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Authors

Mukerjea, Romila

Ghosh, Partho

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Functionally Essential Interaction between *Yersinia* YscO and the T3S4 Domain of YscP

Romila Mukerjea, Partho Ghosh

Department of Chemistry & Biochemistry, University of California, San Diego, La Jolla, California, USA

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.

Gram-negative bacterial pathogens utilize a variety of mechanisms to survive and reproduce within their hosts. One of the most common involves the translocation of specific bacterial proteins directly from the bacterial cytosol into the cytosol of host cells by means of the type III secretion (T3S) system (1, 2). Such transported proteins generally have deleterious and, in many cases, toxic effects on host cells. The T3S system is conserved in *Salmonella*, *Shigella*, and *Yersinia* spp. among other Gram-negative bacteria and is related evolutionarily to the bacterial flagellum (1, 2). Of the ~20 to 25 proteins that comprise the T3S system, ~8 to 10 of these are dually conserved in the T3S and flagellar systems (2). These dually conserved proteins localize to the inner bacterial membrane, either as integral membrane or peripheral membrane-associated proteins that constitute the basal body of the T3S system.

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 synteny 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*

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Address correspondence to Partho Ghosh, pghosh@ucsd.edu.

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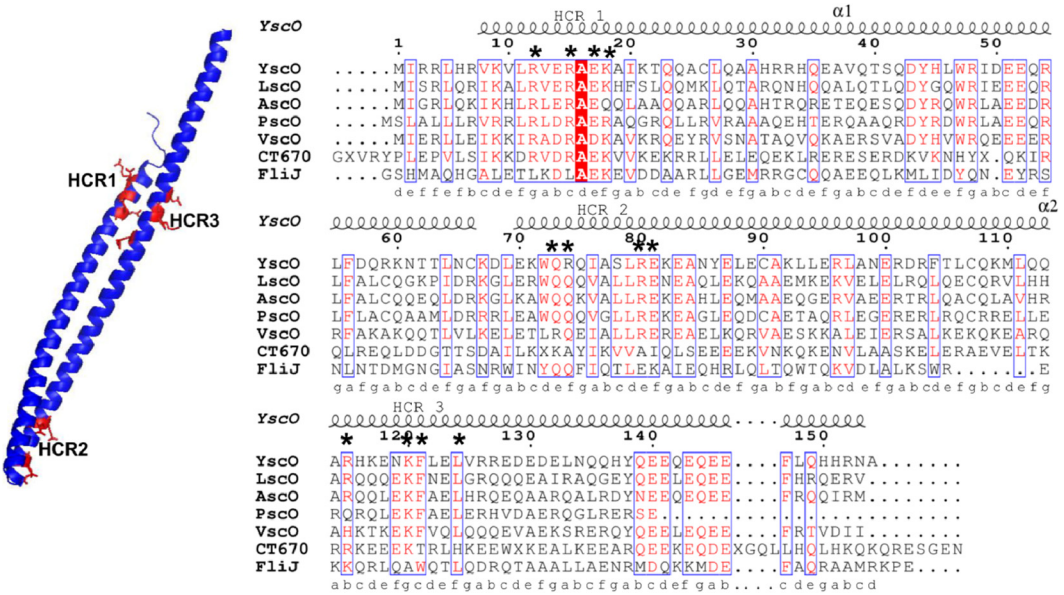

FIG 1 Model of YscO. Left, a model of YscO shown in ribbon representation. The locations of HCR1, -2, and -3 are in red, with residues targeted for mutagenesis shown with side chains in stick representation. Right, multiple sequence alignment of *Y. pseudotuberculosis* YscO, *Photorhabdus luminescens* LscO, *Aeromonas hydrophila* AscO, *Vibrio alginolyticus* VscO, *Pseudomonas aeruginosa* PscO, *C. trachomatis* CT670, and *Salmonella* FliJ. The secondary structure shown above the sequences corresponds to the model of YscO, and the letters below the sequences correspond to heptad register positions predicted for YscO. The stars indicate alanine-substituted residues.

and *Y. enterocolitica* (3, 4). Likewise, both functional and nonfunctional 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 YscP, 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, and later substrates, such as the Yop effector proteins (16). Overall, our results provide evidence that the interaction of YscO with YscP, and in particular the T3S4 domain of YscP, 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 EsPript (19). COILS (20) was used to predict coiled-coil heptad positions.

Construction of *Yersinia pseudotuberculosis* (Δ*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 coelectroporated with pWL204 (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 $CaCl_2$, 50 μg/ml kanamycin, and 5% arabinose; 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 pWL204. 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 1,000 bp upstream and downstream of *aph*.

Complementation of *Y. pseudotuberculosis* (Δ*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 purposes of detection. The resulting plasmids were electroporated into *Y. pseudotuberculosis* (wild type as well as the Δ*yscO*::*aph* strain), and transformants were selected by growing on BHI containing 2.5 mM $CaCl_2$ and 30 μg/ml ampicillin.

To assay secretion, *Y. pseudotuberculosis* strains were cultured by being shook overnight at 26°C in 5 ml BHI with antibiotics as appropriate (50 μg/ml kanamycin or 30 μg/ml ampicillin). A 1:20 dilution was made into fresh BHI containing 10 mM $MgCl_2$ and 10 mM ethylene glycol tetraacetic acid (EGTA), and the cultures were grown with shaking for 2 h at 26°C. For strains expressing *yscO* from the pBAD vector, 0.1% arabinose was added as well. In some samples, 2.5 mM $CaCl_2$ was added in place of 10 mM $MgCl_2$ and 10 mM EGTA in order to verify that the secretion was type III. Cultures were grown at 26°C to an optical density at 600 nm (OD_{600}) of 1.0 and then shifted to 37°C, at which time an additional 0.1% arabinose was added to strains carrying pBAD vectors. Cultures were then grown for an additional 3 h at 37°C. The concentration of bacteria was normalized to an OD_{600} of 1.0 using BHI prior to separation of bacteria from the culture medium by centrifugation (4°C, 10 min, 3,000 × *g*). The supernatant was put through a 0.22-μm-pore-size filter, after which point cold trichloroacetic acid (TCA) was added to a final concentration of 10%. The samples were incubated on ice for 1 h and then pelleted by centrifugation (4°C, 15 min, 20,000 × *g*). The pellet was washed three times with cold acetone, resolubilized in 2× SDS-PAGE sample buffer, and boiled. 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. Horseradish peroxidase (HRP)-conjugated anti-Strep tag II monoclonal antibody (1:1,000; EMD), anti-YopE polyclonal antibodies (1:1,000; a gift from J. Bliska), anti-YscO polyclonal antibodies (MIPA65; 1:1,000; a gift from G. Cornelis) (4), or anti-YscP polyclonal antibodies (MIPA57; 1:1,000; a gift from G. Cornelis) (4) in 5% milk in TBS were incubated with membranes for 16 h at 4°C. Samples for binding assays were treated similarly, except for an additional incubation for 1 h at 25°C with anti-His rabbit polyclonal antibodies (1:1,000; Santa Cruz) in 5% milk in TBS. Membranes were then washed 3 times with 20 ml TBS with 0.5% Tween (TBST) and incubated with HRP-conjugated goat anti-rabbit antibodies (1:5,000; Santa Cruz Biotechnology) in 5% milk in TBS for 30 min at 25°C. Membranes were once again washed 3 times with 20 ml TBST. For detection, SuperSignal West chemiluminescent substrate was used according to the manufacturer's instructions (Thermo Fisher Scientific).

In cases in which the membrane was stripped for probing with a different primary antibody, the membrane was incubated in 67% (wt/vol) guanidine HCl, 50 μM EDTA, 50 mM glycine (pH 10.8), 2.5 mM KCl, and 1.4 mM β-mercaptoethanol (β-ME) for 10 min. The membrane was then blocked and probed as described above, or in the case of probing for RpoA as a loading control, the following protocol was followed. The membrane was blocked for 1 h at room temperature in TBST containing 5% bovine serum albumin (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) 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 polyclonal antibodies (1:2,000; Santa Cruz Biotechnology) 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.

Preparation of His-SycD, YscP-His, and His-YscP T3S4. *Y. pseudotuberculosis* SycD, YscP, and YscP T3S4 (residues 341 to 440) were cloned into pET-28b (Novagen) with an N-terminal His tag (SycD and YscP T3S4) or C-terminal His tag (YscP) and transformed into *Escherichia coli* BL21(DE3). Bacteria were grown at 37°C in Luria broth medium with 30 μg/ml kanamycin to an OD_{600} of 0.6, at which time expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and the bacteria were grown for 16 h further at 20°C. Bacteria were harvested by centrifugation (4°C, 15 min, 3,000 × *g*) and resuspended in (12.5 ml per liter of bacterial culture) lysis buffer (150 mM [for His-SycD] or 500 mM [for YscP-His and His-YscP T3S4] NaCl, 50 mM sodium phosphate buffer [pH 8], and 5 mM [for His-SycD] or 10 mM [for YscP-His] β-ME supplemented with protease inhibitor cocktail [1 Complete tablet per 50 ml lysis buffer; Roche]). Bacteria were lysed by four passages through a high-pressure homogenizer (Emulsiflex-C5; Avestin) and centrifuged (4°C, 30 min, 20,000 × *g*) to remove cell debris. The supernatant, which contained His-SycD, YscP-His, or His-YscP T3S4, was applied to an Ni^{2+}-nitrilotriacetic acid (NTA) column equilibrated with lysis buffer containing 20 mM imidazole. After 10 column volume washes with this buffer, proteins were eluted in 5 column volumes of lysis buffer containing 250 mM (His-SycD) or 500 mM imidazole (YscP-His and His-YscP T3S4). Fractions containing His-SycD, YscP-His, or His-YscP T3S4, as visualized on a Coomassie-stained 15% SDS-PAGE gel, were pooled and dialyzed into lysis buffer. The proteins were then concentrated by ultrafiltration (YM-3K, Pall) and further purified by size exclusion chromatography (Sephadex 200; GE Healthcare) in lysis buffer. His-SycD, YscP-His, and His-YscP T3S4 were concentrated to 45, 25, and 20 mg/ml, respectively, by ultrafiltration (YM-3K; Amicon). Concentrations of these proteins were determined using calculated molar ε_{280} values of 10,720 $M^{-1}cm^{-1}$ for SycD-His, 25,470 $M^{-1}cm^{-1}$ for YscP-His, and 16,244 $M^{-1}cm^{-1}$ for His-YscP T3S4.

Purification of Strep-YscO. *Y. pseudotuberculosis* (Δ*yscO*::*aph*) complemented with *yscO* containing a Strep tag was grown at 28°C to an OD_{600} of 0.6, induced with 2.0% 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 16 h with 1 ml Strep-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*). Strep-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* (Δ*yscO*::*aph*) complemented with various alleles of *yscO* was grown at 28°C to an OD_{600} 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 16 h with 100 μl Strep-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, 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 in 200 μl of buffer A supplemented with 0.1% Triton 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 Ni^{2+}-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). Strep-YscO was incubated with the resin in 100 μl binding buffer for 1 h at 4°C, followed by five washes of 200 μl each of the beads with the same buffer; each wash was followed by a centrifugation step (4°C, 30 s, 3,000 × *g*). The beads were then incubated with binding buffer containing 500 mM imidazole for 10 min followed by centrifugation (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* FliJ (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 (*c*), Glu17 (*e*), and Lys18 (*f*) for HCR1; Gln73 (*e*), Arg74 (*f*), Arg80 (*e*), 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., all 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

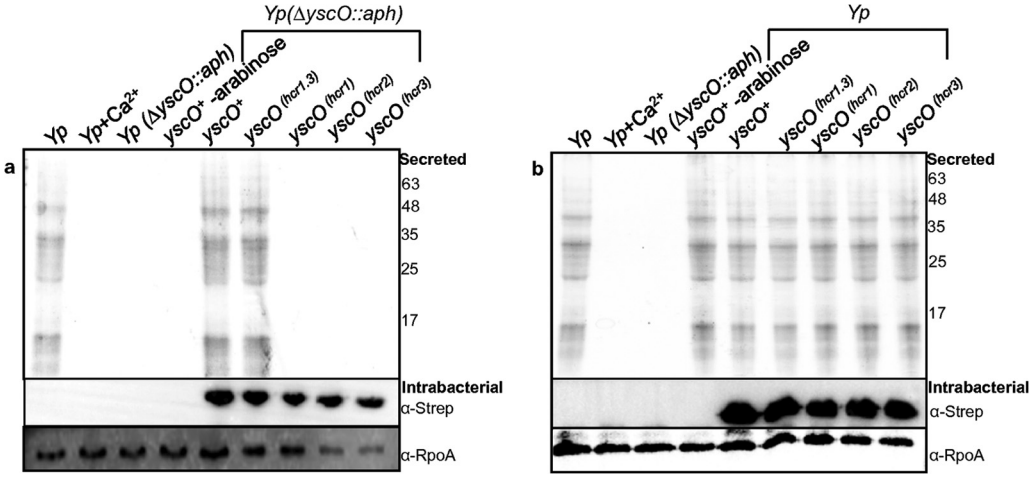

FIG 2 Type III secretion. (a) Top, proteins secreted into the extracellular medium through the T3S system by wild-type *Y. pseudotuberculosis* (Yp), wild-type *Y. pseudotuberculosis* in high calcium concentration (Yp+Ca^{2+}), *Y. pseudotuberculosis* (Δ*yscO*::*aph*), *Y. pseudotuberculosis* (Δ*yscO*::*aph*) transformed with a plasmid encoding inducible wild-type *yscO*, without induction (*yscO*$^+$ −arabinose) or with induction (*yscO*$^+$) of *yscO* expression, and *Y. pseudotuberculosis* (Δ*yscO*::*aph*) transformed with pBAD encoding inducible *yscO* HCR1.3, -1, -2, or -3 (all induced). Proteins were detected by a Coomassie-stained SDS-PAGE gel. Molecular mass markers are indicated at the right. Middle, expression level of intrabacterial, plasmid-encoded Strep-tagged YscO, as visualized by Western blotting using an anti-Strep tag antibody. Bottom, expression level of intrabacterial RpoA, which served as a loading control, as visualized by Western blotting using anti-RpoA antibodies. (b) Same as in panel a, except plasmid-encoded *yscO* was introduced into wild-type *Y. pseudotuberculosis*.

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* (Δ*yscO*::*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 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. pseudotuberculosis* (Δ*yscO*::*aph*) (Fig. 3). However, as described above, YscO was not evident in the type III secreted fraction, which was confirmed to contain YopE. Likewise, YscO was not detected in the secreted fraction of *Y. pseudotuberculosis* complemented with any of the *yscO* HCR mutants. We draw the conclusion from this experiment that YscO is not secreted by *Y. pseudotuberculosis* and further that secretion of YscO is not required for type III secretion. At present, it is not clear why there is a discrepancy with prior results (3, 4). However, the more important point for this study is that the T3S-proficient *yscO* HCR1.3 mutant was indistinguishable from the T3S-deficient *yscO* HCR1, -2, and -3 mutants with regard to YscO secretion.

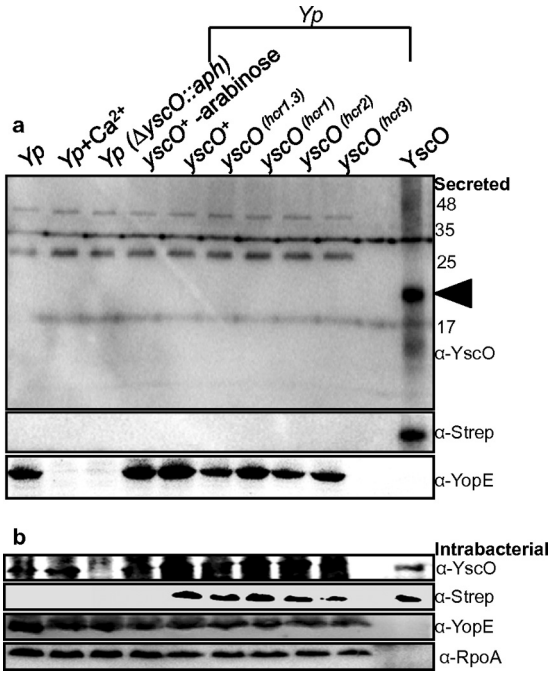

FIG 3 Intrabacterial localization of YscO. (a) Samples shown in Fig. 2b were analyzed by Western blotting using anti-YscO (top), anti-Strep tag (middle), and anti-YopE (bottom) antibodies. The last lane, which is separated from the others by an empty lane, contains purified Strep-YscO, whose position is indicated by the arrowhead. Molecular mass markers are indicated at right. (b) Intrabacterial expression levels of proteins analyzed in panel a using anti-YscO, anti-Strep tag, and anti-YopE antibodies. Additionally, the intrabacterial expression level of RpoA was detected with anti-RpoA antibodies as a loading control.

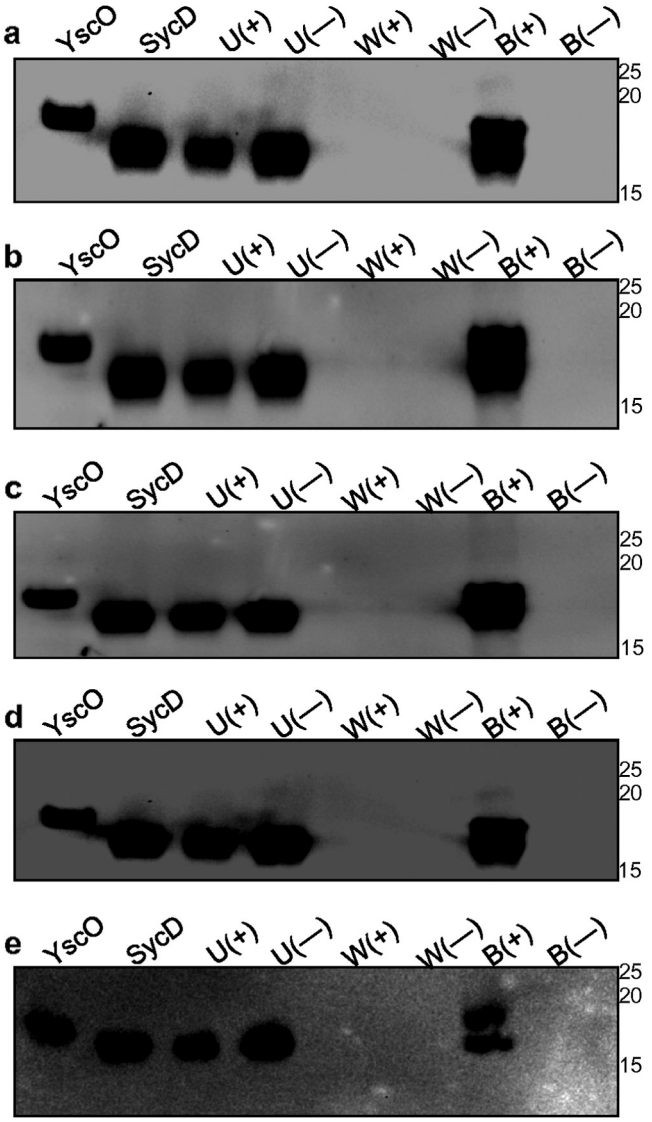

FIG 4 Interaction between YscO and SycD. Lysates from *Y. pseudotuberculosis* that were induced (+) or uninduced (−) for expression of Strep-tagged wild-type YscO (a), YscO HCR1.3 (b), YscO HCR1 (c), YscO HCR2 (d), or YscO HCR3 (e) were applied to Strep-Tactin beads, and purified His-tagged SycD was added to this. After the Strep-Tactin beads were washed, proteins remaining bound to the beads were analyzed by Western blotting using anti-Strep tag and anti-His tag antibodies simultaneously. The first two lanes show the input levels of YscO and SycD in the experiment. "U" signifies the unbound fraction, "W" the final wash fraction, and "B" the bound fraction.

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

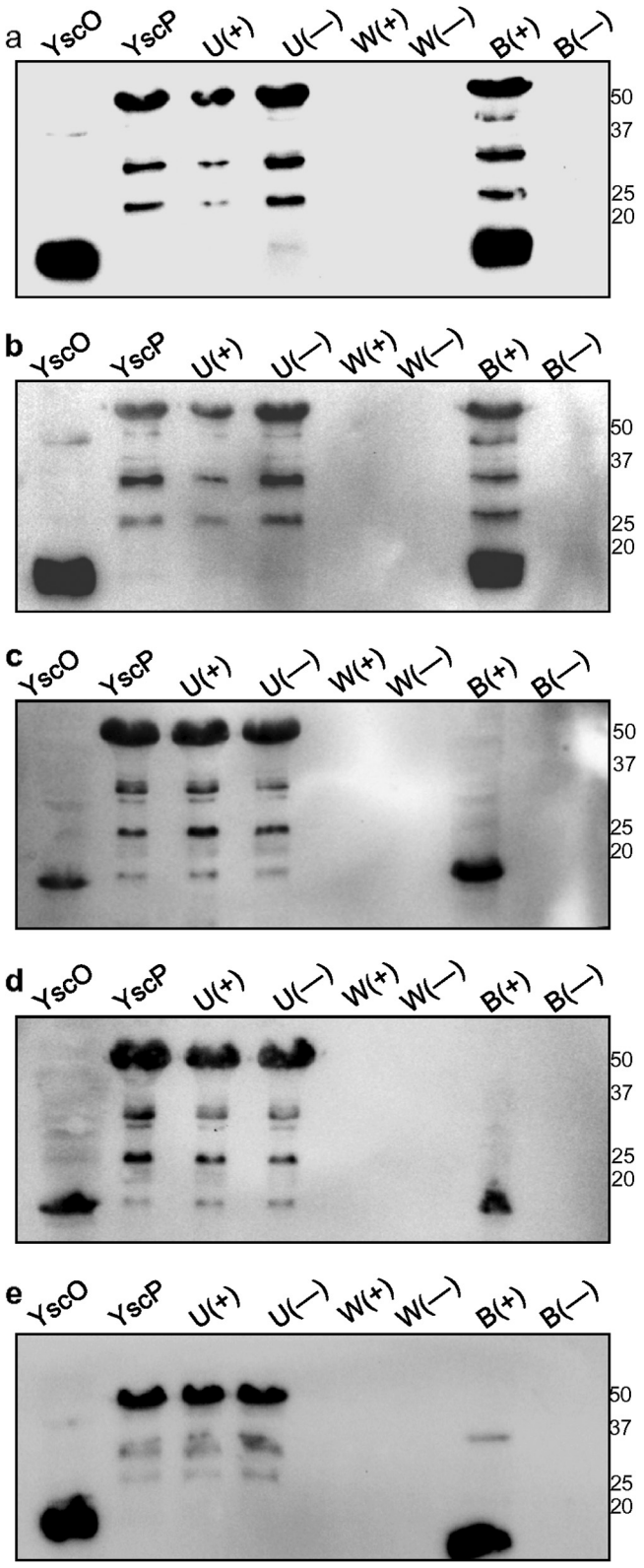

FIG 5 Interaction between YscO and YscP. The format is the same as in Fig. 4, except that His-tagged YscP was used instead of His-tagged SycD.

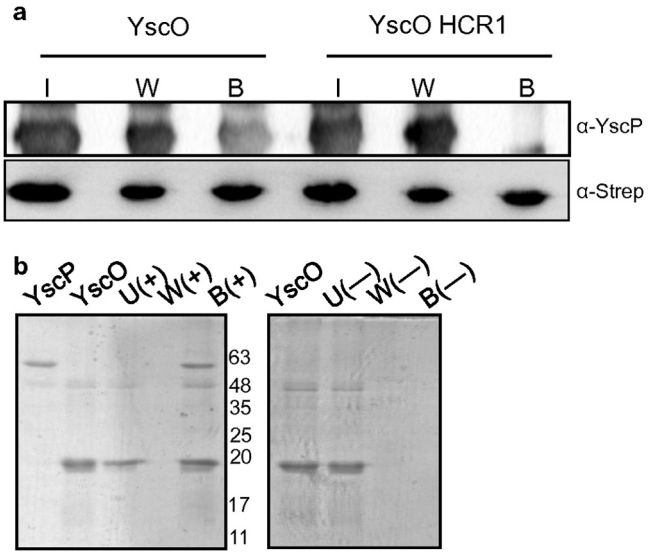

FIG 6 YscO-YscP interaction in *Yersinia* and the interaction detected by Coomassie staining. (a) Lysates from *Y. pseudotuberculosis* that were induced for expression of Strep-tagged wild-type YscO (left) or nonfunctional YscO HCR1 (right) were applied to Strep-Tactin beads. After the Strep-Tactin beads were washed, proteins remaining bound to the beads were analyzed by Western blotting using anti-YscP and anti-Strep tag antibodies. "I" signifies the input, "W" the final wash fraction, and "B" the bound fraction. (b) Purified His-tagged YscP was applied to Ni-NTA beads, and purified Strep-tagged YscO was added to this (+). To serve as a negative control (−), purified Strep-tagged YscO was added to Ni-NTA beads alone. After the beads were washed, proteins were eluted from the beads and visualized on a Coomassie-stained 12.5% SDS-PAGE gel. The YscP and YscO lanes indicate the input levels of each protein used in the experiment. "U" signifies the unbound fraction, "W" the final wash fraction, and "B" the bound fraction. The two panels are from the same gel. Molecular mass markers are indicated between the two panels.

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 YscO HCR1 in *Y. pseudotuberculosis* (Δ*yscO*::*aph*) 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 Ni^{2+}-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

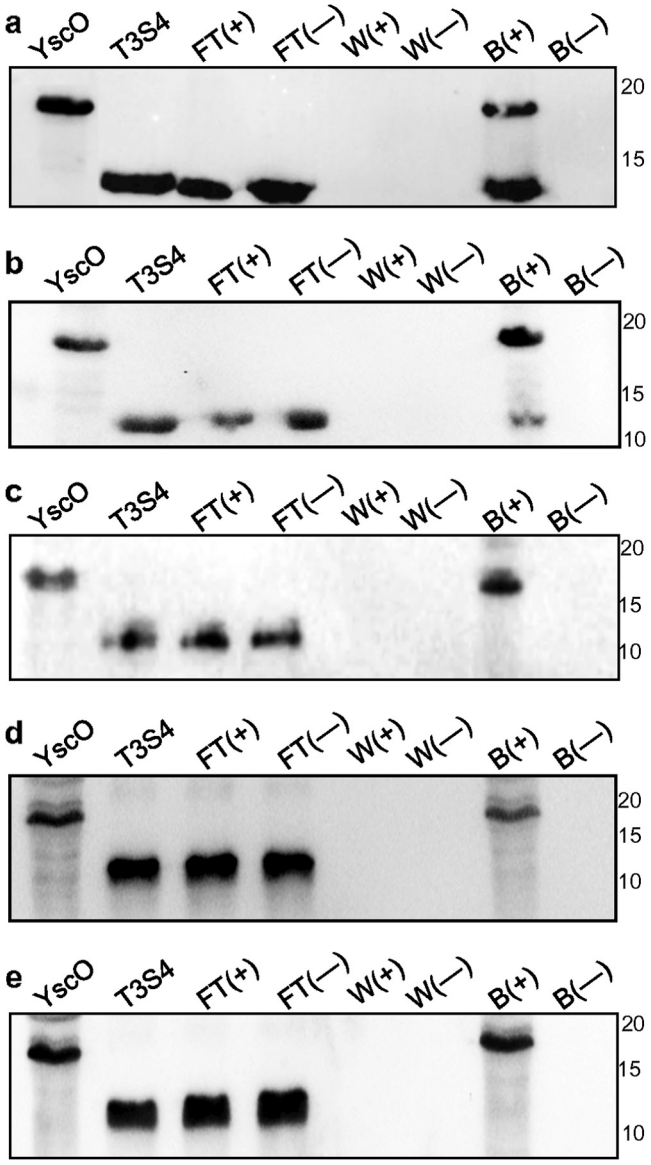

FIG 7 Interaction between YscO and the YscP T3S4 domain. The format is the same as in Fig. 4, except that the His-tagged T3S4 domain of YscP was used instead of His-tagged SycD.

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 deficient 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. Wild-type and HCR1.3 YscO, which were competent for type III secretion, bound recombinant YscP, but HCR1, -2, and -3, which were incompetent for type III secretion, did not. This YscO-YscP interaction was confirmed in *Y. pseudotuberculosis*, and experiments using purified components provided evidence that the interaction was direct. We noticed that 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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