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Homologues of Insecticidal Toxin Complex Genes in *Yersinia enterocolitica* Biotype 1A and Their Contribution to Virulence

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***Yersinia enterocolitica* is an enteric pathogen that consists of six biotypes: 1A, 1B, 2, 3, 4, and 5. Strains of the latter five biotypes can carry a virulence plasmid, known as pYV, and several well-characterized chromosomally encoded virulence determinants. *Y. enterocolitica* strains of biotype 1A lack the virulence-associated markers of pYV-bearing strains and were once considered to be avirulent. There is growing epidemiological, clinical, and experimental evidence, however, to suggest that some biotype 1A strains are virulent and can cause gastrointestinal disease. To identify potential virulence genes of pathogenic strains of *Y. enterocolitica* biotype 1A, we used genomic subtractive hybridization to determine genetic differences between two biotype 1A strains: an environmental isolate, *Y. enterocolitica* IP2222, and a clinical isolate, *Y. enterocolitica* T83. Among the *Y. enterocolitica* T83-specific genes we identified were three, *tcbA*, *tcaC*, and *tccC*, that showed homology to the insecticidal toxin complex (TC) genes first discovered in *Photorhabdus luminescens*. The *Y. enterocolitica* T83 TC gene homologues were expressed by *Y. enterocolitica* T83 and were significantly more prevalent among clinical biotype 1A strains than other *Yersinia* isolates. Inactivation of the TC genes in *Y. enterocolitica* T83 resulted in mutants which were attenuated in the ability to colonize the gastrointestinal tracts of perorally infected mice. These results indicate that products of the TC gene complex contribute to the virulence of some strains of *Y. enterocolitica* biotype 1A, possibly by facilitating their persistence in vivo.**

Three species of the genus *Yersinia*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*, are pathogenic for humans. *Y. pestis* is the causative agent of bubonic and pneumonic plague and is transmitted by flea bites or respiratory aerosols. *Y. pseudotuberculosis* and *Y. enterocolitica* are intestinal pathogens that can produce symptoms such as diarrhea, fever, and abdominal pain if they are ingested in contaminated food or water (8).

Y. enterocolitica is a heterogenous species which is divided into six biotypes: 1A, 1B, and 2 through 5, on the basis of its biochemical behavior (37). Of these biotypes, only 1B and 2 to 5 ever carry the *Yersinia* virulence plasmid (pYV), which encodes approximately 50 proteins, including a surface adhesin, YadA, a type III secretion system, and 12 effector proteins that allow the bacteria to evade phagocytosis and killing by neutrophils and macrophages (7). Strains of biotypes 1B and 2 through 5 also carry chromosomal genes that have been implicated in virulence, including *inv* and *ail*, which mediate invasion of eukaryotic cells; *myf*, which encodes a fimbrial adhesin; and *ystA*, which encodes a heat-stable enterotoxin (Yst-a) (8). In addition to these factors, biotype 1B strains possess iron acquisition genes on a "high-pathogenicity island" and the *ysa* type III secretion apparatus, which also contribute to virulence (5, 18).

Y. enterocolitica strains of biotype 1A do not carry pYV and typically lack Ail, Myf, the *ysa* type III secretion system, and the high-pathogenicity island and seldom produce Yst-a (reviewed in reference 34). Although this suggests that *Y. enterocolitica* biotype 1A strains are not pathogenic, they have been isolated from patients with gastrointestinal symptoms in various countries around the world (34) and in two controlled studies were found to be significantly associated with disease (14, 25). In addition, Burnens et al. (4) reported that the duration and severity of infections with biotype 1A *Y. enterocolitica* are similar to those caused by pYV-bearing strains.

The epidemiological evidence that some strains of *Y. enterocolitica* biotype 1A are able to cause disease is supported by laboratory investigations showing that strains of this biotype can be separated into two groups: pathogenic and nonpathogenic (16, 17). Members of the pathogenic group, comprising strains isolated from humans with gastrointestinal symptoms, possess several virulence-associated properties that are absent from strains obtained from other sources. These properties include a significantly greater capacity to invade HEp-2 and Chinese hamster ovary (CHO) cells, to survive within bone marrow-derived macrophages, to egress or "escape" from HEp-2 cells and macrophages, and to persist within the gastrointestinal tracts of perorally inoculated mice for longer periods than biotype 1A strains from nonclinical sources (16, 17). The factors that allow only some biotype strains to exhibit these characteristics are not known.

The identification of virulence genes in *Y. enterocolitica* biotype 1A would contribute to our understanding of how these bacteria cause disease and provide diagnostic tools to distinguish potentially pathogenic biotype 1A strains from their less

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Y. enterocolitica		
T83	Biotype 1A, O:5, clinical isolate, New Zealand	S. Fenwick
IP2222	Biotype 1A, O:36, water isolate, Japan	G. Wauters
ST5	T83 derivative; *tcaC*::Km^r (pPH1JI)	This study
ST6	T83 derivative; *tccC*::Km^r (pPH1JI)	This study
ST7	T83 derivative; *tcbA*::Km^r	This study
E. coli		
DH1	F$^-$ *supE44 recA1 endA1 gyrA96* (Nal^r) *thi1 hsdR17*(r_k^- m_k^+) *relA1*	19
DH5α	*endA1 hsdR17*(r_k^- m_k^+) *supE44 thi1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80*lac*Δ(*lacZ*)*M15*]	29
LE392	F$^-$ *hsdR574*(r_k^- m_k^+) *supE44 supF58 lacY1 or* Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55*	Promega
SM10 (λ*pir*)	*thi thr tonA lacY supE recA*::RP4 2-Tc::Mu (λ*pir*) Km^r	32
XL1-Blue	F::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17*(r_k^- m_k^+) *supE44 relA1 lac*	Stratagene
Plasmids		
pBAC1	pBluescript II containing the *cat* gene from pACYC184; Ap^r Cm^r	A. J. Pittard
pBluescript II SK$^-$	Cloning vector; Ap^r	Stratagene
pGEM-T Easy	Cloning vector; Ap^r	Promega
pHC79	Cosmid vector; Ap^r Tc^r	22
pKD46	Red recombinase expression plasmid; Ap^r	9
pKD46CAT	pKD46 containing the *cat* gene from pACYC184; Ap^r Cm^r	This study
pPH1JI	*tra IncP1* Cm^r Gm^r Sm^r Sp^r	21
pRK404	Cloning vector; *lacZ tra IncP1* Tc^r	12
pUC4KIXX	pUC4K derivative containing Km^r and Bm^r genes from Tn5	Amersham Biosciences

virulent counterparts. For this study, we used genomic subtractive hybridization, a technique that has been applied to many bacterial pathogens to discover novel virulence genes and targets for diagnostic purposes (38). This approach led to the identification of novel virulence-associated genes of *Y. enterocolitica* biotype 1A, related to the insecticidal toxin complex (TC) genes of other bacterial species.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. In addition to these strains, a sample of *Yersinia* strains from our culture collection was screened for the presence of DNA sequences. This sample consisted of 120 *Y. enterocolitica* isolates (78 clinical biotype 1A, 28 nonclinical biotype 1A, and 14 non-biotype 1A strains) and 2 *Yersinia aldovae*, 3 *Yersinia bercovieri*, 4 *Yersinia frederiksenii*, 6 *Yersinia intermedia*, 4 *Yersinia kristensenii*, 3 *Yersinia mollaretii*, and 13 *Y. pseudotuberculosis* strains. *Yersinia* strains were grown in brain heart infusion (BHI; Oxoid, Hampshire, England) broth or on BHI agar at 28 to 30°C. *Escherichia coli* strains were cultured in Luria-Bertani broth or on Luria-Bertani agar at 37°C. When required, antibiotics were used at a final concentration of 100 μg of kanamycin, 100 μg of ampicillin, 12 μg of tetracycline, 20 μg of chloramphenicol, and 15 μg of gentamicin per ml.

Molecular biology methods. DNA extractions and manipulations were performed according to established protocols (1, 29) or manufacturers' instructions. Colony blot and reverse dot blot hybridizations were performed using digoxigenin (DIG)-labeled probes at 68°C as described in the DIG Application Manual (Roche Diagnostics, Mannheim, Germany). *E. coli* cells were made electrocompetent by washing them with 10% (vol/vol) glycerol (29). Electrocompetent *Y. enterocolitica* cells were prepared as described by Conchas and Carniel (6).

Construction and analysis of a subtracted DNA library. Genomic subtractive hybridization was performed using the Clontech PCR-Select Bacterial Genome Subtraction kit with *Y. enterocolitica* T83, a clinical isolate, as the tester and *Y. enterocolitica* IP2222, an isolate from water, as the driver. The virulence-associated properties of these two strains have been reported previously (17). Briefly, 2-μg samples of *Y. enterocolitica* T83 and *Y. enterocolitica* IP2222 genomic DNA were each digested with RsaI, and two PCR adaptors were ligated to different aliquots of tester DNA. These preparations were denatured and hybridized at 63°C to an excess of denatured driver DNA which binds homologous sequences in the tester DNA (first hybridization). The two DNA pools were mixed together and hybridized at 63°C in the presence of more denatured driver DNA (second hybridization). Tester-specific sequences were amplified in two rounds of PCR using adaptor-specific primers. The subtracted fragments were ligated to pGEM-T Easy and electroporated into *E. coli* XL1-Blue.

To determine if the clones possessed *Y. enterocolitica* T83-specific sequences, reverse dot blot hybridization was performed. Inserts were amplified using adaptor-specific primers, applied to each of two positively charged nylon membranes (Roche Diagnostics) using a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA), and hybridized with DIG-labeled, RsaI-digested *Y. enterocolitica* T83 genomic DNA or DIG-labeled, RsaI-digested *Y. enterocolitica* IP2222 genomic DNA at high stringency. Inserts which hybridized to *Y. enterocolitica* T83 DNA but not to DNA from *Y. enterocolitica* IP2222 were deemed to possess tester-specific sequences.

Cosmid library construction and screening. A cosmid library of *Y. enterocolitica* T83 was constructed using the method described by DiLella and Woo (11). Briefly, 30- to 45-kb fragments of Sau3AI-digested *Y. enterocolitica* T83 genomic DNA were ligated to dephosphorylated pHC79 vector DNA and packaged into bacteriophage heads using the Packagene Lambda DNA Packaging System (Promega Corp., Madison, WI). These were subsequently used to infect *E. coli* LE392 cells according to the manufacturer's instructions. Approximately 800 clones were obtained and screened for the presence of subtracted DNA sequences by colony blot hybridization.

DNA sequencing and analysis. Nucleotide sequencing was performed using an ABI PRISM Big Dye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were analyzed on an Applied Biosystems ABI PRISM 377 DNA sequencer at the Australian Genome Research Facility. Vector-specific primers SP6 (5′-TATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) (26) were used to sequence inserts from subtracted clones. Homology searches were performed using the BLASTN, BLASTX, and BLASTP programs available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The promoter prediction program found at www.fruitfly.org/seq_tools/promoter.html was used to identify putative promoter sequences (28).

Prevalence of *Y. enterocolitica* TC genes. The prevalence of *Y. enterocolitica* T83 *tcbA*, *tcaC*, and *tccC* among 155 *Yersinia* strains was determined by colony blot

hybridization. DIG was incorporated into PCR amplicons using primers 57F (5′-TAATAGTATTTCGAACGGAGAC-3′) and 57R (5′-ACGGTTAACCAC ACCCAGTTC-3′), primers 91′F (5′-TGCCAACAAGTCTTAATGTTCC-3′) and 91′R (5′-TGATATAGCATACCTGGTAGC-3′), and primers 8′F (5′-TCA TAACTGTCACCGATCG-3′) and 8′R (5′-TACCAATTAAGCGCTGGGTC-3′) to produce probes to detect *tcbA*, *tcaC*, and *tccC*, respectively. Probes were hybridized at high stringency to bacteria that had been applied to Hybond N membranes (Amersham Biosciences, Buckinghamshire, England). Data were analyzed using Fisher's exact test (GraphPad Software, San Diego, CA).

Reverse transcription (RT) analysis. RNA was isolated from *Y. enterocolitica* T83 grown to log phase at 30°C and 37°C using the RNeasy mini kit (QIAGEN, Valencia, CA) and the RNase-free DNase set (QIAGEN) and quantified using a spectrophotometer. Equal amounts of RNA were converted into cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. A 580-bp fragment of the *tcbA* gene was amplified using primers 57F and 57R, a 360-bp fragment of the *tcaC* gene was amplified using primers 91′F and 91′R, and a 560-bp fragment of the *tccC* gene was amplified using primers 8′F and 8′R.

Construction of *Y. enterocolitica* T83 TC mutants. The *tcbA*, *tcaC*, and *tccC* genes of *Y. enterocolitica* T83 were inactivated by partial deletion and insertion of a kanamycin resistance (Km^r) gene. Inactivation of *tcbA* was achieved using λ Red recombinase (9). The low-copy-number plasmid pKD46 possesses three arabinose-inducible genes, γ, β, and *exo*, whose products are called Gam, Bet, and Exo, respectively (9). Gam inhibits host RecBCD exonuclease V, while Bet and Exo promote recombination. We cloned a chloramphenicol resistance (Cm^r) gene cassette into pKD46 so that the plasmid could be maintained in *Y. enterocolitica* T83, which is intrinsically resistant to ampicillin. A 1.3-kb Cm^r gene cassette was removed from pBAC1 by digestion with SmaI and HincII and ligated to blunted, NcoI-digested pKD46 to produce pKD46CAT. pKD46CAT was transformed into *Y. enterocolitica* T83 and transformants expressing λ Red recombinase were prepared from bacteria grown in SOB (29) containing 100 mM L-arabinose and chloramphenicol.

A *Y. enterocolitica tcbA*::Km^r construct, ST7, was made as follows. A 2.3-kb region of *tcbA* was amplified from *Y. enterocolitica* T83 genomic DNA using primers TcbAF (5′-TTCTACAGTAATGACTCTATGC-3′) and TcbAR (5′-TC GAACTGGGTGTGGTTAAC-3′) and ligated to pGEM-T Easy. A 525-bp Eco47III fragment was removed from *tcbA* and replaced with a Km^r gene cassette from pUC4KIXX. The 3.2-kb *tcbA*::Km^r construct was excised from this plasmid by digesting it with NotI. Approximately 1.2 μg of linear DNA was electroporated into *Y. enterocolitica* T83 expressing λ Red recombinase. A Km^r transformant, ST7, was obtained and subcultured at 37°C, on medium lacking chloramphenicol to eliminate the temperature-sensitive plasmid pKD46CAT. The partial deletion of native *tcbA* and subsequent integration of the Km^r gene cassette were confirmed by PCR.

A *tcaC* mutant of *Y. enterocolitica* T83, ST5, was constructed by amplifying a 1.5-kb segment of *tcaC* using primers 91′F and TcaCR1 (5′-ACCAACTGCAA CTGAGCGAC-3′) and ligating this fragment to pGEM-T Easy. A 478-bp fragment of *tcaC* was removed following digestion with EcoRV and replaced with a Km^r gene cassette on a 1.4-kb SmaI fragment from pUC4-KIXX. The *tcaC*::Km^r construct was excised from the resultant plasmid using NotI and ligated to Klenow-treated, BamHI-digested pRK404. This plasmid was transformed into *E. coli* SM10 (λ*pir*) and transferred to *Y. enterocolitica* T83 by conjugation. A double-crossover mutant, ST5, was obtained by mating the transconjugants with SM10 (λ*pir*) carrying the gentamicin-resistant (Gm^r) plasmid pPH1JI, which is incompatible with pRK404, and selecting on *Yersinia* selective (CIN) agar (Oxoid) containing kanamycin and gentamicin. Mutant ST5 was Km^r, Gm^r, and tetracycline sensitive (Tc^s) and was shown by PCR to have lost a 480-bp segment of *tcaC* and to have gained the Km^r gene cassette.

A *tccC* mutant of *Y. enterocolitica* T83, ST6, was obtaining by using primers TccCFm (5′-ACTAAGCATTCGCACACTGG-3′) and Tc3′ (5′-ATGTCGTCC AACGCGAAGC-3′) to amplify a 2.6-kb product containing part of the *tccC* gene. This product was ligated to pGEM-T Easy, after which a 1.6-kb BglII/BclI fragment was removed and replaced with a Km^r gene cassette. The *tccC*::Km^r construct was transferred to pRK404 and used to inactivate *tccC* by homologous recombination as described above. The resultant mutant, ST6, was confirmed to possess a deletion or a Km^r gene insertion.

Invasion of epithelial cells. Quantitative assays of bacterial invasion of CHO cells were performed as described previously (17). Briefly, semiconfluent CHO cell monolayers were prepared in 24-well tissue culture trays (Nunc, Roskilde, Denmark). CHO cells were cultured in αMEM (Trace Biosciences, Melbourne, Victoria, Australia) containing 2 mM glutamine, 20 mM HEPES, and 10% (vol/vol) heat-inactivated (56°C for 30 min) fetal calf serum (JRH Biosciences, Lenexa, KS). Approximately 2×10^7 CFU of bacteria grown overnight without shaking were added to each well, and the trays were centrifuged at $860 \times g$ for 8 min. Following incubation for 3 h at 37°C in 5% CO_2, nonadherent bacteria were removed and the cells were washed three times with phosphate-buffered saline (PBS). Cells were incubated for a further 90 min in tissue culture medium containing 100 μg/ml gentamicin to kill extracellular bacteria. This medium was subsequently removed, and the cells were washed with PBS as before. The CHO cells were then lysed with 200 μl of 0.1% (wt/vol) digitonin (Sigma-Aldrich Corp., St Louis, MO) for 5 min, followed by the addition of 800 μl of BHI broth and vigorous pipetting to disrupt the CHO cells. Bacteria released from the CHO cells were then enumerated on agar plates.

Bacterial colonization of mice. Six-week-old female BALB/c mice were inoculated by gavage with 100 μl of a 10% (wt/vol) solution of sodium bicarbonate, followed by 6×10^8 CFU of bacteria suspended in 200 μl of PBS. Two days after inoculation, the mice were killed by CO_2 inhalation and the ileum, cecum, and colon were removed aseptically. The samples were weighed and diluted 1 in 10 (wt/vol) in PBS. Each sample was homogenized using a Polytron homogenizer (Kinematica, Lucerne, Switzerland), and the homogenates were spread on duplicate CIN agar plates to determine the number of viable bacteria.

Statistical analysis. Statistical analysis was performed using InStat version 3.05 (GraphPad Software Inc., San Diego, CA). A two-tailed P value of < 0.05 was taken to indicate statistical significance.

Nucleotide sequence accession number. The *Y. enterocolitica* T83 TC gene sequences were submitted to GenBank and assigned accession number AY647257.

RESULTS

Construction and analysis of a *Y. enterocolitica* T83 subtraction library. Subtractive hybridization of *Y. enterocolitica* strain T83 (the tester strain) with strain IP2222 (the driver strain) yielded a pool of PCR products which were cloned into the vector pGEM-T Easy and electroporated into *E. coli* XL1-Blue to generate a subtracted DNA library. One hundred seventy-three clones obtained in this way were tested for the presence of *Y. enterocolitica* T83-specific inserts by reverse dot blot hybridization. Fifty-three (31%) of these hybridized with DNA from *Y. enterocolitica* T83, but not IP2222, and were deemed to possess *Y. enterocolitica* T83-specific sequences.

Tester-specific inserts were one-pass sequenced from pGEM-T Easy using vector-specific primers SP6 and T7. The sequences obtained ranged from 359 bp to approximately 1.7 kb, with an average size of 680 bp. Three sequences in the subtracted library were present twice, and one was present three times. Therefore, only 48 unique sequences were identified, of which 23 showed homology to genes encoding known proteins, 16 resembled genes for hypothetical proteins, and 9 had no significant matches in the databases.

Identification of *Y. enterocolitica* T83 insecticidal TC gene homologues. Four distinct *Y. enterocolitica* T83-specific sequences showed homology to several insecticidal TC genes which encode high-molecular-weight insecticidal toxins (3) that were first identified in the bacterium *Photorhabdus luminescens*. Sequence SH57 (579 bp) showed 36% (68/185) amino acid identity to toxin A from *P. luminescens*, sequence SH8′ (563 bp) showed 66% (124/186) amino acid identity to a putative insecticidal toxin from *Y. pestis* CO92, sequence SH62′ (376 bp) showed 61% (59/96) amino acid identity to a putative toxin subunit from *Y. pestis* KIM, and sequence SH91′ (359 bp) showed 59% (71/119) amino acid identity to an insecticidal TC from *Y. pestis* CO92. To characterize the genetic regions containing these fragments, a cosmid library of *Y. enterocolitica* T83 was screened for the presence of these sequences by colony blot hybridization. One cosmid clone, 1B12, was identified which hybridized with all four sequences. A 20-kb region en-

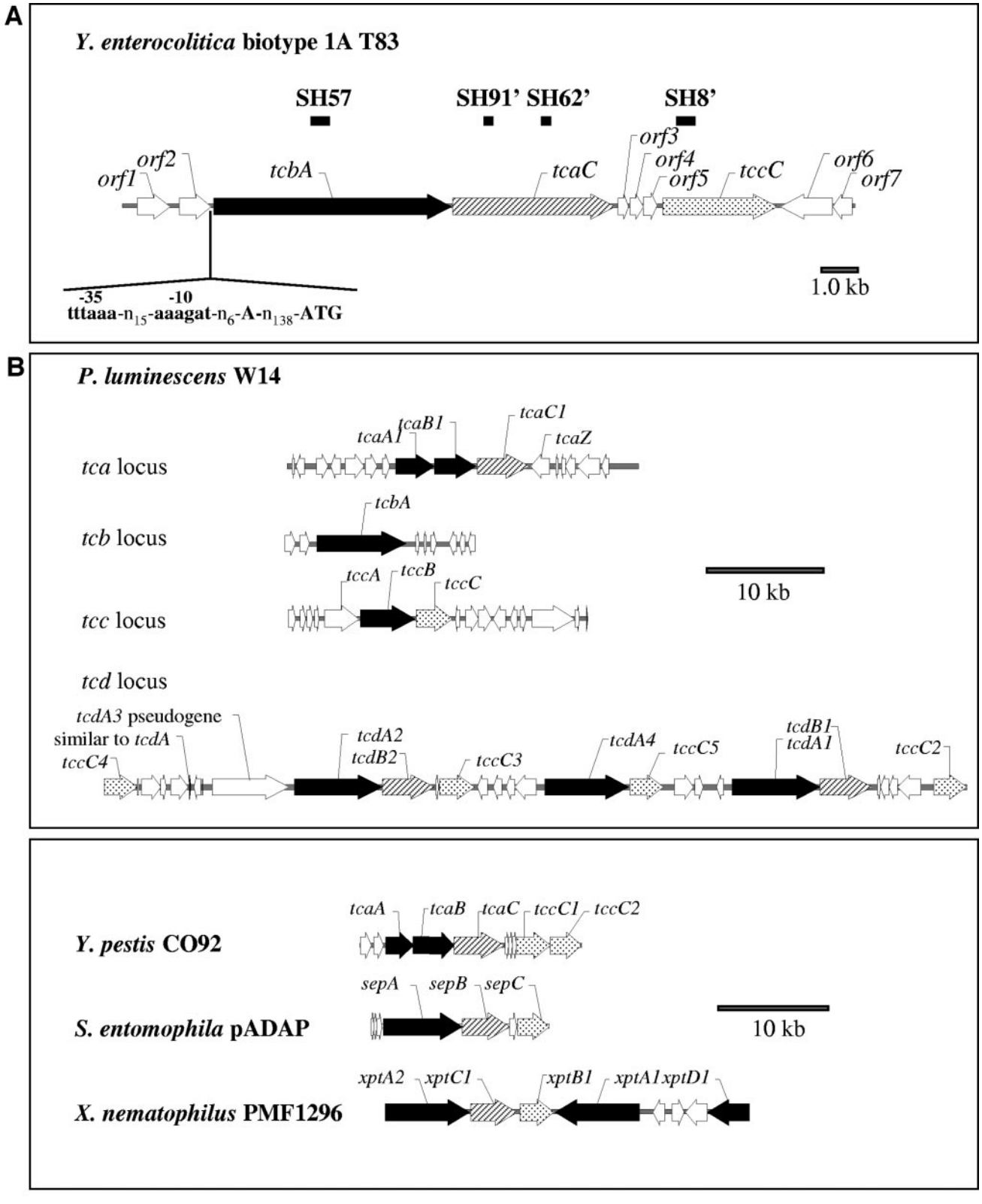

FIG. 1. (A) Schematic representation of the TC gene homologues of *Y. enterocolitica* biotype 1A T83. The regions identified by subtractive hybridization are marked SH57, SH91′, SH62′, and SH8′. A putative promoter upstream of the ATG start codon of *tcbA* is shown. (B) Genetic organization of the TC gene homologues of *P. luminescens* W14, *Y. pestis* CO92, *S. entomophila* pADAP, and *X. nematophila* PMF1296 (24, 36).

TABLE 2. Homology of the predicted ORFs surrounding subtractive hybridization sequences SH57, SH8′, SH62′, and SH91′[a]

ORF	Position (bp)	Size of product (amino acids)	First BLASTP hit	GenBank accession no.	% Amino acid identity (no. identical/total)
orf1	441–1352	303	Probable LysR family transcriptional regulatory protein (*Y. pestis*)	NP_407137	92 (281/303)
orf2	1606–2472	288	Putative LysR family transcriptional regulatory protein (*Y. pestis*)	NP_407136	62 (181/288)
tcbA	2551–9057	2,168	Insecticidal toxin complex protein TcbA (*Photorhabdus luminescens*)	AAC38627	54 (413/760)
tcaC	9118–13563	1,481	Y0185-like protein (*Y. pseudotuberculosis*)	AAS66065	59 (883/1491)
orf3	13641–13952	103	Putative phage-related protein (*Y. pestis*)	NP_407133	64 (63/98)
orf4	13964–14365	133	Hypothetical phage protein (*Y. pestis* KIM)	NP_667530	81 (108/133)
orf5	14362–14721	119	Putative exported protein (*Y. pestis*)	NP_407131	61 (73/119)
tccC	14905–18042	1,045	Insecticidal toxin complex protein TccC (*P. luminescens*)	AAC38630	47 (501/1047)
orf6	18113–19558	481	Putative modulator of DNA gyrase (*Y. pestis*)	NP_407128	98 (472/481)
orf7	19571–20080	169	Putative carbon-nitrogen hydrolase (*Y. pestis*)	NP_407127	88 (144/162)

[a] See Fig. 1.

compassing the four fragments was sequenced in each direction. Analysis of this sequence revealed three large open reading frames (ORFs) that were surrounded by smaller ORFs (Fig. 1). BLASTP homology searches showed that the three large ORFs were homologous to insecticidal TC proteins and were named *tcbA*, *tcaC*, and *tccC* (Table 2).

P. luminescens strains W14 and TT01 possess multiple copies of TC genes scattered over four TC loci, *tca*, *tcb*, *tcc*, and *tcd* (3, 13). However, these genes can be divided into three gene families: (i) *tcaAB*- or *tcb*/*tcdA*-like genes, (ii) *tcaC*- or *tcdB*-like genes, and (iii) *tccC*-like genes (36). *Y. enterocolitica* T83 possessed only one copy of the TC gene complex, as determined by Southern hybridization (data not shown). In this respect, *Y. enterocolitica* T83 is more similar to insect-associated *Y. pestis* and to the insect pathogens *Serratia entomophila* (which causes amber disease in the New Zealand grass grub, *Costelytra zealandica*) and *Xenorhabdus nematophila*, a close relative of *P. luminescens* (23, 24, 27) (Fig. 1).

Prevalence of the *Y. enterocolitica* T83 TC genes. The prevalence of *tcbA*, *tcaC*, and *tccC* from *Y. enterocolitica* T83 among 155 *Yersinia* strains was determined by colony blot hybridization. The results showed that the TC genes of *Y. enterocolitica* T83 were significantly more prevalent among 78 clinical biotype 1A strains than among 77 other *Yersinia* strains, including 28 nonclinical biotype 1A strains (Table 3). None of the TC genes was in *Y. enterocolitica* IP2222 or the well-studied pYV-bearing *Y. enterocolitica* strains W22703 (biotype 2, serotype O:9) and 8081 (biotype 1B, serotype O:8). Furthermore, BLAST analysis of the *Y. enterocolitica* 8081 genome sequence (available at http://www.sanger.ac.uk/Projects/Y_enterocolitica/) revealed that this strain does not possess any TC gene homologues.

Expression of the *Y. enterocolitica* T83 TC genes. RT-PCR was used to determine if *tcbA*, *tcaC*, and *tccC* were expressed by *Y. enterocolitica* T83. The results of this analysis showed that the TC genes are expressed at both 30°C and 37°C (Fig. 2).

Role of the TC genes in virulence. To determine if the *Y. enterocolitica* T83 TC genes contribute to virulence, three different allelic exchange mutants, ST7 (*tcbA*::Km^r), ST5 (*tcaC*::Km^r), and ST6 (*tccC*::Km^r), were constructed and tested for loss of virulence. Firstly, we examined the ability of the mutants to grow in BHI broth at 30°C and in αMEM supplemented with 10% heat-inactivated fetal calf serum and 20 mM HEPES at 37°C in 5% CO_2. Samples taken at 4-h intervals over a 28-h period showed that the TC mutants, ST7, ST5, and ST6, exhibited the same growth kinetics as the wild-type strain under both sets of conditions (data not shown).

We have previously shown that biotype 1A strains of clinical origin are able to invade CHO cells in higher numbers than strains obtained from the environment (16, 17). We tested the ability of the *tcbA* mutant, ST7, to invade CHO cells using a quantitative gentamicin protection assay. The invasive capacity of ST7 (1.83% ± 0.98%) was not significantly different from that of the wild-type strain (1.98% ± 0.05%; $P = 0.80$; Student's *t* test, two tailed). In addition, the ability of mutant ST7

TABLE 3. Prevalence of *Y. enterocolitica* T83 TC genes among other *Y. enterocolitica* biotype 1A strains and other *Yersinia* species

Gene	Hybridization of probe for TC gene to:				P^c
	Clinical biotype 1A strains ($n = 78$)	Nonclinical biotype 1A ($n = 28$)	Non-biotype 1A *Y. enterocolitica* ($n = 14$)	Other *Yersinia* species[b] ($n = 35$)	
tcbA	21 (27)[a]	2 (7)	0	0	<0.001
tcaC	21 (27)	4 (14)	0	0	<0.001
tccC	21 (27)	4 (14)	1 (7)	0	<0.001

[a] No. (%) of strains which hybridized.
[b] This sample consisted of 2 strains of *Y. aldovae*, 3 of *Y. bercovieri*, 4 of *Y. frederiksenii*, 6 of *Y. intermedia*, 4 of *Y. kristensenii*, 3 of *Y. mollaretii*, and 13 of *Y. pseudotuberculosis.*
[c] Comparison of clinical biotype 1A with all other *Yersinia* strains (Fisher's exact test, two tailed).

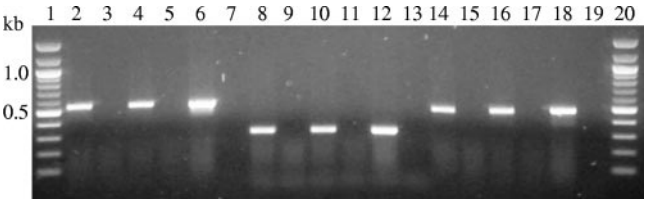

FIG. 2. RT-PCR analysis of the TC genes of *Y. enterocolitica* T83. Expression of *tcbA* (lanes 2 to 7), *tcaC* (lanes 8 to 13), and *tccC* (lanes 14 to 19) was assessed at 30°C (lanes 2, 8, and 14) and at 37°C (lanes 4, 10, and 16). Lanes: 1 and 20, molecular weight markers; 3, 5, 9, 11, 15, and 17, reverse transcriptase-negative controls; 6, 12, and 18, *Y. enterocolitica* T83 genomic DNA-positive controls; 7, 13, and 19, template-negative controls.

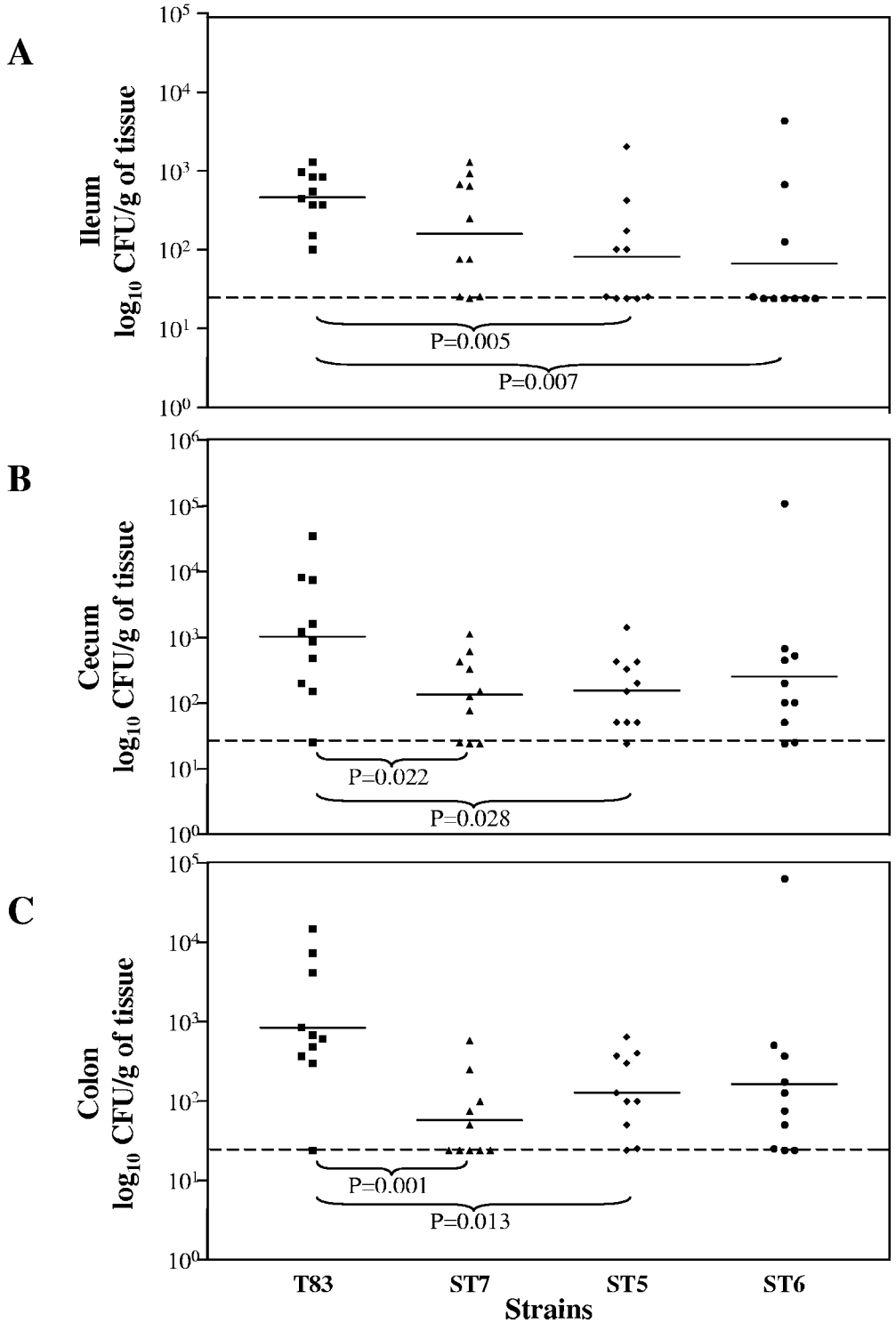

FIG. 3. Colonization of BALB/c mice by TC mutants of *Y. enterocolitica* T83. Ten mice were infected by gavage with strain ST7, ST5, or ST6 and killed after 2 days. The numbers of bacteria in the ileum (A), cecum (B), and colon (C) were compared with those of *Y. enterocolitica* T83 using Student's *t* test (two tailed). The mean values (short horizontal lines) and the detection limit of 25 CFU (horizontal dashed line) are shown. Mice with undetectable levels were assigned a value of 24.

to escape from HEp-2 cells was not different from that of the parent strain (data not shown).

We previously reported that *Y. enterocolitica* biotype 1A strains of clinical origin colonize the gastrointestinal tracts of mice for significantly longer periods than strains isolated from the environment (17). For this part of the study, we administered approximately 6×10^8 CFU of *Y. enterocolitica* T83 and the isogenic *tcbA*, *tcaC*, and *tccC* mutants to 10 6-week-old

BALB/c mice. Two days later, the mice were killed and the numbers of bacteria that had colonized the ileum, cecum, and colon were determined. This time point was chosen because previous investigations had shown that *Y. enterocolitica* biotype 1A strains of clinical origin are at their most abundant in these tissues 2 days after infection (17). All three *Y. enterocolitica* TC mutants generally showed a decreased ability to colonize the intestinal tracts of mice compared to wild-type strain *Y. enterocolitica* T83 (Fig. 3).

DISCUSSION

Although *Y. enterocolitica* biotype 1A strains were once regarded as nonpathogenic, we reported previously that they can be divided into two groups: one comprising strains of clinical origin that possess virulence-associated characteristics and another made up of strains obtained from nonclinical sources that lack virulence-associated characteristics (17). In this study, we used subtractive hybridization to detect DNA sequences in a clinical biotype 1A strain, *Y. enterocolitica* T83, which were absent from a biotype 1A strain isolated from water. Among the genes identified in this way were *tcbA*, *tcaC*, and *tccC*, which were expressed by *Y. enterocolitica* T83, and were significantly more prevalent among clinically sourced biotype 1A strains, suggesting that these genes may contribute to the virulence of some *Y. enterocolitica* strains of biotype 1A.

In *P. luminescens*, the TC genes encode high-molecular-weight toxins capable of killing insects (3). *P. luminescens* colonizes the folds between the extracellular matrix and basal membrane of the midgut epithelium of insects (31). During occupation of this specific niche, the bacteria express TC A (Tca), which induces rounding up of the cells of the midgut epithelium, causing them to detach from the basal membrane and accumulate in the gut lumen (2, 31). The exact role of the different TC proteins in pathogenesis is uncertain. Previous studies have indicated that members of the *tcaAB*- or *tcb*/*tcdA*-like gene family encode the active toxins, whereas the *tcaC*- and *tccC*-like gene families encode proteins involved in toxin activation. There is also evidence to suggest that when multiple *tcaAB*- or *tcb*/*tcdA*-like genes are present in one bacterium, the toxins they encode act on different targets. For example, when XptA1, XptB1, and XptC1 of *X. nematophila* are combined, they act on larvae of the cabbage white butterfly species *Pieris brassicae* and *Pieris rapae*, whereas the combination of XptA2, XptB1, and XptC1 is toxic for the tobacco budworm *Heliothis virescens* (30). These findings support the hypothesis that TcaC- and TccC-like proteins are involved in the delivery or activation of TcaAB- or Tcb/Tcd-like toxins with different host range specificities.

Two other insect pathogens that have TC gene homologues capable of killing insects are *S. entomophila* and *X. nematophila* (23, 24). The insect-associated bacterium *Y. pestis* has also been shown to possess TC gene homologues which are hypothesized to contribute to survival of the bacterium in its arthropod vector (10, 27). However, the *tcaB* gene of *Y. pestis* CO92 possesses a frameshift mutation and the *tcaC* gene has an internal deletion (27). These attenuations are thought to be advantageous to *Y. pestis*, as they may allow the bacteria to survive in the flea midgut without killing the insect. The *tcaC* gene, however, is only believed to possess an internal deletion based on comparisons to TC genes from other bacteria and could conceivably encode an active protein. Furthermore, although the *tcaB* gene of *Y. pestis* CO92 possesses a frameshift mutation, the corresponding *tcbA* homologues in *Y. pestis* strains KIM and 91001 do not (10, 33). The role of these genes in the flea is therefore uncertain.

Interestingly, some bacteria with no known association with insects also possess TC gene homologues, including the enteric pathogens *Y. enterocolitica* biotype 1A and *Y. pseudotuberculosis*, the plant pathogen *Pseudomonas syringae*, the cellulolytic bacterium *Fibrobacter succinogenes*, and the dental pathogen *Treponema denticola* (15, 20). It is possible that the TC genes in these bacteria do not encode insecticidal toxins but serve a different purpose.

Compared to *Y. enterocolitica* T83, isogenic *tcbA*, *tcaC*, and *tccC* deletion mutants exhibited a decreased ability to colonize the ilea, ceca, and colons of mice perorally inoculated with these bacteria 2 days earlier. This was not due to differences in growth kinetics or invasive ability (in the case of the *tcbA* mutant, ST7). Attempts to complement the *Y. enterocolitica* T83 TC mutants with wild-type copies of the TC genes in high-copy-number vectors were unsuccessful, possibly due to toxic effects on *E. coli* intermediates. Difficulties in cloning *tcaAB*- or *tcb*/*tcdA*-like genes in *E. coli* have been reported by other workers (23, 35). Although we were able to clone the three TC genes together on cosmid 1B12, this clone was unstable and could not be used to complement the TC mutants. Despite our inability to complement the TC mutants, it is apparent that the TC genes of *Y. enterocolitica* T83 play a role in the persistence of the bacterium in the gastrointestinal tracts of mice, given that each of the *tcbA*, *tcaC*, and *tccC* insertion-deletion mutants displayed a similar phenotype. The exact roles of the TC proteins in vivo, however, are unknown. We have shown that the *tcbA* gene is not involved in invasion of cells in vitro. Furthermore, *Y. enterocolitica* T83 and other TC-positive biotype 1A strains do not exhibit any cytotoxic effects on mammalian cells in vitro (reference 16 and unpublished data). Possibly, the TC proteins contribute to the enterotoxic activity of some *Y. enterocolitica* biotype 1A strains we have reported previously (17).

In conclusion, we have used subtractive hybridization to identify homologues of insecticidal TC genes in *Y. enterocolitica* biotype 1A. These genes were more prevalent among clinical biotype 1A yersiniae than other *Yersinia* strains but were present in only 27% of clinical biotype 1A strains, indicating that they are not essential for virulence and that they are unsuitable for use in diagnostic applications to identify virulent strains of *Y. enterocolitica* biotype 1A. We also showed that inactivation of the TC genes of *Y. enterocolitica* biotype 1A resulted in mutants which were attenuated in the ability to colonize the gastrointestinal tracts of perorally inoculated mice. Further investigations of these proteins should provide novel insights into the pathogenic mechanisms of some strains of *Y. enterocolitica* biotype 1A.

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