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Contribution of the Major Secreted Yops of *Yersinia enterocolitica* O:8 to Pathogenicity in the Mouse Infection Model

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Pathogenic yersiniae (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) harbor a 70-kb virulence plasmid (pYV) that encodes a type III secretion system and a set of at least six effector proteins (YopH, YopO, YopP, YopE, YopM, and YopT) that are injected into the host cell cytoplasm. Yops (*Yersinia* outer proteins) disturb the dynamics of the cytoskeleton, inhibit phagocytosis by macrophages, and downregulate the production of proinflammatory cytokines, which makes it possible for yersiniae to multiply extracellularly in lymphoid tissue. *Y. enterocolitica* serotype O:8 belongs to the highly mouse-pathogenic group of yersiniae in contrast to *Y. enterocolitica* serotype O:9. However, there has been no systematic study of the contribution of Yops to the pathogenicity of *Y. enterocolitica* O:8 in mice. We generated a set of *yop* gene deletion mutants of *Y. enterocolitica* O:8 by using the novel Red cloning procedure. We subsequently analyzed the contribution of *yopH*, *-O*, *-P*, *-E*, *-M*, *-T*, and *-Q* deletions to pathogenicity after oral and intravenous infection of mice. Here we showed for the first time that a Δ*yopT* deletion mutant colonizes mouse tissues to a greater extent than the parental strain. The Δ*yopO*, Δ*yopP*, and Δ*yopE* mutants were only slightly attenuated after oral infection since they were still able to colonize the spleen and liver and cause systemic infection. The Δ*yopO* mutant was lethal for mice, whereas Δ*yopP* and Δ*yopE* mutants were successfully eliminated from the spleen and liver 2 weeks after infection. In contrast the Δ*yopH*, Δ*yopM*, and Δ*yopQ* mutants were highly attenuated and not able to colonize the spleen and liver on any of the days tested. The Δ*yopH*, Δ*yopO*, Δ*yopP*, Δ*yopE*, Δ*yopM*, and Δ*yopQ* mutants had only modest defects in the colonization of the small intestine and Peyer's patches. The Δ*yopE* mutant was eliminated from the small intestine 3 weeks after infection, whereas the Δ*yopH*, Δ*yopP*, Δ*yopM*, and Δ*yopQ* mutants continued to colonize the small intestine at this time.

Yersiniae that are pathogenic to humans include *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* is the cause of bubonic plague, which is transmitted by fleas, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* cause self-limited food-borne gastrointestinal disease in humans. In the mouse model of infection, however, both *Y. enterocolitica* and *Y. pseudotuberculosis* cause systemic disease. Common to all of these species is the presence of a 70-kb virulence plasmid that harbors a type III secretion system (TTSS) and several secreted and translocated proteins called *Yersinia* outer proteins (Yops) (reviewed by Cornelis [17]). This plasmid-encoded TTSS enables yersiniae to survive and proliferate extracellularly in host lymphatic tissues. *Y. enterocolitica* and *Y. pseudotuberculosis* both translocate a set of at least five effector proteins (YopH, YopO/YpkA, YopP/J, YopE, and YopM), whereas *Y. enterocolitica* is known to translocate a sixth effector called YopT (35). Four of the above-mentioned Yops (YopH, YopE, YopT, and, YopO/YpkA) disturb cytoskeletal dynamics and thereby inhibit phagocytosis by polymorphonuclear leukocytes and macrophages (12, 23, 28, 49). YopH is a phosphotyrosine phosphatase (61) that dephosphorylates focal adhesion kinase (Fak), paxillin, Fyn-binding protein (FYB), p130^{cas}, and SKAP-HOM, thereby disrupting focal adhesions (2, 10, 11, 16, 29, 46, 47), and suppresses the oxidative burst of macrophages (12, 47). YopH has been shown to contribute not only to evasion of the innate immune response but also to the adaptive immune response by impairing T- and B-cell activation (1, 60). YopE is a GTPase-activating protein that acts preferentially on Rac GTPases, which may explain the YopE-associated effect of actin stress fiber destruction (9, 44, 50). YopT is a cysteine protease that preferentially inactivates RhoA GTPases by cleaving at the C-terminal geranylgeranyl-cysteine residue (52, 62). YopO (YpkA in *Y. pseudotuberculosis*) is an autophosphorylating serine/threonine kinase that interacts with RhoA, Rac and actin (6, 22, 62). YopP/J induces apoptosis of macrophages and inhibits activation of the transcription factor NF-κB, thereby inhibiting tumor necrosis factor alpha and interleukin-8 release by macrophages and epithelial cells (14, 19, 20). YopM is a leucine-rich repeat protein that traffics to the nucleus of infected cells (54), but its target and function are as yet unknown. Recently, it was shown that YopM forms a protein complex with the two cellular kinases PRK2 and RSK1 (39). YopQ (YopK in *Y. pseudotuberculosis*) is not one of the translocated effector proteins but has been shown to control the translocation of Yop effectors into eukaryotic cells with a *yopK* mutant hypertranslocating Yops and inducing a larger YopB-dependent pore in eukaryotic cell membranes (33).

Virulence of *Yersinia yop* mutants has been previously studied mostly in *Y. pseudotuberculosis*. It is, however, not possible to generally extrapolate these results to *Y. enterocolitica* since many differences in virulence factor profile and clinical mani-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference or source
Strains		
Yersinia spp.		
WA-314	*Y. enterocolitica* serotype O:8; clinical isolate; pYVO8+	32
WA-C	Plasmidless derivative of WA-314	32
WA-C(pYV::CM)	WA-C harboring pYV::CM	This study
WA-C(pYVΔH)	WA-C harboring pYVΔH	This study
WA-C(pYVΔH+H)	WA-C harboring pYVΔH and pYopH	This study
WA-C(pYVΔO)	WA-C harboring pYVΔO	This study
WA-C(pYVΔP)	WA-C harboring pYVΔP	This study
WA-C(pYVΔE)	WA-C harboring pYVΔE	This study
WA-C(pYVΔM)	WA-C harboring pYVΔM	This study
WA-C(pYVΔT)	WA-C harboring pYVΔT	This study
WA-C(pYVΔQ)	WA-C harboring pYVΔQ	This study
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*lacZ*ΔM15)	30
S17-1λpir	*pir*$^+$ *tra*$^+$ Kan^r	41
Plasmids		
pACYC184	Low-copy vector; Cm^r Tc^r	New England Biolabs
pACYC177	Low-copy vector; Kan^r Amp^r	New England Biolabs
pGPCAT	Suicide vector; R6K replicon; Cm^r Mob$^+$	48
pUC4k	Kan^r cassette	Pharmacia-LKB
pYV::CM	pYV plasmid harboring Cm^r gene from pACYC184 in noncoding region adjacent to *yadA*	This study
pYVΔH	pYV plasmid with *yopH* replaced by Km^r gene from pACYC177	This study
pYVΔO	pYV plasmid with *yopO* replaced by Km^r from pACYC177	This study
pYVΔP	pYV plasmid with *yopP* replaced by Km^r from pACYC177	This study
pYVΔE	pYV plasmid with *yopE* replaced by Km^r from pACYC177	This study
pYVΔM	pYV plasmid with *yopM* harboring Km^r from pUC4k	This study
pYVΔT	pYV plasmid with *yopT* replaced by Km^r from pACYC177	This study
pYopHSycH	pACYC184 harboring *yopH* and *sycH*	57

[a] Amp^r, ampicillin resistance; Tc^r, tetracycline resistance.

festations exist between the two species. Mutations in *yopH*, *ypkA*, *yopJ*, *yopE*, *yopM*, and *yopK* of *Y. pseudotuberculosis* serotype III have been shown to result in various degrees of attenuation (13, 15, 24, 27, 34, 37, 38, 42). In most cases, the 50% lethal dose was determined in order to assess virulence. Several studies also analyzed colonization of mouse tissue after oral infection (9, 26, 27, 34, 42, 55). However, comparison of data is difficult because different inbred mouse strains, different strains of *Yersinia*, and different infection doses and routes of application were used.

The influence of the complete set of Yops on virulence of *Y. enterocolitica* in the mouse model has been only partially studied (35, 43) with *Y. enterocolitica* of serotype O:9. This serotype is only weakly pathogenic for mice (due to lack of the high pathogenicity island encoding the biosynthesis and uptake of the siderophore yersiniabactin [*ybt*] which contributes to mouse virulence), and therefore mice have to be pretreated with an iron chelator such as desferrioxamine, which not only promotes growth of yersiniae by iron delivery via ferrioxamine uptake but also leads to immunosuppression of the host (5). We generated here a set of *yop* deletion mutants of *Y. enterocolitica* of the highly pathogenic O:8 serotype WA-314 (biotype 1B) by the recently reported Red recombination procedure (18) and studied the pathogenicity of these mutants and the course of colonization of the small intestine (SI), Peyer's patches (PP), liver, and spleen over 3 weeks after intravenous and oral infection of C57BL/6 mice.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are listed in Table 1. Bacteria were cultured aerobically in Luria-Bertani (LB) broth or on LB agar plates (Difco Laboratories, Detroit, Mich.) at 27°C (*Yersinia* spp.) or 37°C (*Escherichia coli*). Antibiotics were used at the following concentrations: kanamycin, 25 μg/ml; nalidixic acid, 60 μg/ml; and chloramphenicol, 20 μg/ml.

Nucleic acid manipulations. Plasmid DNA was isolated with Qiagen kits (Hilden, Germany) according to the manufacturer's recommendations. Restriction enzyme digestions, recovery of DNA fragments from agarose gels, ligations, and transformations were performed as previously described (3). Enzymes and deoxynucleoside triphosphates were purchased from Invitrogen (Karlsruhe, Germany). High-fidelity polymerase was obtained from Roche (Mannheim, Germany). Oligonucleotides were synthesized by Thermo (Ulm, Germany).

Red cloning. We generated stable *Y. enterocolitica* Yop mutants by using the λ phage recombinases Redα and Redβ as previously described for *E. coli* (18). Recombinases were expressed directly in *Yersinia*. For this purpose, we transformed WA-314 with plasmid pKD46 harboring recombinases *red*α and *red*β, as well as *red*γ, an inhibitor of bacterial exonucleases. Yersiniae were grown overnight at 27°C, diluted 1:100, and grown to exponential phase in LB medium containing 0.1% arabinose to induce the expression of recombination functions. Yersiniae were made electrocompetent and frozen at −80°C. Recombination fragments consisting of an antibiotic resistance gene with its own promoter, flanked by 50-nucleotide (nt) homology arms, were generated by PCR. The 3′-end 21 nt of each primer were designed to amplify the kanamycin resistance (Kan^r) cassette from pACYC177 (forward, 5-TCACTGACACCCTCATCAGT

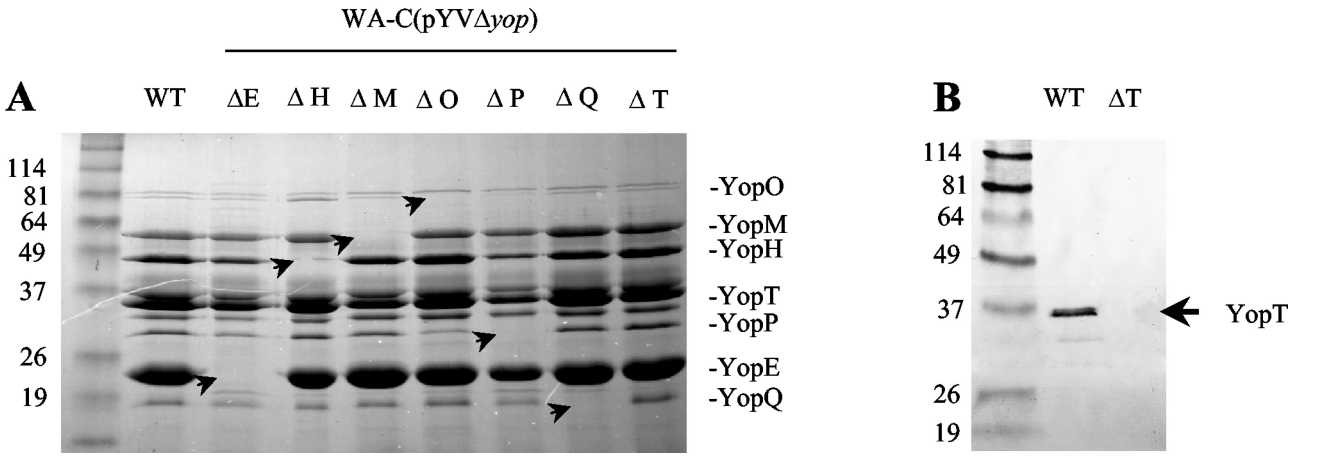

FIG. 1. (A) Coomassie blue-stained SDS-PAGE gel showing secreted proteins of WA-C(pYV::CM) (wild type [WT], lane 1) and Δ*yop* mutants (lanes 2 to 9) precipitated from the culture supernatant. (B) Western blot of secreted proteins from culture supernatant of WA-C(pYV::CM) [WT, lane 1] and WA-C(pYVΔT) (ΔT, lane 2) with a rabbit anti-YopT polyclonal antibody.

G-3′; reverse, 5′-CGTCAAGTCAGCGTAATGCTC-3′) or the chloramphenicol resistance (Cm^r) cassette from pACYC184 (forward, 5′-TGACGGAAGATCA CTTCGCAG-3′; reverse, 5′-TTGAGAAGCACACGGTCAC-3′), whereas the 5′ end of each primer contained the 50-nt homology arms. These were designed so that the entire coding region of each *yop* gene (*yopH*, *-O*, *-P*, *-E*, and *-T*) would be replaced by the antibiotic resistance marker. Homology arms were derived from the sequence of pYVa127/90 (GenBank accession number NC_004564) (25). The Δ*yopQ* mutant used for virulence experiments harbors the first 50 amino acids of YopQ. The Δ*yopM* mutant was constructed by ligating a PstI-restricted 1.2-kb Kan^r cassette cut from pUK4k into the NsiI site of the *yopM* gene, which was cloned into the suicide vector pGPCAT. Mutagenesis was performed as previously described (58). WA-C(pYV::CM) was constructed by inserting a Cm^r cassette amplified from pACYC184 into the noncoding region of the pYV plasmid, upstream of *yadA*, by the Red cloning procedure. This strain was shown to be as virulent as the unmarked parental strain WA-314 (results not shown). All mutated pYV constructs were transferred to a plasmidless WA-C strain. All strains were passaged through mice before virulence experiments were performed by intraperitoneal infection with 10^8 CFU and reisolation of yersiniae from the peritoneum after 20 h.

Analysis of Yop secretion. Secretion of Yop proteins was induced as previously described (57). Yersiniae were grown overnight in LB medium at 27°C, diluted 1:40 in brain heart infusion broth (Difco), and incubated for 2 h at 37°C. Yop expression and/or secretion was induced by the addition of EGTA (5 mM) for Ca^{2+} chelation, $MgCl_2$ (15 mM), and glucose (0.2%). Bacteria were grown at 37°C for 2 to 3 h and centrifuged (10,000 × *g*, 15 min), and proteins were precipitated from the culture supernatant with trichloroacetic acid. Released proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (36) on an 11.5% polyacrylamide gel and then stained with Coomassie blue. Immunoblotting was performed as described previously (31) with nitrocellulose sheets (Schleicher & Schuell). Blocking was performed overnight with 5% bovine serum albumin in phosphate-buffered saline (PBS) at 4°C. Polyclonal rabbit anti-YopO, anti-YopQ, and anti-YopT (1:5,000) antisera, as well as a horseradish peroxidase-conjugated secondary antirabbit antibody (Sigma) diluted 1:5,000, were used for immunostaining.

Mouse infections. Six- to eight-week-old female C57BL/6 mice (Harlan, Winkelmann, Germany) were infected with 10^8 CFU orally or 4×10^4 CFU intravenously from frozen stock suspensions. Stock suspensions were prepared by growing bacteria to stationary phase in LB medium at 27°C, followed by freezing in 15% glycerol. After appropriate dilutions, bacteria were washed twice with PBS, and mice were fed 50 μl by using a microliter pipette, or 100 μl was injected into the lateral tail vein. Mice were subjected to fasting 16 h prior to oral infection. The actually administered dose was determined by plating serial dilutions on Mueller-Hinton agar for 36 h at 27°C. Mice were sacrificed by CO_2 asphyxiation. Liver, spleen, and PP were aseptically removed and homogenized in 5 ml (liver) or 1 ml (spleen and PP) PBS. PP were washed to remove loosely attached bacteria by rinsing them with 2 ml of PBS. The SI was washed with 5 ml of ice-cold PBS. To determine the numbers of CFU/organ, serial dilutions of homogenates were plated on *Yersinia* selective CIN agar (BD Biosciences, Heidelberg, Germany). *P* values were determined by using a two-tailed, unpaired Student *t* test. *P* values of <0.05 were considered significant. For the competition experiment, a two-tailed paired Student *t* test was used.

RESULTS

Generation of stable Yop mutants by Red cloning. *Yersinia yop* mutants were generated by replacing the coding region of each *yop* by a Kan^r cassette. This was accomplished by Redα- and Redβ-mediated homologous recombination between the pYV plasmid and a PCR product consisting of the Kan^r resistance gene flanked by 50-nt homology arms. Correct replacement of the respective *yop* genes by the Kan^r cassette was verified by PCR and SDS-PAGE of secreted Yop proteins and Western blotting (Fig. 1). To rule out any unwanted recombination in the chromosome due to the action of Redα and Redβ, we transferred all mutated plasmids to the previously pYV-cured strain WA-C. Furthermore, we checked that no unwanted recombination in the mutant pYV plasmids had taken place by restriction endonuclease digestion of the resulting Δ*yop*-pYV plasmids with HindIII and BamHI (results not shown). Two of the mutants (Δ*yopQ* and Δ*yopM*) generated by replacement of the entire coding region with a Kan^r cassette turned out to be deregulated when grown at 37°C (temperature-sensitive phenotype) and consequently unable to colonize any mouse tissue after oral infection. Therefore, to perform virulence studies, we generated another Δ*yopQ* mutant by Red cloning, sparing the first 50 amino acids of YopQ. This mutant did not show a growth deficit at 37°C. For *yopM* we used a mutant that we had already constructed by the suicide vector approach. This mutant harbors a 1.2-kb Kan^r cassette at nt 661, expresses a truncated version of YopM of ~25 kDa (verified by Western blotting), and shows Ca^{2+}- and temperature-dependent growth like that of the wild type.

Course of colonization and persistence after oral infection. Groups of six C57BL/6 mice were orally infected with 10^8 CFU of each Δ*yop* mutant (WA-C(pYVΔYop) and WA-C(pYV::CM). The course of colonization was determined by counting the surviving bacteria in the liver, spleen, PP, and SI on days 2, 5, 7, 12, and 21 postinfection by plating tissue homogenates

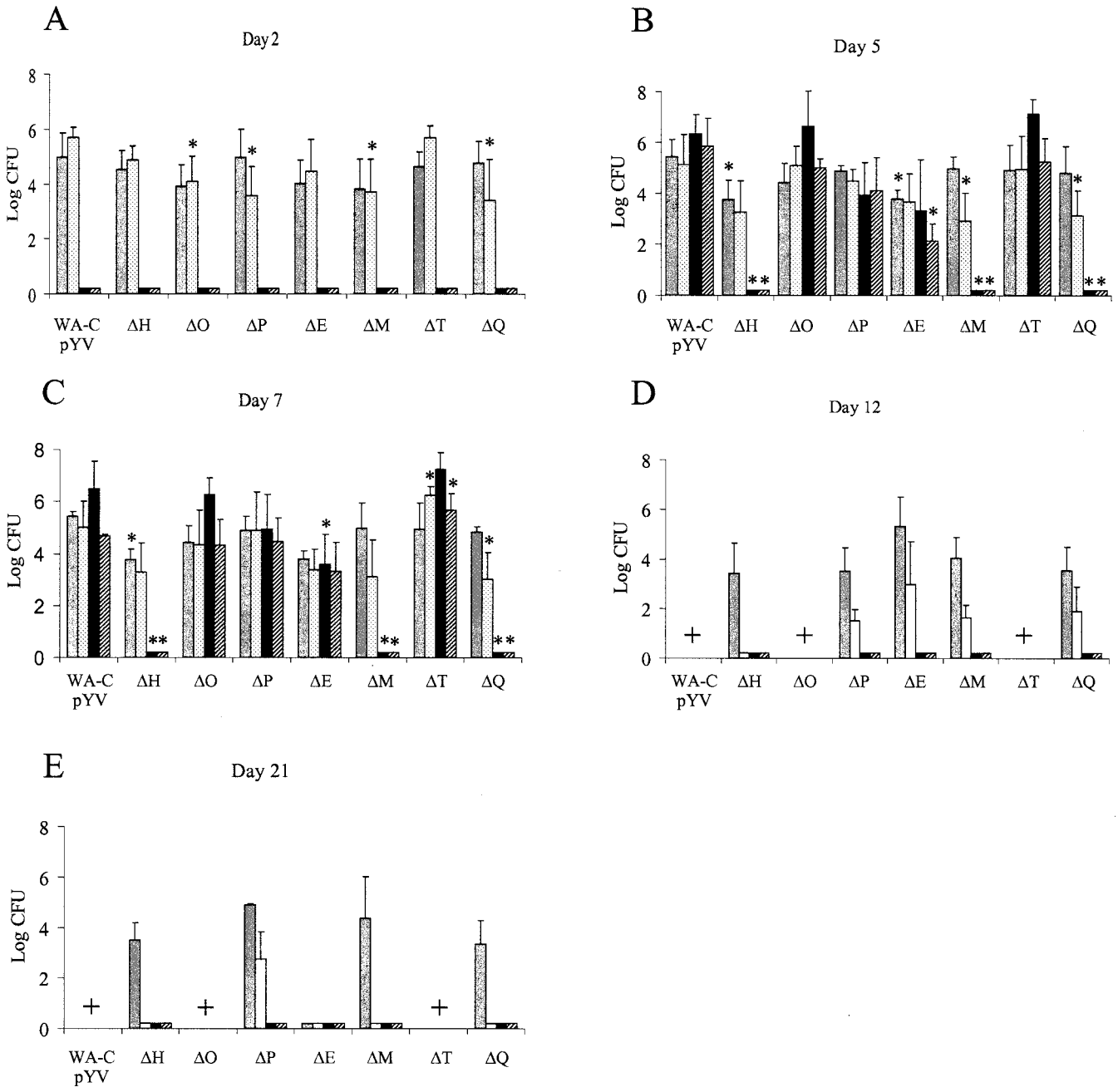

FIG. 2. Time course of colonization and persistence of *Y. enterocolitica* Δ*yop* mutants and WA-C(pYV::CM) after oral infection of C57BL/6 mice with 10^8 CFU. Two (A), five (B), seven (C), twelve (D), and twenty-one (E) days after infection, surviving bacteria in the SI (▨), PP (□), spleen (■), and liver (▨) were determined. Values represent the average log CFU per organ for six mice with the standard errors of the means indicated by error bars. The limits of detection were 10 CFU/organ for PP and spleen and 50 CFU/organ for liver and SI. A "+" indicates that the strain was lethal for mice. Asterisks indicate statistical significance ($P \leq 0.05$) between colonization of WA-C(pYV::CM) and the mutants.

(Fig. 2 and 3). Two days after oral infection, all mutants (Δ*yopH*, Δ*yopO*, Δ*yopP*, Δ*yopE*, Δ*yopM*, Δ*yopT*, and *yopQ*) and WA-C(pYV::CM) were able to efficiently colonize the SI and PP, with Δ*yopH*, *-O*, *-P*, *-E*, *-M*, and *-Q* mutants showing only moderate defects in colonizing these tissues. The course of infection with WA-C(pYV::CM), as well as the Δ*yopO*, Δ*yopP*, Δ*yopE*, and Δ*yopT* mutants, was progressive, with bacteria disseminating systemically and forming abscesses in livers and spleens by day 5. By this time all mice infected with these mutants showed severe signs of illness. The Δ*yopH*, Δ*yopM*, and Δ*yopQ* mutants, on the other hand, were highly attenuated and were not able to significantly colonize spleens and livers on any of the days tested (limit of detection of 10 CFU in the spleen and 50 CFU in the liver). By day 7, the Δ*yopT*, Δ*yopO*, Δ*yopE*, and Δ*yopP* mutants showed the highest colony counts in spleens and livers, with most mice infected with WA-C(pYV::

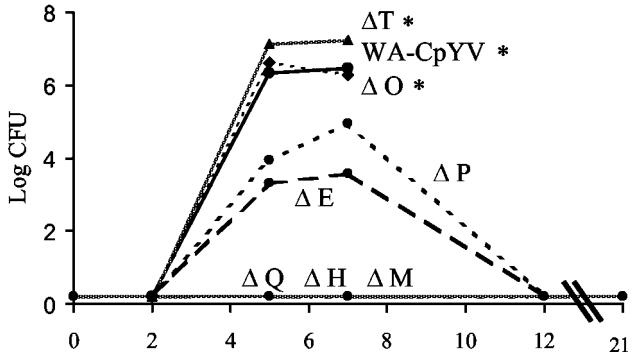

FIG. 3. Colonization and persistence in spleens over 3 weeks by *Y. enterocolitica* Δ*yop* mutants and WA-C(pYV::CM) after oral infection of C57BL/6 mice with 10^8 CFU. Asterisks indicate death of mice between days 7 and 12 postinfection. Δ*yopQ*, -*H*, and -*M* mutants were below the limit of detection (10 CFU/organ). Values indicate the average log CFU per organ for six mice.

CM), Δ*yopT*, and Δ*yopO* mutants dying between days 7 and 12 due to systemic infection and high bacterial load in organs (Fig. 3). The Δ*yopE* and Δ*yopP* mutants were, however, successfully eliminated from spleens and livers by day 12. Furthermore, Δ*yopP*, Δ*yopE*, Δ*yopM*, and Δ*yopQ* mutants were still able to colonize the SI and PP by this time. The Δ*yopH* mutant was able to colonize only the SI but had been eliminated from the PP by this time. At 3 weeks after infection, Δ*yopH*, Δ*yopM*, and Δ*yopQ* mutants were able to colonize the lumen of the SI only, whereas the Δ*yopP* mutant continued to colonize both the SI and the PP. To demonstrate that attenuation of our mutants was not due to polar effects caused by the Kan^r cassette, we complemented the most highly attenuated mutant (Δ*yopH*) with a low-copy plasmid (pACYC184) harboring *yopH* and *sycH* under their natural promoters (57). At 5 days after oral infection, this strain was able to colonize the spleens and livers of infected mice to an extent comparable to WA-C(pYV::CM) (Fig. 4).

Virulence of *yop* mutants after intravenous infection. Groups of five C57BL/6 mice were infected with 4.5×10^4 CFU of the Δ*yop* mutants and WA-C(pYV::CM). Surviving bacteria in livers and spleens were determined on days 2 and 4 postinfection (Fig. 5). WA-C(pYV::CM) showed high colony counts in spleens and livers on day 2 (6.53 ± 0.40 and 4.88 ± 0.37 log CFU) with infection progressing by day 4 (8.33 ± 0.54 and 5.73 ± 0.32 log CFU). The Δ*yopH* mutant was the most highly attenuated mutant, with a 2,100-fold reduction in CFU compared to WA-C(pYV::CM) by day 2. The colony counts were 100-fold lower for Δ*yopQ* and 13-fold lower for Δ*yopM*, whereas the Δ*yopE* and Δ*yopP* mutants (4.7-fold and 2-fold, respectively) were not dramatically attenuated compared to the parental strain ($P > 0.05$). The Δ*yopT* mutant, on the other hand, colonized the spleen to a sevenfold-greater extent on day 2 than did WA-C(pYV::CM). By day 4, infection had progressed for the WA-C(pYV::CM), Δ*yopO*, Δ*yopT*, Δ*yopM*, Δ*yopE*, and Δ*yopP* mutants, showing 63-, 229-, 18-, 17-, 4.3-, and 4-fold-higher CFU counts, respectively, in the spleen than on day 2, whereas the CFU counts in the spleens of mice infected with the Δ*yopH* mutant dropped 40-fold. By day 4 the colony counts in spleens were 219,000-fold lower for the Δ*yopH* mutant than for WA-C(pYV::CM), 6,300-fold lower for the Δ*yopQ* mutant, 50-fold lower for the Δ*yopM* mutant, and 63-fold lower for the Δ*yopE* and Δ*yopP* mutants. The Δ*yopO* mutant showed slightly fewer CFU in the spleen (2.7-fold) but more CFU in the liver (3.2-fold), which was significant only for the liver. The Δ*yopT* mutant showed higher CFU counts in spleens (1.4-fold) and livers (28-fold) than WA-C(pYV::CM), values that were statistically significant only for livers ($P < 0.05$). Generally, the CFU counts of the *yop* mutants and WA-C(pYV::CM) in the livers of infected mice were 10- to

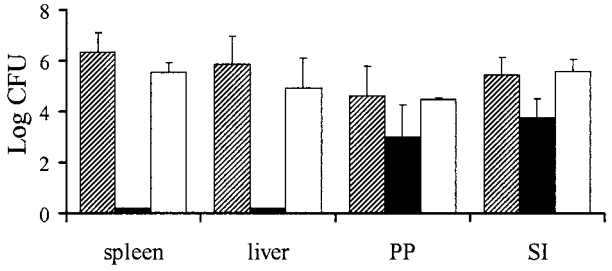

FIG. 4. Colonization of spleens, livers, PP, and SIs of C57BL/6 mice after oral infection with 10^8 CFU of WA-C(pYVΔYopH) (■), WA-C(pYVΔYopH+H) (□), and WA-C(pYV::CM) (▨) on day 5 postinfection.

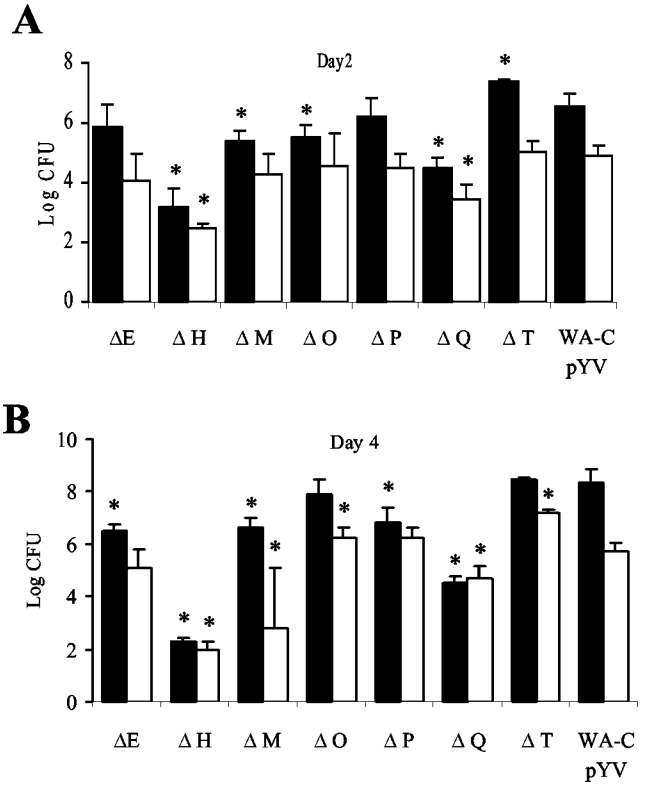

FIG. 5. Colonization of spleen (■) and liver (□) of C57BL/6 mice 2 (A) and 4 (B) days after intravenous infection with 4×10^4 CFU of the indicated *Y. enterocolitica* Δ*yop* mutants and WA-C(pYV::CM). Each bar represents the mean log CFU for five mice. Asterisks indicate statistical significance between colonization of the Δ*yop* mutants and of the WA-C(pYV::CM) ($P \leq 0.05$).

100-fold lower than the CFU counts in the spleens except for the Δ*yopH* and Δ*yopQ* mutants on day 4, which showed comparable numbers of bacteria in the liver and spleen.

The Δ*yopT* mutant outcompetes WA-C(pYV::CM). The oral and intravenous infections suggested that the Δ*yopT* mutant was slightly more virulent than the wild-type strain. To confirm this, we infected six mice with an equal mixture (10^8 CFU) of WA-C(pYV::CMΔYopT) and WA-C(pYV::CM) by the intravenous route. Two days later, the mice were killed, and serial dilutions of organs were plated on kanamycin (for selection of the Δ*yopT* mutant) and chloramphenicol [for selection of WA-C(pYV::CM)] plates. The Δ*yopT* mutant showed a mean log of 4.23 ± 0.99 CFU in the liver, whereas WA-C(pYV::CM) showed a mean log of 3.05 ± 1.03 CFU. Therefore, the Δ*yopT* mutant outcompeted WA-C(pYV::CM) by 1.18 log CFU ($P = 0.0075$). No such difference could be detected for the spleens of mice, with the Δ*yopT* mutant showing a mean log of 6.61 ± 0.36 CFU and WA-C(pYV::CM) showing a mean log of 6.49 ± 0.34 CFU.

DISCUSSION

The objective of this study was to systematically analyze the contribution of seven Yops of *Y. enterocolitica* O:8, one of the highly virulent serotypes of biotype IB (New World strains), to virulence by using the same inbred mouse strain, the same route of application, and the same dose of bacteria. Although several studies have dealt with the effects of Yops on virulence, most of these were performed for *Y. pseudotuberculosis* serotype III, which differs considerably in virulence factor repertoire (e.g., lacking yersiniabactin) and clinical manifestations from *Y. enterocolitica*. This is exemplified by one study showing that even a plasmidless *Y. pseudotuberculosis* strain is able to multiply in the spleen after intravenous infection or to survive in human serum, whereas plasmid-cured *Y. enterocolitica* is rapidly eliminated from this organ or killed by serum (53, 59). Most importantly for the present study, *Y. enterocolitica* harbors the TTSS effector YopT, which is usually absent in *Y. pseudotuberculosis* (35), and several differences in a multitude of other virulence factors exist between the different *Yersinia* strains, e.g., YopP from *Y. enterocolitica* O:8 is substantially more efficient in suppressing the NF-κB pathway and mediating apoptosis than serotype O:9 (51). Invasin-mediated uptake efficiency in cell culture is much higher for *Y. pseudotuberculosis* than for *Y. enterocolitica* (21, 40). YadA and its collagen-binding potential are required for virulence in *Y. enterocolitica* but not in *Y. pseudotuberculosis* (45, 56). Furthermore, differences in serum resistance between the two species have been detected (59), and it has been reported that *Y. pseudotuberculosis* is more resistant to bactericidal cationic peptides (8) and shows increased outer membrane permeability to hydrophobic agents compared to *Y. enterocolitica* (7). It is therefore not justified to generally extrapolate mouse virulence studies obtained with *Y. pseudotuberculosis* to *Y. enterocolitica*.

The study of virulence in the mouse infection model with a naturally high-pathogenicity-island-negative *Y. enterocolitica* of serotype O:9 and O:3 is further complicated by the fact that mice have to be pretreated with desferrioxamine B (DFO) to achieve mouse virulence, and this pretreatment may influence pathogenicity because of the immunosuppressive effect of DFO (4). The virulence of *yopQ* and *yopM* (43) mutants in the mouse model was previously studied by using *Y. enterocolitica* O:9. Both mutants were shown to be attenuated after intravenous infection of mice pretreated with DFO. Both mutants were able to colonize spleens and livers of Swiss mice for at least 4 days at reduced levels compared to the wild-type strain. We were able to confirm these results here and extend them to the oral infection model, showing for the first time that both of these mutants are highly attenuated and able to colonize only the SI and PP but are not able to disseminate to the spleens and livers of orally infected mice. Δ*yopQ* and Δ*yopM* mutants were able to colonize PP for 2 and SI for 3 weeks but the numbers were somewhat lower than for WA-C(pYV::CM). These oral infections are consistent with studies of a *yopK* mutant in *Y. pseudotuberculosis* (34). YopQ fine-tuning of translocation or gating of the YopB-induced pore might be essential for effective immunosuppression of the host and systemic spread of yersiniae as proposed by Holmström et al. (33). Oral infections with Δ*yopM* mutants have not been performed previously. Together with the intravenous infections, our results indicate that YopM and YopQ are mainly involved in the establishment of a systemic infection in mice.

The virulence of Δ*yopH*, Δ*yopO*, Δ*yopP*, and Δ*yopE* mutants has been previously studied only for *Y. pseudotuberculosis*. The most striking difference between the virulence of our mutants and that of *Y. pseudotuberculosis* is that our Δ*yopE* mutant is only slightly attenuated after oral infection and caused systemic disease with regular seeding of yersiniae to spleens and livers. Furthermore, our Δ*yopE* mutant was able to colonize the SI and PP at somewhat reduced levels until day 12 postinfection. *Y. pseudotuberculosis yopE* mutants, on the other hand, were reported to be highly attenuated, being cleared from PP within 4 days and not able to reach the spleens of orally infected mice (34). These differences could be due to the use of a *yadA yopE* double mutant in that study (34). Another study using *Y. pseudotuberculosis* showed a markedly reduced ability of *yopE* mutants to colonize spleens of mice but only minor defects in colonizing the SI and PP (38). Possibly, the additional presence of YopT in *Y. enterocolitica* could also account for the more virulent phenotype of our *yopE* mutant since both of these Yops act on Rho GTPases and are involved in the inhibition of phagocytosis. Presumably, a Δ*yopT* Δ*yopE* double mutant of *Y. enterocolitica* is comparable to a *yopE* mutant of *Y. pseudotuberculosis*.

Our Δ*yopO* and Δ*yopT* mutants behaved most like WA-C(pYV::CM) after oral and intravenous infections, and in fact most mice succumbed to oral infection with these mutants at around day 7 just like the parental strain. For a *yopO* mutant, this is consistent with a recent study with *Y. pseudotuberculosis* (38) but contrasts with another (27) in which a Δ*yopO* mutant failed to cause a systemic infection. The mutant used in the latter study, however, also harbored a mutation in *yadA*, which could have had an effect on colonization of the spleen. There is only scarce evidence for the role of YopT in animal models, with one preliminary study showing that a *yopT* mutant was not affected in its capacity to colonize PP on day 2 after oral infection (35). Surprisingly, our *yopT* mutant was not attenuated and even showed higher colonization of mouse tissues after oral and intravenous infection than WA-C(pYV::CM). This is a surprising finding in light of the fact that YopT and

YopO have a strong influence on phagocytosis resistance in cell culture models (28). YopO binds RhoA and Rac and YopT modifies RhoA and presumably Rac, which may also change binding properties to YopO. Possibly, YopT is redundant in this animal model, and its functions are taken over by other Yops, such as YopO and YopE. Another possibility is that the functions of these effector proteins become apparent only after several weeks of infection, when the adaptive immune system becomes effective. Since C57BL/6 mice succumb to infection after 1 week, it is not possible to study these effects in this model. We are therefore generating double mutants for *yopT* and other *yop* genes to test this hypothesis.

Our *yopP* mutant colonized gut tissues, as well as spleens and livers, at reduced levels but was not impaired in its ability to cause systemic disease, with bacteria regularly colonizing the livers and spleens of all mice after oral infection. By day 12 most mice had eliminated bacteria from spleens and livers, but colonization of gut tissues continued for at least 3 weeks. The role of *yopJ* in virulence has been previously studied only for *Y. pseudotuberculosis*. One study claims *yopJ* to be dispensable for virulence (27), and another shows that a *yopJ* mutant is highly attenuated, with only two of three PP and one of five spleens being colonized after oral infection (42). These differences could be due to the different *Yersinia* species used, the different mouse strains used, or different doses of applied bacteria.

Our Δ*yopH* mutant was by far the most attenuated mutant in both oral and intravenous infections. Δ*yopH* mutants were only slightly deficient in colonizing the SI and PP initially and were not able to disseminate and cause systemic disease. Δ*yopH* mutants were eliminated from the PP by day 12, earlier than the other surviving *yop* mutants, but colonization of the gut lumen continued for at least 3 weeks. Intravenous infections showed that the Δ*yopH* mutant initially implants into spleens and livers, but colony counts dramatically decrease between days 2 and 4. This indicates that YopH plays important roles in PP, as well as during the systemic stage of disease.

In summary, the Δ*yopH*, Δ*yopO*, Δ*yopP*, Δ*yopE*, Δ*yopM*, and Δ*yopQ* mutants had only modest defects in colonization of the SI and PP. This is consistent with results obtained for *Y. pseudotuberculosis* (38) and indicates that no single effector Yop is absolutely necessary for colonizing the SI or translocating to the underlying PP. YopH, YopQ, and YopM of *Y. enterocolitica* are important virulence factors for inducing systemic disease in mice, whereas YopO, YopP, and YopE are dispensable for yersiniae to reach the spleen and liver. The presence of YopT on the other hand even seems to slightly decrease virulence of *Y. enterocolitica* in this model.

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