E. coli* strains were grown routinely in Luria-Bertani (LB) broth or agar medium. Ampicillin (100 μ g ml⁻¹), tetracycline (12 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), sucrose (10%), IPTG (isopropyl- β -D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) were added to media for bacterial selection when necessary. For anaerobic bacterial growth, LB broth was first boiled to remove dissolved air, and liquid paraffin was added to the hot medium. For gene transcription studies, the osmolarity of the LB broth was adjusted by adding 100 mM KCl or 200 mM L-arabinose (final concentrations). The pH of the LB broth was adjusted to 5.5, 7.0, or 8.5 and maintained by supplementing the medium with sulfonate buffers with the appropriate pK_a values: MOPS [3-(*N*-morpholino)propanesulfonic acid], pK_a = 7.2; MES [2-(*N*-morpholino)ethanesulfonic acid], pK_a = 6.1; and TAPS [tris(hydroxymethyl)methyl-aminopropanesulfonic acid], pK_a = 8.4 (29). The pH of each medium was measured before and after bacterial growth to ensure it did not change (<0.1 U).

DNA and RNA preparation, analysis, and hybridization. Genomic DNA extraction and small-scale plasmid DNA isolation were performed as previously described (8). Large-scale plasmid DNA preparations were purified on Qiagen columns according to the manufacturer's recommendations. DNA was digested with the appropriate restriction endonucleases purchased from Life Technologies or Roche Diagnostics, and the resulting fragments were separated by gel electrophoresis. Restriction fragments were eluted from agarose gels with the Qiaquick gel extraction kit (Qiagen GmbH). DNA fragments were transferred from gels to Hybond-N⁺ membranes (Amersham) by the Southern technique. Colony blotting was performed as previously described (36).

Total RNAs were extracted from growing *Yersinia* cells with the SV total RNA isolation kit (Promega) following the manufacturer's instructions. For slot blot analysis, RNA was spotted onto a Hybond-N⁺ membrane.

The DIG hybridization and detection kit (Roche Diagnostics) was used for hybridization. Hybridization was carried out under stringent conditions with a digoxigenin-labeled DNA probe.

Synthetic oligonucleotides. Oligonucleotide primers were custom synthesized (Sigma and Genset) for PCR generation of DNA fragments used for cloning or probing or for reverse transcriptase (RT)-PCR assays. Their nucleotide sequences (5'-3') were as follows: YopH1, CATCGTCAGGTATCTCGA; YopH2, CAATCAGTTGCGCAGTAC; 1f, TCCGTTTCGACAGAATGC; 1r, TTGTGCTGGGCGGTGGTGT; 2f, GCCGAGGTAGGCTCAAC; 2r, GGCTGGCTTCGCAAACA; 3f, GGCTGAAGGGAGTATTG; 3r, AAGGCTGAGTTTTAGCGC; 4f, GGTATAACGGTCTGG; 4r, CGGTAATCTGTTGCGCT; 5f, GGTGTTAGCCAGCATAGA; 5r, GGATAGATTCAGTTGGC; 6f, TGTCGTTG

AGGTCAGT; 6r, CGAACTATCAGCTATACG; 7f, TGTGCATTGCCAGCAA; 7r, ATCGGTATGAACCTCC; 8f, TGAGCGAGTCAACAAGCG; 8r, TAAAGGATACCCAGCCA; 9f, AGGACACTGGCAGAATCG; 9r, GAGGTCATATTGCTGAAC; 10f, AGTCAAGGTCTATGATCC; 10r, AAGCGCGATAAATGCAGC; 11f, CACGCTATTGGACACA; 11r, GCACGTCAGAATA GTAG; 12f, TATGTTGGCCAATGGC; 12r, GTTAGTCGATCAGCTAC; 1, AAAAGAGCTCAGCCACTATGCCATTGGTC; 2, CGCGGATCCCGGATGATAGTAAAGC; 3, CGCGGATCCAGCTATTAGCCTCTGCTGG; 4, AAAACTGCAGGAGCTCTCAGTGTGAGCAATCACTC; pilrs1, TCCTTGC TGACCGAAGGTG; pilrs2, AGGTACGCCACCGACTGA; pil7, AAAGTT TTAACATAATCGC; pil6, CGTTTTTTTATTTCCTCCG; pil5, GCGTTGCCA CCCGTTCCCGC; pil3, ACCATTTCCGGCGGGCATGCTGATA; 5'REG1, CTCTGTTGGCGATGGCTT; 5'REG2, AATGACATTGGCCAGC; 3'REG1, GATCGACGATCTGGTC; 3'REG2, GCAGTAGATGTCAGTG; RRNA1, GAGATTTGATCCCTGGCTCAG; and RRNA2, AAGGAGGTGATCCAGC CG.

PCR. PCR amplification was performed as described elsewhere (37) with AmpliTaq Gold polymerase purchased from Perkin-Elmer Applied Biosystems. Digoxigenin-labeled PCR products were generated with the PCR DIG labeling mix from Roche Diagnostics. PCR products were purified on Dye-ex spin columns or with a Qiaquick PCR purification kit purchased from Qiagen. RT-PCR assays were performed following the manufacturer's recommendations with a kit (Access RT-PCR system) purchased from Promega.

DNA cloning and sequencing. DNA fragments were ligated to endonuclease-restricted vectors according to standard techniques with T4 DNA ligase (Life Technologies). Recombinant plasmid DNA was introduced into *E. coli* by transformation. Chromosomal DNA inserted into the cosmid pHC79 was packaged into bacteriophage lambda head particles with a packaging kit (Giga Pack II) purchased from Stratagene, and *E. coli* NM554 cells were transduced as described elsewhere (36).

DNA was sequenced by the dideoxy chain termination method using the ABI PRISM dichlororhodamine Dye Terminator sequencing kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) according to the manufacturer's instructions. Extension products were analyzed with the Applied Biosystems model ABI 377XL automated DNA sequencer (Perkin-Elmer). The nucleotide sequences obtained were analyzed with Perkin-Elmer software (Sequence Navigator).

Transmission electron microscopy (TEM). Bacteria were scraped from 24-h cultures grown at 37°C on the surface of LB agar and suspended in 30 μ l of 0.05 M Tris-buffered saline (pH 7.4). A carbon-coated Formvar copper grid (200 mesh; Electron Microscopy Sciences) was placed on the bacterial suspension for 2 min. The grid was then stained for 2 min with 4% uranyl acetate, washed twice for 15 s each time with distilled water, and finally air dried. The grid was examined in a Hitachi-7500 transmission electron microscope.

Mouse experimental infection. Six week-old female inbred BALB/c mice (Iffa Credo) were challenged either by the intravenous route (a 0.3-ml bacterial suspension in sterile phosphate-buffered saline) or by the intragastric (i.g.) route (0.2 ml of bacterial suspension in sterile distilled water) using a gastric tube. For gastric inoculation, the mice were first starved for 18 h. Bacterial inoculums were

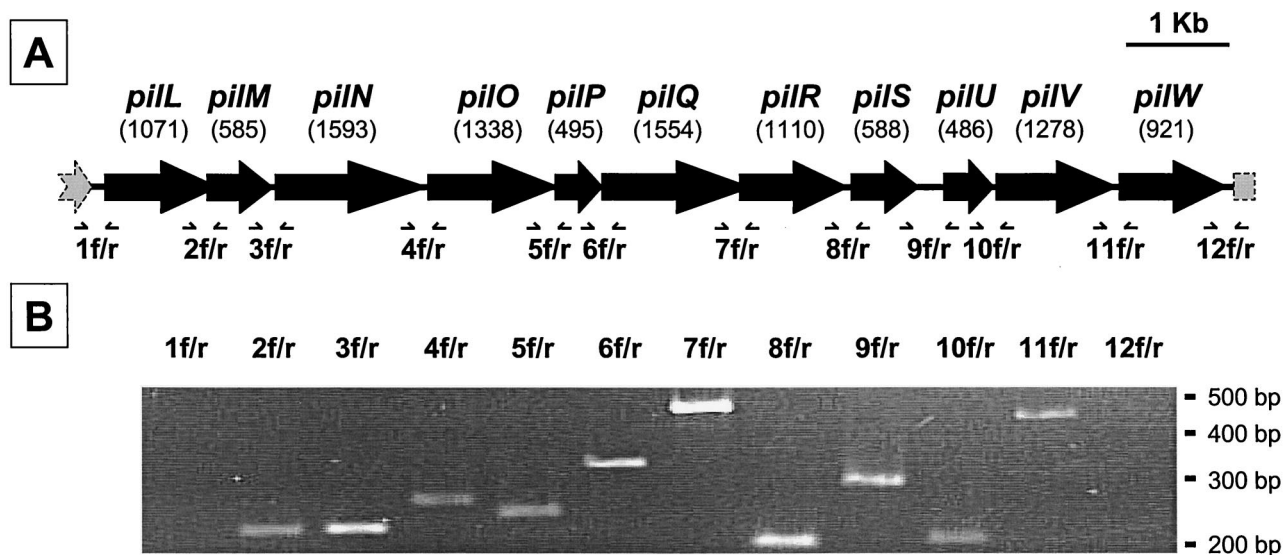


FIG. 1. The *Y. pseudotuberculosis* *pil* operon. (A) Genetic organization of the *pil* gene cluster. The *pil* gene cluster consists of 11 genes (the sizes in base pairs are shown in parentheses) with the same polarity and apparently arranged in an operon-like manner (see the text for details). (B) Transcriptional analysis of the *pil* gene cluster. RNA extracted from exponentially growing *Y. pseudotuberculosis* cultures at 37°C were analyzed by RT-PCR using primer sets 1 to 12. Forward (f) and reverse (r) primers were designed within adjacent genes of the *pil* locus and flanking regions (panel A). The amplification products were analyzed by agarose gel electrophoresis.

prepared from overnight cultures in LB broth at 28°C. The cultures were centrifuged, and the bacterial pellets were washed once and resuspended in distilled water or phosphate-buffered saline. The animals were kept in positive-pressure cabinets during experimentation, and mortality was monitored daily for 21 days after challenge. For each experimental infection, the presence of the virulence plasmid pYV was confirmed by PCR on bacterial thermolysates using the YopH1 and YopH2 primers, which are internal to *yopH*, a gene located on pYV (4). Infected animals were monitored for 3 weeks, and the 50% lethal dose (LD₅₀) was calculated from groups of 5 (intravenous model) or 10 (i.g. model) mice according to the method of Reed and Muench (33).

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the GenBank nucleotide sequence database under accession number AY047316.

RESULTS

***Y. pseudotuberculosis* strain 32777 harbors a type IV pilus gene cluster.** While cloning for another research project, we isolated pLS91.2, a recombinant plasmid bearing a ~3.4-kb *EcoRV* DNA fragment from *Y. pseudotuberculosis* 32777. Analysis of the sequence of the DNA insert revealed the presence of four open reading frames (ORFs) (*orf1* to *-4*), only two of which (*orf2* and *orf3*) were complete. Similarity searches with the deduced amino acid sequences of the proteins encoded by these ORFs showed that ORF1, ORF2, ORF3, and ORF4 displayed 55, 57, 59, and 55% similarity to PilL, PilM, PilN, and PilO, respectively, of *S. enterica*. These Pil proteins are thought to be involved in the biogenesis of type IV pili in this pathogen (19). To identify the complete type IV pilus gene cluster in *Y. pseudotuberculosis*, a cosmid library was constructed from strain 32777 DNA. Briefly, bacterial DNA partially digested with *Sau3A* was ligated to cosmid pHC79 at the *Bam*HI site, and *E. coli* NM554 was transformed. Recombinants were further screened by colony blot hybridization under stringent conditions, using PCR-generated DNA probes at the 5' and 3' ends of the pLS91.2 insert. Three positive

clones, NM554 (pMM2.1), NM554 (pMM2.A6), and NM554 (pMM3.D6), were isolated.

The type IV pilus gene cluster from *Y. pseudotuberculosis* forms a single polycistronic unit. Cosmid pMM2.1 contained an 11-kb locus composed of 11 ORFs, the organization of which is shown in Fig. 1A. In terms of the arrangement of the first 10 genes and their products (see below), the *Y. pseudotuberculosis* gene cluster strongly resembles the *pil* locus recently discovered in *S. enterica* (19, 48). For this reason, we adopted, by analogy, the gene designation put forward for the *Salmonella pil* genes. The *pil* cluster was incomplete in cosmid pMM2.A6 and in pMM3.D6, which contained only *pilLMN* and *pilNOPQRSUVW*, respectively.

The mean G+C content of the *pil* cluster was 50.8%, and for each *pil* gene, the percentage of G+C residues was between 47.6 (*pilW*) and 53.3% (*pilU*). All *pil* ORFs were present on the same strand, and intergenic spaces ranged from -146 to 179 nucleotides. Some *pil* genes were preceded by a putative Shine-Dalgarno sequence. Taken together, these facts suggested that the *pil* gene cluster may be transcribed as a single unit. The instability of the large mRNA transcript made Northern blot analysis impossible. The polycistronic organization of the locus was confirmed by RT-PCR experiments on total RNA extracted from strain 32777 using 12 sets of primers (1 to 12) (Fig. 1 A). No amplification product was obtained with primer sets 1 and 12, whereas primer sets 2 to 11 yielded amplicons of the expected sizes (Fig. 1B). These results indicated that *pilLMNOPQRSUVW* are most likely cotranscribed as a single mRNA, since RT-PCR does not definitively preclude the existence of other nested transcripts. Given the instability of the mRNA, several attempts at primer extension analysis were unsuccessful. Thus, the *pil* operon promoter was further delineated by RT-PCR assays (Fig. 2). The primer pairs pil3-pil5

TABLE 2. Main characteristics of ORFs identified within the 11-kb locus of *Y. pseudotuberculosis* strain 32777

ORF	Size (aa)	Molecular mass (Da)	pI	Homolog as determined by BLAST	% Identity/homology (amino acid overlap)	GenBank accession no.
PilL	356	38,710	8.9	PilL encoded by <i>S. enterica</i> serovar Typhi (unknown function)	43/53 (356)	AF000001
PilM	194	21,620	10.7	Lipoprotein PilM encoded by <i>S. enterica</i> serovar Typhi (unknown function)	42/57 (125)	AF000001
PilN	530	57,189	8.7	Lipoprotein PilN encoded by plasmid R64 from <i>S. enterica</i> serovar Typhimurium (unknown function)	45/59 (528)	D88588
PilO	445	49,158	9.0	PilO encoded by <i>S. enterica</i> serovar Typhi (unknown function)	33/50 (445)	AF000001
PilP	164	17,560	9.2	PilP encoded by <i>S. enterica</i> serovar Typhi (unknown function)	36/54 (149)	AF000001
PilQ	517	57,826	7.3	Nucleotide-binding protein PilQ encoded by <i>S. enterica</i> serovar Typhi (unknown function)	55/70 (495)	AF000001
PilR	369	41,411	8.6	Integral membrane protein PilR encoded by plasmid R64 from <i>S. enterica</i> serovar Typhimurium (unknown function)	44/59 (362)	D88588
PilS	195	20,191	9.7	Prepilin PilS encoded by plasmid R64 from <i>S. enterica</i> serovar Typhimurium	49/62 (189)	D88588
PilU	161	17,461	5.9	Prepilin peptidase PilU encoded by <i>S. enterica</i> serovar Typhi	30/46 (158)	AF000001
PilV	425	45,880	8.8	Minor pilin PilV encoded by plasmid R64 from <i>S. enterica</i> serovar Typhimurium	46/58 (423)	D88588
PilW	306	35,089	6.7	Putative transposase Y1072 from <i>Y. pestis</i> plasmid pMT1	61/78 (306)	AF074611

peptidases; sequence analysis of PilU showed the presence of a pair of aspartate residues at positions 33 and 95. These residues are completely conserved among type IV prepilin peptidases and have been shown to be essential for enzyme activity (23). Finally, the last gene of the *Y. pseudotuberculosis* pil cluster, *pilW*, encodes a product showing 61% identity with a *Yersinia pestis* putative transposase. It is noteworthy that this gene is not present within the type IV pilus gene cluster in *Salmonella* (19, 48).

Expression of the *Y. pseudotuberculosis* pil operon in *E. coli* K-12 reconstitutes bundle-forming pili. Since sequence comparisons showed high homologies between the *Y. pseudotuberculosis* and *S. enterica* pil gene clusters, we investigated whether the *Y. pseudotuberculosis* pil cluster is functional and capable of forming type IV pili. We thus initially analyzed *E. coli* NM554 containing the recombinant plasmids pMM2.1, pMM2.A6, and pMM3.D6; the last two encompass an incomplete pil operon (see above). Two hundred and fifty bacterial cells from each strain obtained after 24-h culture on LB agar at 37°C were observed by TEM. Microscopy was performed without prior knowledge of the identities of the cloned inserts in recombinant plasmids. Bundle-forming pili protruded from the surface in the polar regions of 147 (59%) NM554(pMM2.1) cells (Fig. 3). In contrast, these appendages were absent from all 250 of the NM554(pMM2.A6) and NM554(pMM3.D6) cells examined (data not shown). These results demonstrated that plasmid pMM2.1, which harbors the entire pil gene cluster,

was capable of inducing pilus biogenesis in *E. coli*, in contrast to the truncated pil gene clusters (plasmids pMM2.A6 and pMM3.D6).

These ultrastructural studies strongly suggested that the pil locus governs pilus synthesis. To demonstrate that the pilLMNOPQRSUV cluster is sufficient for the biogenesis of a type IV pilus, a 10.8-kb *NcoI* fragment from cosmid pMM2.1, containing only the pil gene cluster and its putative promoter region, was subcloned into plasmid pACYC184 to yield pMM5. This plasmid was introduced into *E. coli* MC1061, and the bacterial cells were examined by TEM. The recombinant MC1061 strain, carrying only pACYC184, was used as a negative control. As previously observed with strain NM554(pMM2.1), bundles of filaments protruded from a polar end of strain MC1061 (pMM5) whereas the control strain, MC1061(pACYC184), was nonpilated (not shown).

The pil operon in *Y. pseudotuberculosis* is transcriptionally regulated by in vitro environmental factors. In addition to temperature shift, enteropathogenic *Y. pseudotuberculosis* encounters various environmental cues in the ileum lumen prior to invasion of intestinal mucosa: these include low pH, high osmolarity, and low oxygen tension (39). Hence, we investigated whether these signals influence *Y. pseudotuberculosis* pil operon expression. This was achieved by slot blot hybridization of pil transcripts from *Yersinia* grown in vitro under different environmental conditions (Fig. 4).

We initially assessed the phase of the *Y. pseudotuberculosis*

TABLE 3. Comparison of signal sequences of prepilins of the type IVB family

Prepilin	Microorganism	Amino acid sequence of the N terminus of prepilin ^a
prePilS	<i>Y. pseudotuberculosis</i>	MLSPVASRKQPHSG
prePilS	<i>S. enterica</i> serovar Typhimurium	WGLEESGGVALVVIVIAVV
prePilS	<i>S. enterica</i> serovars Dublin and Typhi	MLVENINTTLTGNNKKNPHDKG
prePilV	<i>Y. pseudotuberculosis</i>	MKNETEGKMMNEVSTLNPENRDRG
prePilV	<i>S. enterica</i> serovar Typhimurium	MTFKTRALHRG
prePilV	<i>S. enterica</i> serovars Dublin and Typhi	MKKYDRG
preBfpA	<i>E. coli</i> (EPEC)	MKKQKHDGG
preLngA	<i>E. coli</i> (ETEC)	MVSKIMNKYKYG
preTcpA	<i>V. cholerae</i>	MSLVNRTQKFKAEARKKIAYHELRKQRG
		MQLLKQLFKKKFVKKEHDKKTQEG

^a Deduced from prepilin genes (accession numbers in GenBank, AF004308, AF304486, X74730, AF000001, D88588, and AF247502). The pilin monomers are synthesized as precursor proteins with a hydrophilic leader peptide of variable length that is processed at a consensus cleavage site (arrowhead) by a prepilin peptidase usually recognizing the consensus motif GXXXXE. The signal sequences of prepilins and the first 20 aa (hydrophobic domain) of the mature proteins are shown.

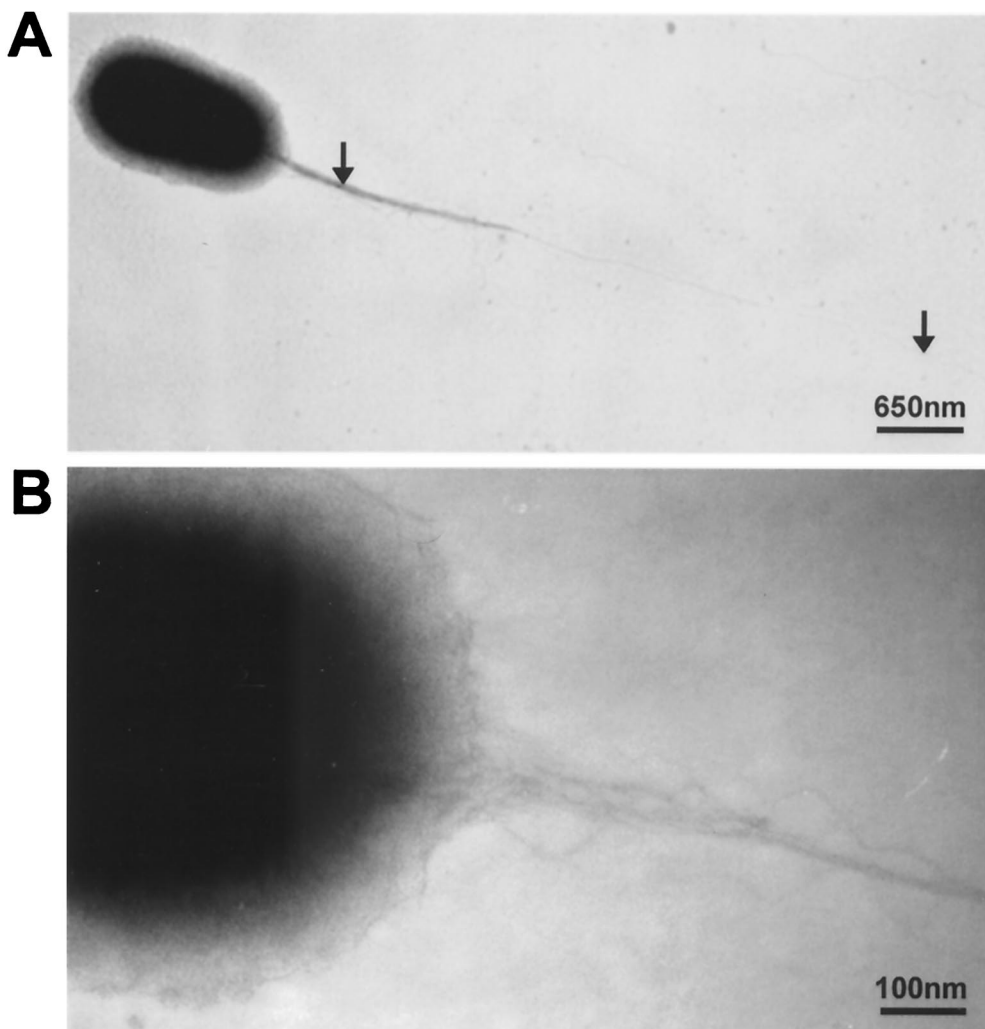


FIG. 3. Electron micrographs of *E. coli* containing the recombinant plasmid pMM2.1, which harbors the *Y. pseudotuberculosis pil* gene cluster. The strain was examined by TEM after uranyl acetate negative staining. A long pilus (6 μ m) emanates from one polar position (A, arrows) and is constituted of bundles of fibers with a diameter of 5 nm (B).

growth cycle during which the *pil* gene cluster was expressed. Strain 32777 was cultivated in standard LB broth, and *pil* transcripts were detected only during the stationary phase at 28°C, whereas they were already synthesized as early as the exponential phase at 37°C. We then studied the influence of oxygen tension levels during exponential growth at 28 and 37°C in LB broth maintained aerobically or anaerobically. At 28°C, anaerobiosis induced expression of the *pil* operon. This was in contrast to *pil* transcription at 37°C. Overall, *pil* expression was both temperature and redox potential dependent.

Additionally, we tested whether, separately, osmotic and pH changes in growth media had effects on *pil* transcription. RNAs were extracted from exponentially growing cultures in LB broth enriched with large amounts of a salt (100 mM KCl) or a sugar (200 mM L-arabinose) to raise the osmolarity of the medium. As depicted in Fig. 4, 100 mM KCl increased *pil* expression at 28°C. Similar results were obtained with L-arabinose (data not shown). In contrast, *pil* transcription was not influenced by pH at either 28 or 37°C.

Deletion of the *pil* gene cluster in *Y. pseudotuberculosis* re-

duces bacterial pathogenicity in the mouse model. The enteropathogenic species *S. enterica*, *E. coli*, and *V. cholerae* produce type IV pili, which contribute to their pathogenicity (1, 9, 48). To determine whether the *pil* gene cluster plays such a role in *Y. pseudotuberculosis*, an isogenic *pil* mutant was engineered from the wild-type strain. A DNA fragment encompassing almost all of the *pil* operon was deleted in the parental strain 32777 and replaced with a kanamycin resistance gene, *aphA-Ia*. This mutation was achieved by mating strain 32777 ($\text{Ap}^s \text{Km}^s$) with *E. coli* strain SM10 λ *pir* harboring the suicide plasmid pMM4. Allelic exchange through homologous recombination, leading to mutant MIV, was carried out in two steps as previously described (4). The genotype of the *pil* mutant was further confirmed by Southern blot hybridization with appropriate DNA probes (not shown).

The virulence of the *pil* mutant was first assessed in an oral model of infection in the BALB/c mouse. The LD₅₀ of this strain was slightly higher than that of the parental strain (10⁸ versus 10^{7.3}). As depicted in Fig. 5, after i.g. inoculation of a lethal dose of *Yersinia*, the death of the animals was delayed

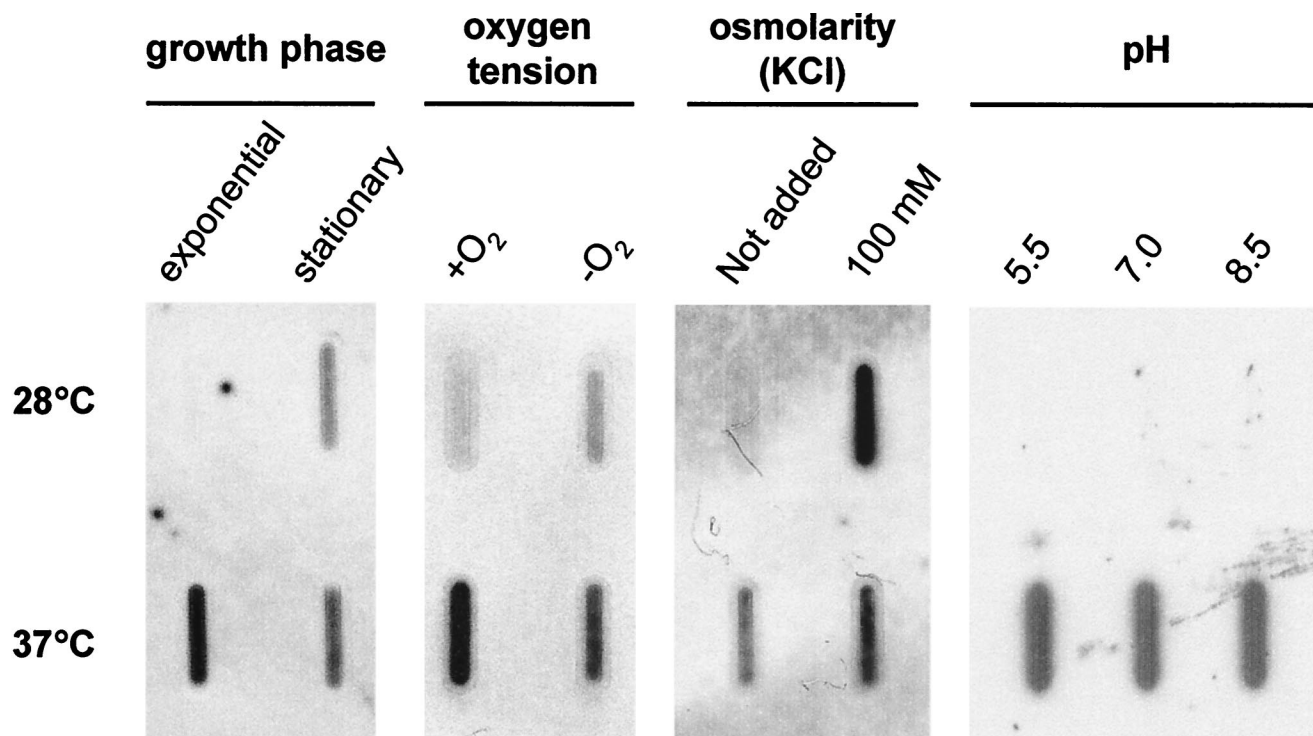


FIG. 4. Transcription of the *pil* operon in *Y. pseudotuberculosis* grown under different environmental conditions. Total RNAs (15 μ g) extracted from bacterial cultures in standard or modified LB broth were spotted onto nylon membranes and hybridized with a labeled 523-bp *pilRS* probe. As a positive control for constitutive gene expression, a ribosomal DNA 16S probe was used (not shown). No signal was detected when RNA samples were pretreated with RNase before hybridization. Experiments were repeated at least twice, and representative results are shown.

when the infecting strain was *pil* deficient; this finding was well correlated with the body weights of mice throughout the infectious process. On the other hand, the *pil* mutant was found to be as virulent as the parental strain when administered intravenously (LD_{50} , $< 10^2$).

The *pil* gene cluster is present in most pathogenic *Y. pseudotuberculosis* strains. We investigated the distribution of the *pil* gene cluster in the *Y. pseudotuberculosis* species. We tested 91 *Y. pseudotuberculosis* strains originating from various countries by colony blot hybridization under stringent conditions with two PCR-generated *pil* probes from strain 32777 DNA: *pilL* (269 bp) and *pilS* (579 bp). Thirty-seven strains hybridized with each of the two probes: 14 of them belonged to serotype I (376/89, 347/89, 72/91, YPT6, YPT7, YPT9, YPT11, 2/91, 33036, 32849, 33053, 417/94, WS53/94, and WS34/94), 5 belonged to serotype II (MA, 2926, 2515, 2929, and WS25/91), 10 belonged to serotype III (304/89, 32945, 32977, 32975, 32992, YPT1, YPT5, 766/95, 1216/93, and 2887), 2 belonged to serotype IV (ST and NT), 4 belonged to serotype V (99/91, 199/90, 2821, and 2843), and 2 belonged to serotype VI (1553 and 1554).

We randomly chose 16 out of the 37 *pil*-positive strains (YPT6, YPT7, YPT9, 33036, WS53/94, MA, 2926, 2929, WS25/91, 304/89, YPT1, YPT5, 1216/93, 199/90, 1553, and 1554) and PCR amplified the entire *pilS* gene encoding a putative prepilin. The PCR products were sequenced, and the *pilS* sequence was found to be identical to the prototype sequence for all except four strains (2926, 2929, WS25/91, and 199/90) in which a nucleotide substitution led to replacement of Tyr₁₃₇ by Ile₁₃₇

in the *pilS* product. Taken together, these results show that at least the *pilS* gene is highly conserved among the species.

DISCUSSION

In this work, we cloned and sequenced an 11-kb chromosomal operon of 11 genes involved in type IV pilus biogenesis in *Y. pseudotuberculosis*. This novel locus, when introduced into *E. coli*, induced the emanation of appendages at one cellular pole that tended to form large bundles of pilus aggregates, an ultrastructural feature of type IV pili. The genetic locus that we discovered in *Y. pseudotuberculosis* strain 32777 was strongly homologous to the *pil* operon of *S. enterica*. In this enteropathogenic bacterial species, two closely related *pil* operons have been characterized: the first, called R64, includes 14 genes (*pilIJKLMNOPQRSTU*) and was originally described in serovar Typhimurium (19); the second lacks the *pilI*, *pilJ*, and *pilK* genes and has been reported in serovar Typhi (48). No homologs of the *Salmonella pilI*, *pilJ*, *pilK*, and *pilT* genes were present in the *Y. pseudotuberculosis pil* operon, but as an *E. coli* strain *trans*-complemented with *Y. pseudotuberculosis pilLMNOPQRSUV* produced pili (Fig. 3), these genes do not seem to be required for pilus formation in this bacterial species. This is in agreement with the study of Yoshida and coworkers (47) showing that of the 14 *pil* genes (*pilIJKLMNOPQRSTU*) carried by the *Salmonella* operon R64, *pilI* and *pilJ* were not required for R64 pilus biogenesis. In addition, as stated above, *pilK* is absent from the type IV pilus gene cluster of *S. enterica*

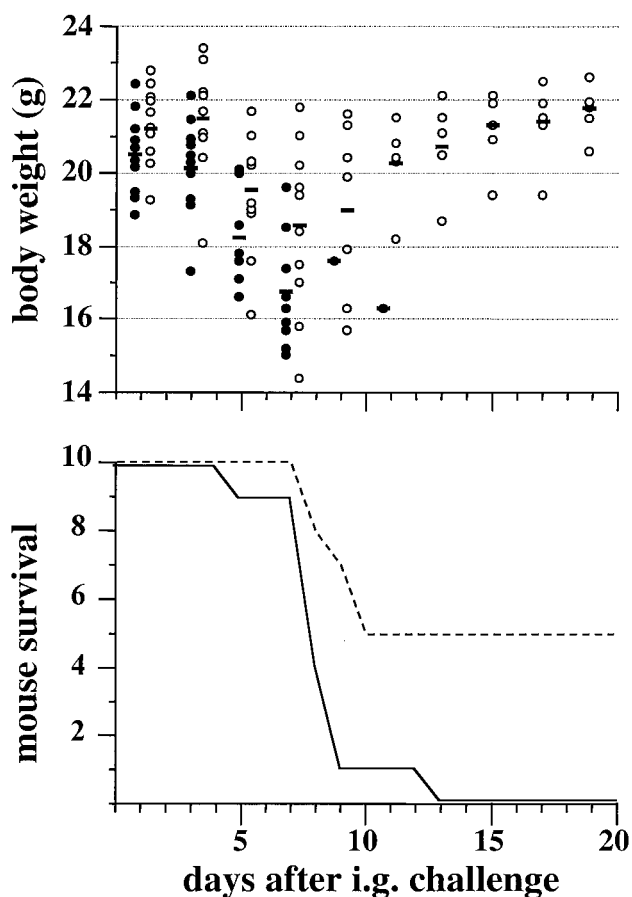


FIG. 5. Impact of the *pil* gene cluster on *Y. pseudotuberculosis* pathogenicity in the mouse model. BALB/c mice (groups of 10) were inoculated by the i.g. route with 10^8 *pil*⁺ or *pil* mutant bacteria. The body weights and survival times of the infected animals were recorded during a 20-day period after bacterial challenge. The experiments were repeated twice, and very similar results were found. Representative data are shown. The bars represent the mean body weights of infected animals. Symbols: ● and solid line, wild-type strain 32777 (*pil*⁺); ○ and dashed line, isogenic mutant MIV (*pil* mutant).

serovar Typhi, even though pili are produced (48). On the other hand, the *pilW* gene present in the *Y. pseudotuberculosis pil* operon is missing in *Salmonella*; however, we demonstrated by a complementation assay in *E. coli* that this gene is not needed for pilus biogenesis. The homology of the *pilW* product with a putative transposase is consistent with the latter finding. Why *pilW*, whose G+C content is, surprisingly, the lowest of the gene cluster, is part of the *pil* operon remains to be elucidated. Sequencing of the flanking DNA environment of the *pil* operon, which is in progress, could shed light on this point.

Some *Y. pseudotuberculosis* Pil proteins (PilN, PilR, PilS, and PilV) are more similar to Pil homologs involved in R64 pilus biogenesis, whereas others (PilL, PilM, PilO, PilP, PilQ, and PilU) are more similar to the corresponding Pil proteins from serovar Typhi (Table 1). Bacterial localization of the Pil proteins involved in R64 pilus biogenesis have been specified, but the functions of most of these proteins are still poorly understood, with the exception of the products of *pilS* and *pilV*, which are prepilins, and that of *pilU*, a prepilin peptidase (19, 35). The putative length of the signal sequence (14 and 11 aa

for prepilins S and V, respectively) and the nature of the first amino acid residue of the mature protein, which is a tryptophan rather than a phenylalanine for both *Y. pseudotuberculosis* PilS and PilV, identified these prepilins as members of the IVB subclass, to which pili from *V. cholerae*, *E. coli* pathovars EPEC and ETEC, and *S. enterica* belong. As for *S. enterica* serovars Typhi and Dublin, the amino acid at position +5 of the putative *Y. pseudotuberculosis* mature protein PilV is a serine. In type IV pilins, the +5 residue is almost always a glutamic acid (24). However, previous studies have shown that the replacement of glutamic acid by another amino acid did not affect prepilin processing by prepilin peptidase (31, 42). Unlike its homolog in *S. enterica*, the *Y. pseudotuberculosis pilV* gene does not contain a clustered inversion region (known as a shufflon) (22), which may generate several different products due to a rearrangement of the DNA in the region encoding the C terminus by a specific recombinase.

We also show in the present study that in vitro expression of the *Y. pseudotuberculosis pil* operon is transcriptionally regulated by temperature, cell density, osmolarity, and oxygen tension. Interestingly, *pil* gene transcription is upregulated under anaerobiosis or high osmolarity and at 37°C. These conditions are encountered by *Y. pseudotuberculosis* in the digestive tract of the host; hence, one can postulate a possible role for the fimbria-encoding gene cluster in bacterial enteropathogenicity. Indeed, inactivation of the *pil* gene cluster was associated with a reduction of *Y. pseudotuberculosis* virulence for the mouse in the oral, but not the systemic, model of infection. This is most likely associated with an impairment of bacterial colonization of the intestinal mucosa. Whether these pili can functionally substitute for other adhesins remains to be elucidated. Further studies of interactions of *Y. pseudotuberculosis pil*⁺ and *pil* mutant strains with reconstituted intestinal epithelial monolayers (38) should provide insight into this question and could indicate whether the *pil* gene cluster contributes to translocation of *Yersinia* across the intestinal barrier.

Two features suggest that *Y. pseudotuberculosis* may have acquired the *pil* operon by horizontal gene transfer. First, the *pil* locus is not uniformly present throughout the species, as only 38 of 92 (41%) nonepidemiologically related strains of diverse origins tested positive for *pil* by stringent hybridization with two specific probes. However, it should be noted that since both DNA probes used in the hybridization assay were extremities of the operon, we may have missed possible deletions in this operon in the different strains. Interestingly, both the strain 32953, which has been used for the *Y. pseudotuberculosis* genome project, and the extensively studied reference strain YPIII were *pil* negative under these detection conditions. Additionally, the *pil* locus was not found in the genome of strains CO92 and KIM5 of the closely genetically related species *Y. pestis* (http://www.sanger.ac.uk/Projects/Y_pestis and <http://www.genome.wisc.edu/sequencing/pestis>). Secondly, whereas the mean G+C content of the *Y. pseudotuberculosis* genome is 47%, that of the *pil* operon is 50.8%. This value is very close to the G+C contents of the genomes of the type IV pilus-producing species *E. coli* and *Salmonella* (51 to 52%). This suggests that *Y. pseudotuberculosis*, which occupies the same ecological niche as these two other enterobacteria, may have acquired the type IV pilus locus from one of these species by genetic exchange in the natural habitat. The two *Salmonella*

pil operons have been found located either on a conjugative plasmid (in serovar Typhimurium) (19) or a large (118-kb) pathogenicity island (in serovar Typhi) (48). Other type IVB pilus gene clusters, in *E. coli* pathovars EPEC and ETEC and *V. cholerae*, were also found to be harbored on either a plasmid (11, 13) or a pathogenicity island (17). In *Y. pseudotuberculosis* 32777, in which the type IV pilus gene cluster has been discovered, no plasmid other than the virulence plasmid pYV was detected. Thus, given the scenarios in *S. enterica*, *E. coli*, and *V. cholerae*, it is tempting to speculate that the *Y. pseudotuberculosis pil* operon could constitute a novel "adaptation-pathogenicity" island, unknown until now, which, in addition to the already described high-pathogenicity island (2), contributes to the virulence of *Y. pseudotuberculosis*.

ACKNOWLEDGMENTS

This work was partly supported by the Conseil Régional Nord-Pas de Calais, the Université René Descartes (Paris V), and the European Regional Development Fund. F. Collyn received a scholar fellowship from the Ministère de l'Enseignement Supérieur de la Recherche et de la Technologie.

We thank S. Aleksić, E. Carniel, D. Caugant, E. Falsen, N. Takeda, and R. Van Noyen for kindly supplying *Y. pseudotuberculosis* strains. We also thank C. Mullet for technical assistance and C. Carnoy for critical reading of the manuscript.

François Collyn and Marie-Annick Léty contributed equally to this work.

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Editor: J. T. Barbieri