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# Delineation and Mutational Analysis of the *Yersinia pseudotuberculosis* YopE Domains Which Mediate Translocation across Bacterial and Eukaryotic Cellular Membranes

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**Pathogenic yersiniae deliver a number of different effector molecules, which are referred to as Yops, into the cytosol of eukaryotic cells via a type III secretion system. To identify the regions of YopE from *Yersinia pseudotuberculosis* that are necessary for its translocation across the bacterial and eukaryotic cellular membranes, we constructed a series of hybrid genes which consisted of various amounts of *yopE* fused to the adenylate cyclase-encoding domain of the cyclolysin gene (*cyaA*) of *Bordetella pertussis*. By assaying intact cells for adenylate cyclase activity, we show that a YopE-Cya protein containing just the 11 amino-terminal residues of YopE is efficiently exported to the exterior surface of the bacterial cell. Single amino acid replacements of the first seven YopE residues significantly decreased the amount of reporter protein detected on the cell surface, suggesting that the extreme amino-terminal region of YopE is recognized by the secretion machinery. As has recently been shown for the *Y. enterocolitica* YopE protein (M.-P. Sory, A. Boland, I. Lambermont, and G. R. Cornelis, Proc. Natl. Acad. Sci. USA 92:11998–12002, 1995), we found that export to the cell surface was not sufficient for YopE-Cya proteins to be delivered into the eukaryotic cytoplasm. For traversing the HeLa cell membrane, at least 49 *yopE*-encoded residues were required. Replacement of leucine 43 of YopE with glycine severely affected the delivery of the reporter protein into HeLa cells. Surprisingly, export from the bacterial cell was also not sufficient for YopE-Cya proteins to be released from the bacterial cell surface into the culture supernatant. At least 75 residues of YopE were required to detect activity of the corresponding reporter protein in the culture supernatant, suggesting that a release domain exists in this region of YopE. We also show that the chaperone-like protein YerA required at least 75 YopE residues to form a stable complex in vitro with YopE-Cya proteins and, furthermore, that YerA is not required to target YopE-Cya proteins to the secretion complex. Taken together, our results suggest that traversing the bacterial and eukaryotic membranes occurs by separate processes that recognize distinct domains of YopE and that these processes are not dependent on YerA activity.**

Protein translocation across biological membranes has been shown in a number of diverse experimental systems to be dependent on signal sequences or on unique structural domains that appear to target the protein to be exported to the site of translocation. For example, proteins exported by the bacterial *sec*-dependent general secretory pathway (alternatively referred to as the type II secretion pathway) contain amino-terminal signal sequences that are 16 to 26 residues in length and that contain a basic amino-terminal domain, a hydrophobic core segment, and a carboxy-terminal domain that encompasses the cleavage site. The basic and hydrophobic domains have been demonstrated to be essential for the initial recognition event between the protein to be exported and components of the secretion complex (for a review, see reference 34). Proteins exported by the *sec*-independent type I secretion system contain no obvious signal sequence at the primary amino acid level; rather, they have been shown to contain domains at their carboxy termini that are necessary for recognition by the secretion complex (for a review, see reference 27). A third secretion pathway, referred to as the type III system, has been described recently for a number of animal and plant bacterial pathogens (2, 14, 15, 19, 21, 23, 35). Type III secretion systems have been shown to recognize the amino-terminal region of the protein to be exported (20), although no obvious sequence similarities in this region have been detected among the various proteins secreted by this pathway. In this report, we identify domains in the *Yersinia pseudotuberculosis* YopE protein that are necessary for both targeting YopE to the type III secretion complex and for eventually delivering the protein to its site of action.

The pathogenic yersiniae deliver a number of effector molecules, referred to as Yops, into the cytoplasm of eukaryotic cells via a type III secretion system (for a review, see reference 7). The YopE, YopH, and YpkA effector proteins have been demonstrated to be delivered into HeLa cells by *Y. pseudotuberculosis* and *Yersinia enterocolitica* that are located on the exterior surface of the eukaryotic cell (12, 22, 26, 29). Although the eukaryotic targets of the Yop proteins remain to be identified, YopE and YopH have been shown to have an antiphagocytic activity which results in maintaining the bacterium on the surface of the phagocytic cell (3, 6, 24, 25). The translocation of Yops from a surface-located bacterium into the cytoplasm of the host cell requires that the Yop proteins traverse the bacterial inner and outer membranes as well as the cytoplasmic membrane of the host cell. Although mutations in a number of different *Y. pseudotuberculosis* genes have been described that abolish the delivery of Yops into host cells (1, 11, 26), very little is known concerning how the Yops interact with the secretion apparatus.

Recently, we reported that residues 49 to 150 of YopH were

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required to detect YopH within cultured HeLa cells following infection (22). YopH proteins lacking this region were still released into the supernatant by *Y. pseudotuberculosis* growing in culture, which suggested that Yop proteins traverse the bacterial and eukaryotic membranes in distinct steps. This is further supported by the fact that bacteria with mutations in either *yopD* or *yopB* secrete the other Yop proteins normally when growing in culture but are unable to deliver the other Yop proteins into HeLa cells (11, 26, 28). This may indicate that the YopD and YopB proteins are involved in translocating the other Yop proteins across the eukaryotic cell membrane.

Sory and Cornelis (29) recently showed that a hybrid protein consisting of the 130 amino-terminal residues of YopE and the 400 amino-terminal residues of the adenylate cyclase-encoding domain of the cyclolysin gene (*cyaA*) of *Bordetella pertussis* was delivered into HeLa cells by *Y. enterocolitica*. The activity of the resulting adenylate cyclase-containing hybrid protein was dependent on calmodulin, a protein found within eukaryotic but not prokaryotic cells. Therefore, following infection, delivery of the YopE-Cya reporter protein into eukaryotic cells could be monitored by assaying for cyclic AMP (cAMP) in cell lysates. We used a similar approach to delineate the regions of YopE that are required for it to be targeted to the secretion complex and eventually delivered into eukaryotic cells. Our results indicate that distinct domains of YopE mediate translocation across the bacterial and host cell membranes and thus are consistent with a model in which the two processes occur in discrete steps.

During the course of these studies, Sory et al. (28) reported that a YopE-Cya fusion protein containing the 15 amino-terminal residues of YopE was secreted by *Y. enterocolitica* growing in culture. They further showed that 50 amino-terminal residues were required for a YopE-Cya protein to be delivered into the cytoplasm of eukaryotic cells. The data presented in this report largely corroborate the findings of Sory et al. (28) and further the analysis by showing that the first 11 residues of YopE are sufficient to target YopE-Cya proteins to the secretion complex and by identifying YopE-derived residues within these domains that are critical for the translocation of YopE-Cya proteins across the bacterial and host cell membranes. Additionally, we characterize the interaction between YopE and its chaperone YerA (alternatively referred to as SycE) (9, 32) and show that YopE-Cya targeting to the secretion complex does not require YerA activity.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Y. pseudotuberculosis* strains YPII-I(pIB102) (wild type [wt]; *yadA*) (4) or YPIII(pIB505) (wt; *yadA yerA*) (9) were used as recipients for the various *yopE-cya* constructs. Bacteria were grown to stationary phase at 26°C in brain heart infusion broth (Oxoid; Hampshire, United Kingdom) supplemented with 5 mM EGTA and 20 mM $MgCl_2$ and induced for Yop expression by fivefold dilution into brain heart infusion broth without supplements and shaking for 30 min at 26°C and then for 60 to 90 min at 37°C. For HeLa cell infections, bacteria were grown to stationary phase in Luria broth at 26°C and diluted 150-fold with Leibovitz L-15 medium containing 10% heat inactivated fetal calf serum without antibiotics. Before addition to HeLa cells, diluted bacteria were shaken for 30 min at 26°C and then for 60 min at 37°C.

**Plasmid constructions.** The *yopEx-cya* deletion series of plasmids were derived from plasmid *yopE130-cya* which was constructed by fusing a PCR-derived fragment containing codons 2 to 399 of the *B. pertussis* cyclolysin gene (with the forward primer 5′-ACAGATCCTGCAGCAGCAATCGCATCAG and the reverse primer 5′-TCAAGGCATGCATAGCCGGAATCCTAGCGTTC, which encompass the calmodulin-dependent adenylate cyclase domain) (10) in frame with *yopE* at the *Pst*I site located at codon 390 on plasmid pAF60 (8). The resulting plasmid was digested with *Bam*HI and *Sph*I, and the 2.64-kb *yopE-cya*-containing fragment (which includes the *yopE* promoter region as well as the entire *yerA* locus [8]) was ligated into pUC19, generating plasmid *yopE130-cya*. Plasmid *yopE130-cya* was used as a template with an upstream primer that encompassed the *Bam*HI site and various downstream primers that contained an in-frame *Pst*I site within the *yopE* coding region (see Fig. 1A). (Primer sequences will be provided by the authors upon request.) The resulting *Bam*HI-*Pst*I PCR-derived fragments were ligated into the *cya*-containing fragment generated by digesting plasmid *yopE130-cya* with *Bam*HI and *Pst*I.

Point mutants were introduced into plasmid *yopE11-cya* by PCR by using it as a template with the *Bam*HI site-containing upstream primer (used as described above) and a downstream primer that spanned the *yopE11-cya* fusion point and that contained the appropriate missense mutation. The resulting *Bam*HI-*Pst*I fragments were ligated back into the *cya*-containing *Bam*HI-*Pst*I *yopE130-cya* fragment. A similar approach was used to introduce point mutations in the plasmid *yopE49-cya*. The plasmid *yopE11*(*yerA*)*-cya* was constructed by PCR by using the plasmid *yopE11-cya* as a template and an upstream primer containing a *Bam*HI site that was complementary to a region between the *yerA* coding region and the *yopE* promoter region and a downstream primer spanning the *yopE11-cya* fusion point, and the resulting *Bam*HI-*Pst*I fragment was ligated back into the *cya*-containing vector plasmid.

**Adenylate cyclase assays.** Induced 2-ml bacterial cultures were pelleted and washed twice with TM (20 mM Tris [pH 8], 10 mM $MgCl_2$) and resuspended in 100 μl of the same buffer. After removal of 10 μl for whole-cell cyclase measurements, the remaining cells were sonicated and the levels of cyclase activity of the cleared lysates were determined. For cyclase measurements, either 10 μl of intact cells, 2 μl of lysate, or 5 μl of supernatant (that had been passed through a 0.45-μm sterile filter) was incubated for 5 min at 20°C in a 50-μl volume that contained 50 mM Tris (pH 8), 6 mM $MgCl_2$, 0.12 mM $CaCl_2$, 2 mM ATP, 0.1 mg of bovine serum albumin per ml, and 1 μg of calmodulin (kindly provided by Stefan Hermann) per ml. Reaction mixtures were then extracted with 2 volumes of ethanol (intact cells were removed by centrifugation prior to ethanol extraction), and, following centrifugation, the supernatant was lyophilized and the pellets were resuspended in 100 to 1,000 μl of TE (10 mM Tris [pH 8], 1 mM EDTA). cAMP measurements were made by using the cAMP [$^3$H] assay system from Amersham (TRK 432).

**Infections.** Induced bacteria were added at a multiplicity of infection of approximately 100 to $2.5 \times 10^5$ HeLa cells plated in 3.5-cm-diameter tissue culture plates in a final volume of 2 ml of drug-free medium. Cultures were incubated at 37°C, and at various times following infections, cells were washed once with cold phosphate-buffered saline, collected in 400 μl of 50 mM HCl by scraping, and placed in a 100°C heating block for 5 min. The lysates were neutralized with NaOH and extracted with 2 volumes of ethanol for 5 min at room temperature. Following centrifugation, the supernatants were lyophilized and the pellets were resuspended in 400 μl of TE. cAMP was measured as described above.

**YerA coprecipitation.** Induced 2-ml cultures were pelleted, washed with TM, and resuspended in 400 μl of the same buffer. The cells were sonicated for 30 s, and the cleared lysates were incubated with 20 μl of calmodulin-agarose (Sigma) for 16 to 20 h at 4°C on a rotary platform. The beads were collected by centrifugation and were washed three times with ice-cold TM. Following the final wash, the beads were resuspended in 20 μl of sodium dodecyl sulfate (SDS) sample buffer, and 5 μl was loaded on a 20% polyacrylamide gel. Following electrophoresis, the gel was electroblotted onto nitrocellulose, which was probed with antibodies raised against YopE130-Cya and YerA (9).

## RESULTS

**Expression, targeting, and secretion of YopE-Cya proteins.** To investigate the region required for secretion of the *Y. pseudotuberculosis* YopE protein, a series of *yopE-cya* hybrid genes were constructed which contained various lengths of the amino-terminus-encoding region of *yopE* fused to the 400 amino-terminal codons of the *B. pertussis* cyclolysin gene that encodes a calmodulin-activated adenylate cyclase (for a review, see reference 5). Expression of the fusion genes was under the control of the native *yopE* promoter, and the genes were designated *yopEx-cya*, where *x* equals the number of amino-terminal *yopE* codons (Fig. 1A). The gene encoding the YopE chaperone YerA (8), which is located immediately upstream of *yopE* and has been shown to be necessary for stable expression of YopE (9, 32), was included in the various constructs. To monitor expression of the fusion genes, cyclase activities were measured in lysates prepared from induced cultures of *Y. pseudotuberculosis* (see Materials and Methods) containing the various *yopEx-cya* constructs. Bacterial lysates prepared from *yopE11-cya*-containing bacteria had a level of cyclase activity approximately twofold higher than that of lysates prepared from *yopE1-cya*-containing bacteria (Fig. 1B, dark columns). The increased expression level of *yopE11-cya* compared with *yopE1-cya* was confirmed on the protein level by Western blot (immunoblot) analysis (Fig. 2A). Increasing the number of

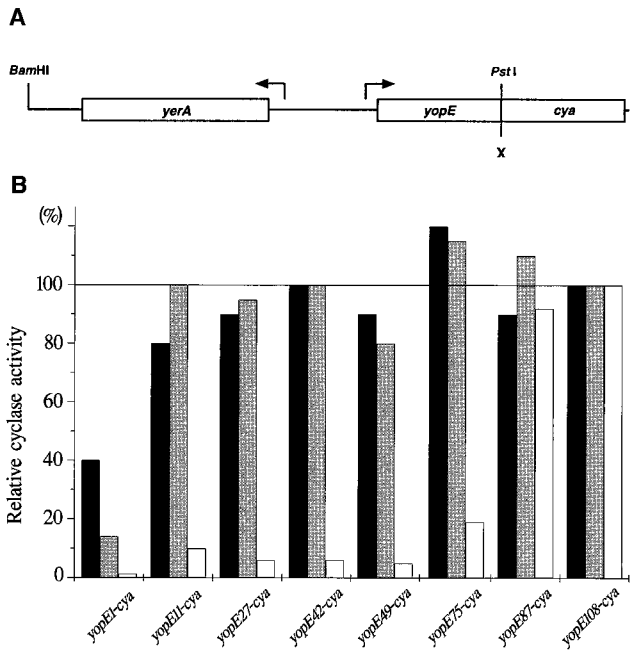


FIG. 1. Activity of the various YopE-Cya hybrid proteins in lysate, intact whole-cell, and supernatant fractions. (A) Diagram of the YopEx-Cya-encoding plasmids. X, the *yopE* codon located at the point of fusion with the gene encoding the calmodulin-activated adenylate cyclase domain of cyclolysin (*cya*). The YopE chaperone-encoding *yerA* gene shares the *yopE* promoter region. (B) Cyclase activities of lysates (dark columns), intact whole cells (grey columns), and supernatants (white columns) prepared from bacteria after 1 h of induction at 37°C (see Materials and Methods). Each point represents the mean of two measurements of a representative experiment and is a percentage of activity compared with that of the *yopE108-cya*-containing strain. The absolute levels of cyclase activity of the lysate, whole-cell, and supernatant fractions of the *yopE108-cya*-containing strain were 320, 4, and 16 pmol of cAMP/min/optical density, respectively.

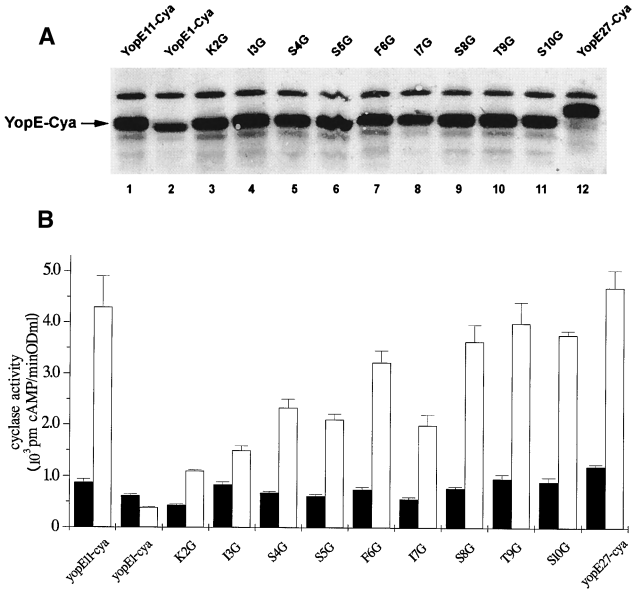


FIG. 2. Expression and activity levels of the various YopE11-Cya proteins containing single-residue replacements. (A) Western blot of lysates prepared from strains harboring the indicated reporter genes probed with anti-Cya antisera. Equal amounts of protein from induced bacterial cultures were loaded in each lane. (B) Whole cells of the indicated strains were assayed for cyclase activity in either the absence (black columns) or presence (white columns) of calmodulin. Bacteria were collected after 1 h of incubation at 37°C in calcium-depleted medium, washed, and resuspended directly in the buffer used to measure cyclase activity. Each point represents the mean of three measurements of a representative experiment ± the standard deviation. pm, pmol.

YopE residues fused to the reporter protein did not significantly affect cyclase activity levels of the bacterial lysates of the respective strains, indicating that the various hybrid genes shown in Fig. 1 (except *yopE1-cya*) were expressed at approximately the same level. For unexplained reasons, YopE-Cya hybrid proteins containing 60 to 70 residues of YopE were not stably expressed in *Y. pseudotuberculosis*, as determined by both cyclase activity measurements and protein levels (not shown).

To determine whether the various YopEx-Cya proteins were translocated across the two bacterial membranes and presented on the exterior surface of the bacterial cell, induced cultures were pelleted, washed, and resuspended directly in the buffer used to measure cyclase activity. We were surprised to observe a level of cyclase activity in intact cells containing *yopE11-cya* significantly higher than that of cells containing the *yopE1-cya* gene (Fig. 1B, grey columns). Increasing the number of YopE residues fused to the reporter protein did not significantly affect the whole-cell cyclase activity levels. The level of cyclase activity of YopE11-Cya- but not YopE1-Cya-containing cells was increased severalfold by the addition of calmodulin (Fig. 2B), indicating that the observed cyclase activities present in intact whole-cell preparations were not due simply to lysed cells, since the sonicated whole-cell lysates prepared from the YopE1-Cya-containing cells had significant levels of both cyclase activity and reporter protein (Fig. 1 and 2A). Additionally, calmodulin activation indicated that the YopE-Cya proteins were located on the exterior surface of the bacterial cell, since calmodulin is a protein with a relative molecular mass of approximately 16 kDa (16) and, accordingly, does not enter the cytosol of the bacteria. Greatly reduced levels of cyclase activity were observed in intact cells, but not bacterial lysates, in *yopE11-cya*-containing strains harboring a polar mutation in *yscJ* (not shown), a gene whose product is required for Yop secretion (25). This indicated that the YopE-Cya proteins are exported via a type III secretion system. These observations suggest that the first 11 residues of YopE are recognized by the type III secretion system of *Y. pseudotuberculosis* and that this recognition event is sufficient for YopE to gain access to the exterior of the cell.

**Release of YopE-Cya proteins from the cell surface.** We then asked whether the various YopEx-Cya proteins that are present on the exterior of the bacterial cell were also released into the culture supernatant. Supernatants from strains harboring reporter genes containing 1 to 49 *yopE* amino-terminal codons had very little cyclase activity compared with supernatants prepared from *yopE87-cya*- and *yopE108-cya*-containing strains (Fig. 1B, white columns). Supernatants prepared from the *yopE75-cya*-containing strain consistently had a low but significant level of cyclase activity. These data suggest that a region located between residues 75 and 87 was required for YopE to be released from the cell surface into the culture supernatant.

**Important residues for YopE11-Cya targeting.** To investigate whether any single YopE-derived residue of YopE11-Cya was especially critical for its delivery to the exterior of the cell, a series of reporter genes were constructed which encoded proteins that replaced, one at a time, the first 10 amino-terminal residues of YopE11-Cya with glycine. Single residue replacements had little effect on the protein levels of the respective reporter proteins (Fig. 2A). Bacterial lysates prepared

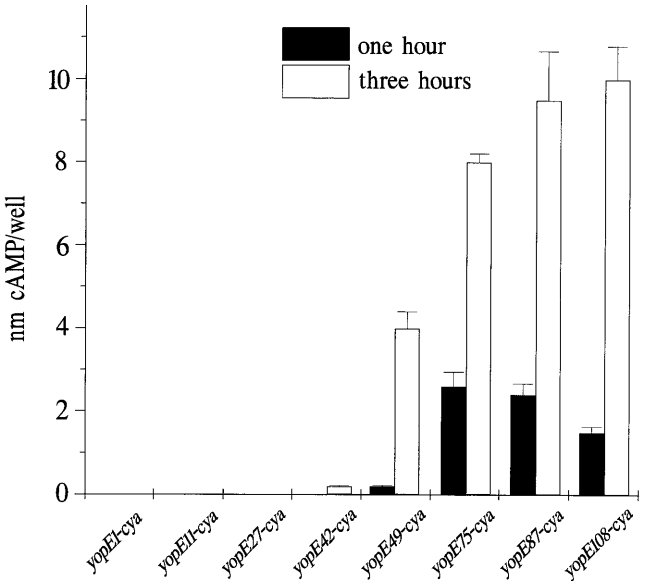


FIG. 3. Levels of cAMP in HeLa cell lysates at various times following infection with strains containing the indicated *yopE-cya* reporter genes. Overnight bacterial cultures were diluted in antibiotic-free tissue culture medium and induced for Yop expression (see Materials and Methods) before being added to HeLa cells. At the indicated times, HeLa cells were washed and lysates were prepared and measured for cAMP. Each point represents the mean of three cAMP measurements of a representative experiment ± the standard deviation. nm, nmol.

from strains carrying the respective point mutants had similar levels of calmodulin-activated cyclase activity (not shown), further suggesting that the altered YopE11-Cya proteins were expressed at approximately the same level.

When intact whole cells were assayed for their level of cyclase activity as described above, there were considerable differences between some of the strains containing mutated reporter proteins and the wt *yopE11-cya*-containing strain (Fig. 2B). Replacement of glycine for either lysine 2 or isoleucine 3 resulted in a three- to fourfold decrease, compared with the wt, in the level of cyclase activity in intact cells of the respective strains. Single-residue replacements of serine 4, serine 5, or isoleucine 7 resulted in an approximately twofold decrease in whole-cell cyclase activity compared with that of the wt, whereas single replacements of the remaining residues had little effect. These data show that single amino acid residues are important for the recognition of YopE by the secretion machinery. Furthermore, our data suggest that the *Y. pseudotuberculosis* type III secretion complex makes critical contacts with the first seven residues of YopE and that these interactions are important for translocating YopE across the bacterial cytoplasmic and outer membranes.

**Delivery of YopE-Cya proteins into HeLa cells.** To test whether the various YopEx-Cya proteins were also delivered into the cytosol of eukaryotic cells during infection, cAMP levels were measured in lysates prepared from HeLa cells following infection with strains containing the various *yopEx-cya* genes. Lysates prepared from HeLa cells infected with strains harboring *yopE1-*, *yopE11-*, *yopE27-*, or *yopE42-cya* had cAMP levels below that which could be detected by the assay (Fig. 3). In contrast, lysates prepared from HeLa cells infected with *yopE75-*, *yopE87-*, or *yopE108-cya*-containing strains had high levels of cAMP, whereas lysates prepared from HeLa cells infected with the *yopE49*-containing strain had a relatively intermediate level of cAMP following infection. The failure to detect increased levels of cAMP following infections with strains carrying the shorter *yopE-cya* genes indicated that although the corresponding proteins were targeted to the secretion apparatus (Fig. 1 and 2), gaining access to the exterior of the bacterial cell was not sufficient for these reporter proteins to be delivered into the eukaryotic cell. These data suggest that YopE contains a domain, with a right-hand boundary between residues 42 and 49, that is necessary for YopE to be translocated across the eukaryotic membrane.

TABLE 1. Levels of cAMP in lysates prepared from HeLa cells infected with *Y. pseudotuberculosis* harboring *yopE49-cya* containing the indicated mutations

| Mutation | nmol of cAMP/well[a] | % wt |
|---|---|---|
| WT | 1.70 (0.31) | 100 |
| L43G | 0.03 (<0.00) | 1.4 |
| A44G | 0.44 (0.04) | 26 |
| R46G | 0.42 (0.06) | 25 |
| T47G | 0.65 (0.07) | 38 |
| E48G | 1.20 (0.10) | 71 |
| S49G | 1.22 (0.22) | 72 |

[a] HeLa cells were collected 1.5 h after infection. The means of three cAMP measurements ± the standard deviations are reported.

We observed that upon infection with strains containing *yopE1-cya*, *yopE11-cya*, *yopE27-cya*, or *yopE42-cya* (i.e., reporter genes encoding nontranslocated YopE-Cya proteins), HeLa cells developed a relatively faster cytotoxic response than that of HeLa cells infected with strains containing reporter genes encoding translocated YopE-Cya proteins (not shown). The cytotoxic response of HeLa cells upon infection with *Y. pseudotuberculosis* has been shown to be primarily mediated by YopE (24). The delayed cytotoxic response of HeLa cells infected with strains containing translocation-competent YopE-Cya proteins suggested that less wt YopE, whose gene is present in the YPIII(pIB102) parental strain, was being delivered into HeLa cells. This may indicate that YopE-Cya proteins which contain YopE residues 42 to 49 compete with YopE for some factor that is required for YopE to gain entry into HeLa cells. Furthermore, the fact that a cytotoxic response is observed in HeLa cells infected with strains expressing nontranslocated YopE-Cya proteins indicated that these proteins, which are present on the exterior of the bacterial cell (Fig. 1B), do not interfere with the translocation of YopE across the bacterial and host cell membranes.

**Critical residues required for YopE49-Cya delivery into HeLa cells.** To identify whether any single residue located between amino acids 43 and 49 of YopE49-Cya was important for its delivery into HeLa cells, a series of point mutants were constructed in which residues 43 through 49 were replaced, one at a time, with glycine. Bacterial lysates prepared from strains of *Y. pseudotuberculosis* harboring either wt *yopE49-cya* or the various mutant *yopE49-cya* constructs had similar levels of cyclase activities, indicating that the introduced mutations did not affect expression levels (not shown). In contrast, there were clear differences among the various point mutants in their abilities to be translocated into HeLa cells (Table 1). There was an approximately 80-fold decrease in the level of cAMP in HeLa cell lysates following infection with strains harboring the *yopE49-cya* gene containing the L43G mutation compared with HeLa cell lysates infected with the wt *yopE49-cya*-containing strain. The A44G, R46G, and T47G replacements resulted in a three- to fourfold decrease in cAMP levels compared with that of the wt, whereas the E48G and S49G replacements had close to wt cAMP levels in HeLa cell lysates following infection

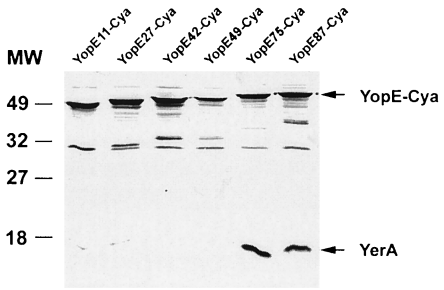


FIG. 4. Calmodulin-agarose precipitations of lysates prepared from various *yopE-cya*-containing strains. Following induction of Yop expression (see Materials and Methods), cells were collected, washed, and sonicated. The cleared lysates were incubated overnight with calmodulin-agarose, at which time the beads were collected, washed thoroughly, and boiled in SDS-containing sample buffer. Samples were separated on a 20% PAGE gel that was blotted and probed with antisera specific for YopE-Cya and YerA. MW, molecular weight.

with the respective strains. These data suggest that YopE residues 43 to 47 interact with factors that are responsible for translocating YopE across the eucaryotic cell membrane.

**Identification of the region of YopE that interacts with YerA.** The *yerA* gene product has been shown to be required for stabilizing the YopE protein within the cytoplasm of the bacterial cell but to be dispensable for targeting YopE to the secretion complex (9). Sory and Cornelis (29) have shown that YerA (alternatively referred to as SycE) coprecipitates with a YopE130-Cya hybrid protein by using calmodulin conjugated to agarose beads. We used a similar approach to determine the minimum length of the YopE amino-terminal region sufficient to coprecipitate YerA. Bacterial lysates prepared from strains harboring the various *yopEx-cya* genes were incubated with calmodulin-agarose, and bound proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a nitrocellulose membrane that was probed with polyclonal antibodies against YopE-Cya and YerA. YopE-Cya proteins that contained 75 or more amino-terminal residues of YopE coprecipitated with YerA, whereas YopE-Cya proteins containing 49 or fewer YopE residues failed to mediate the binding of YerA to calmodulin-agarose (Fig. 4). This shows that YerA binding to YopE-Cya required residues 49 to 75 of YopE and furthermore may indicate the actual region of YopE that binds YerA.

The failure of YerA to coprecipitate with the shorter YopE-Cya proteins suggests that these proteins were targeted to the secretion complex via a mechanism that was independent of YerA. To test this possibility more directly, the YerA-encoding portion was removed from the *yopE11-cya* and *yopE75-cya* constructs, and the resulting plasmids, referred to as *yopE11*(*yerA*)*-cya* and *yopE75*(*yerA*)*-cya*, were introduced into both wt and *yerA* strains of *Y. pseudotuberculosis* (9). There was no significant difference in the levels of cyclase activity in bacterial lysates prepared from wt and *yerA* strains harboring the *yopE11*(*yerA*)*-cya* construct. Similarly, intact whole cells of the *yopE11*(*yerA*)*-cya*-containing wt and *yerA* strains had approximately the same level of cyclase activity, indicating that YerA activity was not required for the export of YopE11-Cya to the exterior of the cell. In contrast, lysates prepared from the *yopE75*(*yerA*)*-cya*-containing *yerA* strain had an approximately twofold decrease in cyclase activity compared with lysates prepared from the *yopE75*(*yerA*)*-cya*-containing wt strain (not shown), suggesting that YerA is required to stabilize YopE. Taken together, these data demonstrate that the amino-terminal domain of YopE does not physically interact with YerA in vitro and furthermore that the targeting of YopE to the secretion complex is not functionally dependent on YerA.

## DISCUSSION

The delivery of Yop effector molecules by surface-located yersiniae into the eukaryotic cytoplasm requires that these proteins cross the bacterial cytoplasmic and outer membranes as well as the eukaryotic cytoplasmic membrane. Genetic and microscopic analyses indicate that Yops most likely traverse the bacterial and eukaryotic cytoplasmic membranes in distinct steps (11, 22, 26, 28). To first gain a better understanding of the interactions that occur between the secretion complex and YopE, we wished to clearly define the domains of YopE that were necessary for it to be targeted to the secretion complex and eventually exported from the bacterial cell. We found that the 11 YopE amino-terminal residues were sufficient to target and export a YopE-Cya hybrid protein to the exterior surface of the bacterial cell. Moreover, singly replacing the first seven YopE-derived residues of YopE11-Cya with glycine significantly reduced the level of the corresponding reporter protein detected on the surface of intact cells, suggesting that the extreme amino-terminal domain of YopE is recognized by components of the secretion machinery. The length of the YopE domain recognized by the *Y. pseudotuberculosis* type III secretion system is significantly shorter than the 16- to 26-residue-long signal sequences recognized by the *sec*-dependent type II secretion machinery (34). Interestingly, it appears that the YopE export domain and the *sec*-dependent signal sequences share at least one attribute; a basic residue at their amino termini. As we have shown here and as others have shown for the *sec*-dependent signal sequences (13, 18), a positive charge at the amino terminus appears to be important for these proteins to enter the respective export pathways. Although we showed that specific residues of YopE were important for routing YopE11-Cya to the export pathway, there are no obvious primary sequence similarities between the amino-terminal region of YopE and the other Yop effector proteins. This may indicate that there exist specific targeting components for each individual type of Yop. Some of the exported Yops have been shown to interact with chaperone-like proteins (31, 32) which have been proposed to target Yops to the secretion complex. Data presented here and previously (9) appear to exclude at least the YopE chaperone-like protein YerA (8, 32) from having a role in either targeting YopE to the secretion complex or from playing a role in exporting YopE from the bacterial cell.

We showed that a YopE11-Cya hybrid protein was not affected in its ability to be exported to the exterior of the bacterial cell when present in a *yerA* strain. Furthermore, YerA coprecipitated in vitro with YopE75-Cya but not YopE49-Cya, which suggests that YerA binds a region between residues 49 and 75 of YopE. Rather than targeting YopE to the secretion complex, we propose that YerA is required to maintain YopE in an export-competent state. The region of YopE between residues 53 and 80 is predicted to contain an amphipathic alpha helix (Fig. 5) which could conceivably result in self-aggregation of YopE in the absence of YerA. This region could possibly be important for YopE activity within eukaryotic cells, since it has been shown that amphipathic alpha helices mediate protein-protein interactions (17). Alternately, this region of YopE may interact with other proteins at the bacterial cell surface, since we have shown that this region was also required for YopE-Cya proteins to be released into the culture supernatant.

By assaying whole bacterial cells for calmodulin-dependent

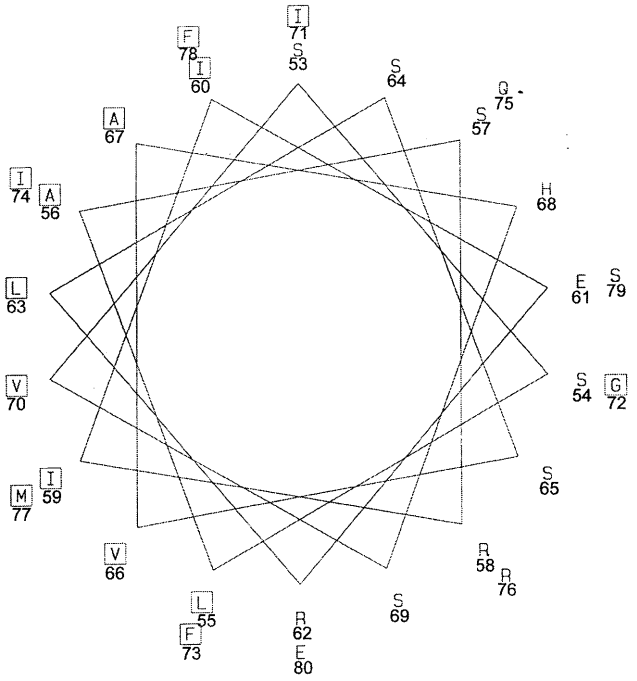


FIG. 5. Helical wheel analysis of the putative YerA-binding region of YopE. YopE residues 53 to 80 are plotted, and the hydrophobic residues are boxed.

cyclase activity, it was shown that a stable pool of YopE-Cya protein exists on the exterior surface of the bacterial cell. The association of YopE-Cya proteins with the cell surface required only the 11 amino-terminal residues of YopE and, furthermore, the level of association, as determined by cyclase activity measurements of whole cells, did not increase with increasing amounts of YopE residues fused to the reporter protein. We have previously shown by electron microscopy that YopE is associated with the cell surface (9). Therefore, it is unlikely that the cell-surface association of YopE-Cya proteins was due to properties of the Cya reporter protein.

The biological role, if any, of surface-located YopE is presently unclear. We propose that a separate pool of YopE exists on the bacterial cell surface that is distinct from YopE molecules that are synthesized in response to contact with eukaryotic cells (26). We base this idea on the fact that the shorter YopE-Cya hybrid proteins, which are present on the bacterial cell surface but are not released into the culture supernatant or delivered into HeLa cells, did not block other Yops from being released into the culture supernatant or delivered into HeLa cells. YopE possesses an antiphagocytic activity within eukaryotic cells by inducing a disruption of the cytoskeletal network (25). Therefore, the surface pool of YopE may serve to protect the bacterium from phagocytosis immediately following attachment of the bacterium to the eukaryotic cell surface before sufficient levels of cell contact-induced YopE expression (26) have been achieved. A similar scenario may occur in the shigellae, in which it has been shown that Ipa effector molecules, which are exported by a type III secretion system similar to that of the yersiniae (1), are present on the bacterial cell surface and released upon contact with the eukaryotic host cell (30). Although the ultimate aims of these two pathogens are dissimilar (the shigellae are invasive, while the yersiniae remain on the exterior of the host cell), a pool of surface-located effector molecules may be necessary for driving the outcome of host-parasite cell contact to the advantage of the pathogen.

Surprisingly, we found that even though YopE-Cya proteins containing fewer than 75 residues of YopE were exported to the exterior surface of the bacterial cell, they were not present at detectable levels in culture supernatants. This implies that a region between residues 75 and 87 was required for YopE-Cya proteins to be released from the cell surface. Our data do not agree with those of Sory and coworkers (28), who reported that cyclase activities levels of culture supernatants prepared from *Y. enterocolitica* harboring similar *yopE-cya* constructs were essentially constant for hybrid proteins containing 15 or more YopE-derived amino-terminal residues. However, in the same report, the authors show that YopE-Cya proteins containing approximately 47 or more YopE residues were secreted into the culture supernatant to a much greater extent than hybrid proteins containing 40 or fewer YopE residues. The reason for the apparent discrepancy between cyclase activities and protein levels was not addressed by Sory et al. (28), and, similarly, we are unable to account for the differences between their results and ours concerning the release of YopE-Cya proteins into the culture supernatant.

Finally, we have shown that a region of YopE between residues 42 and 49 was required for the delivery of YopE-Cya into the cytoplasm of HeLa cells and that particular residues within this region are critical for traversing the eukaryotic cell membrane. Previously, we have shown that residues 49 to 150 of YopH required for it to be delivered into HeLa cells (22). Our results closely match those reported by Sory et al. (28), who mapped the region of *Y. enterocolitica* YopE that was required for its delivery into macrophages. These results are interesting in that they show that the targeting of Yops to the secretion complex and their subsequent export from the bacterial cell are not sufficient for them to be translocated across the eukaryotic cell membrane, suggesting that Yops are delivered into eukaryotic cells in a multistage process. A Yop delivery process consisting of distinct steps is also suggested by mutants that are defective in translocating Yops across the eukaryotic but not bacterial membrane. YopE is not translocated across the HeLa cell membrane in *Y. pseudotuberculosis* harboring mutations in either *yopD* or *yopB* and instead accumulates in the zone of contact between bacterial and eukaryotic cells (11, 26). It remains to be determined whether YopD and YopB are directly responsible for translocating Yops across the eukaryotic cell membrane or whether they work in concert with other bacterial and/or eukaryotic proteins.

The region of YopE that we identified to be important for its delivery into eukaryotic cells is similar to the nuclear export signal of the heat-stable inhibitor of the catalytic subunit of cAMP-dependent protein kinase (cAPK) (33). Specifically, YopE and cAPK each contain hydrophobic residues that have been shown to be critical for traversing the cytoplasmic and nuclear membranes, respectively. Interestingly, no obvious sequence similarity exists between this domain of YopE and other Yop proteins that are also delivered into eukaryotic cells. We wonder whether this may indicate that the various Yop effector molecules traverse the eukaryotic cytoplasmic membrane by distinct pathways, since each type of Yop is presumably targeted to different substrates within the eukaryotic cell.

In summary, we have shown that the 11 amino-terminal residues of YopE were sufficient to translocate the Cya reporter protein across the cytoplasmic and outer bacterial cell membranes via a process that was not dependent on the activity of the chaperone-like protein YerA. The export YopE-Cya hybrid proteins containing fewer than 42 YopE residues was not sufficient for them to be delivered into HeLa cells. Translocation of YopE-Cya hybrid proteins across the eukaryotic membrane required at least 49 amino-terminal YopE residues.

These data suggest that distinct domains of YopE are recognized by separate processes that first export Yop effector molecules from the bacterial cell and then deliver them into the cytoplasm of the eukaryotic cell.

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