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Competition between the Yops of *Yersinia enterocolitica* for Delivery into Eukaryotic Cells: Role of the SycE Chaperone Binding Domain of YopE

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A type III secretion-translocation system allows *Yersinia* adhering at the surface of animal cells to deliver a cocktail of effector Yops (YopH, -O, -P, -E, -M, and -T) into the cytosol of these cells. Residues or codons 1 to 77 contain all the information required for the complete delivery of YopE into the target cell (release from the bacterium and translocation across the eukaryotic cell membrane). Residues or codons 1 to 15 are sufficient for release from the wild-type bacterium under Ca\(^{2+}\)-chelating conditions but not for delivery into target cells. Residues 15 to 50 comprise the binding domain for SycE, a chaperone specific for YopE that is necessary for release and translocation of full-length YopE. To understand the role of this chaperone, we studied the delivery of YopE-Cya reporter proteins and YopE deletants by polymutant *Yersinia* devoid of most of the Yop effectors (ΔHOPEM and ΔTHE strains). We first tested YopE-Cya hybrid proteins and YopE proteins deleted of the SycE-binding site. In contrast to wild-type strains, these mutants delivered YopE<sub>15-77</sub>-Cya as efficiently as YopE<sub>130-77</sub>-Cya. They were also able to deliver YopE<sub>130-77</sub>-SycE was dispensable for these deliveries. These results show that residues or codons 1 to 15 are sufficient for delivery into eukaryotic cells and that there is no specific translocation signal in Yops. However, the fact that the SycE-binding site and SycE were necessary for delivery of YopE by wild-type *Yersinia* suggests that they could introduce hierarchy among the effectors to be delivered. We then tested a YopE-Cya hybrid and YopE proteins deleted of amino acids 2 to 15 but containing the SycE-binding domain. These constructs were neither released in vitro upon Ca\(^{2+}\) chelation nor delivered into cells by wild-type or polymutant bacteria, casting doubts on the hypothesis that SycE could be a secretion pilot. Finally, it appeared that residues 50 to 77 are inhibitory to YopE release and that binding of SycE overcomes this inhibitory effect. Removal of this domain allowed in vitro release and delivery in cells in the absence as well as in the presence of SycE.

The three *Yersinia* species that are pathogenic to humans (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) all share the ability to deliver toxins, called YopE, YopH, YopM, YopT, YopO/YpkA, and YopP/YopJ, into eukaryotic host cells (8). These toxic Yop effectors induce a range of modifications to the normal processes of eukaryotic cells. For example, YopE has a GTPase-activating protein activity which downregulates Rho activity and leads to actin filament disruption and inhibition of phagocytosis by macrophages (21, 22, 28, 31). Together with their complex type III Ysc machinery for export and translocation, the Yops are encoded by a 70-kb virulence plasmid (8). Similar to structures observed in other bacteria endowed with type III secretion, the Yop secretion apparatus—the injectosome—is thought to form a “syringe” directly projecting through the bacterial membranes with a “needle” that connects to the translocation apparatus in the eukaryotic cell membrane (8, 10a). Secretion and translocation of the Yop effectors are normally triggered by contact with a eukaryotic cell. However, secretion can be artificially induced by chelating Ca\(^{2+}\) ions, which leads to a massive release of Yops into the culture supernatant.

A secretion signal for the Yops is located at the 5′ end of the gene (16, 29). It has been proposed that this signal could be in the mRNA, so that the Yops are cotranslationally secreted from the bacteria (1, 2). In the case of YopE, the first 15 codons or amino acids constitute this 5′ secretion signal. In addition, efficient secretion of some Yops requires the assistance of individual cytosolic chaperones, called Sycs (32, 33). These chaperones are small acidic proteins that possess a leucine repeat in their C-terminal moieties. SycE, the chaperone of YopE, binds amino acids 15 to 50 (the chaperone binding domain) of YopE (27, 35), and it prevents the intrabacterial degradation of this Yop (5, 10). The chaperone binding domain is not required for secretion of YopE fusion proteins by the 5′ secretion signal (5, 27, 28). Moreover, in the absence of this chaperone binding domain, SycE becomes dispensable for secretion of YopE, suggesting that it is the presence of the chaperone binding domain that creates the need for the chaperone (35). However, data have been presented to show that hybrid YopE-neomycin phosphotransferase (designated YopE-Npt) proteins lacking the first 5′ secretion signal are still secreted by the Ysc apparatus, suggesting that YopE could contain a second secretion signal (5). This proposed second secretion signal is localized to the site of the chaperone binding domain and, correspondingly, it is only operational in the presence of SycE (5).

Translocation of the effector Yops across the eukaryotic cell membrane was shown by several laboratories (4, 12, 20, 23) to be dependent on YopB and YopD, two other proteins exported by the bacterium, but this view has recently been questioned (15). Translocation of effector Yops can be demonstrated by several methods. A classical approach makes use of...
a calmodulin-dependent adenylyl cyclase (Cya) reporter strategy (29). Translocation of Yop effectors can also be demonstrated by fractionation of the infected cell culture or by indirect immunofluorescence and confocal scanning laser microscopy (23). Demonstration of the translocation turned out to be more difficult with some Yops than with others, and it has been observed that translocation can be improved if expression of the other Yop effectors is abolished (4, 11). This could be due to a decrease in competition between the different Yop effectors for the secretion and translocation machineries. Strains of enterobacteria that carry multiple yop mutations are thus sensitive tools for studying the translocation of Yop effectors.

It has been shown previously that a Cya reporter protein fused to just the first 15 amino acids of YopE (YopE15-Cya) can be released by wild-type (wt) bacteria upon Ca\(^{2+}\) chelation; however, this fusion protein is not delivered into eukaryotic cells. Indeed, at least the first 50 amino acids are required for the reporter protein to be translocated into eukaryotic cells by wt bacteria (27, 28). Therefore, amino acids 15 to 50, which are the residues that bind the SecE chaperone and which constitute the proposed second secretion signal, were thought to be a translocation domain (27, 28), although they are not sufficient, in the absence of the 5’ secretion signal, to direct delivery of YopE by wt bacteria into eukaryotic cells (14). In this study, we investigated the requirement for the two secretion signals for delivery of YopE into eukaryotic cells. We confirmed that SecE and residues 15 to 50 of YopE are required for delivery of YopE by wt bacteria, but we observed that they are dispensable for delivery by a multiantigen system. This suggests that SecE could be a hierarchy factor for YopE delivery. Moreover, we identified a secretion-inhibitory domain between residues 50 and 77.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** Parental wt strain Y. enterocolitica MRS40(pYV40) is an ampicillin-sensitive derivative of serotype O:9 clinical isolate E40(pYV40) (25, 29). Excherichia coli K111, XL-1 Blue, and BL21 were used for plasmid construction and protein expression. Strain SM10pur was used to conjugate plasmids into Y. enterocolitica. The full list of plasmids used in this study is given in Table 1. Bacterial strains were routinely grown in tryptic soy broth and plated on tryptic soy agar. For in vitro induction of plasmids used in this study is given in Table 1. Bacterial strains were routinely grown in tryptic soy broth and plated on tryptic soy agar. For in vitro induction, E. coli SM10pir yopE15-cya was grown in brain heart infusion (BHI) medium supplemented with 20 mM sodium oxalate, 20 mM MgCl\(_2\), and 0.4% (wt/vol) glucose (BHI-Ox). Yop induction under minimal-medium conditions was performed as described previously (5). Selective agents were used at the following concentrations: ampicillin, 200 \(\mu\)g/ml; chloramphenicol, 10 \(\mu\)g/ml; nalidixic acid, 35 \(\mu\)g/ml; streptomycin, 100 \(\mu\)g/ml; sucrose, 5% (wt/vol); and arsenite, 0.4 mM.

**Molecular biology techniques.** Molecular biology techniques were essentially performed as previously described (24). All chemicals were obtained from Sigma unless stated otherwise. Yops were precipitated from culture supernatants by adding an equivalent volume of ethanol and centrifuged at 14,000 × g for 15 min. The supernatant was removed, and the pellets were then washed with 80% ethanol. The pellets were then dissolved in water, and the protein concentrations were determined by the method of Lowry et al. (30).

**Yop translocation assay.** The PU.5-1.8 mouse monocyte/macrophage cell line (ATCC TIB-61) used in these studies was grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% (wt/vol) fetal bovine serum, 2 mM l-glutamine, and 100 U/ml penicillin and streptomycin (no antibiotics). Cells were cultured at a density of 5 × 10\(^4\) cells per ml of medium per well and allowed to adhere for 24 h. After infection with Y. enterocolitica, cells were washed and covered with RPMI 1640 supplemented only with 2 mM l-glutamine. Cytochalasin D was added 30 min before infection, at a final concentration of 5 \(\mu\)g/ml (solution, 2 mg/ml in dimethyl sulfoxide). Cytochalasin D is not toxic to Y. enterocolitica at this concentration (29). A freshly isolated transconjugant colony of Y. enterocolitica was grown overnight at 22°C and diluted the next day to an optical density at 600 nm of 0.2 in 5 ml of BHI medium. After being grown with shaking at 22°C for 2 h, bacteria were washed and suspended in saline. Samples of 100 ml containing about 10\(^5\) bacteria (multiplicity of infection, 20), were added to the monolayer, and the infected cultures were incubated at 37°C for 2 h in a 6% CO\(_2\) atmosphere. Cells were washed and then lysed under denaturing conditions (100°C for 5 min in 50 mM HCl and 0.1% [wt/vol] Triton X-100). The lysate was neutralized by NaOH, and cyclic AMP (cAMP) was measured by an enzyme immunoassay (Biotek Amersham). All experiments were performed three times.

**Cytotoxicity assay.** The HeLa human epithelial cell line (ATCC CCL-2) used in these studies was grown in RPMI 1640 supplemented with 10% (wt/vol) fetal bovine serum and 2 mM l-glutamine, and streptomycin (100 \(\mu\)g/ml) and were exposed to the macrophages as described above except that the HeLa cells were seeded at a density of 7 × 10\(^4\) cells ml\(^{-1}\). Bacteria were pregrown as described above, and cells were infected with Y. enterocolitica at a multiplicity of infection of 70. Two to three hours after infection, the morphology of the cells was observed by phase-contrast microscopy. The cells became rounded as a result of cytotoxicity.
TABLE 1. Plasmids used in this study

| Plasmid | Relevant description | Reference or source |
|---------|----------------------|---------------------|
| pYYV plasmids |                                     |                     |
| pYYV40 | Wild type |                                     |
| pAB4052 | ΔYopE yopE29 | 17 |
| pABB409 | ΔHOPEM yopB | 3 |
| pABL403 | ΔHOPEM yopH32–352 yopO65–558 yopP33 yopE29 | 3 |
| pABB4054 | ΔSycE | This work |
| pABB4055 | ΔSycE | This work |
| pABB40416 | ΔTIEEB | This work |
| pMM426 | yopH32–352 yopO65–558 yopP33 yopE29 yopM23 | This work |
| pMSK49 | ΔHOPEMYopD yopH32–352 yopO65–558 yopP23 yopE29 yopM23 yopH31–165 | 18 |
| pMSK50 | ΔHOPEMYopC yopH32–352 yopO65–558 yopP23 yopE29 yopM23 yopH31–165 yscN355–170 | Sory and Cornelis, unpublished |
| pML41 | ΔSycN yscN355–170 | 28 |
| Other plasmids |                                     |                     |
| pABB24 | pQE-30 containing his-sycE under P23 promoter control | This work |
| pABB26 | pCN26 containing yopE under yopE promoter control | 4a |
| pABB35 | pCN26 containing yopE23–27 under yopE promoter control | This work |
| pABB36 | pCN26 containing yopE112–115 under yopE promoter control | This work |
| pABB17 | pCN26 containing yopE23–27 under yopE promoter control | This work |
| pABB16 | pBluescript KS (containing yopE23–27) plus yscE | This work |
| pABB17 | pBluescript KS (containing yopE112–115) plus yscE | This work |
| pABB18 | pTM100 containing yopE125–254 and yscE | This work |
| pABB19 | pCN26 containing yopE23–27 under yopE promoter control | This work |
| pABB34 | pCN26 containing yopE112–115 under yopE promoter control |This work |
| pABB38 | pBluescript KS (containing yopE112–115) plus yscE | This work |
| pABB40 | pBluescript KS (containing yopE23–27) plus yscE | This work |
| pABB42 | pBluescript KS (containing yopE112–115) plus yscE | This work |
| pABB43 | pBluescript KS (containing yopE23–27) plus yscE | This work |
| pABB44 | pBluescript KS (containing yopE112–115) plus yscE | This work |
| pABB45 | pBluescript KS (containing yopE23–27) plus yscE | This work |
| pABB47 | pTM100 containing yopE130–112–115 yscE | This work |
| pABB48 | pTM100 containing yopE130–112–115 yscE and yscE | This work |
| pABB49 | pTM100 containing yopE130–112–115 yscE and yscE | This work |
| pABB50 | pTM100 containing yopE130–112–115 yscE and yscE | This work |
| pBC18R | Amp4, Pm cards, expression vector | 7 |
| pBC19R | Amp4, Pm cards, expression vector | 7 |
| pBC5 | pBC19R containing yopD yopP yopE and yopO | 7 |
| pBluescript | Amp4, lac Z, cloning vector | Stratagene |
| pCN26 | Amp4, Pm cards, expression vector | 25 |
| pILL14 | pCN26 containing yopE50–77 under yopE promoter control | This work |
| pIM153 | pBC18R containing yopM under yopM and lac promoter control in HisCII fragment | Iriarte and Cornelis, unpublished |
| pNKG101 | Smr, sucBC, suicide vector | 13 |
| pMS111 | pTM100 containing yopE130–115 yscE and yscE | 29 |
| pMSK13 | pCN26 containing yopP under yopE promoter control | 17 |
| pMSL30 | pTM100 containing yopE130–115 yscE and yscE | 35 |
| pMSL56 | pTM100 containing yopE130–115 yscE and yscE | Sory and Cornelis, unpublished |
| pMSL15 | pTM100 containing yopE130–115 yscE and yscE | 28 |
| pPW54 | pNKG101 containing yscE | 33 |
| pOE30 | Amp4, Pm cards, His expression vector | Qiagen |
| pSw6 | pNKG101 containing yscN355–170 | 34 |
| pTM100 | CmR, medium-copy-number cloning vector | 16 |
| pTM163 | pBC18R containing yopE under yopE and lac promoter control | 16 |
| pYO82 | pCN26 containing yopO under yopE promoter control | Guenens and Cornelis |

Staining of actin filaments with phalloidin. Rat 1 fibroblasts grown on coverslips were infected with the different strains. After 2.5 h of infection, the cells were fixed in 2% (wt/vol) parafomaldehyde for 20 min. After being washed with phosphate-buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4 [pH 7.4]), membranes were permeabilized with 0.5% (wt/vol) Triton X-100 in PBS for 10 min. Cells were then incubated for 40 min at 37°C with fluorescein isothiocyanate-conjugated phalloidin. Samples were mounted on Mowiol and examined by fluorescence microscopy.

SycE-binding assays. Native SycE was produced and purified as described in reference 33. His6-SycE was produced in E. coli XL-1 Blue (pAB824) and purified on a His-Trap column by elution with 300 mM imidazole according to the manufacturer's instructions (Pharmacia Biotech). Total cell proteins of Y. enterocolitica were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blockage in PBS plus BSA (PBS plus 0.1% Tween 20 plus 0.5% bovine serum albumin [BSA]), the membrane was incubated with His6-SycE (0.5 μg · ml−1) in PBST plus BSA for 2 h at room temperature. Bound SycE or His6-SycE was revealed with anti-SycE or anti-His antibody (Pharmacia Biotech), respectively, followed by HRP-conjugated secondary antibody and chemiluminescence detection.

RESULTS

Translocation of YopE125-Cya into eukaryotic cells by Yop effector polymutant Y. enterocolitica. It has been previously demonstrated that wt bacteria deliver YopE120-Cya, but not YopE125-Cya, into eukaryotic cells, suggesting that the 5’ se-
cretion signal is not sufficient for YopE translocation (28). We repeated these experiments using HOPEM polymutant bacteria that lack the YopH, YopO, YopP, YopE, and YopM effectors (Table 1). Delivery of YopE15-Cya (encoded by plasmid pMSLE15 [8]) into the PU5-1.8 macrophage-monocyte cell line by HOPEM and by wt Y. enterocolitica was compared. As a control, translocation of YopE130-Cya (encoded by plasmid pMS111 [29]) by the same bacteria was also monitored. In agreement with previously published results (28), wt bacteria delivered YopE130-Cya, but not YopE15-Cya, into eukaryotic cells. In contrast, HOPEM bacteria delivered YopE15-Cya just as efficiently as YopE130-Cya (Table 2). Likewise, YopE30-Cya, YopE24-Cya, and YopE30-Cya (28) were delivered into eukaryotic cells by HOPEM bacteria (data not shown). To confirm that delivery of YopE15-Cya into eukaryotic cells by HOPEM bacteria was due to the type III secretion-translocation system, the delivery of YopE15-Cya by HOPEM YscN bacteria (secretion deficient) and HOPEM YopB and HOPEM YopD bacteria (both translocation deficient) was tested (Table 2). YopE15-Cya was not translocated by these strains, confirming that YopE15-Cya was indeed delivered into eukaryotic cells by the type III injectosome (Table 2). To assess the necessity for the 5' secretion signal, delivery of Cya fused to the first two amino acids of

| Y. enterocolitica strain | Intracellular cAMP accumulation (nmol of cAMP/mg) |
|-------------------------|-----------------------------------------------|
|                         | YopE15-Cya | YopE130-Cya |
| wt                      | 0.4 ± 0.2  | 22.8 ± 4.3  |
| ΔHOPEM                  | 14.3 ± 2.3 | 18.1 ± 3.0  |
| ΔHOPEMYopB              | 0.2 ± 0.1  | 0.1 ± 0.1   |
| ΔHOPEMYopD              | 0.2 ± 0.1  | 0.1 ± 0.1   |
| ΔHOPEMYscN              | 0.1 ± 0.1  | 0.1 ± 0.1   |
| ΔSycE                   | 0.8b       | 3.8 ± 2.2   |
| ΔHOPEMSycE              | 16.5 ± 2.7 | 3.2 ± 0.4   |

a Mean ± SD from three independent experiments.
b Mean of two independent experiments.

mid pMSLE15 [8]) into the PU5-1.8 macrophage-monocyte cell line by HOPEM and by wt Y. enterocolitica was compared. As a control, translocation of YopE130-Cya (encoded by plasmid pMS111 [29]) by the same bacteria was also monitored. In agreement with previously published results (28), wt bacteria delivered YopE130-Cya, but not YopE15-Cya, into eukaryotic cells. In contrast, HOPEM bacteria delivered YopE15-Cya just as efficiently as YopE130-Cya (Table 2). Likewise, YopE30-Cya, YopE24-Cya, and YopE30-Cya (28) were delivered into eukaryotic cells by HOPEM bacteria (data not shown). To confirm that delivery of YopE15-Cya into eukaryotic cells by HOPEM bacteria was due to the type III secretion-translocation system, the delivery of YopE15-Cya by HOPEM YscN bacteria (secretion deficient) and HOPEM YopB and HOPEM YopD bacteria (both translocation deficient) was tested (Table 2). YopE15-Cya was not translocated by these strains, confirming that YopE15-Cya was indeed delivered into eukaryotic cells by the type III injectosome (Table 2). To assess the necessity for the 5' secretion signal, delivery of Cya fused to the first two amino acids of

### TABLE 2. Translocation of YopE15-Cya and YopE130-Cya into PU5-1.8 macrophages by Y. enterocolitica

| Y. enterocolitica strain | Intracellular cAMP accumulation (nmol of cAMP/mg) |
|-------------------------|-----------------------------------------------|
|                         | YopE15-Cya | YopE130-Cya |
| wt                      | 0.4 ± 0.2  | 22.8 ± 4.3  |
| ΔHOPEM                  | 14.3 ± 2.3 | 18.1 ± 3.0  |
| ΔHOPEMYopB              | 0.2 ± 0.1  | 0.1 ± 0.1   |
| ΔHOPEMYopD              | 0.2 ± 0.1  | 0.1 ± 0.1   |
| ΔHOPEMYscN              | 0.1 ± 0.1  | 0.1 ± 0.1   |
| ΔSycE                   | 0.8b       | 3.8 ± 2.2   |
| ΔHOPEMSycE              | 16.5 ± 2.7 | 3.2 ± 0.4   |

a Mean ± SD from three independent experiments.
b Mean of two independent experiments.

mid pMSLE15 [8]) into the PU5-1.8 macrophage-monocyte cell line by HOPEM and by wt Y. enterocolitica was compared. As a control, translocation of YopE130-Cya (encoded by plasmid pMS111 [29]) by the same bacteria was also monitored. In agreement with previously published results (28), wt bacteria delivered YopE130-Cya, but not YopE15-Cya, into eukaryotic cells. In contrast, HOPEM bacteria delivered YopE15-Cya just as efficiently as YopE130-Cya (Table 2). Likewise, YopE30-Cya, YopE24-Cya, and YopE30-Cya (28) were delivered into eukaryotic cells by HOPEM bacteria (data not shown). To confirm that delivery of YopE15-Cya into eukaryotic cells by HOPEM bacteria was due to the type III secretion-translocation system, the delivery of YopE15-Cya by HOPEM YscN bacteria (secretion deficient) and HOPEM YopB and HOPEM YopD bacteria (both translocation deficient) was tested (Table 2). YopE15-Cya was not translocated by these strains, confirming that YopE15-Cya was indeed delivered into eukaryotic cells by the type III injectosome (Table 2). To assess the necessity for the 5' secretion signal, delivery of Cya fused to the first two amino acids of

### FIG. 1. Ca²⁺ chelation-triggered release of YopE130-Cya and YopE15-Cya by wt and HOPEM Y. enterocolitica. The role of Ysc and SycE is shown. (A) Immunoblot probed with calmodulin-biotin and streptavidin-HRP to detect bacteria-associated YopE130-Cya or YopE15-Cya (Bact). A total of 1.5 × 10⁹ Y. enterocolitica bacteria grown under BHI-Ox conditions were loaded in each lane. (B and C) Coomassie blue-stained SDS-PAGE gel of proteins secreted (SN) by various Y. enterocolitica encoding YopE130-Cya or YopE15-Cya and grown under BHI-Ox conditions. Arrowheads indicate YopE130-Cya and YopE15-Cya. The hybrid proteins were encoded by pMS111 (YopE130-Cya plus SycE), pMSL30 (YopE130-Cya only), pMSLE15 (YopE15-Cya plus SycE) and pAPB18 (YopE15-Cya alone). The host strains were MRS40(pYV40) (wt), MRS40(pAPB403) (ΔHOPEM), MRS40(pAPB4054) (ΔSycE), MRS40(pAPB4055) (ΔHOPEM SycE), MRS40(pMSL41) (wt ΔYscN), and MRS40(pMSK50) (ΔHOPEM YscN). In each lane, the proteins released by 1.5 × 10⁹ bacteria were loaded. Arrowheads point to YopE130-Cya or YopE15-Cya.
YopE (encoded by plasmid pMSL56) (Table 1) into eukaryotic cells was measured. This fusion protein was not delivered into macrophages by either wt (0.1 ± 0.1 nmole of cAMP/mg) or ΔHOPEM (0.1 ± 0.1 nmole of cAMP/mg) bacteria, showing that a Yop secretion signal is required for delivery of a protein by ΔHOPEM Y. enterocolitica. These results show that the translocation system of ΔHOPEM bacteria is still specific for the Yops. In conclusion, translocation of YopE-Cya hybrids is possible without the previously described translocation domain, which comprises amino acids 15 to 50, but not without the 5’ secretion signal (residues or codons 1 to 15 and upstream RNA).

Production and secretion levels of YopE 15-Cya by wt and ΔHOPEM bacteria. It was next investigated whether the delivery of YopE 15-Cya by ΔHOPEM, but not wt Y. enterocolitica, could result from differences between strains in the production and secretion of this hybrid protein. Protein levels were analyzed following growth of the bacteria in Ca^{2+}-chelating conditions, which induce Yop production and release. No differences were seen between the two strains in the levels of YopE 15-Cya associated with the bacteria or released into the extracellular medium (Fig. 1A and Fig. 1B, lanes 5 and 6). Secretion of YopE 15-Cya by both strains was strictly dependent on the Ysc system, since no secretion was observed in a yscN background (Fig. 1C). Although Yop release upon Ca^{2+} chelation may not necessarily reflect exactly what occurs upon contact of Y. enterocolitica with eukaryotic cells, these data do show that synthesis of the two fusion proteins and their passage through the bacterial membranes was equally efficient in the two strains and equally dependent on Ysc. The only difference between the two strains with regard to YopE 15-Cya was thus the level of translocation of this protein into eukaryotic cells (Table 2). This suggests that the presence of additional Yops in the wild type directly reduces translocation of YopE 15-Cya and that in order to enter into eukaryotic cells the Yops must thus compete with one another for passage through the secretion-translocation machinery.

Influence of SycE on secretion and translocation of YopE 15-Cya. In order to investigate the requirement for SycE for translocation of YopE 15-Cya and YopE 130-Cya into eukaryotic cells, an sycE mutation was introduced into the wt and ΔHOPEM strains (Table 1). The experiment was carried out with plasmids pMS111 and pMSLE15, which encode SycE along with YopE 130-Cya and YopE 15-Cya, respectively, and with plasmids pMSL30 and pAPBD18, which encode only the YopE-Cya fusion proteins (Table 1). The presence or absence of SycE did not affect the steady-state levels of YopE 130-Cya or YopE 15-Cya associated with the wt or ΔHOPEM bacteria when grown under BHLOx conditions (Fig. 1A). However, SycE was required for efficient secretion and translocation of YopE 130-Cya into eukaryotic cells not only by wild-type but also by ΔHOPEM bacteria (Fig. 1B, compare lanes 1 and 3 and lanes 2 and 4; Table 2). In contrast, the presence or absence of SycE did not influence secretion or translocation of YopE 15-Cya into eukaryotic cells by ΔHOPEM bacteria (Fig. 1B, compare lanes 5 and 7 and lanes 6 and 8; Table 2). Thus, efficient
delivery of YopE15-Cya by ΔHOPEM bacteria occurred in the absence of SycE. We conclude from this that SycE is only required for efficient secretion and subsequent translocation when its binding domain is present. However, when the chaperone binding domain is present, the chaperone is required, irrespective of the presence of other effectors.

Translocation into eukaryotic cells of YopE lacking the SycE chaperone binding domain. In order to confirm that codons or amino acids 1 to 15 of YopE are sufficient to translocate YopE into eukaryotic cells, we removed the chaperone binding domain (YopEΔ17–77) from YopE (Fig. 2), and we checked this removal by a SycE overlay experiment (33). Purified SycE or His6-SycE bound YopE but failed to bind YopEΔ17–77, verifying that the chaperone binding domain had been deleted from the latter protein (Fig. 3B and D). ΔHOPEM bacteria could not be used for cytotoxicity experiments because they still produce the YopT cytotoxin (8). We thus turned to ΔTHE bacteria (Table 1), which do not induce any morphological changes in eukaryotic cells (Fig. 4). YopEΔ17–77 was produced and released by ΔTHE bacteria (Fig. 3A and 3C), and release of YopEΔ17–77 did not occur in a yscN background (Fig. 5), confirming that this release was type III dependent. Delivery was then assayed by monitoring the rounding up of HeLa epithelial cells and by staining the actin of Rat I fibroblasts. ΔTHE Y. enterocolitica strains producing YopE or YopEΔ17–77 were cytotoxic for HeLa epithelial cells (results not shown) and Rat I fibroblasts (Fig. 4), while ΔTHEB bacteria producing YopEΔ17–77 were not cytotoxic, indicating that translocation of YopEΔ17–77 was YopB dependent. This result confirmed that the first 16 amino acids of YopE are sufficient for delivery into
eukaryotic cells and that the chaperone binding domain is not required.

Competition could play an important role in determining the level of translocation. YopE15-Cya was delivered into eukaryotic cells by ΔHOPEM bacteria, but not by wt bacteria, suggesting that competition between the Yops is an important determinant for secretion and translocation and that the chaperone binding domain plays a significant role with regards to this competition. To investigate this theory, the ability of ΔHOPEM bacteria to deliver YopE15-Cya (encoded by pMSLE15) into eukaryotic cells when overproducing another Yop effector in trans was tested. Therefore, the translocation of YopE15-Cya into eukaryotic cells by ΔHOPEM bacteria overproducing YopH, YopO, YopP, YopE, or YopM was measured. ΔHOPEM(pMSLE15)(pBC18R) served as a vector control. In each case, the rate of translocation of YopE15-Cya into eukaryotic cells was lower than that of ΔHOPEM bacteria not overexpressing one of these Yop effectors in trans (Table 3). In contrast, translocation into eukaryotic cells of YopE130-Cya by ΔHOPEM was unaffected by overproducing another Yop in trans. The strongest effect on delivery of YopE15-Cya into eukaryotic cells was lower than that of ΔHOPEM bacteria not overexpressing one of these Yop effectors in trans (Table 3). As a control, we checked the profile of proteins released by these strains upon Ca²⁺ chelation. This control (data not shown) confirmed the overproduction of the Yops encoded in trans. Unfortunately, it also showed a concomitant reduction in the release of the Cya reporter and of the translocators LcrV, YopB, and YopD, indicating that the previous results must be interpreted with caution. To circumvent these difficulties, presumably linked to titration, we tested whether YopE15-Cya could be delivered into cells by Y. enterocolitica bacteria missing only YopE (ΔYopE strain, plasmid pAB4052). Delivery by the ΔYopE strain led to the synthesis of 2.7 ± 0.6 nmole of cAMP/mg of protein, while delivery by the wt strain led only to the synthesis of 0.4 ± 0.2 nmole of cAMP/mg of protein. Thus,
lack of YopE alone significantly increased delivery of YopE

Cya into eukaryotic cells. These results are consistent with the
idea that amino acids 15 to 50 promote translocation of YopE
by wt bacteria by assisting YopE to compete with other Yops
for the secretion-translocation apparatus. If this was so, one
would expect that YopE deprived of its chaperone binding
domain (YopE17–77) would not compete with YopE15-Cya for
delivery into eukaryotic cells. We thus overproduced YopE17–77
in trans, and we monitored translocation of YopE15-Cya. As
expected, overproduction of YopE17–77 did not inhibit trans-
location of YopE15-Cya (Table 3). Thus, amino acids 15 to 50
of YopE, in conjunction with SycE, seem to give YopE a
competitive advantage over the other Yops for the secretion-
translocation process.

Role of proposed second secretion signal in translocation.

Since the first secretion signal (amino acids or codons 1 to 15)
was found to be sufficient for translocation into eukaryotic
cells, we wondered whether the second secretion signal (amino
acids 15 to 100) proposed by Cheng et al. (5) would also be
sufficient to direct translocation into eukaryotic cells by the
Yop effector multmutant strain ΔHOPEM. This second
signal was previously shown to be insufficient for delivery
into eukaryotic cells by wt bacteria (14). Therefore, three
plasmids were constructed encoding YopE proteins lacking
the first secretion signal (amino acids or codons 2 to 15).
Plasmid pAPB35 encodes YopE12–15. Plasmid pAPB36 en-
codes YopE15–112–151, in which amino acids 2 to 15 have
been shifted out of frame by the addition of 1 bp after the ATG
and by compensatory changes before codon 16. A similar construc-
tion has previously been shown to have an inactive first secretion
signal and to be secreted by the proposed second secretion
signal (5). As well, plasmid pAPB37 encodes YopE12–77. The
three constructs were checked first for their capacity to bind
His6-SycE in an overlay assay. As expected, YopE12–15 did
not bind SycE, while YopE12–77 and YopE15–112–151 were rec-
nized by the chaperone (Fig. 3D). Each of the three proteins
was produced by ΔTHE bacteria, but no secretion when grown
in BHI-Ox medium could be detected (Fig. 3A). This result
was expected for YopE12–77, since it lacks both the first 5’
signal and the proposed second secretion signal, but not for
the two others. Surprised by the inability of amino acids 15 to 50
(the proposed second secretion signal) to promote secretion
of YopE12–15 or YopE15–112–151, the secretion of these pro-
teins was tested under the same minimal-medium conditions
as those used by Cheng et al. (5). Under these conditions,
the proteins were produced but not secreted by ΔTHE bac-
teria (Fig. 6). In accordance with their non-secretion pheno-
type, neither ΔTHE encoding YopE12–15, ΔTHE encoding
YopE15–112–151, nor ΔTHE encoding YopE12–77 was cytotoxic
for HeLa cells (data not shown) and Rat I cells (Fig. 4).

In addition, plasmids encoding YopE130-Cya reporter pro-
teins lacking the first 5’ signal sequence were constructed.
Plasmid pAPB50 encodes YopE130(12–15)-Cya and plasmid
pAPB51 encodes YopE130(12–15)-Cya. These proteins were
not translocated into eukaryotic cells by either of these strains
of Y. enterocolitica (intracellular cAMP concentration, 0.1 ±
0.1 ng of cAMP/mg). From the experiments with modified
full-length YopE and YopE130-Cya, we conclude that under
our experimental conditions, the proposed second secretion
signal is not functional and that the only functional secretion
signal for YopE is contained within amino acids or codons 1 to
15.

A secretion-inhibitory sequence localized between residues
50 and 77. While constructing plasmids encoding YopE de-
leted of its SycE-binding site, we constructed pAPBG30, which
encodes YopE17–49 (Table 1). Like YopE17–77, YopE17–49
did not bind SycE in an overlay experiment, since they both
lack the chaperone binding domain at amino acids 15 to 50
(Fig. 3D). Unlike YopE17–77, which was efficiently secreted by
Y. enterocolitica, YopE17–49 was neither secreted (Fig. 3A)
nor delivered into HeLa (data not shown) and Rat I cells (Fig.
4), even though it was well produced (Fig. 3C). This suggested
that the portion of YopE between amino acids 49 and 77
inhibits YopE secretion and that binding of SycE overcomes
this inhibition.

To check this hypothesis, we removed residues 50 to 77 from
YopE, and we monitored in vitro release of YopE in the pres-
ence and in the absence of SycE. As expected, it was released
equally as well as YopE17–77, and this release was indepen-
dent of SycE. This contrasted with wt YopE, which was only
released in the presence of SycE (Fig. 5). Thus, amino acids 50
to 77 of YopE inhibit secretion of YopE in the absence of
SycE. Interestingly, although this construct does not need SycE

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**Table 3. Translocation of YopE15-Cya and YopE130-Cya into PU5-1.8 macrophages by HOPEM strain overexpressing other Yop effector**

| Cya hybrid fusion | Yop overexpressed in trans (Yop expression plasmid) | Intracellular cAMP accumulation (nmole of cAMP/mg) |
|-------------------|-----------------------------------------------------|-----------------------------------------------|
| YopE15-Cya        | YopH (pTM163)                                       | 0.3 ± 0.3                                     |
|                   | YopO (pYOB2)                                        | 0.4 ± 0.3                                     |
|                   | YopP (pMSK13)                                       | 1.6 ± 0.5                                     |
|                   | YopE (pAPB26)                                       | 0.3 ± 0.1                                     |
|                   | YopM (pIM153)                                       | 3.0 ± 0.3                                     |
|                   | None (pBC18R)                                       | 14.06                                         |
|                   | YopE17–77 (pAPB34)                                  | 9.5 ± 3.3                                     |
| YopE130-Cya       | None                                                 | 22.8 ± 4.3                                    |
|                   | YopO (pYOB2)                                        | 25.06                                         |
|                   | YopE (pAPB26)                                       | 15.16                                         |

* Mean ± SD from three independent experiments.

**Figure 6. Lack of detectable release in the absence of residues or codons 1 to 15.** ΔTHE Y. enterocolitica [MRS40(pIM426)] bacteria producing YopE (pAPB26), YopE12–15 (pAPB35), and YopE15–112–151 (pAPB36) were incubated in minimal medium. (Top) Immunoblot with anti-YopE antibodies to detect bacterium-associated YopE proteins. In each lane 2 × 10⁹ bacteria were loaded. (Bottom) Immunoblot with anti-YopE antibodies to detect secreted YopE proteins. In each lane, the proteins released by 2 × 10⁹ bacteria were loaded.
for secretion, it still binds SycE. Thus the secretion-inhibitory domain is distinct from the minimal SycE-binding domain, although this secretion-inhibitory domain must be somehow covered by SycE.

**DISCUSSION**

In this paper, we have analyzed the N-terminal domain of *Y. enterocolitica* YopE in order to clarify its roles in the in vitro release of YopE and its delivery into eukaryotic cells.

The results, summarized in Fig. 7, confirm previous data in showing that residues 1 to 50 of YopE are required for delivery of YopE into eukaryotic cells by wt *Y. enterocolitica* (27, 28). However, the current results also show that delivery of YopE by Yop effector multimutant bacteria does not require amino acids 15 to 50 but rather that the secretion signal encompassing amino acids or codons 1 to 15 is sufficient. This implies that the chaperone binding domain does not need to interact with the Yop translocators for Yop effector translocation. In addition, this suggests that any protein that can be released by the Ysc secretion machinery also has the capacity to be delivered into eukaryotic cells. This conclusion hence implies a continuity between the secretion and translocation apparatuses, so that a Yop can pass through the secretion channel, syringe and needle, and then directly through the translocation apparatus into the target cell.

The requirement for amino acids 15 to 50 for translocation of YopE into eukaryotic cells by wt *Y. enterocolitica*, but not by Yop effector multimutant bacteria, implies that these amino acids give YopE a competitive advantage over the other Yops for the Ysc secretion-translocation apparatus. Due to the competition, only the Yops that are avidly recognized by the Ysc apparatus would be successfully delivered inside eukaryotic cells. Competition between the Yops could determine the order of precedence of Yop entry into eukaryotic cells and/or the relative quantities of each Yop delivered inside a cell.

However, if there is continuity between secretion and translocation, how could one explain that domains 15 to 50 of YopE are required for translocation by wt bacteria but not for release of YopE under Ca$^{2+}$-chelating conditions? This could be due to differences in the structure of the Ysc apparatus when opening is caused by Ca$^{2+}$ chelation and when opening is triggered by contact with eukaryotic cells. It is possible that Ca$^{2+}$ chelation shears the external part of the Ysc apparatus, resulting

![FIG. 7. Schematic representation of the role of SycE binding to amino acids 15 to 50 of YopE in YopE delivery into eukaryotic cells and release under low-Ca$^{2+}$ conditions. *Y. enterocolitica* bacteria are shown attached at the surface of a eukaryotic cell (panels 1, 2, and 3) or incubated under low-Ca$^{2+}$ conditions (panels 4, 5, and 6). Three strains are presented: wt bacteria (panels 1 and 4), sycE mutant bacteria (2 and 5), and ΔHOPEM sycE bacteria (3 and 6). The wt bacteria synthesize full-length YopE, a YopE$_{15}$-X hybrid protein, other effector Yops, and the SycE chaperone. Binding of SycE to amino acids 15 to 50 allows YopE to be delivered into cells (panel 1) or released under low-Ca$^{2+}$ conditions (panel 4). YopE$_{15}$-X, containing the N-terminal 5' secretion signal but lacking the chaperone binding site, is prevented from entering eukaryotic cells (panel 1) but is nevertheless released under low-Ca$^{2+}$ conditions (4). We hypothesize that competition is stronger for delivery into cells (small channel) than for release under low-Ca$^{2+}$ conditions (large channel). In sycE mutant bacteria, the lack of SycE does not affect the pathway followed by YopE$_{15}$-X (panels 2 and 5). However, full-length YopE is neither delivered into cells nor released under low-Ca$^{2+}$ conditions. Removal of the domain encompassing amino acids 50 to 77 (not shown in this figure) allows YopE to be released independently of SycE. We conclude that this domain is inhibitory for release and that this inhibition is prevented by SycE. In ΔHOPEM sycE strains (panels 3 and 6), YopE$_{15}$-X is not only released under low-Ca$^{2+}$ conditions but also delivered into cells. This indicates that the N-terminal 5' secretion signal is sufficient for delivery into cells. YopE and YopE$_{15}$-X are partially degraded when blocked inside bacteria. This representation is based on the results presented in this paper and on previous results which are cited in the text.]
in a secretion channel (syringe) on the surface of the bacteria with an inner diameter that is much wider than that of the channel (needle) bridging the bacteria and the eukaryotic cell (Fig. 7). In support of this hypothesis, Ca\(^{2+}\) chelation leads to the release of some external parts of the Ysc apparatus, such as YscP (19, 30). Thus, passage through the secretion channel under Ca\(^{2+}\)-chelating conditions would be far more abundant and far more permissive than upon bacteria-eukaryotic cell interaction.

In agreement with the observations of Lee et al. (14), domain encompassing amino acids 15 to 50 was not sufficient to direct YopE to the eukaryotic cytosol (14). However, unlike previous data (5), release of YopE to the extracellular milieu by this domain could not be detected, despite the use of various gene constructions, protein systems, and growth conditions. Although the same +1 frame-shift mutation of codons 2 to 15 was used here as that employed by Cheng et al. (5), in the present work the mutation was inserted in yopE and yopE\(_{130-Cya}\), while Cheng et al. (5, 14) tested yopE\(_{npt}\) hybrids. This difference in protein backbone may explain the disparity of our results. In conclusion, the domain encompassing amino acids 15 to 50 is a secretion-translocation enhancer signal that is required for efficient delivery of YopE into eukaryotic cells by wt Y. \textit{enterocolitica}, but it can not be considered as a physiological secretion signal.

Our results indicate that SyeE plays a role as a factor introducing a hierarchical order in effector delivery, by abetting YopE to compete with the other Yops. This role should not be considered as exclusive, as SyeE is required in addition when YopE contains amino acids 50 to 77. Indeed, the presence of this domain creates a need for the chaperone. This fits with older observations that bacteria missing SycE are unable to efficiently release or deliver full-length YopE or YopE\(_{130-Cya}\) but are able to secrete YopE\(_{npt}\)-Cya (35). According to our previous observations, we suggested that it was the Syc-binding domain (residues 15 to 50) that created the need for the chaperone. The more refined present observations indicate that the secretion-inhibitory domain is localized immediately downstream of the minimal domain needed for Syc binding. Although residues 50 to 77 are neither sufficient nor necessary for SycE binding, they are likely to be covered by SyeE. The determination of the three-dimensional structure of the YopE-SyeE complex will clarify this.

The reason why residues 50 to 77 of YopE interfere with secretion of YopE is not clear. These amino acids could interfere with secretion through the Ysc machinery and/or they could affect the stability or solubility of YopE. Recently, Cheng et al. (6) have shown that SyeE fused to glutathione S-transferase was unable to complement ΔSyeE bacteria for delivery of YopE into eukaryotic cells, even though the SyeE hybrid protein bound YopE in the bacterial cytosol and stabilized this Yop (6). These experiments support the results presented here, as they show that in addition to stabilizing YopE in the bacterial cytosol, SyeE is also required for efficient Yop translocation by wt bacteria. It seems that glutathione S-transferase-SyeE fusion proteins do not have this secondary function. In conclusion, the data presented in this paper present a more-complete picture of the functions of the N-terminal domains of YopE for secretion and translocation of this protein. Amino acids or codons 1 to 15 (secretion domain) are sufficient and absolutely necessary to direct translocation of YopE into eukaryotic cells by Yop effector multimutant \textit{Y. enterocolitica}. Amino acids 15 to 50 bind the SyeE chaperone and aid YopE to compete with the other Yops for entry into eukaryotic cells via the secretion-translocation machinery. Finally, amino acids 49 to 77 are inhibitory to YopE secretion, and this inhibition is overcome by binding of SyeE to amino acids 15 to 50. Future crystallography studies of YopE alone and in complex with SyeE will be very beneficial to the further studies of these domains, as would detailed studies on the other Yop-Syc interactions. It will be of great interest to investigate whether these other combinations have properties similar to those of YopE and SyeE described here.

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