INFECTION AND IMMUNITY, May 2002, p. 2708–2714 Vol. 70, No. 5
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.5.2708–2714.2002
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Yersinia pseudotuberculosis Produces a Cytotoxic Necrotizing Factor

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Received 30 November 2001/Returned for modification 16 January 2002/Accepted 1 February 2002

Cell extracts from *Yersinia pseudotuberculosis* induced multinucleation in HEp-2 cells in a manner similar to the effect caused by *Escherichia coli* cytotoxic necrotizing factor (CNF). The activity was not dependent on the *Yersinia* 70-kb virulence plasmid, and the activity was not inhibited by antibodies capable of neutralizing *E. coli* CNF type 1. The nucleotide sequence of the *Yersinia cnf* gene was 65.1% identical to the *E. coli cnf* gene.

Cytotoxic necrotizing factor (CNF) is a protein toxin (110 to 115 kDa) that is prevalent among *Escherichia coli* isolates from humans and domesticated animals with extraintestinal infections (8; reviewed in references 16 and 27). Caprioli and coworkers (11) identified the prototypical *E. coli* CNF type 1 (CNF1), and CNF1 is the best-characterized member of an emergent class of dermonecrotic bacterial toxins that constitutively activate eukaryotic small GTP-binding proteins (GTPases) (21, 31, 42, 46). However, there are independent descriptions of a dermonecrotic protein exotoxin in sterile broth culture supernatants from *Yersinia pseudotuberculosis* (9, 24, 29, 41). This report describes the identification of a CNF from *Y. pseudotuberculosis*. For clarity, the CNF and the gene encoding the toxin from *Yersinia pseudotuberculosis* will be referred to, respectively, as CNF_Y and cnf_Y. The gene and gene product from *E. coli* will be referred to, respectively, as cnf_E and CNF_E.

The following *Yersinia* species were examined: *Y. pseudotuberculosis* (33 strains from serogroups IA, IB, II, III, and V), *Y. enterocolitica* (9 strains from serogroups O3; O4,32; O5,27; O6; O8; O9; O20; O21; and O27), *Y. bercovieri* (3 strains), *Y. frederiksenii* (4 strains), *Y. intermedia* (3 strains), *Y. kristensenii* (3 strains), *Y. mollaretii* (2 strains), and *Y. rohdei* (1 strain). *E. coli* strain J96 ($CNF1^+$) was the source of *E. coli* CNF_E as previously described (39). *E. coli* strain DH5α (Life Technologies, Inc., Gaithersburg, Md.) was the host for cloned *cnf* genes. pHLK102 is a clone of the *E. coli* cnf_E gene (39), and pHLK602 expresses the cnf_Y gene cloned from *Y. pseudotuberculosis* strain YPIII as part of the present work. Bacteria were grown for 18 to 24 h at 26 or 37°C in L broth (30) or tryptic soy broth (Difco Laboratories, Inc., Detroit, Mich.), and on L agar (30). Broth cultures were aerated on a shaker (200 rpm) or on a roller drum. Strains containing recombinant plasmids were cultured in media containing ampicillin (100 μg/ml) or chloramphenicol (30 μg/ml).

CNF biological activity and specific activity were assayed with HEp-2 cells as previously described (34, 39). Tryptic soy broth cultures were centrifuged at 4°C to separate the bacteria from the culture broth supernatants, and broth culture supernatants were filter sterilized through 0.2-μm-pore-size low protein-binding membranes (Millipore Corp., Bedford, Mass.). Bacterial cells were concentrated 10-fold in phosphate-buffered saline containing gentamicin (100 μg/ml) and lysed by sonication or by three cycles of freezing and thawing (35). Bacterial lysates were clarified by centrifugation at 4°C and filter sterilized. Cell-free lysates and sterile supernatants were diluted in 0.1 ml of Eagle minimum essential medium with Earle's balanced salt solution (BioWhittaker, Inc., Walkersville, Md.) containing 10% fetal bovine serum (BioWhittaker, Inc.), gentamicin (100 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) and added to HEp-2 cells cultured in the same medium. The cultures were incubated for 72 h at 37°C in an atmosphere containing 5% CO_2. Culture fluid was then removed, and the cells were fixed and stained with a modified Wright's stain (Hema 3; Fisher Scientific Co.). Stained cells were examined by bright-field microscopy and digitally photographed with a Kodak DC120 camera. The 50% cytopathic dose (CD_{50}) was determined by the dilution of a sample that produced multinucleation in 50% of the cells in a well of the tissue culture plate. Specific activity was defined as the number of CD_{50}s per microgram of protein in a sample. The amount of protein in bacterial cell-free lysates and sterile supernatants was quantitated spectrophotometrically (BCA Protein Assay Kit; Pierce Chemical Co., Rockford, Ill.), and bovine serum albumin was used as a standard. Neutralization of biological activity by specific antibodies was assayed in vitro with HEp-2 cells as previously described (34, 39).

Standard methods were used for DNA purification and cloning (32, 40). Restriction endonucleases (New England Biolabs, Beverly, Mass.) and DNA-modifying enzymes (New England Biolabs and Roche Molecular Biochemicals, Indianapolis, Ind.) were used according to the manufacturers' specifications. Restriction enzyme digestion products and PCR amplification products were transferred from agarose gels to nylon membranes under alkaline conditions (38). Dot blots were prepared by denaturing samples of DNA with 0.5 M NaOH and applying the samples to a nylon membrane filter (Minifold; Schleicher & Schuell, Keene, N.H.). Membranes were hybridized with horseradish peroxidase-labeled probes (described below), washed, and exposed to X-ray film according to the manufacturer's specifications (ECL Direct Labeling and Detection System; Amersham-Pharmacia Biotech, Piscataway, N.J.). Autoradiograms were scanned, and the digital images were annotated with Canvas (Deneba Systems, Inc., Miami, Fla.).

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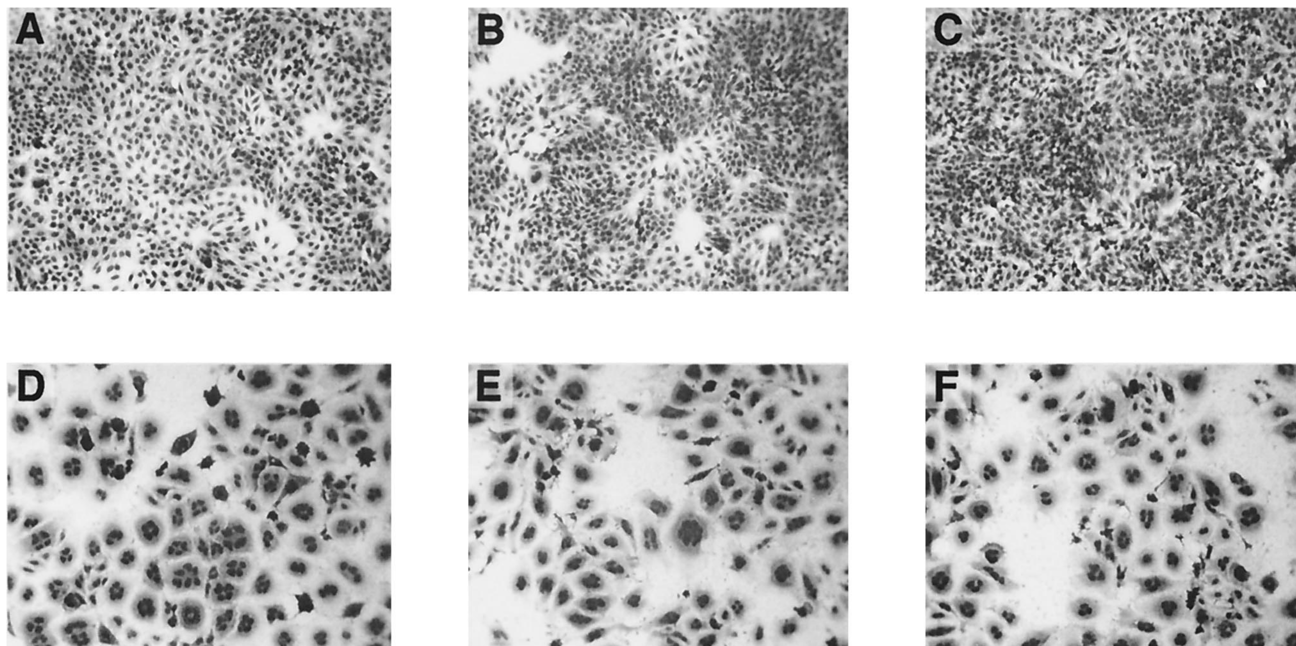

FIG. 1. HEp-2 cell tissue culture assay for CNF biological activity. (A) Untreated control; (B) broth culture supernatant from the CNF_Y^- *Y. enterocolitica* strain 8081v; (C) cell-free lysate from CNF_Y^- *Y. enterocolitica* strain 8081v; (D) cell-free lysate from the CNF_E^+ *E. coli* strain J96; (E) broth culture supernatant from the CNF_Y^+ strain *Y. pseudotuberculosis* strain YPIII; (F) cell-free lysate from the CNF_Y^+ strain *Y. pseudotuberculosis* strain YPIII. All photomicrographs (panels A through F) were taken at the same magnification (10× lens objective) to clearly depict the giant, multinucleated phenotype of cells in panels D to F. Cells were digitally photographed by light microscopy with a Kodak DC120 camera, and the image file was annotated with Canvas.

The cnf_E probe was a 3.0-kb *Bam*HI-*Eco*RI gel-purified fragment from pHLK102 (39), and the cnf_Y probe from pHLK602 was a 2.8-kb *Bsa*I-*Nco*I fragment.

The PCR was done as previously described (39) with commercially synthesized primers (Life Technologies, Inc.) that were designed to amplify *cnf* sequences from *E. coli* (19) and *Yersinia* (37). The following primers were used to amplify *E. coli* cnf_E: forward, 5′-TATTAATCTTCACAGAGGAG-3′; reverse, 5′-CCGGTTATTTATTAAAGGGCTTAG-3′. The following primers were used to amplify *Yersinia* cnf_Y: forward, 5′-TGCATCGTCAATAAAAGGAGTGTT-3′; reverse, 5′-CAATTTGGTTTTACTGGTGGTTCA-3′. The sequences of the *Yersinia* cnf_Y primers correspond to nucleotides 800 to 823 and 2918 to 2941 of the sequence that was determined in this study.

DNA sequencing was performed with dichlororhodamine terminator cycle-sequencing reagents (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's specifications, and automated sequencing was performed on an ABI Prism 377 instrument. Universal primers were used to sequence the ends of cloned DNAs, and custom primers were designed from the *Y. pseudotuberculosis* strain YPIII sequences that were obtained. These custom primers were used to complete the sequencing of the cloned DNA, and they were also used in direct sequencing of genomic DNA. The data reported here were obtained by sequencing both strands of the template. BLAST (3) alignments were performed with data at the National Center for Biotechnology Information (NCBI) (at the website http://www.ncbi.nlm.nih.gov/blast/) and from the Sanger Center (at the website http://www.sanger.ac.uk/Projects/Y_pestis/) (37). Additional analyses of DNA sequences were performed with the software packages Lasergene, version 4.0.3 (DNASTAR, Inc., Madison, Wis.) and the Wisconsin Package, version 10.1-Unix (Genetics Computer Group, Madison, Wis.).

Broth supernatants and cell-free lysates from cultures of different species of *Yersinia* were tested for cytopathic activity. A total of 58 strains of *Yersinia* were examined, including 33 strains of *Y. pseudotuberculosis*. HEp-2 cells treated with extracts from *Y. pseudotuberculosis* strains YPIII (7, 22) and IP2666c (cured of the 70-kb virulence plasmid [45]) developed into giant, multinucleated cells that remained viable and attached to the culture substrate (Fig. 1). *Y. pseudotuberculosis* strain YPIIIc, a derivative of strain YPIII that was cured of the virulence plasmid (7, 22), also expressed CNF_Y activity when grown in vitro at 26 or 37°C. CNF_Y activity was not detected in other strains of *Y. pseudotuberculosis* (Table 1 and data not shown) or from additional *Yersinia* species (Fig. 1B and C). Additional preliminary experiments indicated that CNF_Y activity was constitutively expressed throughout the growth cycle of *Y. pseudotuberculosis* and expression was not regulated by temperature or $[Ca^{2+}]$ (not shown). The morphology of cells treated with extracts from CNF_Y^+ *Y. pseudotuberculosis* was indistinguishable from that caused by *E. coli* CNF_E (compare Fig. 1D with Fig. 1E and F). The specific activity in cell-free lysates of *Y. pseudotuberculosis* strain YPIII was 38-fold higher than that of the CNF_E^+ *E. coli* strain J96, and a substantial amount of CNF_Y activity (10^3 CD_{50}s/μg of protein) was found in the supernatants from broth cultures of CNF_Y^+ *Y. pseudotuberculosis* strains (Fig. 1E). CNF_E activity from *E. coli* strain J96 was cell associated, and no biological activity was detected

TABLE 1. Distribution of cnf_Y among *Y. pseudotuberculosis* strains

Strain[a]	CNF_Y biological activity[b]	Approx size (kb) of cnf_Y allele[c]	HPI[d]	Sero-type	Source (reference)
YPIII	+	3.0	−	III	V. L. Miller (7, 22)
YPIIIc	+	3.0	−	III	V. L. Miller (7, 22)
713425	−	2.1	−	IA	V. L. Miller
722080	−	2.1	−	IB	V. L. Miller
730317	−	2.1	−	I	V. L. Miller
730440-1	−	1.8	+	IB	V. L. Miller
ATCC 29833	−	1.8	ND[e]	ND	ATCC[f]
PTB1	−	1.8	+	ND	K. A. McDonough (25)
PTB4	−	1.8	+	IA	K. A. McDonough (25)
PTB6	−	1.8	+	III	K. A. McDonough (25)
PTB7	−	1.8	+	ND	K. A. McDonough (25)
PTB8	−	1.8	+	III	K. A. McDonough (25)
PTB9	−	1.8	+	IA	K. A. McDonough (25)
PTB13	−	2.1	−	I	K. A. McDonough (25)
PTB16	−	1.8	−	III	K. A. McDonough (25)
PTB20	−	2.1	−	IA	K. A. McDonough (25)
PTB22	−	1.8	−	IA	K. A. McDonough (25)
IP2515c	−	2.1	ND	II	J. B. Bliska (45)
IP2666c	+	3.0	ND	III	J. B. Bliska (45)
IP2775c	−	1.8	ND	I	J. B. Bliska (45)
IP2777c	−	1.8	ND	I	J. B. Bliska (45)
IP2790c	−	1.8	ND	I	J. B. Bliska (45)
IP32953	−	1.8	+	I	E. Carniel (10)

[a] Strains with names ending in 'c' are cured of the *Yersinia* virulence plasmid.
[b] Sterile cell extracts induced the formation of giant, multinucleated HEp-2 cells.
[c] Three cnf_Y alleles were identified in this study: 3.0 is the size of a full-length gene shown in Fig. 2 and 3; 2.1 is the size of a 3′-deleted gene also found in *Y. pestis* strain CO92 (37) and shown in Fig. 3; 1.8 is the size of a variant with 5′ and 3′ deletions.
[d] HPI, *Yersinia* high-pathogenicity island.
[e] ND, not determined.
[f] ATCC, American Type Culture Collection, Manassas, Va.

in filter-sterilized broth-culture supernatants, an observation that was consistent with previous studies of CNF_E (11, 23). *Y. pseudotuberculosis* strains YPIII and IP2666c belong to serogroup III (7, 22, 45), whose strains have been previously reported to produce a dermonecrotic protein exotoxin (9, 24, 26, 29, 41, 47). In the present work, however, additional serogroup III strains did not express CNF_Y biological activity and these strains contained deletions within the cnf_Y gene (described below). Thus, no relationship was found between serogroup and toxin production.

A DNA probe prepared from the cloned *E. coli* cnf_E gene hybridized to a single restriction endonuclease fragment in a Southern blot of total DNA from *Y. pseudotuberculosis* strain YPIII (not shown). However, PCR primers that were designed to amplify cnf_E from *E. coli* (39) did not amplify sequences from *Y. pseudotuberculosis* strain YPIII. A large open reading frame (ORF), with a high level of similarity to the amino acid sequence of *E. coli* CNF_E, was identified by a TBLASTN search of the *Y. pestis* genome sequence (37), and PCR primers designed from the cnf_Y sequence in *Y. pestis* were used to amplify a 2.1-kb product from *Y. pseudotuberculosis* strain YPIII. This PCR product was cloned and used as a probe to identify a 5.4-kb chromosomal fragment from *Y. pseudotuberculosis* strain YPIII DNA that was digested with *Bam*HI and *Sal*I (not shown). This fragment was cloned into pBRKS (44) to create pHLK602, which expressed CNF_Y biological activity in *E. coli* K-12 strain DH5α. Additional evidence that the *Y. pseudotuberculosis* cnf_Y sequence was responsible for the observed biological activity was obtained by inserting an Ω-Cm^r fragment (20) into the chromosomal cnf_Y gene of *Y. pseudotuberculosis* strain YPIII by allelic exchange (17). The derivative with the cnf_Y:: Ω-Cm^r mutation, named strain WEX5000, was confirmed by PCR and Southern blotting and expressed no CNF_Y activity in the HEp-2 tissue culture assay. The mutation in strain WEX5000 was complemented by pHLK602.

Analysis of the sequence of the *Yersinia* DNA cloned in pHLK602 identified a large ORF with a predicted translation product that was highly similar to CNF_E. There are two alleles of *E. coli cnf* (cnf_1 and cnf_2) whose DNA and predicted amino acid sequences are ≥84% identical (19, 36). When the DNA or predicted amino acid sequences of cnf_Y from *Y. pseudotuberculosis* were compared to either of the two *E. coli* sequences, there was ≤1% difference in the results (not shown). Thus, the sequence of cnf_1 (19) was used for the comparisons of cnf_Y and cnf_E reported here. The cnf_Y ORF was 3,045 bp in size and had 65.1% sequence identity with cnf_E. The predicted amino acid sequence of CNF_Y was 60.8% identical and 68.5% similar to that of CNF_E (Fig. 2). The homology between CNF_Y and CNF_E was evenly distributed throughout the entire sequence. The two largest areas of dissimilarity were 5 amino acids (aa) in length, there was one region containing four nonconservative amino acid changes, and all of the remaining dissimilarities were 3 aa in length, or smaller. Cysteine and histidine residues that were essential for the biological activity of CNF_E (43) were conserved in the predicted sequence of CNF_Y (Fig. 2). The DNA sequences flanking cnf_Y had significant similarities to transposases (BLASTX *E* value, 5*e*-36) and oxidoreductases (BLASTX *E* value, 7*e*-40) (Fig. 3). The overall content of guanosine and cytosine (percent G+C) in the DNA that was sequenced in this study was 39% (Fig. 3), but there was a noticeable demarcation. The percent G+C for cnf_Y and the DNA 5′ to the gene was 34%, while 3′ to the cnf_Y gene the sequence contained 51% G+C. Taken together, the sequence data suggested that the cnf_Y gene was introduced into *Y. pseudotuberculosis* from another bacterium.

Despite the considerable identity between the DNA and predicted amino acid sequences of cnf_Y and cnf_E, the CNF_Y biological activity in extracts from *Y. pseudotuberculosis* strain YPIII and *E. coli* K-12 strain DH5α(pHLK602) was not neutralized by goat polyclonal antibodies prepared against purified *E. coli* CNF_E (34). The antibodies completely prevented the multinucleating activity of *E. coli* CNF_E when they were used at a final dilution of up to 1:80, but there was no inhibition of the activity from *Y. pseudotuberculosis* strain YPIII with final antibody dilutions as low as 1:10 (not shown). Preimmune sera had no effect on the activity of either CNF. Identical results were obtained with a mouse monoclonal antibody (33).

The DNA sequence that was determined from *Y. pseudotuberculosis* strain YPIII in this study was compared to a similar sequence in the genome database of *Y. pestis* strain CO92 (37) (Fig. 3). There was ≥99% identity between the DNA sequences that were present in both species. However, there were deletions unique to each species. The sequence from *Y. pestis* contained the first 2,114 bp of the cnf_Y ORF, but there was a 931-bp deletion at the 3′ end of the gene. Distal to the deletion, *Y. pestis* retained the 3′ flanking sequence with sim-

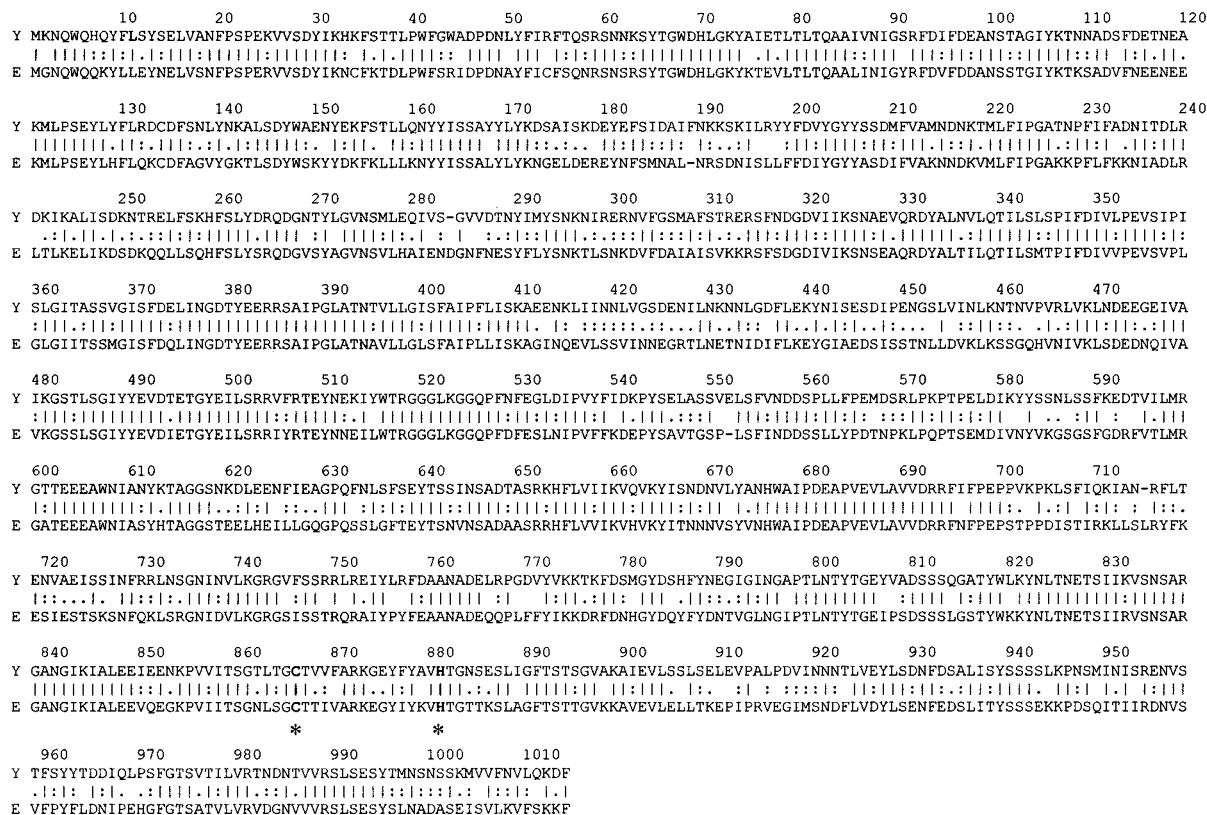

FIG. 2. Alignment of the predicted amino acid sequences of CNF_Y (Y) from strain YPIII and CNF_E (E) by the method of Lipman and Pearson. Conserved amino acids that were shown to be essential for the activity of CNF_E (Cys866 and His881) (43) are bolded and marked by asterisks under those positions, and identical (|) or similar (:, .) residues are indicated.

ilarity to oxidoreductases. The sequences preceding *cnf*$_Y$ in both species also contained deletions in the intervening region between the putative transposase and the start of the *cnf*$_Y$ gene (Fig. 3).

Chromosomal DNAs from pathogenic and nonpathogenic species of *Yersinia* were examined for the presence of the *cnf*$_Y$ gene. All of the isolates of *Y. pseudotuberculosis* (n = 33) that were examined contained *cnf*$_Y$ DNA that hybridized to the *cnf*$_Y$ probe (representative results from seven strains of *Y. pseudotuberculosis* are shown in Fig. 4) or amplified in a PCR with *cnf*$_Y$-specific primers (not shown). The *cnf*$_Y$ gene was detected in *Y. pseudotuberculosis* strain YPIIIc and seven other *Y.*

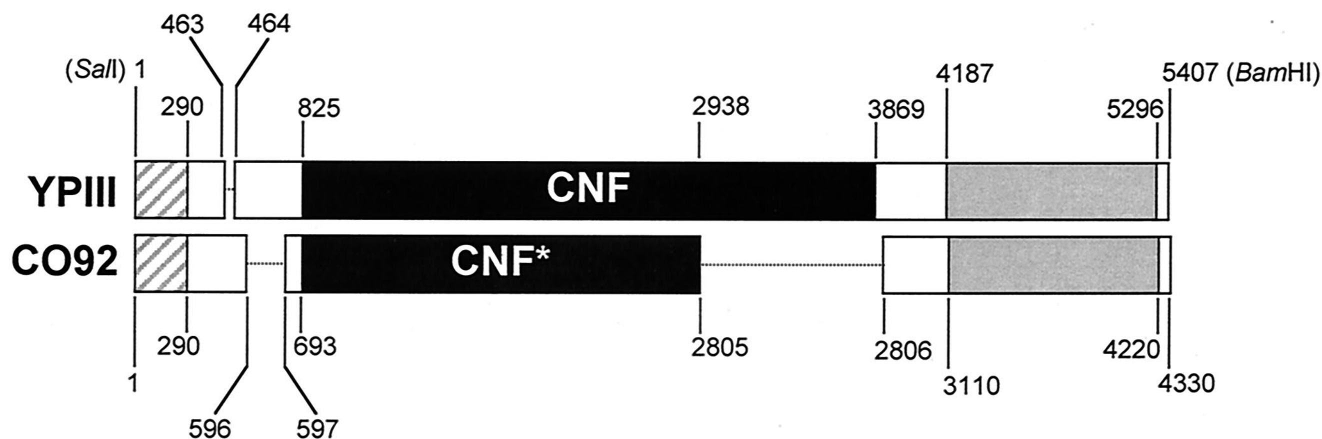

FIG. 3. Comparison of *cnf*$_Y$ DNA sequences from *Y. pseudotuberculosis* strain YPIII and *Y. pestis* strain CO92. Boxes indicate sequences that are ≥99% identical in the two species, and dotted lines indicate deletions. Nucleotide positions are numbered according to the sequence present in each species. The *Bam*HI and *Sal*I sites used in the cloning of *cnf*$_Y$ from strain YPIII also are shown. ▨, putative transposase; □, intervening regions; ■, CNF_Y; ▤, putative oxidoreductase.

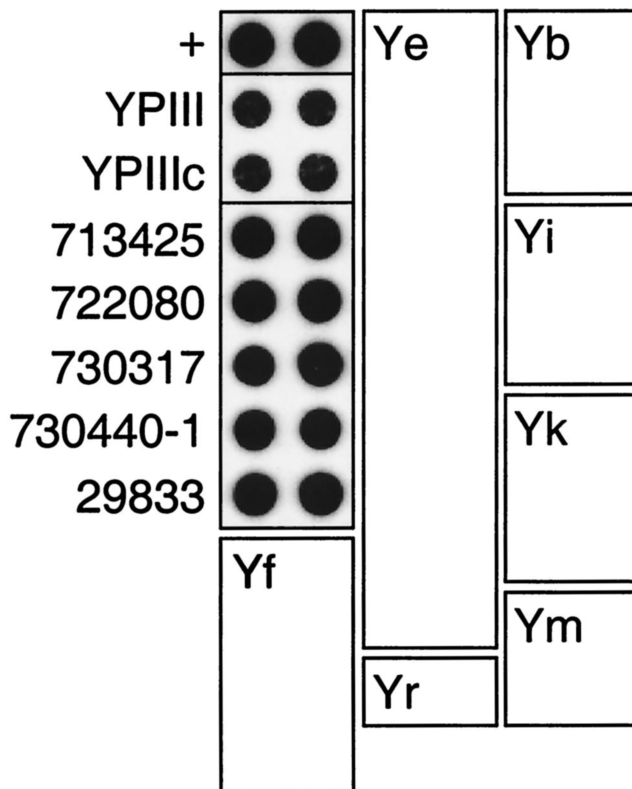

FIG. 4. Dot blot of total DNAs from 33 strains of *Yersinia*. The blot was hybridized at high stringency with a cnf_Y gene probe. Individual strains of *Y. pseudotuberculosis* are listed by strain name or number next to the position of the DNA from that strain; +, positive control, the cloned cnf_Y from *Y. pseudotuberculosis* strain YPIII; Ye, *Y. enterocolitica*; Yb, *Y. bercovieri*; Yf, *Y. frederiksenii*; Yi, *Y. intermedia*; Yk, *Y. kristensenii*; Ym, *Y. mollaretii*; Yr, *Y. rohdei*. All samples were applied to the blot in duplicate. The autoradiogram was scanned, and the digital image file was annotated with Canvas.

pseudotuberculosis strains cured of the 70-kb virulence plasmid. cnf_Y sequences were not detected in *Y. enterocolitica* or in nonpathogenic *Yersinia* species (Fig. 4). A Southern blot of *Y. pseudotuberculosis* DNAs showed polymorphisms at the cnf_Y locus (Fig. 5), and further analysis revealed that *Y. pseudotuberculosis* strains YPIII, YPIIIc, and IP2666c (all $CNF_{Yp}{}^+$) possessed the complete cnf_Y gene shown in Fig. 3. All of the remaining strains of *Y. pseudotuberculosis* carried a single deletion like that in *Y. pestis* strain CO92 (Fig. 3), or they had the *Y. pestis* deletion and a second deletion between nucleotides 1745 and 2065 with respect to the cnf_{Yp} sequence from *Y. pseudotuberculosis* strain YPIII (not shown). The single cnf_Y deletion in *Y. pestis* strain CO92 (Fig. 3) was confirmed in additional independent *Y. pestis* isolates (H. A. Lockman and P. L. Worsham, unpublished observation). The discovery of identical cnf_Y deletions in *Y. pseudotuberculosis* and *Y. pestis* is consistent with the evidence that the plague bacillus is a recently evolved clone of *Y. pseudotuberculosis* (1). However, in an examination of a subset of *Y. pseudotuberculosis* strains, none of the strains that contained a plague-like cnf_Y allele (n = 6) possessed the *Yersinia* high-pathogenicity island (HPI) (12) (Table 1), a feature that distinguished them from *Y. pestis*. In contrast, 8 of 10 *Y. pseudotuberculosis* strains that were HPI^+ also possessed the second cnf_Y deletion (described above) (Table 1) that was not found in *Y. pestis*. It was also evident from this analysis that only those strains of *Y. pseudotuberculosis* with a full-length cnf_Y gene expressed biological activity, and the strains that did not express CNF_Y activity had significant deletions of the cnf_Y ORF.

The discovery of a chromosomally encoded CNF_{Yp} in *Y. pseudotuberculosis* adds a potential virulence factor to the variety of potent cytotoxins that are produced by these bacteria. Altogether, the results of this study indicated that some *Y. pseudotuberculosis* strains express a CNF toxin that may, like the *E. coli* CNF, target eukaryotic small GTPases. Despite the remarkable similarities between the biological activities and the nucleotide and predicted amino acid sequences of the *cnf* genes from *Y. pseudotuberculosis* and *E. coli*, the absence of neutralization of CNF_Y by anti-CNF_E antibodies indicated that there are significant antigenic differences between the proteins. CNF_Y activity was found in the supernatants of broth cultures of *Y. pseudotuberculosis*, a result suggesting that CNF_Y is secreted, unlike the cell-associated *E. coli* CNF_E (11, 23). Pathogenic strains of *Yersinia* also produce plasmid-encoded cytotoxins (Yops) that are substrates of a type III secretion system (2, 13–15). Like CNFs, some Yop cytotoxins profoundly alter the cytoskeletal architecture of mammalian cells by affecting the activity of GTPases. YopE is a GTPase-activating protein that negatively regulates Rho activity (6, 49), and YopT inactivates Rho via a covalent modification (50). YopH is a potent tyrosine phosphatase that acts on $p130^{Cas}$ and FAK, thereby inhibiting formation of focal adhesion complexes (5). YpkA (YopO) is a serine/threonine kinase that is activated by actin and subsequently inhibits Rho activity (18, 28). YpkA also binds directly to Rho and Rac (4). Understanding the combined roles of all of these toxins in the pathogenic lifestyle of *Y. pseudotuberculosis* awaits additional studies, but it is relevant to note that YopT is not expressed by the $CNF_Y{}^+$ *Y. pseudotuberculosis* strain YPIII (48). Thus, different strains of this species may express virulence through alternate mechanisms.

Nucleotide sequence accession number. The cnf_Y DNA sequence reported here, from *Y. pseudotuberculosis* strain YPIII, was deposited in GenBank (NCBI) under accession number AF324349.

We are indebted to Elisabeth Carniel (Institut Pasteur, Paris, France), Kathleen McDonough (State University of New York, Alba-

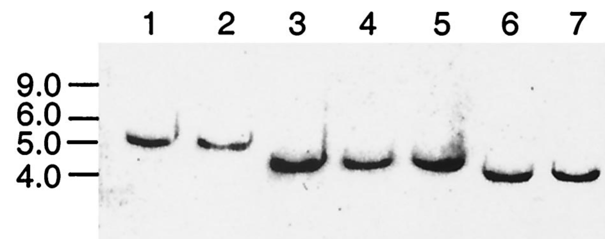

FIG. 5. cnf_Y probe hybridization to a Southern blot of chromosomal DNAs from *Y. pseudotuberculosis* strains. Lane 1, YPIII; lane 2, YPIIIc; lane 3, 713425; lane 4, 722080; lane 5, 730317; lane 6, 730440-1; lane 7, ATCC 29833. One microgram of each DNA was digested with *Nsi*I. The numbers in the left margin indicate molecular size standards in kilobases. The autoradiogram was scanned, and the digital image file was annotated with Canvas.

ny), Virginia Miller (Washington University School of Medicine, St. Louis, Mo.), Karen Birkhead and Nancy Strockbine (Centers for Disease Control and Prevention, Atlanta, Ga.), James Bliska (State University of New York, Stony Brook), and Alexander Sulakvelidze (University of Maryland School of Medicine, Baltimore) for providing the strains of *Yersinia* that were used in this research. We also thank Virginia Miller and James Bliska for helpful discussions. Anti-*E. coli* CNF1 antibodies were generously provided by Karen Meysick and Alison O'Brien (Uniformed Services University of the Health Sciences, Bethesda, Md.). We credit an anonymous reviewer for alerting us about the earlier studies on the *Y. pseudotuberculosis* dermonecrotic exotoxin.

Erica Mersfelder assisted with the DNA sequencing, which was done by the Core Facility at Children's Research Institute and was supported, in part, by National Institutes of Health Grant HD34615.

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Editor: J. T. Barbieri