Structure and Interactions of the Cytoplasmic Domain of the *Yersinia* Type III Secretion Protein YscD

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The virulence of a large number of Gram-negative bacterial pathogens depends on the type III secretion (T3S) system, which transports select bacterial proteins into host cells. An essential component of the *Yersinia* T3S system is YscD, a single-pass inner membrane protein. We report here the 2.52-Å resolution structure of the cytoplasmic domain of YscD, called YscDc. The structure confirms that YscDc consists of a forkhead-associated (FHA) fold, which in many but not all cases specifies binding to phosphothreonine. YscDc, however, lacks the structural properties associated with phosphothreonine binding and thus most likely interacts with partners in a phosphorylation-independent manner. Structural comparison highlighted two loop regions, L3 and L4, as potential sites of interactions. Alanine substitutions at L3 and L4 had no deleterious effects on protein structure or stability but abrogated T3S in a dominant negative manner. To gain insight into the function of L3 and L4, we identified proteins associated with YscD by affinity purification coupled to mass spectrometry. The lipoprotein YscJ was found associated with wild-type YscD, as was the effector YopH. Notably, the L3 and L4 substitution mutants interacted with more YopH than did wild-type YscD. These substitution mutants also interacted with SycH (the specific chaperone for YopH), the putative C-ring component YscQ, and the ruler component YscP, whereas wild-type YscD did not. These results suggest that substitutions in the L3 and L4 loops of YscD disrupted the dissociation of SycH from YopH, leading to the accumulation of a large protein complex that stalled the T3S apparatus.

The virulence of a wide variety of Gram-negative bacterial pathogens requires the transport of select proteins from the bacterial cytosol into host cells by the type III secretion (T3S) system (13, 19). Such transported proteins have diverse and usually deleterious functions within host cells. Transport by the T3S system requires the action of ~20 to 25 proteins that assemble to form the injectisome, a macromolecular apparatus that spans the inner and outer membranes of the bacterial envelope and terminates with an extracellular appendage resembling a needle (23, 28, 50). In the *Yersinia* T3S system, the inner membrane protein YscD (47 kDa) constitutes an essential component of the injectisome (38, 46).

YscD is composed of an N-terminal cytoplasmic domain, a single-pass membrane-spanning region, and a C-terminal periplasmic domain. The periplasmic domain of YscD interacts with the periplasmic domains of the outer membrane secretin YscC and the inner membrane lipoprotein YscJ (49). The complex composed of YscC, YscD, and YscJ appears to form scaffolding for the assembly of the *Yersinia* injectisome (15, 29). The stoichiometry of YscD in this complex is unknown, but equivalent complexes in *Salmonella* and *Shigella* suggest a ring-like assembly of 24 copies of YscD (23, 50). The N-terminal cytoplasmic domain of YscD (residues 1 to 121, ~13 kDa), called here YscDc, is predicted to have a forkhead-associated (FHA) fold and has been shown to be essential for T3S (43, 49). FHA domains are found in a large number of eukaryotic and prokaryotic proteins (24), and while most FHA domains specify binding to phosphothreonine residues (35), a number have been identified to confer phosphorylation-independent interaction (40, 54).

To provide guidance in understanding the role of YscD in T3S, we expressed, purified, and determined the X-ray crystal structure of YscDc. YscDc was found to be monomeric and not prone to oligomerization, and its structure confirmed the predicted FHA fold (44), as has also been demonstrated recently by others (34).

Comparison with other FHA domain proteins, including MxiG, a YscD ortholog from *Shigella flexneri*, whose cytoplasmic domain was recently shown to have an FHA fold by nuclear magnetic resonance (NMR) and X-ray crystallographic techniques (6, 37), highlighted two loop regions as being potentially significant for function. Alanine substitutions at these two loops, L3 and L4, had no deleterious effects on protein structure or stability but abrogated T3S in a dominant negative manner. Proteins associated with YscD were identified by affinity purification coupled to liquid chromatography and mass spectrometry. Wild-type YscD was found to associate with YscJ (38, 51) and the effector YopH (48). Notably, the L3 and L4 substitution mutants of YscD interacted with more YopH than did wild-type YscD. In addition, the specific chaperone of YopH, SycH (56), was found associated with the substitution mutants but not wild-type YscD, as were the ruler component YscP (27) and the putative C-ring component YscQ (10, 18). These results indicate that the L3 and L4 loops are functionally essential. They also suggest that substitutions of these loops disrupted the dissociation of SycH from YopH, leading to the accumulation of a large protein complex that stalled the T3S system.

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MATERIALS AND METHODS

Cloning and expression. The coding sequence of *yscD* was amplified by PCR from the pYV plasmid of *Yersinia pseudotuberculosis* 126 (8). A PCR fragment encoding intact YscD was cloned into the pBAD expression vector (Invitrogen) to yield pBAD-yscD; this construct also included an N-terminal His tag. A second PCR product encoding YscDc (residues 1 to 121) was cloned into the pET28b expression vector (Novagen), yielding pET28b-yscDc. This construct included an N-terminal His tag followed by a PreScission protease cleavage site. Mutants of YscD were constructed by strand overlap extension PCR (22). The integrity of all constructs was verified by DNA sequencing.

Purification of YscDc. YscDc was expressed from pET28b-yscDc in *Escherichia coli* BL21 (DE3). Bacteria were grown at 37°C in lysogeny broth (LB) medium supplemented with 50 mg/liter kanamycin to an optical density at 600 nm (OD_{600}) of 0.5, at which point expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside. Bacteria were then grown for 16 h at 20°C, after which point they were harvested by centrifugation ($5,800 \times g$, 10 min, 4°C). The bacterial pellet was resuspended in 1/100 volume of the original bacterial culture in buffer A (500 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0, 10 mM β -mercaptoethanol [β ME]) supplemented with EDTA-free protease cocktail inhibitor (one tablet per 2 liters of original bacterial culture; Roche) and 20 μ g/ml DNase (Sigma). Resuspended bacteria were lysed using an Emulsiflex-C5 (Avestin) homogenizer with three passes at 15,000 lb/in², and the lysate was clarified by centrifugation ($14,000 \times g$, 10 min, 4°C). The supernatant was applied to a Ni²⁺-nitrilotriacetic acid (NTA) agarose column (Sigma); the column was washed with 25 column volumes of buffer A containing 7 mM imidazole, and bound protein was eluted from the column with three column volumes of buffer A containing 500 mM imidazole. The eluted fractions were concentrated by ultrafiltration using a YM-3 Centricon (Amicon) and further purified by size exclusion chromatography (16/60 Superdex 200; GE Healthcare) in buffer B (50 mM NaCl, 10 mM HEPES, pH 7.5, and 10 mM β ME). Purified YscDc was cleaved with a 50:1 YscDc/PreScission protease mass ratio in buffer B to remove the His tag, and the cleaved sample was reappplied to a Ni²⁺-NTA agarose column in buffer B. PreScission protease carried a His tag and was thus bound to the Ni²⁺-NTA column. Cleaved YscDc was isolated from the flowthrough of the column and concentrated by ultrafiltration using a YM-3 Centricon to 7.5 mg/ml; the concentration of YscDc was determined using a calculated ϵ_{280} of 12,490 M⁻¹ cm⁻¹. In certain cases, the order of steps was varied, such that the size exclusion chromatography step was carried out as the final step, following elution from the Ni²⁺-NTA agarose column, cleavage with PreScission protease, reapplication to the Ni²⁺-NTA agarose column, and isolation of the flowthrough from this column. No difference in purity was observed by this alteration in the order of steps. Selenomethionine (SeMet) was incorporated into YscDc as described previously (55), and SeMet-labeled YscDc was purified as described above.

Crystallization, data collection, and structure determination. Crystals of unlabeled and SeMet-labeled YscDc were grown by the sitting-drop, vapor diffusion method at 20°C by mixing 0.2 μ l of 7.5 mg/ml YscDc in buffer B with 0.3 μ l of 3.5 M NaHCOO. The drop was dispensed by an Oryx8 crystallization robot into a CrystalClearDuo crystallization plate (Hampton), which contained 80 μ l of 3.5 M NaHCOO in the well. Crystals were cryoprotected in 15% glycerol and 3.5 M NaHCOO, mounted in 50- μ m loops (Hampton), and flash cooled in liquid N₂. Native diffraction data were collected from YscDc crystals and single-wavelength anomalous dispersion (SAD) diffraction data from SeMet-labeled YscDc crystals at Beamline 23 ID-B (Advanced Photon Source, Argonne National Laboratory) (see Table S1 in the supplemental material).

Data from native and Se-Met crystals were indexed and scaled using HKL2000 (42). Two molecules of YscDc were located in the asymmetric unit. Four SeMet sites (Met1 and Met50 from each chain) were located using the hybrid substructure search (HYSS) in the program Autosol of the Phenix suite, and phases were calculated and refined using Phenix (1). Automated model building was carried out using Phenix Autobuild,

which produced a chain trace of residues 1 to 105. The model was then refined using the native data set. A random 5% of reflections were omitted from the refinement for calculation of a free residual factor (R_{free}). The model was adjusted manually based on inspection in Coot of σ_A -weighted 2mFo-DFc and mFo-DFc maps prior to subsequent cycles of refinement (17). Each refinement cycle consisted of three rounds of bulk-solvent correction, anisotropic scaling, and refinement of atomic model parameters; default parameters with tight NCS restraints set at 0.2 were used. Waters were added in the later stages of refinement using PhenixRefine or were manually added to $\geq 3\sigma$ Fo-Fc density. The electron density for the main chain was unbroken and modeled from residues 1 to 108. The electron density of all side chains was visible, except for Glu54 in chains A and B. Structure validation was carried out using Molprobit (see Table S1 in the supplemental material) (11).

Molecular figures were made with PyMol (<http://pymol.sourceforge.net>). Structure-based sequence alignments were generated using Ex-presso (16) and displayed using ESPript (20). The electrostatic surface potential was calculated with the Adaptive Poisson-Boltzmann Solver (5) in PyMOL. Structural superpositions were calculated using FATCAT (57) or Dali (25).

Generation of *Y. pseudotuberculosis* (Δ yscD). The entirety of *yscD*, except for 15 bp at the 3' end, was substituted in frame with *aph* (kanamycin resistance) by homologous recombination with a PCR fragment (14). The 15 bp at the 3' end of *yscD* were left intact as they contain a predicted ribosome-binding site for *yscE*. The PCR fragment contained 500 bp of the pYV sequence upstream of *yscD*, followed by *aph*, the terminal 15 bp of *yscD*, and 500 bp of the pYV sequence downstream of *yscD*.

Six hundred nanograms of this fragment was transformed by electroporation into competent *Y. pseudotuberculosis* harboring the plasmid pWL204 (12, 33). Electroporated bacteria were grown in brain heart infusion (BHI) medium at 26°C for 2 h and centrifuged ($3,000 \times g$, 5 min, 25°C), and the pellet was resuspended in BHI supplemented with 3 mM CaCl₂ and 50 mg/liter kanamycin. Bacteria were grown further for 2 h at 26°C under these conditions and were transferred to BHI agar plates containing 3 mM CaCl₂ and 50 mg/liter kanamycin. Plates were grown overnight at 26°C. The integrity of the allelic replacement was verified by sequencing a PCR fragment resulting from primers that anneal 650 bp upstream and downstream of the *yscD* locus. To select for loss of the pWL204 plasmid, bacteria were grown on agar plates as described above but supplemented with 2% sucrose. The loss of pWL204 was confirmed by sensitivity to ampicillin. *Y. pseudotuberculosis* (Δ yscD) was complemented with wild-type or mutant *yscD* expressed from the pBAD plasmid, as described above.

Secretion assay. *Y. pseudotuberculosis* was grown overnight at 28°C in BHI medium with appropriate antibiotics and 2.5 mM CaCl₂. The overnight culture was diluted to an OD_{600} of 0.1 in 10 ml of BHI containing 10 mM EGTA, 10 mM MgCl₂, and appropriate antibiotics. In the case of *Y. pseudotuberculosis* harboring pBAD-yscD, cultures were supplemented with 0.002% arabinose once they reached an OD_{600} of 0.6 and shifted to 37°C once they reached an OD_{600} of 1.0; 0.002% arabinose was subsequently added every 4 h during growth to account for the metabolic depletion of arabinose. Cultures were grown an additional 4 h.

To visualize secreted proteins, the bacterial supernatant was filtered (0.22- μ m-pore-size filter; Millipore) and then precipitated overnight by the addition of 10% trichloroacetic acid (TCA). Precipitated samples were centrifuged ($5,000 \times g$, 5 min, 4°C), and the pellet was washed with ice-cold acetone. Pelleted proteins were air dried, resuspended in SDS-PAGE sample buffer supplemented with 40 mM NaOH, boiled for 5 min, and separated by SDS-PAGE.

For Western blot analysis of expressed proteins, 3 ml of bacterial culture at an OD_{600} of 1.0 was harvested by centrifugation ($3,800 \times g$, 5 min, 4°C). Pelleted bacteria were resuspended in 30 μ l SDS-PAGE sample buffer and lysed by boiling for 5 min. The sample was applied to SDS-PAGE for separation of proteins, and the gel was transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at

room temperature in 5% bovine serum albumin (BSA) (Sigma-Aldrich) in 150 mM NaCl, 50 mM Tris, pH 8.0, and 0.5% Tween 20 (TBST), washed once with TBST, and incubated for 16 h at 4°C with primary antibody (1:500 rabbit anti-YscD polyclonal antibodies) in 5% BSA in TBST. The primary antibodies were generated in rabbits using YscDc as an antigen (Cocalico Biologicals) and were purified by affinity chromatography using YscDc affixed to a solid matrix. Three washes of the membrane in TBST (each 15 min in duration) were carried out, and the membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibodies (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA in TBST for 30 min. Following three washes with TBST (each 20 min in duration), the membrane was developed using SuperSignal West Pico chemiluminescent substrate (Pierce) and visualized with a Bio-Rad ChemiDoc imaging system.

As a loading control, RpoA was detected on the same membrane that had been probed for YscD. After probing for YscD as described above, the membrane was stripped by incubation in 67% (wt/vol) guanidine HCl, 50 μ M EDTA, 50 mM glycine, pH 10.8, 2.5 mM KCl, and 1.4 mM β ME for 10 min. The membrane was then blocked for 1 h at room temperature in TBST containing 5% BSA, washed once with TBST, and incubated for 1 h at 25°C with primary antibody (1:1,000 mouse anti-RpoA monoclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST containing 5% BSA. Three washes of the membrane in TBST (each 10 min in duration) were carried out, and the membrane was then incubated with HRP-conjugated secondary anti-mouse antibodies (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST containing 5% BSA for 30 min. Following three washes with TBST (each 10 min in duration), the membrane was developed and visualized as described above.

CD. Circular dichroism (CD) spectra were collected for wild-type and mutant YscDc at 10 μ M in 100 mM NaF, 10 mM potassium phosphate buffer, pH 7.6, on an Aviv 202 circular dichroism spectrometer using a 1-mm-path-length cuvette (holding 350 μ l) (Hellma). For each sample, the average of three independent spectra recorded from 195 nm to 260 nm at 4°C and 37°C is reported. The scans were performed with 1-nm steps, and the CD signal at each wavelength was averaged for 5 s.

Identification of binding partners of YscD. Wild-type YscD and the L3 and L4 mutants were expressed from pBAD-yScD in *Y. pseudotuberculosis* (Δ yscD). Bacteria were grown as described above for the secretion assay, except that the medium was BHI with appropriate antibiotics and 2.5 mM CaCl_2 throughout to suppress secretion. After 4 h growth of the culture at 37°C, bacteria were harvested by centrifugation ($5,800 \times g$, 10 min, 4°C). The bacterial pellet was resuspended in 1/10 volume of the original bacterial culture in buffer A (100 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0, 10 mM β ME) supplemented with EDTA-free protease cocktail inhibitor (Roche) (one tablet per 250 ml of original bacterial culture). Resuspended bacteria were lysed using an Emulsiflex-C5 (Avestin) homogenizer with three passes at 15,000 lb/in², and the lysate was clarified by centrifugation ($14,000 \times g$, 10 min, 4°C). Bacterial membranes were pelleted by ultracentrifugation ($95,000 \times g$, 4 h, 4°C), and solubilized in 1/100 volume of the original bacterial culture in buffer A containing 20 mM lauryldimethylamine oxide (LDAO). Forty percent of this sample was incubated with Ni^{2+} -NTA agarose beads (0.5 ml per ml of sample). The beads were washed with 10 bead volumes of buffer A containing 7 mM imidazole and 5 mM LDAO and then washed five times with two bead volumes of buffer A alone to remove LDAO.

The protein-bead complexes were prepared for mass spectrometry as described previously (21). They were diluted in TNE (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA) buffer. RapiGest SF reagent (Waters Corp.) was added to the mix to a final concentration of 0.1%, and samples were boiled for 5 min. Tris (2-carboxyethyl) phosphine (TCEP) was added to a 1 mM final concentration, and the samples were incubated at 37°C for 30 min. The samples were then carboxymethylated with 0.5 mg/ml of iodoacetamide for 30 min at 37°C, followed by neutralization with a 2 mM final concentration of TCEP. Samples were digested with trypsin (1:50 trypsin/protein mass ratio) overnight at 37°C. RapiGest was degraded and

removed by treating the samples with 250 mM HCl at 37°C for 1 h, followed by centrifugation ($15,800 \times g$, 30 min, 4°C). The soluble fraction was then added to a new tube, and the peptides were extracted and desalted using Aspire RP30 desalting columns (Thermo Scientific).

Trypsin-digested peptides were analyzed by high-pressure liquid chromatography (HPLC) coupled with tandem mass spectroscopy (LC/MS-MS) using nanospray ionization, as previously described (36) except for the following changes. The nanospray ionization experiments were performed using a QSTAR-Elite hybrid mass spectrometer (ABSCIEX) interfaced with nanoscale reversed-phase HPLC (Tempo) using a 10-cm-by-180- μ m (inside diameter) glass capillary packed with 5- μ m C_{18} Zorbax beads (Agilent Technologies, Santa Clara, CA). Peptides were eluted from the C_{18} column into the mass spectrometer using a linear gradient (5 to 60%) of acetonitrile (ACN) at a flow rate of 400 μ l/min for 1 h. The buffers used to create the ACN gradient were buffer A (98% H_2O , 2% ACN, 0.2% formic acid, and 0.005% trifluoroacetic acid [TFA]) and buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). MS-MS data were acquired in a data-dependent manner in which the MS1 data were acquired at an m/z of 400 to 1,800 Da and the MS-MS data were acquired from an m/z of 50 to 2,000 Da. The collected data were analyzed using MASCOT (Matrix Sciences) and Protein Pilot 4.0 (ABSCIEX) for peptide identifications and Peakview (ABSCIEX) for peak integration and quantification. A peak area of $\sim 1.2 \times 10^3$ was estimated to be the detection limit. This estimate was based on integration of a peak near the noise level; inspection showed that an intensity of ~ 30 was at the noise level, and the integrated peak had an intensity of 38.

Protein structure accession number. The crystal structure and structure factors discussed above have been deposited in the Protein Data Bank (ID code 4D9V).

RESULTS

YscDc has an FHA fold. YscDc was overexpressed in *E. coli*, purified to homogeneity, and crystallized. The crystal structure of YscDc was determined by single-wavelength anomalous dispersion and refined to a 2.52-Å resolution limit (see Table S1 in the supplemental material). The structure revealed that YscDc has a typical FHA domain fold, characterized by a β -sandwich composed of 10 β -strands that form two sheets (Fig. 1). The structure agrees in detail with that recently reported by others (root mean square deviation [RMSD] 0.3 Å, 107 C α) (34). The two sheets, one of which is six-stranded ($\beta 2\beta 1\beta 10\beta 9\beta 7\beta 8$) and the other four-stranded ($\beta 4\beta 3\beta 5\beta 6$), pack against one another through hydrophobic side chains that form the hydrophobic core of the domain. All the strands are antiparallel, except for the short $\beta 4$ strand, which runs parallel to the $\beta 3$ strand. Along with the $\beta 4$ strand, the $\beta 7$, $\beta 8$, and $\beta 9$ strands are also especially short. The only helical portion of the domain consists of a short 3_{10} -helix between strands $\beta 1$ and $\beta 2$. The asymmetric unit of the crystal contains two copies of YscDc that are nearly identical (RMSD 0.25 Å, 108 C α) and related by approximate 2-fold symmetry. However, this association is unlikely to be biologically meaningful, as only ~ 560 Å² of total surface area are buried at the dimer interface. Consistent with this, YscDc was observed to run on a gel filtration column at the size indicative of a globular monomer (data not shown). The N-terminal residues of YscDc were well ordered, while the C-terminal residues (109 to 121), which connect to the transmembrane region of YscD, were unstructured. This suggests that the cytoplasmic domain of YscD is flexibly tethered to the inner membrane, which would enable freedom in interactions with cytoplasmically exposed T3S components.

FHA domains are found in a variety of proteins across multiple kingdoms. The structures most similar to YscDc are the FHA do-

FIG 1 YscDc has an FHA fold. (a) Structure of YscDc (residues 1 to 108) in ribbon representation, with β -strands in red, coils in gray, and helices in blue. Loops L3 and L4 are indicated. (b) Structural superposition of YscDc with closely related structurally characterized FHA domains. YscDc is in red, CT664 (RSCB accession code 3GQ5) purple, EmbR (2FEZ) cyan, Rv0020c (3PO8) green, KIF13 (3FM8) blue, and Ki67 (2AFF) orange. The proteins are shown as α traces. (c) Structure-based sequence alignment of the FHA domains shown in panel b. A red arrow denotes the position of the Ser in loop 4 that is conserved in phosphothreonine-binding FHA domains, and the blue circles denote core residues of YscDc. Secondary structure annotations at the top of the alignment are for YscDc.

main of *Chlamydia trachomatis* CT664 (RMSD 2.0 Å, 96 Cα), *Mycobacterium tuberculosis* EmBR (RMSD 2.2 Å, 93 Cα), *M. tuberculosis* Rv0020c (RMSD 2.0 Å, 92 Cα), *Homo sapiens* kinesin KIF13 (RMSD 2.1, 87 Cα), and *H. sapiens* Ki67 (RMSD 2.3 Å, 93 Cα) (Fig. 1b) (3, 9, 45, 54). However, YscDc has little sequence identity (average, ~18%) to these (Fig. 1c) or other structurally characterized FHA domain proteins. Indeed, the identities of the side chains that make up the hydrophobic core in YscDc are not conserved in these other FHA domain proteins. However, there are several residues that are absolutely conserved in this set of proteins. These include Gly27 and His47 (Fig. 1c): Gly27 initiates loop 3 (L3, the loop connecting the β3 and β4 strands), and His46, which faces inwards to make hydrogen bonds with main chain

atoms of YscDc, terminates loop 4 (L4). L3 and L4 along with loop 6 have been shown to be crucial for target recognition in FHA domain proteins (3, 7, 26, 31).

YscDc lacks phosphothreonine-binding characteristics. Most but not all FHA domains specifically bind phosphothreonine residues. The interaction with phosphothreonine occurs primarily through a serine located on L4 that is three residues upstream of the conserved His (Fig. 1c, red arrow) and secondarily through an arginine found in either L3 or L4. YscDc lacks the conserved Ser on L4 (in its place is Ala43) and has no arginine residues on either L3 or L4. Furthermore, in FHA domains that bind phosphothreonine residues, the surfaces of L3 and L4 are positively charged, while in YscDc the equivalent surface is nega-

tively charged (Fig. 2a). These pieces of evidence suggest that YscDc is unlikely to bind proteins in a phosphorylation-dependent manner. L3 and L4, however, may still be involved in protein-protein interactions but in a phosphorylation-independent manner, as has been observed for the FHA domain of *H. sapiens* KIF13. L3 and L4 of KIF13, which lack the conserved Ser and Arg (Fig. 1c) and have an uncharged electrostatic surface, have been observed to confer binding to the protein CENTA1 in a phosphorylation-independent manner (54).

The FHA domain of YscD has significant sequence similarity to orthologs within its own Ysc subfamily of T3S systems (13). These include *Aeromonas hydrophila* AscD, *Pseudomonas aeruginosa* PscD, and *Photobacterium luminescens* SctD, whose predicted cytoplasmic domains have an average sequence identity of 44% with YscDc (Fig. 2b). The hydrophobic core of YscDc (e.g., Leu49, Val51, Ile56, Leu58) is well conserved among these Ysc subfamily members, indicative of the likely presence of FHA domains in these orthologs. Like YscD, these close family members are missing the Ser and Arg associated with phosphothreonine binding, and thus the weight of evidence suggests that these Ysc subfamily orthologs are likely to interact with their partners in a phosphorylation-independent manner as well.

YscDc has more distant but recognizable sequence identity to the cytoplasmic domains of the well-studied YscD orthologs of the SPI-1 (*Salmonella enterica* PrgH, 17% identity) and SPI-2 (*Escherichia coli* EscD, 24% identity) subfamilies of the T3S system (Fig. 2c) (28, 30, 41, 50, 53). This includes *Shigella flexneri* MxiG, a member of the SPI-1 subfamily. The structure of the cytoplasmic domain of MxiG (called here MxiGc) was recently shown by NMR and X-ray crystallographic techniques to consist of an FHA domain (6, 37). YscDc and MxiGc have 13% sequence identity and an RMSD of 3.08 Å between their two structures (86 Cα) (Fig. 2d). MxiG lacks the conserved His but has a Ser (S61) and Arg (R39) (Fig. 2c, red arrowheads) that have been identified to be important for binding a threonine-phosphorylated peptide that corresponds to a sequence from the putative C-ring protein Spa33 (6, 39). However, a different study using NMR titration reported no interaction between MxiGc and up to 50 mM phosphothreonine (37).

Finally, it is worth noting that the sequences of YscD L3 and L4 are highly similar or nearly identical among orthologs in the Ysc subfamily but quite dissimilar from PrgH, MxiG, and EscD (Fig. 2b and c). Consistent with this, L3 and L4 diverge the most structurally between YscDc and MxiGc (Fig. 2d). Collectively, these observations suggest that interactions between YscDc and its partners are likely to occur at the L3 and L4 region and, further, that these interactions are likely to be conserved with members of the Ysc subfamily but not the SPI-1 or SPI-2 subfamilies.

L3 and L4 are functionally essential. To determine whether L3 and L4 are required for function, we replaced residues in these loops with alanines. Five sequential surface-exposed residues of L3 were replaced with alanines (Ser28, Asp29, Pro30, Leu31, and Gln32), as were four sequential surface-exposed residues of L4 (Asp39, Ser40, Glu41, and Ile42) (Fig. 3a). In addition, single-site alanine substitution mutants were created in L4 at D39 and S40, and owing to the importance of serines in FHA domains for interactions, a single-site alanine substitution was also created at S38.

To assay the functional effects of these mutations, we created a strain of *Y. pseudotuberculosis* lacking *yscD*. As expected, wild-type *Y. pseudotuberculosis* secreted proteins in a low Ca²⁺-dependent

manner that is characteristic of T3S, whereas *Y. pseudotuberculosis* (Δ *yscD*) was deficient for T3S (Fig. 3b) (46). Type III secretion was restored by wild-type *yscD* (carrying an N-terminal His tag), whose expression from a plasmid was induced by arabinose (Fig. 3b), indicating that the deletion of *yscD* was nonpolar. The concentration of arabinose was empirically varied to mimic the level of expression of endogenous YscD, as determined by Western blotting using anti-YscD antibodies (Fig. 3b). The Western blot membrane was also probed with anti-RpoA antibodies, confirming the equal loading of samples. The alanine substitution mutants of YscD (carrying N-terminal His tags) were next introduced into *Y. pseudotuberculosis* (Δ *yscD*). Both the L3 and the L4 multiple alanine substitutions were produced at levels equivalent to those of wild-type YscD but were found to be deficient for T3S (Fig. 3b). The single-site D39A and S40A substitution mutant appeared to be slightly attenuated for T3S, while S38A behaved like wild-type YscD for T3S. These single-site mutants were also produced at levels equivalent to wild-type YscD.

Because the L3 and L4 substitutions are located on surface loops of YscD, it seemed unlikely that substitutions of these loops would affect protein structure or stability. Nevertheless, we examined the physical consequences of the L3 and L4 substitutions. The substitutions were introduced into YscDc, and these mutant proteins were produced in *E. coli*, purified, and subjected to analysis by circular dichroism (CD) (Fig. 3c). YscDc L3 and L4 were found to have CD spectra that were unchanged between 4°C and 37°C and similar to those of wild-type YscDc. Thus, we conclude that the alanine substitutions of L3 and L4 do not have deleterious effects on protein structure and stability.

Interactions of YscD with T3S components. To characterize the defects in T3S caused by the L3 and L4 alanine substitutions, we determined the identity of proteins that associate directly or indirectly with YscD. As described above, wild-type *yscD* and the L3 and L4 substitution mutants (carrying His tags) were expressed in *Y. pseudotuberculosis* (Δ *yscD*). Bacteria were grown under non-secreting conditions (i.e., high calcium concentration), and the membrane fraction was isolated from lysed bacteria and solubilized in detergent. Consistent with the observed stability of YscDc L3 and L4, both YscD L3 and L4 localized to the membrane fraction of *Y. pseudotuberculosis*, just as wild-type YscD did. YscD was captured from this fraction using Ni²⁺-nitrilotriacetic acid (NTA) agarose beads, and the identities of proteins copurifying with YscD were determined by high-pressure liquid chromatography coupled with tandem mass spectroscopy from tryptic peptides. We also subjected *Y. pseudotuberculosis* (Δ *yscD*) to the same analysis to distinguish between specific and nonspecific interactions. Measurements were normalized based on two peptides from EF-Tu that were common to all of the samples (see Table S2 in the supplemental material).

We found that wild-type YscD associated with YscJ, its expected partner, but also the effector protein YopH (48) (Fig. 4). No other associated proteins were identified for wild-type YscD (49). We expected the L3 and L4 substitution mutants to be defective for association but surprisingly found both substitution mutants to have enhanced interactions with YopH compared to wild-type YscD. In addition, the specific chaperone of YopH, SycH (56), associated with the L3 and L4 substitution mutants but not wild-type YscD. The lack of SycH was not due to the lower level of YopH associated with wild-type YscD. While the YscD L3 and L4 substitution mutants had ~4.7-fold more associated

FIG 2 YscDc and T3S orthologs. (a) Electrostatic potential of YscDc mapped to its surface. Red is negative (−10 kT), and blue is positive (+10 kT). (b) Structure-based sequence alignments of YscD and Ysc subfamily members AscD, PscD, and SctD. Secondary structure annotations at the top of the alignments are for YscDc. (c) Structure-based sequence alignments of YscD and T3S orthologs PrgH, EscD, and MxiG. Secondary structure annotations at the top of the alignments are for YscDc and those at the bottom for MxiG. Red arrowheads below MxiG denote MxiG R39 and S61. (d) Structural superposition of YscDc and the FHA domain of MxiG. YscDc is in red and MxiG (2XXS) in blue. The proteins are shown as Cα traces.

FIG 3 L3 and L4 mutants. (a) Positions of residues mutated in the L3 loops (blue) and L4 loops (green). YscDc is shown as a bond trace, with β -strands in red and loops in gray. (b) Top panel, type III secretion as detected by SDS-PAGE of culture supernatants. *Y. pseudotuberculosis* ($\Delta yscD$) was transformed with plasmids expressing wild-type YscD (pHis-YscD) and mutant YscD [pHis-YscD(L3), pHis-YscD(L4), pHis-YscD(S38A), pHis-YscD(S39A), pHis-YscD(S40A)] under the inducible control of arabinose. Yp is wild-type *Y. pseudotuberculosis*; +Ca²⁺ indicates the presence of high calcium concentration, which suppresses T3S; –arabinose indicates lack of arabinose induction. The sizes of molecular mass standards in kDa are indicated at left. Middle panel, level of intrabacterial YscD for each of the samples, as detected by an anti-YscD Western blot. Lower panel, level of intrabacterial RpoA as a loading control, as detected by anti-RpoA blot. (c) Circular dichroism spectra of wild-type YscDc, YscDc(L3), and YscDc(L4) at 4 and 37°C.

YopH than did wild-type YscD, they also had a level of SycH that was ~ 70 -fold higher than the detection limit of the experiment (estimated at $\sim 1.2 \times 10^3$ peak area). Thus, if SycH were associated with wild-type YscD (at the same level relative to YopH as for YscD L3 and L4), it would have been detectable (with an expected peak area of $\sim 1.8 \times 10^4$).

The L3 and L4 substitutions maintained association with YscJ. The continued presence of YscJ suggests that the L3 and L4 mutants are proficient in assembly, consistent with their maintenance of protein structure and stability. In addition, the putative C-ring component YscQ (10, 18) and the ruler component YscP (27) were observed to associate with the L3 and L4 substitution mutants but not wild-type YscD. These results indicate that the defect in secretion for the L3 and L4 substitution mutants was due to increased rather than decreased association between YscD and other T3S components.

The increased association of the L3 and L4 substitution mutants with T3S components raised the possibility that they may act as dominant negatives. To test this hypothesis, we expressed YscD

L3 and L4 as well as wild-type YscD from the pBAD plasmid in a wild-type strain of *Y. pseudotuberculosis*. An increase in the amount of YscD supplied by plasmid-encoded wild-type YscD had no significant effect on secretion (Fig. 5). However, expression of YscD L3 and especially YscD L4 was found to suppress secretion (Fig. 5). This dominant negative behavior suggests that YscD L3 and L4 assemble in the membrane alongside wild-type YscD and that their increased association with T3S components disables the function of wild-type YscD in secretion.

DISCUSSION

YscD and YscJ are predicted, based on analogy to the T3S systems of *Salmonella* and *Shigella* (23, 50), to form a ring-like structure that consists of ~ 24 copies of each protein in the inner bacterial membrane. We found that the cytoplasmic domain of YscD, YscDc, exists as a monomer, with no higher-order structures being evident, even at the high protein concentrations of its crystalline form. This suggests that other portions of YscD are likely to mediate potential oligomerization. A 66-residue region at the very

FIG 4 Proteins associated with YscD. Proteins found associated (indicated at top left of each plot) with wild-type YscD, $\Delta yscD$ (lacking YscD and thus a specificity control), and the L3 and L4 substitutions mutants of YscD. Peak heights of peptides corresponding to these proteins are plotted relative to an internal standard, as identified by mass spectrometry (see Table S2 in the supplemental material).

C-terminal periplasmic end of YscD appears to be the likely determinant (49). The 66-residue region interacts with the secretin YscC (49), a ring-shaped oligomer that appears to be the first component of the T3S apparatus to assemble (15, 29). Other portions of the YscD periplasmic region have been shown to be non-essential for T3S (49), namely, the predicted phospholipid binding (BON) and the so-called ring-building domains, as has the specific sequence of the transmembrane domain (49).

The structure of YscDc confirmed that the cytoplasmic domain

of YscD has an FHA fold (43, 44), as also has been demonstrated by others (34). The FHA fold is strongly but not exclusively associated with phosphothreonine binding (24, 35, 40, 54). While the details of phosphothreonine binding vary considerably among FHA domain proteins, most have in common the participation of a Ser that is three residues upstream of a conserved His on L4 and an Arg on L3 or L4. YscD has neither the Ser nor the Arg. Orthologs of YscD from the Ysc subgroup of the T3S system also lack the Ser and Arg associated with phosphothreonine binding (Fig. 2). Additionally, unlike the positively charged L3 and L4 of phosphothreonine-binding FHA domains, these loops in YscD are negatively charged. These observations suggest that YscD and its orthologs in the Ysc subfamily are unlikely to interact with partners in the phosphothreonine-dependent manner that is characteristic of FHA domains.

YscD has distant but recognizable sequence identity to orthologs in the SPI-1 and SPI-2 subfamilies. This includes the SPI-1 ortholog MxiG, whose cytoplasmic domain was shown to have an FHA fold (6, 37). L4 in MxiG lacks the conserved His but has two serines (S61 and S63), and L3 has an Arg (residue 39). The cytoplasmic domain of MxiG, MxiGc, was shown to bind a phosphothreonine-containing peptide whose sequence corresponds to the putative C-ring component Spa33. This interaction was seen to be phosphorylation dependent, and MxiG R39 and S61 were found to be important for this interaction (6). MxiG and Spa33 have been previously shown to interact (39), but whether Spa33 is phosphorylated *in vivo* remains uncertain. MxiG R39 and S61 were also shown to have a role in type III secretion and epithelial cell invasion (6). In contrast to these findings, a separate study

FIG 5 Dominant negative effect of YscD L3 and L4. Type III secretion detected by Coomassie-stained SDS-PAGE. The format is as in Fig. 3b, with wild-type *Y. pseudotuberculosis* being transformed with plasmids expressing wild-type YscD (pHis-YscD) and mutant YscD [pHis-YscD(L3), pHis-YscD(L4)] under the inducible control of arabinose.

found no evidence for interaction between 50 mM phosphothreonine and MxiGc, and an R39A/S61A/S63A triple alanine substitution mutant was observed to have no loss in type III secretion (37). Thus, whether MxiG binds its partners in a phosphothreonine-dependent manner is currently controversial. The SPI-1 and SPI-2 orthologs PrgH and EscD, respectively, lack the conserved His, and while PrgH has a Ser in predicted L4, neither has arginines in predicted L3 or L4. This suggests that PrgH and EscD are likely to bind their partners in a phosphorylation-independent manner. One T3S ortholog, *C. trachomatis* CT664 from the *Chlamydiales* subfamily of the T3S system (44), does have a typical phosphothreonine-binding motif in L3 and L4. CT664 has a Ser three residues upstream of the conserved His along with an Arg placed appropriately for phosphothreonine interaction (Fig. 1c). The crystal structure of CT664 contains a phosphate bound at these residues, but no direct evidence exists yet for phosphothreonine interaction by CT664 or other *Chlamydiales* subfamily members.

While L3 and L4 in YscDc are unlikely to partake in phosphothreonine-dependent interactions, these same loops have been found in the FHA domain protein KIF13 to participate in phosphorylation-independent interaction (54). To determine whether L3 and L4 in YscD are required for function, we created single and multiple alanine substitutions in these loops. We found that multiple alanine substitutions of either L3 or L4 abrogated T3S but had no deleterious effects on protein structure or stability, as assessed by CD. We sought to understand the basis for the functional defects of the L3 and L4 substitution mutants by characterizing the proteins associated with YscD.

We detected an interaction between wild-type YscD and YscJ, which was expected (49). The YscD L3 and L4 mutants also interacted with YscJ, providing evidence that these mutant proteins assemble in the membrane like wild-type YscD. This conclusion was further supported by the dominant negative behavior of the L3 and L4 substitution mutants. They suppressed secretion by wild-type YscD, consistent with coassembly of wild-type and mutant copies of YscD.

We also found an unexpected association between wild-type YscD and the effector YopH (48). No other effectors were observed to associate with YscD. The action of YopH in host cells is nearly immediate (4), and it is possible that the association between YopH and YscD provides YopH with precedence in the hierarchy of effector secretion. An even stronger association with YopH was observed for the YscD L3 and L4 substitution mutants. Notably, in addition to YopH, the YopH-specific chaperone SycH (56) was found associated with the YscD L3 and L4 substitution mutants but not with wild-type YscD.

It appears that dissociation of SycH from YopH is impaired in the YscD L3 and L4 mutants. This is unlikely to be a direct effect of YscD. Based on analogy to the *Salmonella* T3S (2), it is likely that the *Yersinia* T3S ATPase YscN is responsible for catalyzing the dissociation of SycH from YopH. Therefore, a plausible mechanism for the association of SycH with the YscD L3 and L4 substitution mutants is a defect in the recruitment of YscN by these mutant proteins. We did not observe an association between wild-type YscD and YscN, but this putative interaction is likely to be transient, breaking up after the release of SycH. Impaired dissociation of SycH from YopH would also provide an explanation for the pattern of additional T3S components associated with the YscD L3 and L4 substitution mutants. Among these components is YscQ, which forms the putative C ring (10). YscQ was found

associated with the L3 and L4 substitution mutants but not with wild-type YscD. YscQ may be recruited to YscD through SycH, as both the *Salmonella* and *Chlamydia* orthologs of YscQ, SpaO and CdsQ, respectively, are known to bind T3S chaperones (32, 52). The likely oligomeric nature of YscQ may provide the means by which additional copies of SycH-YopH associate with the YscD L3 and L4 mutants, explaining the increased quantity of YopH associated with these mutants. In addition, the ruler component YscP (27) was also found associated with the L3 and L4 substitution mutants but not with wild-type YscD. This interaction may also occur through YscQ, as YscP has been reported to interact with YscQ (47). The overall picture that emerges is the presence of a large protein complex that is stably associated with the YscD L3 and L4 substitution mutants but missing for wild-type YscD. This protein complex appears to stall the T3S system and prevent it from proceeding through the sequential steps required for protein export.

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