Functionally essential interaction between Yersinia YscO and the T3S4 domain of YscP.

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The type III secretion (T3S) system is essential to the virulence of a large number of Gram-negative bacterial pathogens, including Yersinia. YscO is required for T3S in Yersinia and is known to interact with several other T3S proteins, including the chaperone SycD and the needle length regulator YscP. To define which interactions of YscO are required for T3S, we pursued model-guided mutagenesis: three conserved and surface-exposed regions of modeled YscO were targeted for multiple alanine substitutions. Most of the mutations abrogated T3S and did so in a recessive manner, consistent with a loss of function. Both functional and nonfunctional YscO mutant proteins interacted with SycD, indicating that the mutations had not affected protein stability. Likewise, both functional and nonfunctional versions of YscO were exclusively intrabacterial. Functional and nonfunctional versions of YscO were, however, distinguishable with respect to interaction with YscP. This interaction was observed only for wild-type YscO and a T3S-proficient mutant of YscO but not for the several T3S-deficient mutants of YscO. Evidence is presented that the YscO-YscP interaction is direct and that the type III secretion substrate specificity switch (T3S4) domain of YscP is sufficient for this interaction. These results provide evidence that the interaction of YscO with YscP, and in particular the T3S4 domain of YscP, is essential to type III secretion.

Yersinia YscO (154 residues, 19 kDa) is one such dually conserved protein (3). This small peripheral membrane-associated protein localizes to the inner bacterial membrane and the cytosol (3). YscO has also been reported to be secreted by Yersinia pestis and Yersinia enterocolitica (3, 4). While YscO is essential for type III secretion (3), the basis for its essentiality is unknown. Several Yersinia T3S proteins have been shown to interact with YscO. Among these is SycD (5), which is a chaperone for the T3S translocon proteins YopB and YopD (6). This interaction was detected through a copurification assay using Escherichia coli lysates containing SycD and a glutathione S-transferase (GST)–YscO fusion protein. The same experiment showed that SycT, a chaperone for the translocated protein YopT, also binds YscO, albeit with lower apparent affinity (5). The interactions of YscO with these T3S chaperones mirror interactions observed for FliJ (147 residues, 17 kDa), which is the flagellar ortholog of YscO as defined by synten and common predicted physical properties. FliJ associates with the flagellar chaperones FlgN and FlIT (7), and, notably, these associations occur only when these chaperones are free. These results suggest that FliJ may function by capturing released chaperones for further cycles of binding to cognate targets (7).

The Yersinia protein YscP also interacts with YscO (8). YscP acts as a molecular ruler and sets the precise length of the extracellular needlelike structure of the T3S apparatus (9). The interaction between YscP and YscO was detected in Y. enterocolitica using a YscP-GST fusion protein that blocks the T3S system (8). Several proteins copurified with this blocking YscP-GST protein, including YscO and also YscN (the T3S ATPase) (10), YscL (the negative regulator of YscN) (11), and YscQ (the putative C-ring component) (12). Further evidence for a physical interaction between YscO and YscP comes from the observation that the inhibition of type III secretion due to overexpression of YscP can be relieved by overexpression of YscO (13). Additionally, the Chlamydia trachomatis ortholog of YscO, CT670, has been seen to interact with the C. trachomatis ortholog of YscP, CT671, as determined through bacterial two-hybrid and copurification assays in E. coli (14).

The structures of the YscO orthologs C. trachomatis CT670 (14) and Salmonella FliJ (15) have been determined and reveal simple α-helical coiled-coil hairpins. We used this information to model the structure of YscO and carry out model-guided mutagenesis. We identified three regions in the model that are conserved and surface exposed. Multiple alanine substitution mutagenesis was carried out in these regions, and most of the mutations were found to abrogate T3S. These mutations were recessive to the wild type, consistent with a loss of function. Both functional and nonfunctional YscO mutant proteins were found intrabacterially but not in the secreted fraction. This was also the case for wild-type YscO, in contrast to data reported for Y. pestis.
and Y. enterocolitica (3, 4). Likewise, both functional and non-functional YscO mutant proteins interacted with SycD, suggesting that the mutations had not affected the structure or stability of YscO. The functional and nonfunctional YscO mutant proteins were, however, distinguishable with respect to interaction with YscP. Only wild-type YscO and a T3S-proficient mutant of YscO interacted with YscP, while none of the T3S-deficient mutants of YscO did so. Evidence is presented that this interaction was direct and that the ~100 C-terminal residues of YscP, which form the type III secretion substrate specificity switch (T3S4) domain of YscO, were sufficient for this interaction. The YscP T3S4 domain is so named because its loss affects both the secretion of the early T3S substrate YscF, which polymerizes into the needlelike structure, so named because its loss affects both the secretion of the early T3S and later substrates, such as the Yop effector proteins (16). Over-all, our results provide evidence that the interaction of YscO with YscP, and in particular the T3S4 domain of YscO, is essential to type III secretion.

**MATERIALS AND METHODS**

**Generation of the YscO model.** A model of YscO was generated with Swiss-Model (17) using the YscO ortholog CT670 as a template (PDB accession no. 3K29) (14). The molecular model in Fig. 1 was generated with PyMol (http://www.pymol.org). Sequences were aligned with ClustalW2 (18) and depicted with EsPr ipt (19). COILS (20) was used to predict coiled-coil heptad positions.

**Construction of Yersinia pseudotuberculosis (YscO:aph).** Homologous recombination was used to replace yscO with aph, which confers resistance to kanamycin. A PCR fragment encoding 500 bp of the pYV sequence upstream of yscO, followed by aph, and 500 bp of the pYV sequence downstream of yscO was generated. The PCR product was co-electroporated with pWLV204 (21), which encodes the red recombinase genes and the levansucrase gene sacB (for sucrose counterselection), into competent Y. pseudotuberculosis. Bacteria were grown for 2 h in bovine heart infusion (BHI) medium, and transformants were selected by plating on BHI containing 2.5 mM CaCl₂, 50 µg/ml kanamycin, and 30 µg/ml ampicillin.

**Complementation of Y. pseudotuberculosis (YscO::aph).** Wild-type and mutant yscO alleles, which were generated by the QuikChange method (Agilent), were cloned into the arabinose-inducible pBAD vector. These constructs included an N-terminal Strep tag (WSHPQFEK) for detection. The resulting plasmids were electroporated into Y. pseudotuberculosis, which were grown at 26°C and then shifted to 37°C, at which time an additional 0.1% arabinose was included to induce the expression of the red recombinase genes. Transformants were plated on the above-described medium supplemented with 10% sucrose in order to select for the loss of pWLV204. This loss was confirmed by the sensitivity of colonies to 30 µg/ml ampicillin. The proper substitution of yscO by aph was confirmed by sequencing. Samples were resolved by a Coomassie-stained 12.5% SDS-PAGE gel.
Western blots. Samples were resolved by a 12.5 to 15% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were blocked with 20 ml of 5% milk in TBS (150 mM NaCl, 50 mM Tris [pH 8.0]) for 1 h at 26°C. Peroxidase-conjugated anti-His tag (Promega), anti-Flag antibody, or anti-6xHis antibody was applied to an Ni2+ column equilibrated with 1 mM isopropyl α-D-1-thiogalactopyranoside and 50 mM Tris (pH 8), 1 mM EDTA, and 5 mM β-ME, and lysed by sonication. The lysate was centrifuged (26°C, 20 min, 20,000 × g) to remove insoluble material. The supernatant was incubated at 4°C for 16 h with 1 ml Strept-Tactin beads (IBA) that had been preequilibrated with buffer A. The beads were then washed with 20 column volumes of buffer A, followed by centrifugation (26°C, 30 s, 3,000 × g). Strept-YscO was then eluted from the beads using buffer A containing 2.5 mM desthiobiotin. Eluted fractions containing YscO, as visualized on a Coomassie-stained 15% SDS-PAGE gel, were pooled and concentrated by ultrafiltration (YM-3K; Amicon).

Binding assays. Y. pseudotuberculosis (YscO::aph) complemented with various alleles of YscO was grown at 28°C to an OD600 of 0.6, induced with 0.1% arabinose, and grown further for 4 h. Bacteria were then harvested by centrifugation (4°C, 15 min, 3,000 × g), solubilized in buffer A (300 mM NaCl, 50 mM Tris [pH 8], 1 mM EDTA, and 5 mM β-ME), and lysed by sonication. The lysate was centrifuged (4°C, 20 min, 20,000 × g) to remove insoluble material. The supernatant was incubated at 4°C for 1 h with 100 μl Strept-Tactin beads (IBA) that had been preequilibrated with buffer A. The beads were then washed 2 to 3 times with 200 μl of buffer A, each with a centrifugation step (26°C, 30 s, 3,000 × g) between each wash. His-SycD (3 μM), YscP-His (1 μM), or His-YscP T3S4 (5 μM) was incubated with the beads for 20 μl of buffer A supplemented with 0.1% Triton X-100 for 1 h at 4°C, followed by five washes of the beads with the same buffer; each wash was followed by a centrifugation step (26°C, 30 s, 3,000 × g). The beads were then boiled in 2 × SDS-PAGE sample buffer, and the samples were analyzed by Western blotting.

For the YscO-YscP binding assay using purified components, YscP-His was incubated at 4°C with 50 μl Ni2+-NTA beads (Sigma) for 1 h, which had been equilibrated with binding buffer (300 mM NaCl, 50 mM sodium phosphate [pH 8], 10 mM imidazole, and 10 mM β-ME). Strept-YscO was incubated with the resin in 100 μl binding buffer for 1 h at 4°C, followed by five washes of 20 μl each of the beads with the same buffer; each wash was followed by a centrifugation step (26°C, 30 s, 3,000 × g). The eluted fraction, along with other fractions, was visualized on a Coomassie-stained 15% SDS-PAGE gel.

RESULTS
Mutagenesis of conserved surface-exposed patches. Sequence and structural analyses indicate that YscO is likely to form an α-helical coiled-coil hairpin structure that closely resembles the structures of the YscO orthologs C. trachomatis CT670 (14) and Salmonella Filj (15). Based on this, we generated a structural model for YscO using Swiss-Model (17) and identified residues that are predicted to be solvent exposed and are also conserved within the YscO family of proteins (Fig. 1). We targeted three conserved solvent-exposed patches, called highly conserved regions 1 (HCR1), 2, and 3, for alanine substitutions. HCR1 and -3 flank one another on the coiled coil and are distal to HCR2, which lies at the predicted hairpin turn. Four residues were substituted with alanines at each of the HCRs: Arg12 (predicted to occupy the g position of the heptad repeat), Arg15 (ε), Glu17 (ε), and Lys18 (f) for HCR1; Glu73 (ε), Arg74 (f), Arg80 (ε), and Glu81 (f) for HCR2; and Arg115 (b), Lys120 (g), Phe121 (c), and Leu124 (f) for HCR3. For HCR1, we also constructed a mutant having only the last three residues substituted with alanines (i.e., but Arg12); this mutant was designated HCR1.3.

Type III secretion. To determine if these mutations had affected T3S, we first created a Y. pseudotuberculosis strain lacking
yscO through replacement of this gene with *aph*. Consistent with prior results, the deletion of *yscO* led to loss of secretion (3) (Fig. 2a). By comparison, wild-type *Y. pseudotuberculosis* secreted proteins in a calcium-dependent manner (i.e., at a low but not high calcium concentration), which is diagnostic of T3S in *Yersinia*. The deletion of *yscO* was found to be nonpolar, as *yscO* expressed inducibly from the *araC* promoter of a pBAD plasmid rescued T3S (Fig. 2a). This was shown to be a specific effect, as T3S was not rescued in the absence of arabinose induction of *yscO*. The induced expression of *YscO* was verified through Western blot detection of a Strep tag included with pBAD-borne *yscO*. The α subunit of RNA polymerase (RpoA) was used as a loading control for this experiment, as its expression is independent of the T3S. The *yscO* HCR mutants were introduced into *Y. pseudotuberculosis* (*ΔyscO:aph*) on the pBAD plasmid, and their ability to complement T3S was evaluated. While *yscO* HCR1.3 was fully functional in rescuing T3S, none of the other mutants restored function. Each of the substitution mutants produced quantities of YscO equivalent to that of pBAD-borne wild-type YscO, as detected by Western blotting. These results indicate that the defects in T3S of the *yscO* HCR1, -2, and -3 substitution mutants are not due to changes in expression level. It is worth noting that the functional HCR1.3 substitution mutant differs from the nonfunctional HCR1.3 substitution mutant by a single residue, Arg12. These results thus highlight a critical role for Arg12 in T3S function, along with the surface patches encompassed by HCR2 and -3.

We further characterized the loss of T3S in *yscO* HCR1, -2, and -3 by determining whether these were dominant or recessive with respect to endogenous, wild-type *yscO*. The *yscO* HCR mutants were inducibly expressed in wild-type *Y. pseudotuberculosis*, and their effect on T3S was evaluated. We found that the *yscO* HCR mutants were recessive, as T3S was maintained for all the mutants (Fig. 2b). These data are consistent with the substitutions in HCR1, -2, and -3 resulting in a loss of function.

YscO has been reported to be secreted by *Y. pestis* and *Y. enterocolitica* (3, 4). We wondered whether the loss of T3S in *yscO* HCR1, -2, and -3 resulted from a defect in YscO secretion. Since these mutations led to a loss of secretion, we expressed them in wild-type *Y. pseudotuberculosis* and asked whether the mutant YscO proteins were present in the type III secreted fraction, as detected via their Strep tag. Surprisingly, while the mutant YscO proteins were evident in the intrabacterial fraction, they were not evident in the secreted fraction (Fig. 3). This included both functional (i.e., YscO HCR1.3) and nonfunctional (i.e., YscO HCR1, -2, and -3) mutants. Type III secretion was confirmed to have occurred in these strains, as YopE was detected in the secreted fraction by Western blotting using anti-YopE antibodies. As expected, YopE was also found in the intrabacterial fraction.

Due to the discrepancy between these results showing that YscO is not secreted and prior results showing that it is (3, 4), we next examined secretion of endogenous YscO by *Y. pseudotuberculosis*. For this, we used polyclonal anti-YscO antibodies, which had been used previously for detection of *Y. enterocolitica* YscO (4). Strep-tagged YscO purified from *Y. pseudotuberculosis* was used as a positive control for these experiments. The polyclonal anti-YscO antibodies were cross-reactive, but we were able to detect YscO in the intrabacterial fraction at its correct molecular mass; this protein was not present in *Y. pestis* or *Y. pseudotuberculosis*.

We further characterized the loss of T3S in *yscO* HCR1, -2, and -3 by determining whether these were dominant or recessive with respect to endogenous, wild-type *yscO*. The *yscO* HCR mutants were inducibly expressed in wild-type *Y. pseudotuberculosis*, and their effect on T3S was evaluated. We found that the *yscO* HCR mutants were recessive, as T3S was maintained for all the mutants (Fig. 2b). These data are consistent with the substitutions in HCR1, -2, and -3 resulting in a loss of function.
Interaction with SycD. To identify the mechanistic basis for the defects in HCR1, -2, and -3, we examined interactions between YscO and the chaperone SycD. YscO produced recombinantly in E. coli was found to form insoluble inclusion bodies that were recalcitrant to refolding (data not shown). Therefore, YscO was obtained from Y. pseudotuberculosis for this experiment. We applied lysates of Y. pseudotuberculosis that had been induced for expression of Strep-tagged YscO to Strep-Tactin beads, which were then washed to remove nonspecifically bound proteins. To these beads, we then added His-tagged SycD that had been expressed in E. coli and purified by metal chelation chromatography. The Strep-Tactin beads were once again washed to eliminate nonspecific binding, and proteins remaining bound to the beads were coprecipitated and visualized by Western blotting. We probed the Western blot membrane simultaneously with anti-His and anti-Strep tag antibodies to visualize both YscO and SycD. In agreement with a previous report (5), we found that wild-type YscO interacted with SycD (Fig. 4). This was a specific effect, as no binding of His-tagged SycD to the Strep-Tactin beads occurred in the absence of Strep-tagged YscO being induced. Notably, all of the YscO substitution mutants, both functional (i.e., HCR1.3) and nonfunctional (i.e., HCR1, -2, and -3), were observed to bind SycD (Fig. 4). The maintenance of SycD interaction for these YscO alanine substitution mutants attests to the structural integrity and stability of these proteins. These results also indicate that the defects in T3S for the HCR1, -2, and -3 mutants are not attributable to interaction with SycD.

Interaction with YscP. We next examined the interaction of YscO with YscP, in the same manner as described above using His-tagged YscP which had been expressed in E. coli and purified. We found that YscP was sensitive to proteolytic degradation, resulting in two to three prominent degradation products along with intact YscP, as detected by Western blotting (Fig. 5). Intact YscP and all its visible degradation products were found to interact with wild-type YscO (Fig. 5). Most important, while the functional HCR1.3 substitution mutant interacted with YscP, the nonfunctional HCR1, -2, and -3 substitution mutants did not interact with YscP. These results indicate that the defects in T3S for the
HCR1, -2, and -3 mutants are attributable to the loss of interaction with YscP. Since these experiments were carried out with YscP produced in *E. coli*, we turned to *Y. pseudotuberculosis* to verify the interaction between YscO and YscP. We induced expression of pBAD-encoded wild-type YscO or nonfunctional YscO HCR1 (right) in *Y. pseudotuberculosis* and carried out a coprecipitation assay using Strep-Tactin beads (Fig. 6a). We probed for the presence of YscP in the coprecipitated fraction using anti-YscP antibodies. YscP was observed to interact with wild-type YscO but not YscO HCR1, confirming the results presented above.

We next asked whether the interaction between YscO and YscP was direct. For this, sufficient quantities of *Y. pseudotuberculosis* Strep-YscO were purified to be visualized on a Coomassie-stained SDS-PAGE gel. To this was added purified YscP-His produced in *E. coli*, and the interaction between these two proteins was assayed by an Ni²⁺-NTA coprecipitation assay (Fig. 6b). While there were some impurities (and truncation products in the case of YscP) that copurified with YscO and YscP, these two proteins were the dominant species and found to interact specifically. This result provides strong evidence that the interaction between YscO and YscP is direct.

As the data in Fig. 5 showed that YscO bound the smallest YscP degradation product visible, we asked whether there were domains of YscP that were sufficient for interaction with YscO. Since the His tag on YscP was at its C terminus, we concentrated on the HCR1, -2, and -3 mutants are attributable to the loss of interaction with YscP.

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As the data in Fig. 5 showed that YscO bound the smallest YscP degradation product visible, we asked whether there were domains of YscP that were sufficient for interaction with YscO. Since the His tag on YscP was at its C terminus, we concentrated on the
C-terminal T3S4 domain of YscP (residues 341 to 440). As described above, the YscP T3S4 domain was expressed as a His-tagged protein in E. coli, purified, and incubated with lysates of Y. pseudotuberculosis. Similarly to intact YscP, only wild-type YscO and the functional HCR1.3 mutant interacted with the YscP TS34 domain, while the nonfunctional HCR1, -2, and -3 mutants showed no interaction (Fig. 7).

Collectively, these results indicate that the interaction between YscO and YscP is required for T3S and that the T3S4 domain of YscP is sufficient for this interaction.

DISCUSSION

We have used model-guided mutagenesis to characterize the interactions of YscO with SycD and YscP. YscO is predicted to have a simple α-helical coiled-coil hairpin structure, as seen in its orthologs Chlamydia CT670 (14) and Salmonella FliJ (15). The hairpin structure in FliJ has been noted to resemble part of the γ subunit of FoF1-ATP synthase (15). In accordance with this structural similarity, FliJ was shown to promote hexameric ring assembly of the flagellar ATPase FliI by inserting into the center of the FliI ring (15), just as the γ subunit does in the FoF1-ATP synthase. However, YscO is dissimilar from FliJ with respect to this function. YscO has been shown to be dispensable for the assembly of the Yersinia T3S ATPase YscN, as judged by the formation of fluorescent foci by an enhanced green fluorescent protein (EGFP)-YscN fusion protein in Y. enterocolitica regardless of the presence of YscO (4).

The structural model of YscO made it possible to identify residues that are likely to be conserved and exposed to the surface for interaction with binding partners. We identified three conserved and exposed regions (i.e., HCR1, -2, and -3) and subjected these to multiple alanine substitution mutagenesis. These regions, in the case of HCR1 and HCR3, were near the base of the hairpin or, in the case of HCR2, near the turn of the hairpin. Four alanine substitutions each were created in HCR1, -2, and -3. An additional mutant, HCR1.3, was constructed that contained only three of the four substitutions of HCR1. The HCR1.3 mutant was highly useful for comparison, as it was unaffected for type III secretion. In contrast, HCR1, -2, and -3 were defective for type III secretion. In all cases, equivalent levels of YscO were evident, indicating that the defects in HCR1, -2, and -3 were not due to changes in expression level. These three T3S-deficient mutants were recessive to wild-type yscO, consistent with a loss of function.

In contrast to prior reports that indicated that YscO is itself secreted by Y. pestis and enterocolitica (3, 4), we did not detect secretion of YscO by Y. pseudotuberculosis. This was the case even though we used the same antibodies as had been used for Y. enterocolitica YscO (98% sequence identity between Y. pseudotuberculosis and Y. enterocolitica YscO) (4). The basis for this difference is not clear at present. We note that no functional significance has been attributed to the secretion of YscO, but the more important point for this study is that the functional and nonfunctional versions of YscO were indistinguishable with respect to YscO secretion.

We examined whether the T3S defects of the YscO HCR mutants were attributable to defects in interactions of YscO with SycD and YscP. We found that all the YscO mutants bound recombinant SycD, regardless of whether these mutants were competent for type III secretion. The maintenance of interaction with SycD suggests that the alanine substitutions affected neither the structure nor stability of YscO. These results also suggest that portions of YscO outside HCR1, -2, and -3 are responsible for binding SycD. Because all the YscO mutants bind SycD, these data do not address whether the interaction between YscO and SycD is functionally important; this issue awaits further experimentation.

In contrast to the results with SycD, we found that interactions of YscO with YscP strictly correlated with type III secretion. YscP was prone to proteolytic digestion during purification and that the smallest visible fragment of C-terminally His-tagged YscP bound YscO. This suggested that...
the C-terminal T3S4 domain of YscP may be sufficient to bind YscO. We carried out interaction experiments with recombinant YscP T3S4 and verified that the YscP T3S4 domain is sufficient to interact with YscO. YscO Arg12 appears to be critical to this interaction, as this residue is the only difference between YscO HCR1.3, which binds the YscP T3S4 domain, and HCR1, which does not.

What role might the interaction between YscO and YscP play in type III secretion? One possibility is suggested by the observation that YscO interacts with YscU, a transmembrane protein that is involved in controlling the substrate specificity of type III secretion (22, 23). YscO was found to copurify in Y. enterocolitica with a GST fusion to the cytoplasmic domain of YscU (24). The cytoplasmic domain of YscU undergoes autoproteolytic cleavage (24, 25), and the interaction with YscO was best in a mutant of the C-terminal domain of YscU (G270N) that is incapable of undergoing this cleavage. This suggests that YscO associates with YscU prior to its autocleavage. As noted above, YscP is also involved in controlling substrate specificity (23). While an interaction between YscP and YscU has not been observed, it is noteworthy that the phenotype resulting from loss of yscP is suppressed by a mutation in the cytoplasmic domain of YscU (23). Thus, these results along with our observations raise the possibility that YscO functions as a bridge between the secretion specificity controlling proteins YscP and YscU. With the evidence presented here that the interaction between YscO and the YscP T3S4 domain is essential to type III secretion, this and other possibilities can be tested in greater detail.

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