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**A C-Terminal Region of* Yersinia pestis* YscD Binds the Outer Membrane Secretin YscC**

Julia A. Ross and Gregory V. Plano*

Department of Microbiology and Immunology
University of Miami Miller School of Medicine,
Miami, FL USA

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*corresponding author:  P.O. BOX 016960 (R-138), Miami, FL 33101
phone:  305-243-6310
fax:  305-243-4623
e-mail:  gplano@med.miami.edu
ABSTRACT

YscD is an essential component of the plasmid pCD1-encoded type III secretion system (T3SS) of *Yersinia pestis*. YscD has a single transmembrane (TM) domain that connects a small N-terminal cytoplasmic region (residues 1 to 121) to a larger periplasmic region (residues 143 to 419). Deletion analyses established that both the N-terminal cytoplasmic region and the C-terminal periplasmic region are required for YscD function. Smaller targeted deletions demonstrated that a predicted cytoplasmic forkhead-associated (FHA) domain is also required to assemble a functional T3SS; in contrast, a predicted periplasmic phospholipid binding (BON) domain and a putative periplasmic “ring-building motif” domain of YscD could be deleted with no significant effect on the T3S process. Although deletion of the putative “ring-building motif” domain did not disrupt T3S activity per se, the calcium-dependent regulation of the T3S apparatus was affected. The extreme C-terminal region of YscD (residues 354 to 419) was essential for secretion activity and had a strong dominant negative effect on the T3S process when exported to the periplasm of the wild type parent strain. Co-immunoprecipitation studies demonstrated that this region of YscD mediates the interaction of YscD with the outer membrane YscC secretin complex. Finally, replacement of the YscD TM domain with a TM domain of dissimilar sequence had no effect on the T3S process, indicating that the TM domain has no sequence-specific function in the assembly or function of the T3SS.
INTRODUCTION

The ability of a bacterial pathogen to initiate and sustain an infection is often dependent upon the pathogen’s ability to neutralize host defenses that function to limit bacterial multiplication and/or dissemination. Many Gram-negative bacterial pathogens use a process termed type III secretion (T3S) to inject host cells with effector proteins that target and disrupt critical host defense systems (21). The injection process requires a multicomponent T3S apparatus, termed an injectisome, which transports effector proteins across the bacterial inner and outer membranes as well as across a eukaryotic membrane. The T3S process also plays an essential role in the assembly of the bacterial flagellum (26).

The injectisome consists of a base structure that spans the bacterial inner and outer membranes and of an external needle-like structure that extends 40 to 60 nm from the bacterial cell surface (24). The bacterial flagellum consists of a similar base structure (the basal body) that is topped by the flagellar hook and filament structures (26). The assembly of the injectisome base structure and the flagellar basal body both require a set of approximately ten relatively conserved T3S components (16). Additional essential T3S components are either injectisome- or flagellar-specific or are specific to a limited subset of type III secretion systems (T3SSs).

The T3S apparatus of the human pathogenic yersiniae is assembled from 21 essential \textit{Yersinia} secretion (Ysc) proteins (7, 34). The essential Ysc proteins include conserved components involved in the assembly of the T3S apparatus base structure (YscC, YscD, YscJ and YscW), core inner membrane secretion components (YscR, YscS, YscT, YscU and YscV), cytoplasmic ring (YscK and YscQ), ATPase complex
(YscL and YscN) and needle/rod assembly components (YscE, YscF, YscG, YscI, YscO and YscP). In addition, the YscX and YscY proteins are essential for the T3S process; however, their role in this process is unknown (11). Together, the Ysc proteins direct the ordered assembly of the Ysc T3S apparatus, an essential virulence factor of the human pathogenic yersiniae. The *Yersinia* injectisome directs the secretion of numerous T3S substrates, including six *Yersinia* outer proteins (Yops) that have direct anti-host activities within the host’s cells (41).

The initial T3S apparatus structure assembled is the base, which consists of the YscC, YscD and YscJ proteins in the yersiniae (12, 24). YscC is a member of the secretin family of outer membrane proteins that form ring-shaped oligomers involved in the assembly of type II secretion systems (T2SSs), T3SSs and type IV pili (23, 43). Some secretins, including YscC, require an accessory pilot protein, YscW in the yersiniae, for targeting the secretin to, and insertion in, the outer membrane (5, 14). The assembled YscC oligomer is a stable ring-shaped structure with a central pore-like channel (23).

Completion of *Yersinia* T3S apparatus base assembly also requires the YscJ and YscD proteins. YscJ is an inner membrane lipoprotein that is predicted to multimerize into a ring-shaped structure on the periplasmic face of the inner membrane (38, 45). YscD is an integral inner membrane protein that is predicted to contain a cytoplasmic forkhead-associated (FHA)-containing domain and a large periplasmic domain (32, 33, 35). Like YscJ, YscD is also predicted to multimerize into a ring-shaped structure. Recently, the structures of predicted periplasmic regions of the YscC homolog EscC, YscJ homolog EscJ and YscD homolog PrgH have been determined (39, 45).
Interestingly, these three proteins were found to share a domain with a conserved $\alpha_2\beta_3$ fold that is predicted to function as a conserved “ring-building motif” that may aid in the assembly of the T3S apparatus base structure. In this study, we investigate the role of individual regions and defined domains of the Y. pestis YscD protein in the assembly and function of the Ysc T3S apparatus.
MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α and *Yersinia pestis* KIM strains (see Table 1) were grown in heart infusion broth (HIB) or on tryptose blood agar (TBA) plates (BD-Difco) at 27°C (*Y. pestis*) or 37°C (*E. coli*). The antibiotics ampicillin, kanamycin and streptomycin were used at 50 µg/ml. For standard secretion assays, *Y. pestis* strains were grown in TMH medium in the presence or absence of 2.5 mM CaCl₂ for one hour at 27°C and then shifted to 37°C for the next 5 h as previously described (18). For complementation studies in the ΔyscD background, FLAG-tagged YscD proteins (pFLAG-MAC or pFLAG-ATS vectors) were induced with 0.05 mM IPTG at the temperature shift. Expression of FLAG-tagged YscD proteins in the wild type parent strain (KIM5-3001) (assay for dominant negative phenotype) was induced with 0.05 mM IPTG during the overnight culture as well as for the duration of the assay unless otherwise indicated.

Growth experiments designed to compare the effect of inducing the dominant negative FLAG-YscD354-419 early (before and during T3S apparatus assembly) versus late (following T3S apparatus assembly) used a modified growth and secretion assay. Separate overnight cultures (with and without 0.05 mM IPTG) were grown for each strain. The next day, strains were subcultured (with and without 0.05 mM IPTG as grown overnight), grown 1 h at 27°C and shifted to 37°C for 3 h at which point all cultures were harvested by centrifugation for 3 minutes at 8,000 x g at RT. The 3 h supernatant from each culture was collected (SUP-1) and pellets resuspended with prewarmed TMH media containing the appropriate antibiotics and 0.05 mM IPTG (added to all cultures except CTL cultures). Growth of cultures at 37°C was continued for the next
4 h and bacterial whole cells and supernatants (SUP-2) were separated by centrifugation, collected and analyzed by SDS-PAGE and immunoblot analysis.

**Construction of expression vectors encoding full-length FLAG-YscD, as well as N-terminal and C-terminal truncated FLAG-YscD proteins.** DNA fragments used to construct pFLAG-YscD_{FL}, pFLAG-YscD_{6-419}, pFLAG-YscD_{13-419}, pFLAG-YscD_{47-419}, pFLAG-YscD_{76-419}, pFLAG-YscD_{119-419}, pFLAG-YscD_{143-419}, pFLAG-YscD_{157-419}, pFLAG-YscD_{217-419}, pFLAG-YscD_{283-419}, and pFLAG-YscD_{354-419} were amplified from plasmid pCD1 using the downstream oligonucleotide primer YscD419-BglII-2 with each corresponding upstream primer: YscD-HindIII-1, YscD6-HindIII-1, YscD13-HindIII-1, YscD47-HindIII-1, YscD76-HindIII-1, YscD143-HindIII-1, YscD119-HindIII-1, YscD143-HindIII-1, YscD157-HindIII-1, YscD217-HindIII-1, YscD283-HindIII-1 or YscD354-HindIII-1 (see Table S1 in the supplemental material). The resultant DNA fragments were digested with HindIII and BglIII and inserted into HindIII- and BglIII-digested pFLAG-MAC (Sigma-Aldrich). Additionally, DNA fragments encoding YscD_{157-419}, YscD_{217-419}, YscD_{283-419}, and YscD_{354-419} were inserted into HindIII- and BglIII-digested pFLAG-ATS (Sigma-Aldrich), which encodes the OmpA secretion signal (ss) and a FLAG epitope tag (pssFLAG-YscD_{157-419}, pssFLAG-YscD_{217-419}, pssFLAG-YscD_{283-419}, and pssFLAG-YscD_{354-419}). The DNA fragment inserted in plasmid pssFLAG-YscD_{283-353} (PD3) was amplified from plasmid pCD1 with primers YscD283-HindIII-1 and YscD353-BglII-2. The resultant DNA fragment was digested with HindIII and BglIII and inserted into HindIII- and BglIII-digested pFLAG-ATS.

Expression vectors pFLAG-YscD_{1-353}, pFLAG-YscD_{1-282}, pFLAG-YscD_{1-216}, pFLAG-YscD_{1-156} were constructed using DNA fragments amplified from plasmid pCD1.
using upstream oligonucleotide primer YscD-HindIII-1 with downstream oligonucleotide primers YscD353-BglII-2, YscD282-BglII-2, YscD216-BglII-2, and YscD1-156-BglII-2, respectively. The resultant DNA fragments were digested with HindIII and BglII and inserted into HindIII- and BglII-digested pFLAG-MAC.

Construction of pFLAG-YscD expression vectors with in-frame deletions. In-frame deletions in \textit{yscD} of pFLAG-YscD\textsubscript{FL} were generated by either whole-plasmid PCR (17) or PCR-ligation-PCR (3) techniques. In certain instances, an EagI or KpnI site was created at the joint to facilitate the screening process. The pFLAG-YscD\textsubscript{A24-72} (\textit{ΔFHA}), pFLAG-YscD\textsubscript{A224-277} (\textit{ΔBON}/\textit{ΔPD2}), pFLAG-YscD\textsubscript{A88-113} (\textit{Δ} region between FHA and TM), pFLAG-YscD\textsubscript{A290-315} and pFLAG-YscD\textsubscript{A316-351} constructs were created by whole-plasmid PCR with the following primer sets: pFLAG-YscD\textsubscript{A24-72} (primers YscD23-rev and YscD73-for); pFLAG-YscD\textsubscript{A224-277} (primers YscD223-rev and YscD278-for); pFLAG-YscD\textsubscript{A88-113} (YscD87-rev and YscD114-for); pFLAG-YscD\textsubscript{A290-315} (YscD289-rev and YscD316-for) and pFLAG-YscD\textsubscript{A316-351} (YscD315-rev and YscD352-for). Amplification products were treated with DpnI to digest template DNA, ligated and electroporated into \textit{E. coli} DH5\textalpha.

The pFLAG-YscD\textsubscript{A163-208} (\textit{ΔPD1}) and pFLAG-YscD\textsubscript{A290-351} (\textit{ΔPD3}) constructs were created using the PCR-ligation-PCR technique (3). Initially, PCR fragments upstream and downstream of each deletion were amplified using the following set of primer pairs: pFLAG-YscD\textsubscript{A163-208} (primers YscD162-rev and YscD-HindIII-1; primers YscD209-for and YscD419-BglII-2) and pFLAG-YscD\textsubscript{A290-351} (primers YscD289-rev and YscD-HindIII-1; primers YscD352-for and YscD419-BglII-2). The resultant pair of DNA fragments for each deletion were ligated and reamplified with outside primers.
YscD-HindIII-1 and YscD419-BglII-2. The final DNA products were digested with HindIII and BglII and inserted into HindIII- and BglII-digested pFLAG-MAC.

**Construction of FLAG-YscD-TM24 and FLAG-YscD-TM28.** Plasmids pFLAG-YscD-TM24 and pFLAG-YscD-TM28 encode FLAG-YscD hybrid proteins with TM residues 121 to 142 or 121 to 148 of YscD replaced with sequences encoding the TM domain of PspC of *Y. enterocolitica* (NH\(_2\)-RVMVVLSSLFFGLFVFTIAAYIIVLT-COOH) or *Y. pestis* (NH\(_2\)-RVIMVVLSSLFFGLFVFTVAAYIILAFMLE-C), respectively. These constructs were generated by multiple PCR-ligation-PCR steps. Oligonucleotides Yent-TM-1 and Yent-TM-2 were used to amplify the PspC TM domain of *Y. enterocolitica* from pAJD572 (30), while oligonucleotide primers Yp-TM-1 and Yp-TM-2 were used to amplify sequences encoding the PspC TM domain from *Y. pestis* KIM chromosomal DNA. The PCR fragment encoding YscD residues 143 to 419 was amplified with primers YscD-Yent-TM-for and YscD419-BglII-2 and ligated with the *Y. enterocolitica* pspC PCR fragment, whereas the YscD fragment encoding residues 149 to 419 was amplified with primers YscD-Yp-TM-for and YscD419-BglII-2 and ligated to the *Y. pestis* pspC PCR fragment. Finally, sequences encoding YscD residues 1 to 120 of YscD were amplified with primers YscD-HindIII-1 and YscD-TM-rev. The resultant product was ligated to the amplified DNA fragments encoding the *Y. enterocolitica* or *Y. pestis* PspC TM/C-terminal YscD residues and reamplified with primers YscD-HindIII-1 and YscD419-BglII-2. The resulting fragments encoding full-length YscD with the TM-encoding sequences replaced with those of *pspC* were digested with HindIII and BglII and inserted into HindIII- and BglII-digested pFLAG-MAC, generating plasmids.
pFLAG-YscD-TM24 (\textit{Y. enterocolitica} PspC TM residues) and pFLAG-YscD-TM28 (\textit{Y. pestis} PspC TM residues).

**Construction of yscD::phoAlacZα fusions.** A dual alkaline phosphatase (PhoA) β-galactosidase (LacZα) reporter system developed by Alexeyev and Winkler (1, 2) was utilized to determine the membrane topology of YscD. DNA fragments that encode residues 1-29, 1-54, 1-63, 1-83, 1-110, 1-121, 1-162, 1-180, 1-256, 1-310, and 1-410 of YscD were amplified by PCR using oligonucleotide YscD-SacI-1 with YscD29-ApaI-2, YscD29-ApaI-2 YscD54-ApaI-2, YscD63-ApaI-2, YscD83-ApaI-2, YscD110-ApaI-2, YscD121-ApaI-2, YscD162-ApaI-2, YscD180-ApaI-2, YscD256-ApaI-2, YscD310-ApaI-2, or YscD410-ApaI-2, respectively. The resultant DNA fragments were digested with SacI and ApaI and inserted in-frame with the phoAlacZα reporter gene of SacI- and ApaI-digested plasmid pPHO1 (38). The resulting pPHO1-YscD constructs were electroporated into \textit{E. coli} DH5α and assayed for alkaline phosphatase and β-galactosidase activity as described below.

**β-galactosidase and alkaline phosphatase assays of \textit{E. coli} DH5α carrying yscD::phoAlacZα fusions.** \textit{E. coli} DH5α strains harboring different pPHO1 yscD::phoAlacZα fusion constructs were grown overnight in HIB at 37°C and used to inoculate fresh cultures at an OD\textsubscript{620} of 0.2 the next day. After 1 hour of growth at 37°C, expression of the YscD::PhoA-LacZα hybrid proteins was induced with 0.1 mM IPTG and growth continued for another 2 h. Bacterial cells (100 µl) were added to 500 µl β-Gal assay buffer (100 mM sodium phosphate buffer, pH 7, 10 mM KCl, 1 mM MgSO\textsubscript{4}) or AP assay buffer (1 M Tris-HCl, pH 8) and permeabilized by addition of 25 µl 0.1 % SDS, βME (50 mM final conc.) and 25 µl CHCl\textsubscript{3}. The assays were initiated by addition
of 200 µl of substrate (4 mg/ml ONPG for β-galactosidase assays or 1 mg/ml pNPP for alkaline phosphatase assays). Reactions were stopped by addition of 500 µl of 1M Na₂CO₃ and assay times and OD₄₂₀ readings were recorded and used to calculate units of activity as described previously (38).

**Co-immunoprecipitation of YscJ and YscC with YscD.** *Y. pestis* cultures (25 ml TMH; 2.5 mM CaCl₂; 0.05 mM IPTG) were grown for 1 h at 27°C and for 4 h at 37°C. Bacterial cells were harvested by centrifugation (8,000 x g for 10 min at 4°C) and resuspended in 2 to 3 ml of phosphate buffered saline (PBS), pH 7.4 in accordance with the OD₆₂₀ of each culture at harvest. Protein complexes were stabilized by chemical crosslinking with 0.5 mM dithiobis[succinimidyl propionate (DSP) (Thermo Scientific) for 20 min at RT. Bacterial cells were lysed by passage through a French pressure cell at 20,000 psi. Following bacterial lysis, additional DSP (1 mM total DSP final concentration) was added and the lysate was incubated at RT for 20 min. Crosslinking was terminated by the addition of 100 µl of 1 M Tris-HCl, pH 7.4 for 10 min at RT. Inner and outer membrane proteins were solubilized with Zwittergent 3-14 (Calbiochem). Briefly, 2 ml of bacterial lysate was combined with 0.5 ml of 10% Zwittergent 3-14 in PBS, pH 7.4 (2% final concentration) and incubated at RT for 10 min. Insoluble material was removed by centrifugation at 128,000 x g for 30 min at 8°C. To facilitate antibody binding, 1.5 ml of detergent-soluble supernatant was combined with 13.5 ml of PBS, pH 7.4 (0.2% Zwittergent 3-14 final concentration). Anti-FLAG M2 affinity gel (100 µl) (Sigma-Aldrich) was added to each diluted sample and incubated at 4°C with rotation overnight. The anti-FLAG M2 affinity beads were pelleted by centrifugation at 1,000 x g for 5 min at 8°C, washed twice with 8 ml PBS, 0.2% Zwittergent 3-14, pH 7.4 and eluted
with the same buffer containing 200 µg/ml FLAG peptide (Sigma-Aldrich). Zwittergent 3-14 lysates and anti-FLAG M2 elutions were analyzed by SDS-PAGE and immunoblot analysis with the FLAG M2 antibody (Sigma-Aldrich) and antisera specific for YscJ and YscC.

**SDS-PAGE and immunoblotting.** Cultures of bacteria were harvested by centrifugation at 14,000 x g for 10 min at RT. Pellets of whole cell bacteria and TCA precipitated supernatant proteins were resuspended according to the harvest OD$_{620}$ and analyzed by SDS-PAGE and immunoblotting as previously described (18). YscC, YscJ, YopM and YopN were detected with rabbit polyclonal antisera (1:20,000) raised against the full-length Ysc or Yop proteins. FLAG-tagged proteins were detected with anti-FLAG M2 monoclonal antibodies (1:1,000) or rabbit anti-FLAG polyclonal antibodies (1:1,000).

**RESULTS**

The *Y. pestis* YscD protein is a 419 residue inner membrane protein that is essential for the T3S process (35). It is a member of the EscD/PrgH/YscD family of T3S components, whose members are present in all T3SSs (16). The best studied member of this family is PrgH, a major component of the *Salmonella enterica* SPI-1 T3SS needle complex (22, 39). PrgH and PrgK (a YscJ homolog) are predicted to multimerize and form the inner membrane ring-like structures of the SPI-1 T3S apparatus. PrgH possesses a small cytoplasmic domain, a single TM domain and a large periplasmic domain. The structure of a portion of the periplasmic domain of PrgH has been determined (see Fig. 1B) and shown to contain a conserved domain, termed a “ring-
building motif”, that is also present in the predicted periplasmic regions of EscC (a YscC homolog) and EscJ (a YscJ homolog) (39, 44). Although significant structural information is available for PrgH, no detailed mutational or deletion studies have been completed on an EscD/PrgH/YscD family protein.

**Membrane topology of *Y. pestis* YscD.** Defining the membrane topology of YscD is crucial for understanding the role of this protein in the T3S process. Previous analyses of EscD/PrgH/YscD family proteins suggest that members of this family have a single TM domain. Interestingly, the transmembrane prediction programs TopPred (42), DAS (8) and TMpred (15) all predict YscD to possess two N-terminal TM domains (residues 80 to 97 and 122 to 142). To accurately determine the membrane topology of YscD, a dual *phoAlacZα* gene fusion reporter system (2) was utilized to generate a set of 11 in-frame *yscD::phoAlacZα* gene fusion constructs. These constructs, which encode increasing N-terminal regions of YscD fused in-frame to *E. coli* alkaline phosphatase (without its secretion signal) and the LacZα peptide were electroporated into *E. coli* DH5α and analyzed for both alkaline phosphatase and β-galactosidase (α-complementation) activities (Fig. 2). Alkaline phosphatase will be active if fused to a region of YscD that is localized on the periplasmic side of the inner membrane; in contrast, high β-galactosidase activity will be obtained if the LacZα-peptide is fused to a portion of YscD that is localized in the cytoplasm. Strains expressing YscD::PhoALacZα fusions containing the N-terminal 121 residues of YscD or less (29, 54, 63, 83 or 110 YscD residues) all expressed high β-galactosidase activity and low alkaline phosphatase activities, indicating a cytoplasmic location. These strains included fusions located prior to, within and immediately following the first predicted transmembrane domain (residues...
80 to 97), demonstrating that this region of YscD does not function as a TM domain. Finally, strains expressing YscD::PhoALacZa fusions containing the N-terminal 162 residues of YscD or more (180, 256, 310, 410 YscD residues) all expressed relatively high alkaline phosphatase activities and low β-galactosidase activities, indicating a periplasmic location. These results strongly indicate that YscD contains a single TM domain (residues 122 to 142) that connects a small N-terminal cytoplasmic region (residues 1 to 121) to a large periplasmic region (residues 143 to 419). This membrane topology is similar to that predicted for PrgH and HrcD, two other EscD/PrgH/YscD family members (4, 37).

The N-terminus of YscD is essential for the T3S process. The role of the N-terminal cytoplasmic region of YscD in the T3S process was investigated by constructing a series of deletions in \( yscD \) of pFLAG-YscD, which encodes an N-terminal FLAG-tagged YscD protein. The truncations constructed removed \( yscD \) sequences encoding residues prior to, within, and beyond the previously identified FHA domain (residues 24 to 72) (33) and continued through the YscD TM domain (residues 122 to 142). The derivatives of pFLAG-YscD carrying the deletions were moved into the \( yscD \) deletion strain (35) and the expression of FLAG-tagged YscD proteins, as well as the expression and secretion of YopM and YopN were assessed by growth of the bacteria in the presence and absence of calcium (2.5 mM; blocks Yop secretion) and by analysis of culture supernatant and whole cell proteins by SDS-PAGE and immunoblot analysis (Fig. 3A). The full-length FLAG-tagged YscD protein (FLAG-YscD\(_{FL} \)) restored calcium-regulated secretion of both YopN and YopM. A truncated FLAG-tagged YscD protein (FLAG-YscD\(_{6-419} \)) lacking the N-terminal 5 amino acids showed reduced secretion of
YopM and YopN, while removal of the N-terminal 12, 46, 75, 118 or 142 amino acids completely abolished Yop secretion. These data demonstrate the importance of the N-terminal cytoplasmic domain of YscD in the assembly of a functional T3S apparatus. Removal of even small regions of YscD prior to the FHA domain disrupted the T3S process, indicating that N-terminal regions outside the FHA domain are critical for YscD function or for the proper folding of the cytoplasmic region of YscD.

N-terminally truncated YscD proteins display a dominant-negative phenotype when expressed in a wild type background. YscD is one component of a multiprotein T3S complex and likely mediates functional interactions with other components of this complex (28). Dominant negative mutants are useful tools for defining the function, interactions and interaction domains of a protein. Therefore, we assessed whether expression of the N-terminal truncated FLAG-YscD proteins could impair Yop secretion in a wild-type background (dominant-negative phenotype). Expression of each of the non-functional N-terminally truncated YscD proteins, except for FLAG-YscD_{143-419}, in the wild type parent strain severely impaired the secretion of YopN and YopM (Fig. 3B). The FLAG-YscD_{119-419} protein lacks the entire cytoplasmic domain, suggesting that the dominant negative phenotype associated with this mutant is mediated by the TM domain or the periplasmic region of this protein. Similarly, the lack of a dominant negative effect with the FLAG-YscD_{143-419} is due either to removal of the TM domain or to mislocalization of the periplasmic YscD domain to the cytosol in the absence of the TM domain. Overall, these results demonstrate that YscD mutants with a deletion of 5 or more N-terminal residues are defective for T3S and exhibit a dominant negative effect on the T3S process when expressed in the parent strain.
C-terminal regions of YscD display a dominant-negative phenotype when targeted to the periplasm in the wild type parent strain. To determine if the dominant negative effect associated with expression of the N-terminally truncated FLAG-YscD proteins was dependent upon proper localization of the YscD C-terminus (residues 143 to 419) to the periplasm, vectors encoding the OmpA secretion signal fused to the FLAG epitope tag and various portions of the YscD C-terminal periplasmic region were constructed. Based on amino acid sequence alignment with PrgH (Fig. 1A), the YscD periplasmic region can be divided into four separate regions or domains here termed periplasmic domains (PD) 1 to 4 (PD1, PD2, PD3 and PD4). pFLAG vectors expressing the entire periplasmic region (FLAG-YscD_{143-419}), PD1-4 (FLAG-YscD_{157-419}), PD2-4 (FLAG-YscD_{217-419}), PD3-4 (FLAG-YscD_{283-419}) or PD4 (FLAG-YscD_{354-419}) with (pFLAG-ATS) and without (pFLAG-MAC) the OmpA secretion signal (ss) were generated and moved into the \( yscD \) deletion strain and the parent strain. As expected, all of the truncated YscD proteins were non-functional and failed to restore Yop secretion in the \( yscD \) deletion strain (data not shown). Interestingly, the intact, as well as truncated YscD periplasmic regions with, but not without, the OmpA secretion signal exhibited at least a partial dominant negative effect on the T3S process when expressed in the wild type parent strain (Fig. 4). Periplasmic expression of the isolated PD4 region (\( ss \)FLAG-YscD_{354-419}) had an especially potent dominant negative effect on the secretion of YopM and YopN by the parent strain, suggesting that this region of YscD might compete for a critical interaction site with full-length YscD within the periplasm.

The C-terminus of YscD is essential for the T3S process. To determine if the various C-terminal regions of YscD (PD1, PD2, PD3 or PD4) are necessary for the T3S
process, constructs were generated lacking PD4 (FLAG-YscD\textsubscript{1,353}), PD3-4 (FLAG-YscD\textsubscript{1,282}), PD2-4 (FLAG-YscD\textsubscript{1,216}) or PD1-4 (FLAG-YscD\textsubscript{1,156}). These constructs were expressed in the ysc\textit{D} deletion strain and analyzed for FLAG-YscD expression, as well as Yop expression and secretion (Fig. 5A). The FLAG-YscD\textsubscript{1,216} and FLAG-YscD\textsubscript{1,156} proteins were poorly expressed. Removal of the C-terminal 66 amino acids (PD4) from the C-terminus of YscD resulted in the complete loss of YopN and YopM secretion, confirming an essential role for this region in the T3S process. Interestingly, expression of the FLAG-YscD\textsubscript{1,353} and FLAG-YscD\textsubscript{1,282} proteins in the wild type parent strain had no effect on the secretion of Yops (no dominant negative effects) (Fig. 5B), further indicating that the C-terminal 66 residues (PD4) of YscD may play a critical role in the dominant negative phenotype previously observed with the N-terminally truncated FLAG-YscD proteins. To directly address this possibility, sequences encoding the C-terminal 66 residues of YscD (PD4) were deleted from constructs encoding the dominant negative FLAG-YscD\textsubscript{13-419} and ssFLAG-YscD\textsubscript{143-419} proteins, generating plasmids pFLAG-YscD\textsubscript{13-353} and pssFLAG-YscD\textsubscript{143-353}. The pFLAG-YscD\textsubscript{13-419}, pFLAG-YscD\textsubscript{13-353}, pssFLAG-YscD\textsubscript{143-419} and pssFLAG-YscD\textsubscript{143-353} constructs were moved into the wild type parent strain and the resulting strains were analyzed for Yop expression and secretion (Fig. 6). As suspected, the C-terminal 66 residues of YscD (PD4) were essential for the dominant negative phenotype associated with expression of the FLAG-YscD\textsubscript{13-419} and ssFLAG-YscD\textsubscript{143-419} proteins. These results provide additional evidence that the PD4 region of YscD mediates a critical periplasmic interaction and that defective YscD proteins that carry this domain likely compete with full-length YscD for this interaction.
Expression of ssFLAG-YscD_{354-419} (PD4) blocks the assembly of a functional T3S apparatus. The C-terminal 66 residues (PD4) of YscD were essential for YscD function and generated a strong dominant negative effect on Yop secretion when fused to the OmpA secretion signal and targeted to the periplasm of the parent strain. These results suggest that this region of YscD mediates a critical protein interaction involved in the assembly, regulation and/or function of the T3S apparatus.

To determine if the dominant negative effect of the YscD C-terminal 66 residues (PD4) is mediated through T3S regulatory components (YopN, TyeA or LcrG) (10, 29) that function to block Yop secretion in the presence of calcium, the dominant negative C-terminal 66 residues (PD4) of YscD was expressed in a yopN deletion strain that is defective in the regulation and cannot block secretion in the presence or absence of calcium. Expression of the C-terminal 66 residues (PD4) of YscD blocked YopM secretion from both the wild type parent strain and the yopN deletion strain (Fig. 7A), demonstrating that the dominant negative phenotype is not mediated through the secretion regulatory system.

Dominant negative mutants that function by blocking T3S apparatus assembly must be expressed prior to completion of the assembly process in order to exert their dominant negative effect (9); in contrast, dominant negative mutants that block the function of the assembled apparatus can exert their effects after assembly of the T3S apparatus is complete. Expression of the ssFLAG-YscD_{354-419} (PD4) protein fragment prior to and during T3S assembly resulted in a complete block of Yop secretion (Fig. 7B; ++ IPTG samples); however, induction of the ssFLAG-YscD_{354-419} (PD4) after T3S apparatus assembly (3 h after a shift to 37°C; + IPTG samples) had no effect on Yop secretion.
secretion. These results suggest that periplasmic expression of the C-terminal 66 residues of YscD blocks a critical step in the assembly of the T3S apparatus. Although additional steps in the assembly of the T3S apparatus may still occur in the presence of ssFLAG-YscD$_{354-419}$ (PD4) protein, recent studies by Diepold et al. (12) have demonstrated that in the absence of YscD no further assembly of the T3S apparatus occurs.

**The C-terminal region of YscD (residues 354 to 419; PD4) mediates the interaction of YscD with YscC.** Several recent studies (12, 36, 37) have demonstrated that EscD/PrgH/YscD family proteins interact with both EscJ/PrgK/YscJ family proteins and the assembled outer membrane secretin family proteins (YscC in the yersiniae). To determine if YscD residues 354 to 419 (PD4) mediates the interaction of YscD with YscC or YscJ, anti-FLAG M2 co-immunoprecipitation experiments were conducted with the ssFLAG-YscD$_{354-419}$ (PD4), ssFLAG-YscD$_{283-353}$ (PD3) and full-length FLAG-YscD$_{FL}$ proteins expressed in the ∆yscD and/or ∆yscC background (Fig. 8A). YscC, which primarily migrates as an SDS-resistant high-molecular weight complex (19), co-immunoprecipitated with both ssFLAG-YscD$_{354-419}$ (PD4) and FLAG-YscD$_{FL}$, but not with ssFLAG-YscD$_{283-353}$ (PD3). In contrast, YscJ co-immunoprecipitated with full-length FLAG-YscD$_{FL}$ but not with ssFLAG-YscD$_{354-419}$ (PD4) or ssFLAG-YscD$_{283-353}$ (PD3). These results indicate that the isolated YscD C-terminal region (PD4) interacts with YscC, but not YscJ, and likely mediates its dominant negative effect on T3S apparatus assembly through this interaction.

To further evaluate the role of the individual YscD periplasmic domains in the interaction of YscD with YscC and YscJ, anti-FLAG M2 co-immunoprecipitation studies were carried out with FLAG-YscD proteins specifically deleted for PD1, PD2, PD3 or
PD4 (Fig. 8B). Deletion of DNA sequences encoding PD1, PD2 or PD3 had no effect on the interaction of YscD with YscC or YscJ; however, deletion of sequences encoding PD4 eliminated the interaction of YscD with YscC, but had little effect on the interaction of YscD with YscJ. Together, these results demonstrate that the C-terminal PD4 region of YscD is both sufficient to interact with YscC (Fig. 8A) and essential for this interaction (Fig. 8B). In contrast, the interaction of YscD with YscJ likely involves multiple domains of YscD as deletion of each individual periplasmic domain had no significant effect on the YscD-YscJ interaction.

In-frame deletions of YscD cytoplasmic and periplasmic domains/motifs. Previous bioinformatic analyses of EscD/PrgH/YscD family proteins have provided information on putative structural motifs present in YscD and other members of this family of proteins (33). These include the presence of an FHA motif in the N-terminal cytoplasmic region of YscD (residues 24 to 72) and a BON (phospholipid-binding) domain in the periplasmic region (PD2; residues 224 to 277). In addition, structural studies of PrgH have enabled amino acid sequence alignment-based predictions of the domain structure of the relevant YscD periplasmic region (see Fig. 1) (39). To investigate the function of these individual motifs and/or domains in YscD, in-frame deletions in pFLAG-YscDFL were generated that removed sequences encoding the FHA domain (FLAG-YscDΔ24-72), the region between the FHA domain and TM domain (FLAG-YscDΔ88-113), PD1 (FLAG-YscDΔ163-208), PD2 [BON domain] (FLAG-YscDΔ224-277) or PD3 [putative ring-building motif] (FLAG-YscDΔ290-351). The secretion of Yops by the yscD deletion strains expressing each of these in-frame deletion mutants were analyzed by SDS-PAGE and immunoblot analysis (Fig. 9A). Strains expressing YscD
proteins deleted for the FHA domain, PD1, PD4 (see Fig. 5A) and the region between the FHA domain and the TM domain (Δ88-113) were defective for YopM and YopN secretion, indicating that these regions were required for production of a functional YscD protein. Surprisingly, FLAG-YscD proteins lacking the PD2 (BON domain) or PD3 (putative ring-building motif) secreted YopM and YopN at normal levels in the absence of calcium (secretion permissive conditions); in addition, the strain expressing the ring-building motif mutant (ΔPD3) also secreted YopM and YopN in the presence of calcium, indicating that this mutant had a specific defect in the regulation of Yop secretion. These results indicate that neither the PD2 (BON domain) or PD3 (ring-building motif) domains play an essential role in the assembly or the basic secretory function of the T3S apparatus; although, the PD3 domain may have a role in the regulation of the secretion process. To verify that the regulatory defect associated with the FLAG-YscDΔ290-351 (ΔPD3) protein was not due to multicopy plasmid-based overexpression, the ΔPD3 and a PD4 deletion were moved into plasmid pCD1 (see supplemental data). The resultant strains maintained their unique secretion phenotypes (see Fig. S1), confirming that these phenotypes were not due to multicopy plasmid-based overexpression or to the presence of an N-terminal FLAG tag.

To further assess the role of the periplasmic PD1, PD2 and PD3 domains in the T3S process, we constructed vectors encoding FLAG-YscD proteins deleted for both the PD2 and PD3 domains (FLAG-YscDΔ224-351) or the PD1, PD2 and PD3 domains (FLAG-YscDΔ163-351). Expression of the FLAG-YscDΔ224-351 or FLAG-YscDΔ163-351 proteins in the yscD deletion mutant failed to restore Yop secretion (Fig. 9B), indicating that FLAG-YscD deleted for PD2 and PD3 or PD1, PD2 and PD3 were non-functional.
Interestingly, expression of the FLAG-YscDΔ224-351 protein or the FLAG-YscDΔ163-351 protein in the parent strain still produced a dominant negative phenotype (data not shown), indicating that the mutant YscD proteins were properly inserted with the PD4 domain localized to the periplasm.

The defect in the calcium-dependent regulation of Yop secretion associated with the FLAG-YscDΔ290-351 (ΔPD3)-encoding construct was unexpected and represents the second example of a mutation in a periplasmic-localized T3S component that alters the regulation of the T3S process (36). This phenotype suggests that YscD either has a direct role in the regulation of Yop secretion or that the mutant FLAG-YscDΔ290-351 protein is disrupting the function of another regulatory component, possibly YscJ or indirectly the needle/rod structure that also traverses this region.

Previous studies have demonstrated that some regulatory mutants (constitutive secreting YscF needle mutants) can be suppressed by increasing the amount of extracellular calcium, while other regulatory mutants cannot (constitutive secreting yopN or tyeA mutants) (40). The ability of the FLAG-YscDΔ290-351 (ΔPD3) regulatory mutant to respond to calcium was examined by measuring the expression and secretion of YopM in the presence of 0 mM, 1.25 mM, 2.5 mM, 5 mM and 7.5 mM extracellular calcium (Fig. 10A). Growth of the FLAG-YscDΔ290-351 (ΔPD3) mutant in either 5 mM or 7.5 mM calcium blocked Yop secretion in a manner similar to that previously reported for a YscF D28A point mutant (40), suggesting that the FLAG-YscDΔ290-351 (ΔPD3) protein could mediate its effect on regulation by disrupting the regulatory function of the needle/rod component.
Examination of the available PrgH structure revealed that the PD3 domain is primarily comprised of two anti-parallel alpha helices separated by a beta sheet (39). To further characterize the role of the PD3 domain in the regulation of Yop secretion, two additional in-frame deletions were generated that together deleted the same region as deleted in the ΔPD3 mutant but in two smaller deletions. FLAG-YscDΔ290-315 and FLAG-YscDΔ316-351 were constructed in pFLAG-YscD, moved into the yscD deletion strain and their secretion phenotypes characterized (Fig. 10B). Strains expressing either of the small internal PD3 deletion mutants secreted YopM and YopN in a normal calcium-regulated manner. These results suggest that the PD3 domain likely does not have a direct functional role in the calcium-dependent regulation of Yop secretion; instead, the regulatory defect associated with the original FLAG-YscDΔ290-351 (ΔPD3) is likely due to an indirect effect of this mutant protein on other regulatory components, possibly YscI or YscF.

**Role of the YscD TM domain in the T3S process.** To determine if the TM domain of YscD has an active (sequence specific) role in the secretion process, such as a role in TM signaling, protein interactions or a contribution to the formation of a secretion channel, the TM domain was replaced with TM domains from a protein with a similar topology, but a dissimilar TM amino acid sequence. The DNA sequences encoding the TM domain of FLAG-YscD was replaced with that of the phage shock protein C (PspC) (30) TM domain of *Y. enterocolitica* or *Y. pestis*, generating constructs encoding the different chimeric proteins (pFLAG-YscD-TM24 and pFLAG-YscD-TM28). Interestingly, replacing the TM domain of YscD with a TM domain of dissimilar sequence had no effect on the secretion of Yops (Fig. 11), suggesting that the YscD TM
domain has no distinct sequence specific role in the secretion process, but rather likely functions as an anchor, tether or linker for the soluble cytoplasmic and periplasmic regions of YscD.

**DISCUSSION**

EscD/PrgH/YscD family proteins are conserved T3S components that multimerize to form inner membrane ring-shaped structures critical for the assembly and function of T3S complexes (27, 39). The *Y. pestis* YscD protein is a 419 amino acid inner membrane EscD/PrgH/YscD family protein that is essential for the assembly of a functional plasmid pCD1-encoded T3S apparatus (35). Reporter-based membrane topology studies demonstrated that YscD has a small N-terminal cytoplasmic region (residues 1 to 121), a single TM domain (residues 122 to 142) and a large periplasmic region (residues 143 to 419). A deletion analysis of YscD demonstrated that the N-terminal cytoplasmic region and C-terminal periplasmic regions of YscD are both essential for the production of a functional YscD protein. Removal of the coding sequence for as few as twelve N-terminal amino acids, the central FHA domain or the region between the FHA domain and the TM domain completely blocked all T3S activity. The function of the cytoplasmic region of EscD/PrgH/YscD family proteins is unknown; however, several recent studies suggest that this region may interact with T3S components (EscQ/Spa33/YscQ family proteins) that are implicated in the assembly of a cytoplasmic ring structure (20, 31). Indeed, evidence obtained using a LexA-based bacterial one-hybrid system (13) indicated that the isolated cytoplasmic region of YscD (residues 1 to 121) may dimerize or further multimerize (data not shown) and could form
an oligomeric structure that might serve as a scaffold for the assembly of other cytoplasmic T3S components.

Interestingly, bioinformatic analysis of EscD/PrgH/YscD family proteins (33) and structural information available for PrgH (39) provided intriguing functional leads for the predicted PD2 and PD3 domains of YscD. PD2 was predicted to contain a phospholipid-binding (BON) domain (residues 224 to 277), whereas the PD3 domain was suggested to represent a common “ring-building motif” involved in the multimerization and assembly of EscC/InvG/YscC, EscJ/PrgK/YscJ and EscD/PrgH/YscD family proteins (39, 44). Surprisingly, deletion of sequences encoding almost the entire PD2 or PD3 domains had essentially no effect on the ability of the Ysc T3S apparatus to assemble and export Yop substrates. In contrast, deletion of sequences encoding the PD1 and PD4 domains of YscD disrupted the T3S process. These findings demonstrate that the PD2 (BON domain) and PD3 putative “ring-building motif” do not play an essential role in the assembly of a functional T3S apparatus, whereas the periplasmic PD1 and PD4 domains are essential for YscD function and T3S activity.

The C-terminal periplasmic region of YscD (PD4; residues 354-419) exerted a strong dominant negative effect on the T3S process when targeted to the periplasm of the wild type parent strain. Recent studies with PrgH (37), a Salmonella EscD/PrgH/YscJ family protein, have demonstrated that truncation of only four residues from the PrgH C-terminus significantly weakened the interaction of the InvG secretin ring structure with the remainder of the needle complex. Furthermore, cross-linking of InvG to the C-terminal region of PrgH suggested that these regions are closely approximated in the assembled Salmonella needle complex (36, 37). Consistent with these studies, we
demonstrated that the isolated C-terminal region of YscD (PD4) can independently interact with the outer membrane YscC secretin complex when targeted to the periplasm via the OmpA secretion signal. Furthermore, the interaction between YscD and YscC was eliminated if the PD4 domain was deleted from full-length YscD. Thus, the periplasmic-localized YscD PD4 domain is both sufficient to interact with YscC on its own and essential for the YscD protein to interact with YscC. These findings are also consistent with recent findings by Diepold et al. (12) who used fluorescent hybrid proteins to demonstrate that injectisome assembly initiates with assembly of the outer membrane secretin (YscC) followed by the addition of YscD and then YscJ. Thus, the dominant negative periplasmic FLAG-YscD<sub>353-419</sub> protein would be expected to directly compete with wild type YscD for binding to the YscC secretin and would likely arrest T3S apparatus assembly at this early stage. Interestingly, none of the YscD deletion mutants that lacked the PD4 domain had a dominant negative effect on the T3S process even though these mutants likely contain regions of YscD involved in the interaction with other T3S components, such as YscJ and YscQ. These results further support the hypothesis that YscD must interact/multimerize via interaction with YscC prior to establishing functional interactions with other T3S components (12).

YscD has also been shown to interact with the other major ring-forming periplasmic constituent YscJ (12). YscJ is a lipoprotein that is attached to the inner membrane at its N-terminus via its lipid moiety and at its C-terminus by a single TM domain terminating with a cytoplasmic anchor sequence (38). Recent studies have demonstrated that the N-terminal cytosolic region of PrgH can be cross-linked to the cytosolic C-terminal region of PrgK, a Salmonella EscJ/PrgK/YscJ family protein (37).
In addition, other studies have indicated that the periplasmic regions of these proteins also interact (36). Thus, EscD/PrgH/YscD and EscJ/PrgK/YscJ family proteins likely interact at multiple contact sites involving both the periplasmic and cytoplasmic portions of these proteins. Our results are consistent with this interpretation as YscJ co-immunoprecipitated with full-length FLAG-YscD, as well as with FLAG-YscD proteins deleted for each of the predicted periplasmic domains (Fig. 8) but not with the isolated FLAG-YscD (PD4) or FLAG-YscD (PD3) domains targeted to the periplasmic space.

Surprisingly, sequences encoding the YscD PD2 or PD3 regions could be deleted with essentially no effect on the basic T3S process, indicating that these regions are not essential for the assembly or function of the T3S apparatus. Instead, the essential PD1 and PD4 regions likely mediate the contacts critical for the assembly of a functional T3S apparatus. Alternatively, the PD2 and PD3 domains may serve redundant essential roles in T3S apparatus assembly as elimination of both domains resulted in a non-functional YscD protein.

Although the ΔPD3 YscD mutant secreted normal levels of YopM and YopN under secretion permissive conditions (37°C; -Ca²⁺); this mutant also secreted Yops in the presence of calcium, indicating a defect in the regulation of Yop secretion. This defect could be suppressed by increasing the level of extracellular calcium, similar to what has been reported previously for YscF needle mutants defective in the regulation of secretion (40). The conformation of the needle, which is hypothesized to be controlled, in part, by extracellular calcium levels is further hypothesized to control the activity of the YopN/SycN/YscB/TyeA complex, which controls the export of the Yop effector proteins (10). Importantly, the YscF/YscI needle/rod structure passes through the YscJ
ring of the YscJ/YscD periplasmic structure; thus, we hypothesize that this mutant has an altered conformation that may affect the conformation of the YscD/YscJ ring structure and impinge on the needle/rod structure altering its conformation/signaling function. These findings provide further evidence that the needle/rod-like structure, and/or possibly the YscJ/YscD ring structure itself, plays a role in regulating the T3S process in response to extracellular signals. Multiple smaller deletions in the PD3 domain (FLAG-YscD_{\Delta290-315} and FLAG-YscD_{\Delta316-351}) that together removed the same region had no effect on the regulation or function of the T3S process, indicating that the YscD protein likely has no direct role in the regulation of Yop secretion.

Finally, we demonstrate that the YscD TM domain can be functionally replaced with a sequence dissimilar TM domain from another protein. Similar results were obtained with the single TM domain of YscJ (38). These studies suggest that these TM domains have no direct sequence-specific role in the T3S process and likely are not involved in signaling, protein interactions or channel formation. Thus, the TM domain of YscD functions to tether or anchor the critical N-terminal cytoplasmic and periplasmic regions to the bacterial inner membrane. Together, the studies presented demonstrate that the essential functional regions of YscD are the N-terminal cytoplasmic domain, as well as the periplasmic PD1 and PD4 domains. Future studies will be aimed at further characterizing the specific role of these regions in the assembly and function of the Ysc T3S apparatus.
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FIGURE LEGENDS

Fig. 1. Comparison of the YscD and PrgH periplasmic regions. (A) Amino acid sequence alignment of the periplasmic regions of YscD (residues 155 to 419) and PrgH (residues 170 to 362). Regions corresponding to individual domains of PrgH are labeled periplasmic domains (PD) 1 to 4. Identical residues are marked by an asterisk (*), strongly similar by a colon (:) and weakly similar by a period (.). (B) Ribbon representation of the PrgH\textsubscript{170-362} crystal structure (39) generated using Pymol (http://www.pymol.org).

Fig. 2. Determination of YscD membrane topology. (A) Alkaline phosphatase (AP) activities (measured in AP Units) of \textit{E. coli} DH5\textalpha{} strains expressing YscD::PhoA-LacZ\alpha{} fusion proteins fused after YscD residue 29, 54, 63, 83, 110, 121, 162, 180, 256, 310 or 410. (B) β–galactosidase (BG) activities (measured in Miller Units) from the same \textit{E. coli} DH5\textalpha{} strains expressing YscD::PhoA-LacZ\alpha{} fusion proteins. (C) Model of YscD membrane topology. YscD residues 1 to 121 are located in the bacterial cytoplasm, residues 122 to 142 form a transmembrane (TM) domain and residues 143 to 419 are located in the bacterial periplasm. Activities represent means assayed in duplicate. Error bars represent SD.

Fig. 3. Analysis of YopM and YopN expression and secretion, as well as expression of N-terminal truncated FLAG-YscD proteins. (A) \textit{Y. pestis} Δysc\textit{D} deletion strain carrying pFLAG-YscD plasmids. (B) Wild type \textit{Y. pestis} KIM5-3001 carrying pFLAG-YscD plasmids. Strains were grown in the presence (+) or absence (-) of 2.5 mM CaCl\textsubscript{2}
for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various N-terminal truncated FLAG-YscD proteins.

Fig. 4. FLAG-tagged C-terminal regions of YscD expressed in the periplasm of wild type Y. pestis KIM5-3001 exhibit a dominant negative effect on the T3S process. C-terminal FLAG-YscD proteins were expressed with (pFLAG-YscD constructs) or without (pssFLAG-YscD constructs) an N-terminal OmpA secretion signal (ss). Strains were grown in the presence (+) or absence (-) of 2.5 mM CaCl₂ for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.

Fig. 5. Analysis of YopM and YopN expression and secretion, as well as expression of C-terminal truncated FLAG-YscD proteins. (A) Y. pestis ΔyscD deletion strain carrying pFLAG-YscD plasmids. (B) Wild type Y. pestis KIM5-3001 carrying pFLAG-YscD plasmids. Strains were grown in the presence (+) or absence (-) of 2.5 mM CaCl₂ for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.

Fig. 6. The C-terminal 66 residues of YscD (PD4) are required for the dominant negative effect of FLAG-YscD₁₃₋₄₁₉ and ssFLAG-YscD₁₄₃₋₄₁₉ in wild type Y. pestis.
KIM5-3001. The FLAG-YscD<sub>13-419</sub>, FLAG-YscD<sub>13-353</sub> (PD4 region deleted), ssFLAG-YscD<sub>143-419</sub> and ssFLAG-YscD<sub>143-353</sub> (PD4 region deleted) proteins were expressed in wild type <i>Y. pestis</i> KIM5-3001. Strains were grown in the presence (+) or absence (−) of 2.5 mM CaCl<sub>2</sub> for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.

Fig. 7. The dominant negative ssFLAG-YscD<sub>354-419</sub> (PD4) protein blocks assembly, but not the function, of the T3S apparatus. (A) Expression of the ssFLAG-YscD<sub>354-419</sub> (PD4) protein blocks secretion of Yops by both wild type <i>Y. pestis</i> KIM5-3001 and by a ΔyopN deletion mutant. Strains were grown in the presence (+) or absence (−) of 2.5 mM CaCl<sub>2</sub> for 6 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. (B) Expression of the ssFLAG-YscD<sub>354-419</sub> (PD4) protein blocks secretion of Yops when expression is induced prior to T3S apparatus assembly (IPTG present in overnight cultures and during secretion experiment; ++) but not when added after T3S apparatus assembly (3 h after a shift to 37°C; +). Cultures were grown 1 h at 27°C and then shifted to 37°C for 3 h. At the 3 h time point, all cultures were centrifuged to separate bacterial whole cells (WC) from the supernatant (SUP-1). Pellets were resuspended in pre-warmed TMH media with the appropriate antibiotics and 0.05 mM IPTG (except for – IPTG controls) and growth continued at 37°C for another 4 h. SUP-2 refers to the supernatant collected at the final harvest. Samples were analyzed by SDS-PAGE and immunoblot analysis.
Fig. 8. Co-immunoprecipitation of YscC and YscJ with FLAG-tagged YscD proteins. Zwittergent 3-14-soluble lysates from ΔyscD or ΔyscC Y. pestis expressing the (A) ssFLAG-YscD$_{354-419}$ (PD4), ssFLAG-YscD$_{283-353}$ (PD3) or FLAG-YscD$_{FL}$ proteins or (B) the FLAG-YscD$_{Δ163-208}$ (∆PD1), FLAG-YscD$_{Δ224-277}$ (∆PD2), FLAG-YscD$_{Δ290-351}$ (∆PD3) or FLAG-YscD$_{Δ354-419}$ (∆PD4) proteins were immunoprecipitated with anti-FLAG M2 affinity gel. The starting lysates (L) and elutions (E) were analyzed by SDS-PAGE and immunoblot analysis with the FLAG M2 antibody and antisera specific for YscC and YscJ.

Fig. 9. Analysis of YopM and YopN expression and secretion, as well as expression of FLAG-YscD proteins with internal in-frame deletions. (A) Y. pestis ΔyscD strain expressing FLAG-YscD$_{Δ24-72}$ (∆FHA), FLAG-YscD$_{Δ88-113}$ (∆ region between FHA domain and TM domain), FLAG-YscD$_{Δ163-208}$ (∆PD1), FLAG-YscD$_{Δ224-277}$ (∆PD2/BON), FLAG-YscD$_{Δ290-351}$ (∆PD3), or FLAG-YscD$_{Δ354-419}$ (∆PD4). (B) Y. pestis ΔyscD strain expressing FLAG-YscD$_{Δ224-351}$ (∆PD2-3) and FLAG-YscD$_{Δ163-351}$ (∆PD1-3). Strains were grown in the presence (+) or absence (-) of 2.5 mM CaCl$_2$ for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.

Fig. 10. Characterization of the regulation defect associated with expression of FLAG-YscD$_{Δ290-351}$ (∆PD3). (A) Expression and secretion of YopM by Y. pestis KIM5-
3001.P39 (parent), the ∆yopN strain, the YscF(D28A) strain and the ∆yscD strain expressing FLAG-YscD_{FL} or FLAG-YscD_{Δ290-351} (ΔPD3) grown for 5 h at 37°C in the presence of 0 mM, 1.25 mM, 2.5 mM, 5 mM, or 7.5 mM CaCl\(_2\). Cultures were harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. (B) Analysis of YopM and YopN expression and secretion, as well as expression of the FLAG-YscD_{Δ298-315} and the FLAG-YscD_{Δ316-351} proteins carrying internal in-frame deletions within the PD3 domain. Strains were grown in the presence (+) or absence (-) of 2.5 mM CaCl\(_2\) for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.

**Fig. 11. Analysis of YopM and YopN expression and secretion, as well as expression of the FLAG-YscD-TM24 and FLAG-YscD-TM28 YscD/PspC TM hybrid proteins.**

pFLAG-YscD-TM24 and pFLAG-YscD-TM28 encode FLAG-YscD protein with YscD TM residues replaced with TM residues found in the *Y. enterocolitica* or *Y. pestis* PspC proteins, respectively. *Y. pestis* ΔyscD deletion strains carrying pFLAG-YscD_{FL}, pFLAG-YscD-TM24 or pFLAG-YscD-TM28 were grown in the presence (+) or absence (-) of 2.5 mM CaCl\(_2\) for 5 h at 37°C and harvested by centrifugation to separate whole cell (WC) and supernatant (SUP) fractions. Samples were analyzed by SDS-PAGE and immunoblot analysis. Arrowheads indicate the various FLAG-tagged YscD proteins.
Table 1. Bacterial strains and plasmids used in this study

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<td><strong>E. coli strain</strong></td>
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<td>DH5α</td>
<td>F φ80 lacZ ΔM15, Δ(lacZYA argF) U169, endA1, recA1 hsdR17 deoR supE44 thi-1 gyr96 relA1</td>
<td>(6)</td>
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<td><strong>Y. pestis strains</strong></td>
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<tr>
<td>KIM5-3001 (parent)</td>
<td>SmR pCD1 pPCP1 pMT1</td>
<td>(25)</td>
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<tr>
<td>KIM5-3001.12</td>
<td>SmR pCD1 (ΔyscC) pPCP1 pMT1</td>
<td>(35)</td>
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<tr>
<td>KIM5-3001.14</td>
<td>SmR pCD1 (ΔyscD) pPCP1 pMT1</td>
<td>(35)</td>
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<tr>
<td>KIM5-3001.P39</td>
<td>SmR pCD1 (ΔsycE-yopE::km) pPCP1 pMT1</td>
<td>(10)</td>
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<td>KIM5-3001.P39.F3</td>
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<td>(40)</td>
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<td>KIM5-3001.P62</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pFLAG-MAC</td>
<td>expression vector: encodes FLAG-peptide</td>
<td>Sigma-</td>
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<td>Aldrich</td>
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<tr>
<td>pFLAG-ATS</td>
<td>expression vector: encodes OmpA secretion signal (ss) fused to FLAG-peptide</td>
<td>Sigma-</td>
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<td>Aldrich</td>
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<tr>
<td>pFLAG-YscD&lt;sub&gt;FL&lt;/sub&gt;</td>
<td>HindIII- and BglIII-digested PCR fragment in pFLAG-MAC vector encoding full length FLAG-YscD</td>
<td>This study</td>
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<tr>
<td>Name</td>
<td>Description</td>
<td>Study</td>
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<tr>
<td>pFLAG-YscD&lt;sub&gt;6-419&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;6-419&lt;/sub&gt;</td>
<td>This study</td>
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<td>pFLAG-YscD&lt;sub&gt;13-419&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;13-419&lt;/sub&gt;</td>
<td>This study</td>
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<td>pFLAG-YscD&lt;sub&gt;47-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;76-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;119-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;143-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;157-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;217-419&lt;/sub&gt;</td>
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<td>pFLAG-YscD&lt;sub&gt;283-419&lt;/sub&gt;</td>
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<td><strong>pssFLAG-YscD&lt;sub&gt;283-419&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-ATS encoding ssFLAG-YscD&lt;sub&gt;283-419&lt;/sub&gt;</td>
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<td>HindIII- and BglII-digested PCR fragment in pFLAG-ATS encoding ssFLAG-YscD&lt;sub&gt;354-419&lt;/sub&gt;</td>
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<td><strong>pFLAG-YscD&lt;sub&gt;1-353&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;1-353&lt;/sub&gt;</td>
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<td><strong>pFLAG-YscD&lt;sub&gt;1-282&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;1-282&lt;/sub&gt;</td>
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<td><strong>pFLAG-YscD&lt;sub&gt;1-216&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;1-216&lt;/sub&gt;</td>
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<td><strong>pFLAG-YscD&lt;sub&gt;1-156&lt;/sub&gt;</strong></td>
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<td><strong>pFLAG-YscD&lt;sub&gt;13-353&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;13-353&lt;/sub&gt;</td>
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<td><strong>pssFLAG-YscD&lt;sub&gt;143-353&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD&lt;sub&gt;143-353&lt;/sub&gt;</td>
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<td><strong>pFLAG-YscD&lt;sub&gt;Δ24-72&lt;/sub&gt;</strong></td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the FHA domain (FLAG-YscD&lt;sub&gt;Δ24-72&lt;/sub&gt;)</td>
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This study
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<tr>
<td>pFLAG-YscD&lt;sub&gt;Δ88-113&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the region between the FHA domain and TM domain (FLAG-YscD&lt;sub&gt;Δ88-113&lt;/sub&gt;)</td>
<td>This study</td>
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<td>pFLAG-YscD&lt;sub&gt;Δ163-208&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the PD1 domain (FLAG-YscD&lt;sub&gt;Δ163-208&lt;/sub&gt;)</td>
<td>This study</td>
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<td>pFLAG-YscD&lt;sub&gt;Δ224-277&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the PD2 (BON) domain (FLAG-YscD&lt;sub&gt;Δ224-277&lt;/sub&gt;)</td>
<td>This study</td>
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<tr>
<td>pFLAG-YscD&lt;sub&gt;Δ290-351&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the PD3 domain (FLAG-YscD&lt;sub&gt;Δ290-351&lt;/sub&gt;)</td>
<td>This study</td>
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<td>pFLAG-YscD&lt;sub&gt;Δ163-351&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the PD1, 2, and 3 domains (FLAG-YscD&lt;sub&gt;Δ163-351&lt;/sub&gt;)</td>
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<td>pFLAG-YscD&lt;sub&gt;Δ224-351&lt;/sub&gt;</td>
<td>HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with an in-frame deletion of the PD2 and 3 domains (FLAG-YscD&lt;sub&gt;Δ224-351&lt;/sub&gt;)</td>
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YscD\textsubscript{224-351})

pFLAG-YscD\textsubscript{290-315} HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with a small in-frame deletion within the PD3 domain (FLAG-YscD\textsubscript{290-315})

This study

pFLAG-YscD\textsubscript{316-351} HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD with a small in-frame deletion within the PD3 domain (FLAG-YscD\textsubscript{316-351})

This study

pssFLAG-YscD\textsubscript{283-353} HindIII- and BglII-digested PCR fragment in pFLAG-ATS encoding ssFLAG-YscD\textsubscript{283-353}

This study

pFLAG-YscD-TM-24 HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD\textsubscript{1-120}-PspC\textsubscript{Yent58-81}-YscD\textsubscript{143-419}

This study

pFLAG-YscD-TM-28 HindIII- and BglII-digested PCR fragment in pFLAG-MAC encoding FLAG-YscD\textsubscript{1-120}-PspC\textsubscript{Yp38-65}-YscD\textsubscript{149-419}

This study

pPHO-YscD\textsubscript{1-29} SacI- and ApaI-digested PCR fragment encoding YscD residues 1-29 in pPHO1

This study

pPHO-YscD\textsubscript{1-54} SacI- and ApaI-digested PCR fragment encoding YscD residues 1-54 in pPHO1

This study

pPHO-YscD\textsubscript{1-63} SacI- and ApaI-digested PCR fragment encoding YscD residues 1-63 in pPHO1

This study
<table>
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<th>pPHO-YscD&lt;sub&gt;1:83&lt;/sub&gt;</th>
<th>SacI- and ApaI-digested PCR fragment encoding YscD residues 1-83 in pPHO1</th>
<th>This study</th>
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<tr>
<td>pPHO-YscD&lt;sub&gt;1:110&lt;/sub&gt;</td>
<td>SacI- and ApaI-digested PCR fragment encoding YscD residues 1-110 in pPHO1</td>
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<td>pPHO-YscD&lt;sub&gt;1:121&lt;/sub&gt;</td>
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<td>This study</td>
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<td>pPHO-YscD&lt;sub&gt;1:162&lt;/sub&gt;</td>
<td>SacI- and ApaI-digested PCR fragment encoding YscD residues 1-162 in pPHO1</td>
<td>This study</td>
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<td>pPHO-YscD&lt;sub&gt;1:180&lt;/sub&gt;</td>
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<td>pPHO-YscD&lt;sub&gt;1:256&lt;/sub&gt;</td>
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<td>pPHO-YscD&lt;sub&gt;1:310&lt;/sub&gt;</td>
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<td>pPHO-YscD&lt;sub&gt;1:410&lt;/sub&gt;</td>
<td>SacI- and ApaI-digested PCR fragment encoding YscD residues 1-410 in pPHO1</td>
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REFERENCES


