

## Novel Virulence-Associated Type II Secretion System Unique to High-Pathogenicity *Yersinia enterocolitica*

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***Yersinia enterocolitica* strains comprise an important group of bacterial enteropathogens that cause a broad range of gastrointestinal syndromes. Three groups are distinguishable within this bacterial species, namely, the nonpathogenic group (biotype 1A strains), the low-pathogenicity, non-mouse-lethal group (biotypes 2 to 5), and the high-pathogenicity, mouse-lethal group (biotype 1B). To date, the presence of the high-pathogenicity island (HPI), a chromosomal locus that encodes the yersiniabactin system (involved in iron uptake), defines essentially the difference between low-pathogenicity and high-pathogenicity *Y. enterocolitica* strains, with the low-pathogenicity strains lacking the HPI. Using the powerful tool of representational difference analysis between the nonpathogenic 1A strain, NF-O, and its high-pathogenicity 1B counterpart, WA-314, we have identified a novel type II secretion gene cluster (*yts1C-S*) occurring exclusively in the high-pathogenicity group. The encoded secretin, designated Yts1 (for *Yersinia* type II secretion 1) was shown to be important for virulence in mice. A close examination of the almost completed genome sequence of another high-pathogenicity representative, *Y. enterocolitica* 8081, revealed a second putative type II secretion cluster uniformly distributed among all *Y. enterocolitica* isolates. This putative species-specific cluster (designated *yts2*) differed significantly from *yts1*, while resembling more closely the putative type II cluster present on the genome of *Y. pestis*. The Yts1 secretin thus appears to have been additionally acquired by the high-pathogenicity assemblage for a virulence-associated function.**

The genus *Yersinia* comprises an important group of bacterial pathogens, with *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* representing the species of interest. *Y. pestis* is the etiologic agent of plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens that cause a broad range of gastrointestinal syndromes ranging from acute gastroenteritis to mesenteric lymphadenitis. Central to the pathogenicity of the *Yersinia* is the presence of a 70-kb pYV virulence plasmid whose products mediate, among other things, the resistance of *Yersinia* to phagocytosis by polymorphonuclear leukocytes and macrophages (41). In addition, the chromosomally encoded Ail (for attachment invasion loci), Myf (for mucoid *Yersinia* factor), and Inv (invasin) proteins have been implicated in the virulence of the species to various degrees (3, 38).

Two groups have been identified among the *Y. enterocolitica* strains, which is a focus of the present study: nonpathogenic strains comprising mainly biotype 1A organisms and pathogenic strains carrying the virulence plasmid pYV. The latter group is further subdivided into a low-pathogenicity (LP), non-mouse-lethal group represented by biotypes 2 to 5 and a high-pathogenicity (HP), mouse-lethal group exemplified by biotype 1B strains (8). As a mouse virulence determinant, an HP island (HPI) has been identified, which encodes for synthesis of the siderophore yersiniabactin, an iron-sequestering low-molecular-weight compound invaluable in the iron-limiting environment of the host (8, 25, 36). The presence of the HPI has also been demonstrated in *Y. pseudotuberculosis* and *Y. pestis* (4, 5,

14, 18). The members of the LP group typically lack the HPI but possess all other known virulence factors. In terms of geographical distribution, the LP and HP *Y. enterocolitica* species exhibit some preferences, with the HP organisms being more frequently isolated in the United States (so-called New World strains), whereas the LP isolates (so-called Old World strains) are predominantly isolated in Europe and Japan (9, 10).

We report here the application of representational difference analysis to map out novel chromosomal loci unique to HP *Y. enterocolitica* of serotype O:8 biotype 1B.

This powerful method of suppressive subtractive hybridization has been successfully applied in mapping out crucial genomic differences between closely related bacterial genomes and even isolates of the same species (7, 11, 17, 34). We describe the discovery of a novel type II secretion apparatus with impact on the virulence of the HP *Y. enterocolitica* species. The distribution of this novel secretin is highly similar to that of the recently described chromosomally encoded type III secretion system (TTSS) that was found to be present only in the HP group of *Y. enterocolitica* species (19, 22). This novel transport cluster designated Yts1 (for *Yersinia* type II secretion 1) shares the highest homology with the Eps protein transport cluster of *Vibrio cholerae* (44), the latter mediating the extracellular transport of protease, the cholera toxin, and chitinase.

### MATERIALS AND METHODS

The bacterial strains and plasmids used in the present study are listed in Table 1. Typically, bacterial strains were cultivated in Luria-Bertani (LB) medium or on LB agar plates (Difco Laboratories, Detroit, Mich.) at 27°C for *Yersinia* or 37°C for *E. coli*. The following antibiotic concentrations were used for plasmid maintenance and selection: ampicillin at 100 µg ml<sup>-1</sup> (300 µg ml<sup>-1</sup> for *Yersinia*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<b>Strains</b>		
<i>Y. enterocolitica</i>		
WA-314	<i>Y. enterocolitica</i> O:8 biotype 1B with pYV virulence plasmid	25
WA-C	Plasmidless derivative of strain WA-314; spontaneously resistant to nalidixic acid	25
WA-CS	Derivative of WA-C; spontaneous Sm <sup>r</sup> mutant	This study
8081	Clinical isolate, O:8 biotype 1B	40
WA-CS <i>ytsIE</i> ::Kan <sup>r</sup>	Derivative of WA-CS; Nal <sup>r</sup> , Kan <sup>r</sup> Sm <sup>r</sup> (insertional inactivation of <i>ytsIE</i> by a kanamycin cassette)	This study
Ye1209-79	Clinical isolate, O:13 biotype 1B	25
Ye1223-75-1	Clinical isolate, O:20 biotype 1B	25
Ye737	Clinical isolate, O:21 biotype 1B	25
Y-108	Clinical isolate, O:3 biotype 4	25
Y-96	Clinical isolate, O:9 biotype 2	25
NF-O	Clinical isolate, O:5 biotype 1A	42
MRS-40	Clinical isolate, O:9 biotype 2	25
<i>Y. pseudotuberculosis</i>		
H141/84	Serotype O:1a	S. Aleksic
H457/86	Serotype O:2a	S. Aleksic
IP3295	Serotype O:1	E. Carniel
346	Serotype O:3	S. Aleksic
<i>Y. pestis</i>		
KIM	Biotype Mediaevalis	R. R. Brubaker
KUMA	Biotype Antiqua	R. R. Brubaker
TS	Biotype Orientalis	R. R. Brubaker
<i>E. coli</i>		
C-4441	Enterocaggregative, O:128	47
12860	Enteroinvasive, O:124	47
DH5 $\alpha$	<i>endA1 hsdR17</i> ( $r_{K^{-}} m_{K^{+}}$ ) <i>supE44 thi-1 recA1 gyrA relA1</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15)	23
S17-1 $\lambda$ <i>pir</i>	<i>pir<sup>+</sup> tra<sup>+</sup></i>	48
XL1-Blue MR	$\Delta$ ( <i>mcrA</i> ) 183 $\Delta$ ( <i>mcrCB-hsdSMR-mrr</i> )173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac<sup>c</sup></i>	Stratagene
MOSBlue	<i>endA1 hsdR17</i> ( $r_{K12^{-}} m_{K12^{+}}$ ) <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> [ <i>F' proA<sup>+</sup> B<sup>+</sup> lacI<sup>q</sup>Z</i> $\Delta$ M15:Tn10(Tc <sup>r</sup> )]	Amersham
<b>Plasmids</b>		
Supercos1	Cloning vector; Ap <sup>r</sup> Neo <sup>r</sup>	Stratagene
ScF <i>ytsIE</i> ::Kan <sup>r</sup>	<i>Bgl</i> II/ <i>Bam</i> HI self-religated Supercos1 fragment carrying the mutated <i>ytsIE</i> gene	This study
pKAS 32	Cloning vector with dominant <i>rpsL</i> gene	49
pKAS 32 <i>ytsIE</i> ::Kan <sup>r</sup>	pKAS32 carrying a fragment from the putative type II <i>ytsIE</i> gene inactivated by a kanamycin cassette	This study
pACYC184	Cloning vector; Cm <sup>r</sup> Tc <sup>r</sup>	New England Biolabs
pUC4K	pUC vector carrying the kanamycin cassette from Tn903; Kan <sup>r</sup> Ap <sup>r</sup>	Amersham
pMOSBlue	Cloning vector; Ap <sup>r</sup>	Amersham

<sup>a</sup> Nal<sup>r</sup>, nalidixic acid resistant; Tc<sup>r</sup>, tetracycline resistant; Cm<sup>r</sup>, chloramphenicol resistant.

strains), tetracycline at 40  $\mu$ g ml<sup>-1</sup>, kanamycin at 100  $\mu$ g ml<sup>-1</sup>, streptomycin at 100  $\mu$ g ml<sup>-1</sup>, and chloramphenicol at 20  $\mu$ g ml<sup>-1</sup>.

**Molecular genetic techniques.** Plasmid DNA was prepared by the modified nucleic acid extraction kits from Nucleobond. Chromosomal DNA was prepared as previously described (1). Construction of the cosmid gene library from *Y. enterocolitica* WA-314 serotype O:8 biotype 1B (mouse lethal) was performed with chromosomal DNA *Sau*3AI digested to yield DNA fragments ranging in size from 35 to 40 kb, which were subsequently ligated into the *Bam*HI site of the cosmid vector Supercos1. Ligated DNAs were packaged in vitro into viable phage heads according to manufacturer's instructions (Stratagene) and transduced into *Escherichia coli* XL1-Blue MR. High-stringency Southern blot hybridizations were performed with digoxigenin-labeled probes at 68°C according to standard procedures. To determine the presence of the *yts1D* and *yts2D* genes among different *Yersinia* isolates, the following oligonucleotide primers were employed: Yts1D.for (5'-GCAGTAAAAG-GCAACATCAGCG-3') and Yts1D.rev (5'-AAACAACGCGCATGACTTC-3') for amplification of the *yts1D* gene and 2D.for (5'-GTAGGATATGCGGAGAACTTC-3') and 2D.rev (5'-CGTTTTGCGGGAATAACTTTT-3') for amplification of *yts2D*.

**Construction and analysis of subtractive hybridization library.** The procedure of subtractive hybridization was performed as described in the PCR select genomic subtraction kit from Clontech. In the present study, *Y. enterocolitica* WA-314 genomic DNA was used as the tester, and genomic DNA of *Y. enterocolitica* serotype O:5, biotype 1A (NF-O, nonpathogen), was used as the driver. Briefly, chromosomal DNA was isolated (1) from both tester and driver strains and was *Rsa*I digested. The tester DNA was then ligated with two different oligonucleotide adapters in two separate reactions, and then each adapter-ligation reaction was mixed with an excess of the driver DNA population and subjected to successive rounds of hybridization at 63°C. The subtracted fragments were then enriched through two rounds of PCR-amplification with adapter-specific primers. The secondary PCR products generated, enriched for tester specific sequences, were mobilized into the highly efficient pMOSBlue cloning vector (Amersham) and transformed into *E. coli* MOSBlue-competent cells (Amersham) for sequence analysis. To analyze the efficiency of the subtraction, the entire subtracted fragments were concentrated, and the pool was labeled as a probe to screen the NF-O DNA and DNA representative of other yersiniae in a high-stringency Southern blot hybridization reaction. In a second analysis, NF-O

driver DNA was labeled as a probe and used to screen more than 50 subtracted clones (data not shown). Selected subtracted fragments of interest were digoxigenin-labeled and used to probe the WA-314 cosmid library in high-stringency colony blot reactions. Hybridizing cosmids were isolated and subsequently sequenced (1).

**DNA sequencing and analysis.** DNA Sequencing was performed by the dideoxy-chain terminating method with an automated ABI Prism DNA sequencer. The T7 forward primer was used to initiate sequence reactions with the subtracted clones. Analysis of subtracted fragments was carried out with the BLASTN and BLASTX programs provided by the National Center for Biotechnology Information, The Institute for Genomic Research BLAST Center, and the *Y. pestis* genome sequence from the Sanger Center.

**Reverse transcription analysis.** RNA isolation was carried out with Trizol reagent (Gibco) according to the manufacturer's instructions. RNA was isolated from bacterial cells grown in LB medium at various temperatures (27 and 37°C). As positive controls, the *inv* and *ail* genes were probed in parallel for transcription.

**Construction of a *Y. enterocolitica yts1E* mutant.** To inactivate the *yts1* genes, a site-directed insertional mutation in *yts1E* was generated in *Y. enterocolitica* WA-314. A 1.2-kb *EcoRI-NotI* PCR product was amplified from the original subtracted fragment (n28) that showed homology to the *epsE* gene in *V. cholerae* and cloned into the *EcoRI-NotI* site of a *BglII/BamHI* self-ligated Supercos1 vector fragment (designated ScF). A kanamycin cassette amplified from the pUC 4K plasmid and containing flanking *BamHI* restriction sites was introduced into the *BamHI* site of the amplified n28 fragment. The resulting n28 insert containing the kanamycin cassette was subsequently ligated into the suicide vector, pKAS 32. pKAS 32 harbors the dominant *rpsL* gene which encodes the S12 protein of the ribosomes (37). Thus, insertion of this suicide vector into the chromosome confers a streptomycin-sensitive ( $Sm^s$ ) phenotype to a previously streptomycin-resistant ( $Sm^r$ ) strain. The construct was transformed into *E. coli* S17-1 $\lambda$ pir and subsequently mobilized into *Y. enterocolitica* WA-CS through conjugation. Selection for  $Sm^r$  strains indicated the presence of transconjugants that have excised the plasmid sequences through double allelic exchange. Transconjugants (mutants) were selected on LB agar plates containing kanamycin and streptomycin. The presence of the kanamycin cassette was confirmed through PCR, sequencing, and Southern blot hybridization.

In another experiment, the original n28 fragment was cloned into pKAS32, and the construct was introduced into *Y. enterocolitica* WA-314 *yts1E::Kan<sup>r</sup>* (kanamycin-resistant) strain using the procedure described above. Selection of  $Str^r$ ,  $Kan^s$ , and ampicillin-sensitive ( $Ap^s$ ) clones indicated clones where double allelic exchange had excised the kanamycin cassette and restored the parental wild-type allele. This strain was subsequently designated reconstituted wild type. For transcomplementation, cosmid n28 or cosmid 1D (both harboring complete copies of the *Yts1* secretion) were introduced into electrocompetent *Y. enterocolitica* WA-314 *yts1E* mutant by using standard procedures (1).

**Animal experiments.** Female BALB/c mice (6 weeks old) were infected (groups of five) with yersiniae orogastrically at a dosage of  $5 \times 10^9$  CFU. Mice were monitored twice daily; 48 h after infection the animals were sacrificed, and the average bacterial loads from infected organs (i.e., the liver, spleen, and Peyer's patches) were extrapolated. For the intestinal lavage, 5 ml of ice-cold sterile phosphate-buffered saline was flushed through the small intestine and then recovered, and bacterial loads were determined. For intravenous mouse inoculation,  $10^4$  CFU were inoculated into the mice, which were subsequently monitored for 2 to 3 days before sacrifice.

**Nucleotide sequence accession number.** The sequence of the *Y. enterocolitica* WA-314 *yts1E-S* genes has been submitted to the EMBL database under the accession number AJ344214.

## RESULTS

**Construction of a library of subtracted fragments unique to *Y. enterocolitica* WA-314 and absent from the genome of *Y. enterocolitica* NF-O.** Subtraction of total genomic DNA of *Y. enterocolitica* NF-O (biogroup 1A, nonpathogen) from the genome of its highly pathogenic counterpart, WA-314, was performed by hybridization by using the PCR select genomic subtraction kit from Clontech. The secondary PCR products generated were cloned into pMOSBlue vector (Amersham), and a total of 200 subtracted clones were analyzed. These subtracted fragments, ranging in size from 200 to 1,500 bp, were sequenced and compared at both the nucleotide and the

amino acid levels for homologies with sequences in databanks (provided by the National Center for Biotechnology Information, the Sanger Center, and the Institute for Genomic Research). Generally, the fragments fell into three categories: (i) fragments with sequence similarities to known genes from the *Yersinia* species and other organisms (67%), (ii) fragments with similarities to phages and mobile genetic elements (transposases, etc.) (13%), and (iii) fragments with unknown sequences (20%).

To verify the efficiency of the subtraction procedure, the generated library of subtracted fragments was probed with primers specific for known virulence markers among pathogenic *Yersinia* species. Genomic probes specific for the HPI and the chromosomally encoded *ail* and *inv* genes were utilized in high-fidelity PCRs. The PCRs were positive for the *fyuA*, *irp2*, *ybtA*, and *irp1* gene probes (located on the HPI) and also for the *ail* and *inv* genes, indicating that the subtraction library was sufficiently enriched for tester-specific virulence markers.

**Identification of a novel putative type II secretion pathway in the HP *Y. enterocolitica* WA-314 strain.** The subtracted fragment n28 was highly homologous to the *epsE* gene of *V. cholerae* (44), part of the type II secretion apparatus in this organism. To determine whether this high homology was continuous, the n28 fragment was used to isolate hybridizing cosmids from the cosmid library of *Y. enterocolitica* WA-314 described previously. Two hybridizing cosmids were identified. Cosmid n28 was fully sequenced and its analysis revealed 13 open reading frames (ORFs) with homology to proteins identified in type II secretion clusters. Also, a gene, designated *chiY*, which encodes a putative chitin-binding protein, was identified downstream of this novel type II cluster. The type II secretion gene cluster was designated *yts1C-S*; these letters correspond to the generally accepted nomenclature for the type II secretion pathway identified in different members of the *Enterobacteriaceae* (see first cluster in Fig. 1). Upstream of the *yts1* cluster are sequences highly homologous to the chromosomally encoded *Ysa* TTSS.

The *Yts1* secretion apparatus demonstrates significantly high similarity (for example, 59% homology of *Yts1E* to the *EpsE* protein of *V. cholerae*) to the secretin of *V. cholerae* (*EpsC-N*) required for outer membrane biogenesis and secretion of cholera toxin, along with a protease and a chitinase. The similarity to other homologous secretins was highest for *Yts1D-G*, an observation that confirms previous findings that the *yts1D*, *yts1E*, *yts1F*, and *yts1G* genes typically share the highest identity among the species (60 to 80%) with comparatively less homology (25 to 40%) among the other genes (45, 46). The putative *yts1* cluster described here lacks a *yts1N* gene, in contrast to its counterpart in *V. cholerae*. This is, however, not surprising since many functional type II clusters described to date do not possess a *yts1N* gene, which several studies have shown not to be essential for protein secretion (45).

To determine whether the genes on the *yts1* cluster are transcribed, reverse transcription analysis was carried out for the *yts1D* gene for bacteria grown in LB medium at 27 and at 37°C. As positive controls, the *ail* and *inv* genes (optimally transcribed at 37 and 23°C, respectively) were analyzed in parallel. The *yts1D* gene yielded a positive transcript for culture grown at 37°C, whereas very basal transcription occurred at 27°C (see Fig. 2).

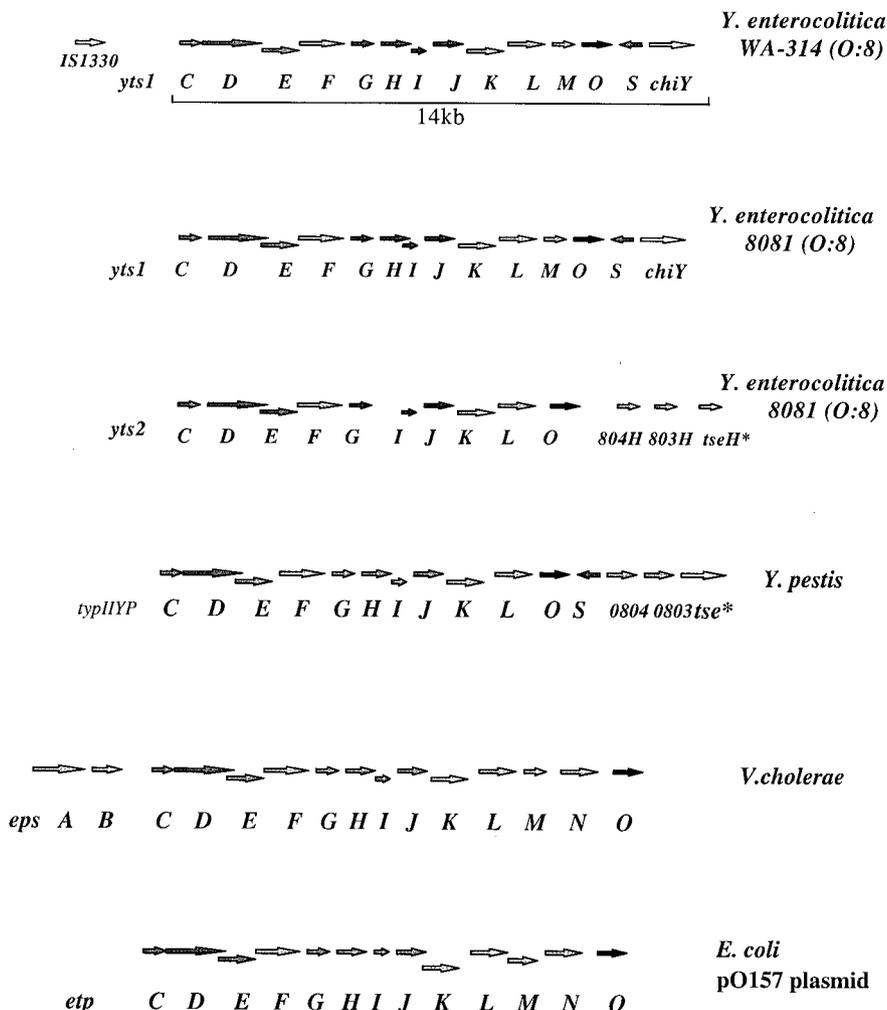


FIG. 1. The *yst1* gene cluster of the HP *Y. enterocolitica* biotype 1B strains and its comparison to related secretons: the *eps* cluster in *V. cholerae*, the *etp* cluster in *E. coli*, and the secreton of *Y. pestis* (designated here *typIIYP*). 0804 and 0803 correspond to ORFs predicted from the genome sequence of *Y. pestis*. 804H and 803H are homologues of 0804 and 0803 on the *Y. enterocolitica* genome. *tseH\** is a homologue of the *Y. pestis tse* gene on the *Y. enterocolitica* genome. *tse\** is the serine sensor receptor.

Next, we determined the presence of the *yst1* genes in diverse *Yersinia* strains. As seen in Fig. 3, it is evident that the Yts1 secreton is exclusive to the HP *Y. enterocolitica* strains (*Y. enterocolitica* serotypes O:8, O:13, O:20, and O:21). The tested LP isolates (*Y. enterocolitica* serotypes O:3 and O:9) and several *Y. pestis* and *Y. pseudotuberculosis* strains did not hybridize to the *yst1E* probe. This observation was consistent with results

from high-stringency PCRs employing primers for several genes on the cluster: among the different yersiniae, only the HP *Y. enterocolitica* strains tested positive (Fig. 4A).

Interestingly, the closely related *Y. enterocolitica* serotype O:8 strains, WA-314 and 8081, showed differences in gene arrangement bordering the *yst1* cluster. In WA-314, a novel IS element, IS1330 (29), is upstream of the *yst1* cluster, whereas in

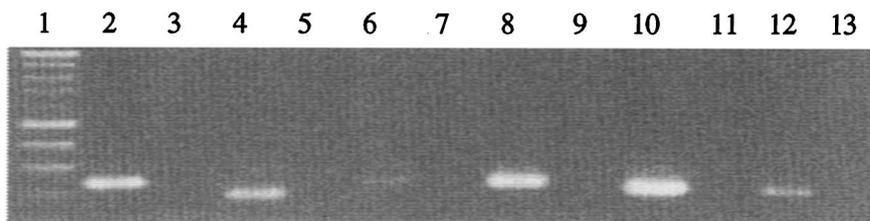


FIG. 2. Reverse transcription analysis of the *yst1D* and *yst2D* genes at 27 and at 37°C. Lanes: 1, marker; 2, *ail* at 37°C; 4, *inv* at 27°C; 6, *yst1D* at 27°C; 8, *yst1D* at 37°C; 10, *yst2D* at 27°C; 12, *yst2D* at 37°C. Lanes 3, 5, 7, 9, 11, and 13, the corresponding negative controls (without reverse transcription).

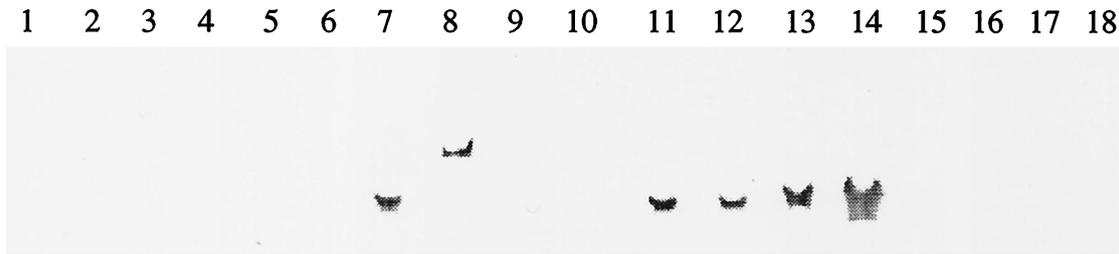


FIG. 3. Distribution of *ytsI* secretion cluster among different *Yersinia* species determined by Southern blot hybridization employing *ytsIE* as probe. Lanes: 1, *Y. pestis* TS; 2, *Y. pestis* KIM; 3, *Y. pestis* KUMA; 4, *Y. pseudotuberculosis* O:1a; 5, *Y. pseudotuberculosis* O:2a; 6, *Y. pseudotuberculosis* O:3; 7, *Y. enterocolitica* O:8 (WA-314); 8, *Y. enterocolitica* WA-314 *ytsIE*::Kan<sup>r</sup>; 9, *Y. enterocolitica* O:5 (NF-O); 10, *Y. enterocolitica* O:9; 11, *Y. enterocolitica* O:8 8081; 12, *Y. enterocolitica* O:21; 13, *Y. enterocolitica* O:20; 14, *Y. enterocolitica* O:13; 15, *Y. enterocolitica* O:9; 16, *Y. enterocolitica* O:3; 17, *E. coli* C-4441; 18, *E. coli* 12860.

strain 8081, it is lacking in the proximity of the *ytsI* cluster (see Fig. 1).

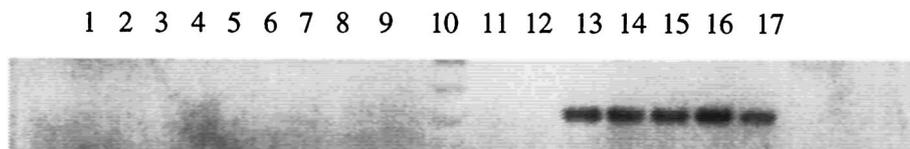
**A species-specific type II secretion cluster distributed among all *Y. enterocolitica* serotypes.** Interest in identifying a second secretion cluster that would uniformly cut across the *Y. enterocolitica* strains without preference to the pathogenic species led us to look closely at the almost-completed sequence genome of *Y. enterocolitica* 8081. A search in the Sanger Center *Y. enterocolitica* database GenBank revealed a putative second secretion cluster designated *yts2* here (Fig. 1). Genomic probing of representative *Yersinia* strains through PCRs revealed the presence of this putative type II secretion cluster among all tested *Y. enterocolitica* strains (nonpathogenic, LP and HP strains alike), whereas *Y. pseudotuberculosis* and *Y. pestis* tested negative for the presence of the genes of this cluster (Fig. 4B). Reverse transcription analysis of the *yts2D* gene (using RNA isolated from cells grown in LB medium at 27 and 37°C) revealed a positive transcript at 27°C, whereas transcription was basal at 37°C (Fig. 2). The ORFs encoded by this cluster bear homology to those previously described except for the

absence of some proteins, notably homologues of the H, M, and S proteins. Figure 1 presents an overview of the two type II secretion clusters identified here and a comparison with other closely related systems in *V. cholerae*, *E. coli*, and *Y. pestis*.

The G+C content of the genes on this species-specific *yts2* cluster differs from that of the *ytsIC-S* cluster (restricted to HP strains), having a 33% versus a 48% G+C content, respectively. Strikingly, the *yts2* cluster shared the greatest homology (50 to 65%) with the type II secretion system (designated in Fig. 1 as *typIIYP*) identified on the *Y. pestis* CO-92 genome (35). For example, the *yts2D* gene was 63% homologous to the *Y. pestis typIIYPD* gene (in contrast, the *yts1D* gene was only 48% homologous to its counterpart on the *Y. pestis* genome). The G+C content of the *typIIYP* cluster was also unusually low (34%); however, only the *yts1M* gene homologue was absent from this cluster, whereas the *yts1H* and *yts1S* gene homologues were present.

**Role of the secreton in virulence.** In order to determine the impact of the Yts1 protein secretion apparatus on the virulence

#### A.



#### B.

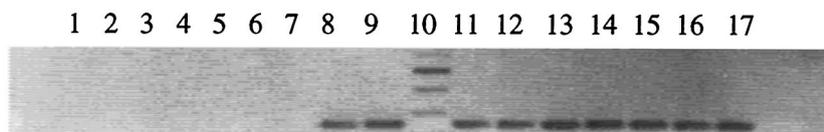


FIG. 4. Presence of the *yts1D* (A) and *yts2D* (B) genes among different *Yersinia* representatives. PCR was performed on purified chromosomal DNA with oligonucleotide primers specific for the *yts1D* and *yts2D* genes, respectively. Lanes: 1, *Y. pseudotuberculosis* H141/84; 2, *Y. pseudotuberculosis* H457/86; 3, *Y. pseudotuberculosis* IP3295; 4, *Y. pseudotuberculosis* 346; 5, *Y. pestis* KIM; 6, *Y. pestis* KUMA; 7, *Y. pestis* TS; 8, *Y. enterocolitica* O:5 (NF-O); 9, *Y. enterocolitica* O:9; 10, 100-bp ladder; 11, *Y. enterocolitica* O:3; 12, *Y. enterocolitica* O:9; 13, *Y. enterocolitica* O:8 (WA-314); 14, *Y. enterocolitica* O:8 8081; 15, *Y. enterocolitica* O:13; 16, *Y. enterocolitica* O:20; 17, *Y. enterocolitica* O:21

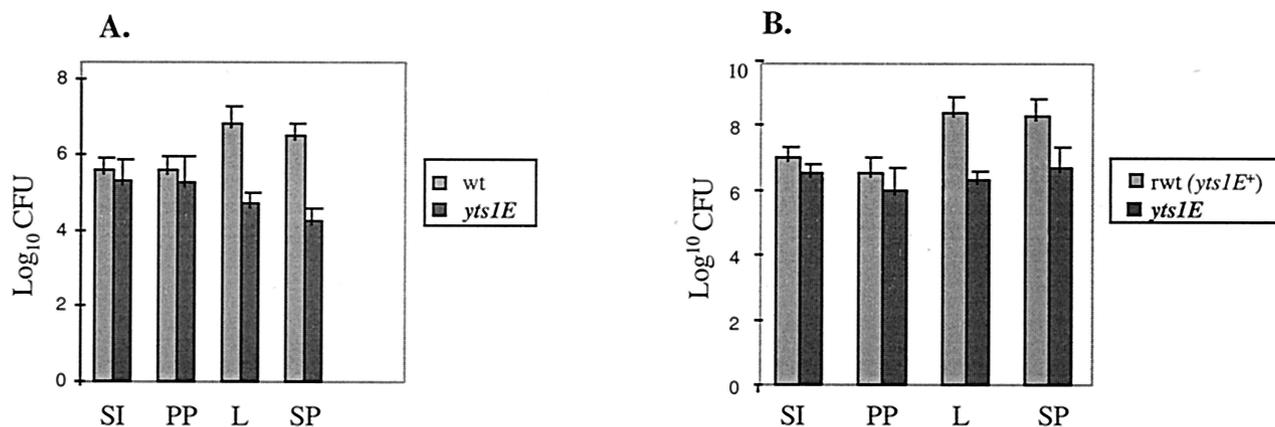


FIG. 5. Pathogenicity of the Yts1 secretin for mice (oral inoculation). BALB/c mice (five per group) were orally challenged with  $5 \times 10^9$  *Y. enterocolitica*. At 48 h after infection, mice were sacrificed, and the bacterial load was determined by plating the intestinal lavage (SI) and tissue homogenates from Peyer's patches (PP), liver (L), and spleen (SP). (A) Comparison of mice infected with strain WA-314 (wt) and *yts1E* mutant; (B) comparison of mice infected with reconstituted wild-type strain (rwt) harboring an intact *yts1E* gene (after gene replacement of the kanamycin-inactivated *yts1E* gene with the wild-type parental allele) and with the *yts1E* mutant. Values are means for five animals, with the standard error of the means indicated by an error bar.

of strain WA-314, the average numbers of bacterial CFU recovered from infected organs were compared for the wild type and the WA-314 *yts1E* mutant in perorally inoculated mice. The results obtained are summarized in Fig. 5A. As depicted in the figure, the bacterial CFU recovered from the small intestine and Peyer's patches were only marginally different for wild-type WA-314 and *yts1E*. In contrast, the bacterial CFU recovered from liver and spleen were 100-fold higher in WA-314 compared to the *yts1E* mutant, representing a highly significant attenuation in the *yts1E* mutant. As an example, the bacterial CFU recovered from infected liver was  $6.3 \times 10^6$  CFU for WA-314 compared to  $5.2 \times 10^4$  CFU for *yts1E*.

In a parallel experiment, survival of *yts1E* mutant bacteria were compared with the reconstituted wild-type strain (in which the mutated *yts1E* gene was replaced with the wild-type allele), the results of which are depicted in Fig. 5B. Typically, the mutant strains presented with significant attenuation in spleen and liver, a finding comparable to the results described above. For example,  $2.3 \times 10^8$  bacterial CFU were recovered from infected liver for the reconstituted wild type compared to the  $1.9 \times 10^6$  CFU for the *yts1E* mutant, the latter representing a 120-fold attenuation in virulence by the mutant bacteria. In contrast, no significant differences in bacterial CFU were observed when the intravenous route of inoculation was used. In a mouse virulence assay, BALB/c mice (five animals per group) were inoculated intravenously with  $10^4$  bacteria. At 48 h after challenge, the mice were sacrificed and the bacterial loads were determined by plating tissue homogenates from the liver and spleen. The results (means  $\pm$  the standard deviation) were as follows: for strain WA-314,  $(1.0 \pm 0.14) \times 10^7$  CFU (liver) and  $(7.5 \pm 0.7) \times 10^7$  CFU (spleen); and for the *yts1E* mutant strain,  $(7.2 \pm 1.0) \times 10^6$  CFU (liver) and  $(4.5 \pm 0.2) \times 10^7$  CFU (spleen).

## DISCUSSION

The potential to secrete proteins into the extracellular medium is generally regarded as an important attribute of pathogenic bacteria. Typically, six bacterial pathways for protein

secretion to the extracellular milieu have been documented (2, 6, 12, 15, 24, 26, 30, 31, 45). These include the signal sequence independent pathway (type I), the main terminal branch of the general secretion pathway (type II), the contact-dependent pathway (type III), the type IV pathway related to bacterial conjugation systems, the two-partner secretion system exemplified by *Bordetella pertussis*, and the autotransporter pathway. Among pathogenic *Yersinia* species, four TTSSs have been discovered, namely, the Ysc TTSS responsible for Yops secretion, the Ysa TTSS of *Y. enterocolitica* species required for the extracellular secretion of a set of proteins designated Ysps, the chromosomally encoded TTSS of *Y. pestis*, and lastly the flagellar biosynthetic system (22, 32, 35, 41, 52, 53). Although the type II secretion pathway has been well described among many representatives of the *Enterobacteriaceae* (45), it has not been previously demonstrated among *Yersinia* species. In the present study, we describe the presence of a secretin, the Yts1 secretion apparatus unique to the HP *Y. enterocolitica* species. The *yts1* transport cluster shares significant homology with the *eps* cluster of *V. cholerae*, with declining identities to the putative type II secretion cluster in *Y. pestis* and the secretins identified in *Erwinia carotovora*, *Erwinia chrysanthemi*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

Originally discovered in *Klebsiella oxytoca* (16), in which it was found to be essential for extracellular secretion of the lipoprotein pullulanase, the type II transport cluster has been identified to date in a broad variety of gram-negative bacteria (31, 43, 44). In the classical studies by Francetic et al. (20, 21), an intact secretin was even identified in the widely used laboratory strain *E. coli* K-12 (nonpathogen). Typically, the interaction of between 12 to 15 gene products have been shown to be essential for protein secretion through this pathway. The encoded proteins form a complex network of interactions spanning the cytoplasmic, inner, and outer membranes. In the Yts1 secretin described here (Fig. 1), all of the interacting partners for a functional secretion are represented, namely, the cytoplasmic, energy-transducing Yts1E protein (which inter-

acts with protein L); the integral inner membrane proteins comprising Yts1C, Yts1M, Yts1K, and Yts1F; the four type 4 pilin-like proteins (pseudopilins), Yts1G, Yts1H, Yts1I, and Yts1J; the two outer member proteins or secretins, Yts1D and Yts1S; and finally, the Yts1O protein, a putative prepilin-like peptidase (51). This is in contrast to the system in *Y. pestis*, in which a Yts1M homologue does not exist (Fig. 1).

The reverse transcription data presented here suggest that the *yts1* genes are preferentially transcribed at 37°C. This is quite interesting since most of the virulence genes unique to *Yersinia* species come under thermoregulation. Transcription of the chromosomally encoded *ail* and many virulence pYV-encoded genes, for example, are temperature induced (3, 13, 39, 50). In contrast, the chromosomally encoded invasins are maximally produced at reduced temperatures (23°C), which makes sense since invasins, which function as adhesins, are important in the early stages of infection, and it would therefore be advantageous for them to be already present on ingested *Yersinia* (3, 28, 37). That the *yts1* genes are preferentially transcribed at 37°C speaks for a possible role of the secretin during the later stages of infection after invasion.

In order to determine the impact of this novel Yts1 secretin on the virulence of the species, mouse virulence tests were conducted that compared the wild-type WA-314 species with a *yts1IE* secretion-defective mutant. The results confirm that the chromosomally encoded Yts1 secretin does enhance the virulence of the *Y. enterocolitica* species as evidenced by a 100-fold attenuation in virulence observed in the *yts1IE* mutant when mice were perorally inoculated. In contrast, the numbers of bacterial CFU after intravenous inoculation of mice were not significantly different for the wild type and the secretion mutant. These results strikingly resemble the observations made by Haller et al. (22) with respect to the Ysa TTSS. These authors reported that the Ysa secretion system appears to play a role in the virulence of *Y. enterocolitica* strains when the bacteria are inoculated into mice perorally, whereas bacteria inoculated intraperitoneally appeared not to benefit from this transport system. Haller et al. concluded that the Ysa TTSS possibly played a role in virulence after the initial invasion of the Peyer's patches but before spread to deeper tissues. In contrast, the Yts1 secretin may be important in the further dissemination of bacteria into deeper tissues (liver and spleen) after initial invasion and colonization of Peyer's patches. The mouse virulence data presented here confirm this hypothesis since the attenuation in virulence for the *yts1IE* mutant was observed only with respect to colonization of liver and spleen, whereas the bacterial counts extrapolated from Peyer's patches were not significantly different between the wild type and the secretion-deficient mutant. Interestingly, as discussed above, preliminary reverse transcription analysis indicating preferential transcriptability of the *yts1* genes at 37°C would support the hypothesis for a virulence-related role of the Yts1 secretin occurring later in the course of infection. More-detailed studies, however, should shed light on the nature of the molecular interaction between the Yts1 secretin and the cognate host tissue it recognizes during the course of bacterial infection.

Sequence analysis of the genome of *Y. enterocolitica* 8081 revealed a second chromosomal locus with great similarity to a type II protein secretion locus (designated Yts2) that was confirmed to be uniformly distributed among all tested *Y. entero-*

*colitica* strains, including both nonpathogenic LP and HP strains (Fig. 4B). Future studies should reveal whether this common, generic Yts2 secretin is functional and will define the possible substrates that could rely on it for secretion.

Comparison of the *yts1* type II cluster with its counterpart on the *Y. pestis* genome reveals a divergence between these two systems with respect to both gene arrangement and sizes of the putative encoded ORFs. The *Y. pestis* secretin, however, shared greater homology with the Yts2 species-specific type II gene cluster common to all *Y. enterocolitica* strains in terms of both G+C content (a low 33 to 34%) and the arrangement of genes bordering the two clusters (see Fig. 1). This finding could argue for a common origin for these two secretion clusters, whereas the Yts1 transport system was independently acquired by the HP *Y. enterocolitica* 1B strains.

The genes encoding certain secretion systems have been assumed to be products of horizontal gene transfer, as evidenced by their different G+C content compared to the average G+C content of the organism (27, 33). The genes of the Yts1 protein secretion apparatus, however, have an average G+C content of 48%, a finding characteristic of the *Yersinia* species and a codon usage in line with the species. This could argue against horizontal acquisition of the transport system or might point to transfer from an organism with similar G+C content. Interestingly, in WA-314, a copy of an IS10-like element, IS1330, flanks upstream the *yts1* cluster, which might be indicative of an insertional event, and analysis of the sequences downstream of the flanking *chiY* gene will yield additional information in this regard. Future studies will therefore shed light on what mechanisms played a role in the acquisition of this additional type II secretion system by the HP *Y. enterocolitica* species.

The Yts1 protein secretion system shares the highest homology with the Eps transport system in *V. cholerae* responsible for the secretion of the cholera toxin, a protease, and a chitinase (44). It is therefore tempting to propose the existence of an additional virulence factor such as a toxin that could rely on the transport cluster for export. Future studies will focus on defining the substrates that are recognized by the Yts1 secretion cluster, a potential candidate being the product encoded by the *chiY* gene that flanks the cluster, and the heat-stable enterotoxin.

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