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YscP and YscU Switch the Substrate Specificity of the *Yersinia* Type III Secretion System by Regulating Export of the Inner Rod Protein YscI▽†

Sarah E. Wood,[1,2] Jin Jin,[1] and Scott A. Lloyd[1]*

Department of Pediatrics, Center for Vaccine Development, University of Maryland at Baltimore, Baltimore, Maryland 21201,[1] and Department of Food Science and Technology, University of Maryland Eastern Shore, Princess Anne, Maryland 21853[2]

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Pathogenic yersiniae utilize a type III secretion system to inject antihost factors, called Yops, directly into the cytosol of eukaryotic cells. The Yops are injected via a needle-like structure, comprising the YscF protein, on the bacterial surface. While the needle is being assembled, Yops cannot be secreted. YscP and YscU switch the substrate specificity of the secretion system to enable Yop export once the needle attains its proper length. Here, we demonstrate that the inner rod protein YscI plays a critical role in substrate specificity switching. We show that YscI is secreted by the type III secretion system and that YscI secretion by a *yscP* mutant is abnormally elevated. Furthermore, we show that mutations in the cytoplasmic domain of YscU reduce YscI secretion by the *yscP* null strain. We also demonstrate that mutants expressing one of three forms of YscI (those with mutations Q84A, L87A, and L96A) secrete substantial amounts of Yops yet exhibit severe defects in needle formation. In the absence of YscP, mutants with the same changes in YscI assemble needles but are unable to secrete Yops. Together, these results suggest that the formation of the inner rod, not the needle, is critical for substrate specificity switching and that YscP and YscU exert their effects on substrate export by controlling the secretion of YscI.

There are three *Yersinia* species pathogenic for humans: *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* is the causative agent of plague, while infection with the enteric pathogen *Y. pseudotuberculosis* or *Y. enterocolitica* generally results in self-limited mesenteric lymphadenitis (8, 44). Despite causing diseases with very different clinical outcomes, pathogenic yersiniae share a common 70-kb virulence plasmid (referred to as pCD, pIB, or pYV) (10). The principle virulence determinant encoded by the 70-kb plasmid is a type III secretion system (T3SS) (11). Pathogenic yersiniae use the T3SS to inject effector proteins called Yops (*Yersinia o*uter *p*roteins) into eukaryotic cells to inhibit phagocytosis (17, 46) and suppress host immune responses (24, 40, 53).

Many of the components of the *Yersinia* T3SS are conserved in the T3SSs of other gram-negative pathogens (15, 22). In addition, nine components of the export apparatus responsible for assembling bacterial flagella are conserved (30). In addition to showing sequence homology to flagella, the T3SSs of gram-negative pathogens share structural features with flagella. For example, the T3SS encoded by *Salmonella* pathogenicity island 1 (SPI1) resembles the flagellar basal body (25, 26). The T3SS of *Shigella flexneri* exhibits a similar structure (6, 54). The surface appendages of the T3SSs of gram-negative pathogens differ from the hook and filament of the flagellum in that they generally consist of hollow needle-like structures that likely serve as conduits for effector proteins destined for the eukaryotic cell cytosol (6, 21, 25, 54).

Virulence-associated T3SSs and flagella are also similar in that the lengths of their surface appendages are regulated. For example, the length of the *Salmonella enterica* serovar Typhimurium flagellar hook is 55 nm (20). The needle structures of the SPI1, *Shigella*, and *Yersinia* T3SSs range from 45 to 80 nm in length (6, 21, 25, 26, 54). The regulation of needle length is critical, as Mota et al. demonstrated that the *Yersinia* needle structure must attain a minimal length in order for Yops to be delivered into eukaryotic cells (38).

As first described for the flagellar system, the type III secretion machinery regulates the lengths of these surface structures by imposing a hierarchy in substrate export. A previous study found that null mutations in *fliK* result in a polyhook phenotype in which hooks sometimes reach several microns in length (42). Unlike normal flagella, these mutant structures do not assemble a filament onto the end of the hook. This study led to the notion that FliK is responsible for switching the substrate specificity of the flagellar export apparatus once the hook reaches its normal length. Such a substrate specificity switching defect has also been observed in the T3SSs of gram-negative pathogens. In an *invJ* mutant, the SPI1 T3SS assembles abnormally elongated needle structures on the cell surface (27) but does not secrete effector proteins (9). Similarly, *Shigella spa32* (54, 55) and *Yersinia yscP* (23) mutants assemble elongated needles but secrete only low levels of effector proteins (31, 43, 49, 55). Although FliK, InvJ, Spa32, and YscP do not show significant amino acid similarity, Agrain et al. used hydrophobic cluster analyses to show that the C termini of these proteins consist of a globular domain, termed T3S4 (*t*ype *III s*ecretion and *s*ubstrate *s*pecificity *s*witch) (1). Mutations within this domain of the FliK (37, 57) and YscP (1) proteins abolish sub-

* Corresponding author. Mailing address: Department of Pediatrics, Center for Vaccine Development, University of Maryland at Baltimore, 685 W. Baltimore St., Baltimore, MD 21201. Phone: (410) 706-5336. Fax: (410) 706-6205. E-mail: slloyd@medicine.umaryland.edu.

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strate specificity switching of the flagellar and *Yersinia* T3SSs, respectively.

FliK (36), InvJ (9), Spa32 (31, 55), and YscP (43, 49) are all secreted proteins, which raises the possibility that they physically measure hook or needle length. By introducing deletions or insertions into the central region of YscP, Journet et al. were able to change the lengths of the needle structures of the *Yersinia* T3SS (23). Specifically, shorter versions of YscP made shorter needles, while elongated versions of YscP made longer needles, thereby suggesting that YscP functions as a molecular ruler. Analogous models to explain the function of FliK in regulating hook length have been put forth (37). However, Marlovits et al. challenged the molecular ruler hypothesis by demonstrating that InvJ is required for the formation of an inner rod structure (32), comprising the PrgJ protein (33), of the SPI1 T3SS. This finding suggests that the formation of the inner rod, not the maturation of the needle structure, is the critical event that triggers substrate specificity switching.

The export apparatus component involved in regulating substrate specificity was first identified in the flagellar system. Specifically, it was shown previously that mutations in *flhB*, which encodes one of the inner membrane proteins that is part of the flagellar export apparatus (30), can partially suppress the phenotype of a *fliK* mutant (20, 28, 57). The presence of these suppressor mutations results in a polyhook-filament phenotype in which a flagellar filament is now assembled onto the end of an elongated hook. Although FlhB is an inner membrane protein, all of the mutations suppressing the *fliK* mutant phenotype map to the cytoplasmic domain. The *Yersinia* T3SS operates in a similar fashion, as Edqvist et al. showed that the phenotype of a *yscP* mutant can be partially suppressed by mutations in the cytoplasmic domain of YscU (13). Specifically, mutations in the cytoplasmic domain of YscU conferred greater levels of Yop secretion on the *yscP* mutant while reducing the export of the needle component YscF.

In the present study, we examined the roles of YscP, YscU, and the inner rod protein YscI in switching the substrate specificity of the virulence plasmid-encoded T3SS of *Y. pseudotuberculosis*. We showed that YscI was secreted by the T3SS and that YscP and YscU regulated the secretion of YscI. Specifically, YscI was secreted in abnormally large amounts by a *yscP* mutant. Mutations in the cytoplasmic domain of YscU, previously shown to suppress the *yscP* mutant phenotype (13), reduced the amount of YscI secretion by the *yscP* mutant. Furthermore, using site-directed mutagenesis, we demonstrated that bacteria expressing one of three YscI mutant forms (those with mutations Q84A, L87A, and L96A) were able to undergo substrate specificity switching yet were defective in needle assembly. When these same YscI mutant proteins were expressed in a *yscP* mutant background, the mutants assembled needles but did not secrete late substrates. This finding suggests that the formation of the inner rod, not the formation of the needle, is critical for substrate specificity switching and that YscP and YscU regulate the substrate specificity of the *Yersinia* T3SS by modulating the secretion of YscI.

MATERIALS AND METHODS

Media and growth conditions. *Escherichia coli* strains were grown in Luria-Bertani broth. *Yersinia* strains were grown in brain heart infusion (BHI) broth supplemented with either 2.5 mM $CaCl_2$ (BHI plus calcium) or 5 mM EGTA and 20 mM $MgCl_2$ (BHI minus calcium); Triton X-100 was present in all media at 0.1% to prevent secreted proteins from adhering to the bacteria. Kanamycin at 50 μg/ml was added to select for the virulence plasmid; ampicillin at 100 μg/ml was added to maintain ColE1 ori plasmids expressing YscI, YscP, and YscU; chloramphenicol at 34 μg/ml was added to maintain a compatible p15a ori YscP-expressing plasmid. Strains and plasmids are listed in Table 1.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
S17-1λ*pir*	Recipient for suicide plasmids	48
BL21(DE3)	Host for protein expression experiments	51
E. cloni	Recipient for cloning experiments	Lucigen
TOP10	Recipient for cloning experiments	Invitrogen
Y. pseudotuberculosis		
SAL1	YPIII(pIB102), wild-type parental strain	7
SAL2	*yscP* mutant	This study
SAL3	*yscP yscU* double mutant	This study
SAL4	*yscI* mutant	This study
SAL5	*yscS* mutant	5
SAL6	*yscI yscP* double mutant	This study
Plasmids		
pSE380	High-copy-no. expression vector	Invitrogen
pSW71	Vector for YscI expression from pSE380	This study
pSW71-P77A	P77A mutation in pSW71	This study
pSW71-L80A	L80A mutation in pSW71	This study
pSW71-M83A	M83A mutation in pSW71	This study
pSW71-Q84A	Q84A mutation in pSW71	This study
pSW71-W85A	W85A mutation in pSW71	This study
pSW71-L87A	L87A mutation in pSW71	This study
pSW71-R89D	R89D mutation in pSW71	This study
pSW71-I90A	I90A mutation in pSW71	This study
pSW71-T91A	T91A mutation in pSW71	This study
pSW71-Q93A	Q93A mutation in pSW71	This study
pSW71-E94K	E94K mutation in pSW71	This study
pSW71-E95K	E95K mutation in pSW71	This study
pSW71-L96A	L96A mutation in pSW71	This study
pSW71-I97A	I97A mutation in pSW71	This study
pSW71-A98V	A98V mutation in pSW71	This study
pSW71-K99E	K99E mutation in pSW71	This study
pSW71-T100A	T100A mutation in pSW71	This study
pSW71-G102A	G102A mutation in pSW71	This study
pSW71-Q106A	Q106A mutation in pSW71	This study
pSW71-N107A	N107A mutation in pSW71	This study
pSW71-E109K	E109K mutation in pSW71	This study
pSW71-T110A	T110A mutation in pSW71	This study
pSW71-L111A	L111A mutation in pSW71	This study
pSW71-K113E	K113E mutation in pSW71	This study
pBAD18	Arabinose-inducible P_{BAD} promoter	18
pBAD33	P_{BAD} promoter; p15a ori	18
pSL327	*yscP* gene in pBAD18	This study
pJJ8	*yscU* gene in pBAD18	This study
pJJ10	*yscP* gene in pBAD33	This study
pSW30	*yscU*(*Y287G*) gene in pBAD18	This study
pSW31	*yscU*(*Y317D*) gene in pBAD18	This study
pGEX-GP-1	GST fusion vector; parent vector	GE Healthcare
pSL303	GST-YscF overexpression vector	This study
pSL310	GST-YscI overexpression vector	This study
pDM4	Suicide vector	35
pSL217	*yscP* deletion in pDM4	This study
pJJ1	*yscI* deletion in pDM4	This study
pJJ2	*yscU* deletion in pDM4	This study

DNA methods. Routine preparations were performed according to the methods of Sambrook et al. (47). Enzymes were obtained from New England Biologicals, except where noted. The sequencing of plasmids was performed by the Biopolymer Core Facility at the University of Maryland School of Medicine.

TABLE 2. Oligonucleotides used in this study

Activity and plasmid (target or product)	Oligonucleotide or oligonucleotide pair[a]	Sequence[b] (restriction endonuclease)
Deletion mutant construction		
pSL217 (*yscP*)	Lloyd249 and Lloyd250	5′-GCC *GCA TGC* GCT TGC AGG CAG CTC ATC GAC-3′ (SphI)
		5′-AGC CTC CCA CTC AGT GGT GAT TTT TAT TCA TTA GGC G-3′
	Lloyd251 and Lloyd252	5′-AAA ATC ACC ACT GAG TGG GAG GCT GAA GAA TGA G-3′
		5′-GGC *TCT AGA* GCT GAG GTT AGC CAG AAG GAG-3′ (XbaI)
pJJ1 (*yscI*)	Lloyd325 and Lloyd326	5′-GCC *TCT AGA* GTG AAT CCG CTG AAA AGA CAC-3′ (XbaI)
		5′-CGA CAA GGT TTC AGC TAT TTC TAT GTT CGG CAT-3′
	Lloyd327 and Lloyd328	5′-ATA GAA ATA GCT GAA ACC TTG TCG AAG GGG GGG-3′
		5′-GGC *CTC GAG* GCC CGT AAT TTC AGA TAA AG-3′ (XhoI)
pJJ2 (*yscU*)	Lloyd316 and Lloyd317	5′-GCC *CTC GAG* CTC AAT CCT GGA CTT GAT AGT C-3′ (XhoI)
		5′-TTC GGA ATG TTG TTG CTC TGT CTT TTC TCC TCC GCT T-3′
	Lloyd318 and Lloyd333	5′-AAG ACA GAG CAA CAA CAT TCC GAA ATG TTA TAA TAG GC-3′
		5′-GCT CAC *GAG CTC* GCA CAG GAG AAA TAC AAT TAC C-3′ (SacI)
Antibody production		
pSL303 (GST-YscF)	Lloyd222 and Lloyd244	5′-GCC *GAA TTC* ATG AGT AAC TTC TCT GGA TTT AC-3′ (EcoRI)
		5′-GGC *CTC GAG* TAA TGG AAC CTC TAT TAA GGG-3′ (XhoI)
pSL310 (GST-YscI)	Lloyd224 and Lloyd225	5′-GCC *GAA TCC* ATG CCG AAC ATA GAA ATA GCT C-3′ (EcoRI)
		5′-GGC *CTC GAG* TCA CCC CCC CTT CGA CAA GGT-3′ (XbaI)
Complementation		
pSL322 (YscP)	Lloyd344 and Lloyd252	5′-GCC GCC *GTC GAC* GCT TGC AGG CAG CTC ATC GAC-3′ (SalI)
		5′-GGC *TCT AGA* GCT GAG GTT AGC CAG AAG GAG-3′ (XbaI)
pSW71 (YscI)	Lloyd325 and Lloyd328	
pJJ3 (YscU)	Lloyd330 and Lloyd333	5′-GC TGA *TCT AGA* TAT TAA TCG CCG CTG TAT TGG C-3′ (XbaI)
		5′-GCT CAC *GAG CTC* GCA CAG GAG AAA TAC AAT TAC C-3′ (SacI)
Site-directed mutagenesis		
pSW30 (YscU Y287G mutant form)	Lloyd544 and Lloyd545	5′-GTA ACA TTC AAA **GG**T ACC GAT GCC CAA G-3′
		5′-CTT GGG CAT CGG TA**C** **C**TT TGA ATG TTA C-3′
pSW31 (YscU Y317D mutant form)	Lloyd548 and Lloyd549	5′-GCC CGT GCT CTT **G**AT TGG GAT GCG CTC G-3′
		5′-CGA GCG CAT CCC AAT **C**AA GAG CAC GGG C-3′
pSW71-P77A	Lloyd359	5′-CAF TTG ATA AT**G** CCA ACG ACC TG-3′
pSW71-L80A	Lloyd360	5′-AAT CCC AAC GAC **GC**G ATG CTA ATG C-3′
pSW71-M83A	Lloyd361	5′-CGA CCT GAT GCT A**GC** GCA ATG GTC AC-3′
pSW71-Q84A	Lloyd362	5′-CCT GAT GCT AAT G**GC** ATG GTC ACT TAT CC-3′
pSW71-W85A	Lloyd363	5′-GAT GCT AAT GCA A**GC** GTC ACT TAT CCG-3′
pSW71-L87A	Lloyd364	5′-ATG CAA TGG TCA **GC**T ATC CGT ATA AC-3′
pSW71-R89D	Lloyd365	5′-TGG TCA CTT ATC **GA**T ATA ACA ATC CAA G-3′
pSW71-I90A	Lloyd366	5′-GTC ACT TAT CCG T**GC** AAC AAT CCA AGA AG-3′
pSW71-T91A	Lloyd367	5′-CTT ATC CGT ATA **G**CA ATC CAA GAA GAA C-3′
pSW71-Q93A	Lloyd368	5′-CGT ATA ACA ATC **GC**A GAA GAA CTT ATC G-3′
pSW71-E94K	Lloyd369	5′-ATA ACA ATC CAA **A**AA GAA CTT ATC GCC-3′
pSW71-E95K	Lloyd370	5′-ACA ATC CAA GAA **A**AA CTT ATC GCC AAG-3′
pSW71-L96A	Lloyd371	5′-ATC CAA GAA GAA **GC**T ATC GCC AAG ACT G-3′
pSW71-I97A	Lloyd372	5′-CCA AGA AGA ACT T**GC** CGC CAA GAC TGC CG-3′
pSW71-A98V	Lloyd373	5′-GAA CTT ATC G**T**C AAG ACT GCC GG-3′
pSW71-K99E	Lloyd374	5′-GAA CTT ATC GCC **G**AG ACT GCC GGG-3′
pSW71-T100A	Lloyd375	5′-TAT CGC CAA G**G**C TGC CGG GCG-3′
pSW71-G102A	Lloyd376	5′-AAG ACT GCC GCG **C**GA ATG AGC-3′
pSW71-Q106A	Lloyd377	5′-GGG CGA ATG AGC **GC**A AAT GTT GAA ACC-3′
pSW71-N107A	Lloyd378	5′-CGA ATG AGC CAA **GC**T GTT GAA ACC TTG-3′
pSW71-E109K	Lloyd379	5′-GCC AAA ATG TT**A** AAA CCT TGT CG-3′
pSW71-T110A	Lloyd380	5′-AAT GTT GAA **G**CC TTG TCG GAA GG-3′
pSW71-L111A	Lloyd381	5′-AAT GTT GAA ACC **GC**G TCG AAG GGG GG-3′
pSW71-K113E	Lloyd382	5′-ACC TTG TCG **G**AG GGG GGG TG-3′

[a] For oligonucleotide pairs, sequences are listed respectively.
[b] Restriction sites used for subcloning are italicized. Complementary sequences for primer pairs used in overlapping PCR are underlined. Nucleotides altered via site-directed mutagenesis are in bold.

Construction of the *yscP* null strain. Overlapping PCR (2) with the primer pairs Lloyd249/Lloyd250 and Lloyd251/Lloyd252 (primers are listed in Table 2) was used to amplify genomic DNA of the wild-type *Y. pseudotuberculosis* strain YPIII(pIB102) (SAL1) and join sequences flanking the *yscP* gene. The resulting PCR product was digested with SphI and XbaI and cloned into the corresponding sites of the sucrose-selectable suicide plasmid pDM4 (35) to yield pSL217. *E. coli* strain S17-1λ*pir* (48) was transformed with pSL217, and pSL217 was conjugated into SAL1 (7) by the plating of bacteria onto cefsulodin-irgasan-novobiocin *Yersinia*-selective agar containing 34 μg of chloramphenicol/ml. Exconjugants were restreaked onto Luria-Bertani plates containing 5% sucrose to eliminate

bacteria still carrying pSL217. Sucrose-resistant colonies were subjected to PCR amplification with the Lloyd249/Lloyd252 primer pair to confirm the presence of the deletion. The resulting *yscP* null strain, SAL2, lacks codons 7 to 449 of the *yscP* gene.

Construction of the *yscP yscU* double null strain. Overlapping PCR (2) with the primer pairs Lloyd316/Lloyd317 and Lloyd318/Lloyd333 was used to amplify and join sequences flanking the *yscU* gene. The resulting PCR product was digested with SacI and XhoI and cloned into pDM4 (35) to yield pJJ2. pJJ2 was conjugated into the *yscP* null strain SAL2 as described above. The resulting *yscP yscU* double null strain, SAL3, lacks codons 9 to 348 of the *yscU* gene.

Construction of the *yscI* null strain. Overlapping PCR (2) with the primer pairs Lloyd325/Lloyd326 and Lloyd327/Lloyd328 was used to amplify and join sequences flanking the *yscI* gene. The resulting PCR product was digested with XbaI and XhoI and cloned into pDM4 (35) to yield pJJ1. pJJ1 was conjugated into the wild-type strain SAL1 as described above. The resulting *yscI* null strain, SAL4, lacks codons 8 to 108 of the *yscI* gene.

Construction of the *yscI yscP* double null strain. pJJ1 was conjugated into the *yscP* null strain SAL2 as described above. The resulting *yscI yscP* double null strain was designated SAL6.

Preparation of YscF antibody. YPIII(pIB102) genomic DNA was PCR amplified using the primer pair Lloyd222 and Lloyd244. The resulting PCR product, which comprised the *yscF* gene, was digested with EcoRI and XhoI and cloned into the corresponding sites of pGEX-6P-1 to yield pSL303. *E. coli* strain BL21(DE3) (19, 51) was transformed with pSL303, which expresses a glutathione *S*-transferase (GST)-YscF fusion protein, and inclusion bodies were isolated, washed, and solubilized in 8 M urea, largely as described previously (29). Solubilized GST-YscF was isolated via preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel slices were excised and used to raise a polyclonal antiserum in rabbits (Lampire Biologicals). The antiserum was used without further purification.

Preparation of YscI antibody. YPIII(pIB102) genomic DNA was PCR amplified with the Lloyd224/Lloyd225 primer pair. The resulting PCR product, encompassing the *yscI* gene, was digested with EcoRI and XhoI and cloned into pGEX-6P-1 to yield pSL310. The resulting GST-YscI fusion protein was isolated from inclusion bodies and used to raise a polyclonal antiserum in rabbits (Lampire Biologicals). The resulting antiserum was affinity purified against membrane-immobilized GST-YscI according to standard protocols (19).

Construction of YscI complementation plasmids. Primer pair Lloyd325/Lloyd328 was used to amplify the *yscI* gene, as well as a total of approximately 400 bp of upstream and downstream sequences. The resulting PCR product was digested with XbaI and XhoI and cloned into the high-copy-number plasmid pSE380 (Invitrogen) to yield pSW71. The expression of YscI is under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *trc* promoter and utilizes the native *yscI* Shine-Dalgarno site.

Sequence alignment analysis of YscI. Pallen et al. first demonstrated that YscI is homologous to the SPI1 T3SS inner rod component PrgJ and other members of this protein family (41). They also determined that inner rod proteins show significant homology to proteins that constitute the needle structures of T3SSs. Using PSI-BLAST searches (4), we confirmed the results of Pallen et al. and identified 24 amino acid residues in the C terminus of YscI that are identical to residues in homologous proteins from the T3SSs of *Aeromonas hydrophila* (AscI), *Photorhabdus luminescens* (LscI), *Pseudomonas aeruginosa* (PscI), and *Vibrio parahaemolyticus* (VscI). The residues are P77, L80, M83, Q84, W85, L87, R89, I90, T91, Q93, E94, E95, L96, I97, A98, K99, T100, G102, Q106, N107, E109, T110, L111, and K113. The sequences of the aforementioned proteins were also aligned with those of the inner rod proteins recombinant Orf8 (*E. coli*), MxiI (*Shigella flexneri*), and PrgJ (*Salmonella* serovar Typhimurium). In addition, alignments included sequences of the needle proteins YscF (*Y. pseudotuberculosis*), MxiH (*Shigella flexneri*), PrgI (*Salmonella* serovar Typhimurium), and BsaL (*Burkholderia pseudomallei*). Alignments were performed using the Clustal server (www.ch.embnet.org/software/ClustalW.html), and the results were printed using the Boxshade server (www.ch.embnet.org/software/BOX_form.html).

Site-directed mutagenesis of *yscI*. Variants with site-directed mutations were constructed using the GeneEditor site-directed mutagenesis kit according to the instructions of the manufacturer (Promega). Plasmid pSW71 served as template DNA. The oligonucleotide primers used are shown in Table S1 in the supplemental material.

Construction of YscP complementation plasmids. Primer pair Lloyd252/Lloyd344 was used to amplify the *yscP* gene, as well as approximately 400 bp of upstream and 400 bp of downstream sequences. The resulting PCR product was digested with SalI and XbaI and cloned into the high-copy-number plasmid pSE380 (Invitrogen) to yield pSL322. The expression of YscP was under the control of the IPTG-inducible *trc* promoter and utilized the native *yscP* Shine-Dalgarno site. pSL322 was introduced into the *yscP* null strain, SAL2, by electroporation; however, complementation was poor. Therefore, pSL322 was digested with EcoRI and XbaI and the *yscP*-expressing insert was cloned into the corresponding sites of pBAD18 (18). The resulting plasmid, pSL327, expresses YscP from the arabinose-inducible P_{BAD} promoter and utilizes the native *yscP* Shine-Dalgarno site. For experiments in which both YscI and YscP were expressed in *trans* in the *yscI yscP* double null strain, a compatible YscP plasmid was constructed by digesting pSL327 with EcoRI and XbaI. The *yscP*-expressing insert was cloned into the corresponding sites of plasmid pBAD33 (18), which utilizes the origin of replication from pACYC184, to yield pJJ10.

Construction of mutations in the cytoplasmic domain of YscU. Primer pair Lloyd330/Lloyd333 was used to amplify the *yscU* gene, as well as approximately 400 bp of upstream and 400 bp downstream sequences, by PCR. The resulting PCR product was digested with XbaI and SacI and cloned into pSE380 to yield pJJ3. The ability of pJJ3 to *trans*-complement a *yscU* null strain was poor. Therefore, pJJ3 was digested with XbaI and HinDIII and the *yscU*-expressing insert was cloned into pBAD18 to yield pJJ8. The YscU Y287G (pSW30) and YscU Y317D (pSW31) mutations were created with the QuikChange II site-directed mutagenesis kit (Stratagene) using pJJ8 as a template and the primer pairs Lloyd545/Lloyd545 and Lloyd548/Lloyd549, respectively.

Type III secretion assay. Overnight cultures of *Yersinia* strains were grown in BHI medium at 26°C. Cultures were diluted to an optical density at 600 nm of 0.2 in 10 ml of either BHI-plus-calcium medium or BHI-minus-calcium medium as appropriate and were grown for 1 h at 26°C and 3 h at 37°C. Cultures were centrifuged at 3,000 × *g* for 15 min. Supernatants containing secreted proteins were passed through a 0.45-μm-pore-size filter and precipitated with 10% trichloroacetic acid (TCA). TCA precipitates were centrifuged at 3,000 × *g* for 15 min. The pellets were resuspended in 200 μl of 2% SDS and precipitated with acetone at −20°C for 30 min. Samples were centrifuged at 15,000 × *g* for 10 min, and the pellets were air dried and resuspended in 100 μl of 8 M urea and an equal amount of 2× sample buffer. Sample volumes were adjusted according to optical density measurements of bacterial cultures. Equal amounts of culture supernatant and cell pellet fractions were separated by SDS–12% PAGE and stained with Coomassie blue or transferred onto polyvinylidene difluoride membranes. Proteins were detected with polyclonal antisera or a mouse monoclonal antibody against the cytoplasmic protein GroEL (Calbiochem) as a control for cell lysis.

Immunoblotting. Samples were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Millipore) by using a semidry transfer apparatus (Bio-Rad). Membranes were blocked for 30 min with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk. Membranes were probed with antisera in 10 ml of blocking buffer for 1 h and washed three times with TBS-T. The membranes were then incubated for 1 h with either anti-rabbit or anti-mouse secondary antibodies (GE Healthcare Life Sciences) in 10 ml of blocking buffer and washed three times with TBS-T. Proteins were detected using the ECL Plus enhanced chemiluminescence detection kit according to the instructions of the manufacturer (GE Healthcare Life Sciences).

Surface localization of YscF. Experiments were performed largely as described by Edqvist et al. (13). Briefly, overnight cultures of *Yersinia* strains were grown at 26°C in BHI medium. Cultures were diluted to an optical density at 600 nm of 0.2 in 10 ml of BHI-plus-calcium medium containing 0.1% Triton X-100 and grown for 1 h at 26°C, followed by 2 h of growth at 37°C. Whole cultures were sheared by five passages through a 22-gauge hypodermic needle. (We [13] and others [39, 52] have used this approach previously to break off the T3SS needle structures from the bacterial surface, thereby providing a better estimate of the amount of the needle component exported by the T3SS.) Culture supernatants were isolated as described above and precipitated with TCA. Sample volumes were adjusted based on optical density measurements of bacterial cultures. Equal amounts of culture supernatant and cell pellet fractions were run on SDS-PAGE gels (16% acrylamide). YscF was detected using a polyclonal antiserum.

Cross-linking of YscF. Experiments were performed largely as described by Torruellas et al. (56). Briefly, overnight cultures of *Yersinia* strains were grown at 26°C in BHI medium. Cultures were diluted to an optical density at 600 nm of 0.2 in 10 ml of BHI medium and grown for 1 h at 26°C, followed by 2 h of growth at 37°C. One-milliliter culture aliquots were washed in phosphate-buffered saline (PBS) and resuspended in 500 μl of PBS. An equal amount of PBS containing 5 mM bis(sulfosuccinimidyl)suberate (BS^3) was then added, and the suspensions were incubated for 30 min at 37°C. Excess cross-linker was then quenched via the addition of 50 μl of 1 M Tris-Cl, pH 8.0, for 15 min. Bacteria were then centrifuged (12,000 × *g* for 5 min), and the bacterial pellet was resuspended in 20 μl of water. An equal volume of 2× sample buffer was added, and the samples were boiled for 2 min. Samples were run on SDS-PAGE gels (12 or 16%

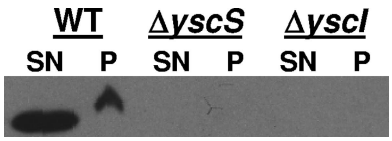

FIG. 1. YscI is a secreted substrate of the T3SS. Wild-type (WT) and *yscS* and *yscI* mutant strains were grown at 37°C in BHI-minus-calcium medium. Secreted supernatant (SN) and cell pellet (P) fractions were separated by centrifugation, and proteins in the supernatant were TCA precipitated. Equal percentages of the two fractions were run on SDS-PAGE gels, and YscI was detected by Western blotting with a polyclonal antiserum.

acrylamide). YscF was detected by Western blotting using a polyclonal antiserum. As shown previously (12, 56), the formation of YscF multimers was dependent upon the presence of BS^3 (data not shown).

RESULTS

YscI is secreted by the T3SS. PrgJ constitutes the inner rod of the SPI1 T3SS (32, 33) and is secreted by the T3SS (52). A recent report by Pallen et al. showed that PrgJ is a member of a family of proteins that are found in other T3SSs and that YscI is the *Yersinia* PrgJ homolog (41). Therefore, we sought to determine whether YscI is secreted by the *Yersinia* T3SS. Wild-type (SAL1) and *yscS* (SAL5) and *yscI* (SAL4) mutant strains were grown under Yop-inducing conditions, and secreted supernatant and cell pellet fractions were isolated (Fig. 1). The results show that YscI was found in both the secreted supernatant and cell pellet fractions of the wild-type strain. YscI expression in the *yscS* strain (5) that lacks a functional T3SS was not observed. An analogous result for PrgJ was observed by Sukhan et al. (52), who showed that PrgJ is not expressed in an *invA* mutant that lacks a functional T3SS. The fact that no bands were observed for the *yscI* mutant confirms that our polyclonal antiserum was indeed specific for YscI. Based on these results, we conclude that YscI is a secreted substrate of the *Yersinia* T3SS.

YscP and YscU regulate YscI secretion. Previous work on the SPI1 T3SS demonstrated that InvJ is required for the assembly of the inner rod component PrgJ (32). In an *invJ* mutant, increased amounts of PrgJ are instead secreted into the culture supernatant (52). Based on this work, we sought to determine whether YscP (the *Yersinia* InvJ homolog) regulates the secretion of YscI. Wild-type and *yscP* null strains were grown at 37°C in either the absence or the presence of calcium, and secreted supernatant and cell pellet fractions were isolated. In the absence of calcium, the wild-type strain, but not the *yscP* mutant (43), induces the low-calcium response (LCR); the induction of the LCR results in the up-regulation of the expression of the T3SS (14, 16, 45, 50). In the presence of calcium, neither strain induces the LCR. The results show that both the wild-type and *yscP* strains secreted large amounts of YscI in calcium-deficient medium, although YscI was more abundant in the wild-type strain due to the induction of the LCR (Fig. 2A). However, when YscI secretion in the presence of calcium was examined, we observed that the *yscP* mutant secreted substantially greater amounts of YscI into the culture supernatant than did the wild-type strain. *Trans*-complementation of the *yscP* null strain with a plasmid expressing native YscP (pSL327) and with a vector control (pBAD18) yielded

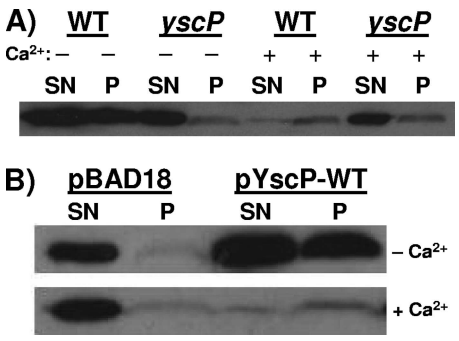

FIG. 2. YscP regulates YscI secretion. (A) Wild-type (WT) strain SAL1 and *yscP* mutant strain SAL2 were grown in either BHI-minus-calcium medium (−; LCR-inducing conditions) or BHI-plus-calcium medium (+; LCR-inhibiting conditions). Secreted supernatant (SN) and cell pellet (P) fractions were separated by centrifugation, and proteins in the supernatant were TCA precipitated. Equal percentages of the two fractions were run on SDS-PAGE gels, and YscI was detected by Western blotting using a polyclonal antiserum. (B) The *yscP* mutant SAL2 was *trans*-complemented with a vector control (pBAD18) or a plasmid expressing YscP (pYscP-WT). Bacteria were grown as described above, and the amounts of YscI in secreted supernatant and cell pellet fractions were determined as previously described.

the same phenotype (Fig. 2B). That is, in calcium-containing medium, YscI secretion in the absence of YscP was substantially elevated but the amounts of YscI secretion by the two strains were comparable when the strains were grown in calcium-deficient medium, with YscI being more abundant in the strain expressing YscP due to the induction of the LCR. We conclude that YscP does in fact regulate the secretion of YscI and that YscI is an early substrate of the T3SS.

In a previous study, we demonstrated that certain mutations in the YscU cytoplasmic domain can partly suppress a *yscP* null phenotype by reducing the amount of surface-located YscF and increasing the amounts of secreted Yops (13). Thus, we sought to determine the effects of some of these YscU mutations on the export of YscI. Either native YscU (pJJ8) or the YscU Y287G (pSW30) or YscU Y317D (pSW31) mutant form was expressed in *trans* under the control of the arabinose-inducible P_{BAD} promoter in a *yscP yscU* double null strain. To confirm that these mutant proteins could reduce YscF export in the absence of YscP, bacteria were grown at 37°C in the presence of calcium and subjected to shearing to release T3SS needle structures (Fig. 3A). The results show that the YscU Y287G mutation slightly reduced the amount of surface-located YscF relative to that in a control strain expressing native YscU. In contrast, the YscU Y317D mutation dramatically reduced the amount of surface-located YscF. As expected, YscF was not observed in the sheared fraction when a vector control (pBAD18) was utilized due to the fact that YscU is required for T3SS function (5). As an additional control for cell lysis, Western blotting was used to detect the cytoplasmic protein GroEL in sheared and cell pellet fractions. The results show that GroEL localized exclusively to the cell pellet fractions, thus demonstrating that the presence of YscF in the sheared fractions was not due to cell lysis (Fig. 3B). These results confirm that mutations in the YscU cytoplasmic domain can suppress the *yscP* null phenotype and that, as noted pre-

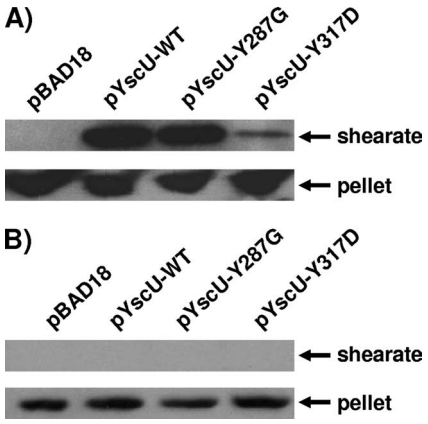

FIG. 3. Mutations in *yscU* reduce YscF export by a *yscP* mutant. (A) Mutations in the cytoplasmic domain of YscU were introduced in *trans* into the *yscP yscU* double null strain SAL3. Bacteria were grown at 37°C in BHI-plus-calcium medium, and YscU expression was induced with 0.2% arabinose. Cultures were sheared to remove T3SS needle structures. Sheared and cell pellet fractions were separated by centrifugation, and proteins in the sheared fraction (shearate) were precipitated with TCA. Equal percentages of sheared and cell pellet fractions were run on SDS-PAGE gels. YscF was detected by Western blotting with a polyclonal antiserum. pBAD18, vector control; pYscU-WT, plasmid expressing wild-type YscU; pYscU-Y287G, plasmid expressing YscU with the Y287G mutation; pYscU-Y317D, plasmid expressing YscU with the Y317D mutation. (B) The same fractions described above were probed with a monoclonal antibody against the cytoplasmic protein GroEL.

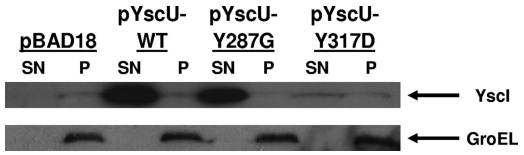

FIG. 4. Mutations in *yscU* reduce YscI export by a *yscP* mutant. Mutations in the cytoplasmic domain of YscU were introduced in *trans* into the *yscP yscU* double null strain SAL3. Bacteria were grown at 37°C in BHI-plus-calcium medium, and YscU expression was induced with 0.2% arabinose. Secreted supernatant (SN) and cell pellet (P) fractions were separated by centrifugation, and proteins in the supernatant were TCA precipitated. Equal percentages of secreted supernatant and cell pellet fractions were run on SDS-PAGE gels. YscI was detected by Western blotting with a polyclonal antiserum (upper panel); the cytoplasmic protein GroEL was detected with a monoclonal antibody (lower panel). pBAD18, vector control; pYscU-WT, plasmid expressing wild-type YscU; pYscU-Y287G, plasmid expressing YscU with the Y287G mutation; pYscU-Y317D, plasmid expressing YscU with the Y317D mutation.

viously (13), the YscU Y317D mutation is a particularly strong suppressor.

Next, we sought to determine the effects of the aforementioned YscU mutations on the export of YscI. Bacteria were grown in calcium-containing medium at 37°C, and secreted supernatant and cell pellet fractions were isolated. The results show that the YscU Y287G mutation slightly reduced YscI secretion but that YscI secretion was dramatically reduced by the YscU Y317D mutation (Fig. 4). Once again, Western blotting to detect GroEL showed that the presence of YscI in secreted supernatant fractions was not due to cell lysis. These results suggest that the ability of the YscU Y317D mutation to suppress a *yscP* mutant phenotype may be due to its ability to down-regulate the secretion of YscI.

Conserved residues in the C terminus of YscI are critical for T3SS function. Sequence alignments revealed that 24 amino acid residues in the YscI C terminus are identical to residues found in the C termini of homologous proteins present in the T3SSs that are most closely related to the *Yersinia* T3SS (34) (see Fig. S1 in the supplemental material). To determine whether these conserved residues are critical for the function of the *Yersinia* T3SS, the YscI gene was cloned into the IPTG-inducible expression vector pSE380. Site-directed mutagenesis was then used to alter these residues individually, and the abilities of the resulting YscI mutant proteins to *trans*-complement a *yscI* null strain were assessed. Bacteria were grown under Yop-inducing conditions in the presence of 20 μM IPTG, and cultures were separated into secreted supernatant and cell pellet fractions. Coomassie staining revealed that the P77A, M83A, T91A, Q93A, K99E, T100A, G102A, Q106A, N107A, E109K, and T110A mutant proteins all restored a wild-type level of Yop secretion to the *yscI* null strain (data not shown). Bacteria expressing any of the other mutant proteins exhibited reduced Yop secretion relative to that of a control strain expressing native YscI (Fig. 5A). Western blotting of secreted supernatant and cell pellet fractions revealed that YscI expression in the mutant expressing the L80A mutant protein (referred to as the L80A mutant) and in the W85A, R89D, I90A, I97A, and K113E mutants was virtually undetectable (Fig. 5B). For the Q84A, L87A, E94K, E95K, L96A, A98V, and L111A mutants, YscI was observed only in cell pellet fractions. Interestingly, no YscI was observed in culture supernatants from the Q84A, L87A, and L96A mutants despite the fact that these mutants secreted significant amounts of Yops.

Therefore, these mutants (as well as the E94K, E95K, A98V, and L111A mutants) were analyzed to determine whether they could assemble T3SS needle structures. First, bacteria were grown at 37°C in the presence of calcium to inhibit the induction of the LCR by the strain expressing native YscI in *trans*. Surface-located YscF was then cross-linked with the cell-impermeable, amine-specific cross-linker BS^3. Previous studies have shown that such treatment cross-links the needle component YscF into a ladder of high-molecular-weight multimers (12, 56). Our results confirm this conclusion, as the control strain expressing native YscI exhibited cross-linked products (Fig. 6A). In contrast, the Q84A, L87A, E94K, E95K, L96A, A98V, and L111A mutants exhibited very little, if any, cross-linked YscF, thus demonstrating that these mutants were defective in needle assembly. This result was surprising given that the Q84A, L87A, and L96A mutants secreted Yops. Therefore, the abilities of these mutants to form needles were reexamined following growth at 37°C in the absence of calcium in order to fully induce the T3SS. Despite the growth of the aforementioned mutants under LCR-inducing conditions, only small amounts of high-molecular-weight cross-linked products were observed (Fig. 6B). We conclude that needle assembly by these mutants was defective but not completely abolished. Note that the additional bands observed in the samples from these mutants were also observed for the vector control (pSE380) strain, which did not express a functional T3SS.

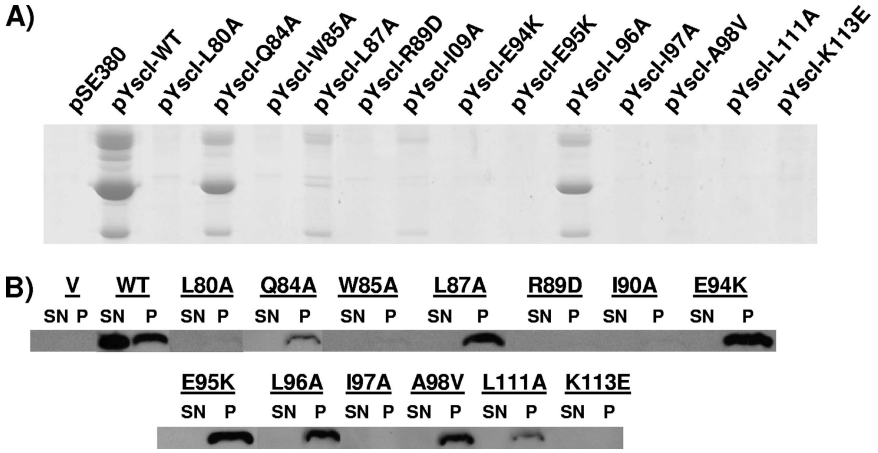

FIG. 5. Conserved residues in the C terminus of YscI are critical for T3SS function. (A) Yop secretion by YscI mutants. Mutations in *yscI* were introduced in *trans* into the *yscI* null strain SAL4. Bacteria were grown in Yop-inducing medium (BHI minus calcium), and YscI expression was induced with 20 μM IPTG. Secreted supernatant fractions were isolated by centrifugation, precipitated with TCA, and run on SDS-PAGE gels. Yops were visualized by staining with Coomassie blue. Higher induction levels did not result in greater Yop secretion (data not shown). pSE380, vector control; pYscI-WT, plasmid expressing wild-type YscI; pYscI-L80A to pYscI-K113E, plasmids expressing YscI proteins with the indicated mutations. (B) YscI expression and secretion. The aforementioned strains were grown as described above. Secreted supernatant (SN) and cell pellet (P) fractions were separated by centrifugation, and proteins in the supernatant were TCA precipitated. Equal percentages of the two fractions were run on SDS-PAGE gels, and YscI was detected by Western blotting using a polyclonal antiserum. Proteins are indicated by the corresponding mutations. V, vector control; WT, wild type.

These bands were due to cross-reactivity with our polyclonal antiserum and were routinely observed upon long exposure.

The aforementioned defects in needle assembly could be due to a defect in the export of the needle subunit YscF or a failure of exported YscF to undergo needle assembly. To test these possibilities, the Q84A, L87A, E94K, E95K, L96A, A98V, and L111A mutants were grown in calcium-deficient medium at 37°C to induce the T3SS and the secreted supernatant fractions were analyzed for the presence of free YscF. The results demonstrate that the E94K, E95K, A98V, and L111A mutants did not secrete YscF (Fig. 7A). In contrast, YscF was observed in the culture supernatants isolated from the Q84A, L87A, and L96A mutants. Western blotting for the cytoplasmic protein GroEL confirmed that the presence of

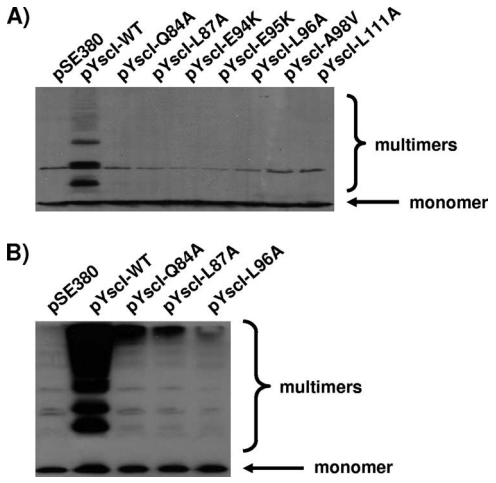

FIG. 6. YscI mutants exhibit defects in needle assembly. (A) Either wild-type YscI or the indicated YscI mutant forms were expressed in *trans* in the *yscI* null strain SAL4. Bacteria were grown in BHI-plus-calcium medium, and surface proteins were cross-linked via the addition of 2.5 mM BS^3. Samples were run on SDS-PAGE gels, and the needle component YscF was detected by Western blotting using a polyclonal antiserum. pSE380, vector control; pYscI-WT, plasmid expressing wild-type YscI; pYscI-Q84A to pYscI-L111A, plasmids expressing YscI proteins with the indicated mutations. (B) Either wild-type YscI or the indicated YscI mutant forms were expressed in *trans* in the *yscI* null strain SAL4. Bacteria were grown in BHI-minus-calcium medium, and YscF was cross-linked and detected as described above.

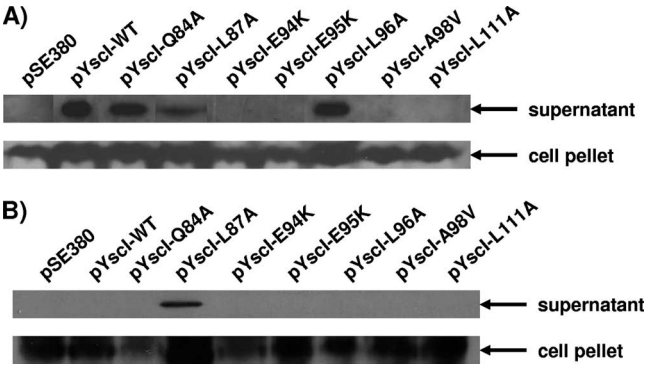

FIG. 7. YscF secretion by YscI mutants. (A) Either wild-type (WT) YscI or the indicated YscI mutants were expressed in *trans* in the *yscI* null strain SAL4. Bacteria were grown in Yop-inducing medium (BHI minus calcium), and YscI expression was induced with 20 μM IPTG. Secreted supernatant and cell pellet fractions were separated by centrifugation. Secreted proteins were precipitated with TCA, and equal percentages of the two fractions were run on SDS-PAGE gel. YscF was detected by Western blotting with a polyclonal antiserum. pSE380, vector control; pYscI-WT, plasmid expressing wild-type YscI; pYscI-Q84A to pYscI-L111A, plasmids expressing YscI proteins with the indicated mutations. (B) The same fractions described above were probed with a monoclonal antibody against the cytoplasmic protein GroEL.

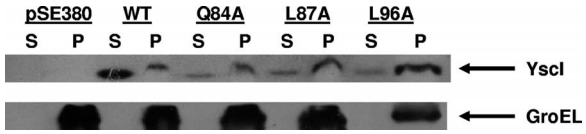

FIG. 8. YscP regulates the secretion of the Q84A, L87A, and L96A mutant forms of YscI. Either wild-type (WT) YscI or the indicated YscI mutant forms (indicated by the corresponding mutations) were expressed in *trans* in the *yscI yscP* double null strain SAL6. Bacteria were grown in Yop-inducing medium (BHI minus calcium), and YscI expression was induced with 20 μM IPTG. Secreted supernatant (S) and cell pellet (P) fractions were separated by centrifugation. Secreted proteins were precipitated with TCA, and equal percentages of the two fractions were run on SDS-PAGE gels. YscI was detected by Western blotting with a polyclonal antiserum (upper panel); GroEL was detected using a monoclonal antibody (lower panel). pSE380, vector control.

YscF in culture supernatants was not due to cell lysis (Fig. 7B), except in the case of the L87A mutant. Low levels of cell lysis were reproducibly observed for this mutant. In this particular case, we cannot unambiguously conclude that the L87A mutant secreted YscF. Note, however, that in later experiments we did observe needle formation by this mutant (see Fig. 9). For the Q84A and L96A mutants, we can conclude that the defect in needle formation was likely due to the failure of exported YscF to undergo assembly.

The Q84A, L87A, and L96A mutants secrete YscI in the absence of YscP. The failure to detect YscI in secreted supernatant fractions of the Q84A, L87A, and L96A mutants was surprising given the fact that these mutants secreted significant amounts of both Yops (Fig. 5) and the needle component YscF (Fig. 7). To determine whether the localization of YscI to the cell pellet fraction in these mutants was dependent upon YscP, plasmids expressing the corresponding mutant proteins were introduced into the *yscI yscP* double null strain SAL6 by electroporation. Bacteria were grown at 37°C in calcium-deficient medium, and secreted supernatant and cell pellet fractions were isolated. In the absence of YscP, a small amount of YscI was now observed in the culture supernatant fraction from each mutant (Fig. 8). To ensure that the small amounts of YscI present in the culture supernatant fractions were not due to cell lysis, culture supernatant and cell pellet fractions were analyzed for the presence of the cytoplasmic protein GroEL. GroEL was detected in cell pellet but not secreted supernatant fractions, thus demonstrating that the presence of YscI in supernatant fractions was not due to cell lysis. We conclude that these mutant forms of YscI are inherently secretable and that their localization to the cell pellet fraction is dependent upon YscP.

The Q84A, L87A, and L96A mutants assemble needles in the absence of YscP. Given the results demonstrating that YscP does in fact regulate the secretion of YscI in the Q84A, L87A, and L96A mutants, we sought to determine whether YscP had an effect on needle assembly by these mutants. Therefore, the corresponding mutant proteins were expressed in *trans* in the *yscI yscP* double null strain SAL6 and needle assembly was assessed by cross-linking with BS^3. The results demonstrate that, in the absence of YscP, the bacteria expressing the YscI Q84A, L87A, and L96A mutant proteins exhibited a degree of needle assembly similar to that observed for the control strain

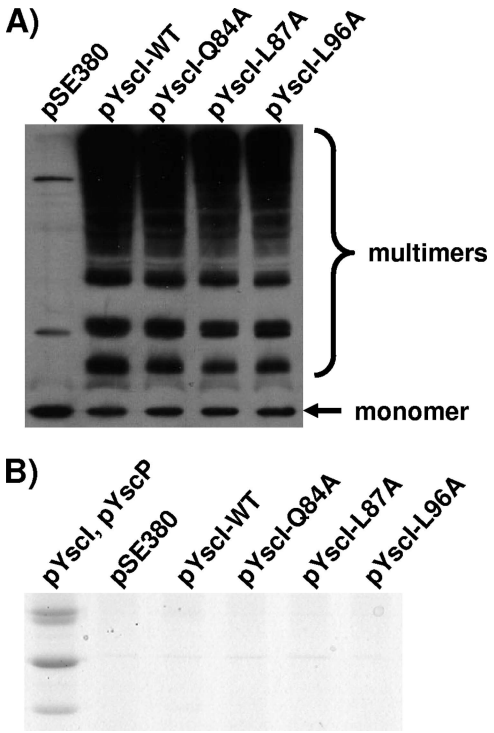

FIG. 9. The YscI Q84A, L87A, and L96A mutant forms assemble needles in the absence of YscP. (A) Either wild-type YscI or the indicated YscI mutant forms were expressed in *trans* in the *yscI yscP* double null strain SAL6. Bacteria were grown in BHI-minus-calcium medium, and YscI expression was induced with 20 μM IPTG. Surface proteins were cross-linked via the addition of 2.5 mM BS^3. Samples were run on SDS-PAGE gels, and the needle component YscF was detected by Western blotting using a polyclonal antiserum. pSE380, vector control; pYscI-WT, plasmid expressing wild-type YscI; pYscI-Q84A to pYscI-L96A, plasmids expressing YscI proteins with the indicated mutations. (B) The aforementioned strains were grown in Yop-inducing medium (BHI minus calcium), and YscI expression was induced with 20 μM IPTG. In addition, a control strain expressing YscI and YscP from two compatible plasmids (pYscI and pYscP) was utilized; YscI expression was induced as described above, and YscP expression was induced with 0.2% arabinose. Secreted supernatant fractions were isolated by centrifugation, precipitated with TCA, and run on SDS-PAGE gels. Yops were visualized by staining with Coomassie blue.

expressing native YscI (Fig. 9A). Note that these results represent much greater levels of needle assembly than those observed for strains expressing YscP together with the same mutant proteins (Fig. 6). To determine whether these mutants could undergo substrate specificity switching, bacteria were grown at 37°C in calcium-deficient medium and secreted supernatant fractions were isolated. Coomassie staining of the supernatant fractions revealed that Yops (late substrates) were in fact not secreted by these mutants. Thus, the mutant proteins behaved identically to native YscI and did not suppress the phenotype of the *yscP* mutant. To demonstrate the levels of Yop secretion expected for a wild-type strain, the *yscI yscP* mutant was *trans*-complemented with two compatible plasmids expressing YscI (pSW71) and YscP (pJJ10). As expected, an abundance of secreted Yops was observed. We conclude that the YscI Q84A, L87A, and L96A mutant proteins do not restore substrate specificity switching in the absence of YscP.

Taken together, these results show that needle assembly is not sufficient to trigger a switch in the substrate specificity of the T3SS.

DISCUSSION

T3SSs are complex macromolecular organelles whose assembly requires the ordered export of their components. For virulence-associated T3SSs, this means that type III effectors destined for the eukaryotic cell cytosol must not be exported while the secretion system is being assembled. Regulating the substrate specificity of the secretion apparatus prevents such unwanted export. In *Yersinia*, YscP and YscU have been shown to play a role in substrate specificity switching. A *yscP* mutant assembles elongated needle structures on the bacterial cell surface (23) but secretes only small amounts of Yop effectors (43, 49). Similar phenotypes corresponding to mutations in the YscP homologs InvJ (SPI1 T3SS) (27), Spa32 (*Shigella* T3SS) (31, 55), and FliK (flagella) (20, 28, 57) have been reported previously. Mutations in the cytoplasmic domain of YscU can partially suppress a *yscP* mutant phenotype, as evidenced by decreased export of YscF (the needle component) and increased Yop secretion (13). Similarly, mutations in the cytoplasmic domain of FlhB (the flagellar YscU homolog) can partially suppress a *fliK* mutant phenotype (20, 28, 57).

Despite these results, the exact nature of the switching event is poorly understood. A previous study proposed that YscP functions as a molecular ruler that switches the substrate specificity of the secretion apparatus once the length of the needle structure matches the length of the extended YscP polypeptide (23). In support of this hypothesis, it was shown that the deletion of the central region of YscP results in shorter needles and that the insertion of additional sequences generates longer needles. However, recent work on the SPI1 T3SS has challenged this hypothesis. Specifically, the investigators showed that an *invJ* mutant, which is defective for substrate specificity switching (9, 27), does not assemble the inner rod structure of the T3SS secreton (32) and secretes excess amounts of the inner rod component PrgJ into cell culture supernatant fractions (52). This finding suggests that inner rod formation is critical for substrate specificity switching.

Based on these results, we sought to determine whether YscI, the inner rod component of the *Yersinia* T3SS (41), plays a direct role in substrate specificity switching. A previous study demonstrated that YscI is required for the secretion of Yop effectors by the T3SS (3). In this work, we have demonstrated that YscI is secreted by the T3SS. Specifically, YscI was found in both the secreted supernatant and cell pellet fractions of a wild-type strain but was not expressed in a *yscS* mutant that lacked a functional T3SS. Analogous results for the SPI1 T3SS were obtained by Sukhan et al., who demonstrated that PrgJ expression in the absence of a functional T3SS is undetectable (52).

Sukhan et al. previously observed that a *Salmonella invJ* mutant secretes large amounts of PrgJ into the culture supernatant but that no PrgJ is observed in cellular fractions enriched with the SPI1 T3SS secreton (52). Our results show that the *Yersinia* T3SS behaves in a similar fashion, as we observed a substantially elevated amount of YscI in the culture supernatant fraction from a *yscP* mutant compared to that in the fraction from a wild-type strain grown under LCR-inhibiting conditions. To date, the *Yersinia* T3SS secreton has yet to be isolated in a purified form. Therefore, we cannot conclude that the increased secretion of YscI into culture supernatants reflects the fact that YscI is not incorporated into an inner rod structure, although this is our favored hypothesis. Ultimately, the purification of the *Yersinia* T3SS secreton will be needed to address this question.

Previous work by Edqvist et al. (13) demonstrated that mutations in the cytoplasmic domain of the inner membrane protein YscU can partially suppress the phenotype of a *yscP* mutant, as evidenced by the fact that these suppressor mutations reduce the amount of surface-located YscF in the *yscP* strain. Here, we have confirmed these results. In addition, we examined the effects of two of these YscU mutations (Y287G and Y317D) on YscI export by the *yscP* mutant. The results paralleled the effects on YscF export observed earlier, that is, the Y287G mutation slightly reduced the amount of YscI exported by the *yscP* mutant, while the Y317D mutation dramatically reduced YscI export. These results confirm that YscP and YscU coordinately regulate the export of early substrates (both YscF and YscI) and that the Y317D mutation is a particularly strong suppressor of the *yscP* mutant phenotype. However, these results did not enable us to determine whether YscF or YscI export is truly critical for substrate specificity switching.

Therefore, to determine whether YscI plays a direct role in substrate specificity switching, we performed an extensive mutational analysis of conserved amino acid residues in the C terminus of YscI. Using this approach, we generated seven mutants (the Q84A, L87A, E94K, E95K, L96A, A98V, and L111A mutants) that exhibited mild to severe defects in Yop secretion (late substrate export) without abolishing YscI expression. Unlike native YscI, which was found in both secreted supernatant and cell pellet fractions, YscI from the aforementioned mutants localized exclusively to the cell pellet fractions. This pattern was particularly striking for the Q84A, L87A, and L96A mutants, which secreted significant amounts of Yops. The fact that YscI is required for Yop secretion suggests that YscI in these mutants does in fact assemble into an inner rod structure. The absence of YscI in secreted supernatant fractions may reflect the fact that the corresponding mutant forms of YscI have a greater propensity to assemble into the inner rod structure than does native YscI.

The cross-linking of surface-located proteins with the cell-impermeable cross-linker BS^3 revealed that the Q84A, L87A, E94K, E95K, L96A, A98V, and L111A mutants exhibited severe defects in the assembly of the T3SS needle structure. This result is quite significant for the Q84A, L87A, and L96A mutants due to the fact that they underwent a substantial degree of substrate specificity switching (as evidenced by the fact that they secreted Yops). This finding suggests that needle assembly is not a prerequisite for substrate specificity switching.

The analysis of secreted supernatant fractions revealed that the E94K, E95K, A98V, and L111A mutants were unable to export the needle component YscF, thus explaining their defect in needle assembly. In contrast, the Q84A and L96A mutants (and, possibly, the L87A mutant) exported significant amounts of YscF yet exhibited severe defects in needle assembly. Therefore, we posit that in these mutants, YscI assembled into the inner rod structure but adopted a conformation that

impaired the assembly of YscF into the needle-like structure that sits atop the inner rod. A role for the inner rod as an anchoring point for the needle structure is not without precedent, as Marlovits et al. (32) demonstrated that the elongated needle structures formed by a *Salmonella invJ* mutant, which is defective for inner rod assembly, are more labile (that is, more easily broken off) than the needle structures formed by a wild-type strain.

Based on the aforementioned hypotheses (namely, that YscI assembly into an inner rod structure is dependent upon YscP and that the YscI Q84A, L87A, and L96A mutant proteins form an inner rod structure that impairs needle assembly), we examined the phenotypes of bacteria expressing the Q84A, L87A, and L96A mutant proteins in a *yscP* null background. The results demonstrated that in the absence of YscP, the mutant forms of YscI were now secreted into culture supernatant fractions. This finding suggests that, in the absence of YscP, these YscI mutant proteins no longer formed the inner rod structure of the T3SS. Consistent with this view is the fact that needle formation by these mutant proteins was now similar to that observed for native YscI. Despite the restoration of needle assembly in the *yscP* background, the Q84A, L87A, and L96A mutants did not undergo substrate specificity switching, as evidenced by their failure to secrete Yops. Together, these results suggest that the inner rod protein YscI, not the needle protein YscF, is critical for substrate specificity switching. In conjunction with the aforementioned roles that YscP and YscU play in coordinately regulating YscI export, we suggest that YscP and YscU switch the substrate specificity of the secretion apparatus by regulating the export of YscI and the assembly of YscI into the inner rod structure of the T3SS. Clearly, future studies will be needed to understand the precise roles of YscI, YscP, and YscU in this process.

Thus, our results are consistent with the view of Marlovits et al. (32) that the formation of the inner rod structure of T3SSs governs substrate specificity switching. Of course, one must reconcile this view with the hypothesis put forth by Journet et al. (23), which posits that YscP acts as a molecular ruler that physically measures the length of the T3SS needle structure and switches the substrate specificity of the export apparatus when the length of the needle equals the length of the extended YscP polypeptide. Recall that in the experiments by Journet et al., deletions within the central region of the YscP polypeptide resulted in shorter needles and insertions of additional amino acid residues generated longer needles. Thus, the physical length of the YscP polypeptide seemingly governs the length of the T3SS needle structure. We must point out that, in addition to the length of the YscP polypeptide, a second variable was implicitly changed in these experiments, namely, the time needed to synthesize YscP. A shorter YscP polypeptide will be synthesized more quickly and accumulate at a greater rate than a longer YscP polypeptide. Therefore, these experiments did not distinguish between molecular ruler and molecular stopwatch mechanisms. If YscP is required for inner rod formation (as we propose), then a truncated version of YscP should increase the rate of inner rod formation due to a higher rate of accumulation. This effect would result in the substrate specificity switch's occurring more quickly by reducing the time permitted for needle assembly and would, therefore, result in shorter needles. The results of Marlovits et al. (32) support this view, as they have demonstrated that the timing of inner rod assembly is critical for substrate specificity switching. Specifically, they demonstrated that the overexpression of the SPI1 T3SS inner rod component PrgJ results in shorter needles (presumably due to faster inner rod assembly) and that the overexpression of the needle component PrgI results in longer needles (presumably because PrgI outcompetes PrgJ for access to the secretion apparatus and thereby delays inner rod formation). Further characterization of the role of YscP homologs will ultimately be required to further our understanding of substrate specificity switching.

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