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Selection and characterization of *Yersinia pestis* YopN mutants that constitutively block Yop secretion

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Summary

Secretion of Yop effector proteins by the *Yersinia pestis* plasmid pCD1-encoded type III secretion system (T3SS) is regulated in response to specific environmental signals. Yop secretion is activated by contact with a eukaryotic cell or by growth at 37°C in the absence of calcium. The secreted YopN protein, the SycN/YscB chaperone and TyeA form a cytosolic YopN/SycN/YscB/TyeA complex that is required to prevent Yop secretion in the presence of calcium and prior to contact with a eukaryotic cell. The mechanism by which these proteins prevent secretion and the subcellular location where the block in secretion occurs are not known. To further investigate both the mechanism and location of the YopN-dependent block, we isolated and characterized several YopN mutants that constitutively block Yop secretion. All the identified amino-acid substitutions that resulted in a constitutive block in Yop secretion mapped to a central domain of YopN that is not directly involved in the interaction with the SycN/YscB chaperone or TyeA. The YopN mutants required an intact TyeA-binding domain and TyeA to block secretion, but did not require an N-terminal secretion signal, an intact chaperone-binding domain or the SycN/YscB chaperone. These results suggest that a C-terminal domain of YopN complexed with TyeA blocks Yop secretion from a cytosolic, not an extracellular, location. A hypothetical model for how the YopN/SycN/YscB/TyeA complex regulates Yop secretion is presented.

Introduction

Yersinia pestis, the etiologic agent of plague, overwhelms its mammalian host with systemic growth by evading phagocytosis and by inhibiting the inflammatory response (Cornelis *et al*., 1998). These properties are associated with a 70 kb plasmid, termed pCD1 (Perry *et al*., 1998), which codes for a virulence-associated type III secretion system (T3SS). The T3SS is comprised of a group of structural proteins that make up the T3S apparatus or injectisome and of a set of secreted effector proteins called *Yersinia* outer proteins (Yops) (Cornelis, 2000). Injectisomes are specialized protein-secretion nanomachines that span the bacterial envelope and are topped by a hollow needle structure that protrudes 40–60 nm from the bacterial surface (Journet *et al*., 2003). The T3S apparatus is dedicated to transporting Yops from the bacterial cytosol to the cytoplasm of phagocytic cells (Rosqvist *et al*., 1994; Cornelis and Wolf-Watz, 1997). This process occurs in two distinct steps: (i) secretion of Yops across the bacterial membranes via the T3S apparatus, and (ii) translocation of Yops across the eukaryotic membrane. This second phase of the delivery process is dependent on three pore-forming bacterial proteins (LcrV, YopB and YopD) that form a translocation complex, or translocon, in the eukaryotic membrane (Nilles *et al*., 1998; Neyt and Cornelis, 1999; Pettersson *et al*., 1999). Once translocated into a eukaryotic cell, effector Yops modulate the cellular functions of professional phagocytes by disrupting the signal-transduction pathways that lead to bacterial uptake and to the production of proinflammatory cytokines (Palmer *et al*., 1998; Schesser *et al*., 1998; Orth *et al*., 1999).

Secretion of T3SS substrates is not constitutive; instead, this process is tightly regulated in response to specific environmental signals. *In vivo*, the T3S pathway is blocked prior to contact with a eukaryotic cell. Upon contact with the surface of a host cell, effector Yops are transported into the cytoplasm of the targeted cell with minimal loss to the extracellular milieu (Rosqvist *et al*., 1994; Cornelis and Wolf-Watz, 1997). *In vitro*, secretion of Yops is blocked at 37°C in the presence of 2.5 mM calcium and is triggered by growth at 37°C in the absence of calcium (Brubaker and Surgalla, 1964; Michiels *et al*., 1990). Secretion of Yops is also accompanied by a cessation of bacterial growth, an event termed growth restriction (Brubaker, 1983). Because of this behaviour, yersiniae's growth at 37°C has traditionally been called calcium-dependent (CD). Two classes of mutants have been identified on the basis of their growth patterns: calcium-independent (CI) mutants, which never enter growth

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restriction nor secrete Yops; and calcium-blind (CB) mutants, which always enter growth restriction and secrete Yops regardless of the calcium concentration (Yother and Goguen, 1985). In this report, we will refer to the CD, CI and CB phenotypes as regulated-secretion (RS), no-secretion (NS) and constitutive-secretion (CS) phenotypes respectively.

Regulation of Yop secretion is dependent on the expression of the secreted YopN protein (Yother and Goguen, 1985; Forsberg *et al*., 1991) and of the cytosolic SycN (Day and Plano, 1998; Iriarte and Cornelis, 1999), YscB (Jackson *et al*., 1998), TyeA (Iriarte *et al*., 1998; Cheng and Schneewind, 2000) and LcrG (DeBord *et al*., 2001; Matson and Nilles, 2001) proteins. Deletion of any one of the genes coding for these proteins results in a CS phenotype and in a loss of polarized translocation after cell contact (Day *et al*., 2003). LcrQ and the SycH chaperone have also been shown to exert an inhibitory effect on the secretion of some, but not all, Yops (Wulff-Strobel *et al*., 2002). The LcrG protein forms a 1–1 complex with LcrV and has been hypothesized to block Yop secretion when present in the cell in excess amount over LcrV (Matson and Nilles, 2001). SycN and YscB are T3S chaperones that interact with one another and together bind to an N-terminal region of YopN. The SycN/YscB complex is required for the stable expression of YopN and for efficient YopN secretion (Day and Plano, 1998). TyeA is a cytosolic protein that binds to a C-terminal domain of YopN (Iriarte *et al*., 1998) and has an inhibitory effect on YopN secretion/translocation (Day *et al*., 2003). YopN is a 32.6 kDa protein whose crystallographic structure in association with the SycN/YscB complex and TyeA has been recently resolved (Schubot and Waugh, 2004; Schubot *et al*., 2005). The structure reveals a seemingly rigid, elongated YopN/TyeA moiety that is highly α-helical in content. The authors propose that the central domain of YopN acts as a molecular ruler that extends the structure of the quaternary complex in a manner that allows its opposing ends, the YopN/SycN/YscB end and the YopN/TyeA end, to interact with equidistant binding sites on the T3S apparatus. Proper engagement of both sites by the quaternary complex would be required to block entry to the T3SS (Schubot *et al*., 2005). Although YopN is translocated into the eukaryotic cell *in vivo* (Lee *et al*., 1998; Day *et al*., 2003), no anti-host function has been ascribed to this protein.

The mechanism by which the YopN/SycN/YscB/TyeA complex blocks secretion of Yops and the physiological signals that release the block of secretion are poorly understood. At this time, two distinct hypotheses have been presented to explain how these proteins regulate secretion of Yops. First, Forsberg *et al*. (1991) have suggested that surface-localized YopN functions as a T3S cap that directly prevents secretion of Yops. This hypothesis is supported by the fact that YopN is seemingly found at the bacterial cell surface prior to the activation of effector Yop secretion (Forsberg *et al*., 1991; Iriarte *et al*., 1998). According to this hypothesis, upon sensing a low-calcium environment or host cell contact, the surface-localized YopN cap is released allowing secretion of Yops to proceed. Alternatively, Cheng *et al*. (2001) have hypothesized that both partial initiation of YopN into the T3S apparatus by the SycN/YscB chaperone complex and TyeA-mediated inhibition of YopN transport across the bacterial envelope function together to control the activity of the T3SS. Upon contact with a eukaryotic cell or in the absence of calcium, an unidentified signalling event allows YopN to complete its secretion, thus freeing the T3S pathway for delivery of the effector Yops.

All previously isolated *Y. pestis* YopN mutants secrete Yops constitutively, suggesting that YopN plays a critical role in preventing Yop secretion in the presence of calcium. To gain mechanistic insights on the YopN-dependent secretory block, we isolated *yopN* point mutants that constitutively blocked (NS phenotype) the secretion of Yops. The identified individual mutations all map to a region of *yopN* encoding residues 133–242 and replace highly conserved residues. The NS YopN proteins required TyeA, but not the SycN/YscB chaperone complex or LcrG, to block secretion of Yops. Correspondingly, NS YopN proteins missing the N-terminal secretion signal and chaperone-binding domain still blocked secretion, whereas NS YopN proteins lacking the TyeA-binding domain could not. These results allowed us to propose an intracellular sublocation for the YopN-dependent secretory block.

Results

Generation of point mutations in yopN *and selection of NS YopN proteins*

Yersinia pestis YopN mutants described previously in the literature display a CS phenotype, characterized by growth restriction and substantial secretion of Yops at 37°C regardless of the calcium concentration in the growth medium (Yother and Goguen, 1985; Forsberg *et al*., 1991). To gain insights into the mechanism by which YopN regulates secretion in the *Y. pestis* T3SS, we isolated YopN mutants that block secretion constitutively at 37°C in the presence or absence of calcium (NS phenotype) and studied their characteristics. A library of randomly mutated *yopN* genes was generated by a modification of the error-prone polymerase chain reaction (PCR) technique used by Leung *et al*. (1989). The resulting *yopN* gene mixture was inserted in vector pBCKS$^-$, generating a plasmid library of pBC-YopN derivatives which were subsequently transformed into *Y. pestis* KIM5-3001.P62, a *yopN* null strain. Individual NS YopN proteins

were selected for by their ability to form colonies at 37°C in the absence of calcium. Several clones were identified that lost the ability to enter growth restriction and secrete Yops at 37°C in the absence of calcium (data not shown), and that displayed, instead, an NS phenotype similar to the one observed in *ysc* null strains (Plano and Straley, 1995).

Identification of the point mutations in yopN *responsible for the NS phenotype*

After DNA sequencing, we found that most of the selected pBC-YopN plasmids [pBC-YopN(NS-1) through pBC-YopN(NS-9), Table 1] carried multiple point mutations in different regions of *yopN* with the exception of pBC-YopN(NS-3), pBC-YopN(NS-6) and pBC-YopN(NS-8) which harboured only one point mutation each in their *yopN* gene. In an effort to eliminate the point mutation(s) not directly responsible for the NS phenotype, we performed restriction-endonuclease fragment exchanges taking advantage of naturally occurring restriction sites HindIII and BglII within the coding sequence of *yopN*. Except for pBC-YopN(NS-9), a single point mutation was found to be responsible for the NS phenotype in each of the pBC-YopN derivatives (Table 1; underlined residues). Each new plasmid, containing only the point mutation or mutations in *yopN* responsible for the NS phenotype, was renamed according to the amino-acid substitution generated by the point mutation it carried (Table 1). The identified point mutations mapped to a region of *yopN* that encodes residues 133–242 and whose function has not yet been defined. Moreover, the amino-acid substitutions generated by the point mutations replace highly conserved residues of YopN. In fact, all the identified amino-acid substitutions that result in an NS phenotype replace residues that are conserved in the four available YopN homologues, including LopN of *Photorhabdus luminescens*, AopN of *Aeromonas hydrophila*, PopN of *Pseudomonas aeruginosa*, and VopN of *Vibrio harveyi* (data not shown).

Recently, Schubot and Waugh (2004) and Schubot *et al*. (2005) have succeeded in resolving the crystal structures of a ternary YopN$^{32–277}$/SycN/YscB–His$_6$ complex and of a binary YopN$^{76–293}$/TyeA complex. By superimposing these two structures, it was possible to build a model of the quaternary YopN/SycN/YscB/TyeA complex (Schubot *et al*., 2005). Interestingly, the residues of YopN that were mutated in the NS proteins (Fig. 1; side chains highlighted in red) are clustered in a central region of the quaternary complex. This region is spatially separated from the secretion targeting elements (both the SycN/YscB complex and the YopN domain interacting with it) and from the TyeA elements (both TyeA and the YopN domain interacting with it). The molecular mechanism by which the YopN/SycN/YscB/TyeA complex blocks secretion of Yops is poorly understood; however, the localization of amino-acid substitutions that allow YopN to constitutively block secretion to the central region of YopN suggests that this region plays an important role in this process.

No-secretion YopN proteins block secretion of YopM and greatly reduce their own secretion and secretion of V antigen at 37°C in the absence of calcium

To test the extent to which the various NS YopN proteins block secretion, we analysed expression and secretion of YopN, YopM and V antigen (LcrV) by the Δ*yopN* mutant strain transformed with plasmid pBC-YopN(WT), expressing wild-type YopN, or with each one of the recombinant plasmids expressing an NS YopN protein [YopN (S133R) through YopN (I180L, D200E)] (Fig. 2A). The parent strain secreted YopN, YopM and LcrV into the culture supernatant when grown at 37°C in the absence of calcium; however, as expected, no secretion of Yops and LcrV was detected in the presence of calcium. A Δ*yscL* mutant, which is defective in the assembly of a functional T3S injectisome, expressed low levels of Yops and LcrV and

Table 1. Residue substitutions in YopN responsible for the NS phenotype

Screened plasmids	Residue substitutions in YopN proteins expressed by the screened plasmids[a]	Plasmids expressing NS YopN proteins[b]
pBC-YopN(NS-1)	I21N, G36E, E37D, S133R, S272R	pBC-YopN(S133R)
pBC-YopN(NS-2)	M52I, I174N, Q246L	pBC-YopN(I174N)
pBC-YopN(NS-3)	L198P	pBC-YopN(L198P)
pBC-YopN(NS-4)	Q33H, E96K, L198P	pBC-YopN(L198P)
pBC-YopN(NS-5)	Y202N, G277R	pBC-YopN(Y202N)
pBC-YopN(NS-6)	F234S	pBC-YopN(F234S)
pBC-YopN(NS-7)	S24N, F234S	pBC-YopN(F234S)
pBC-YopN(NS-8)	D242V	pBC-YopN(D242V)
pBC-YopN(NS-9)	I180L, D200E, L262I	pBC-YopN(I180L, D200E)

a. The residue substitutions uniquely responsible for the NS phenotype are underlined.
b. These plasmids, harbouring only the mutation(s) uniquely responsible for the NS phenotype, were derived from the screened plasmids by restriction-endonuclease fragment exchanges.

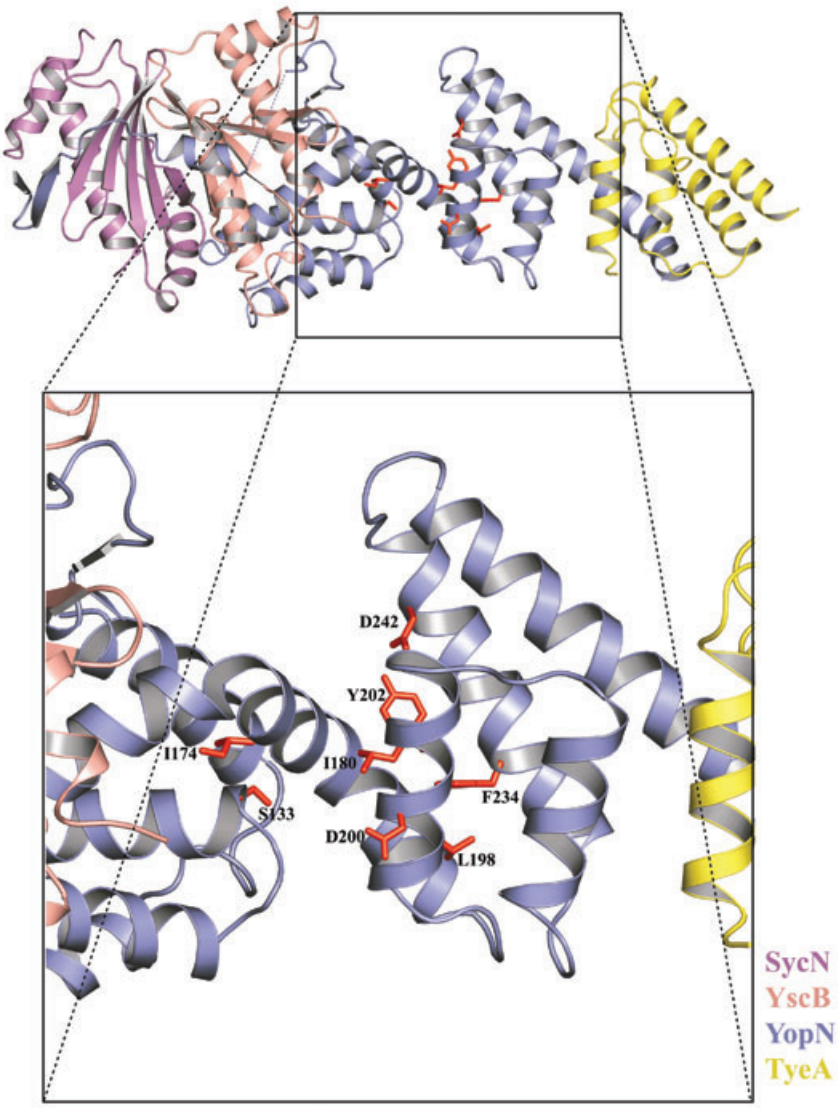

Fig. 1. Model of the quaternary complex composed of YopN (residues 32–293), SycN, YscB and TyeA. This model was constructed by superimposing the structure of the YopN$^{32–277}$/SycN/YscB–His$_6$ complex with that of the YopN$^{76–293}$/TyeA complex. The root mean square deviation for the overlapping regions is only 1 Å, suggesting that the model is a realistic depiction of the macromolecular assembly that regulates T3S in *Y. pestis*. The side chains of the residues that resulted in an NS phenotype when substituted are shown in red. A close-up of the relevant region is shown underneath the quaternary complex.

did not secrete them into the culture supernatant regardless of the calcium concentration in the growth medium. These results agree with published data on other secretion-deficient *ysc* null mutants, which display generalized downregulation of expression of pCD1 virulon genes (Michiels *et al*., 1991; Plano and Straley, 1995). Conversely, the Δ*yopN* mutant grown at 37°C in the presence or absence of calcium expressed and secreted levels of YopM and LcrV similar to those expressed and secreted by the parent strain grown in the absence of calcium. Complementation *in trans* of the *yopN* null mutant strain with a wild-type copy of *yopN* (Fig. 2A) restored RS of YopN, YopM and LcrV. The increased level of YopN secretion is due to its expression from the multicopy pBC-YopN(WT) construct. As expected, transformation of the Δ*yopN* mutant with each one of the plasmids expressing an NS YopN protein [Fig. 2A; YopN (S133R) through YopN (I180L, D200E)] generated strains with blocked secretion of YopM, and reduced or blocked secretion of LcrV and NS YopN. The amounts of Yops and LcrV found associated with these strains' cell pellet fractions were increased or similar to those expressed by the parent strain or the YopN (WT) strain grown in the presence of calcium, indicating that the absence or reduction of Yops and LcrV in the culture supernatant was likely due to a block in secretion and not to a downregulation of Yop and LcrV expression. The YopN (F234S) mutant essentially blocked secretion of all Yops and was initially isolated twice independently (Table 1); therefore, this mutant was used in all further experiments.

To confirm that the NS YopN proteins block secretion of all highly expressed T3S substrates, YopN (F234S) was expressed in a Δ*yopN Y. pestis* strain cured of plasmid pPCP1, which encodes the outer-membrane plasminogen

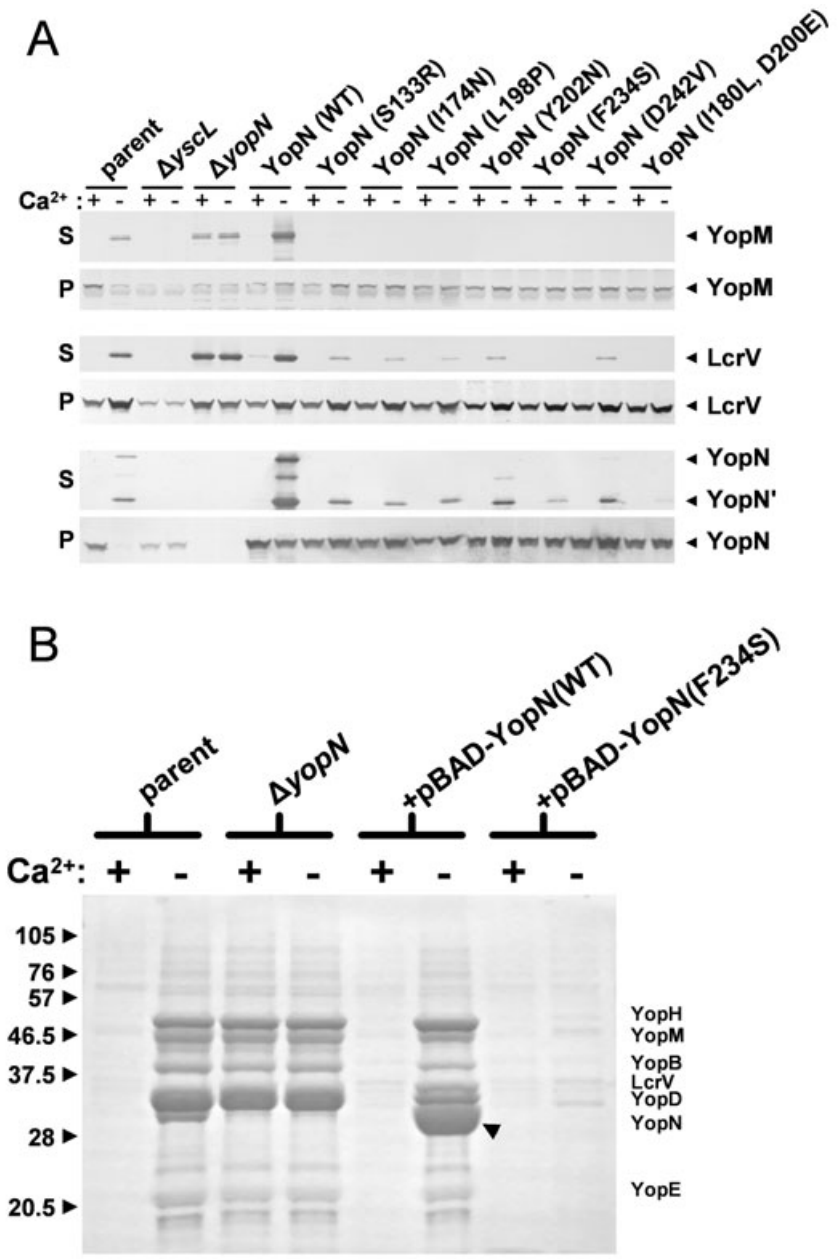

Fig. 2. A. Secretion of YopM, LcrV and YopN by *Y. pestis* strains expressing NS YopN proteins. *Y. pestis* strains KIM5-3001.P39 (parent), KIM5-3001.P67 (Δ*yscL*) and KIM5-3001.P62 (Δ*yopN*) alone and transformed with plasmid pBC-YopN(WT) [YopN (WT)], pBC-YopN(S133R) [YopN (S133R)], pBC-YopN(I174N) [YopN (I174N)], pBC-YopN(L198P) [YopN (L198P)], pBC-YopN(Y202N) [YopN (Y202N)], pBC-YopN(F234S) [YopN (F234S)], pBC-YopN(D242V) [YopN (D242V)] or pBC-YopN(I180L, D200E) [YopN (I180L, D200E)] were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antisera specific for YopM, LcrV or YopN. The locations of YopM, LcrV, YopN and YopN' (secreted YopN clipped by the outer-membrane Pla protease) are shown by arrowheads.
B. Global block of T3S by *Y. pestis* expressing YopN (F234S). Coomassie blue-stained SDS-PAGE of fourfold-concentrated supernatant proteins from bacterial cultures of plasmid pPCP1-cured *Y. pestis* KIM8-3002 (parent) and KIM8-3002.P7 (Δ*yopN*) alone and transformed with a plasmid expressing either wild-type YopN [+pBAD-YopN(WT)] or YopN (F234S) [+pBAD-YopN(F234S)]. Cultures were grown in TMH medium at 37°C for 5 h in the presence (+) and absence (–) of calcium. The positions of different Yops are indicated on the right, and the molecular masses (in kilodaltons) of biotinylated protein standards are indicated on the left. Note that the larger amount of wild-type YopN (arrowhead) secreted by the Δ*yopN* mutant as a result of overexpression from complementing plasmid pBAD-YopN(WT) did not significantly affect the level of secretion of other Yops when this is compared with that of the parent strain.

activator (Pla) protease responsible for the degradation of surface and secreted proteins (Sodeinde and Goguen, 1988; Sodeinde *et al*., 1988). Concentrated culture supernatant proteins were separated by SDS-PAGE and stained with Coomassie Blue R-250 (Fig. 2B). The parent strain, as well as the Δ*yopN* mutant complemented *in trans* with a wild-type copy of *yopN,* secreted large amounts of Yops into the culture supernatant in the absence of calcium. As expected, the Δ*yopN* mutant alone secreted large amounts of Yops regardless of the calcium concentration (CS phenotype). In contrast, the Δ*yopN* mutant transformed with plasmid pBAD-YopN(F234S) displayed no significant secretion of Yops at 37°C in the presence or absence of calcium. These results indicate that expression of YopN (F234S) produces a global block of T3S.

YopN (F234S) assembles needles on the bacterial surface but does not secrete high levels of YscF and YscP at 37°C in the absence of calcium

It has been shown that at least 10 components of the T3SS share sequence homologies with components of the bacterial flagellar hook-basal body (Blocker *et al*., 2003), suggesting an evolutionary relationship. These homologies imply that similar processes are involved in

the assembly and function of these related systems. The flagellar T3SS initially exports only hook-type substrates until the completion of the hook structure, whereupon the substrate specificity switches to filament-type substrates (Macnab, 2003). Likewise, once the base structure of *Yersinia*'s virulence-associated T3SS is formed, it is believed that it initially becomes competent for secretion of YscF, which makes up the needle, and YscP, which regulates the length of the needle (Journet *et al*., 2003). After needle assembly, a substrate specificity switch to Yop-type substrates is thought to occur (Edqvist *et al*., 2003). As per Agrain *et al*. (2005), the proximal C-terminal domain of YscP is responsible for the switch. At this point, the YopN/SycN/YscB/TyeA complex is targeted to the T3S apparatus and blocks secretion until a triggering signal is received (low calcium or cell contact). According to the model outlined above, some YscP and YscF are secreted before the YopN complex blocks T3S; therefore, NS YopN proteins may not prevent initial YscF secretion and needle assembly, but they may block or reduce secretion of excess YscP and YscF subunits after activation of the T3S process. To test this model, secretion of YscP and YscF was analysed by immunoblotting of trichloroacetic acid (TCA)-precipitated culture supernatant proteins in *Y. pestis* strains cured of plasmid pPCP1 (Fig. 3A). The parent strain secreted detectable amounts of YscP and YscF in the culture supernatant fraction in the absence of calcium but not in the presence of calcium. As expected, a strain defective in the assembly of a functional T3S apparatus (Δ*yscF*) did not secrete YscP into the culture supernatant, irrespective of the presence of calcium in the growth medium, whereas a Δ*yscP* mutant, as shown before by Edqvist *et al*. (2003), secreted equal amounts of YscF in the presence and absence of calcium. The Δ*yopN* mutant grown at 37°C exhibited the characteristic CS phenotype, secreting YscP and YscF in the presence and absence of calcium. Providing complementing plasmid pBAD-YopN(WT) to the *yopN* null mutant completely restored RS of YscP and YscF. In contrast, transformation of the *yopN* null mutant with pBAD-YopN(F234S) prevented all detectable secretion of YscP and YscF at 37°C in the presence and absence of calcium.

To determine if bacteria that express the YopN (F234S) protein assemble needles on their surface, we used a cross-linking assay originally developed for the analysis of *Chlamydia trachomatis* T3S needle assembly (K.A. Fields, unpubl.). Bacterial cells grown at 37°C in the presence or absence of calcium were harvested, washed and treated with 5 mM BS^3, a membrane-impermeable, homobifunctional protein cross-linking agent. Cross-linked cell-surface proteins were analysed by SDS-PAGE and immunoblotting with antibody specific for YscF. YscF associated with the cell pellet fraction migrated as an approximately 9 kDa monomer in the absence of BS^3 cross-linking (Fig. 3A and data not shown). BS^3 cross-linking of surface-exposed YscF in the parent, in the Δ*yscP*, in the Δ*yopN* and in the Δ*yopN* strain transformed with pBAD-YopN(WT) or pBAD-YopN(F234S) resulted in the formation of an anti-YscF antibody reactive ladder of bands that corresponded in size to various YscF-YscF multimers (dimers, trimers, and so on) plus a variable number of BS^3 molecules (mw = 572.43). The presence of surface-localized YscF that can be readily cross-linked to itself suggests that an assembled needle is present on the bacterial surface both in the presence and in the absence of calcium, a fact that has been confirmed by electron microscopy in *Yersinia enterocolitica* (Hoiczyk and Blobel, 2001). No cross-linking of YscF was seen in a Δ*yscJ* mutant, which cannot export YscF, confirming that BS^3 cannot enter the bacterial cell. These results suggest that the NS YopN (F234S) protein blocks T3S only after the completion of needle assembly and following a substrate specificity switch from needle-type substrates to Yop-type substrates.

YopN (F234S) blocks translocation of Elk-tagged YopE into HeLa cells

Another known environmental cue that triggers activation of *Yersinia*'s T3SS is contact with a eukaryotic cell. To determine whether NS YopN proteins can also block cell contact-dependent secretion and translocation of Yops into host cells, we used a recently developed reporter system that utilizes a phosphorylatable peptide tag, termed the Elk tag (Day *et al*., 2003), to follow the translocation of tagged proteins. Translocation of an Elk-tagged protein into a eukaryotic cell results in host cell protein kinase-dependent phosphorylation of the tag, which can subsequently be detected with phosphospecific antipeptide antibodies. A Δ*yopN* mutant strain alone and transformed with pBC-YopN(WT) or pBC-YopN(F234S), and a Δ*yopEJB* mutant strain, all carrying plasmid pDHK-$YopE_{129}$-Elk, were used to infect HeLa cell monolayers (Fig. 4). Immunoblot analysis of the infected HeLa cell lysates using the Elk antibody (α-Elk) revealed that all the strains expressed comparable levels of the $YopE_{129}$–Elk hybrid protein. As shown previously (Day *et al*., 2003), the Δ*yopN* mutant translocated a reduced amount of $YopE_{129}$–Elk into HeLa cells when compared with that of the same strain complemented with the plasmid expressing wild-type YopN. In contrast, transformation of the *yopN* null mutant with the plasmid expressing YopN (F234S) prevented phosphorylation of the $YopE_{129}$–Elk hybrid protein, indicating that YopN (F234S) blocked delivery of $YopE_{129}$–Elk into the eukaryotic cytoplasm. As expected, the translocation-defective *yopB* null mutant (Δ*yopEJB*) showed no translocation of the Elk-tagged YopE protein. These data indicate that YopN (F234S) blocks both secretion triggered

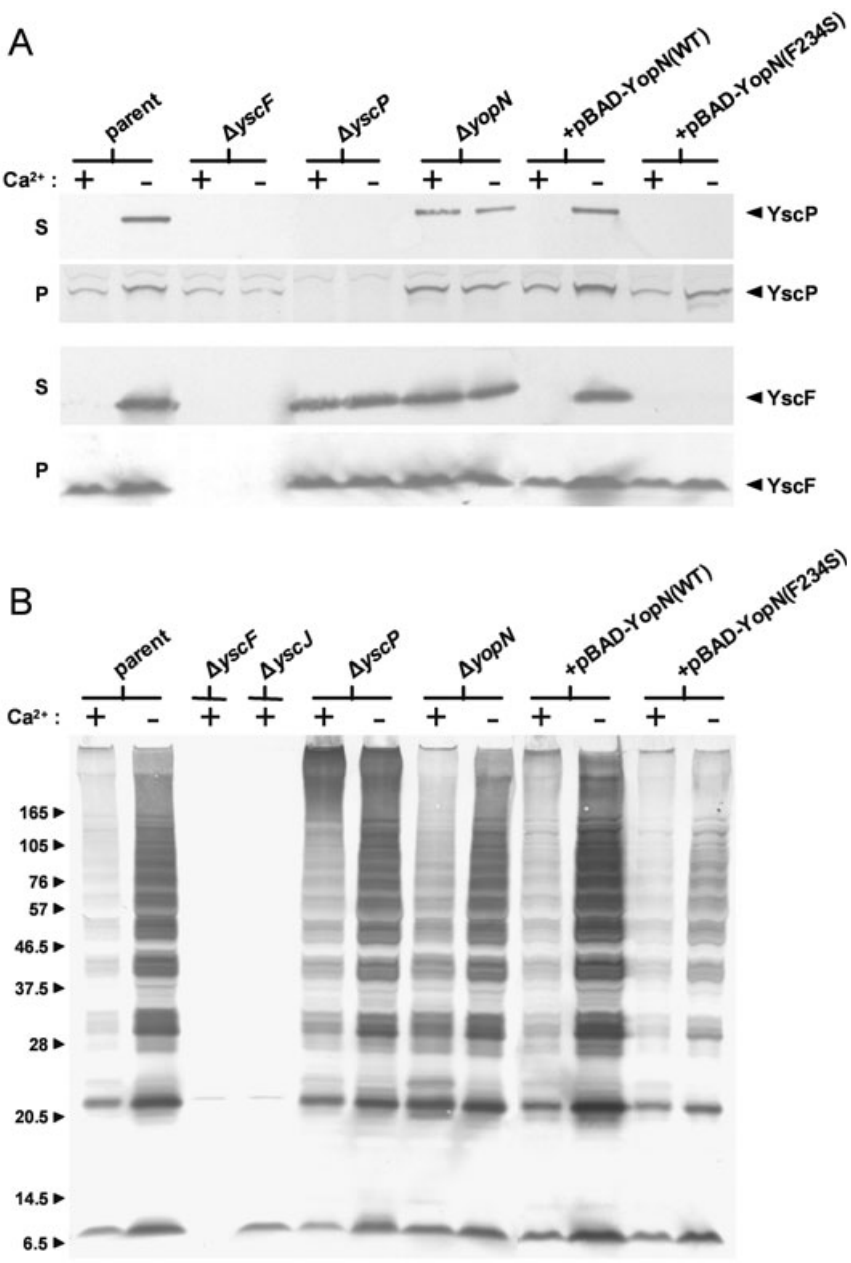

Fig. 3. A. Secretion of YscP and YscF by *Y. pestis* strains expressing wild-type YopN or YopN (F234S). Plasmid pPCP1-cured *Y. pestis* strains KIM8-3002 (parent), KIM8-3002.P61 (Δ*yscF*), KIM8-3002.P66 (Δ*yscP*) and KIM8-3002.P7 (Δ*yopN*) alone and transformed with low-copy number recombinant plasmid pBAD-YopN(WT), expressing wild-type YopN, or pBAD-YopN(F234S), expressing YopN (F234S), were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antisera specific for YscP and YscF (arrowheads).
B. Cross-linking of YscF exposed on the bacterial cell surface. The same strains as in Fig. 3A, plus strain KIM8-3002.P27 (Δ*yscJ*), were grown in TMH medium with (+) or without (–) calcium. After 5 h of growth at 37°C, 1 ml volumes of culture were centrifuged, resuspended in HEPES and cross-linked with membrane-impermeable, non-cleavable cross-linker BS^3. Cell fractions were then collected by centrifugation and resolved by SDS-PAGE and immunoblotting with antiserum specific for YscF. The molecular masses (in kilodaltons) of biotinylated protein standards are indicated on the left.

by low-calcium concentrations *in vitro* and secretion/translocation triggered by cell contact in a tissue culture model.

Secretion of YopM by Y. pestis yopN sycN, yopN yscB, yopN tyeA *and* yopN lcrG *double null strains expressing YopN (F234S)* in trans

The calcium- and cell contact-dependent regulation of Yop secretion is dependent upon the YopN, SycN, YscB, TyeA and LcrG proteins. To investigate the requirements of the NS YopN proteins to block secretion, we moved the plasmid construct expressing YopN (F234S) into different double null *Y. pestis* backgrounds (Fig. 5A). Secretion of YopM was analysed by immunoblotting of TCA-precipitated culture supernatant proteins from the wild-type parent strain, the Δ*yopN* mutant, and the different double null strains, all alone, and transformed with a plasmid expressing either wild-type YopN or YopN (F234S). Panel a in Fig. 5A shows RS of YopM by the parent strain and CS of YopM by the Δ*yopN* strain and by the four double null strains. Complementation of the same strains with plasmid pBC-YopN(WT) fully restored RS in the Δ*yopN* strain and left the parent strain's phenotype unaltered (Fig. 5A, panel b). Conversely, the double null strains expressing wild-type

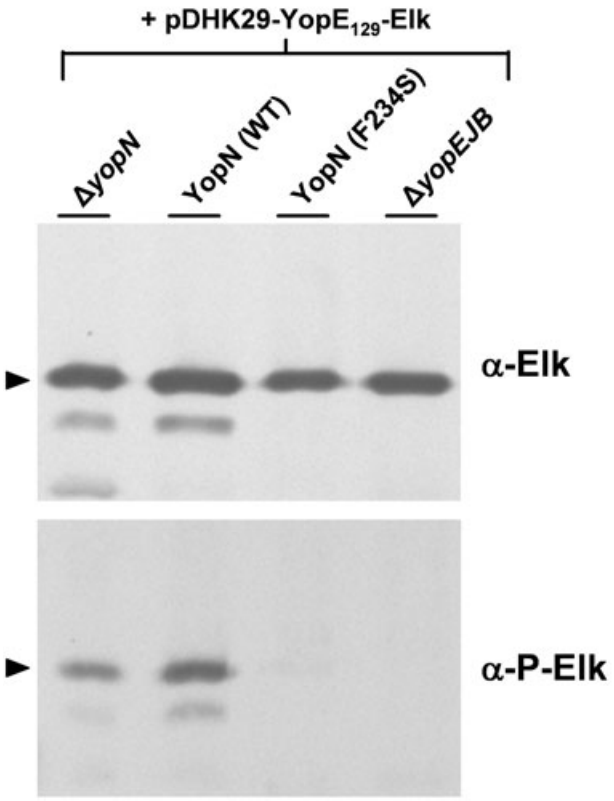

Fig. 4. Translocation of $YopE_{129}$–Elk into HeLa cells. *Y. pestis* KIM5-3001.6 (Δ*yopN*) alone and transformed with pBC-YopN(WT) [YopN (WT)] or pBC-YopN(F234S) [YopN (F234S)] and *Y. pestis* KIM5-3001.P41 (Δ*yopEJB*), all carrying plasmid pDHK-$YopE_{129}$-Elk, were used to infect HeLa cell monolayers at an moi of 30. After 3.5 h at 37°C, infected monolayers were solubilized with SDS-PAGE sample buffer and analysed by SDS-PAGE and immunoblotting with Elk antipeptide antibodies (α-Elk) and Elk phosphospecific antipeptide antibodies (α-P-Elk) as indicated. The position of $YopE_{129}$–Elk is indicated by arrowheads.

YopN lacked the ability to regulate secretion because they were still deleted in one other gene (i.e. *sycN*, *yscB*, *tyeA* or *lcrG*) responsible for the negative regulation of secretion. Expression of pBC-YopN(F234S) effectively blocked secretion of YopM in the Δ*yopN* strain and in the parent strain, suggesting in this last case a strong dominant-negative effect of YopN (F234S) on native wild-type YopN (Fig. 5A, panel c). Furthermore, expression of YopN (F234S) in the *yopN sycN*, *yopN yscB* and *yopN lcrG* double null strains also blocked the secretion of YopM. The fact that the block of Yop secretion mediated by YopN (F234S) is not dependent upon the presence of the SycN and YscB chaperones suggested that efficient chaperone-mediated targeting of YopN (F234S) to the T3SS is not required for YopN (F234S) to block secretion of Yops. Also, the constitutive block of Yop secretion observed in the Δ*yopN* Δ*lcrG* strain expressing YopN (F234S) indicates that the NS YopN proteins do not require LcrG to block secretion. Interestingly, expression of YopN (F234S) in the Δ*yopNtyeA* strain did not block YopM secretion, indicating an absolute requirement for the presence of the TyeA protein in order for YopN (F234S) to block secretion.

The inability of YopN (F234S) to block secretion in the Δ*yopNtyeA* strain suggested that this strain could be used to determine whether the NS YopN proteins could be secreted by strains that express the SycN/YscB chaperone. The Δ*yopN*, Δ*yopNtyeA* and Δ*yopN* Δ*lcrG* strains expressing wild-type YopN showed RS (Δ*yopN*) or CS (Δ*yopNtyeA* and Δ*yopN* Δ*lcrG*) of YopN (Fig. 5B, panel a). No appreciable secretion of YopN (F234S) was observed in the presence or absence of calcium in the Δ*yopN* and Δ*yopN* Δ*lcrG* strains transformed with plasmid pBC-YopN(F234S), confirming the YopN (F234S)-dependent block in T3S (Fig. 5B, panel b). Interestingly, YopN (F234S) was efficiently secreted by the Δ*yopNtyeA* strain, suggesting that the constitutive block of Yop secretion observed in the other strains is not due to the expression of an NS YopN protein that is inherently non-secretable, but is rather the result of a specific enhancement in the ability of the YopN protein to negatively regulate secretion of *Yersinia*'s virulence factors. Equivalent results in all of the above strains were also obtained using pBC-YopN(L198P) (data not shown).

YopN (F234S) deleted for its secretion signal and chaperone-binding domain blocks secretion of YopM

To investigate the role of the N-terminal secretion signal and of the YopN chaperone-binding domain in blocking secretion, we used plasmids pBC-YopN(Δ2-15) and pBC-YopN(Δ2-85) which encode wild-type YopN deleted, respectively, for the N-terminal secretion signal and for both the secretion signal and chaperone-binding domain (Jackson *et al.*, 1998). We also constructed similarly deleted versions of YopN (F234S) in plasmids pBC-YopN(Δ2-15, F234S) and pBC-YopN(Δ2-85, F234S). After transformation of these constructs into a *Y. pestis* Δ*yopN* mutant, immunoblot analysis of TCA-precipitated culture supernatant proteins with YopM polyclonal antibody showed that the strains carrying the deletions in wild-type YopN [YopN (Δ2-15) and YopN (Δ2-85)] displayed a CS phenotype (Fig. 6, panel a), characterized by secretion of YopM in the presence and absence of calcium. Whereas deletion of the secretion signal or both the secretion signal and chaperone-binding domain of wild-type YopN resulted in CS of YopM, the deleted YopN proteins themselves were not secreted [YopN (Δ2-85)] or were secreted inefficiently [YopN (Δ2-15)] (Fig. 6, panel b). These results indicate that wild-type YopN must be efficiently targeted to the T3SS via an N-terminal secretion signal and the SycN/YscB chaperone (Fig. 5A) in order to block Yop secretion. Interestingly, the presence of the F234S substitution in similarly deleted YopN proteins [Fig. 6, YopN (Δ2-15,

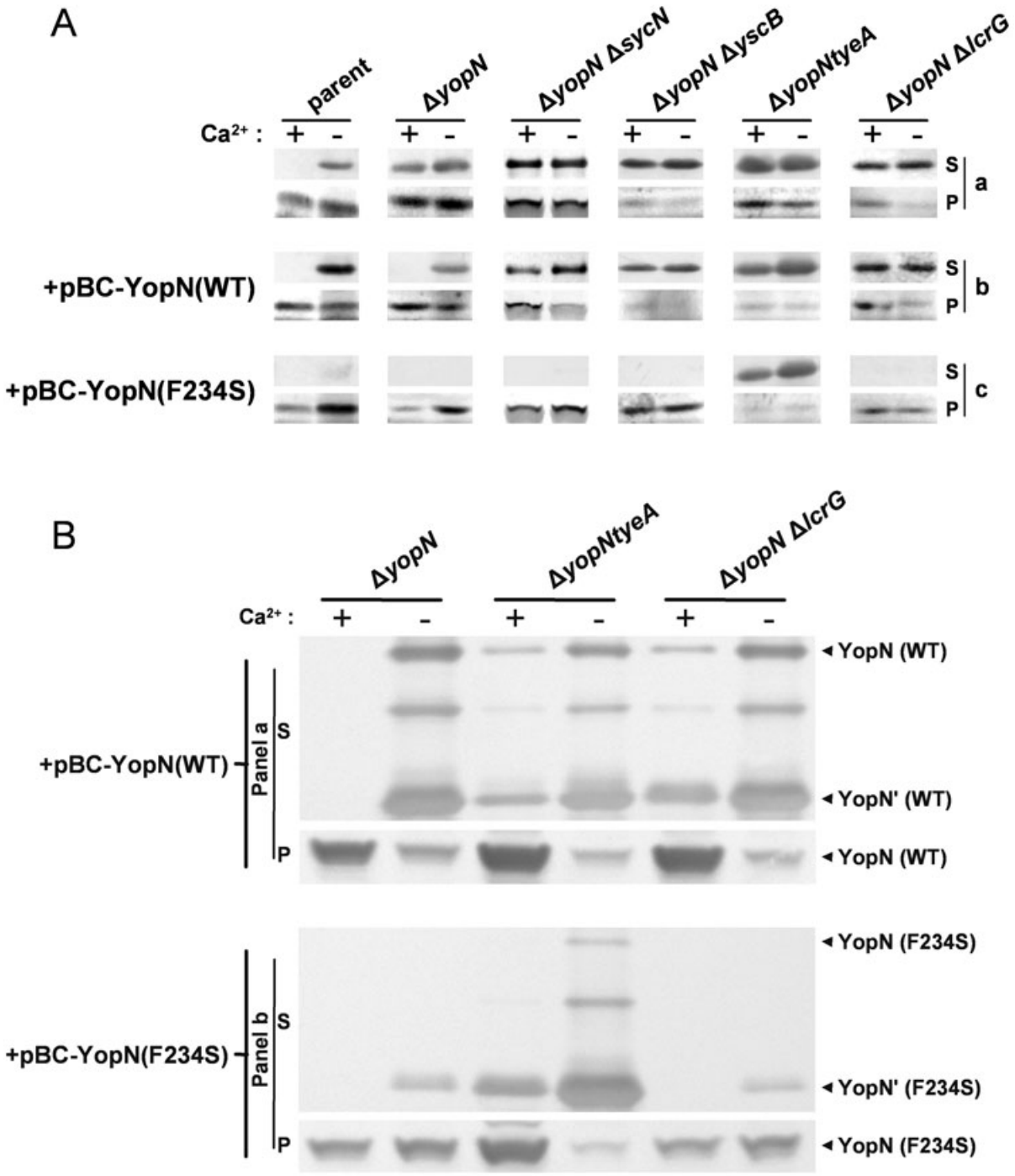

Fig. 5. A. Secretion and expression of YopM by *Y. pestis sycN yopN*, *yscB yopN*, *tyeA yopN* and *lcrG yopN* double null strains transformed with plasmids expressing either wild-type YopN or YopN (F234S). *Y. pestis* strains KIM5-3001.P39 (parent), KIM5-3001.P62 (Δ*yopN*), KIM-3001.P68 (Δ*yopN* Δ*sycN*),KIM5-3001.P70 (Δ*yopN* Δ*yscB*), KIM5-3001.P55 (Δ*yopNtyeA*) and KIM5-3001.P69 (Δ*yopN* Δ*lcrG*), all alone (panel a) or transformed with a recombinant plasmid expressing either wild-type YopN [panel b, + pBC-YopN(WT)] or YopN (F234S) [panel c, + pBC-YopN(F234S)], were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antiserum specific for YopM.
B. Secretion and expression of YopN by *Y. pestis* Δ*yopN*, Δ*yopNtyeA* and Δ*yopN* Δ*lcrG* mutant strains transformed with plasmids expressing either wild-type YopN or YopN (F234S). *Y. pestis* strains KIM5-3001.P62 (Δ*yopN*), KIM5-3001.P55 (Δ*yopNtyeA*) and KIM5-3001.P69 (Δ*yopN* Δ*lcrG*), all transformed with a recombinant plasmid expressing either wild-type YopN [panel a, +pBC-YopN(WT)] or YopN (F234S) [panel b, +pBC-YopN(F234S)], were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antiserum specific for YopN (arrowheads). YopN′ represents secreted YopN clipped by the outer-membrane Pla protease.

F234S) and YopN (Δ2-85, F234S)] was sufficient to bring about a complete block of Yop secretion in the presence or absence of calcium. The above data, together with the lack of requirement for the SycN and YscB chaperones (Fig. 5A), indicate that the NS YopN proteins do not have to be secreted or targeted to the T3S complex by identified secretion signals to block secretion of Yops, suggesting that YopN (F234S) and TyeA are capable of blocking Yop secretion from an intracellular location, namely from the cytosolic compartment. The fact that NS YopN proteins block secretion in the absence of all identified secretion signals indicates that YopN and/or TyeA have a unique secretion signal-independent means to interact with the T3SS and block the secretion of Yops.

YopN (F234S) truncated at its C-terminal domain, and thus unable to interact with TyeA, exhibits a CS phenotype

The fact that NS YopN proteins require the presence of the TyeA protein to block Yop secretion (Fig. 5) suggests that the YopN/TyeA interaction is a prerequisite for wild-type YopN and the NS YopN proteins to block secretion. To confirm the importance of the YopN/TyeA interaction on the ability of wild-type YopN and YopN (F234S) to block secretion, we used plasmids pBC-YopN(Q280Stop), pBC-YopN(F234S, Q280Stop), pBC-YopN(Q274Stop) and pBC-YopN(F234S, Q274Stop), which encode wild-type YopN or YopN (F234S) truncated at codon 280 or 274 of the *yopN* sequence by substitution of a glutamine codon with a stop codon. These YopN proteins lacking, respectively, 14 and 20 residues at their C-terminus are unable to interact with TyeA, as shown by Ferracci *et al*. (2004). The various pBC-YopN constructs were transformed into the Δ*yopN* mutant strain, and SDS-PAGE and immunoblot analysis of cell pellets and TCA-precipitated culture supernatants showed that the Δ*yopN* strains carrying the truncations in YopN (F234S) [YopN (F234S, Q280Stop) and YopN (F234S, Q274Stop)] had a CS pattern that was indistinguishable from that of the Δ*yopN* strains carrying the same truncations in wild-type YopN [YopN (Q280Stop) and YopN (Q274Stop)]. All four truncated YopN proteins appeared to be secreted constitutively in essentially equal amounts (Fig. 7; secreted YopN' clipped by the outer-membrane Pla protease); although, greater amounts of unprocessed YopN* were present in the supernatant from the YopN (Q280Stop) and the YopN (Q274Stop) derivatives than from the F234S derivatives. In contrast, the Δ*yopN* strain carrying a plasmid expressing YopN (F234S)

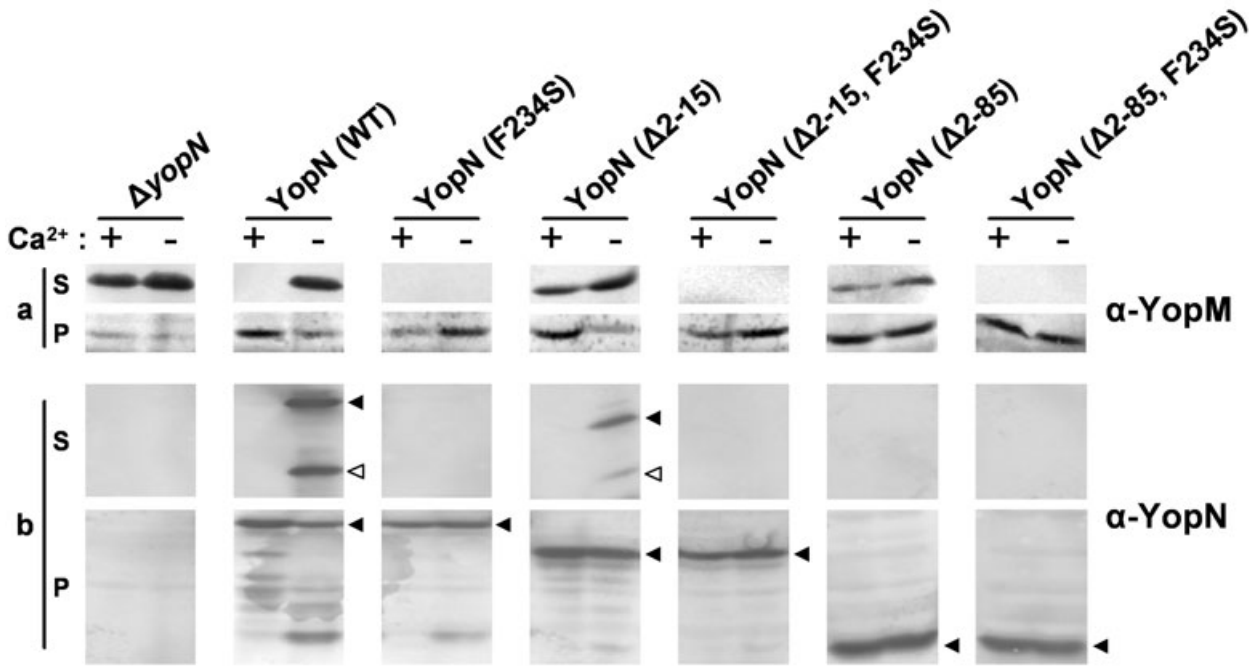

Fig. 6. YopN (F234S) deleted for its secretion signal (Δ2-15) and chaperone-binding domain (Δ2-85) blocks secretion of Yops. Expression and secretion of YopM and YopN by *Y. pestis* KIM5-3001.P62 (Δ*yopN*) alone and transformed with plasmid pBC-YopN(WT) [YopN (WT)], pBC-YopN(F234S) [YopN (F234S)], pBC-YopN(Δ2-15) [YopN (Δ2-15)], pBC-YopN(Δ2-15, F234S) [YopN (Δ2-15, F234S)], pBC-YopN(Δ2-85) [YopN (Δ2-85)], or pBC-YopN(Δ2-85, F234S) [YopN (Δ2-85, F234S)]. Strains were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antisera specific for YopM (panel a, α-YopM) or YopN (panel b, α-YopN). Solid arrowheads in panel b indicate the different-size forms of YopN expressed (P) and secreted (S) by each recombinant plasmid listed above; open arrowheads designate secreted YopN clipped by the outer-membrane Pla protease.

deleted for both its secretion signal and chaperone-binding domain [YopN (Δ2-85, F234S)] blocked secretion constitutively, as seen earlier in Fig. 6 for YopM and YopN and again here in Fig. 7 for YopM, LcrV and YopN. These results indicate that the interaction of YopN (F234S) with TyeA is crucial to effect a constitutive block in Yop secretion, whereas the interaction of YopN (F234S) with cognate heterodimeric chaperone SycN/YscB is not.

Discussion

Secretion of Yop effector proteins by the *Y. pestis* virulence-associated T3SS is triggered by growth in the absence of extracellular calcium (Brubaker and Surgalla, 1964; Michiels *et al*., 1990) or by contact with a eukaryotic cell (Rosqvist *et al*., 1994; Persson *et al*., 1995). The YopN/SycN/YscB/TyeA complex is required to prevent Yop secretion in the presence of calcium and prior to contact with a eukaryotic cell. The mechanism by which these proteins prevent Yop secretion is not known; however, the secreted YopN protein is thought to play a central role in this process (Yother and Goguen, 1985; Forsberg *et al*., 1991; Persson *et al*., 1995). In order to gain insight into the mechanism by which YopN prevents Yop secretion, we isolated and characterized several NS YopN proteins that constitutively block Yop secretion (Table 1).

Expression of the NS YopN proteins in *Y. pestis* blocked high-level secretion of both effector Yops (Fig. 2) and translocator Yops (Fig. 2B) both in the presence and in the absence of calcium. The NS YopN proteins also blocked

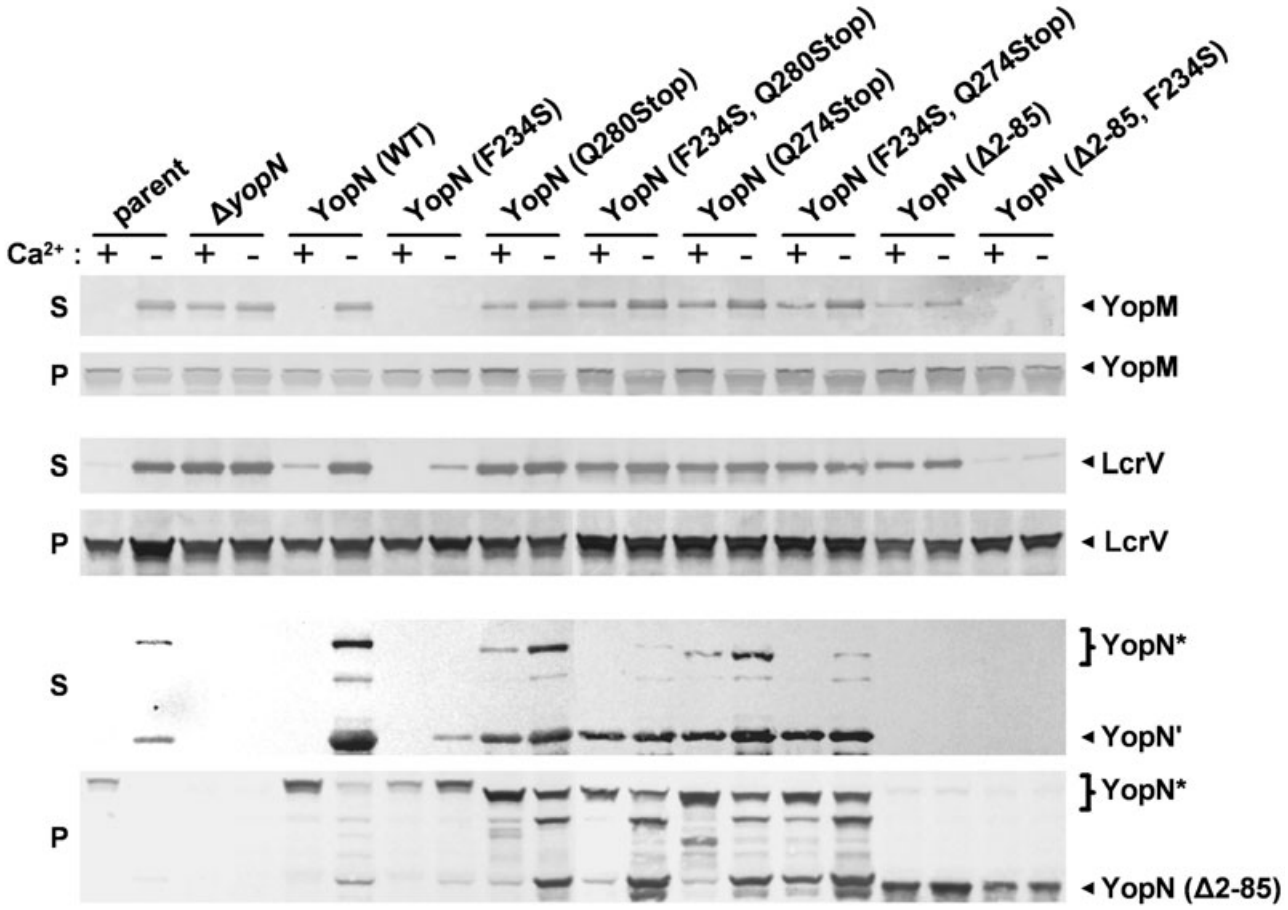

Fig. 7. YopN (F234S) truncated at its C-terminal domain, and thus unable to interact with TyeA, cannot block secretion of Yops. *Y. pestis* strain KIM5-3001.P39 (parent) and strain KIM5-3001.P62 (Δ*yopN*) alone and transformed with plasmid pBC-YopN(WT) [YopN (WT)], pBC-YopN(F234S) [YopN (F234S)], pBC-YopN(Q280Stop) [YopN (Q280Stop)], pBC-YopN(F234S, Q280Stop) [YopN (F234S, Q280Stop)], pBC-YopN(Q274Stop) [YopN (Q280Stop)], pBC-YopN(F234S, Q274Stop) [YopN (F234S, Q274Stop)], pBC-YopN(Δ2-85) [YopN (Δ2-85)], or pBC-YopN(Δ2-85, F234S) [YopN (Δ2-85, F234S)] were grown in TMH medium with (+) or without (–) calcium for 5 h at 37°C. Volumes of culture supernatant (S) proteins and cell pellet (P) fractions corresponding to equal numbers of bacteria were resolved by SDS-PAGE and immunoblotting with antisera specific for YopM, LcrV and YopN (arrowheads). YopN* designates secretion and expression of the different-size forms of YopN, from full-length YopN (WT) and YopN (F234S) to truncated YopN (Q280Stop), YopN (F234S, Q280Stop), YopN (Q274Stop) and YopN (F234S, Q274Stop). YopN′ designates secreted YopN clipped by the outer-membrane Pla protease, whereas YopN (Δ2-85) on the right-hand bottom corner of the figure indicates expression of YopN deleted for both the secretion signal and chaperone-binding domain.

cell contact-dependent secretion and translocation of effector Yops into cultured eukaryotic cells (Fig. 4). The identified amino-acid substitutions that resulted in an NS phenotype mapped to a central region of YopN (Fig. 1) that is not observed to be involved in the interaction of YopN with SycN, YscB or TyeA. The identified amino-acid residues are highly conserved in the various YopN orthologues and are almost entirely buried (Fig. 1). These data suggest that these residues are important and likely contribute to the overall stability of the folded structure. Correspondingly, mutations that specifically alter these conserved residues are likely to cause part of YopN to become unfolded or loosely ordered.

Both wild-type YopN and the NS YopN protein require an intact TyeA-binding domain (Fig. 7) and the TyeA protein (Fig. 5) in order to block secretion. As previously reported, wild-type YopN also requires an intact N-terminal secretion signal, chaperone-binding domain and the SycN/YscB chaperone to block Yop secretion in the presence of calcium (Day and Plano, 1998; Cheng *et al.*, 2001). Unexpectedly, the NS YopN (F234S) protein deleted of its N-terminal secretion signal and chaperone-binding domain still blocked Yop secretion in the presence and absence of calcium (Figs 6 and 7). The YopN (F234S) protein also blocked Yop secretion when expressed in a *sycN*, *yscB* or *lcrG* deletion mutant (Fig. 5). These results suggest that this protein does not have to be targeted to the T3SS via identified N-terminal secretion signals or chaperones in order to block secretion. These data further suggest that the cytosolic YopN/TyeA complex has at least two independent means to interact with the T3S apparatus: (i) via the YopN N-terminal secretion signal and chaperone-binding domain, and (ii) via the YopN C-terminal domain complexed with TyeA. Data presented here suggest that the latter interaction is essential for YopN/TyeA to block secretion. Interestingly, at least one member of the extended YopN family of regulatory proteins, InvE of *Salmonella enterica*, has been shown to be a cytosolic protein that regulates T3S from within the bacterial cell (Kubori and Galán, 2002). This confirms that members of the YopN family of regulatory proteins can regulate T3S from a cytosolic location.

The additional information gained from the characterization of these unique NS YopN proteins has allowed us to develop a modified working model (Fig. 8) for how the YopN/SycN/YscB/TyeA complex may function to regulate Yop secretion. We theorize that following completion of needle assembly, the YopN N-terminal secretion signal and the SycN/YscB chaperone function together to target

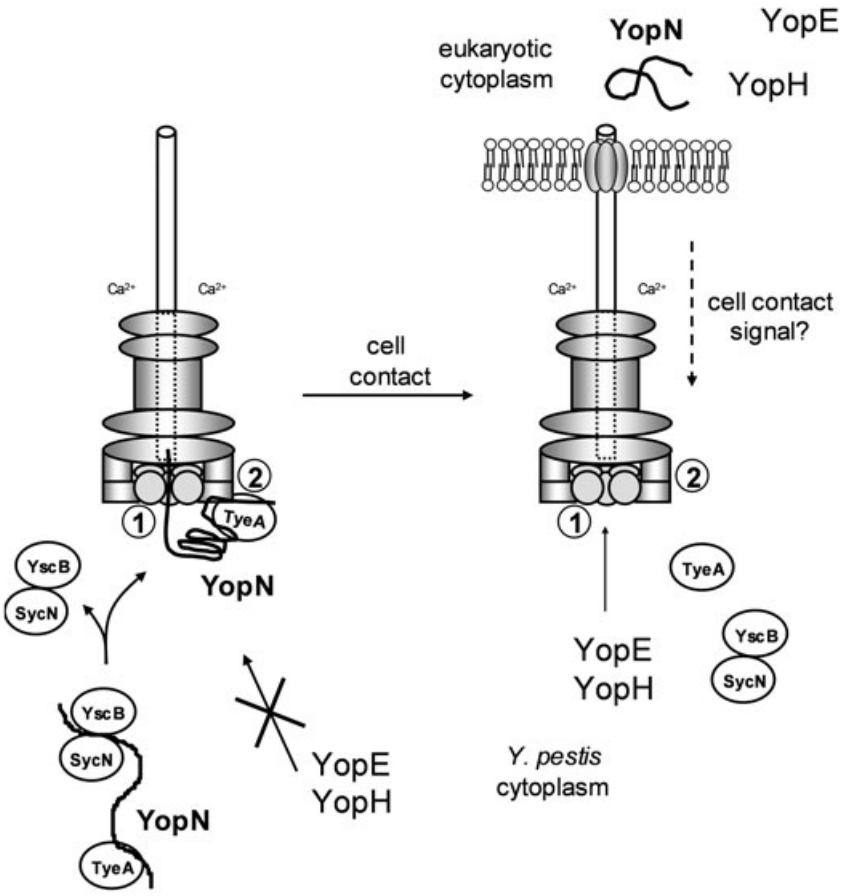

Fig. 8. Hypothetical model for the regulation of Yop secretion in *Y. pestis*. After assembly of the needle, the substrate specificity of the T3SS switches from needle-type substrates to Yop-type substrates. At this point, the YopN N-terminal secretion signal and the SycN/YscB chaperone target the YopN/TyeA complex to the T3SS. In the presence of calcium, the YopN/TyeA complex initiates secretion (site 1); however, a conformational change associated with the unfolding of, or partial secretion of, YopN allows the YopN/TyeA complex to interact with a second unique site on the T3S apparatus (site 2). Interaction of the YopN/TyeA complex at site 2 blocks T3S. Upon contact with a eukaryotic cell (or upon removal of extracellular calcium *in vitro*), an unidentified signal is transmitted to the base of the T3SS and subsequently disrupts the site 2–YopN/TyeA interaction, allowing YopN to complete its secretion. Secretion of YopN opens the secretion pathway to the effector Yops which are secreted and subsequently translocated into the eukaryotic cell. The NS YopN proteins are predicted to be in a conformation that can interact with high affinity at site 2 without having to initiate secretion.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[b]	Source or reference
Strain		
Y. pestis[a]		
KIM5-3001.6 (Δ*yopN*)	Sm^r pCD1 *yopN* pPCP1 pMT1	Plano and Straley (1995)
KIM5-3001.P39 (parent)	Sm^r pCD1 *sycE yopE*::km pPCP1 pMT1	Day *et al.* (2003)
KIM5-3001.P41 (Δ*yopEJB*)	Sm^r pCD1 *sycE yopE*::km *yopJ yopB* pPCP1 pMT1	Day *et al.* (2003)
KIM5-3001.P55 (Δ*yopNtyeA*)	Sm^r pCD1 *yopN tyeA* pPCP1 pMT1	Ferracci *et al.* (2004)
KIM5-3001.P62 (Δ*yopN*)	Sm^r pCD1 *sycE yopE*::km *yopN* pPCP1 pMT1	J. Torruellas *et al.* unpubl.
KIM5-3001.P63 (Δ*tyeA*)	Sm^r pCD1 *sycE yopE*::km *tyeA* pPCP1 pMT1	J. Torruellas *et al.* unpubl.
KIM5-3001.P67 (Δ*yscL*)	Sm^r pCD1 *sycE yopE*::km *yscL* pPCP1 pMT1	E. Silva-Herzog, unpubl.
KIM5-3001.P68 (Δ*yopN* Δ*sycN*)	Sm^r pCD1 *sycE yopE*::km *yopN sycN* pPCP1 pMT1	This study
KIM5-3001.P69 (Δ*yopN* Δ*lcrG*)	Sm^r pCD1 *sycE yopE*::km *yopN lcrG* pPCP1 pMT1	This study
KIM5-3001.P70 (Δ*yopN* Δ*yscB*)	Sm^r pCD1 *yopN yscB* pPCP1 pMT1	This study
KIM8-3002 (parent)	Sm^r pCD1 pPCP1$^-$ pMT1	Williams and Straley (1998)
KIM8-3002.P7 (Δ*yopN*)	Sm^r pCD1 *yopN* pPCP1$^-$ pMT1	Jackson *et al.* (1998)
KIM8-3002.P61 (Δ*yscF*)	Sm^r pCD1 *yscF* pPCP1$^-$ pMT1	J. Torruellas *et al.* unpubl.
KIM8-3002.P27 (Δ*yscJ*)	Sm^r pCD1 *yscJ* pPCP1$^-$ pMT1	Day and Plano (2000)
KIM8-3002.P66 (Δ*yscP*)	Sm^r pCD1 *yscP*::km pPCP1$^-$ pMT1	J. Torruellas *et al.* unpubl.
E. coli		
DH5α	F$^-$ Φ80 d *lacZ* ΔM15, Δ(*lacZYA argF*)U169, *endAI recAI hsdR17 deoR supE44 thi-1 gyrA96 relA1*	Cambau *et al.* (1993)
SY327 λ*pir*	F$^-$ Δ(*lac-pro*) *nalA recA56 araD argE*(Am) *rif* (λ*pir*^{r})	Miller and Mekalanos (1988)
Plasmids		
pBCKS$^-$	Cloning vector; Cm^r	Stratagene
pBAD30	Expression vector; Ap^r	Guzman *et al.* (1995)
pDHK29	Cloning vector; Km^r	Phillips *et al.* (2000)
pBC-YopN(WT)	KpnI-NsiI PCR fragment generated from template pCD1 and cloned into KpnI-PstI-digested pBCKS$^-$	This study
pBC-YopN(S133R)	codon 133 AGT→AGA, aa 133 S→R	This study
pBC-YopN(I174N)	codon 174 ATT→AAT, aa 174 I→N	This study
pBC-YopN(L198P)	codon 198 CTC→CCC, aa 198 L→P	This study
pBC-YopN(Y202N)	codon 202 TAC→AAC, aa 202 Y→N	This study
pBC-YopN(F234S)	codon 234 TTC→TCC, aa 234 F→S	This study
pBC-YopN(D242V)	codon 242 GAT→GTT, aa 242 D→V	This study
pBC-YopN(I180L, D200E)	codon 180 ATA→TTA, aa 180 I→L; codon 200 GAT→GAA, aa 200 D→E	This study
pBAD-YopN(WT)	KpnI-XbaI fragment excised from pBC-YopN(WT) and cloned into KpnI-XbaI-digested pBAD30	This study
pBAD-YopN(F234S)	KpnI-XbaI fragment excised from pBC-YopN(F234S) and cloned into KpnI-XbaI-digested pBAD30	This study
pUK4134.P3	Suicide vector pUK4134 carrying Δ*sycN* (aa 34–65)[c]; Ap^r	Day and Plano (1998)
pUK4134-Δ*lcrG2*	Suicide vector pUK4134 carrying Δ*lcrG* (aa 5–95); Ap^r	Nilles *et al.* (1998)
pUK4134.P1	Suicide vector pUK4134 carrying Δ*yscB* (aa 61–125); Ap^r	Jackson *et al.* (1998)
pDHK-YopE$_{129}$-Elk	BamHI-*Xho*I fragment excised from pYopE$_{129}$-Elk (Day *et al.*, 2003) and cloned into BamHI-*Sal*I-digested pDHK29	This study
pBC-YopN(Δ2-15)	Δ*yopN* (aa 2–15) KpnI-XbaI PCR-ligation-PCR fragment generated from template pBC-YopN(WT) and cloned into KpnI-XbaI-digested pBCKS$^-$	This study
pBC-YopN(Δ2-85)	Δ*yopN* (aa 2–85) KpnI-XbaI PCR-ligation-PCR fragment generated from template pBC-YopN(WT) and cloned into KpnI-XbaI-digested pBCKS$^-$	This study
pBC-YopN(F234S, Δ2-15)	Δ*yopN* (aa 2–15) KpnI-XbaI PCR-ligation-PCR fragment generated from template pBC-YopN(F234S) and cloned into KpnI-XbaI-digested pBCKS$^-$	This study
pBC-YopN(F234S, Δ2-85)	Δ*yopN* (aa 2–85) KpnI-XbaI PCR-ligation-PCR fragment generated from template pBC-YopN(F234S) and cloned into KpnI-XbaI-digested pBCKS$^-$	This study
pBC-YopN(Q280Stop)	BglII-BamHI PCR fragment generated from template pYopN-TyeA (Q280 > Stop) (Ferracci *et al.*, 2004) and cloned into BglII-BamHI-digested pBC-YopN(WT)	This study
pBC-YopN(Q274Stop)	BglII-BamHI PCR fragment generated from template pYopN-TyeA (Q274 > Stop) (Ferracci *et al.*, 2004) and cloned into BglII-BamHI-digested pBC-YopN(WT)	This study
pBC-YopN(F234S, Q280Stop)	BglII-BamHI PCR fragment generated from template pYopN-TyeA (Q280 > Stop) (Ferracci *et al.*, 2004) and cloned into BglII-BamHI-digested pBC-YopN(F234S)	This study
pBC-YopN(F234S, Q274Stop)	BglII-BamHI PCR fragment generated from template pYopN-TyeA (Q274 > Stop) (Ferracci *et al.*, 2004) and cloned into BglII-BamHI-digested pBC-YopN(F234S)	This study

a. All *Y. pestis* strains are avirulent due to deletion of the *pgm* locus (Une and Brubaker, 1984).
b. Native plasmids of *Y. pestis* include pCD1 (Perry *et al.*, 1998), pPCP1 (Sodeinde and Goguen, 1988) encoding the outer-membrane plasminogen activator (Pla) protease that has been shown to degrade exported Yops (Sodeinde *et al.*, 1988), and pMT1 encoding the capsular protein (Protsenko *et al.*, 1983).
c. Amino acids coded for by the deleted DNA sequence.

the YopN/TyeA complex to the T3S apparatus. Initiation of wild-type YopN into the secretion channel (Fig. 8, site 1) results in a conformational change or localized unfolding of the YopN protein which allows the YopN/TyeA complex to participate in a second unique interaction with a cytosolic-exposed portion of the T3S apparatus (Fig. 8, site 2). Interaction of the YopN/TyeA complex at site 2 is hypothesized to directly or indirectly block the T3S process. In the absence of calcium or upon contact with a eukaryotic cell an unidentified signalling event alters or disrupts the site 2–YopN/TyeA interaction and allows YopN to complete its secretion, thus opening the T3S pathway for effector Yop secretion.

The LcrG protein has also been shown to play an essential role in the regulation of Yop secretion. Unbound LcrG, present in excess of LcrV, blocks Yop secretion in a YopN- and TyeA-dependent manner (Matson and Nilles, 2001); however, no direct interaction between YopN or TyeA and LcrG has been identified. Previous studies have suggested that LcrG, like TyeA, works to prevent efficient secretion and translocation of YopN (Day *et al*., 2003). Thus, LcrG could function to regulate effector Yop secretion by stabilizing the binding of the YopN/TyeA complex at site 2, thereby preventing YopN secretion/translocation and subsequent effector Yop secretion/translocation.

We hypothesize that the NS YopN proteins are in a conformation, or are partially unfolded in a manner, that allows these proteins, together with TyeA, to interact at site 2 without having to be targeted to the secretion apparatus via the YopN N-terminal secretion signal and/or the SycN/YscB chaperone. The presence of a cytoplasmic pool of YopN proteins that are all in a conformation that can interact at site 2 without having to be targeted for secretion results in site 2 being consistently occupied and secretion being constitutively blocked. This model provides a plausible explanation for why wild-type YopN requires both a functional secretion signal and the SycN/YscB chaperone in order to block secretion, whereas the NS YopN proteins do not.

The ability of the NS YopN proteins to block at site 2 both in the presence and absence of calcium could be explained by the fact that the NS YopN proteins have a higher affinity for site 2 than the wild-type YopN protein or by the fact that the entire cytoplasmic pool of NS YopN proteins are in a conformation capable of competing for site 2, thus keeping site 2 occupied at all times. In contrast, wild-type YopN is hypothesized to recognize and interact at site 2 only following its initiation into the T3S pathway. Confirmation of these hypotheses awaits identification of site-2 components.

The identity of the T3SS component(s) with which YopN/TyeA interacts to block Yop secretion (site 2) is not known; however, site 2 is most likely an essential cytosolic Ysc protein (YscK, YscL, YscN or YscQ) or an integral inner-membrane Ysc protein that has a domain exposed on the cytoplasmic face of the inner membrane (YscR, YscU or YscV). Future experiments will be aimed at identifying the protein or proteins that constitute site 2. The non-secretable YopN proteins may serve as tools or probes to facilitate the identification of site-2 target proteins via coimmunoprecipitation studies, GST-pulldown experiments or via bacterial or yeast two-hybrid studies. The identification of the T3S component(s) that constitute site 2 will allow us to directly investigate the interaction of both wild-type YopN and the NS YopN proteins with these components.

Experimental procedures

Bacterial strains and culture conditions

Escherichia coli and *Y. pestis* strains used in this report are listed in Table 2. All *Y. pestis* strains in this study are avirulent from peripheral routes of infection due to deletion of the *pgm* locus (Une and Brubaker, 1984). *Y. pestis* and *E. coli* strains were grown routinely in heart infusion broth (HIB) or on tryptose blood agar (TBA) base plates (Difco Laboratories) at a temperature of 27°C or 37°C, respectively, unless otherwise noted. For growth-curve experiments, *Y. pestis* strains were cultured overnight in a shaker bath at 27°C in the defined medium TMH (Goguen *et al*., 1984), and were diluted the next day to an optical density at 620 nm (OD_{620}) of approximately 0.20 in 2 ml of fresh TMH, with or without 2.5 mM calcium chloride. After 1 h of growth at 27°C, cultures were shifted to 37°C and grown for an additional 5 h.

The differential medium TBA magnesium oxalate (TBA MgOX) was prepared according to Higuchi and Smith (1961) by aseptic incorporation of aqueous solutions of sodium oxalate and magnesium chloride (20 mM final concentration) into melted TBA. *E. coli* SY327 λ *pir* (Miller and Mekalanos, 1988) was used to propagate derivatives of the suicide vector pUK4134 (Skrzypek *et al*., 1993), whereas *E. coli* DH5α was used for routine cloning experiments. Bacteria with resistance markers were grown in the presence of the appropriate antibiotic(s) at a final concentration of 25 μg ml^{-1} (chloramphenicol and kanamycin), 50 μg ml^{-1} (ampicillin and streptomycin) or 75 μg ml^{-1} (carbenicillin).

DNA methods

Plasmid DNA was isolated by using the GenElute™ Plasmid Miniprep Kit (Sigma), the HiSpeed™ Plasmid Midi Kit (Qiagen), or the method of Kado and Liu (1981). DNA fragments were precipitated using Pellet Paint® Co-Precipitant (Novagen) or were gel purified using the Qiaex® II Gel Extraction Kit (Qiagen). DNA was routinely eluted with or resuspended in Tris (5 or 10 mM, pH 8). Electroporation of *E. coli* and *Y. pestis* was performed as described previously (Perry *et al*., 1990). The PCR technique was performed using *Taq* (Promega) or *Pfu* Turbo® (Stratagene) DNA polymerases according to the manufacturers' instructions, with modifications as noted. Double-stranded DNA was sequenced by the University of Miami School of Medicine DNA Sequencing

Core Facility using a DyeDeoxy Terminator cycle sequencing kit and an ABI model 373 A DNA sequencer (Applied Biosystems). Nucleotide sequences were analysed with IntelliGenetics computer software (IntelliGenetics).

Generation, selection and isolation of NS point mutations in yopN

The DNA fragment encoding full-length *yopN* and its promoter was PCR-amplified from plasmid pCD1 using high-fidelity *Pfu* Turbo® polymerase and primers 5′-TTTGGTACC GAAAAATAGCCAAGCAGCACT-3′ and 5′-TCCGATGCATTC AGAAAGGTCGTACGCCATTAG-3′. The resulting fragment was digested with KpnI and NsiI (underlined) and inserted into KpnI-PstI-digested plasmid pBCKS$^-$ (Stratagene), generating plasmid pBC-YopN(WT). An error-prone PCR technique (Leung *et al*., 1989) and *Taq* polymerase were used to generate a randomly mutated library of *yopN* with the same template and primers as above. The resulting *yopN* gene mixture was inserted into vector pBCKS$^-$, as described above, generating a plasmid library of pBC-YopN derivatives which were subsequently transformed into *Y. pestis* KIM5-3001.P62 (Δ*yopN*). Three 24-h growth cycles in TMH at 37°C in the absence of calcium enriched the fraction of *yopN* mutants with an NS growth phenotype, while selecting against RS strains and CS mutants (which exhibit growth restriction at 37°C in the absence of calcium). Serially diluted aliquots were then plated on TBA MgOX plates and incubated directly at 37°C. A number of fast-growing, large colonies were individually picked and tested as described in *Results*. After DNA sequencing, we performed restriction-endonuclease fragment exchanges on the isolated plasmids with more then one point mutation by taking advantage of pBCKS$^-$ cloning sites KpnI and BamHI and of naturally occurring restriction sites HindIII and BglII within the coding sequence of *yopN*. Plasmids pBC-YopN(S133R) and pBC-YopN(I174N) were generated by digesting pBC-YopN(NS-1) and pBC-YopN(NS-2), respectively, with HindIII and BglII, followed by insertion of the excised HindIII-BglII fragment into HindIII-BglII-digested pBC-YopN(WT). Plasmids pBC-YopN(L198P) and pBC-YopN(F234S) were generated by digesting pBC-YopN(NS-4) and pBC-YopN(NS-7), respectively, with BglII and BamHI, followed by insertion of the excised BglII-BamHI fragment into BglII-BamHI-digested pBC-YopN(WT). Plasmids pBC-YopN(Y202N) and pBC-YopN(I180L, D200E) were generated by digesting pBC-YopN(NS-5) and pBC-YopN(NS-9), respectively, with KpnI and HindIII, followed by insertion of the excised KpnI-HindIII fragment into KpnI-HindIII-digested pBC-YopN(WT). These were the only fragment exchange combinations that produced plasmids expressing NS YopN proteins.

Construction of Y. pestis *deletion mutants*

Previously constructed suicide vectors pUK4134.P3 (Δ*sycN*) (Day and Plano, 1998) and pUK4134-Δ*lcrG2* (Nilles *et al*., 1998), containing in-frame deletions eliminating, respectively, the coding sequence for amino acids 34–65 of SycN and 5–95 of LcrG, were used to move the *sycN* and *lcrG* deletions into *Y. pestis* KIM5-3001.P62 (Δ*yopN*). Likewise, previously constructed suicide vector pUK4134.P1 (Δ*yscB*) (Jackson *et al*., 1998), containing an in-frame deletion eliminating the coding sequence for amino acids 61–125 of YscB, was used to move the *yscB* deletion into *Y. pestis* KIM5-3001.6 (Δ*yopN*). *Y. pestis* KIM5-3001.P68 (Δ*yopN* Δ*sycN*) contained the correct in-frame deletion in *sycN*, *Y. pestis* KIM5-3001.P69 (Δ*yopN* Δ*lcrG*) contained the correct in-frame deletion in *lcrG*, and *Y. pestis* KIM5-3001.P70 (Δ*yopN* Δ*yscB*) contained the correct in-frame deletion in *yscB*.

Construction of the pBC-YopN(Δ2-15), pBC-YopN(Δ2-85), pBC-YopN(Δ2-15, F234S) and pBC-YopN(Δ2-85, F234S) expression plasmids

Deletions of *yopN* sequences encoding amino-acid residues 2–15 and 2–85 were constructed by the PCR-ligation-PCR technique (Ali and Steinkasserer, 1995). The DNA fragment encoding *yopN*'s promoter and start codon (fragment A) was amplified from plasmid pCD1 using primers 5′-TTTGGTACC CATAAATCCATAATGGTTGAA-3′ and 5′-CATAACTACTCCC TGAGATGAACA-3′. The *yopN* sequences encoding amino-acid residues 16–293 and 86–293 were amplified from both plasmid pBC-YopN(WT) and plasmid pBC-YopN(F234S) using upstream primers 5′-AATGAGCGTCCAGAGATTGCCA GT-3′ or 5′-CAGGTTAATCAATACCTTAGCAAA-3′ and downstream primer 5′-TTTTCTAGATCAGAAAGGTCGTACGCCA TT-3′. These PCR reactions produced four different PCR fragments. Fragment A was then ligated to each one of the four PCR fragments generated above, and the ligation products were reamplified using primers 5′-TTTGGTACCCATAA ATCCATAATGGTTGAA-3′ and 5′-TTTTCTAGATCAGAAAGG TCGTACGCCATT-3′, carrying, respectively, KpnI and XbaI sites (underlined). The resulting fragments were digested with KpnI and XbaI and inserted into KpnI-XbaI-digested pBCKS$^-$, generating plasmids pBC-YopN(Δ2-15), pBC-YopN(Δ2-85), pBC-YopN(Δ2-15, F234S) and pBC-YopN(Δ2-85, F234S).

Construction of the pBC-YopN(Q280Stop), pBC-YopN(Q274Stop), pBC-YopN(F234S, Q280Stop) and pBC-YopN(F234S, Q274Stop) expression plasmids

Previously developed constructs pYopN-TyeA (Q280 > Stop) and pYopN-TyeA (Q274 > Stop) (Ferracci *et al*., 2004), carrying, respectively, a single nonsense mutation at codon 280 or 274 of the *yopN* sequence, were used as templates with upstream primer 5′-CAAGGAATTTATGCGATCTGGAGTGAT-3′ and downstream primer 5′-TTTGGATCCTCAGAAAG GTCGTACGCCATT-3′ to generate two separate PCR products. Both fragments amplified a region from codon 210 of *yopN* to its native termination codon at position 294, and each included a single nonsense mutation (Q280Stop or Q274Stop). After digestion with BglII (naturally occurring restriction site between codons 242 and 243 of *yopN*) and with BamHI (underlined), the fragments were inserted into BglII-BamHI-digested pBC-YopN(WT), generating plasmids pBC-YopN(Q280Stop) and pBC-YopN(Q274Stop), and into BglII-BamHI-digested pBC-YopN(F234S), generating plasmids pBC-YopN(F234S, Q280Stop) and pBC-YopN(F234S, Q274Stop).

SDS-PAGE and immunoblotting

Cell pellets and culture supernatants were separated by centrifugation at 12 200 *g* for 10 min at 4°C. Culture supernatant proteins were precipitated on ice overnight with 10% (v/v) TCA and collected by centrifugation at 12 200 *g* for 15 min at 4°C. Volumes of cellular fractions (whole-cell or secreted proteins) corresponding to equal numbers of bacteria were mixed 1:1 (v/v) with 2× electrophoresis sample buffer and analysed by SDS-PAGE followed by staining with Coomassie blue or immunoblotting, which was performed essentially as previously described (Plano and Straley, 1995). Briefly, samples were separated on NuPAGE™ 12% Bis-Tris acrylamide gels (Invitrogen) using a MES or a MOPS buffer system. Separated proteins were transferred to Immobilon-P Transfer Membranes (Millipore) using a Tris-glycine buffer system, and membranes were blocked with 5% (w/v) non-fat milk (NFM) in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) for at least 30 min at room temperature (RT). Polyclonal antisera or purified antibodies were diluted in TBS containing 1% NFM and 0.05% (v/v) Tween 20 and incubated with the blots overnight at 4°C. Blots were then washed three times with TBS containing 0.1% NFM and 0.05% Tween 20 for 10 min. Secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG) was diluted in TBS containing 1% NFM and 0.05% Tween 20 and incubated with the blots for 1 h at RT. Blots were then washed again as described above and developed with 1-Step™ BCIP/NBT (Pierce). Images of the blots were captured by the ChemiImager 5500 digital imaging system with Alpha Innotech software.

Tissue culture infections

Tissue culture infections of HeLa cells were done as previously described (Day *et al*., 2003).

Cell-surface cross-linking of the YscF needle with BS^3

Cultures were grown at 37°C in 2 ml of TMH with or without 2.5 mM calcium chloride. After 5 h, 1 ml volumes were harvested by centrifugation at 8000 *g* for 5 min at 4°C. Each bacterial pellet from growth in the presence of calcium was gently resuspended in 1 ml of cold 20 mM HEPES, 5 mM calcium chloride (pH 8), whereas bacterial pellets from growth in the absence of calcium were gently resuspended in 1 ml of cold 20 mM HEPES (pH 8) without calcium. Bacterial surface proteins were cross-linked for 30 min at RT with the non-cleavable, membrane-impermeable, amine-reactive cross-linker *bis*(sulphosuccinimidyl) suberate (BS^3) (Pierce) at a final concentration of 5 mM (25 mM stock solution in 20 mM HEPES, pH 8.0). Cross-linking reactions were quenched for 15 min by addition of Tris (pH 8.0) to a final concentration of 20 mM. Cell fractions were collected by centrifugation at 12 200 *g* for 5 min at 4°C and treated as noted in SDS-PAGE and immunoblotting.

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References

Agrain, C., Callebaut, I., Journet, L., Sorg, I., Paroz, C., Mota, L.J., and Cornelis, G.R. (2005) Characterization of a type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. *Mol Microbiol* **56:** 54–67.

Ali, S.A., and Steinkasserer, A. (1995) PCR-ligation-PCR mutagenesis: a protocol for creating gene fusions and mutations. *Biotechniques* **18:** 746–750.

Blocker, A., Komoriya, K., and Aizawa, S.-I. (2003) Type III secretion systems and bacterial flagella: insights into their function from structural similarities. *Proc Natl Acad Sci USA* **100:** 3027–3030.

Brubaker, R.R. (1983) The Vwa$^+$ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca++. *Rev Infect Dis* **5 (**Suppl. 4): S748–S758.

Brubaker, R.R., and Surgalla, M.J. (1964) The effect of Ca++ and Mg++ on lysis, growth, and production of virulence antigens by *Pasteurella pestis*. *J Infect Dis* **114:** 13–25.

Cambau, E., Bordon, F., Collatz, E., and Gutmann, L. (1993) Novel *gyrA* point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. *Antimicrob Agents Chemother* **37:** 1247–1252.

Cheng, L.W., Kay, O., and Schneewind, O. (2001) Regulated secretion of YopN by the type III machinery of *Yersinia enterocolitica*. *J Bacteriol* **183:** 5293–5301.

Cheng, L.W., and Schneewind, O. (2000) *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol* **182:** 3183–3190.

Cornelis, G.R. (2000) Molecular and cell biology aspects of plague. *Proc Natl Acad Sci USA* **97:** 8778–8783.

Cornelis, G.R., and Wolf-Watz, H. (1997) The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23:** 861–867.

Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., *et al.* (1998) The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* **62:** 1315–1352.

Day, J.B., and Plano, G.V. (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol Microbiol* **30:** 777–788.

Day, J.B., and Plano, G.V. (2000) The *Yersinia pestis* YscY protein directly binds YscX, a secreted component of the type III secretion machinery. *J Bacteriol* **182:** 1834–1843.

Day, J.B., Ferracci, F., and Plano, G.V. (2003) Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN*, *tyeA*, *sycN*, *yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. *Mol Microbiol* **47:** 807–823.

DeBord, K.L., Lee, V.T., and Schneewind, O. (2001) Roles of LcrG and LcrV during type III targeting of effector Yops by *Yersinia enterocolitica*. *J Bacteriol* **183:** 4588–4598.

Edqvist, P.J., Olsson, J., Lavander, M., Sundberg, L., Forsberg, Å., Wolf-Watz, H., and Lloyd, S.A. (2003) YscP and YscU regulate substrate specificity of the *Yersinia* type III secretion system. *J Bacteriol* **185:** 2259–2266.

Ferracci, F., Day, J.B., Ezelle, H.J., and Plano, G.V. (2004) Expression of a functional secreted YopN-TyeA hybrid protein in *Yersinia pestis* is the result of a +1 translational frameshift event. *J Bacteriol* **186:** 5160–5166.

Forsberg, Å., Viitanen, A.-M., Skurnik, M., and Wolf-Watz, H. (1991) The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol Microbiol* **5:** 977–986.

Goguen, J.D., Yother, J., and Straley, S.C. (1984) Genetic analysis of the low calcium response in *Yersinia pestis* mu d1 (Ap *lac*) insertion mutants. *J Bacteriol* **160:** 842–848.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177:** 4121–4130.

Higuchi, K., and Smith, J.L. (1961) Studies on the nutrition and physiology of *Pasteurella pestis*. VI. A differential plating medium for the estimation of the mutation rate to avirulence. *J Bacteriol* **81:** 605–608.

Hoiczyk, E., and Blobel, G. (2001) Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. *Proc Natl Acad Sci USA* **98:** 4669–4674.

Iriarte, M., and Cornelis, G.R. (1999) Identification of SycN, YscX, and YscY, three new elements of the *Yersinia yop* virulon. *J Bacteriol* **181:** 675–680.

Iriarte, M., Sory, M.P., Boland, A., Boyd, A.P., Mills, S.D., Lambermont, I., and Cornelis, G.R. (1998) TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *Embo J* **17:** 1907–1918.

Jackson, M.W., Day, J.B., and Plano, G.V. (1998) YscB of *Yersinia pestis* functions as a specific chaperone for YopN. *J Bacteriol* **180:** 4912–4921.

Journet, L., Agrain, C., Broz, P., and Cornelis, G.R. (2003) The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* **302:** 1757–1760.

Kado, C.I., and Liu, S.T. (1981) Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145:** 1365–1373.

Kubori, T., and Galán, J.E. (2002) *Salmonella* type III secretion-associated protein InvE controls translocation of effector proteins into host cells. *J Bacteriol* **184:** 4699–4708.

Lee, V.T., Anderson, D.M., and Schneewind, O. (1998) Targeting of *Yersinia* Yop proteins into the cytosol of HeLa cells: one-step translocation of YopE across bacterial and eukaryotic membranes is dependent on SycE chaperone. *Mol Microbiol* **28:** 593–601.

Leung, D.W., Chen, E., and Goeddel, D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1:** 11–15.

Macnab, R.M. (2003) How bacteria assemble flagella. *Annu Rev Microbiol* **57:** 77–100.

Matson, J.S., and Nilles, M.L. (2001) LcrG–LcrV interaction is required for control of Yops secretion in *Yersinia pestis*. *J Bacteriol* **183:** 5082–5091.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J.-M., and Cornelis, G. (1990) Secretion of Yop proteins by Yersiniae. *Infect Immun* **58:** 2840–2849.

Michiels, T., Vanooteghem, J.-C., Lambert de Rouvroit, C., China, B., Gustin, A., Boudry, P., and Cornelis, G.R. (1991) Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J Bacteriol* **173:** 4994–5009.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires. *Toxr J Bacteriol* **170:** 2575–2583.

Neyt, C., and Cornelis, G.R. (1999) Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: requirement for translocators YopB and YopD, but not LcrG. *Mol Microbiol* **33:** 971–981.

Nilles, M.L., Fields, K.A., and Straley, S.C. (1998) The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. *J Bacteriol* **180:** 3410–3420.

Orth, K., Palmer, L.E., Bao, Z.Q., Stewart, S., Rudolph, A.E., Bliska, J.B., and Dixon, J.E. (1999) Inhibition of the mitogen-activated protein kinase kinase superfamily by a *Yersinia* effector. *Science* **285:** 1920–1923.

Palmer, L.E., Hobbie, S., Galán, J.E., and Bliska, J.B. (1998) YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF-α production and downregulation of the MAP kinases p38 and JNK. *Mol Microbiol* **27:** 953–965.

Perry, R.D., Pendrak, M.L., and Schuetze, P. (1990) Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. *J Bacteriol* **172:** 5929–5937.

Perry, R.D., Straley, S.C., Fetherston, J.D., Rose, D.J., Gregor, J., and Blattner, F.R. (1998) DNA sequencing and analysis of the low-Ca^{2+}-response plasmid pCD1 of *Yersinia pestis* KIM5. *Infect Immun* **66:** 4611–4623.

Persson, C., Nordfelth, R., Holmström, A., Håkansson, S., Rosqvist, R., and Wolf-Watz, H. (1995) Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. *Mol Microbiol* **18:** 135–150.

Pettersson, J., Holmström, A., Hill, J., Leary, S., Frithz-Lindsten, E., von Euler-Matell, A., *et al.* (1999) The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. *Mol Microbiol* **32:** 961–976.

Phillips, G.J., Park, S.K., and Huber, D. (2000) High copy number plasmids compatible with commonly used cloning vectors. *Biotechniques* **28:** 400–402,404,406 *passim*.

Plano, G.V., and Straley, S.C. (1995) Mutations in *yscC*, *yscD*, and *yscG* prevent high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. *J Bacteriol* **177:** 3843–3854.

Protsenko, O.A., Anisimov, P.I., Mozharov, O.T., Konnov, N.P., and Popov Iu, A. (1983) Detection and characterization of the plasmids of the plague microbe which determine the synthesis of pesticin I, fraction I antigen and 'mouse' toxin exotoxin. *Genetika* **19:** 1081–1090.

Rosqvist, R., Magnusson, K.E., and Wolf-Watz, H. (1994) Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *Embo J* **13:** 964–972.

Schesser, K., Spiik, A.K., Dukuzumuremyi, J.M., Neurath, M.F., Pettersson, S., and Wolf-Watz, H. (1998) The *yopJ*

locus is required for *Yersinia*-mediated inhibition of NF-κB activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol Microbiol* **28:** 1067–1079.

Schubot, F.D., and Waugh, D.S. (2004) A pivotal role for reductive methylation in the *de novo* crystallization of a ternary complex composed of *Yersinia pestis* virulence factors YopN, SycN and YscB. *Acta Crystallogr D Biol Crystallogr* **60:** 1981–1986.

Schubot, F.D., Jackson, M.W., Penrose, K.J., Cherry, S., Tropea, J.E., Plano, G.V., and Waugh, D.S. (2005) Three-dimensional structure of a macromolecular assembly that regulates type III secretion in *Yersinia pestis*. *J Mol Biol* **346:** 1147–1161.

Skrzypek, E., Haddix, P.L., Plano, G.V., and Straley, S.C. (1993) New suicide vector for gene replacement in yersiniae and other gram-negative bacteria. *Plasmid* **29:** 160–163.

Sodeinde, O.A., and Goguen, J.D. (1988) Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. *Infect Immun* **56:** 2743–2748.

Sodeinde, O.A., Sample, A.K., Brubaker, R.R., and Goguen, J.D. (1988) Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. *Infect Immun* **56:** 2749–2752.

Une, T., and Brubaker, R.R. (1984) *In vivo* comparison of avirulent Vwa$^-$ and Pgm$^-$ or Pst^r phenotypes of yersiniae. *Infect Immun* **43:** 895–900.

Williams, A.W., and Straley, S.C. (1998) YopD of *Yersinia pestis* plays a role in negative regulation of the low-calcium response in addition to its role in translocation of Yops. *J Bacteriol* **180:** 350–358.

Wulff-Strobel, C.R., Williams, A.W., and Straley, S.C. (2002) LcrQ and SycH function together at the Ysc type III secretion system in *Yersinia pestis* to impose a hierarchy of secretion. *Mol Microbiol* **43:** 411–423.

Yother, J., and Goguen, J.D. (1985) Isolation and characterization of Ca^{2+}-blind mutants of *Yersinia pestis*. *J Bacteriol* **164:** 704–711.