

## Characterization of the *Yersinia enterocolitica* Type III Secretion ATPase YscN and Its Regulator, YscL

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**Type III secretion is a mechanism used by a broad range of gram-negative bacteria to neutralize eukaryotic defenses by enabling translocation of bacterial proteins directly into the cytoplasm of host cells. The bacterial energy source for secretion is ATP, which is consumed by an ATPase that couples ATP hydrolysis to the unfolding of secreted proteins and the dissociation of their chaperones just prior to secretion. By studying the biochemical properties of YscN and YscL of *Yersinia enterocolitica*, we have characterized them as the ATPase and ATPase regulator, respectively, of the type III secretion system of this organism. In vivo, YscL and YscN interact with each other, and the overexpression of glutathione *S*-transferase–YscL abolishes secretion and down-regulates the expression of secretion apparatus components.**

Many pathogenic gram-negative bacteria employ a needle-like structure to secrete proteins that manipulate host defenses, a mechanism that is termed type III secretion (reviewed in reference 17). The shaft of the type III secretion system (TTSS) needle connects the bacterial cytoplasm to the extracellular milieu and can pierce the eukaryotic cell membrane, permitting secretion into the environment or directly into host cells (20, 24, 26, 38). The portion of the TTSS that faces the bacterial cytosol encounters the full complement of bacterial proteins yet is able to specifically recognize secretion substrates (3, 28, 37, 41). How this recognition occurs is a question of great interest (13, 42, 44).

To begin addressing this issue, the substrate-proximal elements required for secretion have been queried in the *Yersinia enterocolitica* TTSS. YopE is a representative secretion substrate, which disables actin polymerization upon injection into eukaryotic cells, thereby preventing phagocytosis (7, 36). An analysis of the sequences within YopE required for its injection has highlighted two elements: (i) a secretion signal within *yopE* codons 1 to 7 that is decoded by features of its RNA sequence and/or amino acid sequence (3, 25, 35) and (ii) amino acids 15 to 75, the binding site for a homodimeric “chaperone,” SycE (6, 8, 10, 45, 51, 53). In the course of an in vitro infection of HeLa cells, both of the intra-YopE elements and the presence of SycE are required for TTSS injection of YopE into the cytoplasm of target cells (24, 45).

These features of the secretion substrate presumably have corresponding recognition elements on the TTSS. One protein that has been implicated to serve such a function in other model systems is YscN (1, 33, 48, 49). YscN is an essential component of the TTSS of yersiniae with characterized homologues, such as FliI of the *Salmonella enterica* serovar Typhimurium flagellar assembly pathway (which is structurally and functionally related to type III secretion) and the *Escherichia coli*, *Pseudomonas syringae*, and *S. enterica* serovar Typhi-

murium type III secretion systems (2, 15, 18, 34, 52). Critically, the *E. coli* and *S. enterica* serovar Typhimurium homologues of YscN have the ability to interact with secreted proteins; YscN could therefore be the component of the TTSS recognizing secretion substrates in yersiniae (1, 48, 49).

Though soluble, YscN more than likely resides at the interface of the TTSS and the bacterial cytoplasm. This is suggested by the observation that the enzymatic activity (to consume ATP) of the *S. enterica* serovar Typhimurium homologue, FliI, is stimulated by 1 order of magnitude in the presence of phospholipids (11). Moreover, in fractionation experiments, YscN homologues are peripherally associated with the membrane and are likely held there through interactions with other components of the TTSS (5, 11, 21, 22, 30, 33, 34, 49). Of special interest among these interacting partners is YscL (21, 22, 32). In the *S. enterica* serovar Typhimurium flagellar system, the YscL homologue FliH not only binds to the YscN equivalent, FliI, but also represses its ATPase activity (32). Intriguingly, a truncation of five carboxy-terminal residues results in a FliH mutant that increases the ATPase activity of FliI fivefold, suggesting that ATPase specific activity can be both increased and decreased by other TTSS components (19). Two additional components of the TTSS, YscQ and YscK, can jointly interact with YscL and perhaps function in such a capacity (21, 22).

The centrality of YscN homologues to type III secretion has been underscored by the recognition that YscN belongs to a class of enzymes that unfold proteins in an ATP-dependent manner (reviewed in reference 40). The *S. enterica* serovar Typhimurium YscN equivalent, InvC, has been demonstrated to be sufficient in vitro for the dissociation of a chaperone-effector complex and the formation of a secretion-competent effector (which is at least partially unfolded) and free chaperone in an ATP-dependent manner (1).

For this report, we investigated the in vivo and in vitro functions of YscN and YscL. YscN and YscL interact with one another in the cytoplasm of *Y. enterocolitica*, and this interaction occurs whether the bacteria are secreting effectors or not. A biochemical analysis of YscN reveals it to be a highly cooperative ATPase whose activity is inhibited by the addition of YscL. A similar phenomenon was observed in vivo, as overex-

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pressed glutathione *S*-transferase (GST)-YscL abolished type III secretion and copurified with YscN.

#### MATERIALS AND METHODS

**Plasmids, bacterial strains, and genetic manipulations.** For the construction of *gst-yscN*, *yscN* was amplified from the virulence plasmid of *Y. enterocolitica* with primers 5'-NNNAGATCTATGCTCTCACTAGATCAGATACC-3' and 5'-NNG AATTCTCATTGGGTCAGCGTCTCC-3' (restriction sites underlined) and digested with BglII and EcoRI. pGEX-2TK (GE Healthcare) was digested with EcoRI and BamHI, and the two fragments were ligated, resulting in pBB2. For selection of this plasmid in *Y. enterocolitica*, pBB2 was digested with EcoRI as was an EcoRI-flanked kanamycin resistance cassette (27); these were ligated, yielding pBB7.

For the construction of a polyhistidine-tagged YscN, *yscN* was amplified with primers 5'-NNNNNCATATGCTCTCACTAGATCAGATACC-3' and 5'-NC TCGAGTCATTGGGTCAGCGTCTCC-3' and digested with XhoI and NdeI, as was pET15b. When the fragments were ligated, pBB5 resulted, which in turn was transformed into *E. coli* strain BL21(DE3) for protein expression, as were all pET derivatives.

For the construction of *gst-yscL*, *yscL* was amplified with primers 5'-AAGGA TCCATGCGCCATTGTCAAATAAT-3' and 5'-AAGAATTCTTATTCC TCTTCTGTAACCTTCA-3' using a chromosomal DNA preparation of *Y. enterocolitica* strain W22703 as the template. The PCR fragment was cloned into pCR2.1 (Invitrogen), and the resulting plasmid, pKER12, was digested with BamHI and EcoRI, as was pGEX-2TK. Ligation of these fragments resulted in pKER15. For selection of this plasmid in *Y. enterocolitica*, *npt* was amplified using primers 5'-AGGTCTGCCTCGTGAAGAAGG-3' and 5'-CGATTTATTCAAC AAGCCG-3' using pHSG299 as the template DNA (46). pKER15 was digested with PstI and blunted with Klenow fragment (New England BioLabs). Insertion of *npt* into the blunted PstI site disrupted *bla* but rendered strains containing plasmid pKER17 kanamycin resistant.

For the expression of decahistidine-tagged YscL, *yscL* was amplified from *Y. enterocolitica* using primers 5'-AACATATGATGCAGCCATTGTCAAATA AT-3' and 5'-AAGGATCCTTATTCTCTTCTGTAACCTTCA-3'. The PCR product was introduced into pCR2.1 (Invitrogen) and digested with BamHI and NdeI, as was pET16b (Novagen), and ligation of these fragments yielded pKER14.

To generate plasmids containing wild-type or K175E mutant *yscN* for insertion into *Y. enterocolitica*, alleles were amplified and cloned into pCR2.1 (Invitrogen). The wild-type allele was amplified with primers 5'-TTATAATTGCAATTACC GCG-3' and 5'-NCTCGAGTCATTGGGTCAGCGTCTCC-3'. The mutant allele was amplified in two steps. The upstream and internal sequences were amplified with 5'-TTATAATTGCAATTACC GCG-3' and 5'-GCAAGCAGTGTACTTTCCCC CCCC GCGGCGCC-3', while internal and downstream sequences were amplified with 5'-GGCCGCGGGGGGGGAAAGTACACTGCTTGC-3' and 5'-NCT CGAGTCATTGGGTCAGCGTCTCC-3', and a subsequent round of PCR amplification ligated these two products together. The alleles were excised from pCR2.1 with EcoRI and XhoI; these restriction enzymes were also used to digest pJS95 (43), into which these fragments were inserted. For insertion of an inducible *yscL* allele into *Y. enterocolitica*, *yscL* was amplified with primers 5'-AACATATGATGCAGC CATTGTCAAATAAT-3' and 5'-AAGGATCCTTATTCTCTTCTGTAACCTTCA-3' and cloned into pCR2.1. The fragment was excised with NdeI and BamHI and inserted into pHSG575 (46), which had been digested with the same restriction enzymes.

All inserts in plasmids described above were confirmed with sequence data generated by the University of Chicago Cancer Research Center DNA sequencing facility. The plasmid complementing *yscN* in *Y. enterocolitica* is pCT142. This plasmid corresponds to pHSG576 with the *tac* promoter inserted between EcoRI and NdeI sites and *yscN* cloned in the NdeI and BamHI sites (4).

*Y. enterocolitica* strain W22703 was used as the wild type (12). The approach to generating the *yscN* and *yscL* mutant strains is described elsewhere (8). The resulting strains contain in-frame deletions, and the *yscL* mutant retains coding sequences corresponding to amino acids 1 to 93 and 208 to 221 with an intervening XbaI site, while the *yscN* mutant retains the coding sequences corresponding to amino acids 1 to 50 and 391 to 439 with an intervening BglII site.

Secretion assays in *Y. enterocolitica* were conducted as described previously (8). Standard methods for electroporation of plasmids into *Y. enterocolitica* have been described elsewhere, as have methods for the *E. coli* DH5 $\alpha$  transformation, selection, and maintenance techniques employed (39).

**Protein purifications.** For purification of histidine-tagged YscL ( $_{\text{His}}$ YscL) under denaturing conditions, *E. coli* BL21(DE3) harboring pKER14 was grown at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 to 0.8, and expression of  $_{\text{His}}$ YscL was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).

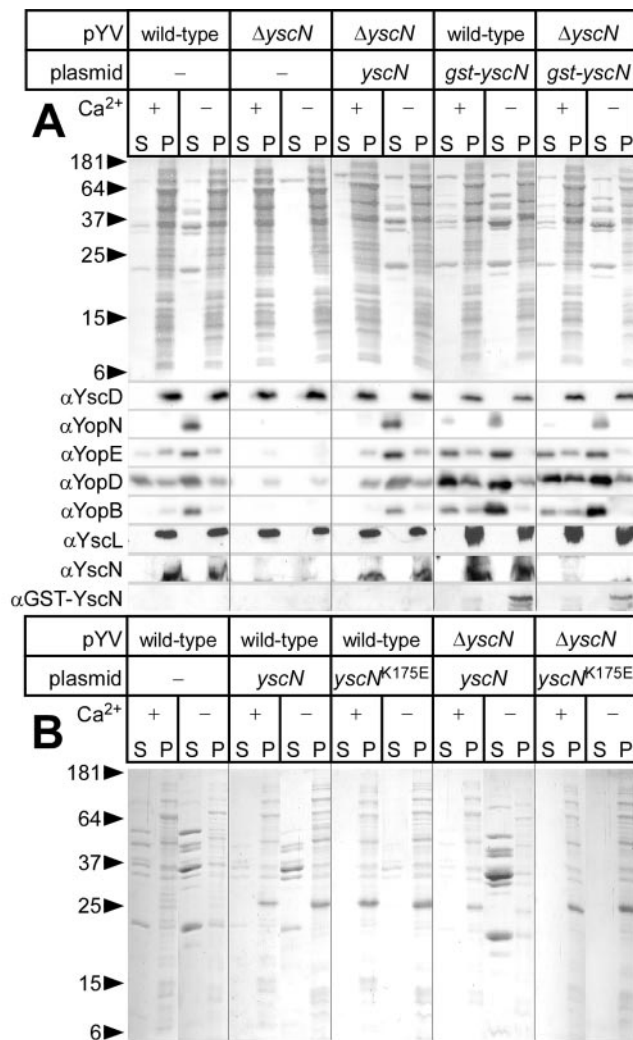


FIG. 1. Effect of GST-YscN on *Y. enterocolitica* type III secretion. (A) Wild-type and *yscN* mutant *Y. enterocolitica* strains bearing plasmids encoding *yscN* or *gst-yscN* were monitored for the functionality of type III secretion. Secretion was monitored by growth in medium supplemented or chelated for calcium (Ca<sup>2+</sup> + or -, respectively), and supernatant (S) and pellet (P) fractions were assayed by Coomassie blue staining, SDS-PAGE, and immunoblotting with the indicated antisera (e.g., anti-YscD [ $\alpha$ YscD]). (B) Wild-type and  $\Delta$ yscN *Y. enterocolitica* strains bearing wild-type or mutant alleles of *yscN* on a plasmid were assayed for secretion by Coomassie blue staining and SDS-PAGE in the presence (+) or absence (-) of calcium. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels.

After 2 hours, the culture was centrifuged for 15 min at 6,000  $\times$  g. The bacterial sediment from 1 liter of cells was suspended in 20 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 8 M urea). Samples were passed through a French pressure cell at 16,000 lb/in<sup>2</sup> twice, and lysate was clarified by centrifugation at 15,000  $\times$  g for 15 min. Clarified lysate was loaded onto a (1-ml) Ni-nitrilotriacetic acid (Ni-NTA) column preequilibrated with lysis buffer. After the column was washed with 10 column volumes of lysis buffer, it was washed with 50 column volumes of renaturing buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0) and left to stand for 24 h. The column was washed again with 10 column volumes of renaturing buffer and eluted with renaturing buffer containing 500 mM imidazole.

For purification of  $_{\text{His}}$ YscN under denaturing conditions, *E. coli* BL21(DE3) harboring pBB7 was grown at 30°C until it reached an OD<sub>600</sub> of 0.5. IPTG was

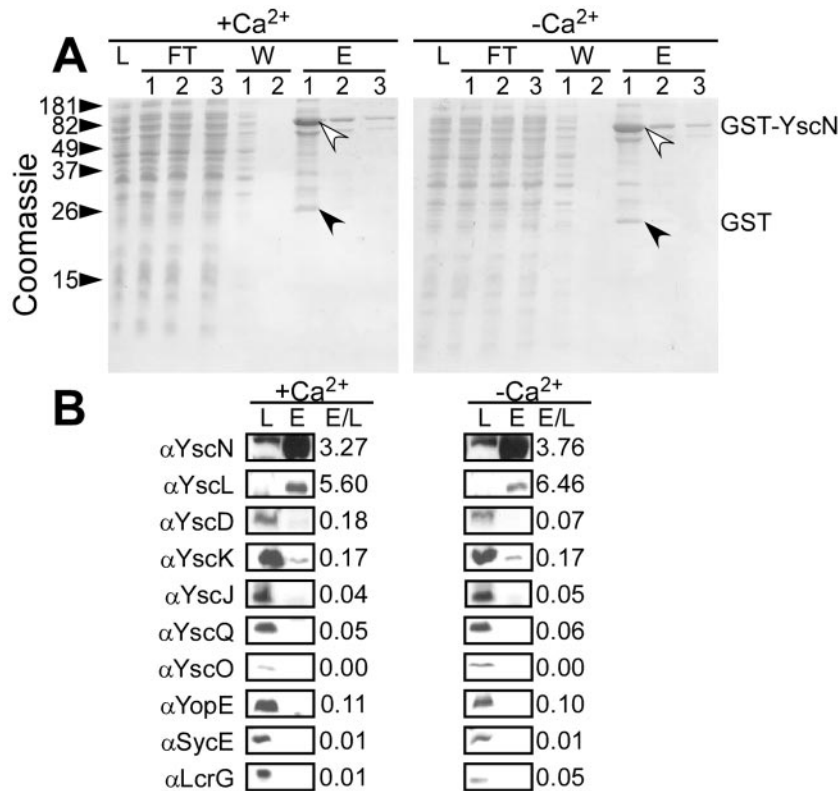


FIG. 2. GST-YscN binds to YscL. (A) *Y. enterocolitica*  $\Delta yscN$  strain bearing a plasmid-encoded *gst-yscN* was grown at 37°C under conditions that induce the type III secretion machine and GST-YscN in the presence or absence of extracellular calcium (+Ca<sup>2+</sup> or -Ca<sup>2+</sup>, respectively). GST-YscN was purified from this strain, and samples corresponding to the lysate (L), flowthrough (FT), wash (W), and eluate (E) fractions were collected and analyzed by Coomassie blue staining and SDS-PAGE. The white arrowhead points to GST-YscN and the filled arrowhead to GST. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels. (B) The lysate (L) and eluate (E) fractions were analyzed by immunoblotting with the indicated antisera (e.g., anti-YscN [ $\alpha$ YscN]). The relative intensity of immunoreactive signals in the lysate and eluate fractions (E/L) is expressed as a ratio to the right of each blot.

added to a final concentration of 0.2 mM and incubated for a further 10 h at 30°C. Cultures were centrifuged for 15 min at 6,000  $\times$  g. Bacterial sediment from 1 liter of culture was suspended in 25 ml of lysis buffer (6 M urea, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 8.0). Samples were passed through a French pressure cell at 16,000 lb/in<sup>2</sup> twice, and the lysate was clarified by centrifugation at 15,000  $\times$  g for 15 min. The clarified lysate was loaded onto a (1-ml) Ni-NTA column preequilibrated with lysis buffer and eluted stepwise with 2 ml of lysis buffer containing sequentially 20, 30, 50, 75, 100, or 200 mM imidazole. The elution fractions were pooled and instantly diluted 20 times in vigorously stirring renaturing buffer (20% glycerol, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 8.0). The resulting solution was loaded onto a (1-ml) Ni-NTA column preequilibrated with renaturing buffer. After the column was washed with 10 volumes of renaturing buffer, His YscN was eluted with 3 column volumes of renaturing buffer containing 500 mM imidazole. The resulting eluate was ultracentrifuged at 100,000  $\times$  g for 30 min, and the supernatant was collected.

For purification of GST fusion proteins from *Y. enterocolitica*, strains containing expression vectors were grown at 26°C with 5 mM CaCl<sub>2</sub> or 5 mM EGTA, pH 7.5, until an OD<sub>600</sub> of 0.6 to 0.8 was reached. Thereupon, cultures were shifted to 37°C. Thirty minutes later, IPTG was added to a final concentration of 1 mM, and the cultures were incubated for a further 2.5 h. Bacterial cultures were centrifuged 15 min at 6,000  $\times$  g. Bacterial sediment from 1 liter of cells was suspended in phosphate-buffered saline, cells were lysed in a French pressure cell twice at 16,000 lb/in<sup>2</sup>, and GST fusions were purified from clarified lysates using glutathione-Sepharose beads (GE Healthcare) according to the manufacturer's recommendations.

For gel filtration chromatography, 0.5-ml samples were loaded onto a Superose 12 column (GE Healthcare), and 0.5-ml fractions were collected. When adenylyl imidodiphosphate AMP-PNP was added to His YscN, it was at a final concentration of 5 mM, and the mixture was incubated 5 min before loading onto the gel. The column was calibrated by monitoring the elution profiles of protein stan-

dards: ovalbumin (43 kDa), aldolase (158 kDa), catalase (232 kDa), and thyroglobulin (669 kDa) (GE Healthcare). The elution profiles of loaded proteins were monitored by using bicinchoninic acid assay standards (Pierce) or by immunoblotting (YscN).

**Malachite green assay.** The malachite green assay was performed essentially as described previously (14). When it was necessary to add His YscL to monitor its effect on His YscN ATPase activity, a constant 90  $\mu$ l of YscL buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0) was added to the reaction mixture, some fraction of which contained His YscL in the same buffer.

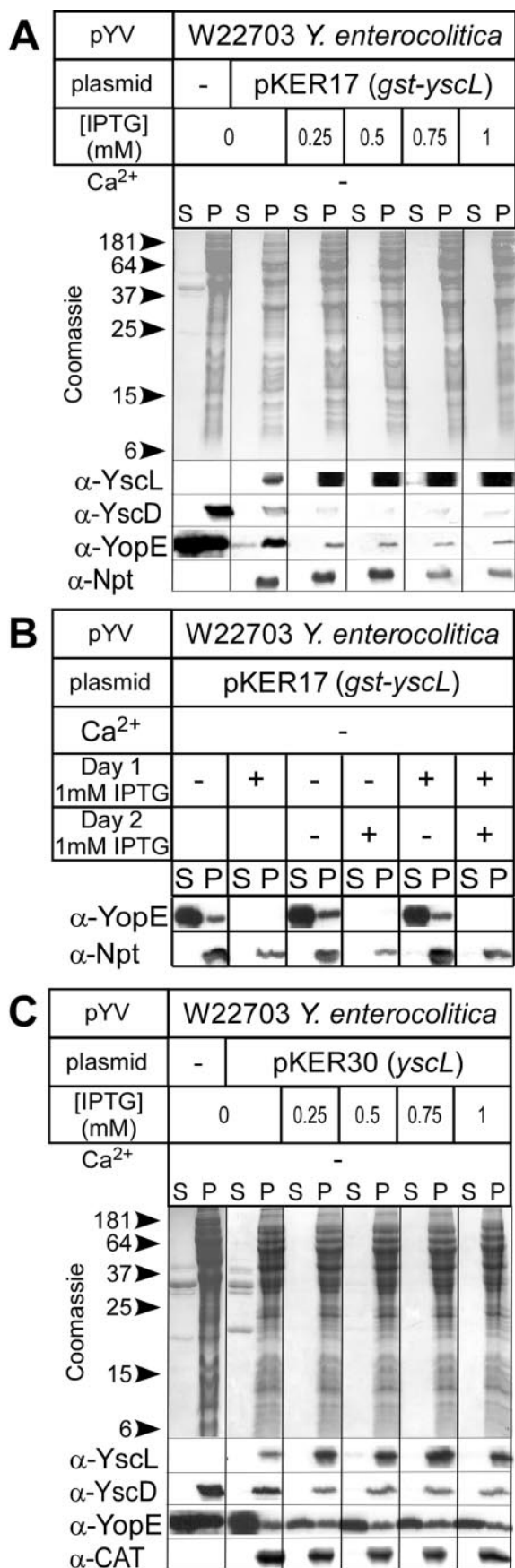
**Protein electrophoresis and immunodetection.** Proteins were resolved by electrophoresis on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels. For immunoblots, proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with antisera as described previously (8).

## RESULTS

**In vivo analysis of *yscN* and *gst-yscN*.** YscL and YscN are essential to the secretion of proteins by the type III pathway (Fig. 1; also see Fig. 3) (29, 52). Moreover, YscL and YscN interact with each other in a yeast two-hybrid assay and with other soluble and membrane-embedded elements of the secretion apparatus (21, 22, 33). To investigate these associations in *Y. enterocolitica*, we introduced a plasmid encoding GST-YscN into wild-type and  $\Delta yscN$  strains and assayed for the ability of GST-YscN to support type III secretion. Secretion was assayed by Coomassie blue staining and SDS-polyacrylamide gel electrophoresis (PAGE) of trichloroacetic acid-precipitated super-







plasmid restored the ability of a *ΔyscL* mutant to secrete effectors (Fig. 3A). Again, we observed a calcium-blind secretion phenotype, as a *ΔyscL* mutant expressing GST-YscL secreted YopB, YopD, YopE, and YopN into the culture medium even in the presence of calcium (Fig. 3A).

When GST-YscL was expressed in wild-type *Y. enterocolitica* W22703, we observed that expression of some machine components (YscD but not YscN) was abolished, and no secretion was observed (Fig. 3A). A previous study of the YscL homologue of the *S. enterica* serovar Typhimurium flagellar system, FliH, has demonstrated that overexpression of FliH can impair secretion of substrates (32). We therefore hypothesized that increased intrabacterial YscL or GST-YscL levels might have impaired type III secretion. To confirm this, where we had previously relied on the low levels of expression of the *tac* promoter to drive expression of *gst-yscL* (Fig. 3A), we monitored secretion over a gradient of increasing amounts of IPTG up to a maximum of 1 mM (Fig. 4A). As expected, increasing intrabacterial GST-YscL led to decreased type III secretion (Fig. 4A). By simultaneously monitoring the production of machine components (YscD), we observed that the secretion defect of bacteria overproducing GST-YscL may be due at least in part to the relatively low levels of secretion machine components in these bacteria (Fig. 4A). A cytoplasmic protein (neomycin phosphotransferase [Npt]) was used to control for a possible global decrease in protein expression, which was not the case (Fig. 4A).

These results raised the possibility that the high copy number of GST-YscL was titrating a factor necessary for secretion. To investigate this possibility, the experiment described above was duplicated, overexpressing YscL in wild-type *Y. enterocolitica* (Fig. 4C). In contrast to the secretion defect of strains producing GST-YscL, overexpression of YscL alone had only a nominal effect on the efficiency of YopE secretion. To dismiss the possibility that GST-YscL expression affected the inheritance of the plasmid encoding the secretion apparatus and its substrates, we examined the ability of bacteria to secrete after they had expressed GST-YscL for a period of several hours (Fig. 4B). Since bacteria several generations re-

FIG. 4. GST-YscL but not YscL affects type III secretion. (A) Secretion of wild-type *Y. enterocolitica* containing a plasmid-encoded *gst-yscL* was monitored as a function of an inducer for GST-YscL. Bacteria were grown in the absence (-) of extracellular calcium, and the supernatants (S) and bacterial sediment pellets (P) of each culture were scored by Coomassie blue staining, SDS-PAGE, and immunoblotting to YscL, YscD (a machine component), YopE (a secreted protein), and Npt (a cytoplasmic protein—our fractionation control). Wild-type bacteria grown without IPTG are shown in the leftmost lanes of the gel as a reference. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. α-YscL, anti-YscL. (B) Wild-type *Y. enterocolitica* bearing a plasmid-encoded *gst-yscL* were grown in the presence (+) or absence (-) of inducer in the absence (-) of calcium and assayed for secretion by immunoblotting. Bacteria were grown for several generations in the presence of calcium and the absence of inducer and assayed again for secretion as described above. (C) Wild-type *Y. enterocolitica* bearing an inducible, untagged *yscL* allele was grown in the absence of calcium and increasing concentrations of inducer. Secretion of proteins was monitored as described above for panel A, except anti-chloramphenicol acetyltransferase (α-CAT) antibodies were used in place of anti-Npt antibodies.

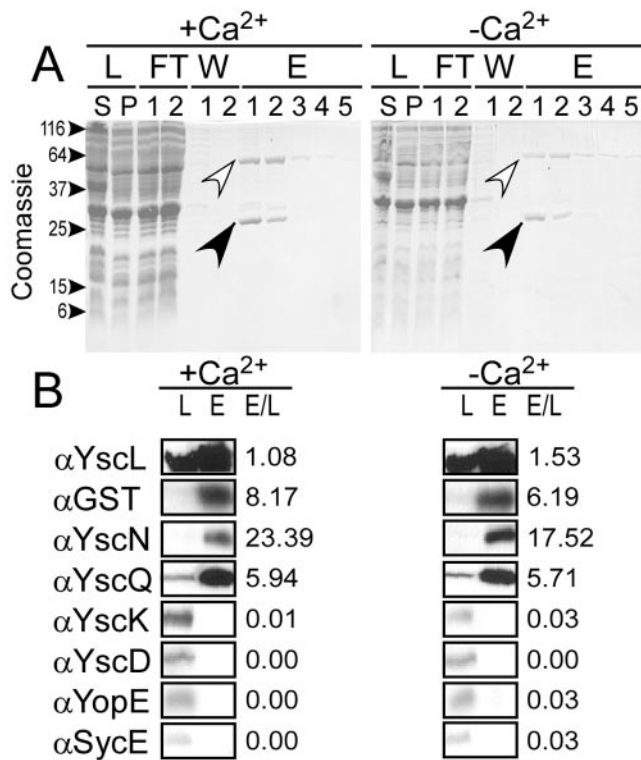


FIG. 5. GST-YscL associates with YscN and YscQ. (A) *Y. enterocolitica*  $\Delta$ yscL strain expressing GST-YscL was grown at 37°C under conditions that induce the type III secretion machine and GST-YscL in the presence or absence of extracellular calcium (+Ca<sup>2+</sup> or -Ca<sup>2+</sup>, respectively). GST-YscL was purified from this strain, and samples corresponding to the lysate (L), flowthrough (FT), wash (W), and eluate (E) fractions were collected and analyzed by Coomassie staining and SDS-PAGE. The white arrowheads point to GST-YscL and the filled arrowheads to GST. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels. (B) Lysate (L) and eluate (E) fractions were analyzed by immunoblotting with indicated antisera (e.g., anti-YscL [ $\alpha$ YscL]). The relative intensity of immunoreactive signals in the lysate and eluate fractions (E/L) is expressed as a ratio to the right of each blot.

removed from bacteria that expressed GST-YscL can secrete YopE at wild-type levels, we conclude that GST-YscL does not affect the stability of the virulence plasmid. We cumulatively conclude that GST-YscL has a dominant-negative effect on secretion which is likely mediated through its repression of the synthesis of machine components.

**GST-YscL associates with YscN and YscQ.** In purifying GST-YscL from a *Y. enterocolitica*  $\Delta$ yscL strain (as described above for GST-YscN), we sought to ascertain the soluble binding partners of YscL in vivo. A Coomassie blue-stained SDS-PAGE analysis of collected fractions revealed selective enrichment of GST-YscL in the eluate as well as degradation products of the fusion protein (Fig. 5A). We immunoblotted lysate and elution fractions with antisera to YscL, YscN, YscQ, YscK, YscD, SycE, and YopE (Fig. 5B). Of these, only immunoblots with GST, YscL, YscN, and YscQ showed substantial enrichment by affinity chromatography (Fig. 5B). We conclude that in addition to YscN, YscL interacts with YscQ in vivo. Since YscQ copurified with GST-YscL but not with GST-YscN, we posit that YscQ and YscN species that copurify with

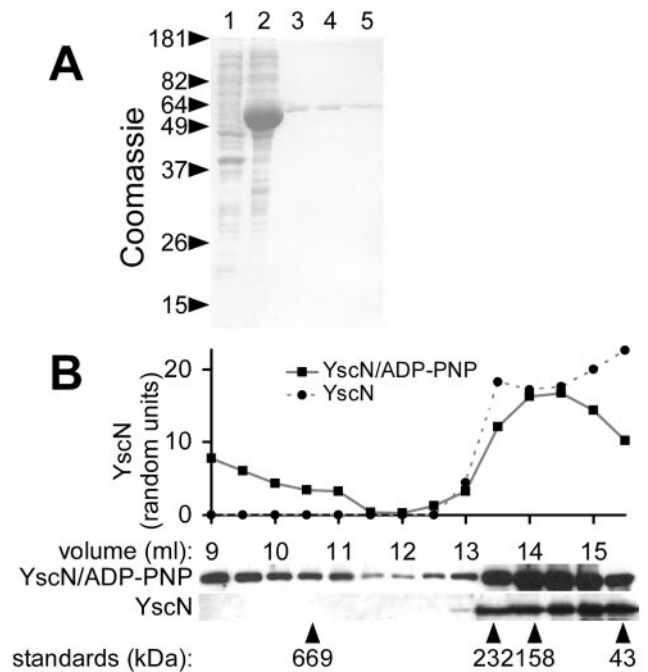


FIG. 6. Purification and gel filtration analysis of His YscN. (A) His YscN purification. *E. coli* capable of His YscN expression was grown in the absence or presence of inducer (lanes 1 and 2, respectively). His YscN was purified on a Ni-NTA column under denaturing conditions (lane 3), renatured, and repurified (lane 4). The elution fractions were ultracentrifuged, and the supernatant was collected (lane 5). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. (B) The gel filtration profile of His YscN was determined in the presence or absence of 5 mM AMP-PNP. Fractions (0.5 ml) were collected and probed for the relative abundance of His YscN by immunoblotting and analysis by densitometry as shown in the graph. The peak elution fractions of calibration standards (in kilodaltons) are indicated below the gels.

GST-YscL represent discrete populations (GST-YscL/YscN as well as GST-YscL/YscQ) but not larger assemblies of GST-YscL/YscN/YscQ.

**Purification of histidine-tagged YscN.** GST-YscN, as purified from *Y. enterocolitica*, is an active ATPase (data not shown). However, the highly heterogeneous nature of this mixture—including as it did a regulator of the ATPase (YscL) and perhaps other proteins—was not suitable for biochemical study of YscN. We therefore expressed an amino-terminal hexahistidine-tagged YscN in *E. coli* (Fig. 6A, lane 2). His YscN was completely insoluble, compelling purification under denaturing conditions (lane 3). His YscN was refolded and concentrated by repurification (lane 4), and we confirmed that this material was soluble by recovering it in the supernatant following ultracentrifugation at 100,000  $\times$  *g* (lane 5).

YscN homologues form higher-order complexes, and these complexes may be stabilized by the addition of nonhydrolyzable ATP analogues (2, 11, 34). To determine the propensity of His YscN for complex formation, we subjected it to gel filtration chromatography in the presence or absence of the nonhydrolyzable ATP analogue AMP-PNP (Fig. 6B). It has been suggested that the hexameric form of the ATPase is the most likely to secrete substrates; a YscN hexamer has an expected molecular mass of ~300 kDa (1, 2, 11). While the



addition of AMP-PNP does lead to the formation of higher-order complexes of  $_{\text{His}}\text{YscN}$ , these are extremely large and irregular as determined by our calibrated gel filtration chromatography and by electron microscopy (Fig. 6B; also data not shown). Nevertheless, the ability of the protein to aggregate upon the addition of an ATP analogue indicated that though renatured,  $_{\text{His}}\text{YscN}$  had demonstrable nucleotide binding properties.

**YscN is a cooperative ATPase.** We sought to characterize the predicted ATPase activity of  $_{\text{His}}\text{YscN}$  with the colorimetric malachite green assay, which measures the release of phosphate (14, 23). By measuring the ATPase activity of  $_{\text{His}}\text{YscN}$  over a range of protein concentrations, we observed a nonlinear increase in the ATPase activity, consistent with positive cooperativity of YscN. The decrease in ATPase activity at high enzyme concentrations is likely the result of enzyme aggregation (Fig. 7A).  $_{\text{His}}\text{YscN}$  ATPase activity was monitored over a range of substrate concentrations (Fig. 7B). When expressed as a Lineweaver-Burk plot, a sigmoidal curve is observed (Fig. 7C). Two phenomena likely generate the observed non-Michaelis-Menten enzyme kinetics: (i) a decrease in activity at high ATP concentrations that likely results from inhibition by high substrate concentrations and (ii) the positive cooperativity of  $_{\text{His}}\text{YscN}$ .

**Noncompetitive inhibition of YscN ATPase activity by YscL.** FliH, the flagellar homologue of YscL, is an inhibitor of the flagellar ATPase FliI (32). To determine whether YscL behaved in a similar manner, we purified histidine-tagged YscL from *E. coli* under denaturing conditions and refolded this polypeptide (Fig. 8A). By increasing the concentration of  $_{\text{His}}\text{YscL}$  in an otherwise identical series of reactions, we observed that the ATPase activity of  $_{\text{His}}\text{YscN}$  decreases (Fig. 8B). The addition of a heterologous protein (bovine serum albumin) did not achieve this effect (data not shown). While the relative amounts of YscN and YscL in the context of yersinial secretion is not known, our data suggest that even one YscL dimer (see below) would have an appreciable impact on the activity of a YscN hexamer (decreasing ATPase activity by 33%); two or three YscL dimers would decrease the activity of a YscN hexamer by approximately 58% and 75%, respectively.

To confirm and characterize the inhibitory effect of YscL, we monitored  $_{\text{His}}\text{YscN}$  ATPase activity over a narrow range of low substrate concentrations (1 to 3 mM) with increasing concentrations of  $_{\text{His}}\text{YscL}$ . In this manner, we avoided inhibition of  $_{\text{His}}\text{YscN}$  under high substrate concentrations. When viewed as a Lineweaver-Burk graph, the plots converge on the axis corresponding to  $1/[S]$ , suggesting that YscL binds to YscN allosterically and impairs its affinity for substrate (*S*) noncompetitively (Fig. 8C). This corroborates the earlier observation that FliH remains associated with FliI as it consumes ATP (32).

**Evidence for YscL dimerization.** The dimerization of *S. enterica* serovar Typhimurium YscL homologues has been described previously (31). To establish that YscL has the capacity for dimerization, we charged glutathione-Sepharose beads with GST or GST-YscL. This column was incubated with a lysate containing  $_{\text{His}}\text{YscL}$ . Retention of  $_{\text{His}}\text{YscL}$  by the column was observed only when GST-YscL was on the column, indicating that the protein interacts with itself, most likely as a dimer (Fig. 9).

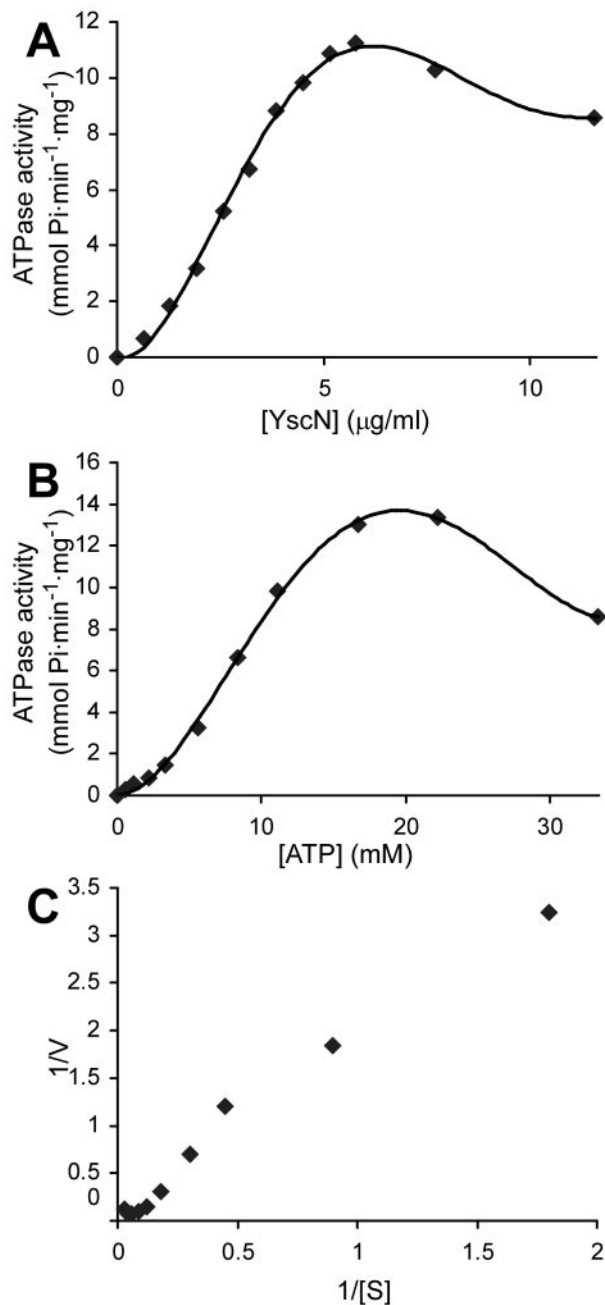


FIG. 7.  $_{\text{His}}\text{YscN}$  functions as an ATPase. (A) ATPase activity of  $_{\text{His}}\text{YscN}$  was measured as a function of increasing concentrations of  $_{\text{His}}\text{YscN}$ . (B) ATPase activity of  $_{\text{His}}\text{YscN}$  was measured as a function of substrate concentrations. (C) Data collected in panel B presented as a Lineweaver-Burk plot. S, substrate; V, velocity of the enzymatic reaction.

## DISCUSSION

YscN, a secretion ATPase, likely plays a central role in recognition and initiation of type III substrates. Using purified recombinant YscN, we were unable to observe hexamerization of the ATPase. In accordance with the observations of Zhu and colleagues, we anticipate that YscN as a hexameric ring sits adjacent to the inner membrane with YscL, receiving chaper-

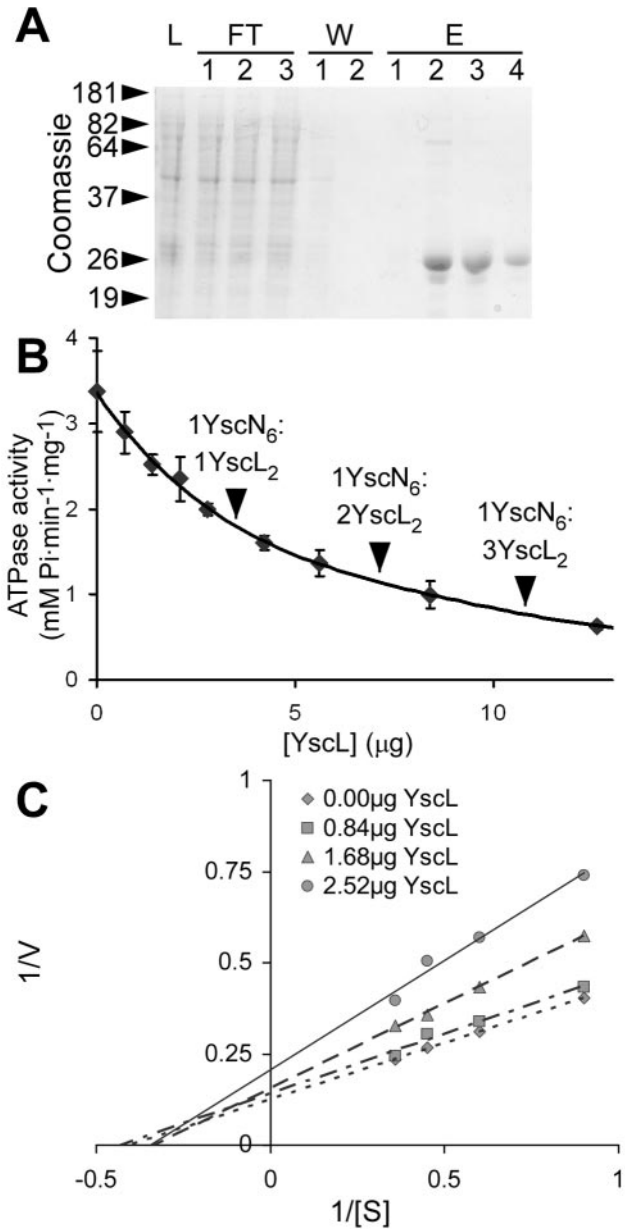


FIG. 8. His YscL purification and noncompetitive inhibition of His YscN. (A) His YscL was purified from *E. coli*, and samples corresponding to the lysate (L), flowthrough (FT), wash (W), and eluate (E) fractions were collected and analyzed by Coomassie blue staining and SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. (B) The effect of His YscL on His YscN activity was measured over the range of indicated concentrations of His YscL. Error bars indicate standard deviations derived from three independent measurements. Arrowheads identify the relative ratios of YscN (assuming it is a hexamer [YscN<sub>6</sub>]) and YscL (assuming it is a dimer [YscL<sub>2</sub>]) along the curve. (C) The inhibition of His YscN activity by His YscL was measured between 1 and 3 mM ATP as a function of the indicated concentrations of His YscL. S, substrate; V, velocity of the enzymatic reaction.

ones bound to effectors and consuming ATP in the context of a larger machine (55). Individual experiments have revealed a network of interactions and biochemical effects that have, however, not yet been elucidated to reveal the cumulative interplay

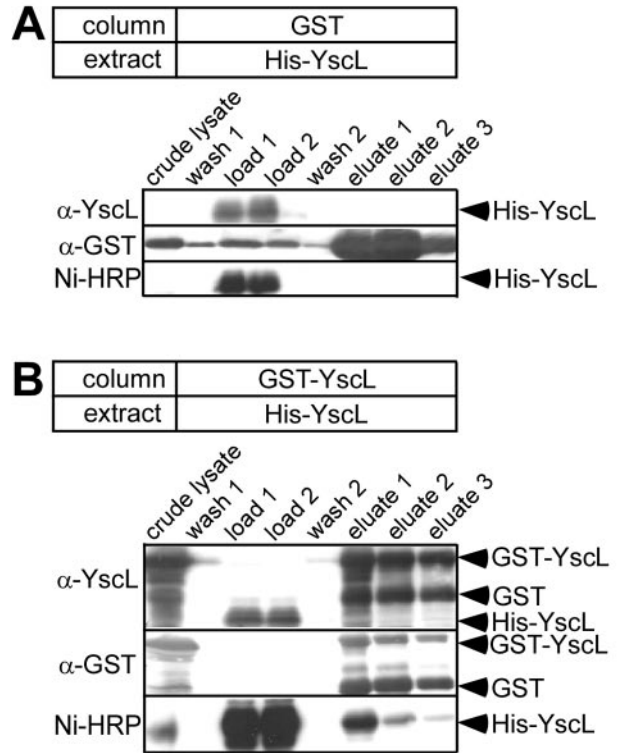


FIG. 9. YscL can interact with itself. (A) Glutathione-Sepharose beads were charged with GST purified from an *E. coli* lysate. This column was incubated with an *E. coli* lysate containing His YscL, and retention of proteins by the beads was monitored in the indicated fractions by immunoblotting to YscL, GST, and the histidine tag.  $\alpha$ -YscL, anti-YscL; Ni-HRP, horseradish peroxidase. (B) Glutathione-Sepharose beads were charged with GST-YscL and then mixed with *E. coli* lysate containing His YscL; His YscL binding to GST-YscL was monitored as described above for panel A.

of their effects. For instance, if YscL is an inhibitor of YscN as is suggested by our experiments (Fig. 8) and those of others (32), does it always inhibit YscN or is it in turn regulated or removed? A likely explanation emerges from the observation that a YscN homologue becomes a more active ATPase (by 1 order of magnitude) in the presence of phospholipids (11). The antagonistic activities of phospholipids and YscL on YscN could mechanistically ensure that the ATPase activity of YscN is maximally active only at the site of protein unfolding and translocation.

If the YscL-YscN complex can maximally consume ATP at the bacterial membrane, additional regulatory mechanisms must ensure that it does so only in the context of type III secretion. The C ring, an antechamber to the secretion channel in the bacterial cytoplasm, may serve such a function (47). Bioinformatic analysis suggests that YscQ is one of the structural components of the antechamber; YscQ interacts with YscL in biochemical (Fig. 5B) and two-hybrid experiments (21). Given these observations, it is conceivable that YscL may serve the additional function of tethering YscN near the secretion channel. Tethering YscN to the machine seems plausible given its presumed low copy number (there are only 1,000 molecules of the flagellar ATPase Flii per cell) (50) Tethering the ATPase to the machine would also imply that the machine



is primed to secrete at all times and could do so the instant the opportunity or the need arises.

The interactions of YscL and YscN and their homologues have previously been reported in the context of in vitro and two-hybrid systems (21, 22, 32). Here, we confirm these observations and extend them in vivo (Fig. 2 and 5). GST fusions to YscN and YscL (when expressed at extremely low levels) fortuitously disrupted the regulation of the *Yersinia* type III machine that keeps secretion in check in the presence of extracellular calcium. Since the fusion proteins did not lose their interactions with any of the anticipated proteins, one must look elsewhere for an explanation. We can only conjecture that perhaps interactions with a negative regulator of secretion, such as YopN-TyeA (9, 16), are destabilized upon low-level GST-YscL expression or in the presence of GST-YscN.

High levels of expression of GST-YscL completely shut off secretion and also abolish expression of some machine components (Fig. 4). Since low levels of GST-YscL permit abundant secretion (Fig. 4), the GST moiety on YscL is probably not responsible for this phenotype. Rather, we hypothesize that this is the result of titration of another soluble component of the machine, perhaps YscN. This does not seem unreasonable, since YscN is likely required to complete the assembly of machine components, and the titration of YscN would therefore lead to incomplete assembly of type III machines.

The inability of GST-YscL or GST-YscN to pull out chaperones or their associated effectors (Fig. 2 and 5) may appear inconsistent with recent reports that ATPases and chaperones interact (1, 18, 48, 49). In all these reports, purified ATPase alone and chaperone-effector complexes were mixed in vitro, and in all cases, interactions were observed or inferred. These experiments differ from the ones reported here. For one, GST-YscN is not very abundant in the cytoplasm of *Y. enterocolitica* (Fig. 2). If only a small portion of GST-YscN was bound to each of the secretion substrates/chaperones at all times, no single chaperone or effector would be enriched by affinity chromatography *ex vivo*. Moreover, in vivo interactions between the secretion machine and substrates are transitory and highly regulated to achieve specific spatiotemporal secretion patterns. Our in vitro experimental results demonstrate that YscL is a potent inhibitor of YscN activity. It is conceivable, though it seems unlikely to us, that YscL alone imposes this regulation on YscN in vivo. Further work is required to reconcile in vitro and in vivo observations, and perhaps this will eventually yield a unified theory of type III secretion.

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