

## A Program of *Yersinia enterocolitica* Type III Secretion Reactions Is Activated by Specific Signals

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**Successful establishment of *Yersinia* infections requires the type III machinery, a protein transporter that injects virulence factors (Yops) into macrophages. It is reported here that the *Yersinia* type III pathway responds to environmental signals by transporting proteins to distinct locations. *Yersinia enterocolitica* cells sense an increase in extracellular amino acids (glutamate, glutamine, aspartate, and asparagine) that results in the activation of the type III pathway. Another signal, provided by serum proteins such as albumin, triggers the secretion of YopD into the extracellular medium. The third signal, a decrease in calcium concentration, appears to be provided by host cells and causes *Y. enterocolitica* to transport YopE and presumably other virulence factors across the eukaryotic plasma membrane. Mutations in several genes encoding regulatory molecules (*lcrG*, *lcrH*, *tyeA*, *yopD*, *yopN*, *yscM1*, and *yscM2*) bypass the signal requirement of the type III pathway. Together these results suggest that yersiniae may have evolved distinct secretion reactions in response to environmental signals.**

Type III secretion systems represent a common pathogenic tool of many gram-negative bacteria (20). Upon bacterial contact with host cells, type III machines deliver protein toxins across the eukaryotic plasma membrane. Once inside the cell, these proteins manipulate host signal transduction pathways, resulting in rearrangement of the cytoskeleton and in induction of an apoptotic program (45, 56). By injecting distinct sets of toxins into the host, each pathogen appears to customize the versatile type III device to suit their unique pathogenic strategy (20). Recent work suggests a temporal and/or spatial regulation of the type III secretion machinery during experimental infections caused by *Salmonella*, enteropathogenic *Escherichia coli* (EPEC), and *Yersinia* species. *Salmonella* species inject two effector proteins, SopE and SptP, which display opposing functions in the host cell (16). SopE, a GTP exchange factor for CDC42 and Rac1, first induces membrane ruffling and facilitates bacterial entry into the host cell (21). Injection of SptP, a GTPase activating factor of CDC42 and Rac1, is thought to restore the cytoskeletal rearrangements once the bacterium is inside the host cell (19). Simultaneous microinjection of both effector proteins prevents cytoskeletal rearrangements, suggesting that a temporal regulation of the type III machine is required to allow the two virulence factors to function independently of each other in the host cell cytoplasm (19).

Pathogenic *Yersinia* species, i.e., *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*, enter their hosts via the intestines or by flea-borne contamination of skin lesions

(11). Once host barriers are breached, *Yersinia* colonizes lymphoid tissues or multiplies in blood to cause septicemic infections (6). The astonishing pathogenic potential of *Yersinia* is executed by a type III secretion machinery that transports 14 Yops (*Yersinia* outer proteins) (11). When bacteria establish contact with macrophages, the type III machinery injects some of these virulence factors (YopEHMOPT, effector Yops) into the cytosol of target cells (44), thereby abolishing the phagocytic process and inducing apoptosis of macrophages (35–37).

In addition to injecting toxins into host cells, the type III machine can deliver substrate proteins to other locations. One example of spatial separation of type III transport is EspA of EPEC. EspA is secreted by the type III machinery and assembled into bacterial surface appendages (27). The formation of EspA filaments is required for the delivery of EspB and Tir into host cells, indicating that the type III machinery must distinguish secretion substrates to allow for differential delivery during infection (26, 27). *Yersinia* species also deliver type III secretion substrates to distinct locations. During infection of tissue culture cells, yersiniae secrete YopB, YopD, and YopR into the extracellular milieu (30), whereas effector Yops (YopE, YopH, YopM, YopO, YopP, and YopT) are injected into the cytosol of host cells (5, 45). Thus, as reported for EPEC, yersiniae may also distinguish between different sets of secretion substrates.

What are the environmental signals that activate secretion via the *Yersinia* type III pathway? Early work established a requirement for calcium to allow growth of pathogenic yersiniae at 37°C on artificial medium (28). Later studies showed that the chelation of calcium together with a temperature shift to 37°C triggers the type III secretion of Yops (18, 33, 49). The calcium concentration of extracellular host fluids (1.2 mM) is well above the threshold required for induction of type III machines (12). Nevertheless, *Yersinia* type III machines are activated during infection, a phenomenon that is referred to as the calcium paradox (12). It has been proposed

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that phagocytosis exposes bacteria to the low-calcium environment of the endocytic pathway, activating type III gene expression and providing a mechanism for *Yersinia* target cell selection (40). This possibility now seems somewhat remote, as the type III injection of effector Yops is believed to be catalyzed by extracellular *Yersinia* (45).

The observation that bacterial contact with host cells causes an increase in virulence gene expression provides a clue to understanding calcium signaling during type III secretion (38). It is thought that receptors on the bacterial surface may interact with specific ligands on the surface of host cells (11). This interaction may activate the type III machinery and remove the YopN-mediated block of the type III pathway (11, 17). However, *Yersinia* surface receptors that are necessary for type III injection or the corresponding ligand on the surface of eukaryotic cells have thus far not been identified. It is reported here that host cells may generate the calcium signal that leads to activation of the type III pathway. A mechanism is proposed whereby yersiniae measure the intracellular calcium of target cells. Further, activation of the *Yersinia* type III pathway is shown to require two other signals, i.e., serum amino acids (glutamate, glutamine, aspartate, or asparagine) and proteins such as albumin. *Yersinia* respond to temperature shift and glutamate with the expression and assembly of the type III machinery. Albumin triggers *Yersinia* type III secretion of YopD into the extracellular milieu. Host cell contact transforms the type III machinery into an injection device that transports YopE across the plasma membrane into the eukaryotic cytosol. Mutations in *Yersinia* regulatory genes (*lcrG*, *lcrH*, *tyeA*, *yopD*, *yopN*, *yscM1*, and *yscM2*) disrupt this type III secretion program and bypass the requirement for specific signals.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Y. enterocolitica* O:9 strain W22703 (13) and isogenic variants with frameshift mutations in  $\Delta(yopB)$  (30),  $\Delta(yopD)$  (32),  $\Delta(yopN)$  (29),  $\Delta(yopQ)$  (2), and  $\Delta(yopR)$  (30) or deletions in  $\Delta(lcrG)$  (15),  $\Delta(lcrV)$  (31),  $\Delta(lcrH)$  (D. M. Anderson, K. Ramamurthi, C. Tam, and O. Schneewind, submitted for publication),  $\Delta(yscH)$  (7),  $\Delta(tyeA)$  (9), and  $\Delta(yscM1/yscM2)$  (7) have been previously described. Plasmids pDA35 and pDA37 have also been previously described (1).

**Type III secretion in DMEM.** *Yersinia* strains were grown overnight at 26°C with shaking. Cultures were diluted 1:20 into 30 ml of fresh Luria broth and incubated at 26°C for 2 h. Bacteria were collected by centrifugation and were washed, and  $2.5 \times 10^8$  CFU was added to 10 ml of Dulbecco's modified Eagle medium (DMEM) in 75-cm<sup>2</sup> tissue culture flasks. Cultures were incubated for 3 h at 37°C and 5% CO<sub>2</sub>. Bacteria were scraped off the flasks, and the culture was transferred to a 15-ml conical tube. *Yersinia* strains were sedimented by centrifugation at 12,000 × g. Five milliliters of the culture supernatant was removed and precipitated with methanol-chloroform. The remaining supernatant was discarded. The *Yersinia* sediment was suspended in 1 ml of phosphate-buffered saline (PBS), and a 0.5-ml aliquot was precipitated with methanol-chloroform. Proteins were collected at the interphase after centrifugation at 12,000 × g. After removal of the aqueous phase, proteins were washed with methanol and sedimented by centrifugation at 12,000 × g. The precipitate was dried, suspended in sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Immunoreactive species were quantified as chemiluminescent signals on X-ray film using laser densitometry scanning.

**Digitonin fractionation.** Overnight cultures of *Yersinia* strains were diluted 1:20 into 30 ml of fresh Luria broth and grown for 2 h at 26°C with shaking. Bacteria were sedimented at 8,000 × g for 10 min and suspended in PBS. HeLa cells were grown to 80% confluency in 75-cm<sup>2</sup> tissue culture flasks with DMEM and 10% fetal bovine serum (FBS). Prior to infection, cells were washed twice with PBS, covered with 10 ml of DMEM, and warmed to 37°C for 30 min.

Aliquots of HeLa cells were counted, and each flask was infected with yersiniae at a multiplicity of infection of 10 and incubated for 3 h at 37°C with 5% CO<sub>2</sub>. Culture media were removed and centrifuged at 32,000 × g for 15 min to separate soluble proteins from nonadherent bacteria in the sediment. HeLa cells as well as adherent bacteria were scraped off the flasks into 10 ml of 1% digitonin in PBS and placed on a rotary shaker for 20 min. Samples were centrifuged at 32,500 × g for 15 min. A 7-ml aliquot was withdrawn and precipitated with methanol-chloroform, while the remaining supernatant was discarded. The sediment was suspended in 10 ml of PBS, and a 7-ml aliquot was precipitated with methanol-chloroform. Protein precipitates were solubilized in sample buffer, separated on SDS-PAGE, and analyzed by immunoblotting with specific antiserum. Immunoreactive species were quantified as chemiluminescent signals on X-ray film using laser densitometry scanning.

**Preparation of environmental signals.** Unless otherwise indicated, glutamate was prepared as a stock of 68 mM monosodium glutamate (Fisher BP378) in water, sterile filtered, and diluted 1:5,000 to yield a final concentration of 135 μM in DMEM. Unless otherwise indicated, purified bovine albumin (Sigma A-7638) was prepared as a stock of 1.5 mM albumin in PBS (10% wt/vol), sterile filtered, and diluted 1:1,000 to yield a final concentration of 1.5 μM in DMEM. DMEM without (GIBCO-BRL 21068-028) or with (GIBCO-BRL 11960-051) 1.8 mM calcium was used in these experiments. Heat-inactivated FBS (Gemini Bioproducts) was stored in frozen aliquots, melted, and diluted to a final concentration of 0.2% (vol/vol). For protease digestion, proteinase K (100 μl of a 100-mg/ml solution in PBS [Roche]) was incubated with 10 ml of FBS for 6 days at 4°C. Protease digestion was quenched by the addition of phenylmethylsulfonyl fluoride to 0.1 mM. Ten milliliters of FBS was dialyzed once against 100 ml of PBS for 12 h at 4°C using a membrane with a permeability barrier of 8 kDa (Spectrum Laboratories 132650); the dialysate was collected and used at a 2% (vol/vol) dilution in DMEM. After two additional dialysis steps, i.e., dialysis against 500 ml of PBS each for 12 h at 4°C using the same membrane, dialyzed FBS was recovered and tested for induction of type III secretion at a dilution of 0.2% in DMEM. Cohn's serum fractions were purchased from Sigma (Cohn I [fibrinogen], F-4753; Cohn II/III [ $\gamma$ -globulin], G-5009; Cohn IV-1, G-8512; Cohn IV-4, G-8637; Cohn V [albumin]; and A-8022). Cohn fractions were prepared as a 10% stock (wt/vol) in PBS, sterile filtered, and diluted 1:1,000 to yield a final concentration of 0.01% in DMEM.

#### RESULTS

***Yersinia* infection of HeLa cells in the presence and absence of calcium.** We wondered whether the specificity of effector Yop injection is controlled by *Yersinia* recognition of a host signal, presumably a change in calcium concentration. Activation of the *Yersinia* type III machinery was measured by infecting and fractionating tissue cultures by the digitonin technique (29). Briefly, HeLa cell cultures were infected with *Y. enterocolitica* W22703 for 3 h. The growth medium was removed and centrifuged to separate nonadherent bacteria from the extracellular medium. HeLa cells and yersiniae adherent to the culture flasks were treated with digitonin, a detergent that disrupts the eukaryotic plasma membrane but not the bacterial envelope (29). Samples were centrifuged to separate the HeLa cell cytosol from the bacterial sediment. During HeLa cell infection in DMEM tissue culture medium with 1.8 mM calcium, *Y. enterocolitica* W22703 secreted YopD into the extracellular medium and injected YopE into the cytosol (Fig. 1A). Small amounts of YopD were also observed in the supernatant of digitonin-extracted HeLa cells. As a control for proper fractionation, p130<sup>cas</sup>, a protein in the HeLa cell cytosol, was solubilized by digitonin extraction of tissue culture cells. In contrast, *Yersinia* Npt (neomycin phosphotransferase, encoded by *npt* carried by pDA37) was not solubilized by digitonin treatment and sedimented with the bacteria after centrifugation of tissue culture extracts. When HeLa cells were infected in DMEM lacking calcium, *Yersinia* strains secreted both YopD and YopE into the extracellular medium (Fig. 1A).

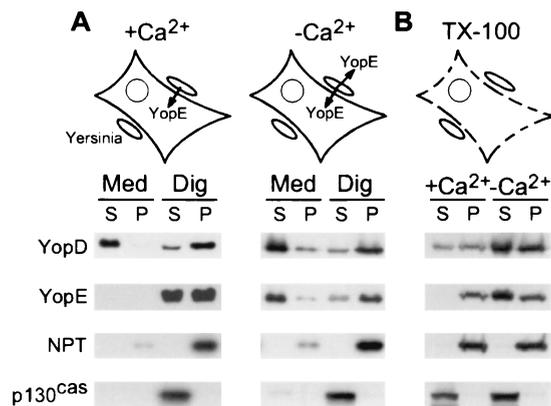


FIG. 1. A calcium signal induces *Yersinia* type III targeting of YopE but not type III secretion of YopD. (A) HeLa cells in DMEM with (+Ca<sup>2+</sup>) or without (-Ca<sup>2+</sup>) 1.8 mM calcium were infected with *Y. enterocolitica* W22703. The culture medium (Med) was decanted and centrifuged, separating the extracellular medium (S, supernatant) from the bacterial sediment (P, pellet). HeLa cells were extracted with digitonin (Dig). After centrifugation, proteins in the cytosol of HeLa cells (S, supernatant) were separated from the bacterial sediment (P, pellet). Samples were analyzed by immunoblotting. p130<sup>Cas</sup> is located in the cytosol of HeLa cells, whereas Npt is located in the bacterial cytoplasm. (B) HeLa cells in DMEM with or without 1.8 mM calcium were lysed with Triton X-100 (TX-100). Cell lysates were infected with *Yersinia* and centrifuged to separate the extracellular medium (S) from the bacterial sediment (P).

These results suggest that the specific injection of YopE via the *Yersinia* type III pathway requires extracellular calcium.

Can the cytosol of HeLa cells generate the calcium signal for the injection of YopE? To address this possibility, HeLa cells were lysed with 0.1% Triton X-100 in DMEM with 1.8 mM calcium, an extraction that also disrupts the calcium gradient across the plasma membrane. As a control, detergent treatment solubilized p130<sup>Cas</sup>, indicating that the plasma membrane, and therefore the calcium gradient across the plasma membrane, had been disrupted (Fig. 1B). Infection of the HeLa cell lysate with yersiniae in the presence of 1.8 mM calcium failed to activate YopE secretion. In contrast, secretion of YopD still occurred, albeit at a greatly reduced rate. To examine whether the removal of extracellular calcium can substitute for the calcium signal that is generated by intact HeLa cells, tissue cultures were lysed with 0.1% Triton X-100 in DMEM without calcium. Infection of lysate without calcium resulted in *Yersinia* type III transport of YopD and YopE (Fig. 1B). Further, the removal of calcium ions from HeLa cell extracts led to an increase in the concentration of YopD and YopE, suggesting that *yop* gene expression may also be stimulated under these conditions (see Table 1 and Table 2 for the regulatory effects of low-calcium signaling). Together these results suggest that intact HeLa cells may generate the calcium signal that activates the transport of YopE. Results similar to those described above were obtained when HeLa cells were lysed by sonication, shearing, or digitonin treatment (data not shown), suggesting that the method of HeLa cell lysis does not affect the calcium signaling in this experiment.

**Calcium concentration required for *Yersinia* type III secretion.** To determine the critical concentration of calcium required to induce type III secretion, *Y. enterocolitica* W22703

TABLE 1. Expression of YopE by *Y. enterocolitica* W22703(pDA35) grown in DMEM at 37°C

| Signal(s) added       | Amt. of YopE in culture with (+) or without (-) Ca <sup>2+</sup> <sup>a</sup> |                                |                                |                                |
|-----------------------|---|--------------------------------|--------------------------------|--------------------------------|
|                       | -Ca <sup>2+</sup> <sup>b</sup>  | -Ca <sup>2+</sup> <sup>c</sup> | +Ca <sup>2+</sup> <sup>b</sup> | +Ca <sup>2+</sup> <sup>c</sup> |
| Glutamate             | 79 (20)   | 55 (7)                         | 74 (26)                        | 65 (18)                        |
| Albumin               | 140 (105)   | 99 (12)                        | 98 (54)                        | 78 (16)                        |
| Glutamate and albumin | 62 (28)   | 80 (32)                        | 64 (8)                         | 75 (15)                        |
| FBS                   | 150 (14)  | 237 (72)                       | 94 (17)                        | 123 (3)                        |
|                       | 150 (14)  | 267 (151)                      | 100 <sup>d</sup>               | 100 <sup>d</sup>               |

<sup>a</sup> Numbers indicate the percent amount of YopE present in *Y. enterocolitica* cultures. Numbers in parentheses represent the standard deviation of the measurement.

<sup>b</sup> Data were generated by trichloroacetic acid precipitating cultures, separating proteins on SDS-PAGE, and immunoblotting with YopE-specific antiserum. Numbers represent averages of three independent experiments. Data were multiplied with a correction factor for the bacterial density, which was determined by measuring the optical density at 660 nm of *Yersinia* cultures.

<sup>c</sup> Data were generated as described above and multiplied with a correction factor for an immunoreactive signal obtained with chloramphenicol acetyltransferase-specific antibodies. *cat* is carried by pDA35 and is not part of the *yop* regulon.

<sup>d</sup> The average amount of YopE in *Y. enterocolitica* W22703 cultures grown in DMEM supplemented with FBS was arbitrarily assigned a value of 100%.

(pDA37) was grown in DMEM supplemented with 0.2% FBS (final concentration) and various amounts of calcium chloride (CaCl<sub>2</sub>) at 37°C for 3 h. Variations in the concentration of calcium were achieved by mixing DMEM without calcium and DMEM with calcium. The concentration of calcium was verified by measuring these ions directly with a calcium electrode. *Yersinia* cultures were centrifuged, and the extracellular medium was separated with the supernatant from the sedimented bacteria in the pellet). Proteins in both fractions were precipitated with chloroform-methanol and analyzed by SDS-PAGE and immunoblotting with specific antisera. In Fig. 2 the proportion of secreted Yop (percent amount of the total) is plotted against the extracellular calcium chloride concentration. We observed a reduction in the amount of secreted YopE when the extracellular calcium chloride concentration reached

TABLE 2. Expression of YopD by *Y. enterocolitica* W22703(pDA35) grown in DMEM at 37°C

| Signal(s) added       | Amt. of YopD in culture with (+) or without (-) Ca <sup>2+</sup> <sup>a</sup> |                                |                                |                                |
|-----------------------|---|--------------------------------|--------------------------------|--------------------------------|
|                       | -Ca <sup>2+</sup> <sup>b</sup>  | -Ca <sup>2+</sup> <sup>c</sup> | +Ca <sup>2+</sup> <sup>b</sup> | +Ca <sup>2+</sup> <sup>c</sup> |
| Glutamate             | 77 (4)  | 55 (12)                        | 73 (4)                         | 68 (32)                        |
| Albumin               | 174 (127)   | 124 (23)                       | 118 (59)                       | 99 (27)                        |
| Glutamate and albumin | 65 (31)   | 85 (39)                        | 54 (14)                        | 63 (22)                        |
| FBS                   | 181 (34)  | 289 (111)                      | 97 (16)                        | 128 (5)                        |
|                       | 113 (29)  | 241 (122)                      | 100 <sup>d</sup>               | 100 <sup>d</sup>               |

<sup>a</sup> Numbers indicate the percent amount of YopD present in *Y. enterocolitica* cultures. Numbers in parentheses represent the standard deviation of the measurement.

<sup>b</sup> Data were generated by trichloroacetic acid precipitating cultures, separating proteins on SDS-PAGE, and immunoblotting with YopD-specific antiserum. Numbers represent averages of three independent experiments. Data were multiplied with a correction factor for the bacterial density, which was determined by measuring the optical density at 660 nm of *Yersinia* cultures.

<sup>c</sup> Data were generated as described above and multiplied with a correction factor for an immunoreactive signal obtained with chloramphenicol acetyltransferase-specific antibodies.

<sup>d</sup> The average amount of YopD in *Y. enterocolitica* W22703 cultures grown in DMEM supplemented with FBS was arbitrarily assigned a value of 100%.

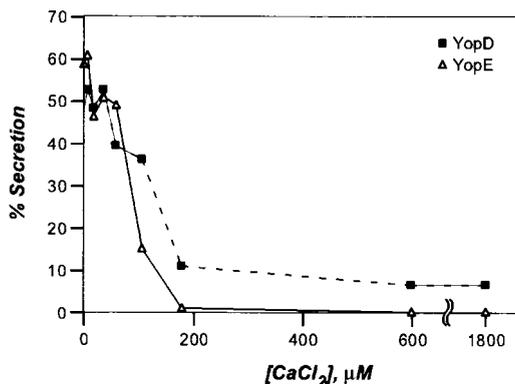


FIG. 2. *Yersinia* type III secretion of YopE and YopD is regulated by extracellular calcium ions. *Y. enterocolitica* W22703(pDA37) was grown in DMEM supplemented with 0.2% fetal bovine serum for 3 h. Cultures were centrifuged, and the extracellular medium was separated with the supernatant from the bacterial sediment. Protein in both fractions was precipitated with methanol-chloroform, separated on SDS-PAGE, and analyzed by immunoblotting with antiserum raised against purified YopD or YopE.

100  $\mu$ M. At 200  $\mu$ M calcium chloride, all *Y. enterocolitica* secretion of YopE was blocked. These observations corroborate the previous finding that *Y. pestis* activates the expression of a *yopK* (*yopQ*)-*lacZ* transcriptional fusion when the calcium chloride concentration drops below 200  $\mu$ M (40). It should be noted, however, that these studies measured reporter protein expression but not type III secretion. These findings are consistent with the observed expression of YopD and YopE shown in Fig. 1B. It therefore appears that the low-calcium signal may regulate *yop* gene expression as well as the activity of the type III machinery. In contrast to the block in secretion of YopE, a low-level secretion of YopD (about 10%) was observed even at a calcium chloride concentration of 1,800  $\mu$ M. Nevertheless, reducing the calcium chloride concentration below 100  $\mu$ M caused *Y. enterocolitica* to increase the secretion of YopD (40 to 50%). During *Yersinia* infection of HeLa tissue cultures or HeLa lysates, comparatively more YopD was secreted in the presence of 1.2 mM calcium. The reason for this discrepancy is unknown. In summary, the critical concentration of calcium required to activate *Yersinia* type III transport of YopE is  $\leq$ 80  $\mu$ M (Fig. 2), a level that is well above the intracellular calcium concentration of HeLa cells (100 nM) (3). We propose that *Yersinia* strains may have evolved a mechanism to measure calcium ions across the eukaryotic plasma membrane. Presumably, this mechanism requires contact between *Yersinia* cells and eukaryotic cells. Hoiczky and Blobel reported that the YscF-containing needle complexes of *Y. enterocolitica* are responsible for the formation of a type III targeting conduit between bacteria and eukaryotic cells (23). These results may have identified the device with which yersiniae sense calcium. As *Yersinia* needle complexes are assembled prior to host cell contact (23), the insertion of this structure into eukaryotic cells may allow bacteria to measure the change in environmental calcium concentration and thereby activate the type III pathway.

**Glutamate activates the *Yersinia* type III machinery.** When bacteria are grown in brain heart infusion or other complex

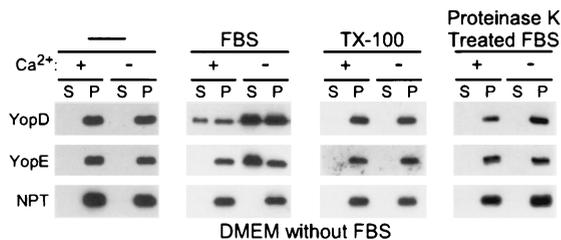


FIG. 3. FBS activates the *Yersinia* type III pathway. *Y. enterocolitica* W22703(pDA37) was grown in DMEM without FBS and with or without calcium. The medium was supplemented with 0.2% FBS or 0.1% Triton X-100 (TX-100) as indicated. Type III secretion was measured as described in the legend to Fig. 2. S, supernatant; P, pellet.

media and incubated at 37°C in the absence of calcium ions, *Yersinia* type III secretion is induced (34). Growth of *Y. enterocolitica* in DMEM tissue culture medium at 37°C without HeLa cells failed to activate the type III transport of YopD and YopE, even in the absence of calcium ions (Fig. 3). This was a surprising result, as the removal of calcium and the incubation at 37°C were hitherto thought to be sufficient as signals for the activation of the *Yersinia* type III pathway (14). Secreted extracellular Yops are known to aggregate and to precipitate on the surface of glass and plastic containers (34). We wondered whether the lack of YopD and YopE secretion observed in Fig. 3 is caused by the aggregation of Yops. *Yersinia* cultures were grown in the presence of 0.1% Triton X-100, a condition that is known to prevent the aggregation of some Yops (1). However, growth of *Yersinia* in the presence of 0.1% Triton X-100 did not result in the appearance of soluble, extracellular YopD or YopE. It seems that YopD and YopE sediment with the bacteria when yersiniae are grown in DMEM without FBS, because the type III secretion machinery is not activated under these conditions.

Supplementation of DMEM with 0.2% FBS restored the ability of *Y. enterocolitica* W22703 to secrete YopD in the presence of calcium as well as YopD and YopE in the absence of calcium (Fig. 3). These data suggest that *Yersinia* requires serum signals to activate the type III pathway. Similar observations have been made for *Salmonella* and *Shigella* species, suggesting that many gram-negative pathogens activate the type III pathway in response to serum components (32, 55). FBS was dialyzed to remove small molecules ( $\leq$ 8 kDa). This treatment removed the signal(s) required to induce *Yersinia* type III secretion (Fig. 4). However, when equal parts of dialyzed FBS and dialysate were mixed, the inducing signal(s) could be reconstituted, as yersiniae were once again activated for YopD and YopE secretion in the absence of calcium as well as for YopD secretion in the presence of calcium (Fig. 4). DMEM contains most of the small molecules (amino acids, carbohydrates, ions, and salts) that are present in serum or extracellular fluids. However, 6 amino acids are absent (glutamine, glutamate, asparagine, aspartate, proline, and alanine). The addition of glutamate, glutamine, aspartate, or asparagine to DMEM without calcium each activated *Yersinia* type III secretion of YopD and YopE, whereas addition of proline or alanine had no effect (Fig. 5A) (data not shown). The critical concentration of amino acids required for activation of type III secretion is  $\geq$ 70  $\mu$ M (data not shown), a level

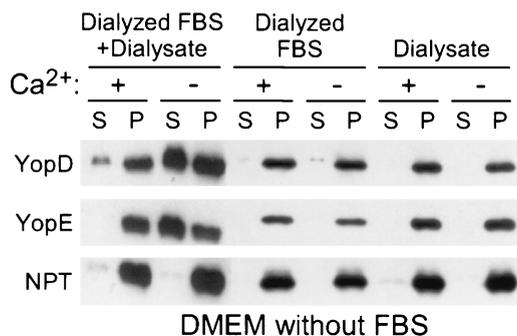


FIG. 4. Dialysis of FBS abolishes the *Yersinia* type III inducing activity. *Y. enterocolitica* W22703(pDA37) was grown in DMEM with or without calcium supplemented with 0.2% dialyzed FBS, the dialysate of this reaction mixture, or a mixture of both. Type III secretion was measured as described in the legend to Fig. 2. S, supernatant; P, pellet.

that is below that of the amino acid concentration in serum (300  $\mu$ M) (48). Glutamate proved to be the most potent inducer of the type III pathway and was used at a final concentration of 135  $\mu$ M for all subsequent experiments. Glutamate and aspartate chelate calcium ions; the concentration at which these amino acids are added to DMEM does not lead to significant changes in the concentration of extracellular calcium ions that could explain the observed activation of the type III pathway (data not shown).

**Albumin activates the type III secretion of YopD.** Digestion of FBS with proteinase K abolished the signal(s) that activates type III secretion (Fig. 3). Addition of glutamate alone to

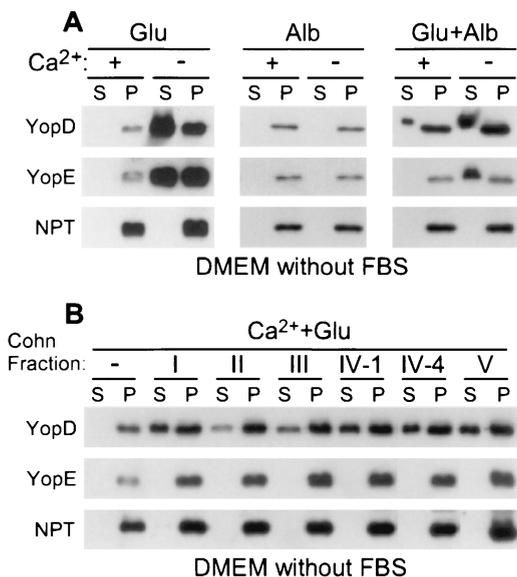


FIG. 5. Glutamate and albumin activate the *Yersinia* type III pathway. (A) *Y. enterocolitica* W22703(pDA37) was grown in DMEM with or without calcium supplemented with glutamate (Glu) and albumin (Alb), alone or in combination, as indicated. Type III secretion was measured as described in the legend to Fig. 2. (B) *Y. enterocolitica* W22703(pDA37) was grown in DMEM with calcium supplemented with glutamate and a Cohn serum fraction (I, II, III, IV-1, IV-4, or V), as indicated. S, supernatant; P, pellet.

DMEM with calcium also did not activate *Yersinia* type III secretion of YopD (Fig. 5). It therefore appears that glutamate, as well as glutamine, aspartate, and asparagine, induces the low-calcium transport of Yops; however, additional serum components seem required to activate type III secretion of YopD in the presence of calcium. When combined with glutamate, dialyzed FBS stimulated the secretion of YopD in DMEM with calcium (data not shown). To test whether serum proteins (>8 kDa) activate type III secretion, we analyzed various Cohn fractions (I through V; Sigma Pharmaceuticals), generated by ethanol precipitation of bovine serum (10). When added to DMEM with glutamate and calcium, each of the five Cohn fractions (100  $\mu$ g of protein/ml) activated type III secretion of YopD (Fig. 5B). This result suggests that many different serum proteins are capable of activating the type III secretion pathway. To test this prediction, albumin, a serum protein that is abundantly present in the blood of many mammals (42), was examined (42). When added to DMEM with glutamate and calcium, bovine albumin induced *Yersinia* type III secretion of YopD (Fig. 5A). The concentration of albumin required for induction of type III secretion ( $\geq 0.3$   $\mu$ M; data not shown) was below that present in human serum (600  $\mu$ M) (42). Extracellular YopD and YopE, secreted by yersiniae growing in the presence of albumin, migrated more slowly on SDS-PAGE than intrabacterial YopD and YopE. This was explained as an overloading of the SDS-PAGE with albumin, a condition that may artifactually alter the mobility of faster-migrating proteins such as YopD and YopE. The molecular properties of serum proteins that activate type III secretion are not known. It is conceivable that peptide sequences, folded structure, or post-translational modifications function as a signal.

The addition of glutamine, albumin, FBS, or calcium to DMEM appears to affect the expression of *yopE* or *yopD*. If so, the regulation of YopD and YopE secretion that is reported here could, in fact, be caused by a regulatory effect of gene expression. To address this possibility, *Y. enterocolitica* W22703 (pDA35) cultures were grown in DMEM with or without various inducers. The cell density was measured, and proteins in the culture were precipitated with methanol-chloroform. After separation of proteins on SDS-PAGE and immunoblotting, YopE and YopD were quantified by chemiluminescent measurement of immunoreactive signals and their expression levels were calculated (Table 1 and Table 2). The addition of glutamate and albumin and the addition of FBS as well as the omission of calcium resulted in a weak stimulation of *yopD* and *yopE* expression. The addition of glutamine and albumin alone had little or no effect. These data suggest that glutamine, albumin, and calcium cause weak regulatory effects on *yopD* and *yopE* expression in addition to regulating the secretion of YopD and YopE polypeptides by the *Yersinia* type III machinery.

**Mutations in *yopD*, *lcrH*, or *yscM1/M2* bypass the glutamate signal.** The type III pathway is modulated by glutamate, albumin, and calcium signals. Presumably, each of these signals modulates the activity of the secretion machinery by a distinct mechanism, requiring the function of regulatory genes. If so, mutations in regulatory genes should abolish the signal requirement of the type III machinery. This prediction was first tested for glutamate. Previous work identified genes that regulate the *Yersinia* type III pathway (4, 8, 14, 25, 41, 43, 47, 54).

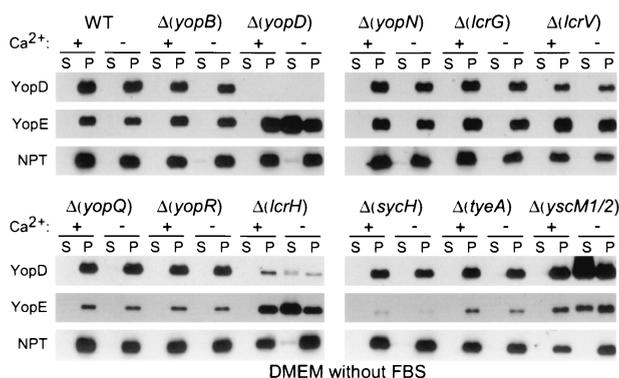


FIG. 6. Knockout mutations in *yopD*, *lcrH*, and *yscM1/M2* bypass the glutamate requirement for *Yersinia* type III secretion. *Y. enterocolitica* strains were grown in DMEM without FBS and without glutamate. Type III secretion was measured as described in the legend to Fig. 2. S, supernatant; P, pellet.

We wondered whether mutations that result in a loss-of-function phenotype for *yopBDNQR*, *lcrGVH*, *sycH*, *tyeA*, or *yscM1/yscM2* abolish the glutamate requirement of the *Yersinia* type III pathway. *Yersinia* were grown in DMEM without calcium, glutamate, albumin, or FBS. Mutations in *yopD*, *lcrH* (*sycD*), or *yscM1/yscM2* allowed yersiniae to secrete YopE, whereas all other mutations had no effect (Fig. 6). *lcrH* and *yscM1/yscM2* mutants failed to secrete YopD and YopE in DMEM with calcium, suggesting that these mutations bypass the requirement for glutamate but not for albumin or calcium (Fig. 6; data for albumin not shown). Furthermore, *yopD* mutants do not secrete YopB in DMEM with calcium (data not shown). YopD, LcrH, and YscM1/YscM2 (LcrQ) seem to control Yop secretion at the same step and appear to function as a switch that allows secretion of type III substrates in response to glutamate. The *lcrH* mutant strain contained reduced amounts of YopD; this can be explained by the lacking chaperone function of LcrH (SycD), a small cytoplasmic protein that binds to YopD (50). The *sycH* mutant *Yersinia* strain synthesized very little, if any, YopD and YopE (Fig. 6). SycH acts as a secretion chaperone and functional inhibitor for YscM1/YscM2, the negative regulators of the *Y. enterocolitica* type III pathway (7, 47). Thus, the *sycH* mutant phenotype can be explained as YscM1/YscM2-mediated inhibition of *yopE* expression (7).

#### Mutations in *yopN*, *lcrG*, and *tyeA* bypass the calcium signal.

When bacteria are grown in rich medium, mutations in *yopN* (*lcrE*) abrogate the calcium signal requirement of *Yersinia* and activate the type III machinery in the presence of 1.8 mM calcium (54). YopN has been proposed to act as both a calcium sensor on the surface of *Yersinia* and as a stop valve that occludes the type III machinery (11, 17). Type III export of YopN itself is regulated in a complex manner. The secretion chaperones YscB and SycN bind to the N-terminal portion of YopN (14). TyeA, a small polypeptide that interacts with the C-terminal end of YopN (24), seems to act as a negative regulator of YopN secretion (9 and L. W. Cheng, O. Kay, and O. Schneewind, submitted for publication). *Yersinia* carrying deletion mutations in *sycN*, *tyeA*, and *yscB* display a calcium-blind phenotype similar to that of *yopN* mutants (14, 25). We asked whether mutations in *yopBDNQR*, *lcrGVH*, *sycH*, *tyeA*,

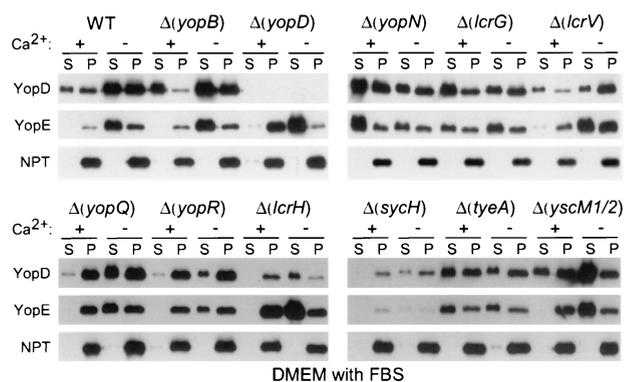


FIG. 7. Knockout mutations in *yopN*, *lcrG*, and *tyeA* bypass the calcium requirement for *Yersinia* type III secretion. *Y. enterocolitica* strains were grown in DMEM with FBS. Type III secretion was measured as described in the legend to Fig. 2. S, supernatant; P, pellet.

or *yscM1/yscM2* abolish the calcium regulation of the *Yersinia* type III pathway. *Yersinia* strains were grown in DMEM with calcium and 0.2% FBS. Mutations in *yopN*, *lcrG*, or *tyeA* allowed yersiniae to secrete YopE in the presence of calcium, whereas all other mutations had no effect (Fig. 7). These data suggest that DMEM supplemented with 0.2% FBS resembles other rich laboratory media and allows induction of type III secretion upon removal of calcium ions. Mutations in *lcrG* are known to cause a calcium-blind phenotype in mutant yersiniae which is corroborated by the data shown in Fig. 7 (15, 46).

*yopN* mutant yersiniae failed to secrete Yops when grown in DMEM without glutamate and calcium (Fig. 8A). Apparently, these mutants are still capable of regulating type III secretion and may require glutamate for induction. Although glutamate allowed secretion in the absence of calcium, *yopN* mutants failed to activate the type III pathway in DMEM with glutamate and calcium (Fig. 8A). Only the addition of glutamate and albumin triggered *yopN* mutants to secrete Yops in the presence of calcium (Fig. 8A). Several conclusions can be drawn from these experiments. (i) Full induction of the type III pathway requires all three signals: glutamate, albumin, and calcium. (ii) The three signals activate *Yersinia* in a sequential manner: glutamate and then albumin and calcium. (iii) When yersiniae are grown in DMEM with glutamate, calcium signaling of *yopN* mutants occurs in a manner similar to that of wild-type strains. This result suggests that YopN cannot function as a calcium sensor for the type III pathway. (iv) As suggested previously, mutations in *yopN*, *tyeA*, *sycN*, and *yscB* bypass the calcium signal requirement of the *Yersinia* type III pathway (17, 24), suggesting that these genes provide a stop valve function for the type III machinery (12). (v) The stop valve is not implemented unless yersiniae receive glutamate and albumin signals.

**Sequential activation of the type III machinery.** The type III pathway appears to be activated by a sequence of signals: glutamate and then albumin and calcium. If so, albumin alone should activate the type III pathway of  $\Delta(yopDN)$  yersiniae, as these mutants are predicted to bypass the *Yersinia* requirement for glutamate and calcium. This was tested, and  $\Delta(yopDN)$  yersiniae secreted YopB and YopE in DMEM with albumin and calcium (Fig. 8B). The  $\Delta(yopDN)$  mutant strain appears to

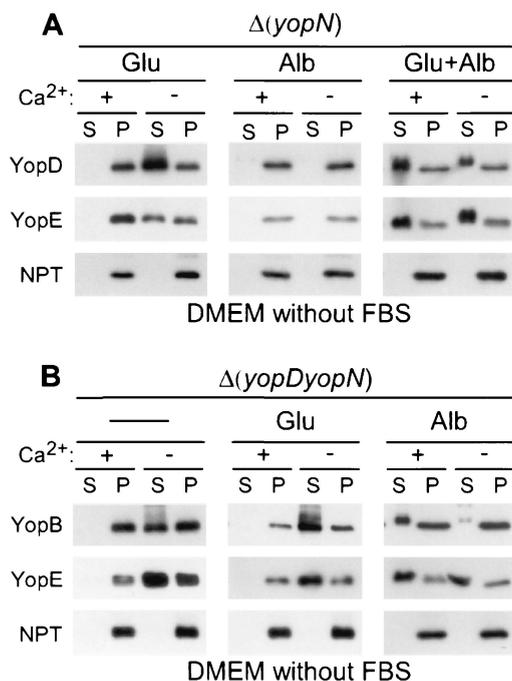


FIG. 8. A program of *Yersinia* type III secretion events is triggered by specific host signals. *Y. enterocolitica* strains were grown in DMEM without FBS and supplemented with glutamate (Glu) and/or albumin (Alb) as indicated. Type III secretion was measured as described in the legend to Fig. 2. (A) Knockout mutations in *yopN*; (B) knockout mutations in *yopD* and *yopN*. S, supernatant; P, pellet.

be capable of sensing calcium, as YopB and YopE secretion in DMEM without albumin occurred only in the absence but not in the presence of calcium (Fig. 8B). Further, the  $\Delta(yopDN)$  mutant secretes YopE in DMEM alone (Fig. 8), similar to *yopD* but unlike *yopN* mutant yersiniae (Fig. 6). These data suggest that the regulatory function of *yopD* is epistatic over that of *yopN*.

**Type III secretion occurs prior to target cell contact.** Glutamate and albumin induce *Yersinia* type III secretion of YopD. Bacterial sensing of these signals should occur immediately upon host entry and before *Yersinia* encounters immune cells. In contrast, type III injection of YopE requires bacterial contact with the target cell and occurs later during infection. We wondered whether the secretion machinery is modified by target cell contact. In this scenario one would expect nonadherent yersiniae to catalyze type III secretion of YopB, YopD, and YopR. Target cell contact should transform the machinery into an injection device that promotes type III targeting but not secretion. To test this, HeLa cells were infected with *Y. enterocolitica* W22703. After 1 h of infection, more than 90% of the yersiniae adhered to tissue culture cells (data not shown). The medium and nonadherent bacteria were removed, and cells were washed and replenished with fresh DMEM. After further incubation for 2 h, the cultures were fractionated by the digitonin technique and analyzed by immunoblotting. Removal of nonadherent yersiniae seemed to reduce the secretion of YopB and YopD, whereas targeting of YopE and YopH was not affected (Fig. 9). These results support the hypothesis that *Y. enterocolitica* transport of Yops may

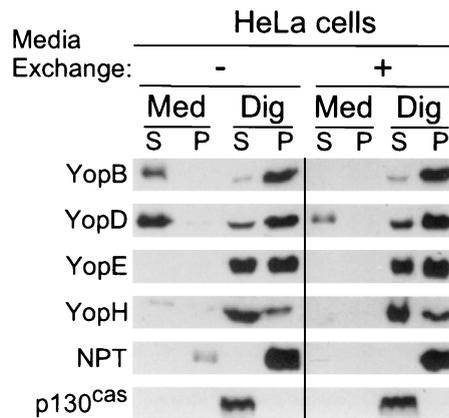


FIG. 9. Type III secretion occurs prior to target cell contact. HeLa cell cultures were infected with *Y. enterocolitica* W22703(pDA37). After 1 h of infection, the medium of HeLa cells was removed to separate nonadherent bacteria from *Yersinia* that had established target cell contact. The samples were washed twice with PBS, and HeLa cells with adherent yersiniae were covered with fresh DMEM and incubated for 2 h. Samples were fractionated and analyzed by immunoblotting. S, supernatant; P, pellet; Med, culture medium; Dig, digitonin.

occur in a sequential manner, beginning with type III secretion of YopBD and followed by type III targeting of YopEH into host cells.

## DISCUSSION

A model is proposed whereby yersiniae enter the host and respond to changes in temperature as well as to glutamate, glutamine, asparagine, or aspartate with the assembly of the type III machinery (Fig. 10). Albumin and other serum proteins activate *Yersinia* to secrete YopB, YopD, and YopR into the extracellular medium. Contact with immune cells transforms the type III machinery into an injection device. The molecular nature of calcium sensing is unknown. Electron microscopy experiments revealed the insertion of type III needles into the plasma membrane of tissue culture cells (23). Such needle insertion may not only serve Yop transport across membranes but could also provide for the measurement of calcium ions. Once stimulated by a low-calcium signal, *Yersinia* transports effector proteins (YopE, YopH, YopM, YopO, YopP, and YopT) across the plasma membrane. Several regulatory genes control this cascade of type III transport reactions. *lcrF* (*virF*) encodes a transcription factor that activates the expression of type III genes when the temperature is shifted to 37°C (22, 51, 53). YopD, LcrH, and YscM1/YscM2 (LcrQ) prevent activation of the type III pathway in the absence of glutamate. Recent work suggests that YopD, LcrH, and YscM1/YscM2 (LcrQ in *Y. pestis* and *Y. pseudotuberculosis*) function in posttranscriptional regulation of *yop* gene expression (D. M. Anderson et al., submitted). As albumin is required to activate type III secretion, other (hitherto unidentified) genes may prevent transport of YopB, YopD, and YopR. YopN, TyeA, SycN, YscB, and LcrG block the type III injection step until *Yersinia* receives the calcium signal.

This model predicts that mutations in regulatory genes may disrupt the *Yersinia* type III pathway at discrete steps. *yopN*, *lcrG*, *sycN*, *yscB*, and *tyeA* mutations cause premature secretion

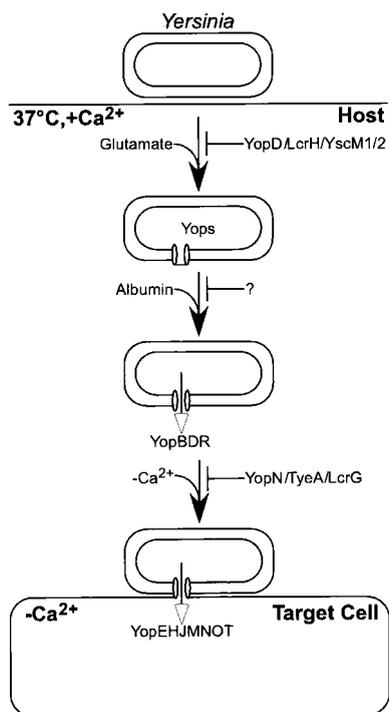


FIG. 10. Host signals trigger a *Yersinia* type III secretion program. The drawing depicts a model for events that occur during *Yersinia* infection. Entry of pathogenic yersiniae into their host causes changes in temperature and extracellular glutamate that induce expression of the type III machinery and *yop* genes. Serum albumin triggers secretion of YopBDR into the extracellular milieu. Contact with host cells halts *Yersinia* secretion and transforms the type III machinery into an injection device. The cytosol of host cells generates the calcium signal that activates injection of effector Yops. Repressor molecules control the activity of type III secretion machines. YopD, LcrH, and YscM are thought to regulate the translation of secretion substrates, whereas YopN, TyeA, SycN, and YscB function as stop valves for the type III secretion machinery. Mutations in repressor genes bypass the requirement of the type III machinery for activation by specific signals. Mutations in regulatory genes that bypass the albumin signal are still unknown (?).

of effector Yops into the extracellular medium (Los phenotype, for loss of type III targeting specificity) (15, 29, 45), consistent with their presumed repressor function for the type III pathway. *yscM1/yscM2* mutant yersiniae transport massive amounts of effector Yops into target cells, whereas *sycH* mutants (*YscM1/YscM2* chaperone) inject only small amounts of YopEMOPT (7). These data suggest that the gene products of *yscM1/yscM2* and *sycH* alter the amplitude of Yop expression without affecting the transport reactions of effector Yops. In contrast, knockout mutations in *yopD* abolish type III targeting without affecting secretion (Not phenotype, for no type III targeting) (30, 45). The Not phenotype cannot be explained by the repression of the type III pathway alone but must be accounted for by the specific function of secreted proteins (45). Thus, in order to advance through the type III program, yersiniae not only receive glutamate, albumin, and calcium signals but also catalyze specific transport reactions, such as the secretion of YopD or the injection of YopN and YscM1/YscM2.

What is the role of serum signals during *Yersinia* infection of a mammalian host? It is presumed here that during host infection, glutamate and albumin provide signals that lead to the activation of the *Yersinia* type III pathway. Further, it is presumed that *Yersinia* receives the serum signals during the infection of tissue culture cells. Experimental verification of these assumptions are hindered by the fact that both animal hosts of *Yersinia* infection as well as cultured human cells or tissues release glutamate, glutamine, and proteins into the extracellular medium (serum) (39, 42). Thus, simple omission of glutamate and serum proteins from the media of tissue culture cells cannot be achieved in an experimental protocol that measures type III secretion and targeting during a prolonged time interval (3 h). It should be emphasized again that many serum proteins in addition to albumin can activate the secretion of YopBDR in the presence of calcium. It appears, therefore, that yersiniae recognize a common property of serum proteins and respond to this signal with type III secretion.

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