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Contribution of YopB to Virulence of *Yersinia enterocolitica*

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The 70-kb virulence plasmid, pYV, of *Yersinia enterocolitica* encodes a number of secreted proteins (Yops) which are essential for virulence. YopD, the 33-kDa product of the *lcrGVHyopBD* operon, appears to be involved in delivering YopE and YopH (the *Yersinia* protein tyrosine phosphatase) into target cells. These proteins then act in concert to cause cytotoxicity in host cells. Previously, we reported that bacteria carrying transposon insertions in *yopD* are not cytotoxic for macrophages, show impaired tyrosine phosphatase activity in host cells, and are avirulent for mice (E. L. Hartland, S. P. Green, W. A. Phillips, and R. M. Robins-Browne, Infect. Immun. 62:4445–4453, 1994). *trans* complementation of *yopD* mutants of *Y. enterocolitica* with the *yopD* gene restores all these properties. In this study, we show that polar mutations in proximal genes of the *lcrGVHyopBD* operon also abrogated bacterial virulence and the capacity to induce cytotoxicity in mouse bone marrow-derived macrophages and HEp-2 epithelial cells. Moreover, *trans* complementation of a *yopBD* mutant with the *yopD* gene alone was not sufficient to restore the ability of the bacteria to cause cytotoxicity. Further work showed that YopB was required for cytotoxicity, dephosphorylation of host proteins, and virulence for mice. These findings indicate that YopB and YopD may serve a related function in *Y. enterocolitica* and that they may act together to deliver intracellularly acting Yops to their respective targets in host cells.

The genus *Yersinia* defines a diverse range of microorganisms, including the three pathogenic species *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. enterocolitica* is an enteric pathogen capable of causing a variety of clinical disorders in humans and animals (7, 11). The severity of the disease caused by *Y. enterocolitica* varies considerably and includes mild diarrhea, mesenteric lymphadenitis, and ileitis, resembling appendicitis (11). Complications of infection include meningitis, endocarditis, osteomyelitis, or septicemia, as well as a variety of autoimmune sequelae (11).

Infections with *Y. enterocolitica* result from the ingestion of contaminated food or water. In the small intestine, the bacteria preferentially invade M cells overlying Peyer's patches, where they have direct access to lymphoid tissue (19). Here the bacteria appear to resist phagocytosis and multiply extracellularly (21, 38).

All pathogenic strains of *Y. enterocolitica* carry a 70-kb plasmid, pYV, which is also found in virulent strains of *Y. pestis* and *Y. pseudotuberculosis* (9, 10). pYV encodes the production and secretion of several proteins, termed Yops, which are essential for virulence and which appear to influence host cell function directly (4, 6, 10, 14, 42). An example of Yops whose function is known is YpkA of *Y. pseudotuberculosis* (the homolog of YopO in *Y. enterocolitica* [2]), which is a serine/threonine kinase with homology to eukaryotic kinases (16). While YpkA has not yet been shown to have a substrate in host cells, it is possible that this protein contributes to virulence by interfering with host cell signal transduction pathways (15). YopM, by contrast, exhibits homology to GP1bα, the α chain of the platelet receptor for thrombin (25). Because inhibition of the binding of thrombin to platelets may interfere with platelet aggregation in the host, YopM may diminish the normal inflammatory response to infection. YopE and YopH appear to act in concert to cause cytotoxic changes in HEp-2 epithelial cells and macrophages (22, 34). YopE evidently acts by disrupting actin filaments of the cytoskeleton, although its substrate and mechanism of action are unknown (34). YopH is a protein tyrosine phosphatase (PTPase) which exhibits close homology to eukaryotic PTPases (43). Since it dephosphorylates host cell proteins, YopH may interfere with signal transduction pathways, leading to the abrogation of various physiological responses to infection, including phagocytosis by macrophages (6, 13).

YopE and YopH are released by *Y. enterocolitica* adhering to the external surface of host cells and must cross the cytoplasmic membrane of these cells to reach their intracellular targets (14, 35, 41). At least one Yop, namely, YopD, a 33-kDa putative transmembrane protein, is thought to assist the transfer of YopE and YopH into cells (14, 22, 35, 41). Thus, YopD is essential for the cytotoxic properties of *Y. enterocolitica* and for virulence in mice (22). YopB, encoded by the *lcrGVHyopBD* operon, is also a putative transmembrane protein which has some homology with YopD (20, 33). The aim of this study was to determine whether YopB and YopD are functionally related and if YopB also contributes to the virulence of *Y. enterocolitica.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Y. enterocolitica* was grown in brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) or on BHI agar. *Escherichia coli* strains were grown in Luria-Bertani broth or on Luria-Bertani agar. Production of Yops was induced at 37°C in BHI broth supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$ (BHI-OX) for 3 h as described previously (26, 30). Kanamycin (100 μg/ml; Boehringer, Mannheim, Germany), ampicillin (100 μg/ml; CSL Ltd., Melbourne, Australia), and tetracycline (30 μg/ml; Sigma Chemical Co., St. Louis, Mo.) were added to bacterial culture media when required. The temperature sensitivity of various strains of *Y. enterocolitica* was monitored in BHI broth supplemented with 2 mM $CaCl_2$ and appropriate antibiotics at 28 and 37°C. Briefly, overnight cultures grown at 28°C were diluted in 10 ml of fresh medium in a 50-ml conical flask to an initial inoculum of 10^3 CFU/ml. Cultures were incubated with shaking at 200 rpm, and aliquots were removed at intervals to determine the number of viable bacteria on duplicate BHI agar plates.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference(s)
Strains		
Y. enterocolitica W22703	Serogroup O:9; pYVe227 Res^- Mod^+ Nal^r	24, 28
Y. enterocolitica W22703c	Derivative of W22703 lacking pYVe227	24, 28
E. coli S17.1	RP4 2-Tc::Mu-Km::Tn*7* Tp^r Sm^r	37
E. coli TG1	*supE hsd*Δ*5 thi*Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	8
Plasmids		
pYVe227	Virulence plasmid of *Y. enterocolitica* W22703	24, 28
pYVA2635	Virulence plasmid of *Y. enterocolitica* A2635	27, 31
pEH37	pYVe227 *yopH*::Tn*2507*	22
pEH79	pYVe227 *lcrV*::Tn*2507*	22
pEH110	pYVe227 *yopB*::Tn*2507*	22
pEH301	pYVe227 *yopD*::Tn*2507*	22
pRK404	Derivative of pRK290; *lacZ* Tc^r	12
pCVD787	pRK404 containing 14-kb *Bam*HI fragment of pYVA2635	31
pCVD777	pRK404 containing 26-kb *Bam*HI fragment of pYVA2635	31
pEH12	pYVe227::Tn*2507*	This study
pPROM20	pBluescript (SK^-) containing the 148-bp *yopH* promoter region[a]	This study
pEH500	pRK404 with *yopH* promoter and *yopD* gene[a]	This study
pEH503	pRK404 with *yopH* promoter and *yopB* gene[a]	This study
pEH504	pRK404 with *yopH* promoter and *yopBD* genes[a]	This study

[a] Genes derived by PCR amplification (see text for details).

DNA methods and construction of plasmids. Plasmid DNA extractions, restriction enzyme digestions, DNA ligations, and transformations into *E. coli* were performed by standard techniques (1, 36). The *yopH* promoter region was amplified by PCR from plasmid pCVD777, which contains a 26-kb region of the virulence plasmid from *Y. enterocolitica* A2635 (27, 31). The oligonucleotide primers for PCR (with *yop* gene sequences underlined) were designated YOPH5 (5′-CGGGATCCTCGATTAACATCAATGAAAATACACGG-3′) and YOPH PROM (5′-CGGGATCCATGGTTCCCTCCTTAATTAAATTAAG-3′). These were designed to introduce *Bam*HI restriction sites into each end of the amplified product and an *Nco*I site into the start codon of the *yopH* gene. PCR was performed with the high-fidelity enzyme Vent polymerase (New England Biolabs, Beverly, Mass.) under the following conditions: 30 cycles at 94°C for 60 s, 42°C for 50 s, and 72°C for 60 s. The 148-bp product of this reaction was cloned into the *Bam*HI site of pBluescript (SK^-; Stratagene, La Jolla, Calif.) to yield pPROM20 (Fig. 1). The *yopB* and *yopBD* genes were amplified from plasmid pCVD787 (31) by PCR using the conditions described above except that the elongation time (third step) was increased to 120 s. The primers for this PCR were YOPB5 (5′-CGGGATCCACATGTGTGCGTTGATAACCCATG-3′) and YOPB3 (5′-CGGGATCCTGCAGTTAAACAGTATGGGGTC-3′), which were also designed to introduce *Bam*HI sites into each end of the PCR fragments, an *Afl*III site into the start codon of *yopB*, and a *Pst*I site into the end of the *yopB* or *yopD* gene. The 1.2- and 2.1-kb amplification products were initially cloned into the *Eco*RV site of pBluescript (SK^-). The *Afl*III-*Pst*I fragments were then cloned into pPROM20 (*Nco*I-*Pst*I) (Fig. 1). A *Bam*HI-*Pst*I fragment containing the *yopH* promoter and *yopB* or *yopBD* genes was then cloned into the broad-host-range plasmid pRK404 (12), which was mobilized into strains of *Y. enterocolitica* from *E. coli* S17.1. These plasmids were named pEH503 (*yopB*) and pEH504 (*yopBD*). The *yopD* gene of *Y. enterocolitica* A2635 was cloned previously by PCR from plasmid pCVD787 (22). An *Afl*III-*Pst*I fragment containing *yopD* was cloned into pPROM20 and then into pRK404 to yield pEH500. Transposon Tn*2507* mutants of *Y. enterocolitica* W22703(pYVe227) were obtained previously (22). The site of insertion of Tn*2507* was determined by PCR amplification of the *cat* gene from Tn*2507* and the upstream or downstream region of the interrupted gene. The size of each product was determined by electrophoresis on a 0.7% agarose gel. The primer used for PCR amplification of the *cat* gene was LOG4 (5′-CCGGGGATCCTTACGCCCCGCCCTGCCACTC-3′). The other primers used in the PCR reactions are described in this study or have been described previously (22), except for LCRG5 (5′-CGGGATCCGCACCGGGT GGTTCTCAC-3′), which was used to map the site of transposon insertion in the *lcrV* gene.

Yop expression and analysis. Preparations of Yops taken 3 h after induction at 37°C in a Ca^{2+}-depleted medium were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) (23). Gels were stained with Coomassie blue or transferred to a Hybond-C nitrocellulose membrane (Amersham Corp.) for Western blotting (immunoblotting). YopD was detected with a mouse monoclonal antibody (diluted 1:500 in 5% skim milk) and incubated with an anti-mouse antibody conjugated to horseradish peroxidase (Silenus, Miami, Fla.). Immunoblots were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Assay for cytotoxicity. HEp-2 epithelial cells were maintained in minimal essential medium (ICN Biomedicals, Seven Hills, New South Wales, Australia) supplemented with 8% fetal calf serum (Sigma), 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Sigma), and 40 μg of gentamicin per ml. For cytotoxicity assays, cells were seeded into 24-well tissue culture trays (Nunc Inter-Med, Roskilde, Denmark) at 10^5 cells per well and incubated overnight in minimal essential medium–5% fetal calf serum–20 mM HEPES at 37°C in air plus 5% CO_2. Each well was inoculated with approximately 10^6 CFU and incubated at 37°C in air plus 5% CO_2 for 3 h. Previous studies have shown that pYV-bearing strains of *Y. enterocolitica* express and secretes Yops under these conditions. Cells were examined for morphologic changes by phase-contrast microscopy (22).

Detection of phosphotyrosine in macrophages. Macrophages derived from femoral precursor cells from CBA mice were cultured for 5 days in six-well trays (Nunc) in RPMI 1640 medium (Flow Laboratories, McLean, Va.) supplemented with 50 mM HEPES, 3 mM glutamine, 10% fetal calf serum, and 20% L-cell-conditioned medium as a source of colony-stimulating factor 1. Macrophages were infected with bacteria at a ratio of 10 bacteria per macrophage and incubated for 3 h as for the cytotoxicity assay. Macrophages were then washed twice with phosphate-buffered saline (PBS) and stimulated with zymosan for 10 min in fresh tissue culture medium to induce tyrosine phosphorylation of cell proteins (17, 18). To extract macrophage proteins, the cells were then washed twice with ice-cold PBS and lysed in Lau lysis buffer for 10 min on ice (Lau lysis buffer: 100 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, 0.5% [wt/vol] deoxycholate, 1% [vol/vol] Nonidet P-40, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 10 mM sodium orthovanadate [pH 7.2]). Lysates were mixed with Laemmli sample buffer and separated by SDS-PAGE. Following electrophoretic transfer to a Hybond-C nitrocellulose membrane (Amersham), phosphotyrosine-containing proteins were revealed by Western blot with an affinity-purified, antiphosphotyrosine monoclonal antibody (PY20) conjugated to horseradish peroxidase (ICN) and detected by enhanced chemiluminescence detection (Amersham) as described previously (18, 22).

Virulence studies. The 50% lethal doses (LD_{50}s) of various derivatives of *Y. enterocolitica* W22703(pYVe227) were determined by intraperitoneal inoculation of BALB/c mice in groups of five with 10-fold dilutions of bacteria. To increase their susceptibility to infection, the mice were pretreated with 5 mg of iron as iron-polymaltose (Hausman Laboratories Inc., St. Gallen, Switzerland) and 5 mg of desferrioxamine B (Ciba-Geigy Ltd., Basel, Switzerland) as described previously (22, 32). Mice were observed for 14 days after inoculation, and the LD_{50} was calculated by the method of Reed and Muench (29). Bacteria carrying complementing plasmids were isolated from the spleens of moribund mice and examined for plasmid content and by Southern blotting to ensure that the *trans*-complementing plasmids had not integrated into the mutated pYV.

RESULTS

Cytotoxicity of *lcrGVHyopBD* mutants for HEp-2 epithelial cells. Previously, we showed that transposon insertions in disparate regions of the *lcrGVHyopBD* operon abrogate the ability of *Y. enterocolitica* W22703 to cause cytotoxicity in HEp-2 epithelial cells (22). We have also shown that *trans* complementation of a *yopD* mutant, *Y. enterocolitica* W22703(pEH301),

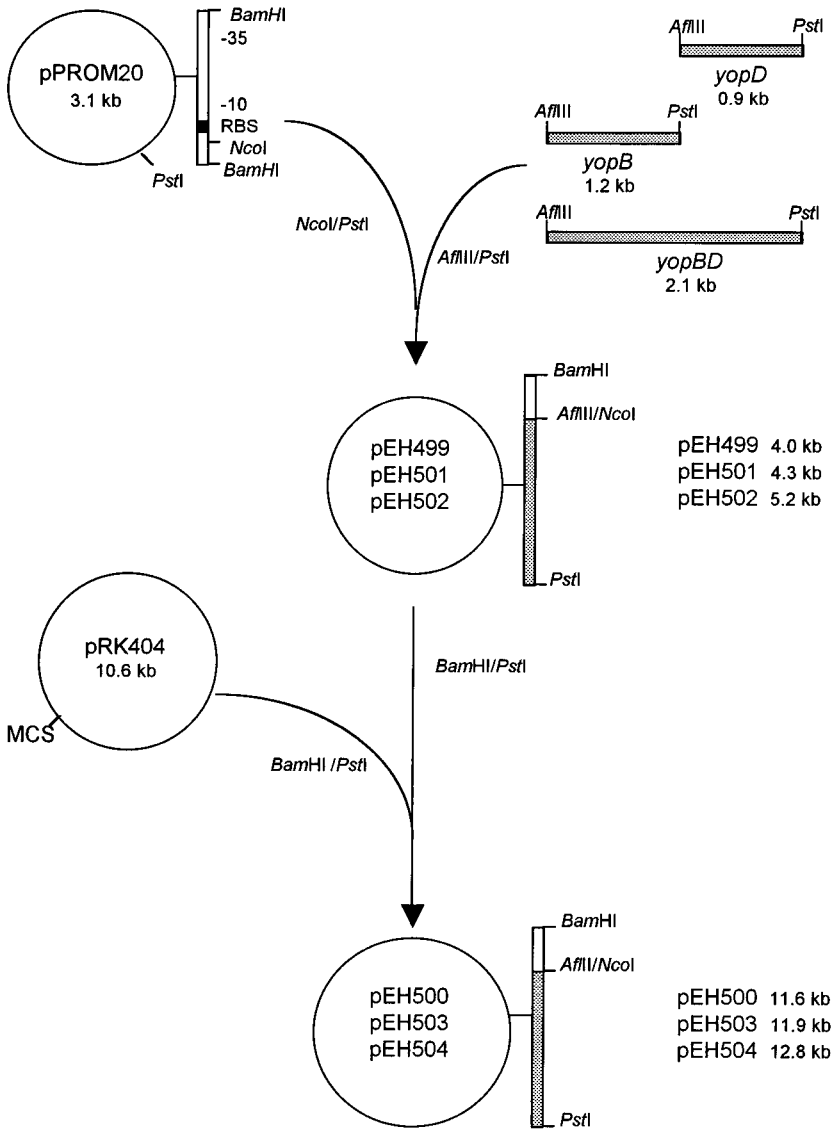

FIG. 1. Construction of *trans*-complementing plasmids pEH500, pEH503, and pEH504. The *yopH* promoter region is represented by an open box, and the ribosome binding site (RBS) is represented by a solid line. *yop* genes are shown as shaded regions. The PCR primers used to amplify the *yop* genes from pCVD787 (31) are indicated. Only restriction enzyme sites used for cloning are shown. Plasmids and DNA fragments are not drawn to scale. The sizes of plasmids and PCR products in kilobases are indicated. MCS is the multiple cloning site of pRK404 (12).

with the *yopD* gene can restore the capacity of the bacteria to induce cytotoxicity, indicating that YopD is essential for this process (22). To determine if other products of the *lcrGVHyopBD* operon are also required for cytotoxicity, we constructed polar mutants in this operon (Fig. 2) and *trans* complemented these mutants with plasmids carrying *yopD* (pEH500), *yopB* (pEH503), or *yopBD* (pEH504) under control of the *yopH* promoter. The sites of Tn*2507* insertion and the Yop profiles of the mutants investigated are shown in Fig. 2.

trans complementation of *Y. enterocolitica* W22703(pEH301) (*yopD*::Tn*2507*) with pEH500 (*yopD*) restored cytotoxicity as expected (Table 2). However, *trans* complementation of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) with pEH500 did not restore cytotoxicity, suggesting that YopB also contributes to the ability of *Y. enterocolitica* to cause cytotoxicity (Table 2). *trans* complementation of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) with pEH504 (*yopBD*) confirmed that *yopB* is required for cytotoxicity (Table 2). In addition, when *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) was *trans* complemented with pEH503 (*yopB*), this strain became cytotoxic, even though the *yopD* gene had not been reintroduced (Table 2). This was in contrast to *Y. enterocolitica* W22703(pEH301, pEH503) (*yopD*::Tn*2507* p*yopB*), which was not cytotoxic and produced YopB but not YopD.

As the absence of YopD in *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) is due to a polar effect on the downstream *yopD* gene, we postulated that *trans* complementation with *yopB* influenced the production of YopD. To determine if *trans* complementation of this mutant with *yopB* increased the amount of YopD secreted into the culture supernatant, we performed Western blotting of Yops prepared from *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) and the *trans*-complemented strains using a monoclonal antibody to YopD. The results showed that YopD was present in low concentrations (detectable by Western blotting only) in the culture supernatant of *Y. enterocolitica* W22703(pEH110, pEH503) (*yopB*::

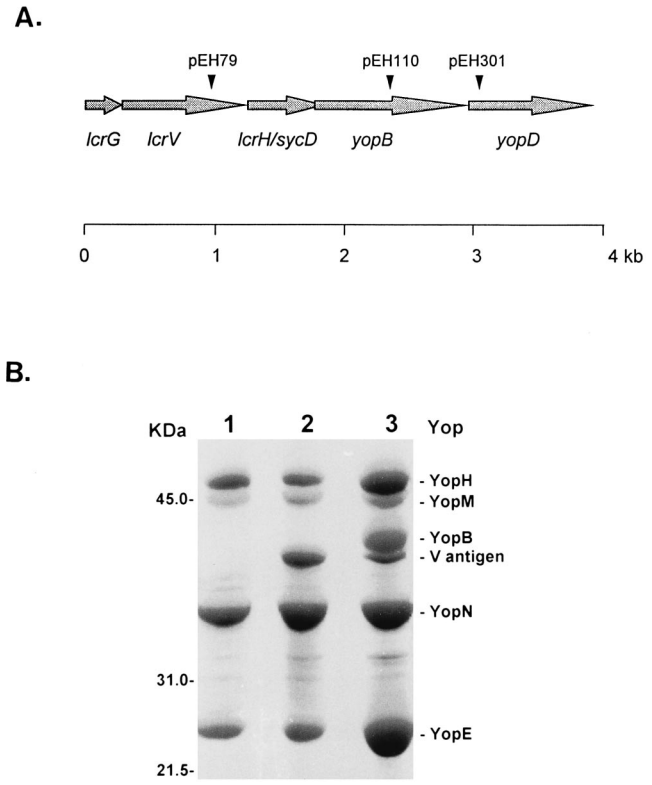

FIG. 2. (A) Schematic diagram of the *lcrGVHyopBD* operon showing the location of Tn*2507* insertions (triangles) in mutants *Y. enterocolitica* W22703 (pEH79), W22703(pEH110), and W22703(pEH301). *lcrH* and *yopB* overlap by 22 bp. Arrows indicate open reading frames and their direction of transcription. (B) SDS-PAGE gel of Yops from *Y. enterocolitica* W22703 carrying various plasmids. Lane 1, pEH79 (pYVe227 *lcrV*::Tn*2507*); lane 2, pEH110 (pYVe227 *yopB*::Tn*2507*); lane 3, pEH301 (pYVe227 *yopD*::Tn*2507*). The gel was scanned with a flatbed scanner using Easy Scan version 3.1.1β (Advanced Vision Research Inc., San Jose, Calif.).

Tn*2507* p*yopB*) but not in that of W22703(pEH110) (*yopB*::Tn*2507*) (Fig. 3). Thus, *Y. enterocolitica* W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*) produced YopB and a small amount of YopD that was sufficient to restore cytotoxicity.

trans complementation of *Y. enterocolitica* W22703(pEH79) (*lcrV*::Tn*2507*) could not be achieved with pEH500 (p*yopD*), pEH503 (p*yopB*), or pEH504 (p*yopBD*) (data not shown). This may be because *Y. enterocolitica* W22703(pEH79) did not produce sufficient amounts of YopB and YopD, even when the gene encoding the chaperone required for the export of these proteins, *sycD* (44), was supplied in *trans* (data not shown). Thus, the question of whether the V antigen contributes directly to the cytotoxic capacity of *Y. enterocolitica* could not be addressed in this study.

Growth of mutant bacteria. Mutations in the *lcrGVHyopBD* operon have been reported to result in thermosensitivity of the bacteria, i.e., an inability to grow at 37°C (28). We reported previously that a *yopD* mutant of *Y. enterocolitica* W22703, namely, *Y. enterocolitica* W22703(pEH301) (*yopD*::Tn*2507*), exhibited a prolonged lag phase when grown at 37°C, but once growth commenced, it proceeded at a rate similar to that of the wild-type strain (22). Furthermore, this growth characteristic did not appear to influence the behavior of *Y. enterocolitica* W22703 (pEH301) (*yopD*::Tn*2507*) in vivo (22). In the present study, we compared the growth characteristics of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*), the *trans*-complemented mutants of this strain, and *Y. enterocolitica* W22703(pEH12). The last strain contains pYVe227 tagged with Tn*2507* in a noncoding region of the virulence plasmid, approximately 3 kb downstream of the *yadA* gene, and retains all attributes associated with virulence. This strain was used in preference to *Y. enterocolitica* W22703(pYVe227) because it carries a selectable marker on the virulence plasmid.

Bacteria were grown at 28 and 37°C in BHI broth supplemented with 2 mM $CaCl_2$ and kanamycin (100 μg/ml). Tetracycline (30 μg/ml) was added for *trans*-complemented strains. All of the strains investigated showed similar growth characteristics at 28°C (Fig. 4). At 37°C, *Y. enterocolitica* W22703 (pEH12) (pYVe227::Tn*2507*) and W22703(pEH110) (*yopB*::Tn*2507*) and the *trans*-complemented mutants *Y. enterocolitica* W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*) and W22703 (pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*) showed similar growth characteristics with no evidence of thermosensitivity. One of the *trans*-complemented mutants, W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*), showed an extended lag phase at 37°C but then grew at a rate comparable to that of the other strains (Fig. 4).

PTPase activity in macrophages. The YopH protein of *Y. enterocolitica* is a PTPase that dephosphorylates macrophage proteins (5, 18, 22). We have reported that a *yopD* mutant of *Y. enterocolitica*, strain W22703(pEH301) (*yopD*::Tn*2507*), is less able than the wild-type strain to cause tyrosine dephosphorylation in macrophages stimulated with zymosan, despite producing YopH (22). This finding suggested that YopD plays a role in the action of YopH, perhaps by assisting delivery of YopH to its intracellular target (22). As YopD and YopB are putative transmembrane proteins which exhibit significant amino acid homology with each other (20), we postulated that YopD and YopB are functionally related. Accordingly, we analyzed the ability of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) and the *trans*-complemented strains to cause tyrosine dephosphorylation of macrophage proteins. *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) did not affect tyrosine phosphorylation in macrophages stimulated with zymosan, suggesting that the action of YopH was inhibited (Fig. 5). *trans* complementation of this strain with the *yopD* gene (pEH500) did not restore its ability to dephosphorylate tyrosine. How-

TABLE 2. Influence of YopB and YopD on the ability of *Y. enterocolitica* W22703 to cause cytotoxicity in HEp-2 epithelial cells[a]

Strain	Presence[b] of:		Cytotoxicity[c]
	YopB	YopD	
W22703(pYVe227)	+	+	+
W22703c (no plasmid)	−	−	−
W22703(pEH301) (*yopD*::Tn*2507*)	+	−	−
W22703(pEH301, pEH500) (*yopD*::Tn*2507* p*yopD*)	+	+	+
W22703(pEH301, pEH503) (*yopD*::Tn*2507* p*yopB*)	+	−	−
W22703(pEH110) (*yopB*::Tn*2507*)	−	−	−
W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*)	−	+	−
W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*)	+	(+)	+
W22703(pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*)	+	+	+
W22703(pEH79) (*lcrV*::Tn*2507*)	−	−	−

[a] Cells were incubated with bacteria for 3 h and observed for morphologic changes.
[b] +, Yop present; −, Yop absent; (+), small amount produced.
[c] +, cytotoxic; −, not cytotoxic.

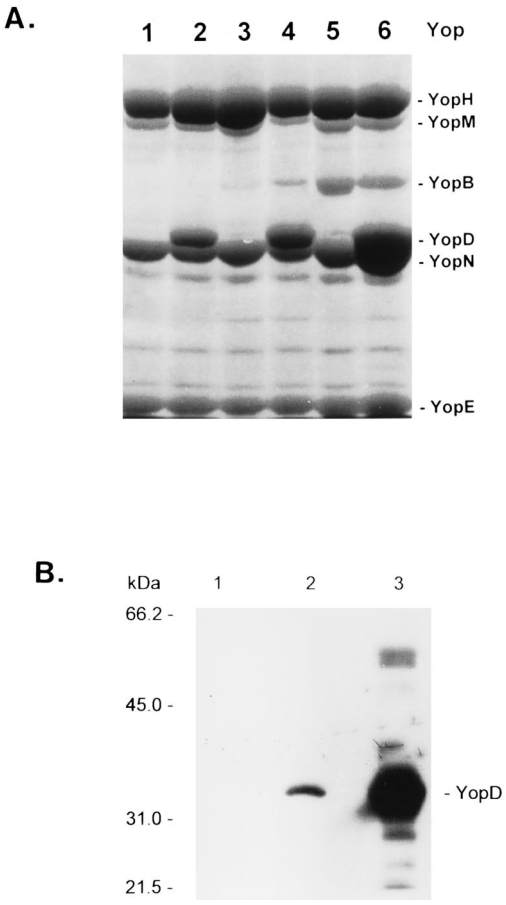

FIG. 3. (A) SDS-PAGE gel of Yops from *Y. enterocolitica* W22703 carrying various plasmids. Yops were prepared 3 h after induction in BHI-OX broth at 37°C. Lane 1, pEH110 (*yopB*::Tn*2507*); lane 2, pEH110 and pEH500 (*yopB*::Tn*2507* p*yopD*); lane 3, pEH110 and pEH503 (*yopB*::Tn*2507* p*yopB*); lane 4, pEH110 and pEH504 (*yopB*::Tn*2507* p*yopBD*); lane 5, pEH301 (*yopD*::Tn*2507*); lane 6, pEH301 and pEH500 (*yopD*::Tn*2507* p*yopD*). The gel was scanned with a flatbed scanner using Easy Scan version 3.1.1β (Advanced Vision Research Inc.). (B) Immunoblot of released proteins from derivatives of *Y. enterocolitica* W22703 carrying various plasmids. Yops were prepared 3 h after induction in BHI-OX broth at 37°C. YopD was detected with a mouse monoclonal antibody and visualized by enhanced chemiluminescence. Lane 1, pEH110 (*yopB*::Tn*2507*); lane 2, pEH110 and pEH503 (*yopB*::Tn*2507* p*yopB*); lane 3, pEH110 and pEH504 (*yopB*::Tn*2507* p*yopBD*). The autoradiograph was scanned with a flatbed scanner using Easy Scan version 3.1.1β (Advanced Vision Research Inc.).

ever, *trans* complementation with the *yopB* and *yopD* genes (pEH504) did restore tyrosine dephosphorylation, indicating that YopB also plays a role in the action of YopH in macrophages, possibly by assisting the translocation of YopH into these cells. *trans* complementation of *Y. enterocolitica*(pEH110) (*yopB*::Tn*2507*) with the *yopB* gene alone (pEH503) partially restored tyrosine dephosphorylation, presumably because of the low level of YopD produced by this strain (Fig. 3B and 5).

For all of these experiments, macrophages were lysed on ice in the presence of 10 mM sodium orthovanadate, a PTPase inhibitor, to ensure that no tyrosine dephosphorylation occurred during lysis. To confirm that YopH activity was inhibited under these conditions and that any tyrosine dephosphorylation occurred before lysis of the macrophages, we demonstrated that wild-type bacteria expressing YopH did not cause tyrosine dephosphorylation when added to macrophages at the time of cell lysis (data not shown).

Virulence for mice. To ascertain if *yopB* was required for virulence in mice, the LD_{50}s of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) and the *trans*-complemented strains were determined by intraperitoneal inoculation of mice pretreated with iron and desferrioxamine B. *Y. enterocolitica* W22703 (pEH301) (*yopD*::Tn*2507*), W22703(pEH110) (*yopB*::Tn*2507*), and W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*) were avirulent, resembling the plasmid-cured strain (Table 3). The LD_{50}s of *Y. enterocolitica* W22703(pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*) and W22703(pEH301, pEH500) (*yopD*::Tn*2507* p*yopD*) were 10^6 and 10^5 CFU, respectively, indicating that both YopB and YopD are required for virulence. As *Y. enterocolitica* W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*) did not kill 50% of mice which received the highest inoculum (5×10^8 CFU), the LD_{50} could not be determined. Nevertheless, this strain caused clinical illness resembling yersiniosis in all mice given 5×10^8 CFU. Thus, *trans* complementation of a *yopBD* mutant with the *yopB* gene alone caused some restoration of virulence, probably due to the small amount of YopD produced by the *trans*-complemented strain (Fig. 3B).

In every case, bacteria recovered from the spleens of moribund mice were indistinguishable from those in the original inoculum, in terms of plasmid content and growth characteristics (data not shown), indicating that restoration of virulence was not due to integration of the *trans*-complementing plasmid into the mutated pYVe227 leading to restoration of the wild type.

DISCUSSION

The *lcrGVHyopBD* operon encodes the V antigen, YopB, and YopD, as well as an 11-kDa secreted protein, LcrG, and LcrH (also known as SycD), the chaperone for YopD and YopB (20, 28, 39, 44). YopD appears to play a critical role in the delivery of YopE and YopH into eukaryotic cells through a mechanism that is not yet understood (14, 22). The 33-kDa

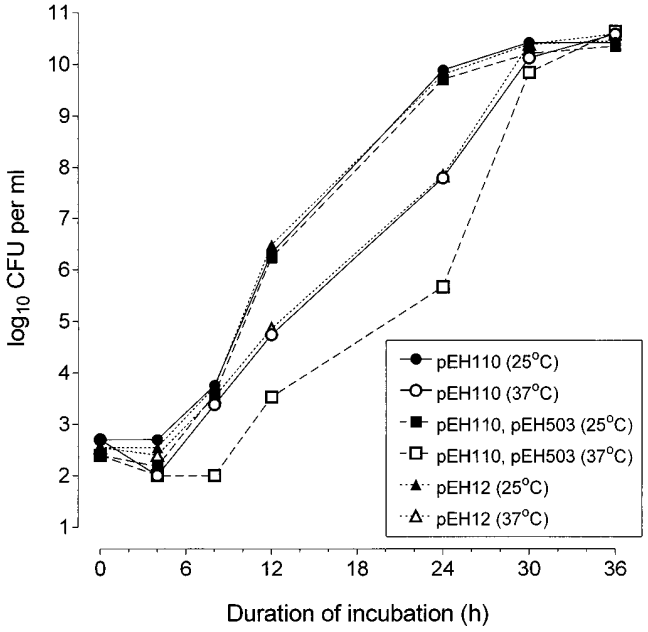

FIG. 4. Growth characteristics of *Y. enterocolitica* W22703(pEH12) (pYVe227::Tn*2507*), W22703(pEH110) (*yopB*::Tn*2507*), and W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*) in BHI broth at 28 and 37°C. Data are a representative sample of three separate determinations. Growth patterns of *Y. enterocolitica* W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*) and W22703(pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*) at 28 and 37°C were similar to that of *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) (data not shown).

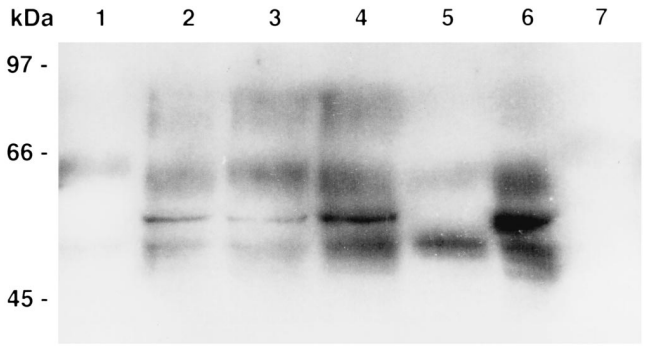

FIG. 5. Effects of various derivatives of *Y. enterocolitica* W22703 on tyrosine phosphorylation in murine bone marrow-derived macrophages. Macrophages were incubated with bacteria for 3 h, stimulated with zymosan for 10 min, and then lysed. Macrophage proteins containing phosphotyrosine were separated by SDS-PAGE and then visualized by Western blotting with a monoclonal antibody to tyrosine phosphate. The autoradiograph was scanned with a flatbed scanner using Easy Scan version 3.1.1β (Advanced Vision Research Inc.). Lane 1, *Y. enterocolitica* W22703(pYVe227); lane 2, W22703c(pYV$^-$); lane 3, W22703(pEH37) (*yopH*::Tn*2507*); lane 4, W22703(pEH110) (*yopB*::Tn*2507*); lane 5, W22703 (pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*); lane 6, W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*); lane 7, W22703(pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*).

YopD protein contains a potential transmembrane region and an amphipathic domain in the carboxyl-terminal end which suggests that YopD forms a pore structure in the eukaryotic cell membrane for the translocation of YopE and YopH into target cells (20). Evidence that YopB, which has approximately 25% amino acid homology with YopD (20), is also required for the translocation process (14) is accumulating, although YopB may also act as a virulence determinant in its own right by suppressing the production of tumor necrosis factor alpha during the course of infection (3).

In this study we have shown that YopB and YopD play an essential role in the cytotoxicity of *Y. enterocolitica* for HEp-2 epithelial cells and are required for virulence in mice. We have also shown that the loss of virulence of the *yopBD* mutant *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) was not due to thermosensitivity, because the growth characteristics of this strain at 37°C were similar to those of fully virulent strains. Cytotoxicity and virulence were restored to the *yopBD* mutant by *trans* complementation with the *yopB* and *yopD* genes but not with *yopB* or *yopD* alone. This indicates that YopB and YopD are not interchangeable and that both are required for virulence, despite their homology. Nevertheless, YopB and YopD appear to fulfill similar functions, presumably because they act in the same pathway. Previous studies with YopD showed that YopH, the *Yersinia* PTPase, requires YopD to cause maximal tyrosine dephosphorylation in macrophages (22). Here we show that YopB is required for the same purpose. Thus, it seems that YopB and YopD may act together to facilitate the translocation of YopH into macrophages.

The *yopBD* mutant *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*) did not produce detectable levels of YopD, because of a polar effect of the Tn*2507* insertion in *yopB* on the *yopD* gene. However, transcomplementation of this strain with the *yopB* gene partially restored production of YopD, suggesting that YopB plays a role in the regulation and/or secretion of YopD. Nevertheless, the observation that a *yopBD* mutant, *Y. enterocolitica* W22703(pEH110) (*yopB*::Tn*2507*), which produced YopD after *trans* complementation with *yopD* (pEH500) (Fig. 3B), showed no detectable PTPase activity (Fig. 5) and remained avirulent for mice (Table 3) indicates that YopB contributes to bacterial virulence in its own right regardless of any possible effects on YopD synthesis or secretion.

The role of the V antigen in the virulence of *Y. enterocolitica* is not clear. Some reports suggest that its predominant role in *Y. pestis* involves regulation of Yop production, while other evidence suggests the V antigen is a virulence factor per se. Recently, Skrzypek and Straley (40) reported the construction of a series of *lcrV* deletion mutants of *Y. pestis* showing that the regulatory and virulence functions of the V antigen are associated with different regions of the protein. Thus, deletion of amino acids 188 to 207 results in reduced expression of the V antigen and other Yops, implying that this region is important for induction of the low-calcium response, while the central region of the protein, amino acids 108 to 125, appears to be required for secretion of the V antigen itself (40). The *lcrV* transposon mutant of *Y. enterocolitica* W22703 examined in this study did not express YopB or YopD, even when the chaperone, *sycD*, was provided with the *yopB* and *yopD* genes in *trans*. The site of Tn*2507* insertion in pEH79 was in the region of amino acids 200 to 220. Thus, the transposon may have interrupted the regulatory region of *lcrV*, which resulted in an inability of this strain to produce YopB and YopD from a *trans*-complementing plasmid. However, *Y. enterocolitica* W22703(pEH79) (*lcrV*::Tn*2507*) could not even be *trans* complemented with the entire *lcrGVHyopBD* operon as a cloned PCR product and was only partially *trans* complemented by the plasmid, pCVD787, which carries this operon and contiguous DNA from *Y. enterocolitica* A2635 (data not shown) (27, 31). Hence, given the overall inability of *Y. enterocolitica* W22703(pEH79) (*lcrV*::Tn*2507*) to express any gene supplied in *trans*, we were unable to address the role of the V antigen in virulence. The construction of nonpolar *lcrV* deletion mutants of *Y. enterocolitica* should allow this difficulty to be overcome.

In this study we have shown that YopB is essential for the virulence of *Y. enterocolitica* and that YopB appears to act in concert with YopD to deliver YopH to its target in macrophages. Although the mechanism by which intracellularly acting Yops, such as YopE and YopH, are transported across the host cell membrane is not known, YopB and YopD could facilitate this process by binding to each other and to other Yops. In this regard, we have preliminary evidence that YopD binds specifically to YopB, YopE, and YopH, supporting the hypothesis that YopB and YopD are involved in the translocation of YopE and YopH. Thus, YopB and YopD may combine to form a pore in the eukaryotic cell membrane through which other

TABLE 3. Virulence of derivatives of *Y. enterocolitica* W22703 for 12-week-old BALB/c mice[a]

Strain	Presence[b] of:		LD_{50} (CFU)[c]
	YopB	YopD	
W22703(pYVe227)	+	+	5×10^4
W22703c (no plasmid)	−	−	$>5 \times 10^8$
W22703(pEH301) (*yopD*::Tn*2507*)	+	−	$>5 \times 10^8$
W22703(pEH301, pEH500) (*yopD*::Tn*2507* p*yopD*)	+	+	1×10^5
W22703(pEH110) (*yopB*::Tn*2507*)	−	−	$>5 \times 10^8$
W22703(pEH110, pEH500) (*yopB*::Tn*2507* p*yopD*)	−	+	$>6 \times 10^8$
W22703(pEH110, pEH503) (*yopB*::Tn*2507* p*yopB*)	+	(+)	$>5 \times 10^8$
W22703(pEH110, pEH504) (*yopB*::Tn*2507* p*yopBD*)	+	+	1×10^6

[a] Mice were inoculated intraperitoneally with a test strain 1 day after treatment with 5 mg of iron and 5 mg of desferrioxamine B and then observed for 14 days.
[b] +, Yop present; −, Yop absent; (+), small amount produced.
[c] Determined by the method of Reed and Muench (29).

Yops pass. Alternatively, they may be cointernalized with other intracellularly acting Yops, which they release at the appropriate subcellular site. Current work in our laboratory involves studies on the interaction of different Yops and an investigation of the subcellular location of YopB and YopD in target cells.

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