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YscP and YscU Regulate Substrate Specificity of the *Yersinia* Type III Secretion System

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Pathogenic *Yersinia* species use a type III secretion system to inhibit phagocytosis by eukaryotic cells. At 37°C, the secretion system is assembled, forming a needle-like structure on the bacterial cell surface. Upon eukaryotic cell contact, six effector proteins, called Yops, are translocated into the eukaryotic cell cytosol. Here, we show that a *yscP* mutant exports an increased amount of the needle component YscF to the bacterial cell surface but is unable to efficiently secrete effector Yops. Mutations in the cytoplasmic domain of the inner membrane protein YscU suppress the *yscP* phenotype by reducing the level of YscF secretion and increasing the level of Yop secretion. These results suggest that YscP and YscU coordinately regulate the substrate specificity of the *Yersinia* type III secretion system. Furthermore, we show that YscP and YscU act upstream of the cell contact sensor YopN as well as the inner gatekeeper LcrG in the pathway of substrate export regulation. These results further strengthen the strong evolutionary link between flagellar biosynthesis and type III synthesis.

There are three pathogenic species of *Yersinia*: *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis. Yersinia pestis* causes plague and is transmitted by flea bites or infectious aerosols, while *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are enteric pathogens that cause gastroenteritis after the ingestion of contaminated food or water (for reviews, see references 3 and 36). After reaching the intestine, enteropathogenic *Yersinia* cells are taken up by antigen-sampling M cells (1). This enables the bacteria to colonize the Peyer's patches, a gut-associated lymphoid tissue. Once in the Peyer's patches, the bacteria are able to inhibit phagocytosis by macrophages (10, 38) and polymorphonuclear leukocytes (52), which allows them to replicate extracellularly (44). In humans, such infections are typically self-limiting, while in rodents, the bacteria are able to colonize other organs, which results in a deadly systemic infection. The ability to cause infection is dependent on the presence of an approximately 70-kb plasmid encoding a type III secretion system (TTSS) that delivers Yop effectors into the cytosol of the target cell. The *Yersinia* TTSS is comprised of about 25 Ysc (Yop secretion) proteins. Nine of these proteins are conserved in the bacterial flagellar export apparatus and in the TTSSs found in a wide variety of gram-negative plant and animal pathogens (for a review, see reference 19). The *Yersinia* type III secretion apparatus assembles a needle-like structure comprised of the YscF protein on the bacterial cell surface prior to eukaryotic cell contact (18). The TTSSs of other gram-negative pathogens form similar structures (7, 21, 22, 48, 49). The concept of substrate specificity switching by TTSSs was first demonstrated in the flagellar system. The bacterial flagellum consists of three parts: the basal body, which is located in the cell wall and membranes of the bacterium; the hook, which is located on the cell surface; and the long flagellar filament, which is assembled onto the end of the hook and serves as a propeller during bacterial motility (for a review, see reference 26). The length of the hook is normally 55 nm. Yamaguchi and colleagues, however, showed that a *fliK* mutant exhibits a polyhook phenotype in which hook elongation proceeds to an abnormal extent but no flagellar filament is built (34). Mutations in *fliK* can be suppressed, with respect to filament assembly, by mutations in the export apparatus protein FlhB (24, 51), which is located in the bacterial inner membrane. Work by Minamino and Macnab has demonstrated that FliK, along with the hook and filament proteins, binds to the cytoplasmic domain of FlhB (30, 31). Therefore, upon the completion of hook assembly, it is possible that FliK switches the substrate specificity of the flagellar export apparatus by altering the conformation of FlhB in order to promote the export of the filament component flagellin. A similar phenomenon has been reported in the TTSS encoded by *Salmonella* pathogenicity island 1 (SPI1). Specifically, an *invJ* mutant assembles a type III secreton with abnormally long needles (23). Interestingly, an *invJ* mutant is unable to secrete effector proteins (5), which suggests that it is defective in substrate specificity switching. Recent findings by Tamano and coworkers (49) showed that Spa32 of *Shigella* spp. is involved in the control of needle length. Spa32 is homologous to InvJ and, interestingly, Spa32 is interchangeable with InvJ of *Salmonella* (49).

Here, we examined the phenotype of *yscP* mutants of the *Yersinia* TTSS. We demonstrate that a *yscP* mutant exports an increased amount of YscF to the bacterial cell surface prior to eukaryotic cell contact. Furthermore, the *yscP* mutant is able to secrete only low levels of the translocator proteins, YopB and YopD, and Yop effectors. Mutations in the cytoplasmic domain of the inner membrane protein YscU can restore a level of Yop effector secretion to the *yscP* mutant higher than that to the corresponding isogenic wild-type strain, while the amount

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
DH5α	Recipient for cloning experiments	17
S17-1λ*pir*	Recipient for suicide plasmids	43
BL21(DE3)	IPTG-inducible T7 RNA polymerase	47
Yersinia		
YPIII(pIB102)	wild-type, parental strain	2
YPIII(pIB69)	*yscP*-null strain	This study
YPIII(pIB75)	*yscU*-null strain	25
YPIII(pIB202)	*yscF*-null strain	This study
YPIII(pIB604)	*yopB*-null strain	16
YPIII(pIB82)	*yopN*-null strain	39
YPIII(pIB60469)	*yopB yscP*-double-null strain	This study
YPIII(pIB6975)	*yscP yscU*-double-null strain	This study
YPIII(pIB8769)	*yopN yscP*-double-null strain	This study
YPIII(pIB70169)	*lcrG yscP*-double-null strain	This study
YPIII(pIB2669)	*lcrQ yscP*-double-null strain	This study
YPIII(pIB62169)	*yopD yscP*-double-null strain	This study
Plasmid		
pBAD33	Contains P_{BAD} promoter	15
pET-22b	T7 overexpression vector	Novagen
pDM4	Suicide vector	28
pSL144	Δ*yscP* in pDM4	This study
pSL340	Δ*yopN* in pDM4	This study
pKK223-3	Contains *tac* promoter	Amersham Pharmacia Biotech
pPE5	*yscU* gene in pET-22b	This study
pPE33	*yscU* gene in pKK223-3	This study
pPE34	*yscP* gene in pBAD33	This study
pPE36	*yscU-A268F* gene in pKK223-3	This study
pPE37	*yscU-Y287G* gene in pKK223-3	This study
pPE38	*yscU-V292T* gene in pKK223-3	This study
pPE39	*yscU-Y317D* gene in pKK223-3	This study
pMMB66EH	Contains *tac* promoter	14
pJO33	GST-YscF overexpression vector	This study
pGEX-5X-3	Cloning vector	Amersham Pharmacia Biotech
pLS52	Δ*yscF* in pDM4	This study
pLS53	YscF expression vector	This study

of YscF present on the bacterial cell surface is reduced. These results suggest that YscP and YscU coordinately regulate secretion of the *Yersinia* TTSS.

MATERIALS AND METHODS

Media and growth conditions. *Yersinia* strains were grown in brain-heart infusion (BHI) broth supplemented with either 5 mM EGTA and 20 mM $MgCl_2$ (BHI minus Ca^{2+}) or 2.5 mM $CaCl_2$ (BHI plus Ca^{2+}). *Escherichia coli* strains were grown in Luria-Bertani broth (LB) (8). *Yersinia* strains were grown in LB containing 50 μg of kanamycin ml^{-1} to maintain selection of the virulence plasmid. *Yersinia* strains carrying YscU-expressing plasmids were grown in LB supplemented with 100 μg of carbenicillin ml^{-1}.

DNA methods. DNA preparations and routine subcloning were performed as described by Sambrook et al. (42). DNA-sequencing reactions were performed by using the ThermoSequenase dye terminator cycle sequencing kit and analyzed with a SEQ4 × 4 sequencer (Amersham Pharmacia Biotech).

Construction of a *yscF* in-frame deletion mutant. PCR was performed with the primer pairs YscF3-YscF4 and YscF5-YscF6, respectively, by using the plasmid pIB102 as template. YscF3 was tailed with an *Xba*I site, and YscF6 was tailed with a *Sac*I site. The PCR yielded one fragment from the upstream region and one fragment from the downstream region of *yscF*. The 5′ ends of the primers YscF4 and YscF5 contain overlapping sequences between the two fragments, and a second PCR with the two fragments as templates and the primers YscF3 and YscF6 resulted in a fragment in which the *yscF* gene, deleted for codons 11 through 69, was flanked by upstream and downstream sequences. After digestion with *Xba*I and *Sac*I, the fragment was cloned into the vector pDM4 (Table 1), resulting in pLS52. This plasmid was transformed into *E. coli* strain S17-1λ*pir*, clones were selected on plates containing chloramphenicol, and the resulting transformants were verified by PCR. *E. coli* containing the plasmid was then used to introduce pLS52 by conjugation into the recipient *Yersinia* strain YPIII (pIB102). Clones in which the plasmid integrated by a single recombination event were selected on *Yersinia* selective agar base (YSAB) plates containing 50 μg of kanamycin/ml and 20 μg of chloramphenicol/ml. The insertion was verified by PCR with the primers from the second PCR. The resulting strain was then counterselected on sucrose to select for clones that had lost the plasmid. The *yscF* deletion was verified by PCR with primers YscF3 and YscF6. The resulting mutant was denoted YPIII(pIB202).

For transcomplementation studies, the *yscF* gene was amplified by PCR with the primers YscF1 and YscF2 tailed with sites for *Eco*RI and *Pst*I and cloned into the vector pMMB66EH in the strain *E. coli* S17-1λpir. After conjugation into the mutant strain on YSAB plates containing 100 μg of carbenicillin/ml and 50 μg of kanamycin/ml, the plasmid (pLS53) was verified by a plasmid mini preparation and PCR with the same primer pair. The primers used and their sequences were as follows: YscF1, 5′-GCT CAG AAT TCG ATG AGT AAC TTC TCT GGA TTT A-3′ (bp 4193 through 4214); YscF2, 5′-CTG ACT CTG CAG TTC ATA TTA TGG GAA CTT CTG T-3′ (bp 4462 through 4441); YscF3, 5′-GCT GA**T CTA GA**C GAA TTG AAT TTC GAG GTG CAA G-3′ bp 3801 through 3822); YscF4, 5′-GAT GCC TTG TCC TTT CGT AAA TCC AGA GAA G-3′ (bp 4222 through 4201); YscF5, 5′-ACG AAA GGA CAA GGC ATC CTA CAG AAG TTC-3′ (bp 4430 through 4450); YscF6, 5′-GCT CAC **GAG CTC** GAG ACG ATT TAA ACG TGA CTC-3′ (bp 4739 through 4719). All YscF primer sequences were from GenBank under accession no. M83225. Restriction sites are shown in boldface.

Construction of the *yscP*-null strain. An in-frame deletion was made in the *yscP* gene by PCR amplifying YPIII(pIB102) genomic DNA with *Pfx* DNA polymerase and the primer pairs SL89-SL90 (which resulted in a fragment complementary to the upstream gene *yscO* and the first six codons of the *yscP* gene) and SL91-SL92 (which resulted in a fragment complementary to the last six codons of the *yscP* gene and the downstream gene *yscQ*). The two fragments were ligated by PCR with the primer pair SL89-SL92. The resulting PCR product was digested with *Xba*I and *Sph*I and cloned into the same sites in the suicide vector pDM4. The resulting construct, pSL144, was then transformed into the *E. coli* strain S17-1λ*pir* (43) and conjugated into the wild-type *Yersinia* strain YPIII(pIB102) by plating on *Yersinia* agar (Difco) plates containing 25 μg of chloramphenicol ml^{-1}. Exconjugants were restreaked onto LB plates containing 5% sucrose in order to counterselect against bacteria still containing the pSL144 plasmid. Sucrose-resistant colonies were PCR amplified with the primer pair SL89-SL92 to confirm the presence of the deletion. The resulting strain, YPIII (pIB69), lacked codons 7 through 449 of the *yscP* gene. The primers used and their sequences were as follows: SL89, 5′-GCC **TCT AGA** TCA GCA AGC TTG CTT GCA GGC; SL90, 5′-CTC CCA CTC CTC ATA CTC AGG TTC TAA TGG GGA; SL91, 5′-GAA CCT GAG TAT GAG GAG TGG GAG GCT GAA GAA; SL92, 5′-GGC **GCA TGC** CCA GAA GGA GAT ATG CGC ATT. Restriction sites are shown in boldface.

Construction of the *yscP yopB*-double-null strain. Plasmid pSL144 was conjugated into the *yopB*-null strain YPIII(pIB604) (19) as described above. The resulting strain was the *yscP yopB*-double-null strain YPIII(pIB60469).

Construction of the *yscP yscU*-double-null strain. Plasmid pSL144 was conjugated into the *yscU*-null strain YPIII(pIB75) as described above.

Construction of the *yopN yscP*-double-null strain. An in-frame deletion in the *yopN* gene was constructed by PCR amplifying YPIII(pIB102) genomic DNA with *Pfx* DNA polymerase and the primer pairs SAL226-SAL227 (which resulted in a fragment complementary to the *yopN* promoter region and the first six codons of the *yopN* coding sequence) and SAL228-SAL229 (which resulted in a fragment complementary to the last four codons of the *yopN* gene and the downstream gene *tyeA*). The two fragments were ligated by PCR with the primer pair SAL226-SAL229. The resulting PCR product was digested with *Sph*I and *Xba*I and cloned into the same sites of the suicide vector pDM4. The resulting construct, pSL340, was conjugated into the *yscP*-null strain YPIII(pIB69) as described above. The resulting strain, YPIII(pIB8769), lacked codons 7 through 289 of the *yopN* gene. The primers used and their sequences were as follows: SAL226, 5′-GCC **GCA TGC** GGC GGC TAC CTA CAA TGC CAT GAC; SAL227, 5′-GAA AGG TCG TAC GTT ATG AAG CGT CGT CAT AAC TAC; SAL228, 5′-ACG CTT CAT AAC GTA CGA CCT TTC TGA GTT TAT GGG; SAL229, 5′-GGC **TCT AGA** GCC AGA TTG AGC CAT CTC TAA TTG. Restriction sites are shown in boldface.

Preparation of YscF antiserum. A fragment of *yscF* generated by PCR with primer pair JO214-JO217 and strain YPIII(pIB102) as template was cloned into pGEX-5X-3 (Amersham Pharmacia Biotech) with the *Bam*HI and *Not*I restric-

tion sites, generating plasmid pJO33. Fusion proteins were expressed, purified, and cleaved at the factor Xa cleavage site according to the manufacturer's instructions. The resulting polypeptide, approximately 8 kDa in size, was used as antigen to raise a polyclonal rabbit antiserum (Agrisera, Umeå, Sweden), which was used in Western blots without further purification. The primers used were JO214 (5′-CG ACA G**GG ATC C**AG ATG AGT AAC TTC TCT GGA TTT) and JO217 (5′-CGA CAG **GCG GCC GC**G TTA TGG GAA CTT CTG TAG GA). Restriction sites are shown in boldface.

Construction of mutations in the cytoplasmic domain of YscU. YPIII(pIB102) genomic DNA was PCR amplified with *Pfx* DNA polymerase and the primer pair SL53-SL83. The resulting PCR product, consisting of the *yscU* gene, was digested with *Eco*RI and *Pst*I and cloned into the same sites in the pKK223-3 vector (Amersham Pharmacia Biotech) such that the expression of YscU was under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter. The resulting construct, pPE33, was used as the template for site-directed mutagenesis, which was carried out according to the procedures in the Gene-Editor in vitro site-directed mutagenesis kit (Promega).

The mutagenic oligonucleotides used were as follows: the A268F mutation (PE40), the Y287G mutation (PE41), the V292T mutation (PE43), and the Y317D mutation (PE46). The primers used and their sequences were as follows: SL53, 5′-GGC **CTG CAG** TTA TAA CAT TTC GGA ATG TTG; SL83, 5′-GCC **GAA TTC** ATG AGC GGA GAA AAG ACA GAG; PE40, 5′-CCG ACC CAT ATT TTC ATT GGT ATT CTT TAC; PE41, 5′-GTA ACA TTC AAA GGT ACC GAT GCC CAA G; PE43, 5′-ACC GAT GCC CAA ACT CAG ACT GTG CGC; PE46, 5′-GCC CGT GCT CTT GAT TGG GAT GCG CTC G. Restriction sites are depicted in boldface.

Yop secretion assay. Overnight cultures of *Y. pseudotuberculosis* strains were grown in BHI medium lacking Ca^{2+} which contained 50 μg of kanamycin ml^{-1} at 26°C. The cultures were diluted to an optical density at 600 nm of 0.2 into 10 ml of fresh medium and grown at 26°C for 1 h and then for an additional 2 h at 37°C to induce secretion. The cultures were then centrifuged at 3,000 × *g* for 15 min. The supernatants containing the secreted Yops 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 20 min, the supernatants were discarded, and the remaining pellets were dried at room temperature. The pellets were resuspended in 250 μl of 2% sodium dodecyl sulfate (SDS) and precipitated with acetone at −20°C for 30 min. Samples were centrifuged at 20,800 × *g* for 10 min, the supernatants were discarded, and the pellets were air-dried. The pellets were then resuspended in 100 μl of 8 M urea and an equal amount of 2× sample buffer. Equal amounts of culture supernatant and cell pellet fractions were separated by SDS-PAGE gels stained with Coomassie blue or transferred to a nitrocellulose membrane. Yop proteins were detected by using a polyclonal anti-Yop antiserum raised against cell-secreted Yops (11).

Immunoblotting. Samples were separated by SDS–12% PAGE and electroblotted (Trans Blot SD; Bio-Rad) onto a nitrocellulose transfer membrane (Protran; Schleicher and Schuell) by using a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was blocked for 1 h with Tris-buffered saline plus 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk. The membrane was probed for 1 h with the appropriate polyclonal antiserum in 10 ml of the blocking buffer and was then washed three times at 5 min each with TBS-T. The membrane was incubated for 1 h with an anti-rabbit antibody (Amersham Pharmacia Biotech) in 10 ml of blocking buffer, followed by washing with TBS-T. Proteins were detected by using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Surface localization of YscF. Overnight cultures of the wild-type *Y. pseudotuberculosis* strain YPIII(pIB102), the *yscF*-null mutant YPIII(pIB202), and the *yscP*-null mutant YPIII(pIB69), expressing different YscU constructs, were grown at 26°C in BHI medium containing calcium (2.5 mM) supplemented with 50 μg of kanamycin ml^{-1} (to select for the virulence plasmid) and 100 μg of carbenicillin ml^{-1} (to select for YscU-expressing constructs). The cultures were diluted to an optical density at 600 nm of 0.2 in 10 ml of fresh medium and grown for 1 h at 26°C, after which the cultures were shifted to 37°C for 2 h to induce secretion. Whole cultures were sheared by five passages through a hypodermic needle (23Gx1, 0.6 by 25 mm; B. Braun), which released surface proteins and organelles from the bacterial surface. The cultures were centrifuged at 1,800 × *g* for 15 min. The pellets were resuspended in 100 μl of H_2O and an equal amount of 2× sample buffer. The supernatants were precipitated with 10% TCA and incubated on ice for 30 min and thereafter centrifuged at 1,800 × *g* for 20 min. Next, the supernatants were discarded and the pellets were resuspended in 250 μl of 2% SDS and precipitated with acetone at −20°C for 30 min. The samples were then centrifuged at 15,000 × *g* for 10 min, the supernatants were discarded, and the pellets were dried, followed by resuspension in 100 μl of 8 M urea and an equal amount of 2× sample buffer. Two microliters of the pellet, diluted 1:10, and 5 μl of undiluted supernatant were separated by 15% Tris-tricine SDS-PAGE and transferred to a nitrocellulose membrane. YscF was detected with a polyclonal anti-YscF antibody.

Cytotoxicity assay. Overnight cultures of the wild-type strain YPIII(pIB102), the *yscP*-null mutant YPIII(pIB69), and the *yopB yscP*-double-null mutant YPIII (pIB60469) were grown in LB containing 50 μgof kanamycin ml^{-1}; for strains expressing different YscU constructs, 100 μg of carbenicillin ml^{-1} was also added. Cytotoxicity was assayed as described previously (39). Pictures were taken with a phase-contrast microscope (Zeiss).

RESULTS

Phenotypic characterization of a *yscP* mutant. To investigate the possible role of YscP in regulating the secretion of the *Yersinia* type III needle protein YscF, a *yscP*-null mutant, YPIII(pIB69), and the isogenic wild-type strain YPIII(pIB102) (Table 1) were grown at 37°C in either the presence or absence of calcium. The bacterial supernatants were first investigated for the presence of YscF. No YscF could be detected in these fractions, showing that YscF was not released to the culture medium during growth (data not shown). The bacterial pellets were sheared to release surface proteins and organelles from the bacterial surface. When the sheared fraction of the bacterial pellet was investigated, the *yscP*-null mutant was found to release larger amounts of YscF to the bacterial surface than the wild-type strain, irrespective of the calcium concentration of the medium (Fig. 1A). Since no YscF was found in the culture supernatants, we concluded that the YscF protein recovered from the sheared fractions originated from the bacterial surface. In addition, the levels of YscF in the bacterial pellets recovered after shearing were similar (Fig. 1A), which suggested that the increased level of YscF released by shearing that was found in the *yscP*-null mutant was not due to increased expression of the YscF protein. Moreover, we could also confirm the findings of others that the *yscP* mutant secreted much lower levels of Yops than the wild-type strain (Fig. 1B) (35, 46).

YopE is a Rho-GTPase-activating protein (53) that, when translocated into the eukaryotic cell cytosol, induces a cytotoxic effect by disrupting the actin cytoskeleton of host cells (40). Since the *yscP* mutant was defective in Yop secretion, we tested whether it was capable of inducing a cytotoxic response in eukaryotic cells. HeLa cells were infected with either the *yscP* mutant YPIII(pIB69) or the wild-type strain YPIII(pIB102), and cytotoxicity was monitored by phase-contrast microscopy (Fig. 2). The results demonstrate that, consistent with the *yscP* mutant's defect in Yop secretion, the cytotoxic effect induced by the *yscP* mutant was delayed relative to that induced by the wild-type strain. The control strain, a *yscP yopB* double mutant which lacks the essential translocator protein YopB (16, 33, 45), did not induce a cytotoxic response. These results suggest that YscP is not required for the translocation of effector proteins into the cytosols of eukaryotic cells but rather exerts its effect at the level of Yop secretion.

Mutations in YscU partly suppress the *yscP* mutant phenotype. Previous studies demonstrated that a *fliK* mutant, which exhibits a polyhook phenotype, could be partially suppressed by mutations in the cytoplasmic domain of the inner membrane protein FlhB (24, 51). Homologues of FlhB are essential components of all TTSSs (for a review, see reference 19), and the *Yersinia* FlhB homologue is YscU. Guided by the suppres-

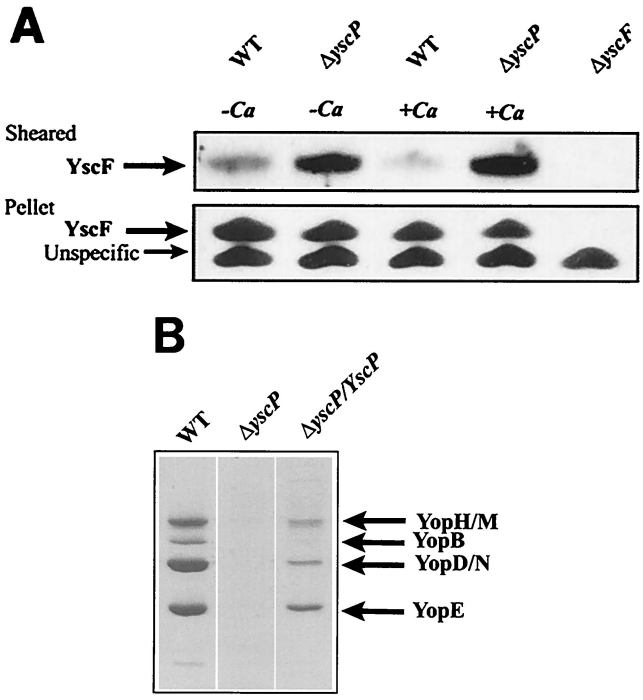

FIG. 1. YscP regulates the substrate specificity of the *Yersinia* TTSS. (A) Surface localization of YscF in the *yscP*-null strain. The wild-type strain YPIII(pIB102) (WT) and the *yscP*-null mutant YPIII (pIB69) were grown at 37°C in BHI medium with or without calcium. The bacteria were harvested by centrifugation, and the pellets were sheared to release surface organelles from the bacterial surface. The bacteria were then centrifuged to separate cell pellet and sheared fractions. Two microliters of the pellet, diluted 1:10, and 5 μl of undiluted supernatant were separated on SDS-PAGE gels, and Western blots were performed by using an anti-YscF antiserum. (B) Yop secretion by the *yscP*-null strain. Bacteria were grown at 37°C in BHI medium without calcium to induce secretion. Bacteria were centrifuged to separate cell pellet and supernatant fractions. Equal amounts of supernatant fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue. The *yscP*-null strain was transcomplemented by pPE34, which expresses YscP under the control of an arabinose-inducible promoter.

sor mutations isolated by Macnab and colleagues, we used site-directed mutagenesis to introduce four individual missense mutations into the cytoplasmic domain of YscU (Fig. 3A). The mutations included the following: A268F(pPE36), Y287G (pPE37), V292T(pPE38), and Y317D(pPE39). The YscU mutants were first expressed in *trans* under the control of the IPTG-inducible *tac* promoter in the *yscU*-null strain YPIII (pIB75). Bacteria were grown at 37°C in the absence of calcium in order to induce Yop secretion. The results show that the level of Yop secretion by the A268F and V292T mutants was equivalent to that of wild-type YscU, while the Y317D mutant secreted much lower levels of Yop (Fig. 3B). The Y287G mutant secreted Yops at an intermediate level (Fig. 3B).

Each of the YscU mutants was then expressed in the *yscP yscU*-double-null mutant to investigate whether it could partially suppress the *yscP* phenotype in comparison with FlhB. Bacteria were grown at 37°C in medium lacking calcium, and Yop secretion was measured. The results indicate that all the YscU mutants restored an increased level of Yop secretion when expressed in the *yscP yscU* double mutant compared to wild-type YscU (Fig. 4A). Consistent with these results, these same *yscU* mutants also restored a more rapid cytotoxic response to the *yscP yscU* strain (Fig. 4B). Thus, these *yscU* mutants could suppress the *yscP* mutation and, similar to the corresponding *flhB* mutants, the suppression was only partial. Next, the surface localization of YscF was examined. Bacteria were grown at 37°C in calcium-containing medium and sheared to release surface-located proteins. The results showed that expression of both YscU-Y287G and YscU-Y317D reduced the amount of surface-localized YscF in the *yscP yscU* double mutant, whereas the other YscU mutations did not (Fig. 4C). The YscU-Y317D suppressor mutation exhibited the strongest suppressor phenotype with respect to both Yop and YscF secretion (compare Fig. 3 with 4A and C). Together, these results confirmed that mutations in YscU can suppress the phenotype of the *yscP*-null mutant, which strongly argues for the fact that YscP and YscU coordinately regulate substrate export by the *Yersinia* TTSS.

YscP acts upstream of YopN, LcrG, LcrQ, and YopD. Previous studies indicated that the LcrG (9, 27, 32) and YopN (4, 12) proteins might act as stop valves or gatekeepers to inhibit Yop secretion prior to eukaryotic cell contact. This is based on the fact that mutations in any of these genes result in a calcium-blind derepressed phenotype in which Yops are secreted in the presence of calcium. To determine the order in which YscP, YscU, and YopN act during the regulation of substrate export by the *Yersinia* TTSS, we constructed a *yopN yscP* double mutant. This strain was grown at 37°C in either the presence or absence of calcium, and Yop secretion was measured. The results showed that Yop secretion was impaired in both the presence and absence of calcium (Fig. 5A). The amount of

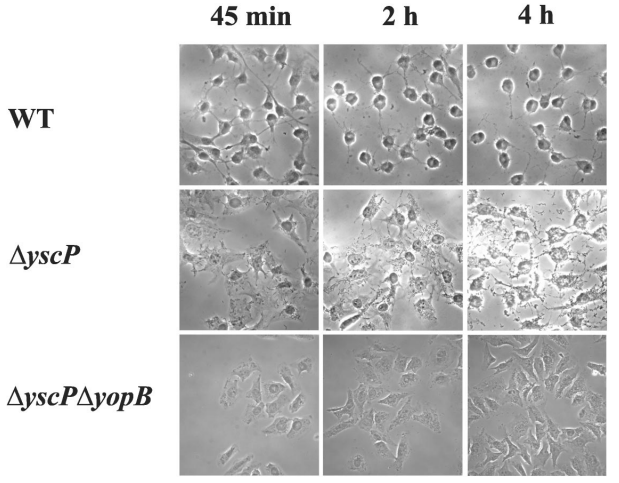

FIG. 2. Cytotoxicity of the *yscP*-null strain. HeLa cells were infected with either the wild-type strain YPIII(pIB102) (WT), the *yscP*-null mutant YPIII(pIB69), or the *yopB yscP*-double-null mutant YPIII (pIB60469) as described in Materials and Methods, and the effect on HeLa cells was recorded. Photographs were taken at the indicated times. A delay in cytotoxicity was seen in the *yscP*-null mutant compared to that seen in the wild-type strain. The *yopB yscP*-double-null strain was used as a negative control, since it lacks the essential translocator protein YopB.

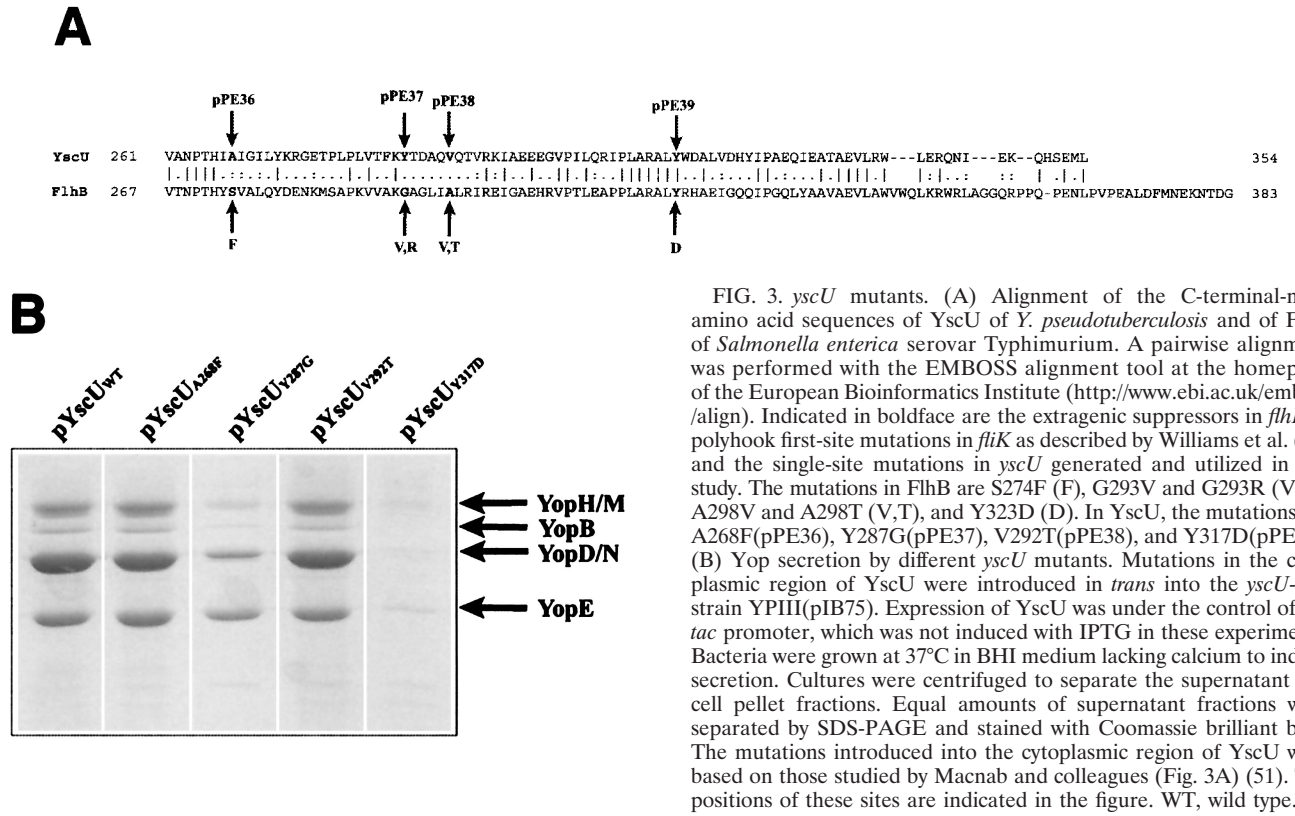

FIG. 3. *yscU* mutants. (A) Alignment of the C-terminal-most amino acid sequences of YscU of *Y. pseudotuberculosis* and of FlhB of *Salmonella enterica* serovar Typhimurium. A pairwise alignment was performed with the EMBOSS alignment tool at the homepage of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align). Indicated in boldface are the extragenic suppressors in *flhB* of polyhook first-site mutations in *fliK* as described by Williams et al. (51) and the single-site mutations in *yscU* generated and utilized in this study. The mutations in FlhB are S274F (F), G293V and G293R (V,R), A298V and A298T (V,T), and Y323D (D). In YscU, the mutations are A268F(pPE36), Y287G(pPE37), V292T(pPE38), and Y317D(pPE39). (B) Yop secretion by different *yscU* mutants. Mutations in the cytoplasmic region of YscU were introduced in *trans* into the *yscU*-null strain YPIII(pIB75). Expression of YscU was under the control of the *tac* promoter, which was not induced with IPTG in these experiments. Bacteria were grown at 37°C in BHI medium lacking calcium to induce secretion. Cultures were centrifuged to separate the supernatant and cell pellet fractions. Equal amounts of supernatant fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue. The mutations introduced into the cytoplasmic region of YscU were based on those studied by Macnab and colleagues (Fig. 3A) (51). The positions of these sites are indicated in the figure. WT, wild type.

the needle component YscF exported to the bacterial cell surface by the *yopN yscP*-null strain was also examined. Bacteria were grown at 37°C in the presence of calcium and were sheared to release YscF from the bacterial cell surface. The results showed that the *yopN yscP* strain exported an abnormally large amount of YscF to the bacterial cell surface (Fig. 5B). The fact that the phenotype of the *yopN yscP* double mutant is similar to that of the *yscP* mutant alone, with regards to the levels of YscF and Yop secretion, indicates that YopN did not directly regulate the substrate specificity of the *Yersinia* TTSS but rather acted downstream of YscP and YscU to regulate substrate export in response to eukaryotic cell contact.

LcrG has been suggested to act as an inner gatekeeper, working at the cytosolic side to regulate substrate export via the TTSS (32), and a *lcrG* mutant expresses and secretes Yops also in the presence of Ca^{2+}. An interesting question was whether LcrG acted upstream or downstream of YscP in the regulatory hierarchy. Therefore, a double *lcrG yscP* mutant was constructed and analyzed. In comparison with the *yopN yscP* double mutant, the *lcrG yscP* mutant did not secrete Yops but instead exported YscF in large amounts to the surface of the cell (data not shown). Thus, YscP and YscU are epistatic over LcrG.

Two additional proteins, LcrQ and YopD, have also been implicated in Yop regulation, since the corresponding mutants in contrast to the wild-type strain showed high levels of Yop expression when incubated at 37°C in the presence of Ca^{2+} (13, 37, 50). We wanted to investigate the possibility that these two proteins affected the YscP-YscU substrate switch. Therefore, we analyzed the phenotypes of the *lcrQ yscP* and *yopD yscP* double mutants. Both double mutants had lost their ability to secrete Yops but had retained their ability to secrete YscF in large amounts to the bacterial surface (data not shown), indicating that neither LcrQ nor YopD affects the substrate specificity switch of the *Yersinia* TTSS.

DISCUSSION

In this work, we have confirmed and extended previous findings regarding the role of YscP in the TTSS by *Yersinia* (35, 46) and shown that *yscP*-null mutants were severely affected in their ability to secrete Yop effector proteins. In contrast, the YscF protein was secreted in elevated amounts. YscF subunits build up a surface-located pilus-like structure, the needle structure, which protrudes from the *Yersinia* TTSS (18). Similar structures have also been identified in *Salmonella* and in *Shigella* (21, 22, 49). YscP shows homology to InvJ of *Salmonella* and Spa32 of *Shigella*. Mutants in either *invJ* or *spa32* assemble extended needles, and these mutants are also unable to secrete effector molecules to the culture supernatant (23, 49). Thus, *yscP*-null mutants show a phenotype similar to that of the *invJ* and *spa32* mutants, indicating that YscP, InvJ, and Spa32 exhibit similar functions in regulating the secretion of effector proteins in the three different species. Work by Kutsukake, Macnab, and coworkers has shown that FliK, which is essential for regulating the hook length of the flagellum, is involved in regulating the subunit secretion of the flagellum export apparatus (24, 51). Interestingly, FliK shows homology with YscP, InvJ, and Spa32 (19), and Galan and coworkers have suggested that InvJ is, in comparison to FliK, involved in regulating substrate specificity (23). Our findings support this view, and we suggest that YscP has a similar function by regulating a

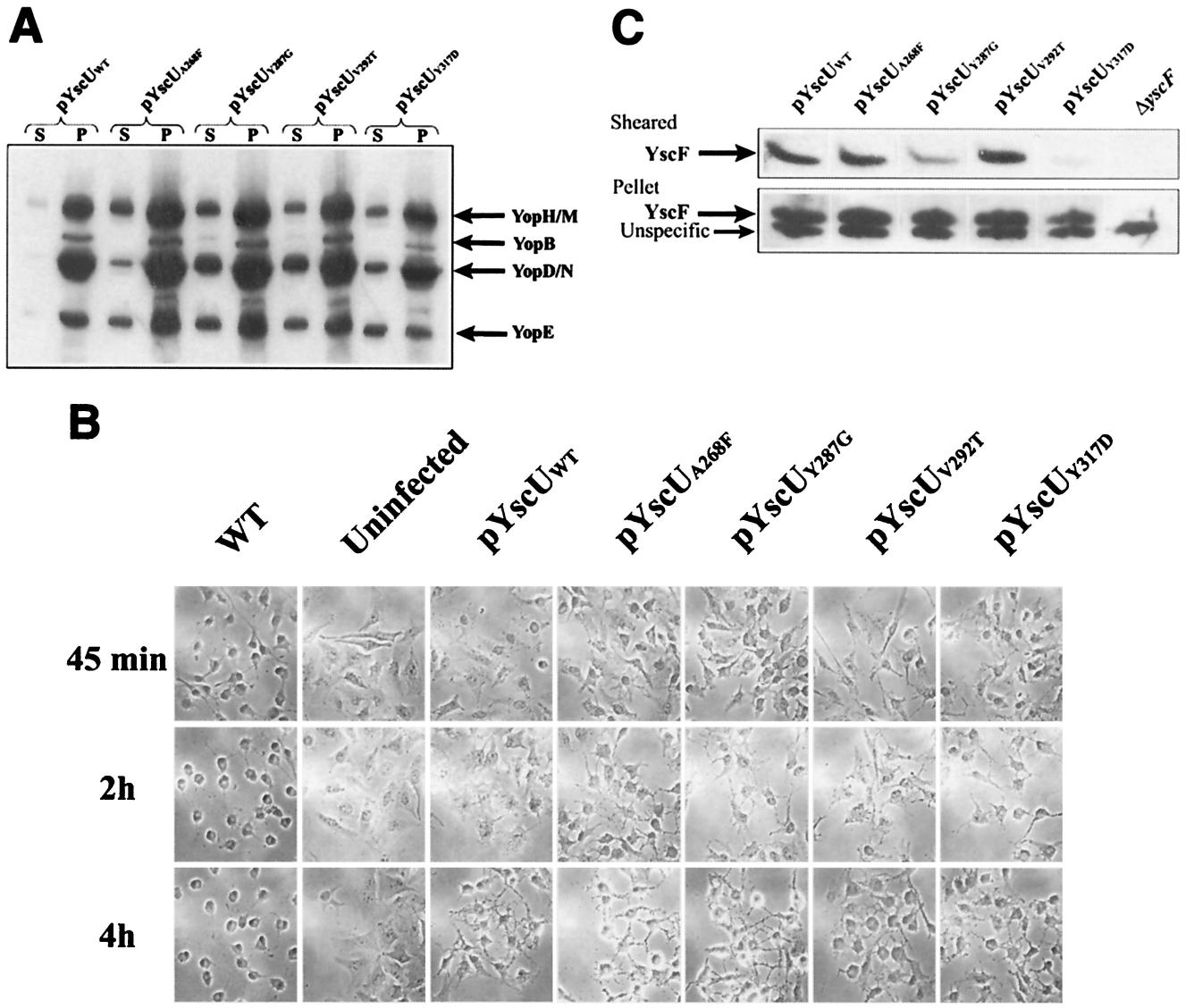

FIG. 4. Mutations in *yscU* suppress the *yscP* mutant phenotype. (A) Mutations in YscU increase Yop secretion by the *yscP* mutant. Mutations in the cytoplasmic region of YscU were introduced in *trans* into the *yscP yscU*-double-null strain. Expression of YscU was under the control of the *tac* promoter, which was not induced with IPTG in these experiments. Bacteria were grown at 37°C in BHI medium lacking calcium to induce secretion. Cultures were centrifuged to separate cell pellet (P) and culture supernatant (S) fractions. Equal percentages of each fraction were separated by SDS-PAGE, and Western blots were performed by using an anti-total Yops antiserum. WT, wild type. (B) Mutations in YscU increase the cytotoxicity of the *yscP* mutant. Mutations in the cytoplasmic domain of YscU were introduced in *trans* into the *yscP yscU*-double-null strain. HeLa cells were infected with these strains as described in Materials and Methods, and the effect on HeLa cells was recorded. Photographs were taken at the indicated times. (C) Mutations in YscU reduce the surface localization of YscF by the *yscP* mutant. Mutations in the cytoplasmic region of YscU were introduced in *trans* into the *yscP yscU*-double-null strain. Bacteria were grown at 37°C in BHI medium plus calcium and were sheared to release surface organelles from the bacterial surface as described in Materials and Methods. Cultures were centrifuged to separate cell pellet and sheared fractions. Two microliters of the pellet, diluted 1:10, and 5 μl of undiluted supernatant were separated by SDS-PAGE, and Western blots were performed by using an anti-YscF antiserum.

switch in secretion from YscF to Yop effectors after the TTSS has been activated by eukaryotic cell contact. How this switch is regulated is at present unclear. Our results show, however, that the inactivation of YscP results in elevated YscF secretion and reduced Yop secretion. This suggests that YscP is inactive prior to contact between the pathogen and the eukaryotic cell. Consequently, YscP is activated upon target cell contact, which results in elevated Yop secretion and subsequent Yop translocation. A potential problem with this model is the fact that YscP is secreted (unpublished results) (35, 46). However, both InvJ and FliK are secreted, and it seems therefore likely that these proteins fulfill their regulatory functions prior to or during secretion and that secretion per se is not a prerequisite for their function (6, 29). The *yscP*-null mutant shows a leaky phenotype with regards to Yop secretion, since the mutant is still cytotoxic for HeLa cells. This is in contrast to *fliK* flagellar mutants and *invJ* mutants of *Salmonella*, which are impaired in substrate secretion (41, 51). The reason behind this difference

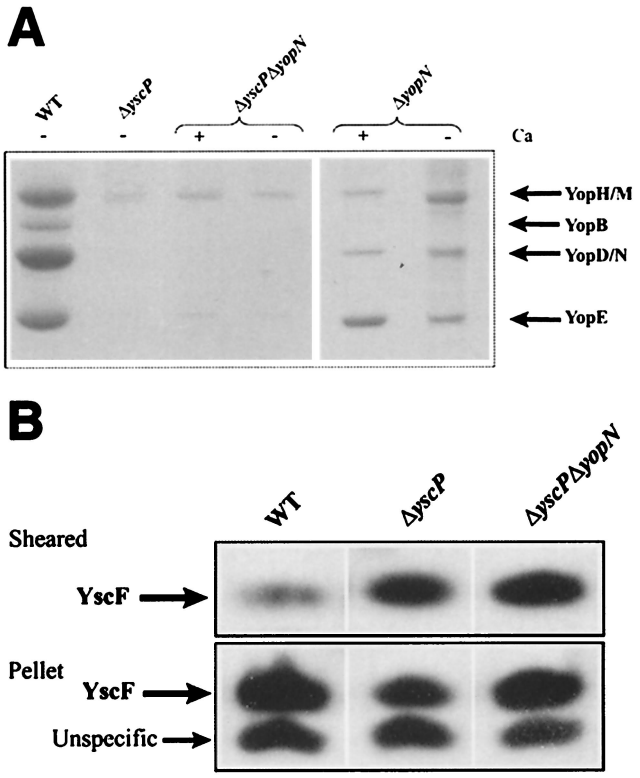

FIG. 5. YscP acts upstream of YopN. (A) The *yopN yscP* double mutant is impaired in Yop secretion. The *yopN yscP*-null strain YPIII (pIB8769) and the *yopN*-null strain YPIII(pIB82) were grown at 37°C in BHI medium with or without calcium to induce secretion. Bacteria were centrifuged to separate cell pellet and supernatant fractions. Equal amounts of supernatant fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue. WT, wild type. (B) Surface localization of YscF in the *yopN yscP* mutant. Bacteria were grown at 37°C in BHI medium plus calcium and were sheared to release surface organelles from the bacterial surface as described in Materials and Methods. Cultures were centrifuged to separate cell pellet and sheared fractions. Two microliters of the pellet, diluted 1:10, and 5 μl of undiluted supernatant were separated on SDS-PAGE gels, and Western blots were performed by using an anti-YscF antiserum.

is unclear, but it is possible that our cytotoxic assay was more sensitive than the assays used to determine the phenotypes of the *fliK* and *invJ* mutants. Another possibility is that the other type III systems of *Yersinia* encoded by chromosomal genes can partially complement the loss of secretion by a *yscP*-null mutant and thus cause an artificial situation.

Interestingly, Kutsukake, Macnab, and coworkers have isolated suppressor mutants that partially restore the *fliK* phenotype with regards to filament assembly. These extragenic suppressors were localized to *flhB* and were found to be substitutions in the carboxy-terminal end of FlhB (Fig. 3A) (24, 51). FlhB is homologous to YscU of *Yersinia*, and when the corresponding amino acids were changed in YscU, partial suppression of the *yscP*-null mutant was obtained. This was evidenced by an increased level of Yop secretion in vitro and by the induction of a more rapid cytotoxic response in infected HeLa cells in vivo. The suppression of the *yscP* mutant phenotype by the YscU-Y317D mutant was particularly impressive given that this mutant secreted much lower levels of Yop than wild-type YscU when expressed in the *yscU*-null strain (compare Fig. 3B with 4A). In addition, the YscU-A268F and YscU-Y317D mutations reduced the amount of YscF present on the bacterial surface in a calcium-containing medium. Given that most of the mutations in YscU involve the replacement of hydrophobic residues with polar residues or larger hydrophobic residues, we suspect that these mutant proteins may have slightly altered conformations that serve to mimic the conformation of YscU that normally occurs when YscP is present. In any event, this suppression suggests that YscU, together with YscP, has a role in regulating the secretion of Yop effectors as well as YscF in the *Yersinia* TTSS. Like FlhB (30), the C-terminal cytoplasmic part of YscU is organized into two domains, as it was demonstrated that YscU was specifically proteolyzed in vivo (25). Although there is no evidence that the proteolysis of YscU per se is important for the function of the *Yersinia* TTSS, it clearly reflects the conformation of the YscU cytoplasmic domains. Specifically, deletion of the conserved proteolytic site of YscU, comprised of amino acid residues 263 through 266, did not abolish the proteolysis of YscU but resulted in a larger C-terminal proteolytic fragment. This result is consistent with the YscU-Δ263-266 mutant having an altered conformation (25). Significantly, this mutant restored only minimal Yop secretion to the *yscU*-null strain, which suggests that the conformation of the cytoplasmic domains of YscU is important for Yop secretion (25). Since YscP and YscU coordinately regulate secretion by the *Yersinia* TTSS, we suggest that YscP may serve to regulate the conformation of YscU in the cytosol of the bacterial cell.

It has been suggested that the LcrG (9, 27, 32) and YopN (4, 12, 20) proteins function as gatekeepers to prevent Yop effector secretion prior to eukaryotic cell contact. Specifically, these mutants display a calcium-independent phenotype in which Yop secretion occurs, even when the bacteria are grown in a medium that contains calcium. LcrG is thought to act intracellularly (9, 27, 32), while YopN (12) is thought to act on the bacterial cell surface. The question is whether these proteins act downstream of YscP and YscU or whether they have a direct role in regulating the substrate specificity of the *Yersinia* TTSS. Our results showed that both a *yopN yscP* and a *lcrG yscP* double mutant exhibit a phenotype essentially the same as that of the *yscP* mutant alone. That is, the double mutants exported an increased amount of the needle component YscF to the bacterial cell surface in the presence of calcium but were unable to efficiently secrete Yops in either the presence or absence of calcium. These results suggest that YopN and LcrG act downstream of YscP and YscU. Thus, these proteins may serve as a safety mechanism to prevent the premature secretion of Yop effectors until eukaryotic cell contact occurs.

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