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YscU, a *Yersinia enterocolitica* Inner Membrane Protein Involved in Yop Secretion

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Pathogenic yersiniae secrete antihost Yop proteins by a recently discovered secretion pathway which is also encountered in several animal and plant pathogens. The components of the export machinery are encoded by the *virA* (*lcrA*), *virB* (*lcrB*), and *virC* (*lcrC*) loci of the 70-kb pYV plasmid. In the present paper we describe *yscU*, the last gene of the *virB* locus. We determined the DNA sequence and mutated the gene on the pYV plasmid. After inactivation of *yscU*, the mutant strain was unable to secrete Yop proteins. The topology of YscU was investigated by the analysis of YscU-PhoA translational fusions generated by Tn*phoA* transposition. This showed that the 40.3-kDa *yscU* product contains four transmembrane segments anchoring a large cytoplasmic carboxyl-terminal domain to the inner membrane. YscU is related to Spa40 from *Shigella flexneri*, to SpaS from *Salmonella typhimurium*, to FlhB from *Bacillus subtilis*, and to HrpN from *Pseudomonas solanacearum*.

Pathogenic bacteria of the genus *Yersinia* (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) cause rodent and human diseases ranging from enteritis to fatal septicemia, essentially by invading the host tissues. They harbor the closely related pYV plasmids which are essential for virulence (Fig. 1). At 37°C and in the absence of Ca^{2+} ions, these plasmids cause a growth arrest and the release of large amounts of proteins called Yops. These Yops are designated by a letter which is identical for the homologous proteins in the three *Yersinia* species. For recent reviews on the Yops, see the work of Cornelis (13), Straley et al. (52), and Forsberg et al. (21).

The *yop* genes are scattered around the pYV plasmid (Fig. 1A). Their transcription requires both a temperature of 37°C and the presence of an activator of the AraC family encoded by the *virF* gene (11, 27, 29).

The Yops are secreted via a recently discovered pathway that does not involve the cleavage of a classical N-terminal signal sequence (33, 35). The secretion signal is nevertheless localized in the amino-terminal domain, and the system readily secretes hybrid proteins made of the N terminus of YopH or YopE fused to various prokaryotic or even eukaryotic proteins (33, 34, 51). This export system appears to be an archetype of a new family of systems recently encountered in bacteria which are pathogenic for animals (*Shigella flexneri*, *Yersinia* spp., and *Salmonella typhimurium*) and plants (*Pseudomonas solanacearum*, *Pseudomonas syringae*, *Xanthomonas campestris*, and *Erwinia amylovora*) (for recent reviews, see the work of Salmond and Reeves [45], Van Gijsegem et al. [54], and Forsberg et al. [20]). Related genes are also encountered in the loci involved in flagellum synthesis and assembly (1, 9). In *S. flexneri*, this pathway is devoted to the secretion of the Ipa proteins (3–5, 55). The system is also encountered in *S. typhimurium* (22, 24), but the secreted products have not been identified yet. *P. syringae*, *E. amylovora*, and *P. solanacearum* use the same pathway to secrete the elicitor of the hypersensitive reaction observed in plants (6, 18, 23, 26, 58, 59, 62).

The Yop secretion system, encoded by the pYV plasmid, appears to be a complex mechanism which requires the products of multiple genes of the *virA* (*lcrA*), *virB* (*lcrB*), and *virC* (*lcrC*) loci (15). The *Y. enterocolitica* 8.5-kb *virC* operon is composed of 13 genes called *yscA* to *yscM* (for Yops secretion). So far, only *yscD*, *yscJ*, and *yscL* have been definitely shown to be required for secretion (34). Parts of this operon have been described in *Y. pestis* (25) and in *Y. pseudotuberculosis* (44). The inner membrane protein LcrD, encoded by the *virA* locus, is another component of the secretion machinery (41, 42). YscN, encoded by the first gene of the *virB* locus, is an ATP-binding protein which could act as an energizer of the secretion process (60). The products of the neighboring *yscQ*, *yscR*, and *yscS* genes in the *Y. pestis lcrB* locus are also involved in the Yop secretion (19). One particular feature of the Yop secretion system is its use of cytoplasmic chaperones which are specific for individual Yops (57). These chaperones, called Syc (for specific Yop chaperone), presumably recognize the export signals of their nascent Yop partner proteins and lead them to the translocon (57).

The *virB* locus has not been completely characterized so far. In the present work we describe YscU, a novel protein of the Yop secretion machinery which is encoded by the last gene of the *virB* locus. It is the homolog of Spa40 from *S. flexneri*, a protein involved in the secretion of the Ipa invasin (48). It is also related to SpaS from *S. typhimurium* (24), to FlhB from *Bacillus subtilis* (9), and to HrpN from *P. solanacearum* (23).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) of the serotype O:9 strain W227 (14). Strain W22703(pBM33) carries a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*) inserted into the *yopQ* gene which allows the transcription of *yopQ* to be monitored (38). *Escherichia coli* LK111 and DH5α lambda *pir*$^+$ were used for standard genetic manipulations. *E. coli* SM10 lambda *pir*$^+$, constructed by Miller and Mekalanos (37), was used to deliver mobilizable plasmids to *Y. enterocolitica*. *E. coli* CC102F′ (carrying the Tn*phoA* transposon in the chromosome) and KS272 (Δ*phoA*) (received from C. Parsot, Institut Pasteur,

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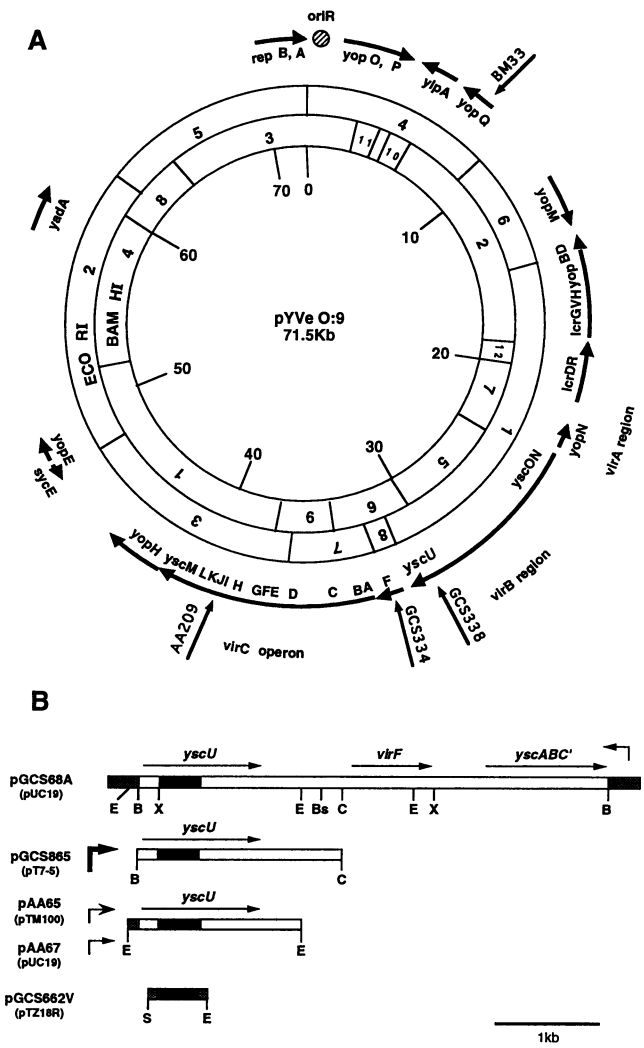

FIG. 1. (A) Genetic map of the pYV227 plasmid integrating previous data (13). The arrows (oriented to the center) indicate the positions of the mutants used in this study. The position of *yscU* is shown at the end of the *virB* region. (B) Structure of plasmids carrying *yscU*, *virF*, and the beginning of the *virC* operon (*yscABC'*). The plasmids are shown in linear form in which *Y. enterocolitica* DNA is indicated by open bars (black bars represent the 400-bp internal DNA fragment of *yscU* that has been used for the construction of the *yscU* mutant). The DNA vector in pGCS68A (not shown to scale) is indicated by broken lines; the vector names are indicated in parentheses. The thin arrows represent the *lac* promoter (pGCS68A and pAA67) and the *cat* promoter (pAA65); the thick arrows correspond to the ϕ10 promoter of bacteriophage T7. Arrows indicate the positions and extents of *yscU*, *virF*, and *yscABC'*. The positions of selected restriction sites are shown: B, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; S, *Sph*I; and X, *Xho*I.

Paris, France) were used for Tn*phoA* mutagenesis and alkaline phosphatase assays, respectively.

The strains were routinely grown in tryptic soy broth (Oxoid, Basingstoke, England) and plated on tryptic soy agar (Oxoid), sometimes supplemented with 20 mM $MgCl_2$ and 20 mM Na-oxalate (MOX). For the induction of the *yop* regulon, *Y. enterocolitica* was grown in brain heart infusion (Difco, Detroit, Mich.) supplemented with 4 mg of glucose ml^{-1}, 20 mM $MgCl_2$, and 20 mM Na-oxalate (BHI-OX). M9 medium ($MgSO_4$, 1 mM; $CaCl_2$, 100 μM; NaCl, 86 mM; Na_2HPO_4, 420 mM; KH_2PO_4, 220 mM; NH_4Cl, 190 mM; and glucose, 4 mg ml^{-1}) was used for the labeling of YscU. Antibiotics were used at the following concentrations: ampicillin, 300 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; gentamicin, 5 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and nalidixic acid, 35 μg ml^{-1}.

Plasmids are listed in Table 1.

Molecular cloning and sequencing procedures. Plasmid DNA purification, restriction, separation by gel electrophoresis, transfer, hybridization, ligation, and transformation of *E. coli* strains were performed according to standard methods (46). Nucleotide sequences were determined by the dideoxy chain termination procedure (47) on single- or double-stranded DNA. The sequence of *yscU* was determined on plasmids pGCS68A and pGCS68B and on a set of overlapping deletion mutants of pGCS661 and pGCS662 generated with T4 DNA polymerase (17) by using the Cyclone system of International Biotechnologies Inc. (New Haven, Conn.). Both DNA strands were completely sequenced.

Identification of the *yscU* gene product. Specific labeling of YscU was achieved in *E. coli* by using the T7 expression system (53). *E. coli* LK111 containing both pGP1-2 and either pT7-5 or pGCS865 (Fig. 1B) was grown in 10 ml of tryptic soy broth with ampicillin and kanamycin. At an optical density at 600 nm of 0.5, 1 ml of culture was centrifuged and bacteria were washed in 1 ml of M9 medium. Cells were then resuspended in 5 ml of M9 medium supplemented with thiamine (20 μg ml^{-1}), 19 amino acids (minus methionine) (each at final concentration of 100 μg ml^{-1}), ampicillin, and kanamycin and incubated with shaking at 28°C in a 100-ml conical flask for 180 min. The temperature was then shifted to 42°C for 15 min. Rifampin (Lepetit, Milan, Italy) was subsequently added to a final concentration of 400 μg ml^{-1}. The incubation temperature was maintained at 42°C for an additional 10 min and afterwards shifted down to 37°C for 20 min. The samples were then labeled with 20 μCi of [^{35}S]methionine (Dupont NEN Research Products, Brussels, Belgium) for 15 min at 37°C, and the cells were finally centrifuged, resuspended in sample buffer, and loaded on a sodium dodecyl sulfate (SDS)–14% polyacrylamide gel electrophoresis (PAGE) gel.

Induction of the *yop* regulon and analysis of Yop production and Ca^{2+} dependency. The Yops were prepared and analyzed by SDS-PAGE and Western blotting (immunoblotting) as described by Cornelis et al. (15) and by Sory and Cornelis (50). For analysis of the intracellular proteins, 7×10^{10} bacteria were disrupted by sonication in 800 μl of phosphate-buffered saline (50 mM Na-phosphate, 150 mM NaCl [pH 7.4]). Undisrupted cells were removed by three rapid centrifugations (1 min each) at 10,000 $\times$ *g*. Membranes were then obtained by a 30-min centrifugation at 10,000 $\times$ *g*. Aliquots of the soluble fraction, containing the cytoplasmic and periplasmic proteins and membranes of 2.5×10^8 bacteria, were applied to SDS–14% PAGE gel. Immunoblotting procedures were carried out using rat monoclonal antibodies 6G1 (anti-YopE) and 13A4 (anti-YopD), described by Bodeus et al. (7). The Ca^{2+} requirement for growth at 37°C was monitored by plating the bacteria on tryptic soy agar supplemented with 5 mM $CaCl_2$ and on MOX agar at 37°C.

Construction of a *yscU* mutant (pGCS338). The *Eco*RI-*Sph*I DNA fragment of pGCS662V containing nucleotides 285 to 680 of *yscU* (Fig. 1B and 2) was cloned into the suicide vector pGP704, giving pGCS907. Since *Y. enterocolitica* is resistant to ampicillin (12), we introduced a gentamicin resistance gene (39) into pGCS907. *E. coli* SM10 lambda *pir*$^+$ carrying the resulting plasmid, pGCS908, was then mated overnight at 32°C with *Y. enterocolitica* W22703(pBM33). The recombinant *Y.*

TABLE 1. Plasmids used in this study

Plasmid	Genetic structure	Source or reference
pAA65	pTM100 + 1,680-bp *Eco*RI fragment of pGCS68A (kb 31.0–32.6)	This paper
pAA67	pUC19 + *yscU* expressed from *lacp* (*Eco*RI deletion of pGCS68B)	This paper
pAA73	pAA67 *yscU* codon 80::Tn*phoA*	This paper
pAA85	pAA67 *yscU* codon 58::Tn*phoA*	This paper
pAA106	pAA67 *yscU* codon 36::Tn*phoA*	This paper
pAA110	pAA67 *yscU* codon 175::Tn*phoA*	This paper
pAA111	pAA67 *yscU* codon 63::Tn*phoA*	This paper
pAA113	pAA67 *yscU* codon 130::Tn*phoA*	This paper
pAA114	pAA67 *yscU* codon 76::Tn*phoA*	This paper
pAA115	pAA67 *yscU* codon 103::Tn*phoA*	This paper
pAA116	pAA67 *yscU* codon 337::Tn*phoA*	This paper
pAA117	pAA67 *yscU* codon 29::Tn*phoA*	This paper
pAA209	pYVe227 *yscJ*::*apha3* (nonpolar mutation)	2
pBC18R	pTZ18R + *oriT*	10
pBM33	pYVeO:9 *yopQ*::Tn*2507* (*yopQ-cat* operon fusion)	38
pCL5	pKNG78-*virF*$^+$ (*tacp virF*)	29
pGCS68A	pUC19 + *Bam*HI fragment 6 of pYVeO:9 (kb 31.0–36.5) (oriI)	11
pGCS68B	pUC19 + *Bam*HI fragment 6 of pYVeO:9 (kb 31.0–36.5) (oriII)	11
pGCS660	pTZ18R + 2,640-bp *Xho*I fragment of pYVeO:9 (kb 31.2–33.9) (oriI)	This paper
pGCS661	pBC18R + 2,640-bp *Xho*I fragment of pYVeO:9 (kb 31.2–33.9 (oriII)	This paper
pGCS662	pBC18R + 1,590-bp *Xho*I-*Bst*EII fragment of pYVeO:9 (kb 31.2–32.8)	This paper
pGCS662V	deletion of pGCS662 by T4 DNA polymerase (kb 31.2–31.6)	This paper
pGCS334	pBM33 *virF*::pGCS904	29
pGCS338	pBM33 *yscU*::pGCS908	This paper
pGCS865	pT7-5 + 2,110-bp *Bam*HI-*Cla*I fragment of pYVeO:9 (kb 31.0–33.1)	This paper
pGCS907	pGP704 + 400-bp *Eco*RI-*Sph*I fragment (kb 33.1–33.7) from pGCS662V	This paper
pGCS908	pGCS907 + *Xba*I fragment containing *aadB* (gentamicin) from Tn*732* (39)	This paper
pGP1-2	pBR322 + *c*I857 + T7 RNA polymerase from promoter p_L	53
pGP704	ori_{6K} $oriT_{RK2}$ bla^+	37
pTM100	pACYC184 + *oriT* from RK_2	33
pTZ18R	pUC18 + T7 RNA polymerase ϕ10 promoter	Pharmacia
pT7-5	Contains T7 RNA polymerase ϕ10 promoter oriented opposite to the *bla* gene	53

enterocolitica W22703(pGCS338) carrying pGCS908 integrated in the pYV derivative pBM33 was selected on gentamicin and nalidixic acid. The disruption of *yscU* was confirmed by Southern blot analysis (data not shown).

Tn*phoA* mutagenesis. For Tn*phoA* mutagenesis, the target plasmid pAA67, carrying the *yscU* gene (Fig. 1B), was first introduced in *E. coli* CC102F′, in which the Tn*phoA* transposon is integrated in the chromosome. Ten colonies were separately inoculated in 1 ml of tryptic soy broth. After 1 h of incubation at 37°C, 9 ml of the same medium containing 100 μg of ampicillin ml^{-1} and 400 μg of kanamycin ml^{-1} was added and the colonies were further incubated overnight. Plasmid DNA was then extracted and used to transform *E. coli* KS272 (Δ*phoA*). In-frame fusions of the transposon in pAA67 were identified on plates containing XP (5-bromo-4-chloro-3-indolyl-phosphate), the chromogenic substrate of alkaline phosphatase, at a final concentration of 40 μg ml^{-1}. The Tn*phoA* insertion sites were determined by DNA sequencing using oligonucleotide MIPA178 (5′ AATATCGCCCTGAGCA 3′), which hybridizes to nucleotides 71 to 87 of the 5′ end of Tn*phoA* (31).

Enzymatic assays. CAT and alkaline phosphatase (PhoA) were assayed as described by Shaw (49) and Manoil and Beckwith (31), respectively. The alkaline phosphatase activity was expressed in milli-optical density units at 420 nm per minute per optical density unit at 600 nm (36).

Sequence analysis. Identity scores between proteins were calculated with the FASTA program based on the method of Pearson and Lipman (40). The multiple alignments were made with the MULTALIN program with the Dayhoff matrix (16). The isoelectric point was calculated with the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis software package. The signal sequence was researched by using the SIGSEQ program (43) implementing to the rules of von Heijne (56). The hydropathy index was calculated by the method of Kyte and Doolittle (28), using a window of 19 amino acids.

Nucleotide sequence accession number. The sequence of *yscU* has been submitted to the GenBank Nucleotide Sequence Data Libraries under accession number U08019.

RESULTS

Sequence analysis of *yscU*. The sequence of the *Bam*HI-*Eco*RI DNA fragment spanning kb 31.0 to 32.6 of pYV439-80 (11, 30) is presented in Fig. 2. It contains an open reading frame (ORF) extending to nucleotide 1102 at the 3′ end. The ORF extends in the 5′ direction, outside the given sequence. However, at position 40, there is an ATG codon which is preceded by a fairly good, adequately spaced ribosome binding site (TGGAGA). This ATG overlaps a stop codon in another reading frame, a situation reminiscent of the *virC* operon (34). This suggested that the ATG at position 40 starts an ORF which belongs to a multicistronic operon of the *virB* locus. This ORF is followed by a 359-bp noncoding region containing from 7 up to 11 stop codons in the three reading frames. This DNA segment includes a 42-nucleotide palindrome (from nucleotide 1461 to 1503) which could be a transcriptional terminator, suggesting that the ORF presented in Fig. 2 is the last gene of the *virB* locus. This hypothesis was confirmed by the genetic analysis presented below. According to a common nomencla-

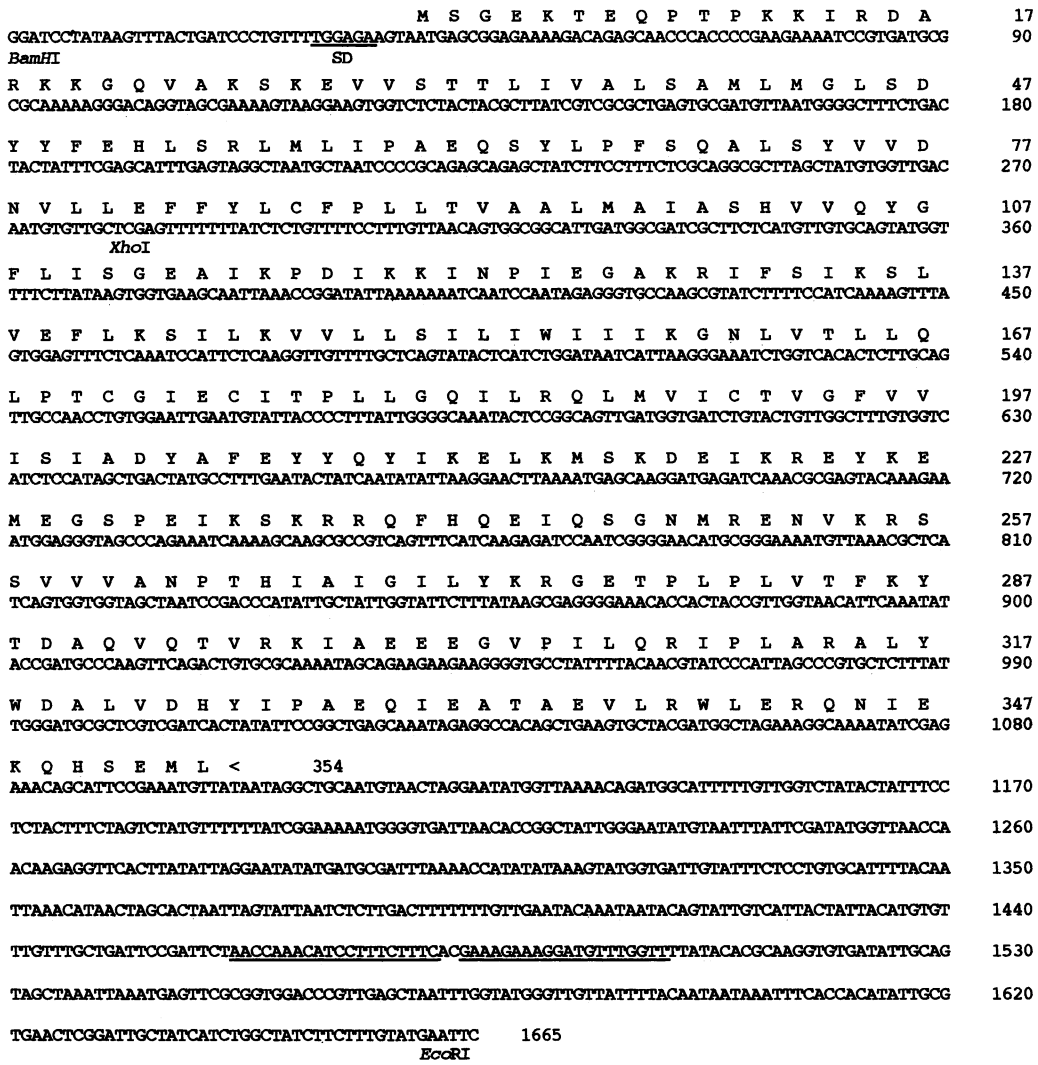

FIG. 2. Nucleotide sequence of the 1,650-bp *Bam*HI-*Eco*RI DNA fragment (kb 31.0 to 32.6) of the pYV plasmid (11, 30) containing *yscU* and deduced amino acid sequence of YscU. SD indicates a putative Shine-Dalgarno sequence. Important restriction endonuclease sites are shown. The putative terminator is underlined.

ture for *virB* (*lcrB*) proposed by H. Wolf-Watz (University of Umeä, Umeä, Sweden), this ORF was designated *yscU*.

yscU encodes a 354-amino-acid-residue polypeptide with a calculated molecular mass of 40.3 kDa. Its isoelectric point is predicted to be 9.11. According to the von Heijne algorithm (56), no classical N-terminal signal sequence was present in this putative YscU protein. Hydropathy analysis of the predicted amino acid sequence of YscU showed four hydrophobic regions, suggesting that YscU could be anchored in the inner membrane (data not shown).

Visualization of the *yscU* gene product. In order to visualize the predicted YscU protein, we used pGCS865, a plasmid in which *yscU* is cloned downstream of the bacteriophage T7 gene ϕ10 promoter (Fig. 1B). Whereas no protein was detected in the strain carrying pT7-5 (Fig. 3, lane 1), expression from pGCS865 resulted in the synthesis of several polypeptides (Fig. 3, lane 2). The molecular mass of the largest protein was 40.3 kDa, which corresponds to the size expected from *yscU* translation. The other bands could represent either degradation products of YscU or proteins resulting from the use of alternative translation start sites located within *yscU*.

Similarity analysis of YscU. The amino acid sequence of YscU was compared with the protein sequences available from GenBank. This comparison revealed extensive similarity between YscU and proteins from animal or plant pathogens known to contain elements of the new secretion system.

YscU has 33.3% identity to Spa40 from *S. flexneri* (48), 33.2% identity to SpaS from *S. typhimurium* (24), and 31% identity to HrpN from *P. solanecearum* (23). FlhB, a component of the flagellar assembly apparatus from *B. subtilis*, has 33.6% identity to YscU (9). The alignments of the YscU, FlhB, HrpN, Spa40, and SpaS sequences are presented in Fig. 4.

The similarity detected between YscU and these proteins encompasses their entire length, but some regions are much more conserved than others (Fig. 4). Moreover, the hydrophobicity profile is also highly conserved (data not shown); this suggests that all of these proteins perform similar functions. Since Spa40 has been shown to be involved in the export or surface presentation at the cell surface of the *S. flexneri* Ipa invasin (48), YscU could be a component of the Yop secretion machinery.

Construction and characterization of a *yscU* mutant. To inves-

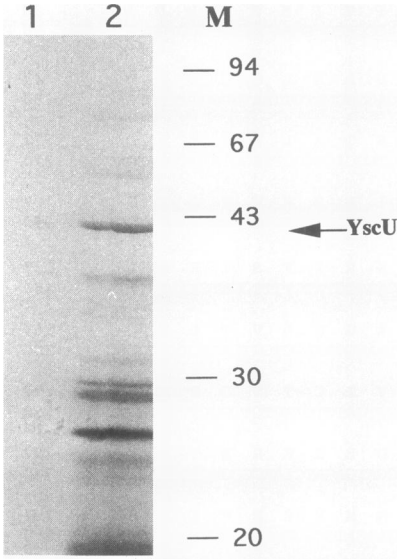

FIG. 3. Expression of the *yscU* gene with T7 RNA polymerase (53). Lanes: 1, *E. coli* LK111(pT7-5)(pGP1-2); 2, *E. coli* LK111(pGCS865)(pGP1-2). The positions of molecular size markers (in kilodaltons) are indicated. The arrow points to YscU.

tigate the role of YscU, we disrupted the *yscU* gene by integrating pGCS908, a suicide vector containing an internal DNA fragment of *yscU*, in the pYV plasmid. The resulting *yscU* mutant strain, *Y. enterocolitica* W22703(pGCS338), was independent of Ca^{2+} for its growth at 37°C and did not release Yops upon thermal induction in Ca^{2+}-depleted medium (Fig. 5I, lane B). To ensure that this phenotype was indeed due to the *yscU* mutation, we introduced pAA65, a low-copy-number plasmid carrying *yscU* transcribed from the *cat* promoter of the pTM100 vector (Fig. 1B). Strain W22703(pGCS338)(pAA65) required Ca^{2+} for its growth at 37°C and secreted approximately the same amount of Yops as the wild-type strain (Fig. 5I, lane C). YopD, however, was not secreted as well by W22703(pGCS338)(pAA65) as by the wild-type bacteria. As a control, we introduced the pTM100 vector into the *yscU* mutant, which still displayed the mutated phenotype. Hence, the cloned *yscU* gene complemented the mutation, showing that the defect of Yop secretion in strain W22703(pGCS338) was solely due to the inactivation of the *yscU* gene. The complementation also confirmed that the *yscU* gene was complete in the *Bam*HI-*Eco*RI DNA fragment presented in Fig. 2 and suggested that *yscU* is the last gene of the *virB* operon. The poor secretion of YopD by W22703(pGCS338)(pAA65) was not investigated further. It could result from some imbalance in the relative amounts of Ysc proteins.

To decide at what level the production of the Yops was compromised, we monitored the presence of YopD and YopE among the intracellular proteins by immunoblotting using monoclonal anti-YopD and anti-YopE antibodies. There was clearly much less YopD and YopE in both the soluble (cytoplasmic and periplasmic) and membrane fractions of the *yscU* mutant (Fig. 5II, lanes B) than in the corresponding fractions of the wild-type bacteria (Fig. 5II, lanes A). The introduction of pAA65 in the *yscU* mutant restored normal amounts of YopD and YopE. This suggested that the lack of YscU resulted in a reduction in Yop synthesis. In this respect, the *yscU* mutant was different from the *yscJ* mutant (*virC*) (2), which was also unable to secrete Yops (Fig. 5I, lane F), but YopE and YopD were nevertheless detectable (Fig. 5II, lanes F). In order to assay the effect of *yscU* on *yop* transcription, we took advantage of a *yopQ-cat* transcriptional fusion present on pBM33 (38), the pYV plasmid used for the construction of the *yscU* mutant. We monitored transcription of *yopQ* by assaying CAT activity in W22703(pBM33) (*yscU*$^+$ *virF*$^+$), in W22703(pGCS338) (*yscU virF*$^+$), and in W22703(pGCS334) (*yscU*$^+$ *virF*). The low CAT activity detected in the *yscU* mutant (Table 2) confirmed that the lack of YscU reduced *yop* transcription. Therefore, it was not clear whether YscU is directly involved in Yop secretion. However, the similarity detected between YscU and proteins involved in protein secretion (23, 24, 48) suggested that in addition to its role in Yop synthesis, YscU could be a component of the Yop secretion system. To test this hypothesis, we decided to overcome the reduction in Yop production by overexpression of VirF, the transcriptional activator (11, 29). We introduced pCL5, a multicopy plasmid harboring *virF* under the control of the *tac* promoter (29), into the *yscU* mutant W22703(pGCS338). We induced *virF* and *yop* transcription by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C and monitored the presence of YopD and YopE by immunoblot analysis. As shown in Fig. 5II, lanes E, YopD and YopE appeared in membrane and intracellular fractions but not in the culture supernatants (Fig. 5I, lane E). This result unambiguously demonstrates that YscU is also involved in Yop secretion. We concluded that YscU is a physical part of the secretion machinery which, in addition, has an indirect effect on *yop* transcription, supporting our previous hypothesis of a feedback transcriptional regulation by the export system (15).

Construction of YscU-PhoA protein fusions. The analysis of the predicted amino acid sequence of YscU revealed the presence of four hydrophobic domains, suggesting that YscU is a transmembrane protein (data not shown). To test this hypothesis, we constructed and analyzed YscU-PhoA hybrid proteins using Tn*phoA*. In-frame fusions of *phoA* with the region coding for a periplasm-facing segment of a membrane protein allow export of the PhoA portion of the hybrid protein, resulting in high alkaline phosphatase activity. In contrast, fusions of PhoA to segments of the target protein normally located on the cytoplasmic side of the membrane exhibit a low phosphatase activity (8, 31, 32). Transposon mutagenesis was carried out in *E. coli* CC102F′ on plasmid pAA67 (Fig. 1B). After Tn*phoA* mutagenesis, several colonies giving different intensities of blue color on plates containing XP were analyzed. The Tn*phoA* insertion sites were determined by sequencing the junctions. Ten different Tn*phoA* insertion sites within *yscU* were identified. Tn*phoA* was found to be inserted after codons 29 (pAA117), 36 (pAA106), 58 (pAA85), 63 (pAA111), 76 (pAA114), 80 (pAA73), 103 (pAA115), 130 (pAA113), 175 (pAA110), and 337 (pAA116).

The alkaline phosphatase activity expressed by the plasmids carrying the various *yscU*::*phoA* fusions was assayed in *E. coli* KS272 (Table 3); the obtained levels of alkaline phosphatase activity allowed us to propose the model presented in Fig. 6 for the transmembrane topology of YscU.

DISCUSSION

The Yop secretion process has been shown to involve the *virA*, *virB*, and *virC* loci of the virulence plasmid pYV (19, 21, 34, 44). In the present study, we characterized YscU, the product of the last gene of the *virB* locus. YscU has significant similarity to other secretion apparatus components, including HrpN of *P. solanacearum* (23) and Spa40 and SpaS, produced by the last gene of the *spa* locus of *S. flexneri* (48) and *S. typhimurium* (24), respectively. YscU is also related to FlhB from *B. subtilis*, a protein involved in flagellum synthesis and assembly (9). The *spa* locus is surprisingly well conserved in *S.*

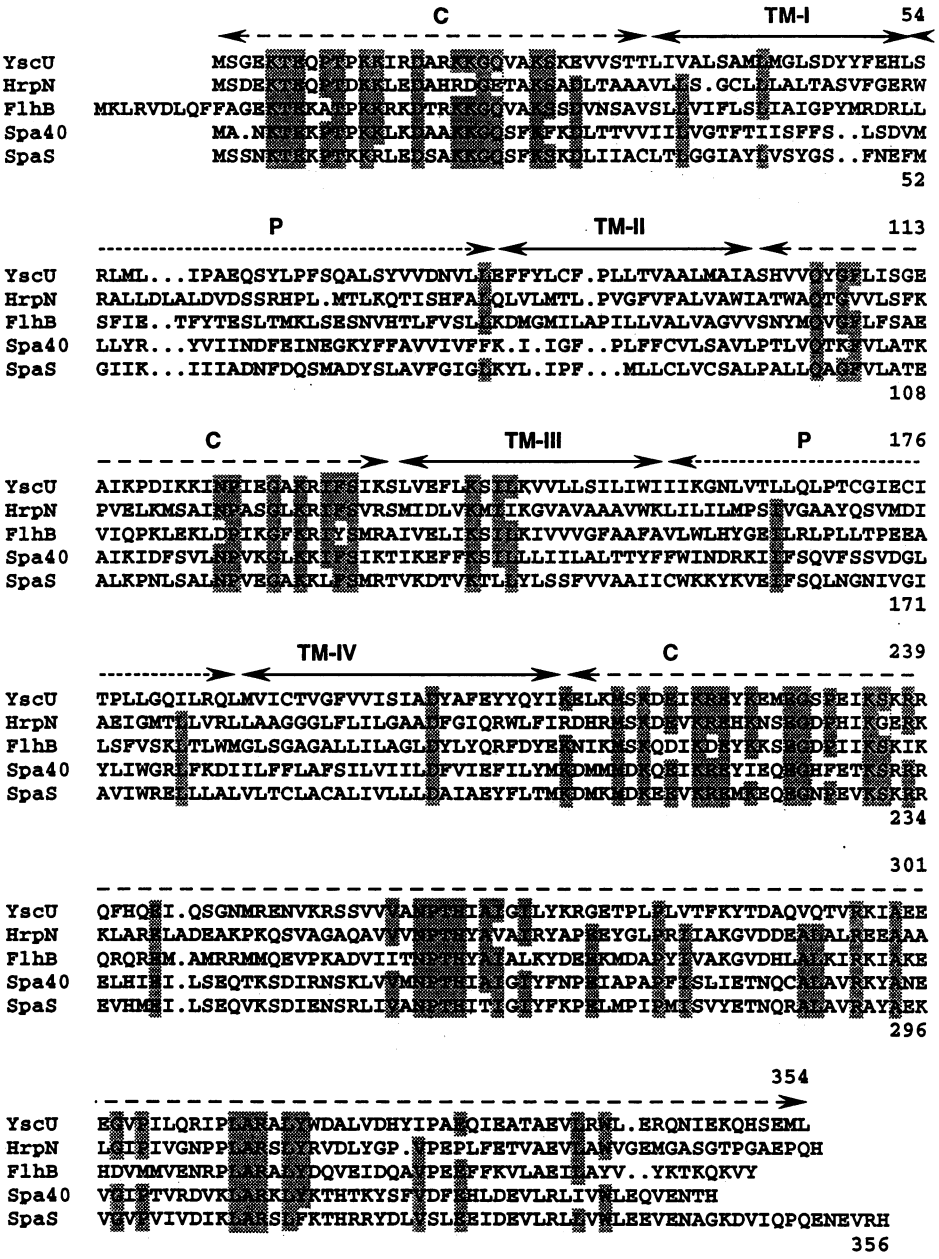

FIG. 4. Amino acid sequences of YscU, HrpN (*P. solanacearum*) (23), FlhB (*B. subtilis*) (9), Spa40 (*S. flexneri*) (48), and SpaS (*S. typhimurium*) (24). The alignment was done using the MULTALIN program (16) with a gap penalty of 8. Residues conserved in at least four proteins are shaded. C, putative cytoplasmic domains of YscU; TM, putative transmembrane domains of YscU; P, putative periplasmic domains of YscU.

flexneri (48, 55) and in *S. typhimurium* (24). The degree of conservation varies between 20 and 50% identity for each individual gene, but every gene has its counterpart in both species at the same relative position. The distance separating the proximal *spa47* (*spaL*) from the distal *spa40* (*spaS*) is 4.5 kb. This length exactly matches the distance separating *yscN* from *yscU*, the first and last genes of the *virB* operon. This unavoidably suggests that the whole *virB* (*lcrB*) locus of *Y. enterocolitica* could be the counterpart of the *spa* loci in *S. flexneri* and *S. typhimurium*. Wolf-Watz and colleagues made the same observation in *Y. pseudotuberculosis*, and they proposed the nomenclature that was adopted in this paper (61). All this suggests that YscU must also be a part of the Yop secretion machinery. In order to test this hypothesis, we inactivated the *yscU* gene on the pYV plasmid and showed that the resulting bacteria were defective in Yop secretion. As previously observed for the *virC* and the *yscN* mutants (34, 60), the *yscU* mutant produced less YopD and YopE than the wild-type bacteria, which suggested that Yop synthesis was affected by the inactivation of the *yscU* gene. To confirm the role of *yscU* in secretion, we introduced a plasmid carrying the *virF* gene on multicopy plasmid in the *yscU* mutant. By overproducing the transcriptional activator VirF, transformants regained their ability to produce Yops but were unable to secrete them. These results clearly demonstrate that YscU is indeed a component of the secretion machinery. Thus, YscU

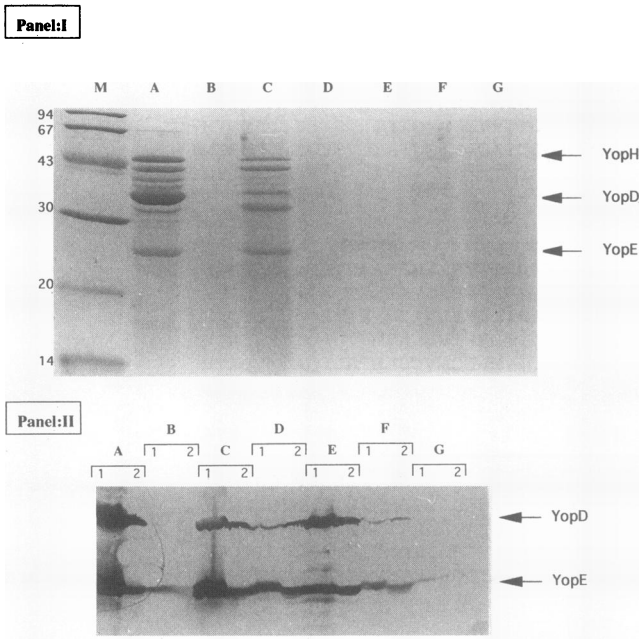

FIG. 5. Analysis of Yop secretion and synthesis. (I) *Y. enterocolitica* W22703 containing the following plasmids: lane A, pBM33 (*yscU*$^+$ *virF*$^+$) (38); lane B, pGCS338 (*yscU*); lane C, pGCS338 and pAA65; lane D, pGCS338 and pCL5 (without IPTG); lane E, pGCS338 and pCL5 (with IPTG); lane F, pAA209 (*yscJ*) (2); lane G, pGCS334 (*virF*) (29). Culture supernatants were separated by SDS-PAGE and stained with Coomassie brilliant blue. The positions of YopH, YopD, and YopE are indicated by arrows. (II) Western blot analysis of YopD and YopE. Membrane (lanes 1) and soluble (periplasmic and cytoplasmic) (lanes 2) subcellular fractions of *Y. enterocolitica* W22703 harboring plasmids described for panel I in the same order (lanes A to G) were transferred onto nitrocellulose (after SDS-PAGE) and incubated with a mixture of monoclonal antibodies directed against YopD and YopE. The positions of YopD and YopE are indicated by arrows.

seems to play a dual role: a direct one in secretion and a probable indirect one on transcription. The latter, which remains to be explained, was not due to a polar effect on *virF* transcription, because the wild-type phenotype could be restored in the *yscU* defective strain only by the introduction of a plasmid carrying *yscU* but not *virF*.

Translation of the DNA sequence gave a protein devoid of a classical N-terminal signal sequence but possessing four hydrophobic domains. This suggested that YscU could be localized in the inner membrane. To test this hypothesis, we mutagenized plasmid-borne *yscU* with Tn*phoA* and analyzed

TABLE 2. Activity of the *yopQ* promoter in *yscU* mutants[a]

Strain	Genotype	CAT (U)	
		$-Ca^{2+}$	$+Ca^{2+}$
W22703(pBM33)	*yscU*$^+$ *virF*$^+$	2,531 ± 450	82 ± 36
W22703(pGCS338)	*yscU virF*$^+$	92 ± 32	68 ± 25
W22703(pGCS334)	*yscU*$^+$ *virF*	19 ± 20	8 ± 12

[a] Bacteria grown overnight at room temperature in brain heart infusion were inoculated to an optical density at 600 nm of 0.1 in a conical flask containing 10 ml of BHI-OX. Cultures were incubated with rotary shaking (150 rpm) for 2 h at room temperature and then shifted for 4 h to 37°C. Bacteria were washed, resuspended in 10 ml of water, and sonicated. CAT activity was assayed as described by Shaw (49). Values presented are the averages for three experiments.

TABLE 3. Alkaline phosphatase activity expressed by *yscU*::*phoA* fusions in *E. coli*[a]

Plasmid (fusion[b])	PhoA (U)
pSF22[c]	420 ± 30.55
pAA117 (29)	40 ± 18.56
pAA106 (36)	70 ± 5.50
pAA85 (58)	378 ± 12.16
pAA111 (63)	350 ± 15.27
pAA114 (76)	404 ± 17.90
pAA73 (80)	205 ± 27.84
pAA115 (103)	50 ± 5.29
pAA113 (130)	39 ± 20.01
pAA110 (175)	340 ± 36.05
pAA116 (337)	25 ± 24.01

[a] Alkaline phosphatase activity was assayed as described elsewhere (31) in transformed *E. coli* KS272 (Δ*phoA*) cells grown to the stationary phase. Values presented are the averages for three independent experiments.

[b] For each plasmid, the last amino acid of the YscU moiety of the hybrid PhoA is indicated in parentheses.

[c] Plasmid containing the *S. flexneri* hybrid lipoprotein MxiJ-PhoA, which expressed a high alkaline phosphatase activity (3).

10 YscU-PhoA fusion proteins. The analysis of their primary structures and enzymatic activities supports the hypothesis of a protein spanning the inner membrane. It also suggests that two loops are exposed in the periplasm while the N terminus, the C terminus, and one loop are cytoplasmic. The long cytoplasmic C-terminal domain could be an effector of the putative regulatory function of YscU. Figure 6 gives the hypothetical topology model of YscU. Since the homologs of YscU in the related systems all have the same hydrophobicity pattern (data not shown), they also probably have the same topology as that proposed for YscU.

If one accepts that *virC* probably contains 10 genes involved in Yop secretion, this phenomenon probably requires at least 19 genes, excluding the *syc* genes encoding the individual chaperones. Some of their products can be tentatively localized in the cell. YscC has been assigned to the outer membrane (34). The lipoprotein YscJ is also very likely to be anchored in the outer membrane (34), while LcrD, YscR, and YscU span the inner membrane (19, 41). According to its putative ener-

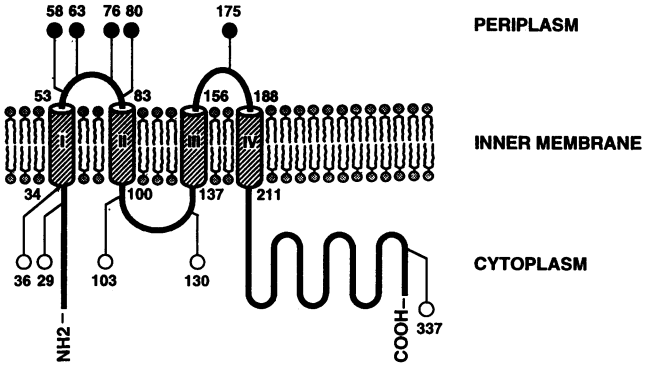

FIG. 6. Schematic two-dimensional model of YscU and locations of YscU-PhoA fusions. The four predicted membrane-spanning α-helices are represented by cylinders. Numbers near the cylinders indicate the amino acid residues at each end. Solid circles correspond to fusions with high alkaline phosphatase activity (>200 U). Open circles correspond to low-activity fusions (<100 U). Numbers above or below each circle indicate the amino acid residue in which the fusion joint is located.

gizer role, YscN could be a cytoplasmic protein attached to the inner membrane via an integral membrane protein. We hypothesize that YscN, a highly conserved piece of the machinery, binds to a protein which is also highly conserved among the various homologs of the family. If this proposal is correct, one would exclude LcrD because the homologs of the LcrD family greatly vary in their C-terminal cytoplasmic domains. In contrast, YscU could be a candidate for YscN binding: its cytoplasmic domains are highly conserved, while the transmembrane domains and the loops exposed in the periplasm differ significantly. Of course, other inner membrane proteins may well appear among the products of others *ysc* genes of the *virB* and the *virC* loci.

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