The EMBO Journal vol.13 no.4 pp.964–972, 1994

Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells

Roland Rosqvist[1,2,3], Karl-Eric Magnusson[2] and Hans Wolf-Watz[1]

[1]Department of Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, [2]Department of Medical Microbiology, University of Linköping, S-581 85 Linköping and [3]National Defence Research Establishment, S-901 82 Umeå, Sweden

Communicated by S.Normark

Pathogenic bacteria of the species *Yersinia*, including *Yersinia pestis*, block phagocytosis by macrophages. This process involves the YopE protein, which induces disruption of the host cell actin microfilament structure. Here, we show that the contact between the pathogen and the mammalian cell induces expression and then polarized transfer of YopE into the eukaryotic cell. While the bacteria remain at the surface of the target cell, the YopE cytotoxin is transferred through the host cell plasma membrane and YopE is only recovered within the cytosol of the target cell. The results suggest that the pathogen senses cell structures and focuses the transfer of YopE to occur solely at the interaction zone between the bacterium and the eukaryotic cell. The regulation of this process is shown to involve surface-located YopN sensor protein of the bacterium.

Key words: HeLa cell interaction/polarized translocation/ virulence/*Yersinia*/*yop* gene regulation

Introduction

Yersinia is the genus of bacteria of the causative agent in plague or the Black Death. Virulent yersiniae possess a common virulence plasmid of ~70 kb which encodes a number of virulence determinants (for reviews see Cornelis, 1992; Straley *et al.*, 1993). Four of these, YopH, YopE, YopM and YpkA, have been shown by site-directed mutagenesis to be indispensable for virulence (Bölin and Wolf-Watz, 1988; Forsberg and Wolf-Watz, 1988; Leung *et al.*, 1990; Galyov *et al.*, 1993). YopM, which shares high homology with GpIbα, the α-chain of the platelet receptor of the von Willebrand factor, prevents platelet aggregation by interaction with thrombin (Reisner and Straley, 1992). YpkA exhibits a Ser/Thr phosphokinase activity that shows extensive homology with corresponding enzymes of eukaryotic origin (Galyov *et al.*, 1993). YopH and YopE obstruct the primary host defence by inhibition of phagocytosis (Rosqvist *et al.*, 1988, 1990). YopH has a tyrosine phosphatase activity of eukaryotic type, suggesting that this protein acts by dephosphorylation of host structures (Guan and Dixon, 1990; Bliska *et al.*, 1991). YopE mediates a contact dependent cytotoxic activity which leads to depolymerization of the actin microfilament network of the target cell (Rosqvist *et al.*, 1991). Interestingly, while the pathogen remains bound to the target cell surface (Rosqvist *et al.*, 1990, 1991), the YopE mediated activity requires that YopE is translocated through the plasma membrane of the target cell. This may suggest that virulent *Yersinia* exhibit a specific mechanism to translocate and direct YopE to its intracellular target.

These plasmid encoded virulence determinants belong to a class of proteins (Yops), which are regulated positively by the stimuli temperature and negatively by the calcium concentration (Bölin *et al.*, 1985; reviews: Cornelis, 1992; Straley *et al.*, 1993). In response to an increase in temperature, synthesis of an AraC-homologous protein, LcrF(VirF), is induced (Cornelis, 1992). LcrF is an activator of *yop* transcription that was shown recently to bind to the *yop* promoters (see model, Figure 1) (Lambert de Rouvroit *et al.*, 1992). At 37°C in the absence of Ca^{2+}, *yop* transcription is derepressed and high amounts of Yops are produced and secreted into the growth medium. If the pathogen is incubated at 37°C in the presence of millimolar concentrations of Ca^{2+}, the *yop* genes are repressed and Yop expression is greatly reduced (Cornelis, 1992; Straley *et al.*, 1993). The surface-located YopN protein is involved in sensing the Ca^{2+} concentration and in transmitting the signal to regulatory factors located intracellularly (Forsberg *et al.*, 1991). Thus, YopN is at the top of the regulatory hierarchy. LcrH, which is at the bottom of the regulatory hierarchy, has recently been suggested to be the transcriptional repressor (Figure 1), since overproduction of LcrH in certain strains leads to repression of *yop* transcription (Price and Straley, 1989; Bergman *et al.*, 1991). In parallel to this transcriptional regulation, the Yops are secreted to the culture medium by a specific Ca^{2+}-regulated plasmid encoded secretion system (Figure 1) (Rosqvist *et al.*, 1990; Michiels *et al.*, 1991; Haddix and Straley, 1992).

We show here that *Yersinia* can overcome the Ca^{2+} repression at 37°C if the pathogen is allowed to interact with target cell surfaces. This interaction leads to derepression of Yop expression followed by polarized transfer of the YopE cytotoxin, into the target cell. These events are mediated by a YopN triggered signal. The results suggest that the pathogen senses the junction between the target cell and the bacterium and translocates YopE only at this point. This mechanism of virulence gene induction followed by the transfer of effector molecules may be a common feature among several pathogens. This seems likely since the mammalian pathogens *Salmonella*, *Shigella* (Andrews and Maurelli, 1992; Ginocchio *et al.*, 1992; Allaoui *et al.*, 1993), and the plant pathogens of the genera *Erwinia*, *Pseudomonas* and *Xanthomonas* (reviewed by Salmond and Reeves, 1993) exhibit transport systems analogous to *Yersinia*.

Results

Contact between the pathogen and the HeLa cells induces expression of YopE

We have recently shown that the YopE cytotoxin must be presented inside the target cell to execute its activity which

© Oxford University Press

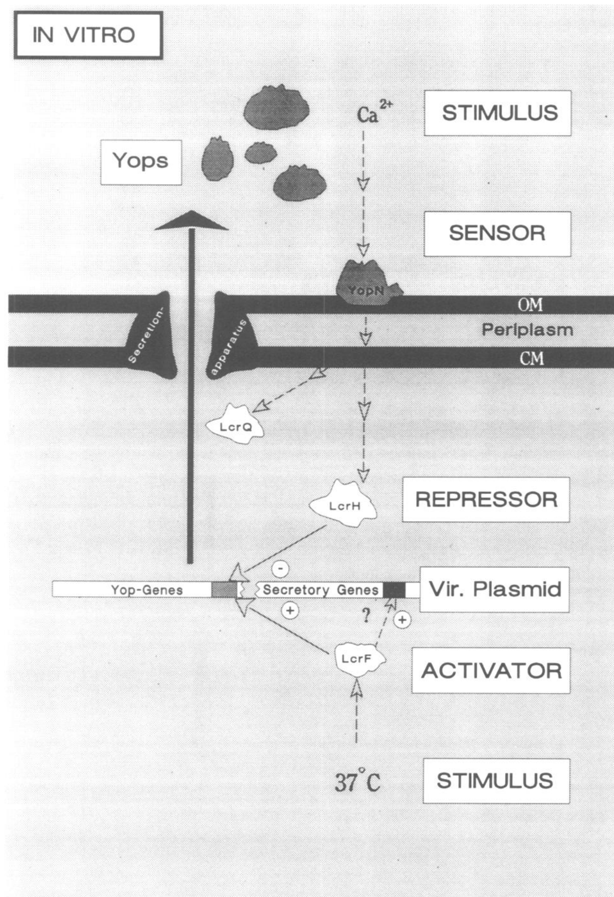

Fig. 1. *In vitro* model of Yop expression and secretion. In response to an increase in temperature the LcrF activator is expressed and interacts with the *yop* promoters (Lambert de Rouvroit *et al.*, 1992) to enhance transcription of the *yop* genes. The surface-located YopN protein senses the Ca^{2+} concentration and transmits the signal to regulatory proteins located inside the bacterium (Forsberg *et al.*, 1991). This signal results in repression of Yop expression in the presence of Ca^{2+} via the LcrH repressor (Price and Straley, 1989; Bergman *et al.*, 1991). In the absence of Ca^{2+} derepression of Yop expression occurs resulting in high expression and secretion of Yop proteins. The Yop secretion apparatus is regulated similarly to the Yops. This model is based on data compiled from the three pathogenic species of *Yersinia* (Forsberg *et al.*, 1991; Cornelis, 1992; Straley *et al.*, 1993).

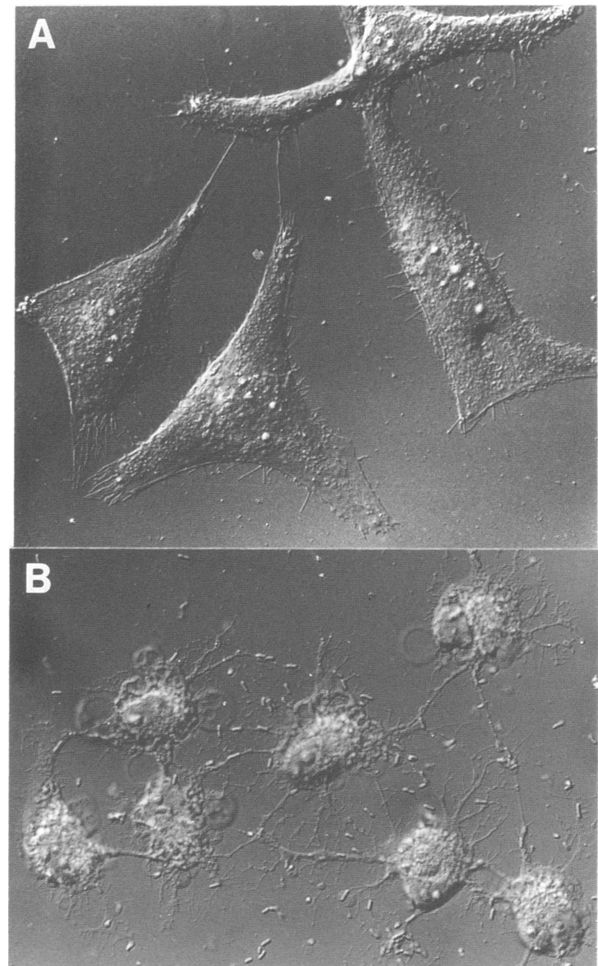

Fig. 2. Differential interference contrast micrographs of HeLa cells. HeLa cells were infected with different strains of *Y.pseudotuberculosis*. Photo taken 1.75 h after infection. (**A**) Non-infected HeLa cells showing normal morphology. (**B**) HeLa cells infected with the wild-type strain YPIII(pIB102), showing cytotoxic affected cells.

results in blockage of phagocytosis (Rosqvist *et al.*, 1988, 1991). Although *Y.pseudotuberculosis* is able to invade HeLa cells, intracellularly located bacteria do not promote any cytotoxic effect and they are in addition unable to replicate in this environment (Rosqvist and Wolf-Watz, 1986). Accordingly such bacteria are unable to express the Yop proteins in detectable amounts (Rosqvist *et al.*, 1990). Thus, the pathogen remains at the surface of the target cell while the YopE protein is likely to be transported into the mammalian cell. This process requires the presence of viable bacteria (Rosqvist *et al.*, 1990). Cultured HeLa cells were used as the model system to explore the possibilities that YopE was translocated from *Y.pseudotuberculosis* into mammalian cells by a specific mechanism.

When the HeLa cells were infected with the wild-type strain YPIII(pIB102), the cells showed a YopE mediated cytotoxic response within 2 h of infection (Figure 2). In comparison with the wild-type strain, a *yopN* mutant induced a delayed cytotoxic response, which could be detected ~4 h after infection, suggesting that this mutant was defective in the YopE promoted cytotoxic response. Thus, the difference in this cytotoxic response could be due to differences in YopE expression and/or YopE translocation. Both strains were therefore grown in tissue culture medium (containing 1 mM Ca^{2+}) and in a medium known to induce high Yop expression and secretion *in vitro* [brain heart infusion broth containing 5 mM EGTA (BHI)]. The bacteria and the growth medium were separated and collected. The bacterial pellets were resuspended in SDS-sample buffer, the Yops secreted to the supernatants were immunoprecipitated, and the amount of YopE was determined in each fraction. A non-cytotoxic *yopE* deletion mutant, YPIII(pIB522), was included as a negative control for the specificity of the antiserum used in these experiments (Figure 3). The *yopN* mutant expressed and secreted YopE at about the same level irrespective of the growth medium (Figure 3). In contrast, the wild-type strain showed a much lower expression and secretion of YopE when grown in tissue culture medium as compared with the BHI medium (Figure 3B). These results showed that YopE expression is repressed in the wild-type strain but derepressed in the *yopN* mutant when grown in the tissue culture medium. Although the wild-type strain showed a repressed expression of YopE in the tissue culture medium,

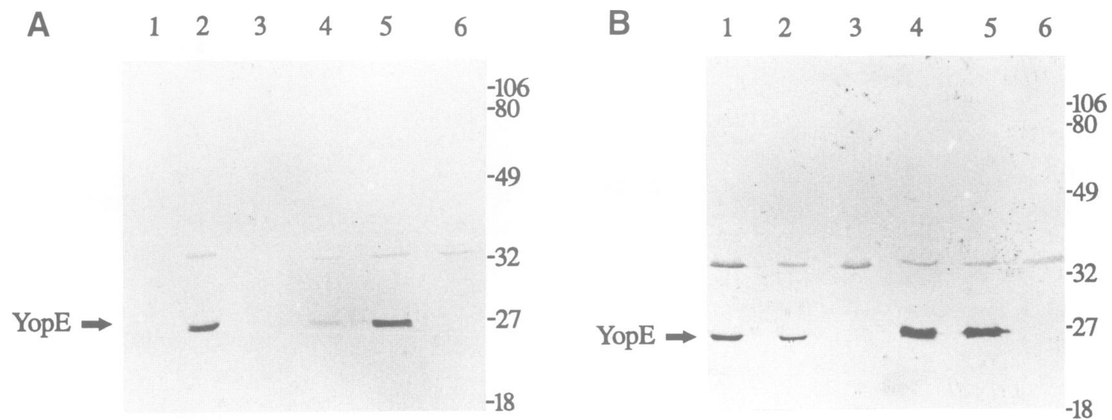

Fig. 3. *In vitro* expression and secretion of YopE after incubation in different growth media. YopE expression and secretion in the wild-type strain YPIII(pIB102), the *yopN* mutant YPIII(pIB82) and the *yopE* mutant YPIII(pIB522) of *Y.pseudotuberculosis* grown in (**A**) tissue culture medium or (**B**) brain heart infusion broth containing 5 mM EGTA for 4.5 h at 37°C and then analysed by Western blot. Lanes: YopE was immunoprecipitated from the growth medium of the wild-type strain YPIII(pIB102) (lane 1) or the *yopN* mutant YPIII(pIB82) (lane 2) or the *yopE* mutant YPIII(pIB522) (lane 3). Bacteria were recovered from the growth medium of the wild-type strain YPIII(pIB102) (lane 4) or the *yopN* mutant YPIII(pIB82) (lane 5) or the *yopE* mutant YPIII(pIB522) (lane 6). Samples representing 5% of each fraction were added to each lane.

this strain induced an earlier YopE mediated cytotoxic effect on the HeLa cells than the *yopN* mutant. This indicated that YopE expression of the wild-type strain was stimulated by the presence of the HeLa cells and that the difference between the two strains was not due to differences in YopE expression.

To analyse whether this difference between the two strains could be caused by differences in the ability to translocate YopE into the HeLa cells, the distribution of YopE in the tissue culture medium and within infected HeLa cells was determined. Cultured HeLa cells were infected with either the wild-type strain or the *yopN* mutant, and incubated at 37°C. The infected HeLa cell cultures were collected and divided into four different fractions. The first fraction was the non-adherent bacteria separated from the tissue culture medium. The second fraction was the bacteria-free tissue culture medium. The third fraction consisted of the released cytoplasmic content of Triton X-100 lysed infected HeLa cells and the fourth fraction was the pellet obtained after lysis of the HeLa cells with Triton X-100. Non-adherent bacteria, the tissue culture medium, the cytoplasmic content, and the pellet were analysed for the amount of YopE present in these different fractions (Figure 4). YopE was found in the cytoplasmic fraction of the wild-type strain infected HeLa cells, whereas barely detectable amounts of YopE had been secreted to the tissue culture medium (Figure 4, lanes 3 and 1). This was in clear contrast to HeLa cells infected with the *yopN* mutant. In this case, YopE was found in the cytoplasmic fraction as well as in the tissue culture medium (Figure 4, lanes 4 and 2). Thus, for the wild-type strain, YopE expression required contact between the pathogen and the HeLa cells and most of the YopE protein was in this case translocated solely into the target cell. The *yopN* mutant did not require this contact for YopE expression and showed a non-polarized secretion of YopE both to the tissue culture medium and into the HeLa cells. Moreover, the *in vivo* and *in vitro* experiments also indicate that the level of YopE expression is tightly regulated in the wild-type strain during infection, since the *yopN* mutant expressed higher levels of YopE *in vivo* than the wild-type strain (Figure 4), while *in vitro*, the wild-type strain expressed equal or even higher amounts of YopE than the *yopN* mutant (Figure 3B).

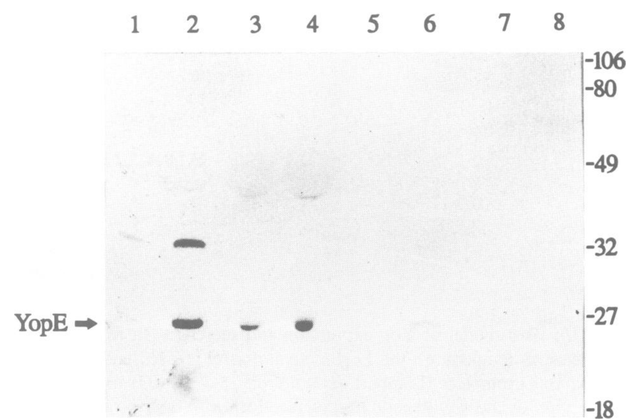

Fig. 4. *In vivo* expression and translocation of YopE after infection of HeLa cells with the wild-type strain and the *yopN* mutant. Western blot analysis showing the relative distribution of the *Yersinia* YopE cytotoxin in HeLa cells and in the tissue culture medium 4.5 h after infection with different strains of *Y.pseudotuberculosis*. Lanes: YopE was immunoprecipitated from the tissue culture medium of HeLa cells infected with the wild-type strain YPIII(pIB102) (lane 1) or the *yopN* mutant YPIII(pIB82) (lane 2). YopE was immunoprecipitated from the released cytoplasmic content of lysed HeLa cells infected with the wild-type strain YPIII(pIB102) (lane 3) or the *yopN* mutant YPIII(pIB82) (lane 4). The pellet of lysed HeLa cells infected with the wild-type strain YPIII(pIB102) (lane 5) or the *yopN* mutant YPIII(pIB82) (lane 6). Non-adherent bacteria isolated from the tissue culture medium of HeLa cells infected with the wild-type strain YPIII(pIB102) (lane 7) or the *yopN* mutant YPIII(pIB82) (lane 8). Samples representing 5% of each fraction were added to each lane.

Polarized transfer and intracellular targeting of the YopE cytotoxin

To study further the translocation of YopE into HeLa cells, immunofluorescence and confocal laser scanning microscopy analyses were performed using HeLa cells infected with different strains of *Y.pseudotuberculosis*. When the wild-type strain was used to infect the HeLa cells a strong immunofluorescence signal was seen in the HeLa cells 1.75 h after infection, showing that the YopE protein was translocated into the cells. However, no bacteria were visualized by the immunofluorescence staining, although 15–20 bacteria per HeLa cell were seen using phase contrast

microscopy (data not shown). To study a possible subcellular localization of YopE in the HeLa cells the specimens were analysed by confocal microscopy. YopE was found to be enriched in the perinuclear region of the HeLa cells (Figure 5A). The *yopE* deletion mutant YPIII(pIB522), which does not induce any cytotoxic response, was used in parallel experiments as a control for background staining, confirming the specificity of the anti-YopE antibodies used (Figure 5B).

In contrast, the *yopN* mutant did not induce a cytotoxic effect on the HeLa cells after 1.75 h, and no transfer of the YopE cytotoxin into the target cell could be detected (Figure 6A). However, the HeLa cells were cytotoxically affected after prolonged incubation (3.75 h) and at this time point, the YopE protein could be detected in the HeLa cells (Figure 6B). In addition, YopE was also discerned on the surface of the HeLa cell bound *yopN* mutant (Figure 6).

The results presented above indicate that *Yersinia* has an efficient export machinery which, upon contact with the HeLa cell, specifically transfers the YopE cytotoxin in a

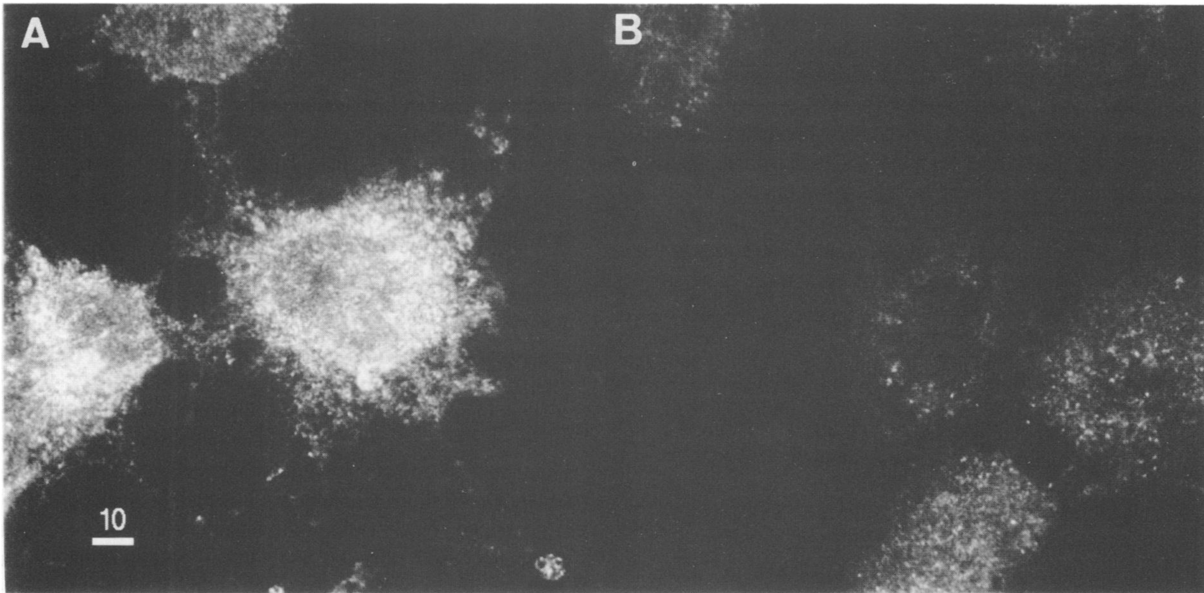

Fig. 5. The YopE cytotoxin is translocated into the cytosol of the HeLa cells after infection with the wild-type strain of *Y.pseudotuberculosis*. Confocal fluorescence micrographs of HeLa cells infected with different strains of *Y.pseudotuberculosis*. Samples were taken out and analysed for the presence of YopE using indirect immunofluorescence techniques. (**A**), analysed 1.75 h after infection with the wild-type strain YPIII(pIB102) showing enrichment of YopE in the perinuclear region of the target cells. Note that no bacteria are visualized by the anti-YopE antibodies. (**B**), analysed 1.75 h after infection with the *yopE* mutant YPIII(pIB522) showing background level of staining.

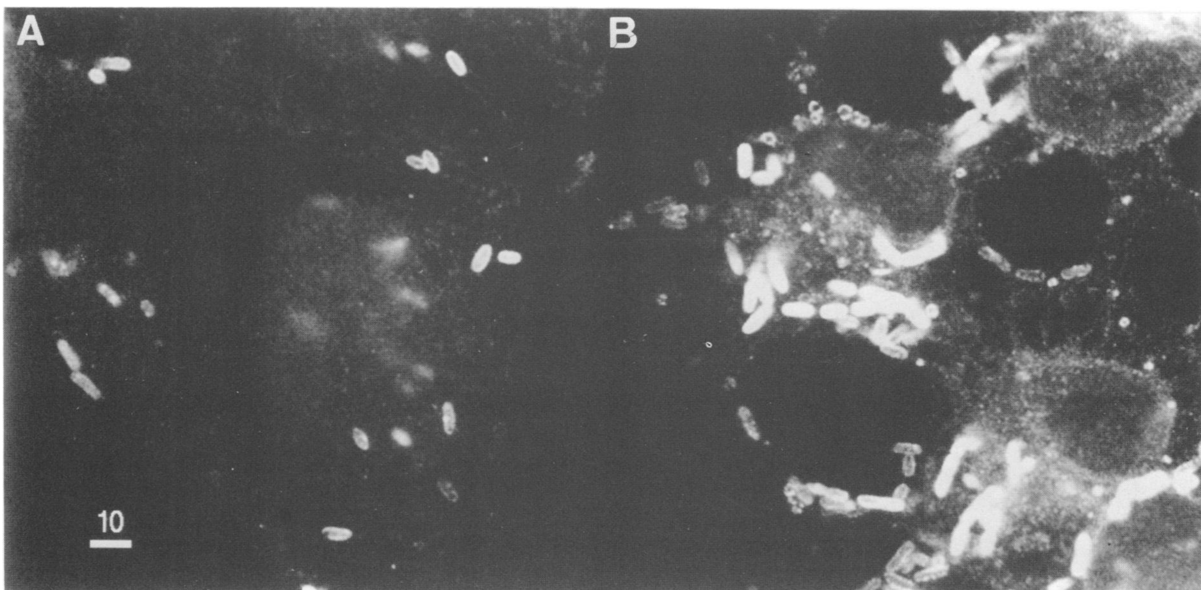

Fig. 6. The YopE cytotoxin is deposited on the bacterial surface of the *yopN* mutant after infection of HeLa cells. Confocal fluorescence images of HeLa cells infected with the *yopN* mutant YPIII(pIB82) of *Y.pseudotuberculosis*. At different time points samples were taken out and analysed for the presence of YopE using indirect immunofluorescence. (**A**) When analysed 1.75 h after infection with the *yopN* mutant YPIII(pIB82) no detectable transfer of YopE into the HeLa cells was displayed. Many cell-associated bacteria have detectable amounts of YopE. (**B**) When studied 3.75 h after infection with the *yopN* mutant YPIII(pIB82), showing transfer of YopE into the HeLa cells. A large proportion of the HeLa cell-associated bacteria are strongly stained, indicating the presence of YopE on the surface of the pathogen.

polarized manner from the bacterium into the host cell during the infectious process. In contrast, the *yopN* mutant secretes YopE into the tissue culture medium as well as translocating YopE into the target cell.

We have previously shown that adherence of *Y.pseudotuberculosis* to the HeLa cells is a prerequisite to induce a cytotoxic response (Rosqvist *et al.*, 1990). The adherence is mediated either by invasin or the YadA protein. Thus, a *invA*/*yadA* double mutant is unable to bind to the HeLa cells and does not induce cytotoxicity (Rosqvist *et al.*, 1990). In this respect this double mutant shows similarities to the *Y.pestis* strain EV76, since this latter strain lacks functional invasin and YadA. However, if functional *yadA* is provided *in trans* EV76 can bind to the surface of the HeLa cell and the strain induces a YopE mediated cytotoxicity (Rosqvist *et al.*, 1990). In this case >95% of the infected HeLa cells had on average about three bacteria located on the surface and no intracellular bacteria. The YadA expressing *Y.pestis* strain EV76p(pAMS2) was examined for YopE translocation. This strain showed the same behaviour as the wild-type strain YPIII(pIB102), i.e. YopE was found only within the cytosol of the HeLa cells (data not shown).

To investigate further the importance of the binding of the pathogen to the target cell, we asked if a non-adherent *yopN* mutant of *Y.pestis* [EV76Cp(pIB82)] induced a cytotoxic response on HeLa cells. This *yopN* mutant did not induce cytotoxicity while the isogenic strain carrying the YadA adhesin, which mediates binding to the HeLa cells, did so (Table I), showing that freely diffusible YopE did not induce cytotoxicity. However, this experiment did not exclude the possibility that freely diffusible YopE present in the tissue culture medium could be translocated with the aid of an adherent bacterium. Therefore a mixed infection experiment using the non-adherent *yopN* mutant [EV76Cp(pIB82)] and the adherent *yopE* mutant [EV76Cp(pIB522, pAMS2)] was carried out. No YopE mediated cytotoxicity could be recorded (Table I), indicating that translocation of YopE into the target cell can only be accomplished by the bacterium that binds to the target cell.

Table I. Ability of different *Y.pestis* strains to induce a YopE mediated cytotoxic response on cultured HeLa cells

Strain	Relevant genotype	Cytotoxicity on HeLa cells
EV76Cp	plasmid cured	−
EV76Cp(pIB82)	*yopN*, *yadA*	−
EV76Cp(pIB82, pAMS2)	*yopN*, *yadA*$^+$	+
EV76Cp(pIB522, pAMS2)	*yopE*, *yadA*$^+$	−
EV76Cp(pIB522, pAMS2) EV76Cp(pIB82)	mixed infection	−

YopD is involved in the translocation process of YopE into HeLa cells

We have previously isolated a mutant in the *yopD* gene [YPIII(pIB15)], which shows the same YopE expression and secretion as the wild-type strain *in vitro* (Rosqvist *et al.*, 1991). This mutant does not mediate a cytotoxic effect, presumably due to its inability to translocate YopE through the target cell membrane (Rosqvist *et al.*, 1991). When this mutant was studied with the immunofluorescence techniques employed here, no YopE was found within the HeLa cells. However, YopE was found to be deposited at localized spots on ~10–20% of the bacterium adhering to the HeLa cells (Figure 7). No YopE was expressed from bacteria not associated with the HeLa cells (Figure 7). This localized YopE staining pattern was not observed when the wild-type strain [YPIII(pIB102)] was analysed (compare Figure 5). The infected HeLa cell shown in Figure 7 was also stained with Texas Red conjugated wheat germ agglutinin (WGA–Texas Red) to visualize the surface of the HeLa cell. One bacterium (marked with an arrow in Figure 7) giving a localized YopE staining pattern was chosen for further

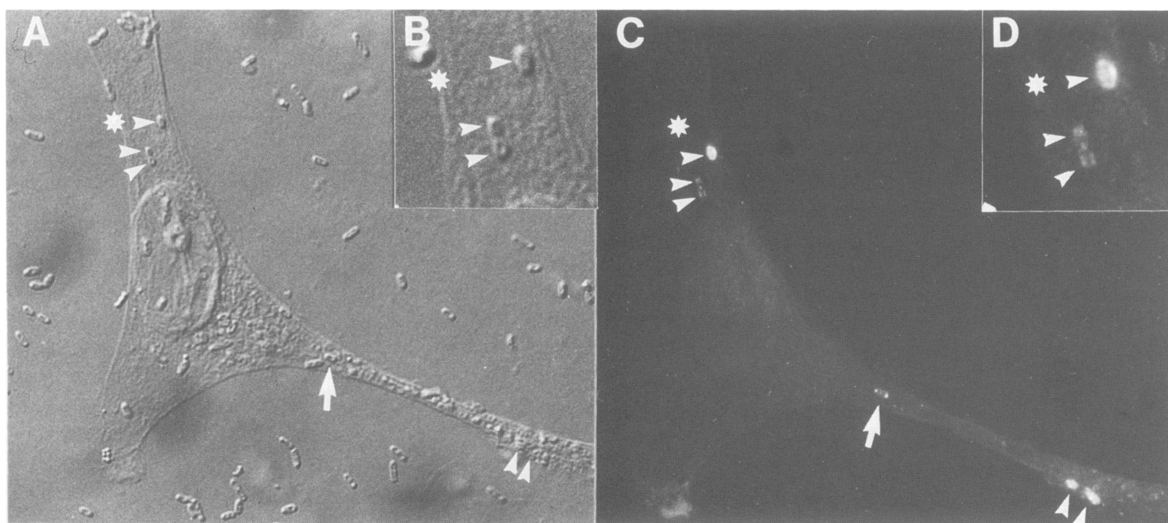

Fig. 7. A *yopD* mutant is blocked in the YopE translocation process. Differential interference contrast (**A** and **B**) and epifluorescence (**C** and **D**) images of HeLa cells infected with the *yopD* mutant YPIII(pIB15). After 1.75 h samples were withdrawn and analysed for the presence of YopE using indirect immunofluorescence. The membrane of the HeLa cell was visualized using WGA–Texas Red (see Figure 8). The same view field was analysed using differential interference contrast and epifluorescence microscopy. Notable is the enrichment of YopE at localized sites on some of the cell-associated bacteria (arrowhead). (B) and (D) show the indicated area at higher magnification. No staining is seen on bacteria adhering to the glass coverslip. An area containing one bacterium with localized YopE staining (indicated by an arrow) was further analysed using confocal laser scanning microscopy (see Figure 8).

analysis using confocal laser scanning microscopy. The obtained data-sets were used to generate a three dimensional (3D) reconstruction of the HeLa cell surface and the distribution of YopE. With the help of volume visualization techniques (Kaufman, 1991) applied on the 3D-reconstructed data it was found that the bacterium was adhering to the HeLa cell surface and YopE was localized to certain regions of the bacterium (Figure 8A). A side view projection of the same bacterium shows its localization at the HeLa cell surface (Figure 8B). In this latter projection a low density value for YopE was chosen to allow visualization of the bacteria. To determine the spatial localization of YopE in this area the opacity of the HeLa cell membrane was increased to allow look-through inside the HeLa cell. In this case a high density value for YopE was chosen to allow visualization of high concentration of YopE (Figure 8C). This side view projection shows that a high concentration of YopE can be found in the lower part of the bacterium, suggesting that the bacterium is focusing the transfer of YopE to the zone of contact between the bacterium and the target cell. These results suggests that the *yopD* mutant is blocked in a discrete step in the YopE translocation process. Moreover, the results also show that the secretion of YopE is localized to certain regions of the bacterium and that also in this case contact between the bacterium and the cell membrane is a prerequisite for YopE expression.

Discussion

One important virulence function of pathogenic *Yersinia* is their ability to resist phagocytosis (Burrows and Bacon, 1956; Cavanaugh and Randall, 1959). The pathogen adheres to the surface of the phagocyte and inactivates the phagocytic process by the action of at least the two proteins YopE and YopH (Rosqvist *et al.*, 1990). YopE exhibits a cytotoxic activity involving actin microfilament depolymerization, which can also be monitored using cultured HeLa cells. This activity can only be induced by bacteria bound to the target cell surface. Intracellularly located bacteria are unable to induce cytotoxicity as well as unable to express the Yop proteins (Rosqvist and Wolf-Watz, 1986; Rosqvist *et al.*, 1990). In accordance with this, intracellularly located bacteria are rarely observed during infection, and the bacteria are predominantly found at an extracellular location (Lian *et al.*, 1987; Hanski *et al.*, 1989; Simonet *et al.*, 1990).

In this study we show that the pathogenic bacterium *Y.pseudotuberculosis* induces virulence gene expression upon contact between the pathogen and the target cell. Gene expression is followed by a polarized transfer of the YopE virulence determinant through the plasma membrane of the eukaryotic cell. The surface-located YopN protein of the pathogen exhibits a key role in this process and our results suggest that YopN senses target-cell surface structures and transmits this signal via the *yop*-regulatory proteins (Figure 1). Moreover, the transfer of YopE may be suggested to occur only at the contact zone between the pathogen and the target cell. After transfer, YopE is enriched in the perinuclear region of the host cell where it most likely exerts its activity, resulting in disruption of the actin microfilament structure of the host cell. How YopE is transported to this region is at present not understood but it may be speculated that this process is guided by other virulence involved Yop proteins, which are known to contribute to the cytotoxic activity (Rosqvist *et al.*, 1991).

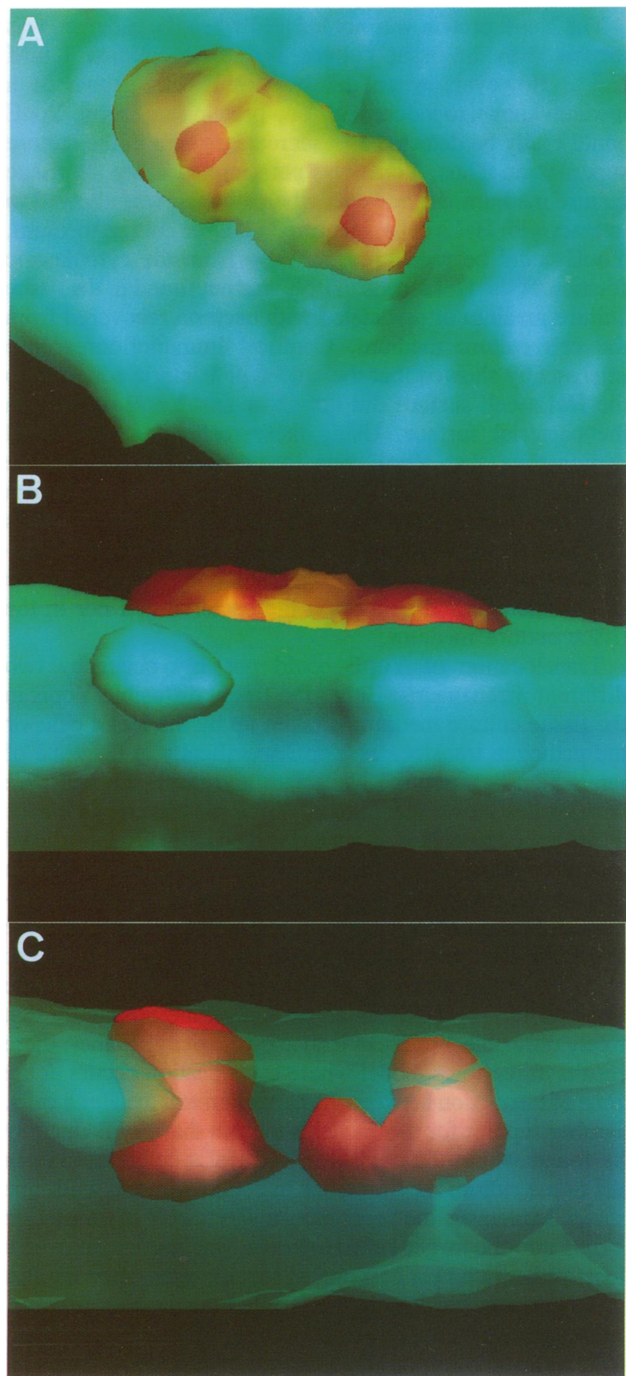

Fig. 8. Three dimensional reconstruction of the area indicated by an arrow in Figure 7, showing the *yopD* mutant YPIII(pIB15) adhering to the HeLa cell membrane and that YopE is localized in the lower part of the bacterium directed into the HeLa cell. **(A)** Top view, showing the bacterium adhering to the HeLa cell surface (green colour) and YopE (red colour) localized to certain regions of the bacterium. **(B)** Side view. By using a low density value for YopE, visualization of the upper part of the bacterium was possible. **(C)** Side view. The opacity value for the HeLa cell membrane was increased allowing look-through into the cell. The density value for YopE was increased allowing only high concentrations of YopE (red colour) to be visualized. The area indicated in Figure 7 (arrow) was subjected to confocal laser scanning microscopy analysis. The images (30 sections with image size 256 × 256) of the HeLa cell membrane (stained with WGA–Texas Red) and YopE (visualized with anti-YopE antibodies and a FITC-conjugated secondary antibody) were scanned simultaneously with 0.07 μm pixel size and 0.2 μm step size. After digital reversion of the colours, the red colour indicates the highest concentration of YopE and the yellow colour a low concentration. The HeLa cell surface is shown by a green colour.

The YopE protein was not induced when the pathogen was incubated in the tissue culture medium containing 1 mM Ca^{2+}. However, when the bacterium was grown in a medium known to induce high Yop protein expression, YopE could easily be detected as a secreted protein in the culture supernatant. Thus, YopE was not secreted using the tissue culture medium. If, however, the pathogen was allowed to interact with HeLa cells, incubated in tissue culture medium, YopE could be recovered but only from the cytoplasmic fraction of the HeLa cells. YopE was not found in the tissue culture medium or associated with bacteria recovered from the cell culture medium. Confocal immunofluorescence microscopy showed that the wild-type bacteria adhering to the HeLa cell surface did not show any YopE protein on the bacterial surface. Using these techniques, we could demonstrate that YopE was localized solely within the cytosol of the target cell. Thus, both the results obtained from the experiments using cell fractionation and the results obtained using immunofluorescence microscopy demonstrated that, after infection, YopE is found solely within the eukaryotic cell and neither on the bacterial cell nor in the tissue culture medium.

Translocation of YopE could possibly occur by two different mechanisms. Either the freely diffusible YopE in the tissue culture medium is taken up by the HeLa cells or the pathogen translocates YopE specifically at the zone of interaction between the bacterium and the HeLa cell. We have earlier shown that purified YopE protein cannot induce a cytotoxic effect *in vitro* unless it is specifically introduced into the cytosol of the HeLa cell (Rosqvist *et al.*, 1991). In this study, we have extended this observation. A non-adherent strain able to express and secrete YopE into the tissue culture medium did not induce cytotoxicity, either alone or in the presence of a *yopE* mutant binding to the HeLa cells. These results argue against the possibility that freely diffusible YopE can induce cytotoxicity. Rather, these results argue for a model where translocation occurs at the zone of interaction between the bacterium and the HeLa cells. Further support for this model is the fact that YopE can only be found within the cytosol of the HeLa cells, and not in the tissue culture medium after infection with the wild-type strain. Moreover, the *yopD* mutant was unable to translocate YopE into the target cell. However, this mutant showed a localized enrichment of YopE at specific sites, showing that this mutant is blocked in the YopE translocation process. Furthermore, 3D-reconstruction and volume visualization techniques applied on the images obtained from confocal laser scanning microscopy showed that the enrichment of YopE was localized towards the HeLa cell. It is likely that these sites reflect the zone of YopE transfer. Thus, all these results favour a model suggesting that translocation of YopE occurs at the site of interaction between the pathogen and the target cell.

Earlier studies have shown that the YopN protein of *Yersinia* is involved in the regulation of *yop* gene expression and Yop secretion (Yother and Goguen, 1985; Forsberg *et al.*, 1991). *yopN* mutants show derepressed *yop* expression and Yop secretion at 37°C independent of the Ca^{2+} concentration. Interestingly, Forsberg *et al.* (1991) demonstrated that the YopN protein was surface located indicating a role for YopN as a receptor signal transducing protein (see model, Figure 1). When the *yopN* mutant, YPIII(pIB82), was allowed to infect HeLa cells, the mutant generated a delayed cytotoxic activity compared with the wild-type strain YPIII(pIB102). Cell fractionation and immunofluorescence analyses of the infected HeLa cell cultures revealed that YopE was secreted to the HeLa tissue culture medium as well as deposited on the surface of the pathogen. In addition, YopE could also be found in the cytosol of the cells, showing that the *yopN* mutant could translocate the YopE cytotoxin into the target cell, albeit at a reduced efficiency.

These observations indicated that the YopN protein exhibits a key function in the polarized translocation process of YopE into the target cell. We believe that YopN exhibits two roles in this regulatory system. First, YopN acts as a surface receptor for the pathogen and transmits the 'target cell–pathogen interaction signal' leading to derepressed *yop* expression. Second, YopN directs the formation of the Yop secretion apparatus to be assembled in the interaction zone between the target cell and the pathogen. This also excludes Ca^{2+} as a signal molecule during infection. From the results presented here, it is highly likely that the effects of Ca^{2+} are an *in vitro* artifact. It is possible that the YopN protein requires Ca^{2+} to maintain its 3D structure, as for example has been shown for some prokaryotic proteases (Thayer *et al.*, 1991). Thus, removal of Ca^{2+} *in vitro* would result in conformational changes which would mimic *in vivo* signals.

Interestingly, recent findings have indicated that there are homologous proteins involved in protein secretion in a number of non-related organisms (Salmond and Reeves, 1993; Van Gijsegem *et al.*, 1993). Of special interest in this context is the observation that the LcrD protein of *Yersinia* (Plano *et al.*, 1991) shows considerable homology (41% identity) to the FlbF protein of *Caulobacter crescentus* (Ramakrishnan *et al.*, 1991; Sanders *et al.*, 1992), and the FlhA protein of *Bacillus subtilis* (Carpenter *et al.*, 1992). The FlbF and the FlhA proteins are involved in flagellum biosynthesis, a process which shows similarities with the polarized transfer of YopE. Thus, it is possible that *Yersinia* has adopted the polarized transfer of proteins from similar mechanisms to those which have evolved to promote flagellum biosynthesis. Moreover, the plant pathogens of the genera *Xanthomonas, Pseudomonas* and *Erwinia* also have proteins similar to those involved in Yop secretion (Salmond and Reeves, 1993; Van Gijsegem *et al.*, 1993). For the plant pathogens, export proteins have been identified through the isolation of specific mutants of the corresponding genes. It therefore seems likely that the plant pathogens have a similar system of transfer of virulence proteins to those described herein. This could explain why the effector proteins have been difficult to study (Lindsay *et al.*, 1993) since in these cases the virulence determinants would be localized solely within the plant cell. It is perhaps fortunate that *Yersinia* shows a Ca^{2+} response *in vitro* otherwise the Yop-virulence determinants would not have been so easily discovered.

A model (Figure 9), which encompasses the present findings on the infectious process of virulent *Yersinia* can be described as follows. When *Yersinia* infects the mammalian host it encounters an environmental temperature of 37°C and millimolar levels of Ca^{2+}. Upon contact with target cells, expression and polarized transfer of YopE are induced via a YopN-mediated signal. YopE, and possibly other Yops, are efficiently and selectively translocated into the target cell. YopE then disrupts the filamentous actin network of the macrophage-target cell, leading to blockage of phagocytosis. This model could also provide an

explanation for Yop expression and secretion. In the absence of Ca^{2+}, the YopN protein adopts an induced conformation and the bacteria are then exposed to multiple YopN signals. This leads to derepressed *yop* expression followed by a non-polarized Yop secretion to the culture medium. Hence, our results presented here strongly support previous results obtained from *in vitro* studies.

Materials and methods

Bacterial strains, HeLa cells and the infectious procedure

The bacterial strains of *Y.pseudotuberculosis* and *Y.pestis* used in this study are listed in Table II. The bacterial strains were grown overnight in Luria Broth (LB) medium on a rotary shaker at 26°C and the bacterial density was determined by measuring the optical density at 550 nm in a spectrophotometer.

Cultivation and infection of HeLa cells have been described in detail elsewhere (Rosqvist *et al.*, 1990). Briefly, HeLa cells were grown as monolayers to semi-confluence on 12 mm coverslips in a 24 well tissue culture plate or in a 80 cm^2 tissue culture flask in Leibovitz L-15 medium with 10% heat inactivated fetal calf serum and 100 IU/ml penicillin, at 37°C in a humidified atmosphere. 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. After infection the HeLa cells were centrifuged for 5 min at 400 *g* to facilitate contact between the bacteria and the HeLa cells, whereafter the incubation were continued at 37°C.

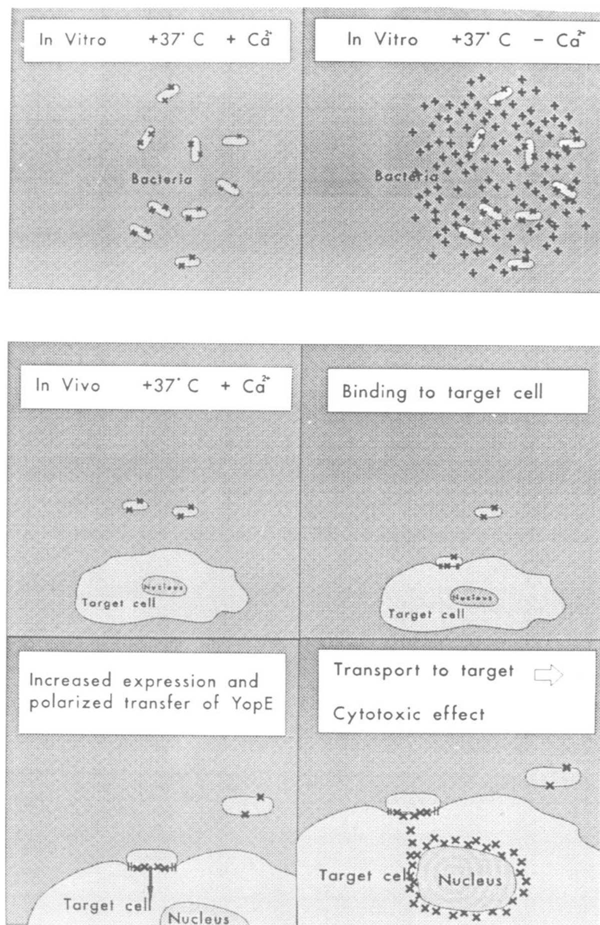

Fig. 9. Model of YopE expression and translocation *in vitro* and *in vivo*. See Discussion for further details.

Expression and secretion of YopE in vitro

About 1 × 10^8 bacteria from an overnight culture (26°C in Luria broth) of the wild-type strain YPIII(pIB102) or the *yopN* mutant YPIII(pIB82) were inoculated in 10 ml of tissue culture medium (L-15 medium containing 10% heat inactivated fetal calf serum) and 10 ml of brain heart infusion broth medium containing 20 mM $MgCl_2$ and 5 mM EGTA and incubated at 37°C. After 4.5 h the bacteria were separated from the growth medium by centrifugation for 10 min at 1700 *g*. The bacterial pellets were resuspended in 200 μl SDS-sample buffer. The growth media were passed through a 0.45 μm sterile filter and 10 μg of gentamicin was added. The bacteria-free media were then subjected to immunoprecipitation, as described earlier (Forsberg *et al.*, 1987), using a rabbit anti-Yop antiserum followed by incubation with protein A−Sepharose 4B beads to concentrate the precipitate. After washing, the protein A−Sepharose beads were resuspended in 100 μl SDS-sample buffer. Ten μl samples representing 5% of each fraction were analysed with SDS−PAGE. The gel was subjected to Western blot analysis using a purified mouse anti-YopE antibody and an alkaline phosphatase conjugated secondary anti-mouse antibody.

Expression and secretion of YopE in vivo

HeLa cells (5 × 10^6 per 80 cm^2) were infected with ~1 × 10^8 bacteria from an overnight culture (26°C in Luria broth). Thirty minutes after infection, the HeLa cell cultures were gently washed with 5 ml pre-warmed phosphate buffered saline (PBS) pH 7.3 to remove non-adherent bacteria and 10 ml of Leibovitz L-15 medium with 10% heat-inactivated fetal calf serum without any antibiotics added. After 4.5 h at 37°C, the tissue culture medium was collected and the HeLa cells were washed twice with ice-cold PBS. The collected medium and the washes were pooled (20 ml altogether) and centrifuged 10 min at 1700 *g*. The bacterial pellet was resuspended in 200 μl SDS-sample buffer. The supernatants were passed through a 0.45 μm sterile filter and gentamicin (10 μg/ml) and Triton X-100 (0.1%) were added. Yop proteins in 10 ml of the medium were then immunoprecipitated, as described above. The HeLa cell monolayers were lysed in 0.1% Triton X-100 and harvested with a cell scraper. Gentamicin (10 μg/ml) was added and the incubation was continued for 2 h at room temperature on a rotary shaker. The lysed HeLa cells were centrifuged for 10 min at 1700 *g*. The pellet of the lysed HeLa cells, consisting of bacteria, HeLa cell nuclei and cytoskeletons, was dissolved in 200 μl SDS-sample buffer. The supernatant, consisting of the released cytoplasmic content, was subjected to immunoprecipitation as described for the secreted Yop proteins using the same volume (10 ml) of antigens. Thus, half of the total released cytoplasmic content was subjected to immunoprecipitation. After washing, the protein A—Sepharose beads were resuspended in 100 μl SDS-sample buffer.

Table II. *Yersinia* strains used in this study

Strain	Relevant genotype	Reference to strain/plasmid
Yersinia pseudotuberculosis		
YPIII(pIB102)	wild-type	Bölin and Wolf-Watz (1984)
YPIII(pIB522)	*yopE*	Forsberg and Wolf-Watz (1990)
YPIII(pIB82)	*yopN*	Rosqvist *et al.* (1990)
YPIII(pIB15)	*yopD*	Rosqvist *et al.* (1991)
Yersinia pestis		
EV76Cp	plasmid cured	Rosqvist *et al.* (1990)
EV76Cp(pIB82)	*yopN*, *yadA*	This study
EV76Cp(pIB82, pAMS2)	*yopN*, *yadA*$^+$	This study, Rosqvist *et al.* (1990)
EV76Cp(pIB522, pAMS2)	*yopE*, *yadA*$^+$	This study, Rosqvist *et al.* (1990)

Ten μl samples representing 5% of each fraction were analysed by SDS–PAGE. The gel was subjected to Western blot analysis as described above.

Immunofluorescence staining

HeLa cells grown on coverslips ($\sim 0.5 \times 10^5$) were infected with different strains of *Y.pseudotuberculosis* ($\sim 2 \times 10^6$ bacteria per well). At various times after infection the cell monolayers were washed twice in PBS and fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100 and further processed for indirect immunofluorescence labelling (for details see Rosqvist *et al.*, 1991) using biotin-conjugated anti-rabbit antibodies and finally FITC-conjugated streptavidin. In double labelling experiments infected HeLa cells were stained 1.75 h post-infection with wheat germ agglutinin (WGA) conjugated to Texas Red (25 μg/ml for 5 min at 24°C), whereafter the specimens were fixed and permeabilized and incubated with affinity purified rabbit anti-YopE antibodies followed by FITC-conjugated anti-rabbit antibodies.

Fluorescence microscopy and image processing

The specimens were analysed using an epifluorescence microscope (Nikon, Diaphot) or a confocal laser scanning microscope equipped with dual detectors and an argon-krypton (Ar/Kr) laser for simultaneous scanning of two different fluorochromes (Multiprobe 2001, Molecular Dynamics, Sunnyvale, CA). Confocal microscopy was used to analyse YopE localization in the *yopD* mutant YPIII(pIB15) when binding to the HeLa cell surface. Sets of fluorescent images were acquired simultaneously for Texas Red and fluorescein-tagged markers. Companion images (30 sections with image size 256 × 256) were scanned with 0.07 μm pixel size and 0.2 μm step size. In the images obtained in the red channel (excitation 568 nm, WGA–Texas Red staining) background noise was subtracted. The images were then subjected to 3D reconstruction using surface shading to create an image of the HeLa cell membrane. The images obtained in the green channel (excitation 488 nm, YopE staining with fluoresceinated antibodies) were also processed to reduce background noise, and then presented as a look-through projection. By the use of volume visualization techniques (Kaufman, 1991) it was possible to highlight different aspects of the interaction between the *yopD* mutant and the HeLa cell surface. A low opacity value for the HeLa cell membrane (no look-through) and a low density value for YopE concentration allowed visualization of the upper part of the bacterium, showing that parts of the bacterium is outside of the HeLa cell (Figure 8B). In Figure 8C a high opacity value for the HeLa cell membrane and a high density value for the YopE concentration was used to determine the spatial localization of YopE.

Acknowledgements

We thank Debra Milton and Bernt-Eric Uhlin for critical reading of the manuscript. Peter Vestman, Fredrik Gustavsson and Phillip Dale, Department of Information Processing, University of Umeå, Sweden for image processing and the 3D reconstructions. This research was supported by the Swedish Natural Research Council, the Swedish Medical Research Council, the King Gustaf Vth 80 year Foundation, the Professor Nanna Svartz Foundation, the Swedish Society against Rheumatism, the Swedish Research Council for Engineering Sciences, the Erna and Victor Hasselblad Foundation, Magn. Bergvall Foundation and from Syskonen Perssons donations fund.

References

Allaoui,A., Sansonetti,P.J. and Parsot,C. (1993) *Mol. Microbiol.*, **7**, 59–68.
Andrews,G.P. and Maurelli,A.T. (1992) *Infect. Immun.*, **60**, 3287–3295.
Bergman,T., Håkansson,S., Forsberg,Å., Norlander,L., Macellaro,A. and Wolf-Watz,H. (1991) *J. Bacteriol.*, **173**, 1607–1616.
Bliska,J.B., Guan,K., Dixon,J.E. and Falkow,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 1187–1191.
Bölin,I. and Wolf-Watz,H. (1984) *Infect. Immun.*, **34**, 72–78.
Bölin,I. and Wolf-Watz,H. (1988) *Mol. Microbiol.*, **2**, 237–245.
Bölin,I., Portnoy,D.A. and Wolf-Watz,H. (1985) *Infect. Immun.*, **48**, 234–240.
Burrows,T. and Bacon,G.A. (1956) *Br. J. Exp. Pathol.*, **37**, 286–299.
Carpenter,F.B., Ordal,G.W. and Hanlon,D.W. (1992) *Mol. Microbiol.*, **6**, 2705–2713.
Cavanaugh,D. and Randall,R. (1959) *J. Immunol.*, **83**, 348–363.
Cornelis,G. (1992) In Hormaeche,C.W., Penn,C.W. and Smythe,C.J. (eds), *Molecular Biology of Bacterial Infections*. Cambridge University Press, Cambridge, UK, pp. 231–265.
Forsberg,Å. and Wolf-Watz,H. (1988) *Mol. Microbiol.*, **2**, 121–133.
Forsberg,Å. and Wolf-Watz,H. (1990) *J. Bacteriol.*, **172**, 1547–1555.
Forsberg,Å., Bölin,I., Norlander,L. and Wolf-Watz,H. (1987) *Microb. Pathogen.*, **2**, 123–137.
Forsberg,Å., Viitanen,A.-M., Skurnik,M. and Wolf-Watz,H. (1991) *Mol. Microbiol.*, **5**, 977–986.
Galyov,E.E., Håkansson,S., Forsberg,Å. and Wolf-Watz,H. (1993) *Nature*, **361**, 730–732.
Ginocchio,C., Pace,J. and Galán,J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5976–5980.
Guan,K. and Dixon,J.E. (1990) *Science*, **249**, 553–556.
Haddix,P.L. and Straley,C.S. (1992) *J. Bacteriol.*, **174**, 4820–4828.
Hanski,C., Kutschka,U., Schmoranzer,H.P., Naumann,M., Hahn,H., Menge,H. and Riecken,E.O. (1989) *Infect. Immun.*, **57**, 673–678.
Kaufman,A. (1991) In Kaufman,A. (ed.), *Volume Visualisation*. IEEE Computer Society Press, Washington DC.
Lambert de Rouvroit,C., Sluiters,C. and Cornelis,G.R. (1992) *Mol. Microbiol.*, **6**, 395–409.
Leung,K.Y., Reisner,B.S. and Straley,S.C. (1990) *Infect. Immun.*, **58**, 3262–3271.
Lian,C.-J., Hwang,W.S., Kelly,J.K. and Pai,C.H. (1987) *J. Med. Microbiol.*, **24**, 219–226.
Lindsay,W.P., Lamb,C.J. and Dixon,R.A. (1993) *Trends Microbiol.*, **1**, 181–186.
Michiels,T., Vanooteghem,J.-C., Lambert de Rouvroit,C., China,B., Gustin,P. and Cornelis,G.R. (1991) *J. Bacteriol.*, **173**, 4994–5009.
Plano,G.V., Barve,S.S. and Straley,S.C. (1991) *J. Bacteriol.*, **173**, 7293–7303.
Price,S.B. and Straley,S.C. (1989) *Infect. Immun.*, **57**, 1491–1498.
Ramakrishnan,G., Zhao,J. and Newton,A. (1991) *J. Bacteriol.*, **173**, 7286–7292.
Reisner,B.S. and Straley,S.C. (1992) *Infect. Immun.*, **60**, 5242–5252.
Rosqvist,R. and Wolf-Watz,H. (1986) *Microb. Pathogen.*, **1**, 229–240.
Rosqvist,R., Bölin,I. and Wolf-Watz,H. (1988) *Infect. Immun.*, **56**, 2139–2143.
Rosqvist,R., Forsberg,Å., Rimpiläinen,M., Bergman,T. and Wolf-Watz,H. (1990) *Mol. Microbiol.*, **4**, 657–667.
Rosqvist,R., Forsberg,Å. and Wolf-Watz,H. (1991) *Infect. Immun.*, **59**, 4562–4569.
Salmond,G.P.C. and Reeves,P.J. (1993) *Trends Biochem. Sci.*, **18**, 7–12.
Sanders,L.A., Van Way,S. and Mullin,D.A. (1992) *J. Bacteriol.*, **174**, 857–866.
Simonet,M., Richard,S. and Berche,P. (1990) *Infect. Immun.*, **58**, 841–845.
Straley,S.C., Skrzypek,E., Plano,G.V. and Bliska,J.B. (1993) *Infect. Immun.*, **61**, 3105–3110.
Thayer,M.M., Flaherty,K.M. and McKay,D.B. (1991) *J. Biol. Chem.*, **266**, 2864–2871.
Van Gijsegem,F., Genin,S. and Boucher,C. (1993) *Trends Microbiol.*, **1**, 175–180.
Yother,J. and Goguen,J.D. (1985) *J. Bacteriol.*, **164**, 704–711.

Received on August 30, 1993; revised on October 7, 1993