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Type III machines of pathogenic yersiniae secrete virulence factors into the extracellular milieu

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Summary

Gram-negative bacteria use type III machines to inject toxic proteins into the cytosol of eukaryotic cells. Pathogenic *Yersinia* species export 14 Yop proteins by the type III pathway and some of these, named effector Yops, are targeted into macrophages, thereby preventing phagocytosis and allowing bacterial replication within lymphoid tissues. Hitherto, YopB/YopD were thought to insert into the plasma membrane of macrophages and to promote the import of effector Yops into the eukaryotic cytosol. We show here that the type III machines of yersiniae secrete three proteins into the extracellular milieu (YopB, YopD and YopR). Although intrabacterial YopD is required for the injection of toxins into eukaryotic cells, secreted YopB, YopD and YopR are dispensable for this process. Nevertheless, YopB, YopD and YopR are essential for the establishment of *Yersinia* infections in a mouse model system, suggesting that type III secretion machines function to deliver virulence factors into the extracellular milieu also.

Introduction

During infection of their mammalian hosts, Gram-negative bacteria require type III secretion machines to inject virulence factors into the cytosol of eukaryotic cells (Rosqvist *et al*., 1991). Some of these pathogens, such as *Salmonella* and *Shigella*, use the type III pathway to invade specific host cells (Menard *et al*., 1996; Collazo and Galan, 1997). Once inside, bacteria are shielded from the immune system and begin to multiply. Other microorganisms, for example yersiniae, dock onto the surface of macrophages (Isberg, 1996) and inject virulence factors to kill their target cells (Cornelis and Wolf-Watz, 1997; Cornelis, 1998). Type III injection of Yop proteins allows yersiniae to escape phagocytosis and to replicate within lymphoid tissues of the animal host. *Pseudomonas aeruginosa* as well as enteropathogenic *Escherichia coli* deliver toxins into epithelial cells (Frank, 1997; Nataro and Kaper, 1998). By damaging their local environment these microbes aim to spread and infect new tissues. Although these infections result in fundamentally different pathological lesions, the type III-mediated injection of toxins is a common strategy of many Gram-negative pathogens (Hueck, 1998).

Type III machines are assembled from more than 20 polypeptides and translocate virulence factors across the double membrane envelope of Gram-negative bacteria (Michiels *et al*., 1990; Plano *et al*., 1991; Straley *et al*., 1993; Allaoui *et al*., 1994; 1995; Bergmann *et al*., 1994). The protein components of type III machines are conserved between many different pathogens, however their secretion substrates are not (Hueck, 1998). *Yersinia* species export some 14 different Yop proteins by the type III pathway (Straley *et al*., 1993). Six of those, named effector Yops, are targeted into eukaryotic cells (YopE, YopH, YopM, YopO, YopP and YopT) (Rosqvist *et al*., 1991; 1994; Boland *et al*., 1996; Hakansson *et al*., 1996a; Mills *et al*., 1997; Monack *et al*., 1997; Persson *et al*., 1997; Iriarte and Cornelis, 1998). *Yersinia* strains carrying mutations in any of the *ysc* genes loose the ability to target effector Yops (Sory and Cornelis, 1994; Sory *et al*., 1995). The same phenotype has been reported for strains carrying mutations in the *yopB* or *yopD* genes (Rosqvist *et al*., 1994; Sory and Cornelis, 1994; Boland *et al*., 1996; Hakansson *et al*., 1996a). YopB/YopD have been viewed as a protein translocation pore that inserts into the eukaryotic plasma membrane and recognizes effector Yops as import substrates (Cornelis and Wolf-Watz, 1997; Cornelis, 1998). Mutations in other *Yersinia* genes (e.g. *yopQ*$^-$, *lcrG*$^-$ and *yopN*$^-$) either altered the amount of targeting or caused the secretion of effector Yops into the extracellular environment (Holmstrom *et al*., 1997; Boyd *et al*., 1998; Sarker *et al*., 1998). Thus, yersiniae are thought to dedicate their entire type III pathway to the delivery of bacterial toxins into eukaryotic cells (Silhavy, 1997).

Previous work measured type III targeting in two ways, one of which is the detection of Yop proteins in the cytosol of HeLa cells by immunofluorescence microscopy (Rosqvist *et al*., 1991). Although this experimental approach revealed the presence or absence of Yops within HeLa cells, it did not permit quantitative measurements of targeting. This was overcome by using Yop fusions to a reporter protein, *Bordetella pertussis* Cya (Sory and Cornelis, 1994). Cya requires binding to calmodulin in the eukaryotic

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cytoplasm to synthesize cAMP, and an increased amount of cAMP within HeLa cells previously infected with yersiniae is a measure of Yop–Cya targeting. Neither of the two experimental strategies allowed the detection of Yops in all cellular and extracellular compartments. We therefore established the digitonin fractionation technique for the rapid and direct detection of Yop proteins in all compartments (Lee *et al*., 1998). This approach revealed that during *Yersinia enterocolitica* infections of HeLa cells YopB, YopD and YopR are secreted into the extracellular milieu (Lee *et al*., 1998). This was a surprising result because YopB/YopD were hitherto thought to be located in the eukaryotic plasma membrane. Nevertheless, our work left unresolved whether or not secreted Yops play a role in type III targeting as measured by digitonin fractionation.

We report now that YopB, YopD and YopR are secreted into the extracellular milieu of all *Yersinia* species and strains examined. Secreted YopB, YopD and YopR are dispensable for the type III targeting of effector Yops into the cytosol of HeLa cells. *Yersinia* strains carrying mutations in any one of the three genes, *yopB*, y*opD* and *yopR* displayed reduced virulence in a mouse model system, suggesting that the secretion of YopB, YopD and YopR into the extracellular milieu is essential for the pathogenesis of *Yersinia* infections.

Results

Secretion of YopB, YopD, and YopR into the extracellular milieu

To understand the role of Yops in type III targeting, we wished to determine their location during infection and used an experimental scheme that allowed fractionation of *Yersinia*-infected HeLa cell cultures (Lee *et al*., 1998). The growth medium was removed and centrifuged to sediment non-adherent bacteria from the extracellular medium. HeLa cells and yersiniae adherent to the culture flasks were treated with digitonin, a detergent that disrupts the eukaryotic plasma membrane but not the bacterial envelope. Samples were centrifuged to separate the HeLa cell cytosol from the bacterial sediment. As a control, a duplicate sample of infected HeLa cell culture was treated with SDS to solubilize all eukaryotic and bacterial membranes. Yop proteins were located either in the extracellular milieu (YopB, YopD and YopR), injected into the eukaryotic cytosol (YopE, YopH, YopM, and YopN), or remained associated with the bacteria (YopQ) (Fig. 1). Similar results were obtained for *Y. enterocolitica* strains W22703 (O9 serotype, European isolate) (Lee *et al*., 1998) and 8081 (O8, American isolate) as well as *Y. pseudotuberculosis* YPIII, indicating that these observations were neither strain nor species specific (Fig. 1A and B).

To examine whether the observed secretion and targeting of Yop proteins are catalysed by the type III machines of yersiniae, we tested *Y. enterocolitica* strain KUM1 (Cheng *et al*., 1997) in the digitonin fractionation assay. The mutant strain carries the *lcrD1* allele, which abolishes synthesis of the integral membrane protein LcrD, an essential component of type III machines (Plano *et al*., 1991), thereby interfering with all Yop secretion (Cheng *et al*., 1997). After infection with strain KUM1 and digitonin fractionation of HeLa cells, all Yops examined sedimented with the bacteria (Fig. 1C). YopD, YopE and YopH were also observed in bacteria that sedimented from the tissue culture medium (non-adherent yersiniae). Small amounts of YopB were only detected when HeLa cells and adherent bacteria were extracted with SDS. Together these data suggest that the type III machines of yersiniae catalyse both the secretion of YopB, YopD and YopR into the extracellular milieu and the targeting of YopE, YopH, YopM and YopN into the cytosol of HeLa cells.

After fractionation, small amounts of YopB and YopD can be observed in the supernatant of digitonin-extracted HeLa cells (Fig. 1A and B). These polypeptides could be located either in the eukaryotic cytosol or on the surface of HeLa cells in a manner that permits their solubilization with detergent. Thus, if indeed the type III machinery directs Yops to two different locations, the addition of protease to infected HeLa cells should digest extracellular Yops, whereas effector Yops should be protected by the eukaryotic plasma membrane. This was tested, and proteinase K degraded YopB, YopD and YopR (more than 95%), whereas YopE and YopN were resistant to this treatment (Fig. 1D). Membrane solubilization with detergent rendered all Yops sensitive to proteinase K. If cytosolic YopE is protected from protease, extracellular YopE should be sensitive. *Yersinia* VTL1 (*yopN1*) secretes all effector Yops into the extracellular milieu (Lee *et al*., 1998), and the addition of proteinase K to HeLa cells infected with strain VTL1 degraded all YopE (Fig. 1E). These results demonstrate that YopE and YopN are located in the eukaryotic cytosol, whereas YopB, YopD and YopR are mostly secreted into the extracellular milieu. Because small amounts of YopB and YopD are protected from extracellular protease, we cannot exclude the possibility that these species are located inside HeLa cells. Similar observations have been made for *Y. pseudotuberculosis* infections of HeLa cells, and small amounts of YopD are thought to be translocated into the eukaryotic cytosol (Francis and Wolf-Watz, 1998).

Mutations in yopB, yopD and yopR

We wondered what role extracellular YopB, YopD and YopR might play for the type III targeting of other Yop proteins and constructed strains with a nonsense mutation followed by a frameshift inserted after codon 8 in either of

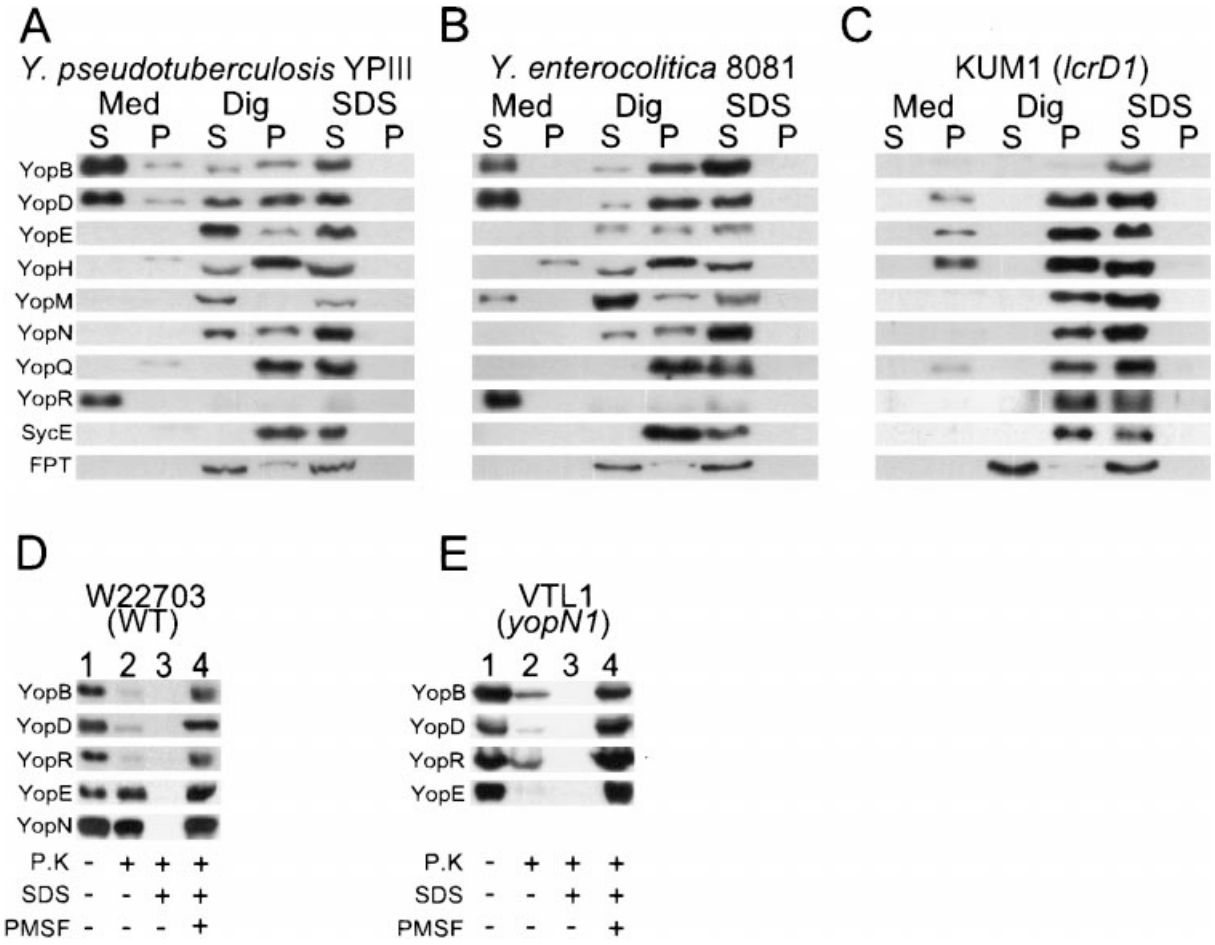

Fig. 1. Localization of Yop proteins during HeLa cell infections of *Y. pseudotuberculosis* YPIII (A), *Y. enterocolitica* 8081 (O8) (B) and *Y. enterocolitica* KUM1, an *lcrD1* mutant of strain W22703 (C). Media (Med) were decanted from infected HeLa cells and centrifuged to sediment non-adherent bacteria (P, pellet) and separate them from secreted Yops in the supernatant (S). HeLa cells and adherent yersiniae were extracted with digitonin (Dig) to disrupt the eukaryotic plasma membrane. Bacteria, membranes and eukaryotic organelles were sedimented by centrifugation (P) and separated from the HeLa cytosol in the supernatant (S). As a control for solubilization of all membranes, samples were extracted with SDS and centrifuged. Proteins were precipitated with chloroform/methanol, separated on SDS–PAGE, electroblotted and immunodetected with specific antisera. HeLa cell farnesyl protein transferase (FPT) and bacterial SycE were used as controls for the selective disruption of the eukaryotic plasma membrane by digitonin. Protease sensitivity of extracellular YopB, YopD and YopR during HeLa cell infection with *Y. enterocolitica* O9 strain W22703 (D) or strain VTL1 (*yopN1*) (E). Infected tissue cultures were left untreated (1) or incubated with either (extracellular) proteinase K (P.K) (2), SDS and proteinase K (3) or SDS, proteinase K and PMSF inhibitor (4). After incubation, proteolysis in all samples was quenched by the addition of PMSF. Proteins were precipitated with methanol/chloroform, solubilized in sample buffer and separated on SDS–PAGE before immunoblotting with specific antisera.

the three genes (Fig. 2). To characterize the mutant strains for the synthesis and secretion of Yop proteins, *Yersinia* cultures were induced for type III secretion by temperature shift to 37°C and growth at low calcium concentration. Cultures were centrifuged and bacterial cells in the sediment were separated from the supernatant (extracellular milieu). Proteins in both fractions were precipitated with TCA and analysed by immunoblotting. *Yersinia* strains carrying the above-mentioned mutations in either *yopB* (*yopB1*), *yopD* (*yopD1*) or *yopR* (*yopR1*) displayed a defect in the synthesis of the encoded Yop protein. The *yopB* and *yopD* genes are located at the 3′ end of the *lcrGVHyopBD* operon (Bergmann *et al*., 1991). Neither the *yopB1* nor the *yopD1* mutation interfered with the expression of other genes in this operon by the mutant strains MC4 and VTL1. The *yopR* gene is located within the *virC* operon (Allaoui *et al*., 1995) and the *yopR1* mutation did not alter expression of a downstream gene, *yscM* (Stainier *et al*., 1997) (Fig. 2). Expression of plasmid-encoded wild-type *yopB*, *yopD* or *yopR* restored the low calcium-induced synthesis and secretion of YopB, YopD and YopR by mutant strains MC4, VTL1 and MC1 respectively (data not shown). Together these data suggest that the *yopB1*, *yopD1* and *yopR1* mutations are non-polar for the expression of other genes in the *virC* and *lcrGVHyopBD* operons.

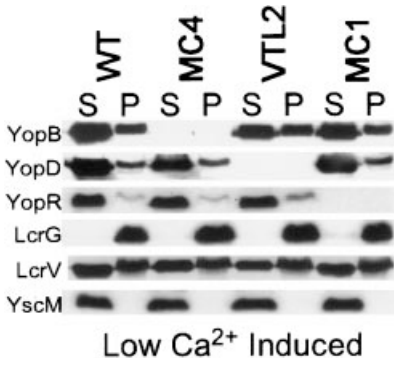

Fig. 2. *Yersinia enterocolitica* strains carrying non-polar mutations in *yopB*, *yopD* or *yopR*. Mutations generated non-polar null alleles of *yop* genes via the insertion of a stop codon followed by frameshift mutation after codon 8 of each open reading frame. Wild-type and mutant *Yersinia* strains were analysed for the expression and secretion of Yops in low Ca^{2+}-induced cultures. Samples were centrifuged to separate Yops secreted into the extracellular medium from proteins that sedimented with the bacterial cells. Proteins were precipitated with TCA, solubilized and analysed by immunoblotting with specific antisera.

YopB and YopR are not required for type III targeting

Mutant *Yersinia* strains were analysed for their defects in type III secretion and/or targeting by digitonin fractionation of infected HeLa cells. The *yopB1* mutant strain MC4 secreted YopD and YopR into the extracellular medium while YopE, YopH, YopM and YopN were solubilized by digitonin extraction (Fig. 3, and Table 1). Expression of plasmid-encoded, wild-type *yopB* from the *lcrG* promoter restored YopB expression to wild-type levels. These results are in disagreement with previous reports that *yopB* mutations abrogate all Yop targeting (Boland *et al*., 1996; Hakansson *et al*., 1996a). We therefore sought to analyse Yop targeting further and examined the subcellular location of YopE with protease protection and immunofluorescence assays. When HeLa cells were infected with the *yopB1* mutant strain MC4, 100% of YopE was protected from extracellular protease. YopE was visualized by indirect immunofluorescence in the cytosol of HeLa cells infected with strain MC4, similar to YopE delivered by wild-type yersiniae (Fig. 5). No YopE staining was observed during infections with the *yopE* null strain LC1

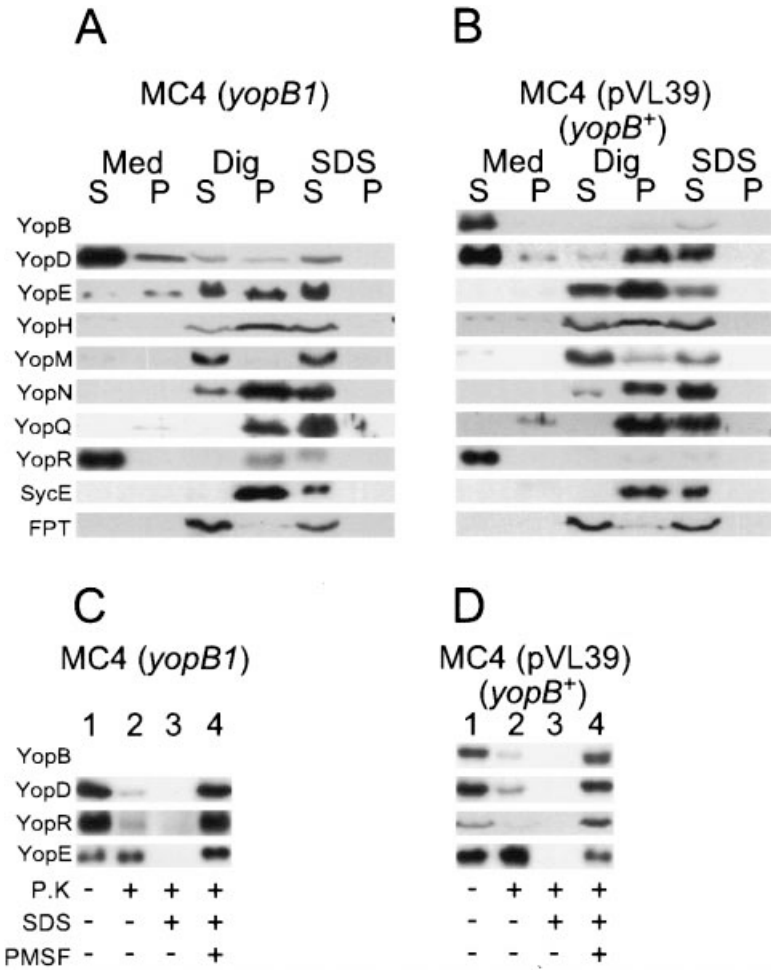

Fig. 3. Localization of Yop proteins during HeLa cell infections of *Y. enterocolitica* strains MC4 (*yopB1*) (A) and MC4 (pVL39)(*yopB*$^+$) (B). Infected HeLa cells were fractionated as described in the legend to Fig. 1A and B. Protease sensitivity of secreted, extracellular YopB, YopD and YopR during HeLa cell infections of *Y. enterocolitica* strains MC4 (*yopB1*) (C) and MC4 (pVL39)(*yopB*$^+$) (D). Infected tissue cultures were left untreated (1) or incubated with either (extracellular) proteinase K (P.K) (2), SDS and proteinase K (3) or SDS, proteinase K and PMSF inhibitor (4). After incubation, proteolysis in all samples was quenched by the addition of PMSF. Proteins were precipitated with methanol/chloroform, solubilized in sample buffer and separated on SDS–PAGE before immunoblotting with specific antisera.

Table 1. Targeting of YopE and YopH during the infection of HeLa cells with *Y. enterocolitica W22703* (wild-type) and isogenic mutant strains

Y. enterocolitica strain	Per cent soluble after digitonin extraction of infected HeLa cells			
	YopE	YopH	FPT	SycE
W22703 (wild-type)	45 (±7)	82 (±26)	88 (±18)	2 (±2)
MC4 (*yopB1*)	59 (±34)	47 (±23)	88 (±17)	0 (±1)
MC4, pVL39 (*yopB*$^+$)	41 (±5)	66 (±27)	97 (±1)	1 (±1)
MC1 (*yopR1*)	16 (±5)	65 (±13)	96 (±1)	0[a]
MC1, pVL5 (*yopR*$^+$)	37 (±1)	81 (±4)	97[a]	0[a]
VTL2 (*yopD1*)	5 (±4)	0 (±0)	100 (±0)	0 (±0)
VTL2, pVL40 (*yopD*$^+$)	32 (±10)	15 (±5)	97 (±3)	0[a]
VTL2, pVL41 (*yopD*$^+$)	43 (±6)	36[a]	100[a]	0[a]
VTL2, pDA255 (*gst-yopD*)	31 (±5)	24[a]	84[a]	0[a]

HeLa cells were infected with *Y. enterocolitica* strains as indicated, then fractionated and finally extracted with digitonin. Targeting of YopE and YopH into the cytosol of HeLa cells was measured as the percentage amount of polypeptide that is solubilized by extraction with digitonin, a detergent that disrupts the eukaryotic plasma membrane but not the bacterial envelope. Farnesyl-protein transferase (FPT) is located in the cytosol of HeLa cells and served as a control for the solubilization of the plasma membrane. SycE is located in the *Yersinia* cytoplasm and was used as a control for bacterial integrity during this procedure. Unless indicated otherwise, data were obtained from 3–5 independent experiments. The average percentage amount of polypeptide in the supernatant of digitonin extracts compared with the total amount of polypeptide in all fractionation samples was calculated. Standard deviations were determined and are indicated in parenthesis (±).

a. These data were collected from a single experiment.

(*yopE1*). Thus, although the *yopB1* mutation abolished all YopB synthesis, it did not interfere with either type III secretion or type III targeting of strain MC4.

The *yopR1* mutation abrogated all YopR synthesis (Fig. 2). During the infection of HeLa cells, *Y. enterocolitica* strain MC1 (*yopR1*) secreted YopB and YopD into the extracellular milieu, whereas YopE, YopH, YopM and YopN were solubilized by digitonin extraction (Fig. 4). Compared with wild-type yersiniae, strain MC1 injected somewhat less YopE into HeLa cells (Table 1). Expression of *yopR* from the *yopE* promoter (pVL5) restored YopR synthesis of strain MC1 and raised the amount of YopE targeting to levels measured for wild-type yersiniae (Table 1). These observations suggest that the *yopR1* mutation does not abolish type III targeting or secretion by strain MC1.

Secreted YopD is not required for type III targeting

Yersinia enterocolitica VTL2 carrying the *yopD1* mutation displayed a unique phenotype. Small amounts of YopE and YopM were located in the extracellular milieu but not in the digitonin extract of HeLa cells (Fig. 6 and Table 1). YopQ, which normally remains associated with bacterial

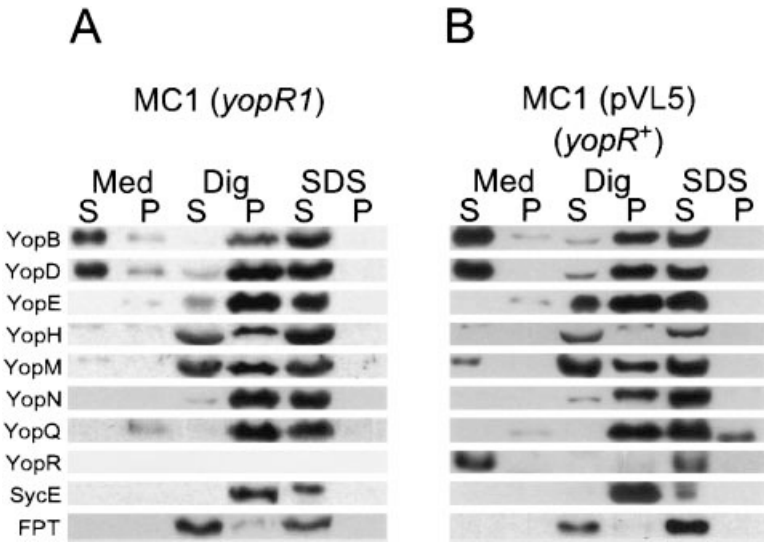

Fig. 4. Localization of Yop proteins during HeLa cell infections of *Y. enterocolitica* strains MC1 (*yopR1*) (A) and MC1 (pVL5)(*yopR*$^+$) (B). Infected HeLa cells were fractionated as described in the legend to Fig. 1A and B.

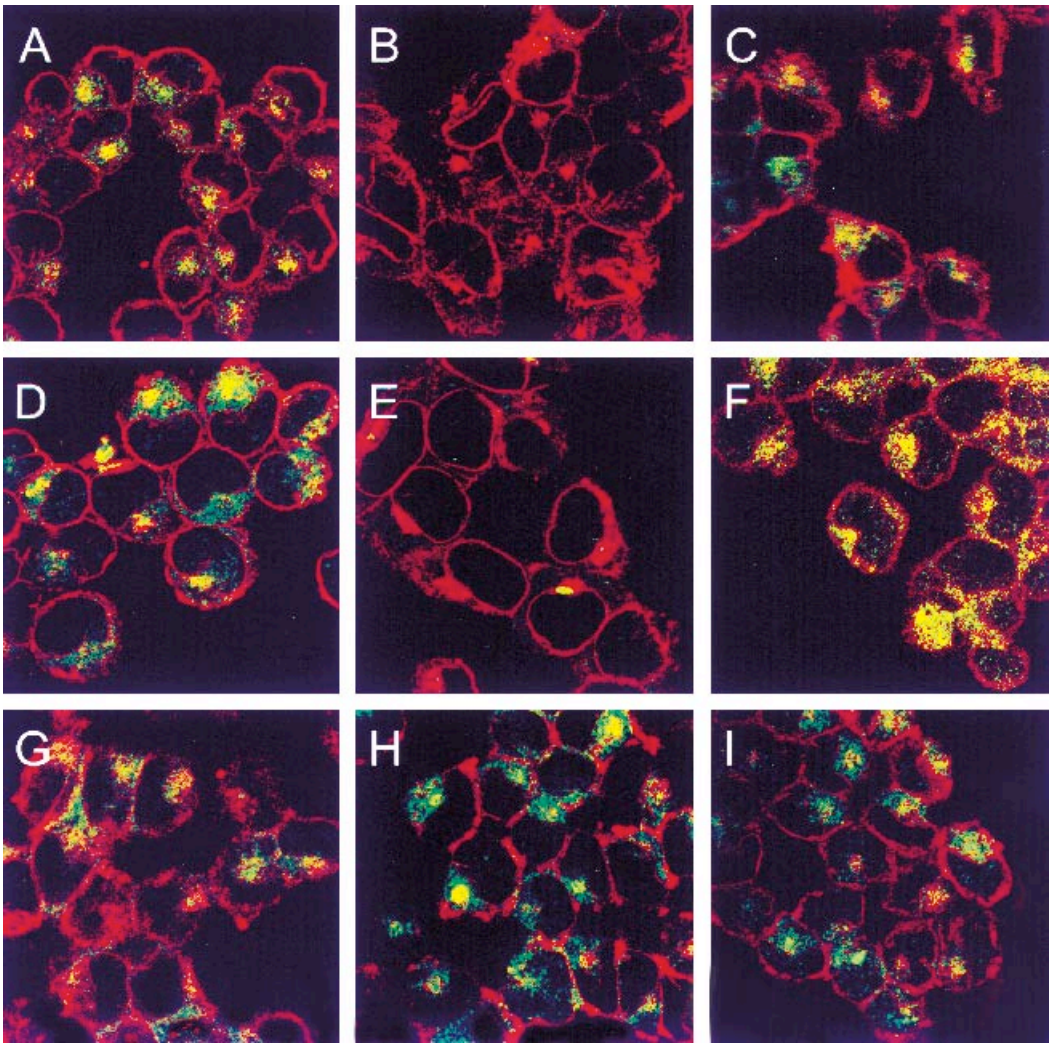

Fig. 5. Immunofluorescent detection of YopE during HeLa cell infections of *Y. enterocolitica* W22703 (A) or its isogenic mutant strains LC1 (*yopE1*) (B), MC4 (*yopB1*) (C), MC4 (pVL39, *yopB*$^+$) (D), VTL2 (*yopD1*) (E), VTL2 (pVL41, *yopD*$^+$) (F), VTL2 (pDA255, *gst-yopD*) (G), MC1 (*yopR1*) (H), and MC1 (pVL5, *yopR*$^+$) (I). Infected HeLa cells were fixed, membranes permeabilized with Triton X-100 and incubated with Texas red-conjugated wheat germ agglutinin. YopE was detected with rabbit antiserum and Oregon green-conjugated secondary antibody. Samples were viewed under a confocal laser microscope. Strong intrabacterial YopE staining was observed regularly for strain VTL2 (*yopD1*); because of the proximity to the stained eukaryotic membrane these mutants appear yellow (E).

cells, was also found in the culture medium, as were YopB and YopR. Strikingly, YopN, a regulatory molecule required for efficient targeting, as well as YopH remained associated with the *yopD1* cells and were neither secreted nor targeted. These results confirmed previous observations that the *yopD* mutants are unable to promote targeting of effector Yops (Rosqvist *et al*., 1994). When cloned under control of the *lcrG* promoter (pVL40), *yopD* was expressed at a very low level and this plasmid did not fully complement the targeting defect of strain VTL2 (Table 1). *yopD1* mutant cells expressing plasmid-encoded, wild-type *yopD* from the inducible *tac* promoter (pVL41) injected YopE, YopH, YopM and YopN into the cytosol of HeLa cells (Figs 5 and 6 and Table 1). However, overexpression of *yopD* from the *tac* promoter also caused a marked decrease in the synthesis and secretion of YopB, whereas the relative amount of polypeptide in the digitonin supernatant appeared to increase (Fig. 6B). We asked whether YopB and YopD were accessible to extracellular proteinase K in a protease protection experiment during HeLa cell infection with strain VTL2 (pVL41). YopB (94%), YopD (99%) and YopR (94%) were degraded by extracellular proteinase K, whereas YopE, YopH, YopM and YopN were protected (Fig. 6D). Thus, although the overexpression of the *yopD* gene reduced the total amount of YopB, this strain secreted Yops (YopB, YopD and YopR) into the extracellular milieu as observed for wild-type *Yersinia*. We think it is likely that YopB/YopD are in part bound to the surface

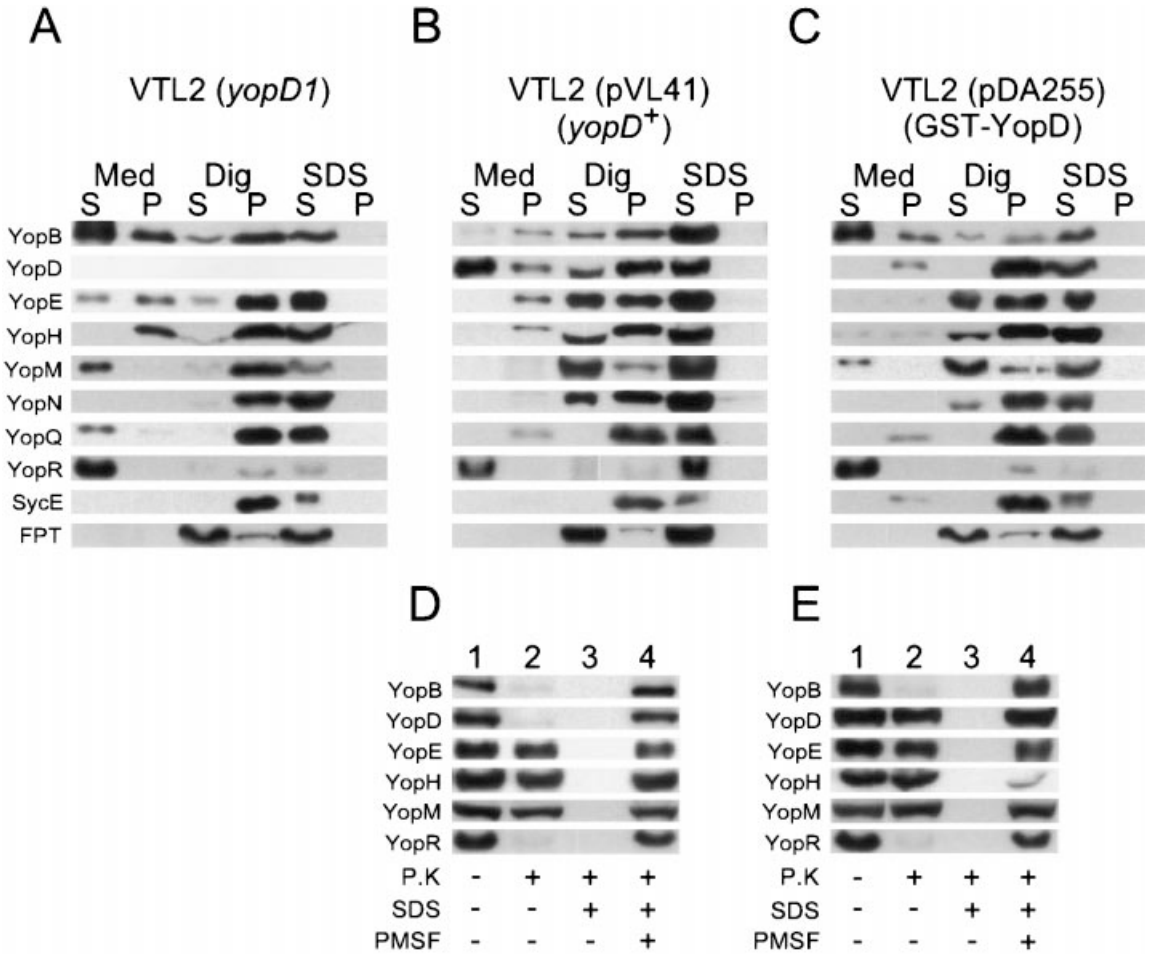

Fig. 6. Localization of Yop proteins during HeLa cell infections of *Y. enterocolitica* strains VTL2 (*yopD1*) (A), VTL2 (pVL40)(*yopD*$^+$) (B) and VTL2 (pDA255)(*gst-yopD*)(C). Infected HeLa cells were fractionated as described in the legend to Fig. 1A and B. Plasmid pVL40 encodes the wild-type *yopD* gene, whereas pDA255 specified for a translational fusion between the structural gene of glutathione-S-transferase (*gst*) and *yopD*. Protease sensitivity of secreted, extracellular YopB and YopR and protection of intracytoplasmic YopE, YopH, YopM and YopN during HeLa cell infections of *Y. enterocolitica* strains VTL2 (pVL40)(*yopD*$^+$) (D) and VTL2 (pDA255)(*gst–yopD*) (E). The Gst–YopD fusion protein was not secreted but remained intrabacterial and is thus protected from extracellular proteinase K. Infected tissue cultures were left untreated (1) or incubated with either (extracellular) proteinase K (P.K) (2), SDS and proteinase K (3) or SDS, proteinase K and PMSF inhibitor (4). After incubation, proteolysis in all samples was quenched by the addition of PMSF. Proteins were precipitated with methanol/chloroform, solubilized in sample buffer and separated on SDS–PAGE before immunoblotting with specific antisera.

of HeLa cells and solubilized by extraction with digitonin. Together these data reveal that plasmid pVL41 (*tac–yopD*) complemented the defect of strain VTL2 (*yopD1*) in targeting YopE, YopH, YopM and YopN.

We sought to determine whether extracellular YopD is required for type III targeting and fused the N-terminal end of YopD to the C-terminus of glutathione-S-transferase (Gst–YopD). Hybrid proteins in which the reporter domain is fused to the C-terminus of Yops generally can be exported by the type III pathway, whereas hybrids to the N-terminus cannot (Anderson and Schneewind, 1997). As expected, Gst–YopD remained in the bacterial cytoplasm and was not secreted during *Yersinia* infections of HeLa cells (Fig. 6C). Expression of Gst–YopD from the *tac* promoter (pDA255) complemented the *yopD1* mutation of strain VTL2 and restored targeting of YopE, YopM and YopN into the cytosol of HeLa cells (Figs 5 and 6). During a protease protection experiment, YopB (95%) and YopR (94%) were degraded by extracellular proteinase K, whereas Gst–YopD, YopE, YopH, YopM and YopN were not (Fig. 6E). Thus, it appears that intrabacterial YopD is required for targeting, whereas the secreted, extracellular species is dispensable for the injection of Yops into the eukaryotic cytosol.

Yop secretion into the extracellular milieu is essential for pathogenicity

To determine whether secreted YopB, YopD or YopR play a role in *Yersinia* pathogenicity during animal infection, we

Table 2. Pathogenicity of *Y. enterocolitica W22703* (wild-type) and isogenic mutant strains in a mouse model system.

Y. enterocolitica strains	Bacteria with plasmid (%)	LD_{50}
W22703 (wild-type)	–	1.4×10^3
MC4 (yopB1)	–	1.9×10^7
MC4 (yopB1), pVL39	35	1.9×10^4
VTL2 (yopD1)	–	$> 6.5 \times 10^7$
VTL2 (yopD1), pVL40	100	1.2×10^7
VTL2 (yopD1), pVL41	25	4.1×10^7
VTL2 (yopD1), pDA255	11	8.0×10^6
MC1 (yopR1)	–	4.8×10^4
MC1 (yopR1), pVL5	75	8.8×10^3
KUM1 (lcrD1)	–	1.8×10^8

Mice were injected intraperitoneally first with iron dextran and 24 h later with various numbers of yersiniae. The course of infection was monitored over a 14 day period. Plasmid stability was measured by counting bacteria in the spleen of infected animals (after their death) by dilution and colony formation on selective media. LD_{50} reports the number of colony-forming units of a bacterial strain required to cause a fatal infection in half of all experimental animals.

measured the virulence of the mutant strains as a lethal dose of infection in a mouse model system (Robins-Browne and Prpic, 1985; Hartland *et al*., 1994; 1996) (Table 2). The *lcrD1* mutant strain KUM1 is defective for all type III export and displayed a greater than 10^5-fold reduction in pathogenicity compared with the wild-type strain W22703. Although yersiniae carrying the *yopB1* allele promote type III targeting, strain MC4 was 10^4 times less virulent than the wild type. When complemented with the wild-type *yopB* gene, virulence was restored almost to same level as *Y. enterocolitica* W22703. The *yopD1* mutation reduced virulence by 5×10^4. Plasmid-encoded, wild-type *yopD* expressed from either the *lcrG* or the IPTG inducible *tac* promoter failed to restore pathogenicity to wild-type levels. We do not know the reason why plasmid pVL41 restores type III targeting yet cannot complement the defect in pathogenicity of strain VTL2. Perhaps the reduced synthesis and secretion of YopB by VTL2 (pVL41) may account for the reduction in virulence. VTL2 cells expressing the non-secretable Gst–YopD displayed a 5×10^3 reduction in virulence compared with wild-type *Yersinia*, suggesting that secreted YopD is also necessary for pathogenicity. The *yopR1* mutation reduced virulence 33-fold and was complemented by a plasmid-encoded wild-type *yopR* allele. Thus, in addition to the targeting of effector Yops, yersiniae require the secretion of YopB, YopD and YopR into the extracellular milieu for pathogenicity in mice.

Discussion

The type III injection of effector Yops has been thought to occur by a mechanism of discontinuous protein translocation (Cornelis and Wolf-Watz, 1997; Cornelis, 1998). Type III machines have been proposed to transport effector Yops across the bacterial envelope and a YopB/YopD complex in the plasma membrane might then import these proteins into the macrophage cytosol (Boland *et al*., 1996; Hakansson *et al*., 1996b; Francis and Wolf-Watz, 1998; Frithz-Lindsten *et al*., 1998). We show here that secreted YopB/YopD are not essential for the targeting of effector Yops. Furthermore, data presented here and elsewhere failed to detect extracellular translocation intermediates of effector Yops (Lee *et al*., 1998). Thus, it appears that effector Yops may be injected by a different mechanism, during which the type III machinery transports polypeptides continuously across both the bacterial envelope and the macrophage plasma membrane. The elements required for the injection of effector Yops are still elusive, but this function is probably provided by some of the genes encoded on the *Yersinia* virulence plasmid.

Our results are in disagreement with several other reports that YopB is essential for the injection of effector Yops (Rosqvist *et al*., 1994; Sory and Cornelis, 1994; Boland *et al*., 1996; Hakansson *et al*., 1996a,b; Frithz-Lindsten *et al*., 1998). The *yopB* gene is positioned immediately upstream of *yopD* in the *lcrGVHyopBD* operon. *yopB* mutants have previously been reported for both *Y. pseudotuberculosis* and *Y. enterocolitica* (Boland *et al*., 1996; Hakansson *et al*., 1996a). However, these studies did not demonstrate that the targeting defect of *yopB* mutants could be complemented by plasmid-encoded wild-type *yopB*. Hence, one likely explanation for the discrepancy between our results and the previous work is that these studies used *yopB* mutations with a polar effect on *yopD*, which is required for type III targeting. Herein the targeting of Yops was measured by immunoblotting of digitonin-fractionated HeLa cells. This technique yields reliable measurements of as little as 5–10% targeting (i.e. 5–10% of all Yop protein in the digitonin-soluble fraction of extracted HeLa cells, Table 1) and is more sensitive than either immunofluorescence microscopy or measurements of Cya reporter protein activity. We think that the phenotype of at least some of the known *ysc* and *yop* mutants should be reconsidered with this assay.

Our results demonstrate for the first time that the type III machinery has not only evolved to inject toxic proteins into eukaryotic cells but also to deliver virulence factors into the extracellular milieu. Although it has been established that type III targeting of yersiniae is essential for bacterial escape from phagocytic killing, the precise functions of type III secretion remain to be elucidated. Previous work revealed that YopB inhibits synthesis of tumour necrosis factor (TNF)-α by murine macrophages and is required for bacterial colonization of intestinal lymphoid tissues of mice (Beuscher *et al*., 1995). When mice were immunized with YopB before infection, murine TNF-α production was

not affected and the Peyer's patches of immune mice were less colonized by *Y. enterocolitica*, indicating that YopB acts as a diffusible substance that can be inactivated by a specific antibody. These data are consistent with our hypothesis that yersiniae may secrete YopB, YopD and YopR to modulate the host's immune response at a distance from the site of infection.

Experimental procedures

Bacterial strains and plasmids

Yersinia enterocolitica strains 8081 (Portnoy *et al*., 1981), W22703 (Cornelis and Colson, 1975) as well as *Y. pseudotuberculosis* YPIII (Forsberg and Wolf-Watz, 1990) have been described previously. The *yopB1*, *yopD1* and *yopR1* mutant alleles have a stop codon followed by a single nucleotide and *Bam*HI site inserted after codon 8 of the respective open reading frame. Mutations were introduced by allelic replacement according to a standard protocol (Cheng *et al*., 1997). Mutant strains were verified by PCR amplification of the mutant alleles, restriction digestion and sequencing of the PCR products. Complementing plasmids were constructed by amplifying DNA sequences from pYV227 virulence plasmid with the primers YopB–Nde (5′-AACATATGAGTGCGTTGATAACCCAT-3′) and YopB–Bam (5′-AAGGATCCAACAGTATGGGGTCTGCCG-3′), YopD–Nde (5′-AACATATGACAATAAATATCAAGACAGA-3′) and YopD–Bam (5′-AAGGATCCGACAACACCAAAAGCGGCTT-3′), as well as YopR–Nde (5′-AACATATGACGGTTACCCTTAATAGA-3′) and YopR–Bam (5′-AAGGATCCTGTCTCCATATCAATTTGATGG-3′). After *Nde*I–*Bam*HI digestion, the fragments were cloned into similarly cut low-copy-number plasmids under the control of either the *lcrG* or the IPTG-inducible *tac* promoter (Amann *et al*., 1983). For the N-terminal GST fusion to YopD, we replaced the *Nde*I–*Bam*HI fragment of YopD with two DNA fragments joined at a *Kpn*I site (YopD–Kpn 5′-AAGGTACCACAATAAATATCAAGACAGA-3′). Gst–YopD contains the entire YopD polypeptide fused to the C-terminus of Gst. GST was PCR amplified from pGEX-T7 with the primers (GST1 5′-AACATATGTCCCCTATACTAGGTTATTGGA-3′ and GST2 5′-AAGGTACCAACAGATGCACGACGAGATC-3′) (Kaelin *et al*., 1992). Constructs were confirmed by DNA sequencing.

Digitonin fractionation assay

Overnight cultures of *Yersinia* were diluted 1:20 into fresh Luria broth and grown for 2 h at 26°C with shaking. Bacteria were sedimented at 8000 x *g* for 10 min and suspended in phosphate-buffered saline (PBS). HeLa cells were grown to 80% confluency in 75 cm^2 tissue culture flasks with Dulbecco's modified Eagle medium (DMEM) and 10% fetal bovine serum. Before infection, the cells were washed twice with PBS, covered with 10 ml of DMEM and warmed to 37°C for 30 min. Aliquots of HeLa cells were counted; each flask was infected with yersiniae at a multiplicity of infection (MOI) of 10 and incubated for 3 h at 37°C with 5% CO_2. Bacterial protein synthesis was stopped by the addition of 30 μg of chloramphenicol or 100 μg of kanamycin. Culture medium was removed and centrifuged at 32 500 × *g* for 15 min to separate soluble proteins from non-adherent bacteria in the sediment. HeLa cells as well as adherent bacteria were scraped off the flasks into 10 ml of 1% digitonin in PBS and vortexed for 15 s every 5 min for 20 min. As a control for Yop solubility, a duplicate plate was treated with 10 ml of 1% SDS in PBS. Samples were centrifuged at 32 500 × *g* for 15 min. A 7 ml aliquot of supernatant was withdrawn and precipitated with methanol/chloroform while the remaining supernatant was discarded. The sediment was suspended in 10 ml of 1% SDS in PBS, and a 7 ml aliquot was precipitated with methanol/chloroform. Protein precipitates were solubilized in sample buffer, separated on SDS–PAGE and analysed by immunoblotting with rabbit or mouse antiserum. Immunoreactive species were quantified as chemiluminescent signals on X-ray film with laser densitometry scanning.

Proteinase K protection assay

Infected HeLa cells were treated with 30 μg of chloramphenicol or 100 μg of kanamycin to stop all bacterial protein synthesis. Four simultaneously infected flasks were treated with either (i) a mock digest, or (ii) 100 μg of proteinase K, or (iii) 1% SDS and 100 μg of proteinase K, or (iv) 1% SDS, 1 mM PMSF, and 100 μg of proteinase K and incubated at room temperature for 20 min. All proteinase K was inactivated with the addition of PMSF to 1 mM, and cells were lysed with 1% SDS. Proteins were precipitated with methanol/chloroform, washed with methanol, suspended in sample buffer containing PMSF, and analysed by SDS–PAGE and immunoblotting.

Immunofluorescence

HeLa cells were grown on 12 mm glass coverslips to ≈ 8×10^5 cells, washed twice with PBS, covered with 1 ml of DMEM, and warmed to 37°C in a tissue culture incubator. HeLa cells were infected with yersiniae at a MOI of 10 and incubated for 3 h at 37°C and 5% CO_2. Cells were washed with PBS, fixed with 3.7% formaldehyde for 20 min and washed again with PBS. Excess formaldehyde was quenched with 100 mM glycine in PBS for 5 min. Cells were permeabilized with 1% Triton X-100 in PBS for 30 min, blocked with 5% non-fat milk, 0.05% Tween-20 in PBS for 15 min. Cells were probed with a 1:100 dilution of YopE antiserum in PBS–Tween–milk for 20 min. Unbound antibodies were removed by four washes with PBS–Tween. Cells were stained with anti-rabbit IgG-Oregon 488 conjugate and wheatgerm agglutinin–Texas red conjugate for 20 min (both diluted 1:500 in PBS–Tween–milk). Unbound fluorescent probes were removed by four washes with PBS–Tween. Coverslips were dried for 1 h and mounted onto glass slides with 50% glycerol solution. Samples were viewed under Leica confocal laser fluorescence microscope.

Mouse infections

One day before infection, BALB/c adult female mice were injected intraperitoneally (i.p.) with 0.1 ml of a solution containing 50% (v/v) iron dextran (100 mg ml^{-1} iron) and 50 mg ml^{-1} desferrioxamine B mesylate (Robins-Browne and Prpic,

1985). If infected with yersiniae-expressing genes under the *tac* promoter, mice were fed 10 mM IPTG water, beginning 24 h before infection (Wu *et al*., 1997). Mice were injected i.p. with 0.1 ml of a bacterial dilution and observed for 2 weeks. The lethal dose 50 (LD_{50}) was calculated from a series of 10-fold dilutions (10^3–10^8 colony-forming units) by administering each dilution to five mice (Reed and Muench, 1938).

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