

YscO of *Yersinia pestis* Is a Mobile Core Component of the Yop Secretion System

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The *Yersinia pestis* low-Ca²⁺ response stimulon is responsible for the temperature- and Ca²⁺-regulated expression and secretion of plasmid pCD1-encoded antihost proteins (V antigen and Yops). We have previously shown that *lerD*, *yscC*, *yscD*, *yscG*, and *yscR* encode proteins that are essential for high-level expression and secretion of V antigen and Yops at 37°C in the absence of Ca²⁺. In this study, we characterized *yscO* of the Yop secretion (*ysc*) operon that contains *yscN* through *yscU* by determining the localization of its gene product and the phenotype of an in-frame deletion. The *yscO* mutant grew and expressed the same levels of Yops as the parent at 37°C in the presence of Ca²⁺. In the absence of Ca²⁺, the mutant grew independently of Ca²⁺, expressed only basal levels of V antigen and Yops, and failed to secrete these. These defects could be partially complemented by providing *yscO* in *trans* in the *yscO* mutant. Overexpression of YopM and V antigen in the mutant failed to restore the export of either protein, showing that the mutation had a direct effect on secretion. These results indicated that the *yscO* gene product is required for high-level expression and secretion of V antigen and Yops. YscO was found by immunoblot analysis in the soluble and membrane fractions of bacteria growing at 37°C irrespective of the presence of Ca²⁺ and in the culture medium in the absence of Ca²⁺. YscO is the only mobile protein identified so far in the *Yersinia* species that is required for secretion of V antigen and Yops.

The genus *Yersinia* contains three species that are pathogenic for humans: *Y. pestis*, the causative agent of bubonic plague, and *Y. pseudotuberculosis* and *Y. enterocolitica*, which cause gastroenteritis and mesenteric lymphadenitis. A requirement for the pathogenesis of yersiniae is the ability to both express and secrete *Yersinia* outer proteins (Yops) and V antigen (also called LcrV) (10, 11, 50). The genes that encode Yops and V antigen, the proteins which regulate their expression, and the proteins of the Yop-specific secretion apparatus (Ysc) are carried on a ca. 70-kb plasmid found in all virulent isolates of pathogenic yersiniae (referred to as pCD1 in *Y. pestis* KIM) (6, 19). Yops and V antigen have antihost functions that subvert innate defenses (e.g., phagocytosis) and cellular signaling and cytokine expression necessary for the mobilization of an effective immune response (14). They are crucial for the survival and multiplication of yersiniae in lymphoid tissues (14, 50, 74). Yops exert their antihost effects within eukaryotic cells. When yersiniae contact a eukaryotic cell, at least four Yops (YopE, YopH, YopM, and YpkA) are vectorially targeted into the cell at the point of contact (9, 28, 52, 59, 71) by a process requiring YopB, YopD, and YopK (29, 31, 59, 71). In *Y. pestis*, the secreted Yops and V antigen are subject to degradation by Pla, a plasminogen activator protease, present on the bacterial surface and encoded by the *Y. pestis*-specific plasmid pPCP1 (69, 70). Despite its ability to rapidly degrade Yops on the cell surface (60, 61, 69), Pla has little effect on the expression, secretion, or vectorial targeting of Yops (46, 55, 56, 66–68, 72).

High-level expression of Yops and V antigen, concomitant with secretion, is called the low-Ca²⁺ response (LCR). In vitro

this occurs at 37°C in the absence of Ca²⁺ and is accompanied by growth cessation (termed restriction). Ca²⁺ is required in millimolar concentrations for growth in certain media at temperatures above 34°C (80). The LCR is believed to be manifest in vivo upon yersinial contact with a eukaryotic cell (14). It is believed that growth restriction does not occur in vivo (23) but that Yops and V antigen expression and secretion are regulated and that the absence of Ca²⁺ in vitro mimics cell contact (14, 73). The genes that are coordinately downregulated (73) by Ca²⁺ are referred to as the LCRS (low-Ca²⁺ response stimulon) (72).

A cluster of genes occupying ca. 25 kb on the *Yersinia* LCR plasmid is responsible for the regulation of expression, secretion, and postsecretion targeting of Yops (13, 50). Most of these (at least 22 gene products) encode the type III, or contact-dependent Yop secretion mechanism, Ysc (13, 14, 43). This is one of the best characterized of a relatively new class of secretion mechanisms present in a wide variety of gram-negative pathogens of mammals and plants (37). The Ysc mechanism is environmentally modulated in its activity at 37°C, and this modulation is indirectly responsible for the Ca²⁺ regulation of *yop* transcription seen in vitro. A current model for this effect (14) holds that under conditions that do not activate the Ysc (presence of Ca²⁺ and absence of contact with a eukaryotic cell), proteins necessary for negative regulation (LcrQ [53, 58] and YopD [77]) are retained in the bacterial cytoplasm, where they permit only a low expression of Yops and V antigen. However, this small pool of virulence proteins is available for immediate targeting upon imposition of Ysc-activating conditions (5). Upon cell contact, the Ysc is activated locally between the eukaryotic and bacterial cells and the effector Yops are vectorially targeted into the eukaryotic cell by the postsecretion delivery mechanism containing YopB, YopD, and YopK. The negative regulator LcrQ also is secreted out of the bacterial cell (53, 58), and this permits upregulation of *yop*

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transcription (14, 53). In vitro, the absence of Ca^{2+} is thought to activate all secretion channels and cause strong *yop* induction, through the massive secretion of YopD and LcrQ. Up-regulation of translation of at least some Yops may also occur upon the activation of the Ysc and be coordinated with targeting of the newly expressed Yops to the Ysc (5). This also would ensure a rapidly available supply of additional Yops for targeting and a greater degree of amplification of Yops expression than that expected from transcriptional induction alone (5).

The Ysc secretes Yops, without processing, through both bacterial membranes. Although some Yops can be obtained in small amounts in periplasmic fractions (46), there is no evidence for a true, obligatory periplasmic phase of Yop secretion. By analogy to the type III secretion system of centisome 63 in *Salmonella typhimurium* (35), the Ysc probably spans both membranes as a supramolecular complex. Three operons encode the secretion mechanism proper: *lcrDR* (55), *ysoA* to *ysoM* (27, 43), and *ysoN* to *ysoU* (3, 7, 20, 78); VirG appears to promote insertion of the secretin YscC into the outer membrane (1, 34). Additional genes, including those that encode LcrE (also called YopN), LcrG, LcrV, and TyeA, modulate the activity of the Ysc in response to environmental inputs such as Ca^{2+} and cell contact (22, 32, 46, 66, 67, 79). Finally, members of the *lcrGVH-yopB-yopD* operon function in the postsecretion targeting of Yops (14).

Homologies to components of the flagellar basal body have been noted for LcrD (54, 55) and all except YscP of the products of the eight-member *ysoN* to *ysoU* operon (3, 7, 20, 78). All of these except YscO, YscP, and YscQ have been shown or assumed on the basis of predicted sequences to be inner membrane proteins or inner membrane-associated proteins (in the case of the putative energizer protein YscN). The only other known inner membrane component is YscD (not a flagellar component homolog) (43, 56). It has been speculated that these homologies could reflect a supramolecular analogy between the flagellar basal structure and the inner membrane and membrane-spanning regions of the type III mechanism, and this is supported by the recently demonstrated shape of the *Salmonella* type III mechanism (35).

Other than VirG, which probably is located in the outer membrane, the only proven outer membrane component of the Ysc is the secretin YscC, which oligomerizes to form a ring-shaped structure with a central pore (34). This has been assumed to serve as a channel for Yop secretion through the outer membrane (34). Interestingly, the homolog in *Salmonella*, InvG, was found to be one of three main components of a needle-like projection from the putative outer membrane ring of the isolated type III complex (35).

In *Y. pestis*, the LcrD, YscC, YscD, YscG, and YscR products have been shown to be required for Yop secretion (20, 27, 56). In *Y. pseudotuberculosis* or *Y. enterocolitica*, YscC to YscG, YscI to YscL, YscN, YscQ, YscR, and YscU have been shown to be essential for the secretion of Yops (2, 3, 7, 43, 78). All are thought to be core (essential) components of the Ysc and to exert an indirect inductive effect on *yop* operon expression through their requirement for the secretion of negative regulators such as LcrQ and YopD out of the bacterial cell (14, 53, 77). Mutations in any of the essential Ysc components abolish the Ca^{2+} requirement for growth (a phenotype referred to as Ca^{2+} independence), limit the induction of LCRS operons at 37°C to the level typical for the presence of Ca^{2+} , and block the secretion of LCRS proteins (20, 25, 55, 56).

ysoN and *ysoQ* through *ysoT* have a high sequence similarity to counterparts in loci for virulence protein secretion in *Salmonella* pathogenicity island 1 (SPI1) at centisome 63 (*spa* or

inv), SPI2 at centisome 30 (*ssa*), *Shigella flexneri* (*spa*), and enteropathogenic *Escherichia coli* (*esc*) (18, 24, 26, 30, 63). The *Yersinia* and both *Salmonella* loci are similar in the size and arrangement of the *ysoN* to *ysoU* homologs. However, the predicted sequences of *ysoO* and *ysoP* have at best weak similarity to homologs in type III systems and no similarity to any non-type III-related proteins outside the *Yersinia* spp. For example, YscO is 21% identical (37% similar) to SpaM/InvI of the *Salmonella* SPI1 and 20% identical (23% similar) to *Shigella* Spa13. This suggests that YscO and YscP may play a specific role in the recognition, secretion, or targeting of the *Yersinia*-specific Yops or V antigen and prompted us to initiate their characterization. In this study, we have focused on *ysoO*. We constructed a mutant with essentially a complete deletion of *ysoO* and characterized it for the expression and secretion of V antigen and Yops. The data show *ysoO* to be essential for high-level expression and secretion of V antigen and Yops, with a direct effect on secretion. We show that under LCR-inductive conditions, YscO is weakly expressed and found in all bacterial fractions. To our knowledge, this is the first mobile core component of the Ysc.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used in this study and their relevant properties are listed in Table 1, and some are shown in Fig. 1. *Y. pestis* KIM5-3001 (LCR⁺) was the parent strain (wild type [wt]) used in these studies. It contains three naturally occurring *Y. pestis* plasmids: pCD1 (containing the genes encoding the LCR phenotype) (19, 25), pPCP1 (encoding the plasminogen activator [Pla] responsible for the degradation of Yops [69]), and pMT1 (encoding the F1 capsular protein) (57). *Y. pestis* KIM8-3002 (LCR⁺, Pla⁻; wt Pla⁻) was used in experiments in which degradation of proteins would have affected the analysis of the results. *E. coli* strains were typically grown in Luria-Bertani (LB) broth or on LB agar (15). *Y. pestis* strains were routinely grown in heart infusion broth or on tryptose blood agar base plates (Difco Laboratories, Detroit, Mich.) at 26°C. For physiological studies, *Y. pestis* strains were grown in TMH defined liquid medium (72) supplemented with 2.5 mM CaCl_2 as indicated. The medium was inoculated to an optical density at 620 nm of ca. 0.1 from a culture that had been growing exponentially at 26°C with shaking at 200 rpm for about seven generations. Cultures were started at 26°C and then shifted to 37°C when the optical density reached ca. 0.2. Cells and secreted proteins were harvested at 5 or 6 h after the temperature shift. All bacteria with antibiotic resistances were grown in the presence of the appropriate antibiotic(s) (100 µg/ml for ampicillin and streptomycin).

DNA methods. Cloning methods, including the use of restriction endonucleases, T4 DNA ligase, isolation of plasmid DNA (8, 33, 41), and electroporation (51), were as previously described. The PCR technique (44) was performed with 20 to 30 cycles of amplification; the denaturing, annealing, and extending conditions were 94, 55, and 72°C for 30 s each in a model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Double-stranded DNA was sequenced by the method of Sanger et al. (62) with the Sequenase 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio) and [α -³⁵S]dATP (NEN Research Products, Boston, Mass.) or by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington).

Site-directed mutagenesis of *ysoO*. To study an effect of *ysoO* on the LCR, we used a variation of two site-directed in vitro mutagenesis protocols (11a, 56a) to construct an in-frame deletion in *ysoO* that effectively created a null mutation. A single-stranded DNA (ssDNA) template of pYscOP.2 (Table 1; Fig. 1) coding strand was prepared by using the helper phage M13K07, as described previously (41). The mutagenesis reaction involved annealing a selection oligonucleotide and two mutagenic oligonucleotides to the ssDNA template. The 5'-phosphorylated selection oligonucleotide (Trans Oligo *AlwNI/SpeI*; Clontech) changed *AlwNI* to *SpeI* in the vector region of pYscOP.2. The two mutagenic oligonucleotides used to introduce *AvrII* sites into *ysoO* at bp 881 and 1321 were 5'-ACTTTAACCCGGCCCTAGGCGCGGTATC-3' and 5'-TCATTAGGCGTTCCTAGGATGCTGTAG-3', respectively. (The underlined portions represent *AvrII* sites; the bold sequences represent bases that were changed during mutagenesis; nucleotide numbering corresponds to the numbering of GenBank accession no. L25667). The mutagenic oligonucleotides were 5'-end phosphorylated (Promega) and annealed to ssDNA at 100°C for 3 min with a 200-fold molar excess of each of the three oligonucleotides over the ssDNA template (Clontech). The mutant strand was synthesized with T4 DNA polymerase to prevent strand displacement and allow the annealing of all three oligonucleotides in one reaction (42, 48). After ligation (Clontech), any nonmutagenized parental pYscOP.2 DNA remaining in the mixture was selectively linearized by digestion with *AlwNI* to decrease its transformation efficiency, and the DNA was transformed into a repair-defective strain of *E. coli* (BMH 71-18 *mutS*). The use of

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
<i>E. coli</i>		
BMH 71-18 <i>mutS</i>	<i>thi supE</i> $\Delta(lac-proAB)$ [<i>mutS::Tn10</i>] [<i>F' proAB laqI^qZAM15</i>]	Promega
DH5 α	<i>end-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> $\Delta(lacIZYA-argF)U169 deoR[\phi80dlac\Delta(lacZ)M15]$	GIBCO-BRL
<i>Y. pestis</i> ^a		
KIM5-3001 (wt)	Sm ^r ; pCD1 (LCR ⁺), pPCP1(Pla ⁺) pMT1	38
KIM5-3001.16 ($\Delta yscO$)	Sm ^r ; pCD1 <i>yscO</i> ($\Delta 6-151$), ^b pPCP1(Pla ⁺), pMT1	This study
KIM6-3001	Sm ^r ; pCD1 ⁻ (LCR ⁻), pPCP1(Pla ⁺)	38
KIM8-3002 (wt Pla ⁻)	Sm ^r ; pCD1, pPCP1 ⁻ (Pla ⁻), pMT1	75
KIM8-3002.3 ($\Delta yscO$ Pla ⁻)	Sm ^r ; pCD1 <i>yscO</i> ($\Delta 6-151$), ^b pPCP1 ⁻ (Pla ⁻), pMT1	This study
Plasmids		
pBluescript II SK+	Ap ^r ; cloning vector	Stratagene
pBluescript II SK-	Ap ^r ; cloning vector	Stratagene
pYP-F2	Ap ^r ; <i>Bam</i> HI F fragment cloned from pBGCD1 carrying <i>yscN'OPQRS</i> , with frameshift mutation in <i>yscR</i> , was cloned into pBluescript II SK- with the insert oriented with <i>lac</i> promoter	20
pYscOP.2	Ap ^r ; 2.38-kb <i>Bpu</i> 1102I fragment of pYP-F2 carrying <i>yscO</i> and <i>yscP</i> was filled in with Klenow and cloned into <i>Xho</i> I-digested/Klenow blunt-ended pBluescript II SK+ with the insert oriented with the <i>lac</i> promoter	This study
pYscOP	Ap ^r ; same as pYscOP.2 with insert oriented with the T7 promoter	This study
pYscO.2	Ap ^r ; <i>Sph</i> I- <i>Kpn</i> I (<i>Kpn</i> I site in vector) digestion of pYscOP.2 followed by blunt ending with T4 polymerase and religation resulting in the elimination of <i>yscP</i> and carrying <i>yscO</i>	This study
pYscO	Ap ^r ; <i>Cla</i> I (one site in vector) digest of pYscOP followed by religation of plasmid, carries <i>yscO</i>	This study
p $\Delta yscO$	Ap ^r ; <i>Avr</i> II ^c -digested <i>pyscOP.2</i> was filled in with Klenow and religated, resulting in a deletion of <i>yscO</i> ($\Delta 6-151$) ^b	This study
pYscN' Δ OP	Ap ^r ; 400-bp <i>Sac</i> II fragment of pYP-F2 ligated with ~5.0-kb <i>Sac</i> II fragment of p $\Delta yscO$ carrying <i>yscN'</i> , <i>yscO</i> ($\Delta 6-151$), ^b and <i>yscP</i>	This study
pYscP	Ap ^r ; 1.8-kb <i>Ava</i> I fragment of pYP-F1 (20) carrying <i>yscP</i> , filled in with Klenow and cloned into <i>Eco</i> RV site of pBluescript II SK+ with insert oriented with the T7 promoter	This study
pHTV	Ap ^r ; expression vector carrying <i>lcrV</i> ; translationally fused to a leader encoding 19 residues, including 6 histidines	21, 45
pTRCM.2	Ap ^r ; expression vector carrying <i>yopM</i> behind the <i>trc</i> promoter	56
pGEX-3X	Ap ^r ; GST fusion expression vector using the <i>tac</i> promoter	Pharmacia
pGST-YscO	Ap ^r ; 1.9-kb <i>Mlu</i> I- <i>Eco</i> RI (vector site) fragment of <i>pyscO</i> filled in with Klenow following <i>Mlu</i> I digestion and directionally cloned into <i>Sma</i> I- <i>Eco</i> RI-digested pGEX-3X (expresses fusion protein of GST and aa 13-154 of YscO)	This study
pUK4134	Ap ^r ; suicide vector <i>oriR6K oriT cos rpsL</i>	65
pUK $\Delta yscO$	Ap ^r ; ~2.3-kb <i>Xba</i> I (in vector site)- <i>Age</i> I fragment of <i>pyscN'ΔOP</i> filled in with Klenow and cloned into the <i>Eco</i> RV site of pUK4134, carries <i>yscO</i> ($\Delta 6-151$) ^b	This study
pCVD442	Ap ^r Suc ^s ; suicide vector	16
pCVD442 $\Delta yscO$	Ap ^r Suc ^s ; same insert as in pUK $\Delta yscO$ cloned into the <i>Sma</i> I site of pCVD442	This study

^a All *Y. pestis* strains are Pgm⁻ (76).

^b Numbers in parentheses give the amino acids deleted from the protein product.

^c *Avr*II sites introduced by site-directed mutagenesis (see Materials and Methods).

BMH 71-18 *mutS* prevented repair of the newly synthesized unmethylated strand and provided high mutation efficiency. A 100- μ l volume of the transformation mix was subjected to 60 min of incubation in LB broth without antibiotics and then to overnight selection in the presence of ampicillin, and plasmid DNA was isolated and subjected to a second digestion with *Alw*NI to enrich for mutant plasmid DNA (undigested). Following desalting with a Qiaex kit (Qiagen Inc., Studio City, Calif.), the plasmid digestion mix was introduced into *E. coli* DH5 α . Plasmid DNA from selected transformants was sequenced to verify the introduction of *Avr*II sites at bp 881 and 1321. Plasmid DNA containing both *Avr*II sites was digested with *Avr*II, filled in with Klenow, and religated to form p $\Delta yscO$, which encoded only 9 of the original 154 amino acids of *yscO* but retained the ribosome binding site of the downstream *yscP* gene. This was transformed into *E. coli* DH5 α . Again, use of the correct in-frame deletion was confirmed by sequencing the double-stranded DNA (dsDNA) of transformants.

DNA from a clone with the correct in-frame deletion was digested with *Sac*II to produce a 72-bp fragment and a ~5.0-kb fragment. The ~5.0-kb fragment carried the 3' end of *yscN*, the *yscO* deletion, and *yscP*. The dephosphorylated ~5.0-kb *Sac*II fragment and a 400-bp *Sac*II fragment of pYP-F2, containing additional *yscN* sequences, were ligated to form pYscN' Δ OP. This *Y. pestis* DNA insert contained 870 bp upstream of the *yscO* start codon, the mutant *yscO*

sequence, and the coding sequence of *yscP*. The resulting clones were checked for proper orientation of the *Sac*II fragments by PCR amplification with primers that annealed to portions of both *Sac*II fragments. The ends of a *Xba*I-*Age*I fragment excised from pYscN' Δ OP were filled in with Klenow, and the resulting ~2.3 kb fragment containing the mutant *yscO* sequence and ~900 bp on either side of the sequence was cloned into the *Eco*RV site of pUK4134 to form pUK $\Delta yscO$ and into the *Sma*I site of the suicide vector pCVD442 (16) to form pCVD442 $\Delta yscO$. *Y. pestis* KIM5-3001.16 ($\Delta yscO$) was created by allelic exchange with pUK $\Delta yscO$, as previously described (65). *Y. pestis* KIM8-3002.3 ($\Delta yscO$ Pla⁻) was created by allelic exchange with pCVD442 $\Delta yscO$. Plasmid pCVD442 $\Delta yscO$ was electroporated into *Y. pestis* KIM8-3002. Selection for re-solvants of the plasmid was performed by growth on medium containing 5% sucrose. Allelic exchange was confirmed by checking the size of the $\Delta yscO$ -containing fragment on a *Hind*III digestion of pCD1 and by PCR analysis with primers outside the region mutagenized. Additionally, we sequenced a PCR fragment of the regions ~500 bp upstream and downstream of $\Delta yscO$ in *Y. pestis* KIM5-3001.16 to verify that the deletion and adjacent sequences were correct after allelic exchange.

Antibody preparation. To determine the location of YscO in bacterial fractions, antibody was raised in rabbits against a fusion protein of glutathione

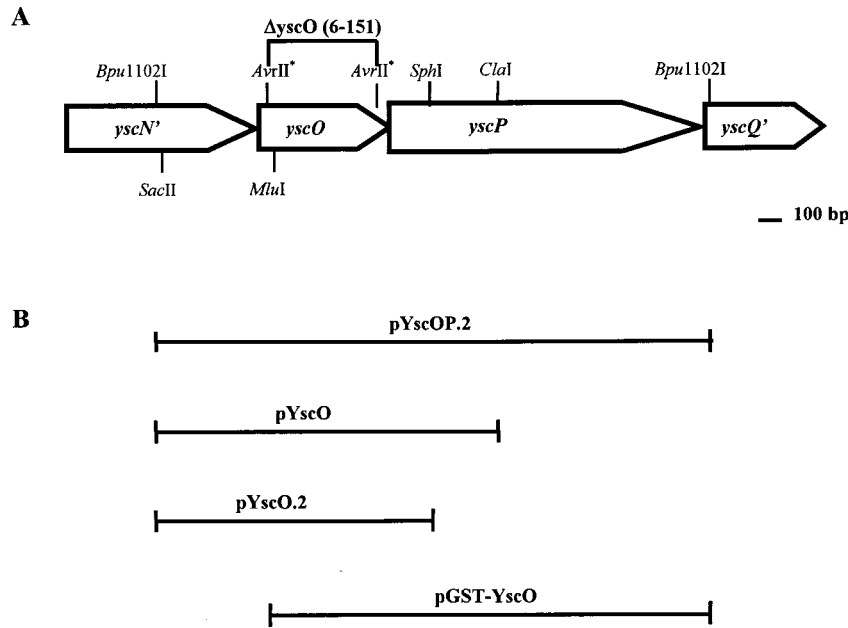


FIG. 1. Physical and genetic map of the region of pCD1 that encompasses *yscO* and *yscP*. (A) Coding regions for *yscO* and *yscP* and parts of *yscN* and *yscQ* carried on pYsoOP.2, as well as selected restriction sites. Asterisks denote restriction sites introduced by site-directed mutagenesis. (B) Regions included in selected clones.

S-transferase (GST) and amino acids 12 to 154 of YscO. Plasmid pGST-YscO was transformed into *E. coli* DH5 α (Table 1; Fig. 1). The soluble GST-YscO fusion protein was expressed and purified as specified by the manufacturer for use of the pGEX-3X vector (Pharmacia Biotech Inc., Piscataway, N.J.) and used to raise antibodies in New Zealand White rabbits as previously described (54).

Cell fractionation and immunoblot analysis. Cells were pelleted by centrifugation, and the top half of the culture medium containing secreted proteins was removed from the tubes. The bacterial pellet was washed and resuspended in 100 mM Tris-HCl (pH 7.4)–1 mM EDTA and lysed by a single passage through a chilled French pressure cell at 20,000 lb/in². Unlysed cells and cellular debris were removed by centrifugation at 8,800 \times g for 5 min at 4°C. Total soluble proteins (cytoplasmic plus periplasmic) were separated from membranes of the cleared lysates by ultracentrifugation at 417,000 \times g for 15 min in a TLA 100.4 rotor (Beckman, Inc., Palo Alto, Calif.). The membranes were resuspended in 100 mM Tris-HCl (pH 7.4)–1 mM EDTA. Secreted proteins were precipitated with 5% (vol/vol) trichloroacetic acid for 2 h to overnight on ice. After centrifugation (14,000 \times g for 30 min at 4°C) to pellet the precipitated proteins, the pellet was neutralized with 1 M Tris-HCl (pH 8.0), resuspended in electrophoresis sample buffer (60 mM Tris-Cl [pH 6.8], 2.3% [wt/vol] sodium dodecyl sulfate [SDS], 20% [vol/vol] glycerol, 5% [vol/vol] β -mercaptoethanol, 0.01% [wt/vol] bromophenol blue), and stored at –20°C. Bacterial fractions were analyzed on denaturing SDS-polyacrylamide gels (12 to 15% [wt/vol] acrylamide) (36) followed by transfer to Immobilon-P (Millipore Corp., Bedford, Mass.) using Towbin transfer buffer (75). The gels were loaded such that each lane contained proteins corresponding to equal numbers of bacteria. *Y. pestis* LCR proteins were visualized on the membranes by using protein-specific primary antibody and a secondary antibody (goat anti-rabbit or goat anti-mouse [Sigma]) conjugated to either alkaline phosphatase or horseradish peroxidase. The primary rabbit polyclonal antibodies included anti-ECP, against total proteins in the culture medium of *Y. pseudotuberculosis* 43 (pCD1 *yopKL::Mu d1* [Ap^r *lac*]) (45); anti-HTV, against full-length V antigen having a 19-residue leader containing 6 His residues fused to its N terminus (21, 45); anti-YopM, against purified YopM (45); anti-LcrD, against an LcrD peptide coupled to the carrier protein bovine serum albumin (54); anti-YscD, against a fusion protein of GST and YscD (56); and anti-YscP, against a fusion protein of GST and amino acids (aa) 328 to 455 of YscP (49). Rabbit anti-YopE was a generous gift from Gregory V. Plano (University of Miami, Miami, Fla.). Mouse anti-YopE and anti-YopH were a generous gift from Gerard P. Andrews and Arthur M. Friedlander (U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick, Md.).

DNA sequence analysis. DNA and predicted protein sequences were analyzed with PCGene (IntelliGenetics, Inc., Mountain View, Calif.), IntelliGenetics Suite (IntelliGenetics, Inc.), and Coils 2.1 (39, 40). The deduced amino acid sequences were compared with available sequences in the GenBank database via the National Center for Biotechnology Information BLAST (4) mail server. The *yscQRS* nucleotide sequence of *Y. pestis* (20) has been updated to include the sequences of *yscO* and *yscP* (accession no. L25667).

RESULTS

Analysis of *yscO* of *Y. pestis* KIM. We extended the sequence upstream of *yscQRS* (20) in *Y. pestis*. This encompassed the 3' end of *yscN* and the 5' end of *yscQ*. The predicted amino acid sequences of YscO and YscP were 98 and 100% identical to those predicted for *Y. pseudotuberculosis* but not significantly similar to those outside the *Yersinia* spp. Computer analysis of *yscO* predicted a 154-residue protein with a molecular mass of 18.9 kDa and a predicted isoelectric point of 7.89. The predicted protein sequence does not contain either a putative signal sequence or any hydrophobic domain. Secondary-structure analysis predicted a predominantly alpha-helical structure with a high probability of existing as a coiled-coil protein. Interestingly, *Salmonella* SpaM (also called InvI) (12) and *Shigella* Spa13 are also predicted by the same analysis to have a large percentage of charged residues and potentially exist as coiled-coil proteins. The similarity of size, charges, and potential secondary structure suggests that these proteins may have similar functions in their respective bacterial strains but have no similarity in sequence because of the differences in the proteins with which they interact.

Mutational analysis. To determine the role of YscO in the LCR and its presumed role in secretion, we compared the growth (Fig. 2) and secretion (Fig. 3) of *Y. pestis* KIM5-3001 (wt), KIM5-3001.16 (Δ *yscO*), and KIM5-3001.16 carrying *yscO* or *yscO* and *yscP* in *trans* (Δ *yscO/O* or Δ *yscO/OP*) in TMH defined medium under both inductive and noninductive conditions of the LCR.

The *yscO* mutant has a similar growth phenotype and secretion pattern to that in previously characterized *Y. pestis* Ysc mutants (20, 25, 55, 56). In the presence of Ca²⁺, all strains exhibited full growth yield at 37°C (Fig. 2). The mutant and parent displayed similar expression patterns and low levels of V antigen and YopE (Fig. 3B); YopM was undetectable in both strains (data not shown). Additionally, there was no secretion in the presence of Ca²⁺, which was expected for the

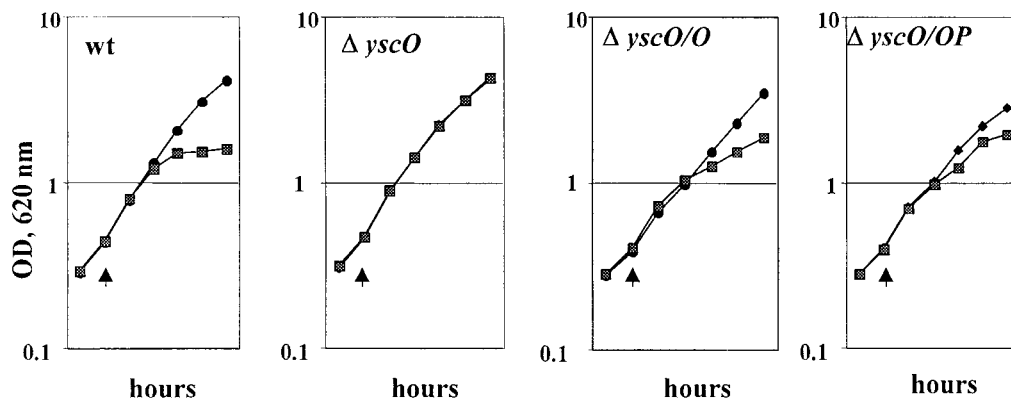


FIG. 2. Growth of *Y. pestis* KIM5-3001 (wt); KIM5-3001.16, the *yjcO* mutant ($\Delta yjcO$); and the mutant carrying *yjcO* in *trans* ($\Delta yjcO/O$) and ($\Delta yjcO/OP$). *Y. pestis* strains were grown at 37°C in the presence or absence of Ca^{2+} in TMH defined medium. The temperature was shifted from 26 to 37°C (temperature shifts are denoted by arrowheads). Symbols: ●, + Ca^{2+} ; □, - Ca^{2+} .

parent. *Y. pestis* KIM5-3001 (wt) displayed a normal Ca^{2+} -dependent phenotype in which bacteria undergo restriction at 37°C in the absence of Ca^{2+} (Fig. 2), and the growth restriction was accompanied by expression and secretion of YopE, YopM, and V antigen (Fig. 3A, lanes wt). Expression was higher for YopE, YopM, and V antigen than in the presence of Ca^{2+} (compare Fig. 3A and B, lanes wt). The *yjcO* mutant ($\Delta yjcO$) failed to show growth restriction irrespective of the presence of Ca^{2+} and was therefore characterized as having Ca^{2+} -independent growth (Fig. 2). Under LCR-inductive conditions (without Ca^{2+}), the mutant failed to secrete YopM, V antigen, YopE, or YopD and expressed clearly reduced levels of these proteins compared to the parent (Fig. 3A, compare $\Delta yjcO$ and wt [not shown for YopD]). Significantly, YscP was detected in the whole-cell fraction of the mutant (Fig. 3D). As with Yops and V antigen, its expression was lower in the mutant than in the parent, and it was not secreted in the mutant as it was by the parent (49). This indicated that the *yjcO* mutation did not have a polar effect on the expression of *yjcP*.

We wanted to know whether the mutant was selectively impaired in the secretion of some but not all LCR proteins, as reported for both a *virG* mutant and a *yjcF* mutant (1, 2). Therefore, we analyzed the culture medium of the mutant for secreted proteins by using antibody raised against a mixture of secreted *Yersinia* proteins (anti-ECP). No proteins were detected, although the parent (wt) secreted several (Fig. 3C). These results show that *yjcO* is required for the secretion of V antigen and Yops.

Growth restriction and secretion were partially restored to the mutant by providing *yjcO* in *trans* (Fig. 2 and 3A, lanes $\Delta yjcO/O$). The extent of complementation varied from experiment to experiment, and we were unable to determine what variables were responsible for these differences. We tried providing *yjcO* and *yjcP* together in *trans* on pYscOP-2 to see if the complementation would be stronger. However, the complementation of growth and secretion phenotypes turned out to be weaker (Fig. 2 and 3A, lanes $\Delta yjcO/OP$), probably due to an effect of the extra copies of *yjcP* (49). There was no complementation by a plasmid carrying *yjcP* in *trans* (data not shown). Accordingly, we believe that the phenotype of the *yjcO* mutant is due to the absence of *yjcO* and not to a polar effect on downstream genes in the operon.

Secretion of V antigen and YopM expressed from plasmids with non-LCR, inducible promoters. To test the hypothesis that *yjcO* affects secretion directly and to show that low levels

of substrate available in the mutant were not responsible for the lack of secretion, we analyzed secretion in *Y. pestis* KIM5-3001 (wt) and *Y. pestis* KIM5-3001.16 ($\Delta yjcO$), each carrying V antigen or YopM expressed in *trans* from an inducible, non-LCR-regulated promoter. Plasmid pHTV, which encodes histidine-tagged V antigen (HTV), and pTRCM.2, which encodes YopM, were introduced into wt and $\Delta yjcO$ *Y. pestis* strains. Both plasmids contain an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter. At 5 h before harvest, the expression of YopM or HTV was induced by the addition of IPTG (1 mM). The expression and secretion of YopM and HTV were monitored by immunoblot analysis (Fig. 4).

The parent strain expressed and secreted YopM and V antigen. When carrying pTRCM.2, the parent expressed and secreted higher levels of YopM. The parent strain carrying pHTV expressed both native V antigen and HTV, which ran slightly above the native V antigen due to its His₆-containing leader sequence. These data indicate that YopM and HTV expressed from plasmid pTRCM.2 and pHTV, respectively, were competent for secretion. We think the extra protein expressed in the parent carrying either pTRCM.2 or pHTV overwhelmed the secretion system and resulted in a backup of the respective proteins (especially of YopM) in the soluble fraction of these strains in comparison to the situation in the parent not carrying a plasmid in *trans*.

The *yjcO* mutant carrying pHTV expressed high levels of HTV but was unable to secrete any. A very small amount of the strongly expressed YopM was seen in the culture medium of the mutant carrying pTRCM.2. This may indicate that the *yjcO* mutant is not 100% blocked in secretion but that the small amount of Yops that gets through is not visible unless a huge amount is overexpressed. Our results suggest that the low LCRS protein expression in the *yjcO* mutant was secondary to the secretion defect in this mutant and was not the primary cause of the lack of secretion.

Since it is possible that the secretion defect is due to an indirect effect of *yjcO* on other Ysc proteins, we analyzed membrane fractions of the *yjcO* mutant for two Ysc components, LcrD and YscD, each encoded by one of the other two operons encoding components of the Ysc. We saw comparable levels of the two proteins in the mutant compared to the parent (Fig. 5). We were not able to analyze the expression of all known Ysc proteins; therefore, we cannot rule out the possibility that YscO affects the expression or localization of a component not tested. However, these results indicate that the

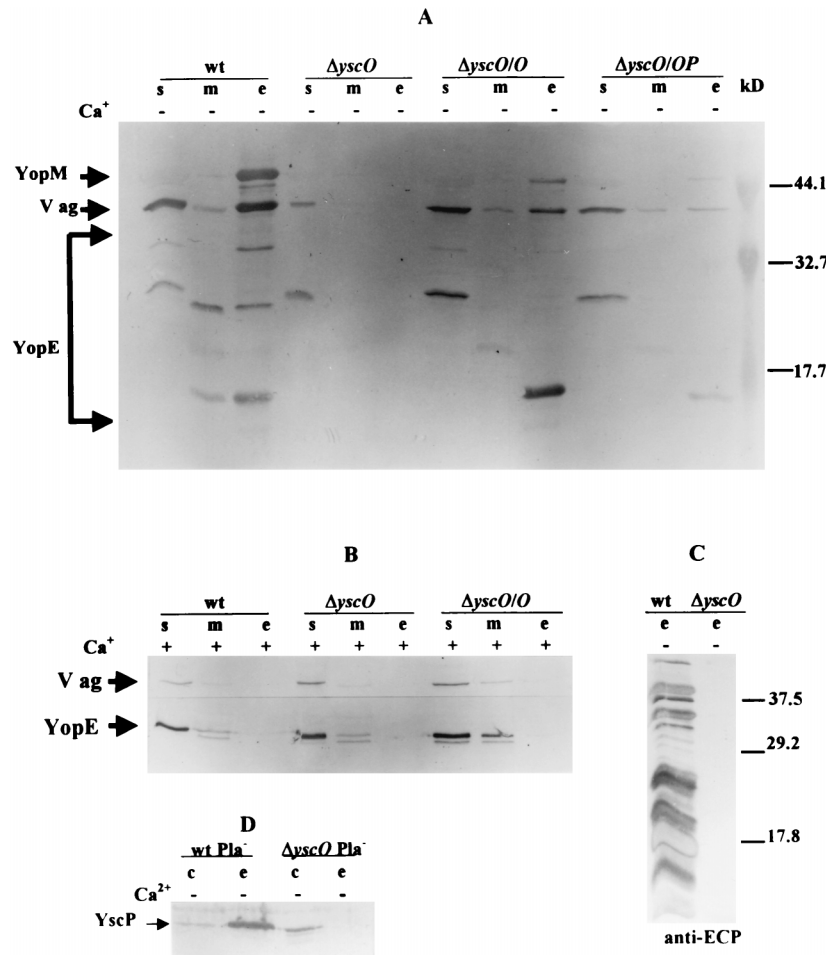


FIG. 3. Secretion profile of $\Delta yjcO$ *Y. pestis* grown in the presence or absence of Ca^{2+} . Shown is an immunoblot analysis of proteins expressed and secreted from *Y. pestis* KIM5-3001 (wt); KIM5-3001.16, the *yjcO* mutant ($\Delta yjcO$); and the mutant carrying pYscO.2 or pYscOP.2 in *trans* ($\Delta yjcO/O$ and $\Delta yjcO/OP$). Bacteria were grown in TMH with (+) or without (-) Ca^{2+} , and proteins from bacterial fractions were separated by SDS-PAGE (A, B, and D, 12% [wt/vol] acrylamide; C, 15% [wt/vol] acrylamide). Proteins from soluble (s), membrane (m), whole-cell (c), and culture medium (e) fractions were visualized with polyclonal antibodies specific to YopE, YopM, V antigen, and YscP and with antibody raised to a mixture of extracellular *Yersinia* proteins (ECP). The secondary antibody used was conjugated to alkaline phosphatase. Arrows denote the positions of proteins. (A) YopE was visualized with mouse anti-YopE (YopE and its Pla-generated degradation products are enclosed in brackets). (B) YopE was visualized with rabbit anti-YopE. (C) The complexity of the protein pattern reflects degradation products from multiple Yops due to the Pla protease. (D) YscP was visualized with antibody to a GST-YscP fusion protein. Molecular masses (in kilodaltons) of prestained molecular mass standards (Bio-Rad) are denoted to the right in panels A and C.

yjcO mutant is defective in some aspect of the secretion process, and they support a direct role for *yjcO* in the secretion of LCR virulence proteins by *Y. pestis*.

Identification of the *yjcO* gene product. Since we had established a direct role for *yjcO* in secretion, we wanted to gain insight into its possible function by determining its bacterial location. YscO was visualized on immunoblots of SDS-polyacrylamide gel electrophoresis (PAGE)-separated proteins from fractionated *Y. pestis* cultures by using a polyclonal antiserum that was directed against the GST-YscO fusion protein containing aa 13 to 154 of YscO (Fig. 6).

YscO detection proved to be difficult, because the protein is expressed very weakly and the antiserum reacted with several proteins in the parent and *yjcO* mutant strains (data not shown). The antibody identified a 21-kDa protein in all fractions of the parent, *Y. pestis* KIM5-3001 (wt) grown at 37°C in the absence of Ca^{2+} (Fig. 6A, lanes wt), and this was approximately the size expected (19 kDa) for YscO. We hypothesize that this is YscO, since it was not observed in the *yjcO* mutant under the same conditions (lanes $\Delta yjcO$) but was seen in the

mutant complemented with *yjcO* (lanes $\Delta yjcO/O$ and $\Delta yjcO/OP$). Furthermore, the intensity of the band was increased when *yjcO* was expressed in *trans* in the parent (Fig. 6B, compare wt Pla⁻ and wt Pla⁻/OP), although Yop expression was not changed in wt/OP (49). This supports the idea that the 21-kDa band represents YscO and is not a cross-reacting Yop. Less YscO was expressed in the presence of Ca^{2+} (compare Fig. 6C with Fig. 6A and B), which is consistent with previous observations of weak Ca^{2+} regulation in this operon (20). It is expected that a core secretion component would have to be present under noninductive conditions (in the presence of Ca^{2+}) and be ready to function rapidly upon contact induction. Indeed, in the presence of Ca^{2+} , YscO was localized to the membranes of KIM5-3001 (Fig. 6C).

In the course of visualizing YscO, we determined that it was subject to degradation by Pla. When grown at 37°C in the absence of Ca^{2+} , the *Y. pestis* parent lacking Pla, KIM8-3002 (wt Pla⁻), yielded more YscO than did the parent containing Pla, KIM5-3001 (wt) (Fig. 6B, compare lanes wt to lanes wt Pla⁻), presumably due to the absence of Pla activity in *Y. pestis*

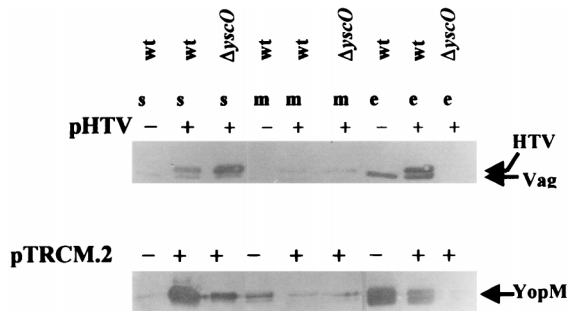


FIG. 4. Immunoblot analysis of YopM, V antigen, and HTV in the soluble (s), membrane (m), and culture medium (e) fractions from *Y. pestis* KIM5-3001 (wt) and KIM5-3001.16 ($\Delta yscO$) with (+) or without (-) pHTV or pTRCM.2. The *Y. pestis* strains were grown at 37°C in the absence of Ca^{2+} . Expression of HTV from pHTV and YopM from pTRCM.2 was induced by the addition of IPTG to 1 mM 5 h prior to harvest. The proteins were separated by SDS-PAGE (12% [wt/vol] acrylamide). Polyclonal antibody to HTV or YopM was used to detect HTV and V antigen, and YopM, respectively. Secondary antibody was conjugated to alkaline phosphatase. In the lower panel, YopM was detected as two closely migrating species.

KIM8-3002. Overall, the distribution pattern of YscO resembled that of Yops: more YscO was present in the culture medium than in the cellular fraction whether Pla was present or not. In some experiments, we unexpectedly detected YscO (Fig. 6C) as well as low levels of some Yops (not shown) in the culture medium in the presence of Ca^{2+} , if the *Y. pestis* strain was Pla⁻. We believe that the absence of Pla unmasks a very low level of Yop secretion which occurs in the presence of Ca^{2+} but is not detected in Pla⁺ yersiniae due to degradation of these very small amounts by Pla.

In the Pla⁻ (but not Pla⁺) parent and the parent carrying *yscO* in *trans*, we visualized a 24-kDa protein more strongly in the culture medium than in the membrane fraction (Fig. 6B, lanes wt Pla⁻ and wt Pla⁻/OP). This band is of similar intensity in the two strains, and we think it represents a rapidly degraded LCRS protein (which would be expressed more strongly in strains having a functional secretion system than in the *yscO* mutant). In some experiments, we also saw a 16-kDa protein in either the soluble and/or membrane fraction but never in the culture medium (Fig. 6B).

DISCUSSION

The present study characterized *yscO* of the *Y. pestis yscN* to *yscU* operon. The lack of significant similarity of YscO to the corresponding type III proteins in *Salmonella* and *Shigella* sug-

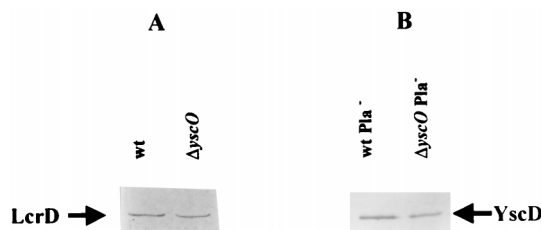


FIG. 5. Detection of LcrD and YscD in $\Delta yscO$ *Y. pestis*. *Y. pestis* strains were grown in TMH at 37°C in the absence of Ca^{2+} . The proteins in membrane fractions were separated by SDS-PAGE (12% [wt/vol] acrylamide), transferred to Immobilon P, and analyzed by immunoblotting with antibodies specific to LcrD or YscD. (A) *Y. pestis* KIM5-3001 (wt) and *Y. pestis* KIM5-3001.16 ($\Delta yscO$) were analyzed with anti-LcrD. (B) *Y. pestis* KIM8-3002 (wt Pla⁻) and *Y. pestis* KIM8-3002.3 ($\Delta yscO$ Pla⁻) were analyzed with anti-YscD. The secondary antibody used was conjugated to alkaline phosphatase. Arrows indicate each protein.

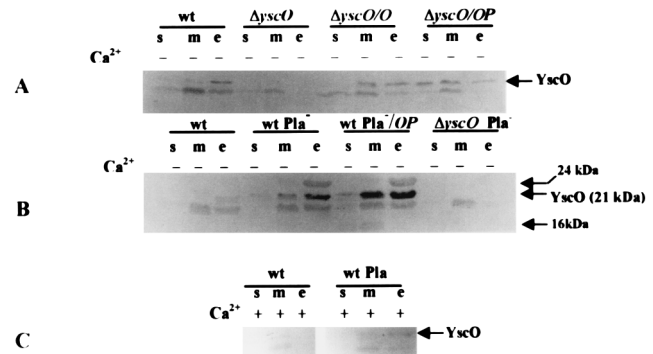


FIG. 6. Localization of YscO by immunoblot analysis. *Y. pestis* KIM5-3001 (wt), KIM5-3001.16 ($\Delta yscO$), KIM8-3002 (wt Pla⁻), and KIM8-3002.3 ($\Delta yscO$ Pla⁻) are shown. Strains carrying plasmids are denoted /O or /OP for pYscO.2 or pYscOP.2, respectively. Bacteria were grown at 37°C in TMH with (+) or without (-) Ca^{2+} for 5 h prior to harvest. Proteins from bacterial fractions were separated by SDS-PAGE (15% [wt/vol] acrylamide). Antibody raised against a GST-YscO fusion protein was used to detect YscO in soluble (s), total membrane (m), whole-cell (wc), or culture medium (e) proteins. All panels were analyzed with alkaline phosphatase. Arrows indicate the various proteins that were potential candidates for YscO.

gests that YscO could function in recognition of *Yersinia* secreted effector proteins, and our data point to a role for YscO in Yop secretion.

At 37°C in the absence of Ca^{2+} , our *yscO* mutant failed to undergo growth restriction and was unable to export LCRS proteins (V antigen and Yops). We believe that this phenotype was an effect of the loss of *yscO* only, since we observed expression of YscP in the mutant and since provision of only *yscO* in *trans* complemented the mutant. A His-tagged fusion of V antigen was not secreted when overexpressed from pHTV in the *yscO* mutant, and only a small amount of YopM was seen in the culture medium when overexpressed in the mutant from pTRCM.2. These results indicated that the *yscO* deletion directly prevented secretion of LCRS proteins in *Y. pestis*. This type of defect is similar to those described for other *Yersinia* proteins found to be components of the Ysc secretion mechanism, including the known or predicted inner membrane proteins, LcrD (55), YscD (43, 55), and YscR, YscS, and YscU (3, 7, 20); the outer membrane PulD homolog YscC (56); the lipoprotein YscJ (2); and the cytoplasmic protein YscN (78), thought to be associated with the inner membrane and a likely candidate for providing the energy needed for secretion. Other proteins that are essential for secretion but whose function and subcellular location are not known include YscE to YscG and YscI to YscL (2, 27, 43). For all of these essential Ysc components, mutations block the secretion of LCRS proteins and prevent full transcriptional induction of LCRS operons, probably by blocking release of the LCR negative regulator(s) from the cytosol through the type III secretion system. The similarity in growth and secretion defects in the *yscO* mutant suggests that *yscO* also is a core (essential) component of the *Y. pestis* LCRS export apparatus.

Surprisingly, we found that YscO was present in the culture medium in the absence of Ca^{2+} . This differs from the localization of the corresponding type III protein in *Salmonella* called SpaM or InvI. SpaM/InvI was not detected in the culture medium but is essential for *Salmonella* entry into cultured epithelial cells (12). The corresponding protein in *Shigella*, Spa13, has been shown to be essential for invasion and presumably for the secretion of Ipa proteins, but its bacterial location has not been determined (63).

Secretion and translocation of Yops have been shown to occur in distinct steps (64) and to require recognition by some component of the type III secretion apparatus. There is no picture yet for how the ca. 22 Ysc components are arranged and function to move Yops through the two bacterial membranes. We would expect that each type III system would have unique proteins or adapted domains of conserved proteins for the recognition and/or transport of the system-specific secreted proteins. If YscO serves this purpose, it is unlikely that it interacts and moves with Yops in a 1:1 ratio, because YscO does not appear to be abundant enough for such a role. YscO evidently is a peripheral protein that is largely associated with or released from the membrane, suggesting that its function does directly correlate with Yop conduction through the membranes. Consistent with this idea, no YscO was found in the culture medium at 26°C, a situation in which no bulk Yop secretion was taking place (data not shown). YscO was sometimes observed in culture medium of the Pla⁻ parent at 37°C in the presence of Ca²⁺, a condition in which low levels of Yops were also seen in the culture medium. Our findings suggest the possibility that the type III Ysc mechanism has a dynamic moving core and that YscO is part of this. Such an image is consistent with the developing picture for protein secretion through the Sec system, in which cycles of the piston-like pumping of SecA and the orientation inversion of SecE are essential to the mechanism of protein secretion (17, 47). We do not yet know the role of YscO release in the Yops secretion process. It is conceivable that this is an artifact of our in vitro system, which lacks the localized activation of secretion upon contact with a mammalian cell and the coupling of secretion with translocation into the cell. However, it is also possible that the release of YscO into the medium in our studies reflects a role that links the secretion of Yops with their subsequent targeting into a host cell.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI21017. We acknowledge Mike Russ, University of Kentucky Macromolecular Structure Analysis Facility, for the synthesis of some of the oligonucleotides used in this study.

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