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Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae

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Virulent bacteria of the genera *Yersinia*, *Shigella* and *Salmonella* secrete a number of virulence determinants, Yops, Ipas and Sips respectively, by a type III secretion pathway. The IpaB protein of *Shigella flexneri* was expressed in *Yersinia pseudotuberculosis* and found to be secreted under the same conditions required for Yop secretion. Likewise, YopE was secreted by the wild-type strain LT2 of *Salmonella typhimurium*, but YopE was not secreted by the isogenic *invA* mutant. Secretion of both IpaB and YopE required their respective chaperones, IpgC and YerA. In addition, *yopE*-containing *S.typhimurium* expressed a YopE-mediated cytotoxicity on cultured HeLa cells. YopE was detected in the cytosol of the infected HeLa cells and the amount of translocated YopE correlated with the degree of cytotoxicity. Both translocation and cytotoxicity were prevented by the addition of gentamicin. Treatment of HeLa cells with cytochalasin D prior to infection prevented internalization of bacteria, but translocation of YopE was still observed. These results favour the hypothesis that YopE is translocated through the plasma membrane by surface-located bacteria. We propose that virulent *Salmonella* and *Shigella* deliver virulence effector molecules into the target cell through the utilization of a functionally conserved secretion/translocation machinery similar to that shown for *Yersinia*.

Keywords: Ipa/*Salmonella*/*Shigella*/*Yersinia*/Yop

Introduction

Several Gram-negative animal and plant pathogens have evolved similar molecular strategies to present anti-host factors during the infectious process (Van Gijsegem *et al.*, 1993, 1995; Bergman *et al.*, 1994; Forsberg *et al.*, 1994). Pathogenic *Yersinia* express a class of secreted proteins, Yops (*Yersinia* outer proteins), known to be important in the virulence process. These proteins are encoded by a 70 kb plasmid common to all human pathogenic *Yersinia*. In addition, the plasmid encodes proteins involved in both regulation and secretion of the Yop proteins. Maximal expression and subsequent secretion of Yops *in vitro* occur at 37°C in a medium devoid of Ca^{2+} (Cornelis 1992; Straley *et al.*, 1993a,b; Forsberg *et al.*, 1994). Virulent *Shigella flexneri* strains contain a 220 kb virulence plasmid that encodes a set of secreted immunodominant proteins denoted Ipas (invasion protein antigens) (Sansonetti *et al.*, 1982; Hale *et al.*, 1985; Oaks *et al.*, 1986). Analysis of non-polar mutants in the genes encoding IpaB, IpaC and IpaD identified these proteins as essential for entry of *S.flexneri* into epithelial cells (Ménard *et al.*, 1993). These antigens are exported by a virulence plasmid-encoded secretion system involving the *mxi* and *spa* gene clusters (Andrews *et al.*, 1991; Allaoui *et al.*, 1992, 1993; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). Interestingly, *Salmonella typhimurium* was recently shown to encode two secreted proteins, SipB and SipC, which showed significant homology with IpaB and IpaC respectively (Kaniga *et al.*, 1995). By analogy with IpaB and IpaC of *S.flexneri*, SipB and SipC are essential for the invasion of *S.typhimurium* into cultured epithelial cells (Kaniga *et al.*, 1995). A third secreted protein, InvJ, has also been shown to be involved in the invasion process of *S.typhimurium* (Collazo *et al.*, 1995). Secretion of these proteins involves the *inv* and *spa* gene clusters, which show extensive homologies with the *mxi* and *spa* gene clusters of *S.flexneri* (Gálan and Curtiss, 1989; Gálan *et al.*, 1992; Groisman and Ochman, 1993; Eichelberg *et al.*, 1994; Kaniga *et al.*, 1994).

The export systems mediating secretion of Yops, Ipas, Sips and InvJ have been classified as type III secretion systems (Salmond and Reeves, 1993). Proteins secreted by this pathway lack typical signal sequences, are not processed during transfer and do not have a periplasmic intermediate. It has been shown that Yops carry an undefined secretion signal in the N-terminal part of the protein (Michiels and Cornelis, 1991) and it is likely that the other secreted proteins also have a specific signature which is required for secretion.

YopE and YopH are both dependent on specific cytoplasmic chaperones, YerA (SycE) and SycH respectively, for secretion and each Yop protein has been suggested to have its own chaperone (Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994; Frithz-Lindsten *et al.*, 1995; Persson *et al.*, in preparation). The detailed function of the Syc chaperones has not yet been determined, but two alternative models have been suggested; either the chaperone acts as a pilot protein and targets the Yop to the secretion organelle or the chaperone binds to the Yop to retain the protein in a secretion-competent form (Wattiau *et al.*, 1993, 1994; Frithz-Lindsten *et al.*, 1995). IpgC (IppI) of *S.flexneri* has a similar function in the secretion of IpaB and IpaC as the Syc proteins in Yop secretion (Ménard *et al.*, 1994). IpgC stabilizes IpaB in the bacterial cytoplasm and *ipgC* mutants show lower levels of IpaB in the culture supernatant (Ménard *et al.*, 1994). A protein homologous to IpgC, SicA, has also been identified in *S.typhimurium* and this protein has been postulated to

be involved in secretion of SipB and SipC (Kaniga *et al.*, 1995).

Three different operons, *lcrDR*, *yscA-M* and *yscN-U*, are involved in Yop secretion (Rosqvist *et al.*, 1990; Michiels *et al.*, 1991; Rimpiläinen *et al.*, 1992; Plano and Straley, 1993; Allaoui *et al.*, 1994; Bergman *et al.*, 1994; Fields *et al.*, 1994; Woestyn *et al.*, 1994). LcrD of *Yersinia* is a 75 kDa cytoplasmic membrane protein that shows high homology to MxiA of *S.flexneri* and InvA of *S.typhimurium* (Plano *et al.*, 1991; Andrews and Maurelli, 1992; Gálan *et al.*, 1992). These related proteins play an essential role in surface presentation of the virulence determinants and are likely to serve a similar function in the different species. Some of the gene products of the *yscA-M* operon of *Yersinia* show high homology to proteins involved in secretion of Ipas and Sips (Rosqvist *et al.*, 1990; Michiels *et al.*, 1991; Rimpiläinen *et al.*, 1992; Allaoui *et al.*, 1993; Plano and Straley, 1993; Fields *et al.*, 1994; Kaniga *et al.*, 1994). The *yscN-U* gene cluster encodes proteins which show striking homology with the *inv/spa* gene products of *S.typhimurium* and the Spa proteins of *S.flexneri* (Venkatesan *et al.*, 1992; Groisman and Ochman, 1993; Sasakawa *et al.*, 1993; Allaoui *et al.*, 1994; Bergman *et al.*, 1994; Eichelberg *et al.*, 1994; Fields *et al.*, 1994; Woestyn *et al.*, 1994). The *spa* gene products of *S.flexneri* are involved in secretion of the Ipa proteins (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). The homologous *inv/spa* gene cluster of *S.typhimurium* is required for secretion of the SipB, SipC and InvJ proteins (Collazo *et al.*, 1995; Kaniga *et al.*, 1995).

The secretion system of *Yersinia* is a central component in the virulence character of this pathogen. *Yersinia* secretes and translocates the Yop effector proteins YopE and YopH through the plasma membrane only at the zone of contact between the bacterium and the target cell (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, in preparation). Although this is conducted by cell surface-bound bacteria, neither YopE nor YopH is secreted into the cell culture medium during the process, which has been defined as polarized translocation (Rosqvist *et al.*, 1994; Persson *et al.*, in preparation). Both YopE and YopH induce a cytotoxic response after infection of HeLa cells with a virulent strain of *Yersinia pseudotuberculosis* (Rosqvist *et al.*, 1990). The molecular target of YopE is unknown, but the activity of YopE leads to disruption of actin microfilaments and a characteristic rounding up of the cells (Rosqvist *et al.*, 1990). To exert its activity YopE must be introduced into the cytosol of the target cell and extracellular addition of YopE to a cell culture has no effect (Rosqvist *et al.*, 1991).

In this study we have addressed the question whether *S.typhimurium* and *Y.pseudotuberculosis* allow secretion and translocation of heterologous proteins via the conserved type III secretion system. We found that YopE is secreted by a secretion-competent strain of *S.typhimurium* and that *Y.pseudotuberculosis* secretes IpaB. In both cases the secretion is dependent on the specific chaperones YerA and IpgC respectively. Moreover, we also present results showing that *S.typhimurium* has the ability to translocate the YopE cytotoxin into HeLa cells from cell surface-bound bacteria via a process similar to that of *Y.pseudotuberculosis*.

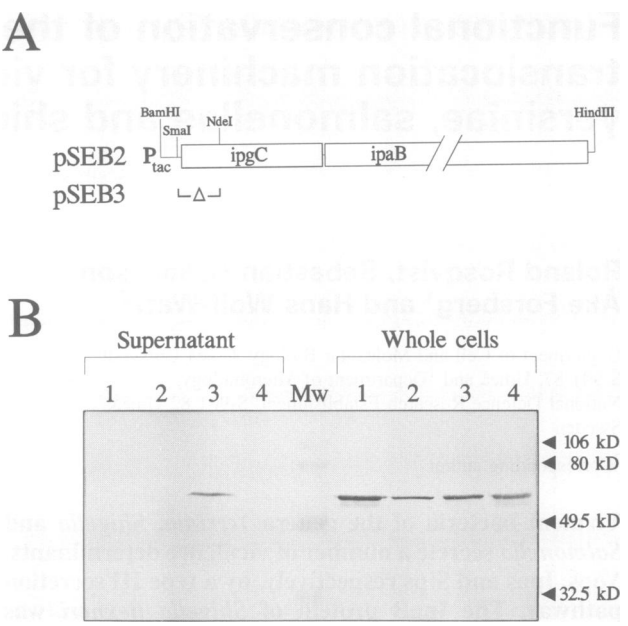

Fig. 1. (**A**) Schematic map of the *ipgC* and the *ipaB* genes of plasmids pSEB2 and pSEB3. P_{tac} represents the IPTG-inducible *tac* promoter of pMMB66EH. The Δ indicates the deletion made to generate pSEB3. The restriction enzyme sites used are indicated. (**B**) Western immunoblotting analysis of IpaB expression in the wild-type strain YPIII(pIB102) carrying pSEB2 or pSEB3 and the *yscT* mutant YPIII(pIB61) strain carrying pSEB2. The bacteria were grown at 37°C for 3 h in Ca^{2+}-containing (+) or Ca^{2+}-depleted (–) medium with 1 mM IPTG. Samples obtained from the same number of bacteria were subjected to immunoblotting analysis with an anti-Ipa rabbit antiserum. Lanes 1, YPIII(pIB61, pSEB2) grown at 37°C (–); lanes 2, YPIII(pIB102, pSEB3) grown at 37°C (–); lanes 3, YPIII(pIB102, pSEB2) grown at 37°C (–); lanes 4, YPIII(pIB102, pSEB2) grown at 37°C (+).

Results

Ysc-dependent secretion of IpaB in Y.pseudotuberculosis

To investigate whether *Y.pseudotuberculosis* was capable of secreting IpaB of *S.flexneri,* we cloned a PCR-derived DNA fragment encompassing *ipaB* and the upstream *ipgC* gene. The latter gene was included since it has recently been shown that IpgC functions as an IpaB-specific chaperone and is required for efficient secretion of IpaB (Ménard *et al.*, 1994). The genes were placed under the control of the IPTG-inducible *tac* promoter of plasmid pMMB66EH, generating pSEB2 (Figure 1A). This plasmid was introduced into the wild-type strain YPIII(pIB102) and into the Yop secretion-deficient *yscT* mutant YPIII(pIB61) (Bergman *et al.*, 1994). These strains were incubated under different conditions and the expression and secretion of IpaB and the Yops were examined by Western blot analysis (Figure 1B). In both strains IpaB was expressed only after the addition of IPTG (data not shown). However, IpaB was found in the culture supernatant only when expression was induced in strain YPIII(pIB102, pSEB2) at 37°C in the absence of Ca^{2+} (Figure 1B) (i.e. under conditions permitting Yop secretion), indicating that IpaB was secreted via the Ysc-mediated pathway. This was confirmed when the secretion-deficient *yscT* mutant strain YPIII(pIB61, pSEB2) was examined (Figure 1B). No IpaB secretion was observed, although the strain expressed IpaB to the same extent as the wild-type strain (Figure 1B).

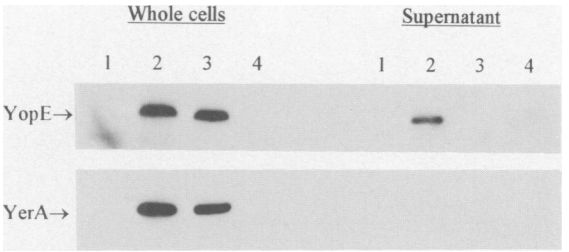

Fig. 2. Western blotting analysis of whole cell fractions and culture supernatants of *S.typhimurium* strains harbouring recombinant plasmids encoding *yerA* and *yopE* of *Y.pseudotuberculosis* using specific anti-YerA and anti-YopE antisera. The different strains were grown as described in Materials and methods. Lanes 1, *S.typhimurium* strain LT2; lanes 2, *S.typhimurium* strain LT2 (pAF19); lanes 3, *S.typhimurium* strain STA1(pAF19); lanes 4, *S.typhimurium* strain LT2(pAF20).

To explore whether IpgC was necessary for secretion of IpaB expressed in *Y.pseudotuberculosis*, part of the *ipgC* gene of pSEB2 was deleted between the restriction endonuclease sites *Sma*I and *Nde*I, generating plasmid pSEB3 (Figure 1A). The expression and secretion of IpaB were examined in the wild-type strain carrying pSEB3. No IpaB protein could be detected in the culture supernatant and the amount of IpaB recovered in the whole cell fraction was lower compared with the IpgC-expressing strain [YPIII(pIB102, pSEB2)] (Figure 1B). This was probably an effect of increased sensitivity of IpaB to proteolytic degradation in the absence of its chaperone, as has been shown to be the case in *S.flexneri* (Ménard *et al.*, 1994).

IpaB secretion did not affect Yop secretion and in all experiments described above the pattern of Yop expression and secretion was not affected (data not shown). To exclude the possibility that IpaB was released by lysis of the bacteria, the localization of the cytosolic YerA protein (Frithz-Lindsten *et al.*, 1995) was examined by Western blot analysis with monospecific anti-YerA antibodies. No YerA could be found in any of the culture supernatants (data not shown). Thus IpaB is secreted in a IpgC-dependent manner by the same machinery designed for Yop secretion in *Y.pseudotuberculosis*.

YopE is secreted by S.typhimurium via the Inv pathway

The *inv/spa* locus of *S.typhimurium* is essential for invasion of cultured epithelial cells by the pathogen and it was recently shown that the *inv* genes encode a type III secretion machinery (Collazo *et al.*, 1995; Kaniga *et al.*, 1995). To investigate whether the *inv/spa* locus of *S.typhimurium* allowed secretion of heterologous proteins, the *yopE* gene was expressed in the wild-type strain LT2 from plasmid pAF19. This plasmid also encodes the YerA chaperone, which is essential for YopE secretion in *Y.pseudotuberculosis* (Frithz-Lindsten *et al.*, 1995). Both genes were transcribed from their native promoters. YopE expression and secretion were analysed by Western blotting using a monospecific YopE antiserum. YopE was detected in both the whole cell fraction and in the culture supernatant (Figure 2). The appearance of YopE in the culture medium was not a result of lysis of the bacteria, since the cytosolic YerA protein was only detected in the whole cell fraction (Figure 2). An essential part of the *yerA* gene was removed, generating plasmid pAF20. No YopE was secreted when this plasmid was expressed in the wild-type strain LT2 (Figure 2). Analogous to the situation in *Y.pseudotuberculosis*, YopE was barely detected in the whole cell fraction when YerA was absent, indicating that YerA also stabilizes YopE in *S.typhimurium* (Figure 2).

To show that YopE secretion in *S.typhimurium* was mediated by the *inv/spa*-encoded secretion system, an *invA* mutation was introduced into strain LT2(pAF19), generating STA1(pAF19). YopE was not detected in the culture supernatant when strain STA1(pAF19) was employed (Figure 2). Thus this result showed that the *inv* gene cluster was involved in secretion of YopE.

YopE-mediated cytotoxicity and translocation by S.typhimurium strain LT2(pAF19)

It has previously been shown that YopE mediates a cytotoxic effect on HeLa cells after infection with a virulent strain of *Y.pseudotuberculosis* (Rosqvist *et al.*, 1990). Therefore, we asked if *S.typhimurium* LT2(pAF19) also elicited a YopE-induced cytotoxic response on cultured HeLa cells. Four hours after onset of infection >90% of the HeLa cells were cytotoxically affected, showing a characteristic YopE response, whereas the *invA* mutant STA1(pAF19) and the YerA-deficient strain LT2(pAF20) did not induce any detectable cytotoxicity (Figure 3). The cytotoxic effect correlated with the presence of YopE in the HeLa cell cytoplasm, as deduced by confocal laser scanning microscopy (Figure 4A). No YopE could be detected when the YopE secretion-incompetent strains STA1(pAF19), LT2(pAF20) and LT2 were analysed (data not shown).

We have recently shown that YopE of *Y.pseudotuberculosis* is translocated by extracellular cell-bound bacteria into the cytosol of the target cell, while intracellularly located bacteria do not contribute to the cytosolic presence of YopE (Rosqvist *et al.*, 1994). In support of these findings, YopE has been shown to counteract the internalization of *Y.pseudotuberculosis* into cultured epithelial cells (Bliska *et al.*, 1993). To investigate whether YopE also affected the internalization of *S.typhimurium* into HeLa cells, we followed the kinetics of invasion of the isogenic strains LT2(pAF19) (*yopE*$^+$, *yerA*$^+$) and LT2(pAF20) (*yopE*$^+$, *yerA*) into cultured HeLa cells (Table I). Similarly to *Yersinia*, YopE expression had an effect on the uptake of *S.typhimurium* and after 4 h infection 48% of the cell-associated YopE-secreting bacteria of strain LT2(pAF19) remained surface located. The YerA-deficient strain was more efficiently taken up, resulting in only 8% of the cell-associated bacteria bound to the surface of the cells (Table I). This result favours the idea that invasion of *S.typhimurium* was reduced due to YopE delivered by extracellular bacteria. However, it could not be excluded that this reduction was caused by intracellular bacteria.

Gentamicin is commonly used to study intracellular bacteria, since the drug only inactivates extracellularly located bacteria (Vaudaux and Waldvogel, 1979). To investigate whether a cytotoxic effect could be mediated by intracellular bacteria, gentamicin was added at different time points after onset of infection of HeLa cells with strain LT2(pAF19) and analysed after 4 h. When gentamicin was

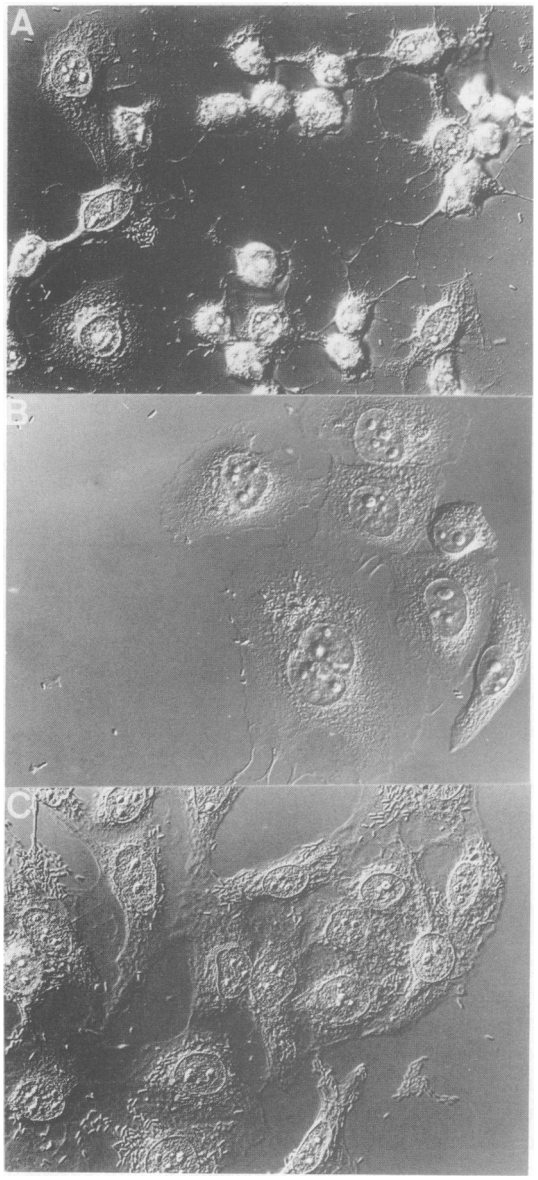

Fig. 3. Differential interference contrast micrographs of infected HeLa cells. HeLa cells were infected with different strains of *S.typhimurium*. The photos were taken after 4 h infection using a ×40/1.0 Nikon oil lens. (**A**) HeLa cells infected with strain LT2(pAF19) (*yopE*$^+$, *yerA*$^+$) showing cytotoxically affected cells. (**B**) HeLa cells infected with strain LT2(pAF20) (*yopE*$^+$, *yerA*). (**C**) HeLa cells infected with strain STA1(pAF19) (*invA*, *yopE*$^+$, *yerA*$^+$).

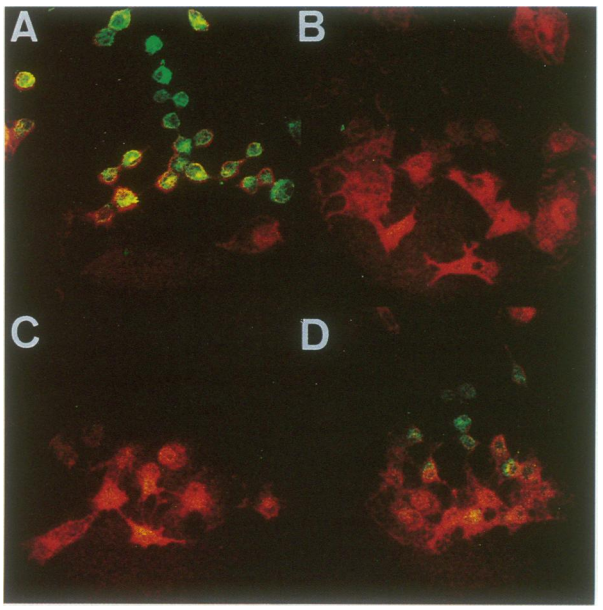

Fig. 4. Confocal micrographs showing translocation of YopE into HeLa cells by surface-located *S.typhimurium*. The *S.typhimurium* LT2 strains were grown overnight at 37°C under oxygen-limited conditions and used to infect a HeLa cell monolayer. In all cases the samples were analysed 4 h after onset of infection and gentamicin (4 μg/ml final concentration) was added at different time points. (**A**) No gentamicin addition. More than 90% of the HeLa cells show a cytotoxic response and YopE (green colour) is found inside the HeLa cell membrane (red colour). (**B**) Gentamicin added after 30 min.. All HeLa cells show normal morphology and no YopE is found inside the HeLa cells. (**C**) Gentamicin added after 1 h. Some HeLa cells are cytotoxically affected by YopE, but only small amounts of YopE are seen inside the cells. (**D**) Gentamicin added after 2 h. A majority of the HeLa cells show a YopE-mediated cytotoxic response and YopE is found inside the HeLa cells. The HeLa cell membrane was visualized with Texas red-conjugated wheat germ agglutinin and YopE by indirect immunofluorescence using an affinity purified rabbit anti-YopE antibody followed by donkey anti-rabbit IgG conjugated to FITC. The fluorescent images were collected simultaneously using a confocal laser scanning microscope equipped with dual detectors and an argon/krypton laser. The images were scanned at 0.42 μm pixel size and a pinhole setting of 100 μm. The images represent one scanned section.

added after 30 min the cytotoxic effect was completely inhibited and no YopE was detected in the cytosolic compartment (Figure 4B). Addition of gentamicin 1 h after onset of infection resulted in a moderate increase in the fraction of cytotoxically affected HeLa cells, while no YopE was detected within the cells (Figure 4C). A cytotoxic effect was observed when the drug was added after 2 h. About 50% of the HeLa cells were rounded up and exhibited a typical YopE-mediated cytotoxicity that also correlated with appearance of the cytotoxin within the HeLa cells, albeit to a lesser extent than for infected HeLa cells not treated with gentamicin (Figure 4D). The number of intracellular bacteria per HeLa cell was determined in each sample after 4 h infection using a double immunofluorescence assay (Table I). The numbers were similar, six to seven intracellular bacteria per HeLa cell in the non-treated culture and cultures treated with gentamicin 1 or 2 h after infection. Addition of gentamicin after 30 min reduced the number of intracellular bacteria to about one to two per cell, suggesting that the process of invasion occurs mainly within the first hour of infection. Although the numbers of intracellular bacteria were the same, gentamicin addition 1 or 2 h after the onset of infection had an inhibitory effect on cytotoxicity, as well as the amount of YopE found within the eukaryotic target cell. Prolonged incubation (up to 16 h) in the presence of gentamicin added after 30 min did not result in intracellular growth or YopE-mediated cytotoxicity (data not shown). These results indicated that the YopE-mediated cytotoxicity is mainly due to extracellular bacteria

Cytochalasin D has been shown to block invasion of *S.typhimurium* into cultured epithelial cells (Kihlström and Nilsson, 1977; Finlay and Falkow, 1988). This drug prevents elongation of actin microfilaments (Flanagan and Lin, 1980), which results in a rounding up of the treated cells. To investigate if YopE is delivered to the target cell cytoplasm by surface-located bacteria, HeLa cells were

Table I. Effects of gentamicin addition on HeLa cells infected with different *S.typhimurium* strains

Plasmid	Time of gentamicin addition after onset of infection (h)	Bacteria per infected HeLa cell	Intracellular bacteria per infected HeLa cell	Percent surface-located bacteria of total cell-associated bacteria	Cytotoxic response on HeLa cells
pAF19 (*yopE*$^+$, *yerA*$^+$)	0.5	5.8	1.3	78	–
pAF19 (*yopE*$^+$, *yerA*$^+$)	1.0	11.0	6.3	43	+
pAF19 (*yopE*$^+$, *yerA*$^+$)	2.0	12.3	7.4	40	++
pAF19 (*yopE*$^+$, *yerA*$^+$)	no addition	12.8	6.6	48	++++
pAF20 (*yopE*$^+$, *yerA*)	no addition	15.0	13.8	8	–

The different *S.typhimurium* LT2 strains were grown overnight at 37°C under oxygen-limited conditions and used to infect a HeLa cell monolayer. At the indicated times gentamicin at a final concentration of 4 μg/ml was added to the infected HeLa cells to inhibit protein synthesis of the surface-located bacteria, whereafter the incubation was continued until 4 h post-infection at 37°C. The specimens were then fixed and analysed for localization of HeLa cell-associated bacteria by the double-fluorescent antibody test described in Materials and methods. + and – refer to the level of cytotoxicity, see also Figure 4. The same results were obtained in two separate experiments.

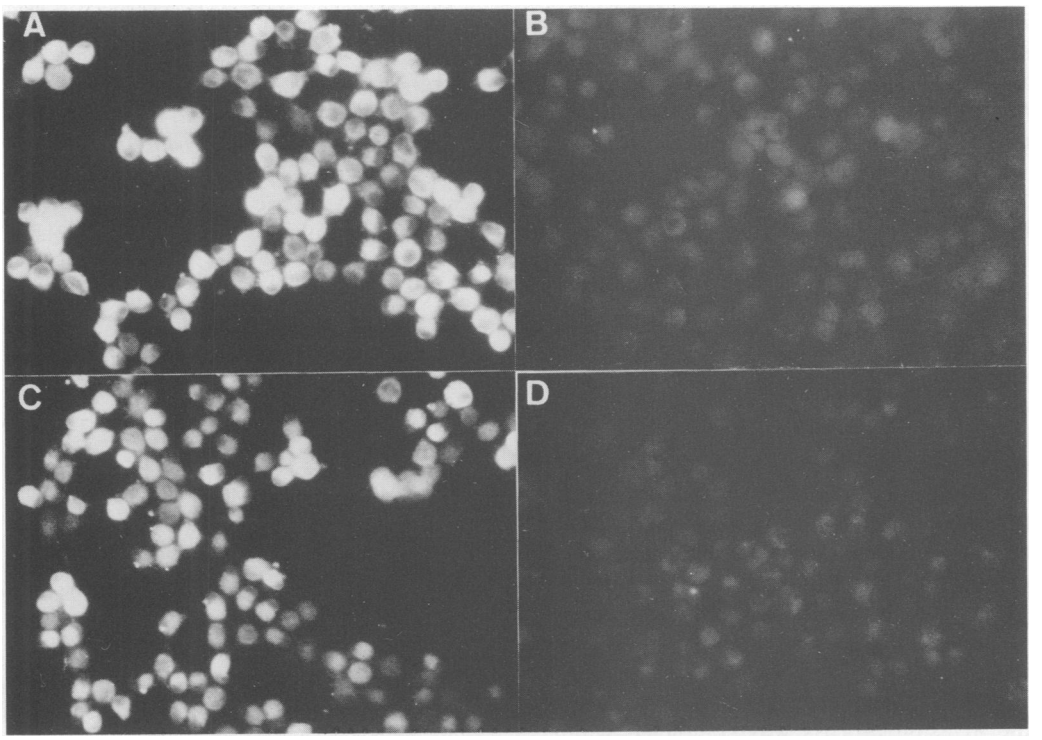

Fig. 5. Translocation of YopE into HeLa cells in the presence or abscence of cytochalasin D. **(A)** LT2(pAF19) (*yopE*$^+$, *yerA*$^+$). No cytochalasin D treatment. About 90% of the HeLa cells showed intracellular YopE staining. **(B)** LT2(pAF20) (*yopE*$^+$, *yerA*). No cytochalasin D treatment, showing background staining. **(C)** LT2(pAF19) (*yopE*$^+$, *yerA*$^+$). Cytochalasin D (0.5 μg/ml final concentration) was added 30 min prior to infection. Approximately the same number of HeLa cells as in (A) displayed intracellular YopE staining. Note that cytochalasin D affected the amount of YopE translocated, although the number of adherent bacteria were the same in the two cases. **(D)** LT2(pAF20) (*yopE*$^+$, *yerA*). Cytochalasin D was added 30 min prior to infection, showing background staining. The translocation was analysed by epifluorescence microscopy using indirect immunofluorescence staining of YopE. The specimens were treated identically and analysed 4 h after onset of infection. Exposure time on Kodak Tri-X 400 ASA was 30 s for (A) and (C) and 45 s for (B) and (D) in order to visualize the cells.

infected with LT2(pAF19) in the presence or absence of 0.5 μg/ml cytochalasin D. Treatment with this drug mediated a 2-fold reduction in the number of cell-associated bacteria. Therefore, the inoculum was increased two times. More than 70% of the HeLa cells infected in the presence of cytochalasin D were found to lack intracellular bacteria. Still, YopE staining of the intracellular compartment could be detected in 85% of the treated cells (Figure 5C). The number of YopE stained HeLa cells was similar to that of cells not treated with cytochalasin D, albeit the intensity of the YopE staining was lower in the treated cells (compare Figure 5A and C). We cannot explain why cytochalasin D reduces the amount of YopE found in the cytoplasm, but this phenomenon is also observed for HeLa cells infected with *Y.pseudotuberculosis*.

The fact that cytochalasin D does not block the appearance of YopE within the target cell cytosol strengthens the hypothesis that surface-associated *S.typhimurium* translocate YopE through the plasma membrane of the target cell via a molecular mechanism similar to that recently described for *Y.pseudotuberculosis* and *Y.enterocolitica* (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994).

Discussion

YopE secretion by *S.typhimurium* strain LT2 required gene products encoded by the *inv* gene cluster. Likewise,

secretion of IpaB of *S.flexneri* by *Yersinia pseudotuberculosis* involved the *ysc*-encoded type III secretion system. It was recently shown that InvA of *S.typhimurium* and MxiA of *S.flexneri* were functionally interchangeable. However, the *Yersinia* homologue LcrD did not complement an *invA* mutant of *Salmonella* (Ginocchio and Gálan, 1995). The fact that YopE was secreted by *S.typhimurium* indicated that although some of the individual components of the secretion systems are not interchangeable, the molecular principles governing secretion are the same. Secretion of IpaB in *Yersinia* and YopE in *Salmonella* both required the respective chaperones IpgC and YerA. By analogy with what has been shown in homologous systems, the presence of the chaperones prevented degradation of the proteins destined for secretion (Ménard *et al.*, 1994; Frithz-Lindsten *et al.*, 1995). Additional roles for these chaperones have been suggested to be either targetting of the secreted proteins to the secretion organelle or maintenance of the secreted proteins in a secretion-competent conformation (Wattiau *et al.*, 1994). On the basis of our results we favour the latter idea, since heterologous secretion is allowed, although some of the individual components of the secretion system are not interchangeable, suggesting that the chaperones do not interact with the secretion apparatus. This conclusion is also in agreement with the recent finding that YopE in *Y.pseudotuberculosis* is also targetted to the secretion apparatus in a *yerA* mutant (Frithz-Lindsten *et al.*, 1995).

YopE was not only secreted by *S.typhimurium*, but also inferred cytotoxicity on HeLa cells identical to that of *Y.pseudotuberculosis*. Our results strongly suggest that extracellular *S.typhimurium* translocated YopE through the target cell membrane in a similar fashion to that demonstrated for *Y.pseudotuberculosis*. This conclusion is based on three independent observations: (i) gentamicin treatment blocks both YopE-mediated cytotoxicity and the appearance of YopE in the cytosol of HeLa cells; (ii) intracellular staining of YopE was observed after cytochalasin D treatment; (iii) expression of YopE in strain LT2 interferes with uptake of the pathogen into HeLa cells. Since the invasion process was completed within 1 h after onset of infection, the fact that gentamicin addition 1 and 2 h after onset of infection had a profound effect on cytotoxicity, as well as YopE translocation, argues for the concept that YopE delivery is mediated by surface-located bacteria. Gentamicin had no effect on the viabilty of the internalized bacteria. Incubation for up to 16 h in the presence of the drug did not result in any detecable YopE-mediated effects on HeLa cells, suggesting that internalized bacteria do not contribute to this effect. This conclusion was further supported by the observation that prevention of uptake of bacteria by cytochalasin D treatment did not block the apperance of YopE in the cytosol of the target cell. Similarly to what has been shown for *Y pseudotuberculosis*, expression of YopE in *S.typhimurium* LT2 reduces uptake of the bacteria into epithelial cells (Rosqvist *et al.*, 1990; Bliska *et al.*, 1993). Thus expression of YopE in *S.typhimurium* results in the same biological activities as has been reported for *Y.pseudotuberculosis* (Forsberg *et al.*, 1994).

In *Y.pseudotuberculosis* YopE translocation requires the YopD protein (Rosqvist *et al.*, 1994), which is secreted by the *ysc*-encoded type III secretion system. Infections using mutants not able to adhere but secreting YopE into the cell culture medium had no YopE-mediated cytotoxic effect (Rosqvist *et al.*, 1994). Moreover, addition of purified YopE to cultured HeLa cells has no effect unless the protein is introduced into the cytosolic compartment by artificial means (Rosqvist *et al.*, 1991). This excludes the possibility that uptake of YopE is independent of adherent bacteria. Hence, it can be predicted that *S.typhimurium* possesses a protein(s) exhibiting an analogous function to YopD (Rosqvist *et al.*, 1994). Preliminary results obtained in our laboratory suggest that YopB is also involved in Yop translocation (Håkansson *et al.*, in preparation). Interestingly, SipB of *S.typhimurium* and IpaB of *S.flexneri* share homology with YopB of *Y.pseudotuberculosis* and it is possible that these proteins serve the same function in the three different species (Håkansson *et al.*, 1993; Kaniga *et al.*, 1995).

Taken together, the results presented here favour the hypothesis that the basic mechanisms of contact-dependent secretion and translocation are conserved in *Y.pseudotuberculosis* and *S.typhimurium*. Based on the facts that IpaB is secreted in *Y.pseudotuberculosis* and that the secretion systems are conserved, we suggest that *S.flexneri* also exhibits a similar translocation mechanism.

Materials and methods

Bacterial strains and growth conditions

Yersinia pseudotuberculosis strain YPIII harbouring different recombinant plasmids are listed in Table II. The *Escherichia coli* strains used were DH5α [φ80d*lacZ*Δ*M*15 *end*A1 *rec*A1 *hsd*R17 (r_k^- m_k^-) *thi*-1 *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*F)U169] (Bethesda Research Laboratories) and S17-1 (*thi pro hsd*R mutant *hsdM*$^+$ *rec*A RP4-2.TC::Mu-Km::Tn*7*) The *S.typhimurium* strain used was LT2, with the different recombinant plasmids listed in Table II. The *invA* mutation of strain χ3642 (Gálan and Curtiss, 1989) was introduced into strain LT2 by P22 HT*int* transduction, as described (Davies *et al.*, 1980). The *invA* derivative of LT2 was denoted STA1. The liquid growth medium for *Y.pseudotuberculosis* strains was brain/heart infusion broth (Oxoid; Unipath Ltd, Basingstoke, UK) supplemented with 5 mM EGTA and 20 mM $MgCl_2$, or Luria broth. The solid medium was blood agar base supplemented with 2.5 mM $CaCl_2$. *Escherichia coli* and *S.typhimurium* strains were grown in Luria broth or on Luria agar.

DNA methods

Preparations of plasmid DNA, restriction enzyme digestions, ligations and transformations were performed as previously described (Sambrook *et al.*, 1989).

PCRs were performed with deoxynucleoside triphosphates (dNTPs) purchased from Pharmacia Biotech and Taq polymerase from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). The PCR mix consisted of 1× Taq polymerase buffer (supplied by the manufacturer), 0.2 mM each dNTP, 10 pmol each primer, 0.5 μg template DNA and 2.5 U Taq polymerase in a total volume of 50 μl. The reaction profile consisted of denaturation at 94°C for 5 min, annealing at 50°C for 1 min and extension at 72°C for 1 min for five cycles, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min in a DNA thermal cycler (Perkin-Elmer Cetus Gene Amp system 9600).

Cloning of the ipgC and ipaB genes into plasmid pMMB66EH

The vector pMMB66EH (Fürste *et al.*, 1986) was used to clone the PCR-amplified *ipgC* and *ipaB* genes under the control of the *tac* promoter. The *S.flexneri* strain M4243 (Hale and Bonventre, 1979) was used to provide template DNA for the PCR amplification of *ipgC* and *ipaB*. A *Bam*HI site and a *Hin*dIII site were introduced at the ends of the PCR fragment, thereby allowing cloning of the PCR product into the corresponding sites of pMMB66EH, generating plasmid pSEB2. The cloned 2.2 kbp PCR fragment of *ipgC* and *ipaB* spans from 15 bp

Table II. Plasmids used in this study

Plasmid	Description	Reference
pIB102	*yad*A::Tn*5* (wild-type) of the virulence plasmid pIB1 of *Y.pseudotuberculosis*	Bölin and Wolf-Watz (1984)
pIB61	*ysc*T::Tn*5* of pIB1	Forsberg and Wolf-Watz (1988)
pMMB66EH	p*tac* expression vector	Fürste *et al.* (1986)
pSEB2	*ipcG*, *ipaB* cloned into the expression site of pMMB66EH	this study
pSEB3	*Sma*I/*Nde*I deletion of pSEB2	this study
pAF19	*Bam*HI–*Sph*I fragment encoding *yerA* and *yopE* cloned into pUC19	this study
pAF20	*Eco*RI–*Sph*I fragment encoding *yopE* cloned into pUC19	this study

upstream of the initiation codon of *ipgC* to 15 bp downstream of the *ipaB* stop codon. The primers used were as follows:

ipgC1, 5′-GTAAAGGATCCAAAAAGGAGACCTTATG-3′;
ipaB2, 5′-TTTCCATAAGCTTCTCCTTATTTG-3′.

The unique *Sma*I site of the multiple cloning cassette of pMMB66EH and the *Nde*I site of the *ipgC* gene (Baudry *et al.*, 1988; Venkatesan *et al.*, 1988; Sasakawa *et al.*, 1989) of pSEB2 were used to construct a deletion within *ipgC* (Figure 1A). pSEB2 was digested with *Sma*I and *Nde*I and the *Nde*I site was blunt ended using T4 polymerase. The plasmid was re-ligated, generating a deletion spanning the Shine–Dalgarno sequence and the first 41 codons of *ipgC*. The construct carrying the deletion was confirmed using PCR and restriction enzyme analysis. The construct was denoted pSEB3 (Figure 1A). The two constructs were introduced into *Y.pseudotuberculosis* by transformation.

Plasmid pAF19 contains a 1.4 kb *Bam*HI–*Sph*I fragment encoding both the *yerA* and the *yopE* genes (Forsberg and Wolf-Watz, 1990) cloned into pUC19. Two thirds of the *yerA* gene was deleted by excising a 0.4 kb *Bam*HI–*Eco*RI fragment, thereby generating plasmid pAF20.

Analysis of protein expression

Yersinia strains were grown at 26°C to an optical density at 550 nm of 0.1. The cultures were then shifted to 37°C, with or without the addition of IPTG to a final concentration of 1 mM, and grown for an additional 3 h before being harvested. The secreted proteins of the culture supernatant were filtered through 0.22 μm filters and precipitated with 10% trichloroacetic acid. The whole cell and supernatant fractions were subjected to SDS–PAGE and immunoblotting analysis as described earlier (Forsberg *et al.*, 1987), using Ipa rabbit antiserum EIEC 0143 (Pál *et al.*, 1985) kindly provided by Dr B.-E.Uhlin.

Overnight cultures of *S.typhimurium* strains were diluted 1:100 in fresh Luria broth medium and cultured for 16 h in 10 ml tubes (filled to the top) without shaking at 37°C before being harvested. The secreted proteins were subjected to filtration through 0.22 μm filters and precipitated with 10% trichloroacetic acid. The whole cell and supernatant fractions were subjected to SDS–PAGE and immunoblotting analysis as described above, using monospecific anti-YopE (Forsberg *et al.*, 1987) and anti-YerA antibodies (Frithz-Lindsten *et al.*, 1995).

Cultivation and infection of HeLa cells

The *S.typhimurium* bacterial strains were grown in Luria broth statically overnight at 37°C to mid-exponential phase (75 klett) under oxygen-limited conditions. Aliquots (500 μl) of the overnight cultures were pelleted at 12 000 *g* for 2 min and resuspended in 1 ml Leibovitz L-15 medium containing 10% heat-inactivated fetal calf serum. Samples (100 μl) of the resuspended bacterial suspensions (corresponding to ~1×10^7 bacteria) were used to infect the HeLa cell cultures. When cytochalasin D (0.5 μg/ml final concentration) was used the inoculum was increased two times.

Cultivation and infection of HeLa cells have been described in detail elsewhere (Rosqvist *et al.*, 1990). The HeLa cells were seeded (0.6×10^5 per well) in a 24-well tissue culture plate in Leibovitz L-15 medium with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin at 37°C in a humidified atmosphere. For immunofluorescence studies the HeLa cells were grown on 12 mm coverslips placed in a 24-well tissue culture plate. Before infection the HeLa cells were washed free of the penicillin, and Leibovitz L-15 medium containing 10% heat-inactivated fetal calf serum without any antibiotics was added. In experiments using cytochalasin D the drug was added 30 min prior to infection. After infection the HeLa cells were centrifuged for 5 min at 400 *g* to facilitate contact between the bacteria and the HeLa cells, followed by continued incubation at 37°C. In all experiments, except when gentamicin was used, the coverslips were washed twice in phosphate-buffered saline (PBS) (37°C) after 2.5 h infection to remove excess bacteria.

Translocation of YopE into HeLa cells by S.typhimurium

HeLa cells grown on coverslips (~0.6×10^5 cells) were infected with different strains of *S.typhimurium* (~1×10^7 bacteria/well). In some experiments gentamicin was added to a final concentration of 4 μg/ml at various times after onset of infection to inhibit protein synthesis by extracellular bacteria. After 4 h infection the cell monolayers were washed twice in PBS and stained with wheat germ agglutinin conjugated to Texas red (Molecular Probes, Eugene, OR) (10 μg/ml for 10 min at 24°C), whereafter the specimens were fixed in 2% paraformaldehyde for 10 min. The cells were then permeabilized with 0.5% Triton X-100 in a buffer containing 1 mM EGTA, 4% polyethylene glycol 6000 and 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.9, and further processed for indirect immunofluorescence labelling (for details see Rosqvist *et al.*, 1991) using affinity purified rabbit anti-YopE antibodies followed by FITC-conjugated anti-rabbit antibodies. The specimens were finally mounted in a mounting media containing Citifluor (The City University, London, UK) as an anti-fading agent.

The specimens were analysed either by epifluorescence microscopy (Zeiss Axioscope 50) or by using a confocal laser scanning microscope equipped with dual detectors and an argon/krypton laser for simultaneous scanning of two different fluorochromes (Multiprobe 2001; Molecular Dynamics, Sunnyvale, CA). Laser power and gain were set by using cells labelled with either fluorochrome alone, so that there was no crossover of green to red or red to green channels. Sets of fluorescent images were acquired simultaneously for Texas red (the HeLa cell membrane) and fluorescein-tagged (YopE) markers using a ×40/1.0 plan-apochromate Nikon oil immersion lens. Companion images (15 sections with image size 512×512) were scanned at 0.42 μm pixel size and 1.0 μm step size and a pinhole setting of 100 μm.

Discrimination between intracellular and surface-located bacteria

To be able to distinguish between bacteria located on the surface of the HeLa cell and bacteria on the inside of the cells a double-fluorescent antibody test described earlier was used (Rosqvist *et al.*, 1988). At various times after infection the specimens were washed and coverslips were overlaid with anti-*S.typhimurium* lipopolysaccharide rabbit antiserum (Calbiochem) diluted 20 times in PBS and incubated on ice for 1 h. Excess antiserum was washed off by dipping the coverslips in PBS, whereafter the samples were fixed in methanol at –15°C for 2 min. The coverslips were overlaid with lissamine rhodamine-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:100 in PBS and incubated for 1 h at 37°C to stain extracellularly located bacteria. After washing the samples were again overlaid with the rabbit anti-*S.typhimurium* lipopolysaccharide antiserum diluted 1:100 in PBS and incubated for 1 h at 37°C. During this step the antibodies were able to react with both intracellular and extracellular bacteria. After washing the monolayers were overlaid with FITC-conjugated donkey anti-rabbit IgG antiserum diluted 1:200 and incubated at 37°C for 1 h. The specimens were again rinsed in PBS and finally mounted in a mounting media containing Citifluor (The City University, London, UK) as an anti-fading agent. The specimens were examined with a fluorescent microscope (Zeiss Axioskop 50) with epifluorescence illumination and a ×100/1.4 plan-apochromate oil immersion lens (final magnification ×1000). In each experiment 25 HeLa cells were counted from randomly selected fields without preview. The number of extracellular bacteria was counted with a rhodamine filter system and the total number of cell-associated (intracellular plus extracellular) bacteria was determined with a fluorescein filter system.

Viable count of intracellular bacteria

HeLa cell infections were treated with gentamicin at a final concentration of 100 μg/ml for 1 h and incubation was prolonged for different times in the presence of 10 μg/ml of gentamicin. At different time points the cultures were washed to remove the gentamicin and the cells were lysed in 0.1% Triton X-100 in PBS. The number of viable bacteria were determined by plating.

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