

YscP of *Yersinia pestis* Is a Secreted Component of the Yop Secretion System

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The *Yersinia pestis* low-Ca²⁺ response stimulon is responsible for the environmentally regulated expression and secretion of antihost proteins (V antigen and Yops). We have previously shown that *yscO* encodes a secreted core component of the Yop secretion (Ysc) mechanism. In this study, we constructed and characterized in-frame deletions in the adjacent gene, *yscP*, in the *yscN-yscU* operon. The $\Delta P1$ mutation, which removed amino acids 246 to 333 of YscP, had no effect on Yop expression or secretion, and the mutant protein, YscP1, was secreted, as was YscP in the parent. In contrast, the $\Delta P2$ strain expressed and secreted less of each Yop than did the parent under the inductive conditions of 37°C and the absence of Ca²⁺, with an exception being YopE, which was only minimally affected by the mutation. The YscP2 protein, missing amino acids 57 to 324 of YscP, was expressed but not secreted by the $\Delta P2$ mutant. The effect of the $\Delta P2$ mutation was at the level of Yop secretion because YopM and V antigen still showed limited secretion when overproduced in *trans*. Excess YscP also affected secretion: overexpression of YscP in the parent, in either *yscP* mutant, or in an *lcrG* mutant effectively shut off secretion. However, co-overexpression of YscO and YscP had no effect on secretion, and YscP overexpression in an *lcrE* mutant had little effect on Yop secretion, suggesting that YscP acts, in conjunction with YscO, at the level of secretion control of LcrE at the bacterial surface. These findings place YscP among the growing family of mobile Ysc components that both affect secretion and themselves are secreted by the Ysc.

The pathogenic *Yersinia* species, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, cause human diseases ranging from the systemic bubonic plague to a variety of gastroenteric symptoms collectively referred to as yersiniosis. Despite the differences in disease severity, these bacteria share several virulence properties that enable them to resist the nonspecific host immune response (10). Key among these is the ability to secrete a set of virulence proteins, V antigen and Yops, which function to incapacitate host cells and thus enable yersiniae to survive and multiply in lymphoid tissue (10).

V antigen has functions both to regulate the delivery of Yops and as an antihost protein (10, 31, 32, 34, 36, 48). Yops appear to fall in two categories: ones that have direct antihost function and ones whose function relates to the delivery of the antihost Yops (10). Upon cell contact, six Yops (YopE, YopH, YopJ, YopM, YopT, and YpkA) are directly transferred (targeted) into the eukaryotic cell at the point of contact (10). The intracellular activities of these Yops require their secretion and targeting. Secretion and targeting are dependent on a functional Yop secretion mechanism (Ysc); in addition, targeting requires YopB, YopD, and YopK (10).

The genes that encode V antigen and Yops, most of the proteins that regulate their expression, and the Ysc are located on a virulence plasmid (pCD1 in *Y. pestis* (10, 39) and have been referred to as the Yop virulon for their function (10) and as the low-Ca²⁺-response stimulon (LCRS) for their regulation in vitro (18, 52). The transcription of LCRS operons is thermally regulated by the transcriptional activator LcrF (10). The extent of activation is determined by environmental conditions. At 37°C, eukaryotic cell contact induces maximal expression of Yops (40). In vitro, the presence of millimolar

concentrations of Ca²⁺ limits the activation of LCRS expression whereas the lack of Ca²⁺ mimics cell contact and results in strong expression of Yops (52). Under these conditions, there is high-level Yop expression and secretion accompanied by cessation of bacterial growth (referred to as growth restriction), collectively termed the low-Ca²⁺ response (LCR) (52). Growth restriction probably does not occur in vivo (17) but has been a useful marker of Yop induction (51).

In the absence of cell contact or in the presence of Ca²⁺ in vitro, LcrE (also called YopN) and TyeA at the bacterial surface and LcrG in the cytoplasm are thought to block the putative Ysc secretion pore at the outer and inner membranes, respectively. LcrE is thought to act at the surface as a Ca²⁺ sensor, although that activity has not been directly demonstrated (16, 57). TyeA interacts with LcrE and appears to function both at the level of secretion control and in the targeting of a subset of Yops (24). LcrG is located mostly in the cytosol, although a significant amount also is membrane associated; a small amount is secreted in the absence of Ca²⁺ (36, 49). The block of the secretion pore prevents the secretion of LcrQ, which, in conjunction with YopD, acts as a negative regulator of Yop and V-antigen expression (44, 55). Mutations in LcrE, TyeA, or LcrG result in strong Yop expression and secretion independent of Ca²⁺ levels or in the absence of host cell contact, presumably due to the secretion of LcrQ and the loss of control over Yop release (16, 24, 49). Upregulation of Yop expression is thought to occur when cell contact or the absence of Ca²⁺ relieves the block of LcrE and TyeA at the bacterial surface. Some LcrQ is then secreted, which results in the increased expression of V antigen and Yops. LcrG is thought to be maximally titrated away from the Ysc by directly interacting with V antigen when elevated levels of V antigen are produced upon LCR induction (36).

The Ysc mechanism is composed of 20 identified protein products of genes encoded by multiple operons (*yscVlcrR*, *yscBCDEFGHIJKLM*, *yscNOPQRSTU*, and *yscW*) (1–3, 6, 10,

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14, 20, 29, 37, 39, 41, 42, 56). Mutants with mutations of essential Ysc components are unable to secrete Yops and thus are not induced for high-level Yop expression, presumably due to the inability to secrete LcrQ (14, 18, 40–42, 44). These mutants are characterized by Yop expression at the level seen in the presence of Ca^{2+} and by failure to undergo growth restriction regardless of Ca^{2+} levels (a growth phenotype referred to as Ca^{2+} independence). Ysc-related secretion mechanisms exist in several pathogenic gram-negative bacteria and are involved in the direct delivery of bacterial proteins into eukaryotic cells (23). Most of the proteins encoded by the *Yersinia yscN-yscU* operon share a high sequence similarity to their Spa counterparts in *Salmonella typhimurium* (where the locus is also called *inv*) and *Shigella flexneri* (6, 14, 23). However, the YscO and YscP proteins have little similarity to their Spa counterparts, which suggests that they have a *Yersinia*-specific function. We have previously characterized *yscO* (37). The present work focuses on *yscP*. We constructed two non-polar deletions in *yscP*, and the resulting mutants were characterized for the expression and secretion of V antigen and Yops. Under LCR-inductive conditions, one mutation had no effect on either the growth of the strain or the expression and secretion of Yops. The second *yscP* mutation caused a decrease in the secretion and expression of some but not all Yops. YscP itself was found to belong to the LCRS and to be secreted by the Ysc. It appears to be a mobile component of the Ysc itself and may act in conjunction with YscO at the level of LcrE function.

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 used in these studies. It contains three naturally occurring *Y. pestis* plasmids: pCD1 (carrying the genes necessary for the LCR phenotype) (12, 18), pPCP1 (encoding the plasminogen activator [Pla] responsible for degradation of Yops) (50), and pMT1 (encoding the F1 capsular protein) (43). *Y. pestis* KIM8-3002 (LCR⁺, pPCP1⁻; parent Pla⁻), was used in experiments in which degradation of proteins by the surface protease Pla would have affected the analysis of results. *Escherichia coli* strains were typically grown in Luria-Bertani broth or on Luria-Bertani agar (11). *Y. pestis* strains were routinely grown in heart infusion broth (HIB) or on tryptose blood agar base plates (Difco Laboratories, Detroit, Mich.) at 26°C. For physiological studies, *Y. pestis* strains were grown in the defined liquid medium TMH (51), supplemented with 2.5 mM CaCl_2 as indicated. The medium was inoculated to an optical density at 620 nm (A_{620}) 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 A_{620} reached ca. 0.2. Cells and secreted proteins were harvested 5 or 6 h after the temperature shift. All bacteria with antibiotic resistances were grown in the presence of the appropriate antibiotics, ampicillin and/or streptomycin, at 100 µg/ml.

DNA methods. Cloning, including the use of restriction endonucleases and T4 DNA ligase, was performed essentially as described previously (28). Plasmid DNA was isolated by an alkali lysis procedure (7), by the method of Kado and Liu (25), by cesium chloride gradients (28), or with Qiagen columns (Qiagen, Inc., Studio City, Calif.). Certain DNA fragments were isolated and purified from agarose gels by using a Qiaex DNA purification kit (Qiagen). Transformation of *E. coli* was done by a standard CaCl_2 procedure (28). Electroporation of *E. coli* and *Y. pestis* was done as previously described (38). The PCR technique (30) was performed with 20 to 30 cycles of amplification. The denaturing, annealing, and extending conditions were 94, 55, and 72°C, respectively, for 30 s each with a 480 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Nucleotide primers synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington, Ky.) or Integrated DNA Technologies (Coralville, Iowa) were used for PCR.

Mutagenesis of *yscP*. To study the effect of *yscP* on the LCR, we made two in-frame deletions in *yscP*. Plasmids pYscP1 and pYscP2 were constructed by restriction endonuclease digestions as described in Table 1. Restriction endonuclease fragments that contained the deleted sequence flanked by homologous DNA were subcloned into the *EcoRV* site of the suicide vector pUK4134, generating pUKΔP1 and pUKΔP2 (Table 1). Plasmids pUKΔP1 and pUKΔP2 were introduced into *Y. pestis* KIM5-3001 and pUKΔP2 was introduced into *Y. pestis* KIM5-3002 by electroporation, and recipient bacteria that had integrated the clone into pCD1 by homologous recombination were selected for their

resistance to ampicillin as previously described (47). Following passage under nonselective conditions to allow a second crossover, clones which had resolved the cointegrate by excision of the vector sequences were selected by growth on streptomycin and screened for ampicillin sensitivity, and the presence of the correct deletion was confirmed by PCR and restriction endonuclease digestion. *Y. pestis* KIM5-3001.17 (ΔP1), KIM5-3001.18 (ΔP2), and KIM8-3000.4 (ΔP2 Pla⁻) contained the correct in-frame deletions (Table 1) and were used in this study.

T7 promoter-polymerase expression system. The T7 promoter-polymerase expression system in *E. coli* was used to determine if the predicted coding sequence of *yscP* expressed a protein. *E. coli* XL1-Blue was transformed with pBluescript II SK+ (vector-only control) and plasmids carrying *yscP* and its derivatives. Plasmid pYscP carried *yscP* oriented for expression from the T7 promoter, and pYscP.2 carried *yscP* oriented with the *lac* promoter and against the T7 promoter, an orientation that would prevent transcription in the T7 system. The clone-specific and control (pBluescript II SK+) proteins were expressed by using bacteriophage mGP1-2 to supply T7 RNA polymerase as previously described (5). The proteins expressed by the T7 polymerase were labeled with Tran³⁵S-label (³⁵S-Met and ³⁵S-Cys; ICN Pharmaceuticals). Equal numbers of cells as determined by measurement of the A_{620} of the labeled culture were pelleted in a microcentrifuge at 4°C and solubilized in 100 µl of electrophoresis sample buffer (37). Portions of each sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% [wt/vol] acrylamide) and visualized by autoradiography.

Virulence tests. To assess the importance of *yscP* for virulence, we used a systemic plague model in intravenously infected mice. Bacterial inocula were prepared by growing the bacteria for 10 generations at 26°C in HIB containing streptomycin. The cells were sedimented by centrifugation at room temperature. They were washed once with room temperature (RT) phosphate-buffered saline (PBS) (135 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 [pH 7.2]) and resuspended in RT PBS before use. Serial 10-fold dilutions of each strain (10^5 and/or 10^3 dose) were spread in triplicate onto tryptone blood agar for later determination of viable numbers (CFU). Groups of five female BALB/c mice, 6 to 8 weeks old and caged separately, were anesthetized with Metofane and injected intravenously retro-orbitally with 0.1 ml of bacterial suspensions containing 10^3 CFU of *Y. pestis* KIM5-3001 (parent) or 10^3 or 10^5 CFU of the *yscP* mutants *Y. pestis* KIM5-3001.17 (ΔP1) and KIM5-3001.18 (ΔP2). For the high-dose infection group of each mutant, an extra mouse was inoculated and euthanized 24 h after infection for recovery from macerated spleen and verification by plasmid profile that the challenge strain was unaltered by growth in vivo. The other mice were observed until they succumbed to infection or for a maximum of 14 days (ΔP1) or 33 days (ΔP2).

Antibody preparation. To determine the localization of YscP in bacterial fractions, antibody was raised in rabbits against fusion proteins of glutathione S-transferase (GST) and a portion of YscP (amino acids [aa] 328 to 455). Plasmid pGST-YscP (Table 1; Fig. 1), which encoded the fusion protein, was transformed into *E. coli* DH5α. The fusion protein, GST-YscP, was expressed as specified by the manufacturer for use of the pGEX-3X vector (Pharmacia-LKB, Piscataway, N.J.). After harvest, cell pellets were resuspended in PBS and lysed by two passages through a chilled French pressure cell at 20,000 lb/in². Unlysed cells, large debris, and inclusion bodies were removed by centrifugation at $8,800 \times g$ for 5 min at 4°C. The GST-YscP fusion protein was found almost exclusively in the $8,800 \times g$ pellet, indicating that this protein was in inclusion bodies. The GST-YscP-containing inclusion bodies were resuspended in PBS containing 1% (vol/vol) Triton X-100 and then subjected to centrifugation at $8,800 \times g$ for 5 min at 4°C. This treatment, which did not solubilize the inclusion bodies, would remove most of the contaminating soluble and membrane proteins from the inclusion body preparation and was performed a total of three times. GST-YscP fusion protein was then purified from the inclusion bodies by solubilization in electrophoresis sample buffer and SDS-PAGE. The protein was isolated from excised gel pieces by electroelution with a Centrilon microelectroluter (Amicon), concentrated in a Centricon-10 concentrator (Amicon), and stored at -20°C until use. A sample of GST-YscP and known amounts of the similar-sized bovine serum albumin separated by SDS-PAGE were compared for their intensity of staining by Coomassie brilliant blue to estimate the concentration of GST-YscP in the sample.

Two female New Zealand White rabbits were injected subcutaneously at multiple sites along the back with a total of 0.1 mg of GST-YscP which had been eluted from a gel and emulsified 1:1 (vol/vol) in Freund's complete adjuvant. At 4 and 8 weeks, the rabbits were boosted with 0.1 mg of the same fusion protein preparation mixed 1:1 in Freund's incomplete adjuvant. Antiserum was collected and applied to a protein A-Sepharose column (Pharmacia-LKB) to purify the immunoglobulin G fraction.

Cell fractionation and immunoblot analysis. Samples for analysis of protein content by immunoblotting were prepared from *Y. pestis* strains grown in TMH (with and without 2.5 mM Ca^{2+}). Cell pellets and culture supernatants were separated by centrifugation at 4°C. Cell pellets were washed in ice-cold 100 mM Tris-HCl (pH 7.4)-1 mM EDTA. Whole-cell fractions were prepared by resuspending the pellet in 2× sample electrophoresis buffer. Cell extracts were made by disintegration of the Tris-EDTA-resuspended cell pellets in an ice-cold cell of a French pressure cell (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

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	<i>end-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacIZYA-argF</i>)U169 <i>deoR</i> [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15]	GIBCO-BRL
XL1-Blue	<i>end-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1</i> λ <i>recA1 gyrA96</i> (Nal ^r) <i>relA1</i> (Δ <i>lac</i>)[F' <i>proAB</i> ⁺ <i>lacI</i> ^s Z Δ M15::Tn10 (Tc ^r)]	Stratagene
<i>Y. pestis</i> ^a		
KIM5-3001 (parent)	Sm ^r ; pCD1 (LCR ⁺), pPCP1(Pla ⁺) pMT1	27
KIM5-3001.5	Sm ^r ; pCD1 <i>lcrG</i> (Δ 39–53) ^b , pPCP1(Pla ⁺), pMT1	49
KIM5-3001.6	Sm ^r ; pCD1 <i>lcrE</i> (Δ 48–197), pPCP1(Pla ⁺), pMT1	42
KIM5-3001.17 (Δ P1)	Sm ^r ; pCD1 <i>yscP</i> (Δ 246–333), pPCP1(Pla ⁺), pMT1	This study
KIM5-3001.18 (Δ P2)	Sm ^r ; pCD1 <i>yscP</i> (Δ 57–324), pPCP1(Pla ⁺), pMT1	This study
KIM5-3401	Sm ^r ; pCD1 <i>lcrH::cat yopJ::Mu d1734</i> [Km ^r Lac ⁺] [LcrH ⁻ , YopB ⁻ , YopD ⁻ , YopJ ⁻], pPCP1, pMT1	42
KIM8-3002 (parent Pla ⁻)	Sm ^r ; pCD1 (LCR ⁺), pPCP1 ⁻ (Pla ⁻), pMT1	34
KIM8-3002.1	Sm ^r ; pCD1 <i>yopB</i> (Δ 8–399), pPCP1 ⁻ (Pla ⁻), pMT1	13
KIM8-3002.4 (Δ P2-Pla ⁻)	Sm ^r ; pCD1 <i>yscP</i> (Δ 57–324), 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 the <i>lac</i> promoter	14
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	37
pYscOP	Ap ^r ; same as pYscOP.2 with the insert oriented with the T7 promoter	37
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 elimination of <i>yscP</i> and carrying <i>yscO</i>	37
pYscP	Ap ^r ; 1.8-kb <i>Ava</i> I fragment of pYP-F1 (14) carrying <i>yscP</i> , filled in with Klenow and cloned into the <i>Eco</i> RV site of pBluescript II SK+ with the insert oriented with the T7 promoter	This study
pYscP.2	Ap ^r ; same as pYscP but oriented with the <i>lac</i> promoter	This study
pYscP1	Ap ^r ; <i>Bcl</i> II digest and religation of plasmid pYscP, resulting in deletion of <i>yscP</i> (Δ 246–333)	This study
pYscP2	Ap ^r ; pYscOP digested with <i>Age</i> I, filled in with Klenow followed by digestion with <i>Sly</i> I and blunt ending with mung bean nuclease; religation of plasmid resulted in deletion of <i>yscP</i> (Δ 57–324)	This study
pHT-V	Ap ^r ; expression vector carrying <i>lcrV</i> ; translationally fused to a leader encoding 23 residues, including 6 histidines	15, 33
pTRCM.2	Ap ^r ; expression vector carrying <i>yopM</i> behind the <i>trc</i> promoter	42
pGEX-3X	Ap ^r ; GST fusion expression vector with the <i>tac</i> promoter	Pharmacia
pGST-YscP	Ap ^r ; <i>Bam</i> HI (in vector site)- <i>Age</i> I digest of pGST-YscO (37) filled in with Klenow followed by religation of plasmid (expresses fusion protein of GST and aa 328–455 of YscP)	This study
pUK4134	Ap ^r ; suicide vector <i>oriR6K oriT cos rpsL</i>	47
pUK Δ P1	<i>Xho</i> I digest of pYscP1 filled in with Klenow followed by <i>Sma</i> I (vector site) digest; <i>Xho</i> I- <i>Sma</i> I fragment cloned into <i>Eco</i> RV site of pUK4134	This study
pUK Δ P2	<i>Mlu</i> I digest of pYscP2 filled in with Klenow followed by digestion with <i>Eco</i> RV (vector site); <i>Mlu</i> I- <i>Eco</i> RV band cloned into <i>Eco</i> RV site of pUK4134	This study

^a All *Y. pestis* strains are Pgm⁻ (54).

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

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

(cytoplasmic plus periplasmic) were separated from membranes of the cleared lysates by ultracentrifugation at 417,000 \times g for 15 min at 4°C in a TLA 100.4 rotor (Beckman, Inc., Palo Alto, Calif.). Membranes were resuspended in ice-cold 100 mM Tris-HCl (pH 7.4)–1 mM EDTA. Soluble and membrane fractions were stored at –20°C. Before electrophoresis, the samples were thawed and diluted 1:1 with 2 \times electrophoresis sample buffer. Secreted proteins were precipitated with 5% (vol/vol) trichloroacetic acid (TCA) 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, and stored at –20°C. All samples were boiled for 2 min before being loaded onto gels for electrophoresis. YscP was subject to degradation by Pla, even with immediate processing and storage at –20°C.

Accordingly, samples to be analyzed for YscP were separated by SDS-PAGE on the day when the bacteria were grown. Gels were loaded such that each lane contained proteins corresponding to equal numbers of bacteria. Bacterial fractions were analyzed by denaturing SDS-PAGE (12 to 15% [wt/vol] acrylamide) (26) followed by transfer to Immobilon-P (Millipore Corp., Bedford, Mass.) with Towbin transfer buffer (53). Specific proteins were visualized on the membranes by using the polyclonal antibodies specific for the proteins (37) and a secondary antibody (goat anti-rabbit or goat anti-mouse [Sigma]) conjugated to either alkaline phosphatase or horseradish peroxidase.

Chemical cross-linking of proteins. To determine if YscP was part of a protein complex or associated with other *Yersinia* proteins, proteins in whole cells of *Y. pestis* strains growing in TMH (lacking Ca²⁺) were cross-linked with either

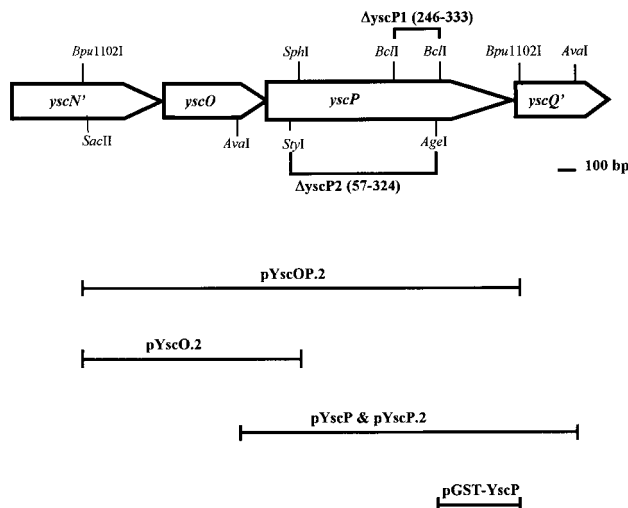


FIG. 1. Physical and genetic map of the region of pCD1 that encompasses *yjcP*. The coding regions for *yjcP*, *yjcO*, and parts of *yjcN'* and *yjcQ'* carried on pYjcOP.2, as well as selected restriction sites, are shown. Regions included in selected clones are diagrammed below the map.

disuccinimidyl suberate (DSS; spacer arm, 11.4 Å) or 1,4-di-[3'-(2'-pyridylidithio)-propionamido]butane (DPDPB; spacer arm, 16 Å) (Pierce) at 1 mM as specified by the manufacturer. The optimal concentration of each crosslinker was determined in separate experiments with various concentrations of DSS and DPDPB (0.5, 1, 2, and 5 mM, and 0.1, 1, and 2.5 mM, respectively). Each *Y. pestis* strain was grown in TMH (lacking Ca^{2+}), and after ca. 5 h of growth, a portion representing 2 OD₆₂₀ ml was transferred to a 2.5-ml prewarmed flask in a shaking water bath. For each strain tested, cultures were cross-linked by the addition of either DSS or DPDPB (final concentration, 1 mM). Additionally, a sample from each strain was mock treated with dimethyl sulfoxide at a concentration equal to that in samples containing cross-linker. After 30 min at 37°C, the DPDPB-cross-linked cultures were removed to ice. The DSS-cross-linked cultures were quenched by the addition of Tris-HCl (pH 8.0) (final concentration, 50 mM), allowed to react for 15 min, and then removed to ice. Each sample was lysed in a French pressure cell, and soluble proteins were separated from the cellular debris by low-speed centrifugation. TCA was added to the soluble fraction to a final concentration of 5% (vol/vol), and the proteins were allowed to precipitate overnight on ice. Proteins and cross-linked protein complexes were collected by centrifugation (543,300 × *g* for 10 min at 4°C) in a Beckman 100.4 TLC rotor. After being air dried, each protein pellet was neutralized, and the mock-treated and DSS-cross-linked proteins were resuspended in electrophoresis sample buffer lacking β-mercaptoethanol (BME) and boiled for 2 min. The proteins were separated by SDS-PAGE and subjected to immunoanalysis with anti-YscP. Duplicate samples were run for DPDPB, one in the sample buffer lacking BME and one with BME added to a final concentration of 10% to cleave any sulfhydryl cross-links. In other experiments, *Y. pestis* cultures grown as described above were fractionated into soluble proteins, membranes, and culture medium, and these were subjected to chemical cross-linking and SDS-PAGE.

Yop targeting studies. To assess the effect of *yjcP* on the targeting of Yops into eukaryotic cells, we used a tissue culture model of *Yersinia* infection to induce Yop targeting. We detected the amount of Yops released into the tissue culture medium, the amount targeted (that in the soluble fraction of eukaryotic cells), and the amount associated with the bacteria and the surface of eukaryotic cells (that in cellular debris of lysed eukaryotic cells with adherent bacteria) by immunoanalysis of SDS-PAGE-separated fractions.

HeLa and J774 murine macrophage-like cells were seeded in 3.5-cm-diameter tissue culture wells (~2.5 × 10⁵ cells/well) and grown to semiconfluence (5–8 × 10⁵ cells/well) as previously described (46). The bacteria for these targeting studies were grown at 26°C in HIB, diluted into warm RPMI 1640 (Life Technologies, Grand Island, N.Y.), and immediately added at an infectious dose of 10 per eukaryotic cell. The cluster dishes containing infected eukaryotic cells were centrifuged in a Beckman TJ-6 centrifuge (200 × *g* for 5 min at room temperature) to facilitate bacterial contact with the eukaryotic cells and then incubated for 30 min at 37°C under a 5% CO₂ atmosphere. Nonadherent bacteria were removed by washing twice with 2 ml of warm PBS. Then 2 ml of warm RPMI (with 5 μg of cytochalasin D per ml for J774 cells, to prevent phagocytosis of the nonencapsulated 26°C-grown yersiniae) was added to the appropriate wells, and the incubation was continued for 3.5 h (for a total of 4 h). After infection, one replicate well of HeLa and J774 cells per infecting strain was treated for 5 min at 37°C with trypsin (final concentration, 100 μg/ml) to differentiate between

surface (trypsin-susceptible) and cytosol-localized (trypsin-nonsusceptible) proteins. After a maximum 5-min treatment, protease inhibitors (Pefabloc and leupeptin; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were added (final concentration, 100 μg/ml each) to stop the trypsin treatment. To each well that had not been treated with trypsin, 2 μg of each of the two protease inhibitors per ml was added. The contents of each well were harvested and fractionated as previously described (46). Briefly, the RPMI 1640 was removed from each well and placed in a separate capped 5-ml syringe. A 1-ml volume of RT PBS was used to gently wash the cells adhering to the wells, and this was added to the syringe. The combined supernatant and wash from each well was filtered into a tube on ice. Infected eukaryotic cells remaining in the wells after removal of the supernatant were gently washed a second time in RT PBS, and this wash was discarded. The cells were completely lysed by addition of 1 ml of ice-cold distilled water containing protease inhibitors (Pefabloc and leupeptin, each at a final concentration of 2 μg/ml) followed by incubation on ice for ~30 min. Complete lysis of cells was achieved by scraping and vigorous pipetting of well contents. The soluble fraction (eukaryotic cytosolic contents including any targeted Yops) from each well was separated from the cellular debris (nonlysed eukaryotic cells, nonlysed large organelles, large pieces of membrane or cytoskeleton, and intact bacteria) by centrifugation (20,800 × *g* for 15 min at 4°C). The soluble and cell-free supernatant fractions were precipitated (10% [vol/vol] TCA) overnight on ice, neutralized in 1 M Tris-HCl (pH 8.0), and resolubilized in no more than 50 μl (total volume) of electrophoresis sample buffer. The debris (low-speed pellet) was washed in cold PBS and then solubilized in 50 μl of electrophoresis sample buffer. Proteins were analyzed by immunoblotting after separation by SDS-PAGE.

DNA sequence analysis. The *yjcP* nucleotide sequence of *Y. pestis* was previously submitted (accession no. L25667) (37). The DNA and predicted protein sequences of *yjcP* were analyzed by using PCGene (IntelliGenetics, Inc., Mountain View, Calif.) and IntelliGenetics Suite (IntelliGenetics, Inc.) software. The deduced amino acid sequence was compared with available sequences in the GenBank database via the National Center for Biotechnology Information BLAST (4) mail server (blast@ncbi.nlm.nih.gov).

RESULTS

Analysis of *yjcP* of *Y. pestis* KIM. The focus of this study is *yjcP* of *Y. pestis*, the third gene in the *yjcN*–*yjcU* operon. Computer analysis of *yjcP* predicted a 455-residue protein having a molecular mass of 50.4 kDa and an isoelectric point (pI) of 5.23; the predicted sequence does not contain any hydrophobic domain, remarkable secondary structure, or protein motif. The corresponding Spa proteins in *Salmonella* (SpaN, also called InvJ) (9, 19) and *Shigella* (Spa32) (45) are predicted to be proteins of 36.4 and 32.9 kDa with pIs of 6.3 and 5.6, respectively. There was no ca. 32-kDa region of YscP that showed significant similarity to either Spa protein, and the predicted amino acid sequence of *yjcP* is not significantly similar to any other protein outside the *Yersinia* spp.

T7 expression of *yjcP* in *E. coli*. Products were expressed from the cloned *yjcP* gene on plasmid pYscP and its derivatives (Fig. 1; Table 1) in *E. coli* by using a bacteriophage T7 promoter-polymerase system (Fig. 2). This system provides high-level transcription of cloned genes, while background expression is minimized due to inhibition of *E. coli* RNA polymerase by rifampin. pYscP expressed a unique ca. 60-kDa protein product, which was a higher molecular mass than expected for YscP (predicted 50.4 kDa) (Fig. 2, lane 2). Plasmids pYscP.2 (with *yjcP* oriented opposite the T7 promoter) and pYscP1 did not express this protein, suggesting that the 60-kDa band was the product of *yjcP* (lanes 3 and 4). pYscP1 expressed a unique ca. 48-kDa protein (lane 4) that was higher than the predicted molecular mass of 40.7 kDa.

Identification of *yjcP* in *Y. pestis* KIM. In an effort to identify a role for YscP, it was of interest to determine its cellular location. We were also interested to see if YscP would be expressed in *Y. pestis* at the same molecular mass as in *E. coli*. Polyclonal antiserum directed against a GST fusion protein containing the C terminus of YscP (aa 328 to 455) was used to identify the *yjcP* gene product in immunoanalysis of bacterial fractions of *Y. pestis*.

We found that in *Y. pestis* KIM 5-3001, YscP was subject to

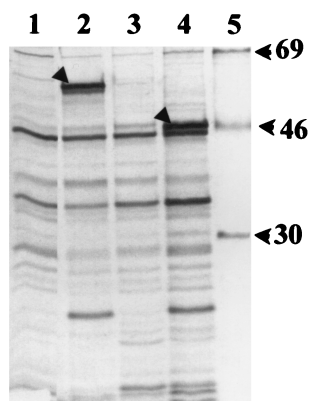


FIG. 2. T7 promoter/polymerase expression of cloned *yscP* in *E. coli*. Radio-labeled proteins were separated by SDS-PAGE (12% [wt/vol] polyacrylamide), and the gels were dried and autoradiographed. Lanes 1 to 4 were loaded with equivalent numbers of *E. coli* XLI-Blue cells carrying (left to right) pBluescript II SK+, pYscP, pYscP.2, and pYscP1. Lane 5 was loaded with ^{14}C -labeled protein markers (sizes are given in kilodaltons to the right). Arrowheads in lanes 2 and 4 indicate putative full-length and mutant YscP products, respectively.

rapid degradation and was seen predominantly as a 26-kDa band, even if the bacterial fractions were prepared and electrophoresed on the same day (data not shown). At least some of the degradation spared the carboxyl terminus, because putative degradation products as small as ca. 7 kDa were detected by our antiserum against the C terminus of YscP. The degradation was suspected to be *Yersinia* specific and to be due to Pla, since no degradation products of YscP were observed in *E. coli* (Fig. 2). Thus, fractions of the Pla⁻ *Y. pestis* strain KIM8-3002 (parent Pla⁻) were analyzed for the presence of YscP (Fig. 3). At 37°C in the presence of calcium, a single band corresponding to the putative YscP was detected in the soluble fraction of *Y. pestis* KIM8-3002 (parent Pla⁻) and in very small amounts in the membrane fraction. It migrated as a ca. 60-kDa protein, greater than the expected molecular mass (50.4 kDa) but similar to that seen in *E. coli*. These data suggest that Pla, which is known to degrade Yops (50), had been the major cause of YscP degradation in Pla⁺ *Y. pestis*. YscP1 also was found as degradation products in fractions from Pla⁺ *Y. pestis* KIM5-3001.17 (Δ P1) (data not shown). However, in whole-cell

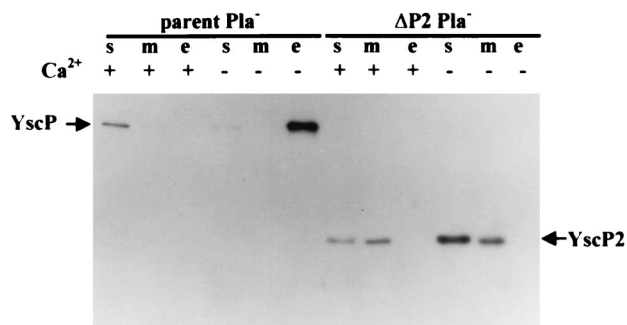


FIG. 3. Localization of YscP in Pla⁻ *Y. pestis*. An immunoblot analysis of *Y. pestis* KIM8-3002 (parent Pla⁻) and *Y. pestis* KIM8-3002.4 (Δ P2 Pla⁻) is shown. Bacteria were grown in TMH with (+) and without (-) Ca²⁺. Proteins from the soluble (s), membrane (m), and culture medium (e) fractions were separated by SDS-PAGE (12% polyacrylamide gels) and transferred to Immobilon P. The proteins were detected with polyclonal antibody specific to a fusion protein of GST-YscP and a secondary antibody conjugated to horseradish peroxidase and were visualized by enhanced chemiluminescence.

preparations, it was observed as a 48-kDa protein, the same mass as in *E. coli* (predicted, 40.7 kDa) (see Fig. 7). In *Y. pestis* KIM8-3002.4 (Δ P2 Pla⁻) grown in the presence of Ca²⁺, the putative YscP2 was detected in the soluble and membrane fractions as a 22-kDa species, the expected molecular mass (21.1 kDa) for this mutant protein (Fig. 3). In Pla⁺ *Y. pestis*, we also saw smaller putative YscP2 degradation products in the same bacterial fractions (data not shown).

The aberrant migration of YscP and YscP1 but not YscP2 might be due to aa 57 to 233, since this region is present in YscP and YscP1 but lacking in YscP2. Computer analysis did not reveal any posttranslational modification site in aa 57 to 233 that would account for these differences; therefore, the effect may be due to the protein conformation.

In the absence of calcium, both YscP and YscP2 were detected at higher levels (Fig. 3). This shows that *yscP* belongs to the LCRS stimulon, as previously shown for *yscO* (37) and *yscR* (14). YscP was present almost exclusively in the culture medium of the parent (parent Pla⁻), whereas YscP2 still was observed in the soluble and membrane fractions of *Y. pestis* KIM8-3002.4 (Δ P2 Pla⁻). This suggests that YscP is a secreted protein. Its lack of a signal sequence suggested the possibility that it was being secreted by the Ysc mechanism, and indeed, we had previously found that YscP was not secreted by a *yscO* mutant (37).

LCR phenotype of *yscP* *Y. pestis*. Ca²⁺-independent growth, decreased Yop and V-antigen expression, and blocked secretion of Yops and V antigen have been previously described for *yscO* and *yscR* mutants when grown under LCR-inductive conditions (14, 37), and we anticipated that a *yscP* mutant would be similar. To determine the LCR phenotype of our *yscP* strains, we compared the growth and secretion phenotypes of the parent and mutant strains (Δ P1 and Δ P2) growing in TMH under both inductive and noninductive conditions for the LCR (see Fig. 4 for growth and Fig. 5 for secretion).

All strains exhibited full growth yield at 37°C in the presence of Ca²⁺ (Fig. 4). In the absence of Ca²⁺, the parent, *Y. pestis* KIM5-3001, showed growth restriction typical for media like TMH (17) following a temperature shift from 26 to 37°C. *Y. pestis* KIM5-3001.17 (Δ P1) grew in the same manner as the parent. *Y. pestis* KIM5-3001.18 (Δ P2) displayed a growth phenotype that ranged from a slight amount of growth restriction to Ca²⁺-independent growth (Fig. 4, Δ P2). Growth restriction has previously been used as a marker for LCR induction, and strains that display Ca²⁺-independent growth under inductive conditions generally also express less Yop and V antigen than does the parent and do not secrete these proteins. The Δ P1 mutant, in keeping with the growth pattern of the parent, expressed and secreted YopM (Fig. 5) YopH, and V antigen (data not shown) at a similar level. However, for the Δ P2 strain, the growth response was not a sensitive indicator of induction: although there was some decrease in the abundance of some secreted proteins, this mutant showed significant Yop expression and secretion (Fig. 6). *Y. pestis* KIM8-3002.4 (Δ P2 Pla⁻) expressed and secreted less V antigen, YopD, YopM, and YopH than did the parent (Fig. 6A). The negative regulators LcrE and LcrQ were detected only in the culture medium fraction for either the parent or the mutant, as expected under LCR-inductive conditions (Fig. 6B). The amount of LcrQ was smaller for the Δ P2 mutant than for the parent, whereas LcrE secretion by the mutant was slightly decreased. The significance of these differences is not obvious at present. Interestingly, YopE abundance was not affected in the Δ P2 mutant, and secretion of YopE occurred at near wild-type levels (Fig. 6A). This phenotype is believed to be due to the mutation in *yscP* alone and not to a polar effect on downstream

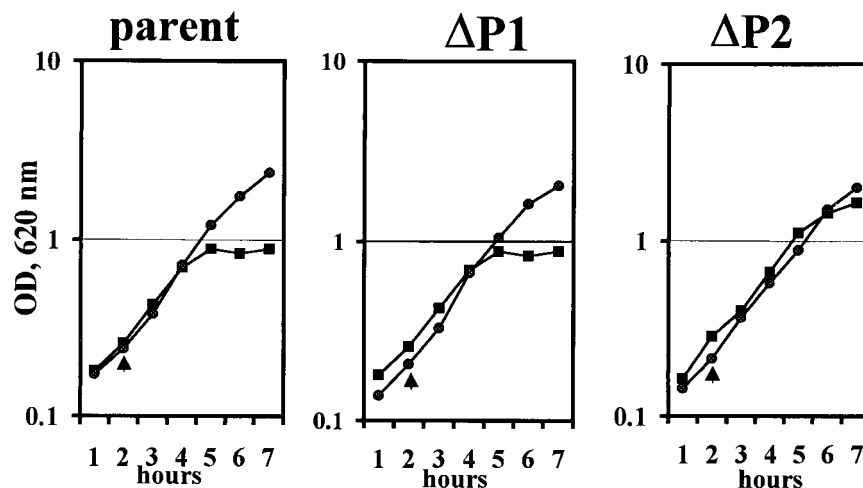


FIG. 4. Growth phenotype of *y_{sc}P* *Y. pestis*. *Y. pestis* KIM5-3001 (parent), KIM5-3001.17 (Δ P1), and KIM5-3001.18 (Δ P2) were grown at 37°C in the presence or absence of Ca^{2+} in the defined medium TMH. The temperature was shifted from 26 to 37°C (temperature shifts are denoted by arrowheads). Symbols: circles, $+\text{Ca}^{2+}$; squares, $-\text{Ca}^{2+}$. OD, optical density.

genes, because YscR, the product of the second gene downstream of *y_{sc}P* in the same operon, was detected at similar levels in membranes of the parent and the Δ P2 mutant (Fig. 6B).

No proteins were detected in the culture medium of the parent or either *y_{sc}P* mutant at 37°C in the presence of Ca^{2+} (data not shown; the antibodies used were to YopE, YopH, YopM, V antigen, and total secreted proteins). This showed that the mechanism for blocking secretion through the Ysc in response to Ca^{2+} was functional in the *y_{sc}P* mutants.

Complementation of the Δ P2 mutant. To test whether the Δ P2 mutation could be complemented, we expressed YscP *in trans* from the *lac* promoter on pYscP.2 in the Δ P2 *Y. pestis* KIM5-3001.18. To our surprise, this caused Yop secretion to be almost completely blocked (Fig. 5, Δ P2/P). A similar secretion phenotype was also observed for the Δ P1 *Y. pestis* KIM5-3001.17 carrying pYscP.2 *in trans* (Fig. 5, Δ P1/P). These effects were not due to the vector, since introduction of vector only into the parent and both *y_{sc}P*-mutant *Y. pestis* strains did not affect the secretion phenotype of the respective strains (data not shown). Unlike pYscP.2, pYscP was not expected to have any effect on the phenotype of either mutant, since in this construct *y_{sc}P* is oriented against the *lac* promoter and with the T7 promoter, which is not functional in *Yersinia*. Indeed, there was little effect on the secretion of Yops when pYscP was introduced into the Δ P1 mutant (Fig. 5, compare Δ P1/ P^{T7} and Δ P1); however, the Δ P2 mutant was fully complemented by

this plasmid and secreted Yops and V antigen similarly to the parent (Fig. 5, compare Δ P2/ P^{T7} and parent). This indicated that pYscP in fact did express some functional YscP. In a separate experiment, the putative YscP (ca. 60 kDa) and YscP1 (ca. 48 kDa) were observed in fractions of *Y. pestis* KIM5-3001.17 carrying pYscP (Δ P1/ P^{T7}) (results not shown). It may be that low-level extra expression of YscP is tolerated without a significant effect on Yop secretion. Accordingly, Yop secretion by *Y. pestis* KIM5-3001.17 (Δ P1) was similar to that by Δ P1 carrying pYscP *in trans* (Δ P1/ P^{T7}) (Fig. 5). In contrast, in *Y. pestis* KIM5-3001.17 carrying *y_{sc}P* oriented with the *lac* promoter (which is constitutively expressed in *Y. pestis*) (Δ P1/*P*), YscP and YscP1 were detected in whole cells (data not shown) but none was secreted (Fig. 7, Δ P1/*P*, lane e), and Yops were not secreted (Fig. 5, Δ P1/*P*). Indeed, we have found that the *lac* promoter provides stronger expression of YscP than does the putative *y_{sc}P* promoter (data not shown). The data suggest that YscP expression is tightly regulated in *Y. pestis* and show that excess YscP blocks secretion. The block in secretion could be due to excess YscP sterically blocking the secretion pore. Alternatively, YscP could associate with other *Y. pestis* proteins, and in strains expressing excess YscP, the extra YscP could sequester one or more factors into nonfunctional complexes, resulting in a loss of function and no Yop secretion. Either alternative explains the results obtained for Δ P1 or the parent carrying pYscP or pYscP.2 and also explains the complementation of Δ P2 by pYscP but not pYscP.2

Secretion of V antigen and YopM expressed from plasmids with non-LCR-inducible promoters. The data suggest that the secretion defect of *Y. pestis* KIM5-3001.18 (Δ P2) could be due to the effect of YscP on the secretion mechanism. However, in the LCR, maximal *yop* expression is dependent upon a functional Ysc. We have previously shown that mutations in *y_{sc}C*, *y_{sc}D*, *y_{sc}G*, and *y_{sc}O* indirectly affect expression by directly blocking secretion (37, 42). To distinguish these two potential effects of *y_{sc}P* and to test the hypothesis that *y_{sc}P* affects secretion directly, secretion was analyzed in *Y. pestis* KIM5-3001 (parent) and *Y. pestis* KIM5-3001.18 (Δ P2), each carrying V antigen or YopM expressed *in trans* from an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible, non-LCR regulated promoter on, respectively, pHT-V, which produces histidine-tagged V antigen (HT-V) or pTRCM.2, which encodes YopM.

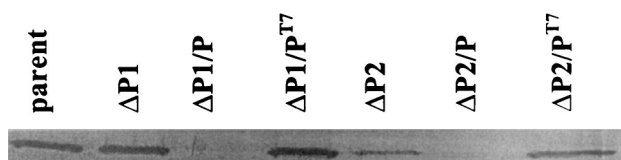


FIG. 5. Secretion of YopM by *y_{sc}P* *Y. pestis*. An immunoblot analysis of YopM secreted from *Y. pestis* KIM5-3001 (parent), KIM5-3001.17, (Δ P1), KIM5-3001.18 (Δ P2), and the mutants carrying *y_{sc}P* *in trans* on pYscP.2 or pYscP (*P* and P^{T7} , respectively) is shown. Bacteria were grown in TMH at 37°C without Ca^{2+} . Proteins from the culture medium were separated by SDS-PAGE (12% polyacrylamide gels), transferred to Immobilon P, and visualized with antibody specific to YopM and a secondary antibody conjugated to alkaline phosphatase.

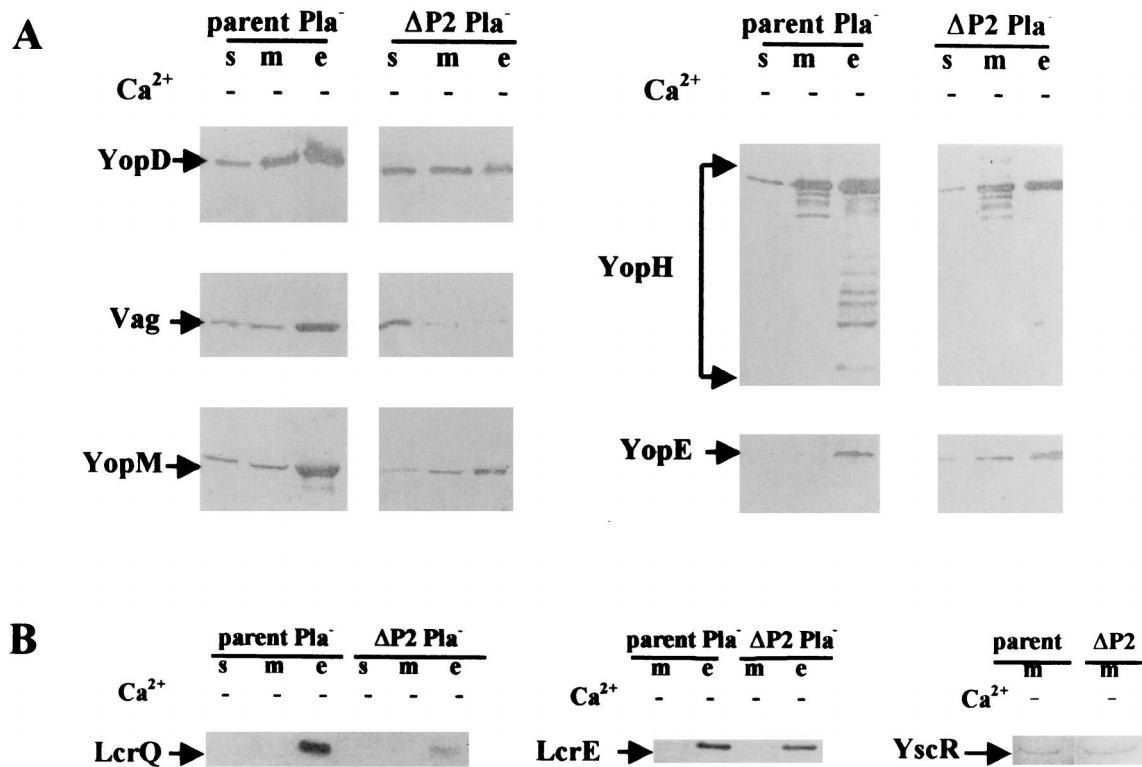


FIG. 6. Expression and secretion of LCRS proteins in *yscP* *Y. pestis*. An immunoblot analysis of proteins expressed and secreted from *Y. pestis* KIM5-3001 (parent) and KIM5-3001.18 (Δ*P2*) or their *Pla*⁻ counterparts. KIM8-3002 (parent *Pla*⁻) and KIM5-3002.4 (Δ*P2 Pla*⁻), respectively, is shown. Bacteria were grown in TMH at 37°C without Ca²⁺. Proteins from soluble (s), membrane (m), and culture medium (e) fractions were separated by SDS-PAGE (12% polyacrylamide gel) and transferred to Immobilon P. Primary antibodies were used that were specific to YopE, YopD, YopH, YopM, LcrE, LcrQ, YscR, and V antigen (Vag), and the secondary antibody was conjugated to alkaline phosphatase. Arrows denote the positions of the respective proteins.

Five hours prior to harvest, the expression of YopM or HT-V was induced by the addition of IPTG. The expression and secretion of YopM and HT-V were monitored by immunoblotting (Fig. 8).

When pTRCM.2 was present, the parent strain expressed and secreted higher levels of YopM than it did without pTRCM.2. With pHT-V, the parent strain expressed both native V antigen and HT-V, which ran slightly above the native V antigen due to its His₆-containing leader sequence. The Δ*P2* mutant carrying pHT-V expressed HT-V and secreted some of

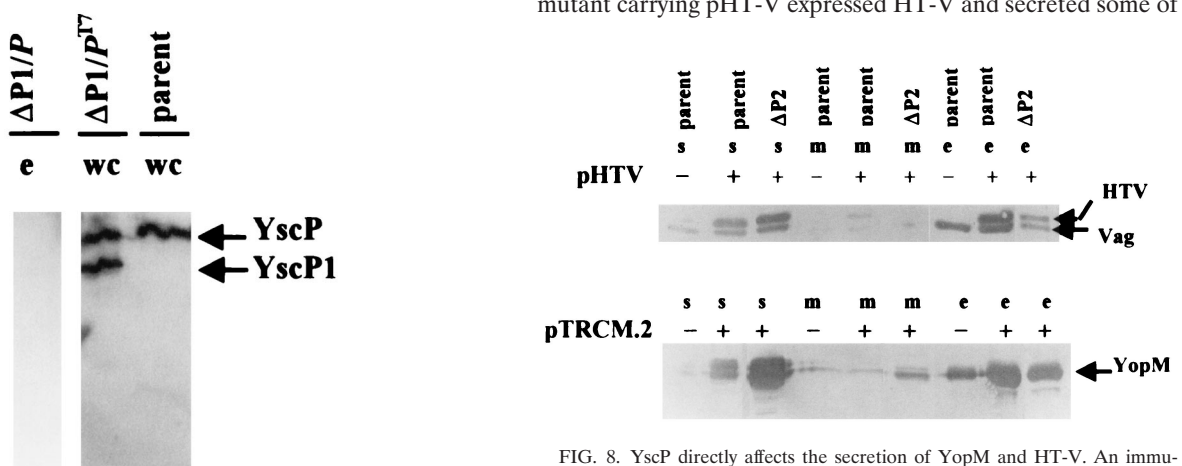


FIG. 8. *YscP* directly affects the secretion of YopM and HT-V. An immunoblot analysis of YopM, V antigen, and HT-V in the soluble (s), membrane (m), and culture medium (e) fractions from *Y. pestis* KIM5-3001 (parent) and *Y. pestis* KIM5-3001.17 (Δ*P2*) with (+) and without (-) pHT-V or pTRCM.2 is shown. *Y. pestis* strains were grown in the defined medium TMH at 37°C in the absence of Ca²⁺; expression of HT-V from pHT-V and YopM from pTRCM.2 was induced by addition of IPTG to 1 mM at 5 h prior to harvest. Proteins from each fraction were separated by SDS-PAGE (12% [wt/vol] acrylamide) and transferred to Immobilon P. Polyclonal antibody to HT-V or YopM was used to detect HT-V and YopM, respectively. Secondary antibody was conjugated to alkaline phosphatase. In the lower panel, YopM was detected as two closely migrating species.

FIG. 7. Evidence of an independent promoter for *yscP*. An immunoblot analysis of the *Pla*⁻ *Lcr*⁺ parent strain of *Y. pestis* (KIM8-3002, parent) and the *Pla*⁺ Δ*P1 Y. pestis* KIM5-3001.17 carrying *yscP* in *trans* on pYscP.2 or pYscP (Δ*P1/P* and Δ*P1/P*¹⁷, respectively) is shown. Bacteria were grown in TMH without Ca²⁺. Proteins from the whole-cell (wc) and culture medium (e) fractions were separated by SDS-PAGE and transferred to Immobilon P. They were detected by using polyclonal antibody specific to the fusion protein GST-YscP and a secondary antibody conjugated to horseradish peroxidase and were visualized by enhanced chemiluminescence.

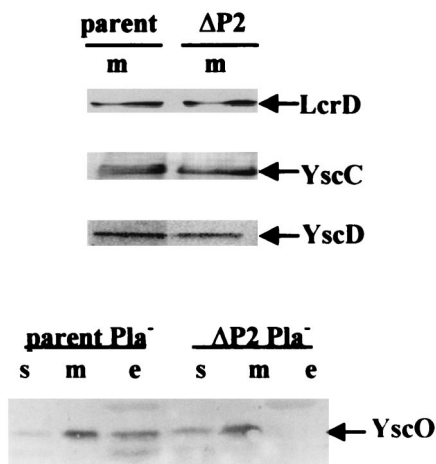


FIG. 9. Detection of LcrD, YscD, YscC, and YscO in *yjcP* *Y. pestis*. *Y. pestis* strains were grown in the defined medium TMH at 37°C in the absence of Ca²⁺. The proteins in the soluble (s), membrane (m), and culture medium (e) fractions were separated by SDS-PAGE (12% [wt/vol] acrylamide), transferred to Immobilon P, and analyzed by immunoblotting. (Top) Pla⁺ *Y. pestis* KIM5-3001 (parent) and Pla⁺ *Y. pestis* KIM5-3001.18 (Δ P2) were analyzed with antibodies specific to LcrD, YscC, or YscD. (Bottom) *Y. pestis* KIM8-3002 (parent Pla⁻) and *Y. pestis* KIM8-3002.4 (Δ P2 Pla⁻) were analyzed with anti-YscO. The secondary antibody used was conjugated to alkaline phosphatase. Arrows indicate each protein.

it, but most of the HT-V was retained in the soluble fraction. Similarly, despite the high levels of YopM expressed in the mutant from pTRCM.2, over half of the YopM expressed was retained in the soluble fraction of the bacteria. These results indicate that the mutation in *Y. pestis* KIM5-3001.18 (Δ P2) causes a defect in some aspect of the secretion process and supports a direct role for *yjcP* in the secretion of LCR virulence proteins by *Y. pestis*.

It is possible that *yjcP* affects the expression or localization of other Ysc proteins. However, immunoanalysis of proteins from membrane fractions of the parent and the *yjcP* mutant *Y. pestis* KIM5-3001.18 (Δ P2) showed that at least LcrD, YscC, YscD, and YscO were present in comparable amounts in both strains (Fig. 9). YscO normally is secreted under inductive conditions (37), but the Δ P2 mutant did not secrete any (Fig. 9). This suggests that YscO, like YscP, depends on the Ysc for its own secretion. We do not yet know the significance of the secretion of YscO; however, it is not likely that the Δ P2 phenotype reflects nonfunctional YscO, because a *yjcO* null mutant has a completely inactive Ysc (37) whereas the Δ P2 strain still secretes some Yops.

Effect of extra copies of *yjcP* in other *Y. pestis* backgrounds. We performed several tests to identify a potential target for the secretion-blocking effect of excess YscP. First we tested whether YscP would still block secretion when overexpressed along with YscO, the other Ysc protein that lacks sequence homology to Spa counterparts in *Salmonella* and *Shigella* and hence that may have the same *Yersinia*-specific adaptation that we postulate for YscP. Plasmids pYscP.2, pYscOP.2, and pYscO.2 were introduced into the parent *Y. pestis* KIM5-3001, creating the strains parent/P, parent/OP, and parent/O, respectively, and the culture medium of each strain was analyzed for the secretion of YopM, V antigen, and YopE (Fig. 10). Secretion of these proteins was similar in the parent and the parent carrying either pYscO.2 (parent/O) or pYscOP.2 (parent/OP). In contrast, the parent expressing *yjcP* secreted less YopE than did the parent, and levels of YopM and V antigen were barely

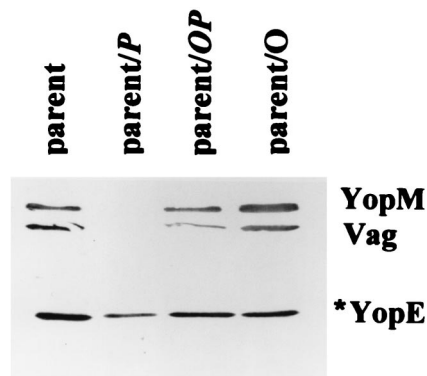


FIG. 10. Overexpression of YscP does not block secretion if YscO also is overexpressed. An immunoblot analysis of proteins expressed and secreted from the Pla⁺ *Y. pestis* KIM5-3001 (parent) and the parent expressing *yjcO*, *yjcP*, or both in *trans* (parent/O, parent/P, and parent/OP, respectively) is shown. Bacteria were grown in TMH at 37°C without Ca²⁺. Proteins from bacterial fractions were separated by SDS-PAGE (12% acrylamide gels) and transferred to Immobilon P. Proteins from the culture medium were detected with polyclonal antibodies specific to YopM, V antigen (Vag), and YopE. The secondary antibody used was conjugated to alkaline phosphatase. Due to the activity of Pla, only the major degradation product of YopE (YopE*) was observed. The proteins are identified by labels to the right.

detectable, a phenotype similar to that of the Δ P2 mutant. This indicates that, as in the phenotypically wild-type Δ P1 mutant, overexpression of YscP in the parent strain imposes a secretion defect. However, it is not extra YscP per se but extra YscP unbalanced by YscO that causes the defect, because overexpression of YscO together with YscP did not affect secretion of V antigen and Yops. This may indicate that an interaction occurs between YscP and YscO.

We next determined where the secretion block from YscP fits in the secretion/regulatory pathway by testing if the negative effect of excess YscP would be dominant over an *lcrG* or *lcrE* defect. *Yersiniae* with mutations of *lcrE* or *lcrG* are constitutively induced for the LCR because they constitutively secrete the negative regulator(s). Plasmid pYscP.2 was introduced into both mutants, and the resulting strains were checked to see if extra YscP would block secretion (Fig. 11). As expected, both mutants expressed and secreted YopM irrespective of Ca²⁺ and expression of YopM was higher in the mutants than in the parent irrespective of Ca²⁺. Introducing *yjcP* in *trans* into the *lcrE* mutant (Δ *lcrE*/P) decreased the expression of YopM to the parental level but did not block secretion. This shows that excess YscP could not have its full secretion-blocking effect in the absence of *lcrE* function. However, introduction of *yjcP* in *trans* into the *lcrG* mutant (Δ *lcrG*/P) resulted in a block in the secretion of YopM, showing that the effect of extra YscP did not require functional LcrG. In a current model for secretion, loss of either LcrG or LcrE would open the secretion pore at the inner or outer membrane, respectively, allowing the secretion of LcrQ and resulting in the upregulation of Yop expression. Our results show that the secretion block due to excess YscP can occur in the absence of LcrG but not of LcrE and suggest the hypothesis that the normal function of YscP involves an interaction with the mechanism of LcrE for modulating secretion system activity. YscP may act in conjunction with YscO and LcrE to control the opening of the secretion "pore."

Interactions of YscP and other *Y. pestis* proteins. We performed a direct test of the postulated interactions of YscP with other proteins, such as YscO and LcrE. We cross-linked bac-

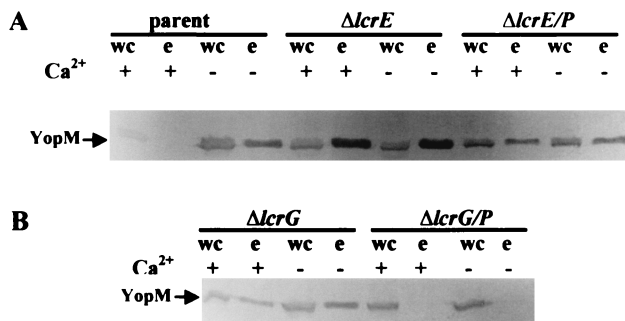


FIG. 11. Effects of YscP overexpression in *lcrE* and *lcrG* *Y. pestis*. An immunoblot analysis of proteins expressed and secreted from *Y. pestis* strains carrying *yscP* in *trans* is shown. Bacteria were grown in TMH at 37°C without Ca²⁺. Proteins from whole cells (wc) and the culture medium (e) were separated by SDS-PAGE (12% polyacrylamide gels) and transferred to Immobilon P. Proteins from the culture medium were detected with polyclonal antibodies specific to YopM. The secondary antibody used was conjugated to alkaline phosphatase. Arrows denote YopM. (A) *Y. pestis* KIM5-3001 (parent), KIM5-3001.6 (Δ *lcrE*), and KIM5-3001.6 carrying *yscP* in *trans* on pYscP.2 (Δ *lcrE/P*). (B) *Y. pestis* KIM5-3001.5 (Δ *lcrG*) and KIM5-3001.5 carrying pYscP.2 (Δ *lcrG/P*).

terial fractions and growing whole cells of the Pla⁺ parent, *Y. pestis* KIM5-3001, and the Pla⁻ parent, *Y. pestis* KIM8-3002, by using two cross-linkers, one that was sulfhydryl reactive and one that was amine reactive. No novel bands were observed in any cross-linked reactions with either cross-linker at any of the concentrations tested (data not shown).

Targeting of Yops in the Δ P2 mutant. To gain a more complete picture of the role of YscP in Yop deployment by *Y. pestis*, we wanted to determine if the mutation in *yscP* affected

the targeting of Yops into HeLa and J774 cells. We found that the Δ P2 mutant was not as strongly cytotoxic as the parent, and when we tested for the presence of YopE and YopH in the soluble fraction of infected HeLa cells, we found that less had been targeted by the Δ P2 mutant than by the parent (Fig. 12A, soluble, compare lanes 1 and 2 [data not shown for J774 cells]). It was surprising that there was a significant effect on the targeting of YopE, since we had seen little effect of the Δ P2 mutation on YopE secretion. However, during the course of these experiments, separate studies in our laboratory suggested that V-antigen secretion may be required for targeting (34, 35); therefore, the reduction in the amount of Yops targeted by this mutant could be an indirect effect due to the defect in V-antigen secretion by the Δ P2 *Y. pestis*. YscP and YscP2 themselves were detected in the *Yersinia*-containing low-speed pellet of eukaryotic cells infected with the respective *Y. pestis* strains but not in the corresponding soluble fractions (Fig. 12B, data shown for HeLa cells). This shows that YscP is expressed in the tissue culture infection model but that YscP itself is not targeted.

Virulence of the *yscP* mutants in mice. To determine if the *yscP* mutants were defective in virulence, a test was performed for lethality in BALB/c mice by using doses of 10³ and 10⁵ of each *yscP* mutant. As expected, a nominal dose of 10³ (800 CFU) of the parent, *Y. pestis* KIM5-3001, was lethal for all mice tested. (The intravenous 50% lethal dose of the parent strain is 4.2 × 10¹ [27].) The 50% lethal dose of *Y. pestis* KIM5-3001.17 (Δ P1) was near 10³ (880 CFU), since three of five mice died from infection with this dose. All five mice infected with the higher dose died. Mice infected with Δ P1 succumbed to infection 1 to 2 days later than did those infected

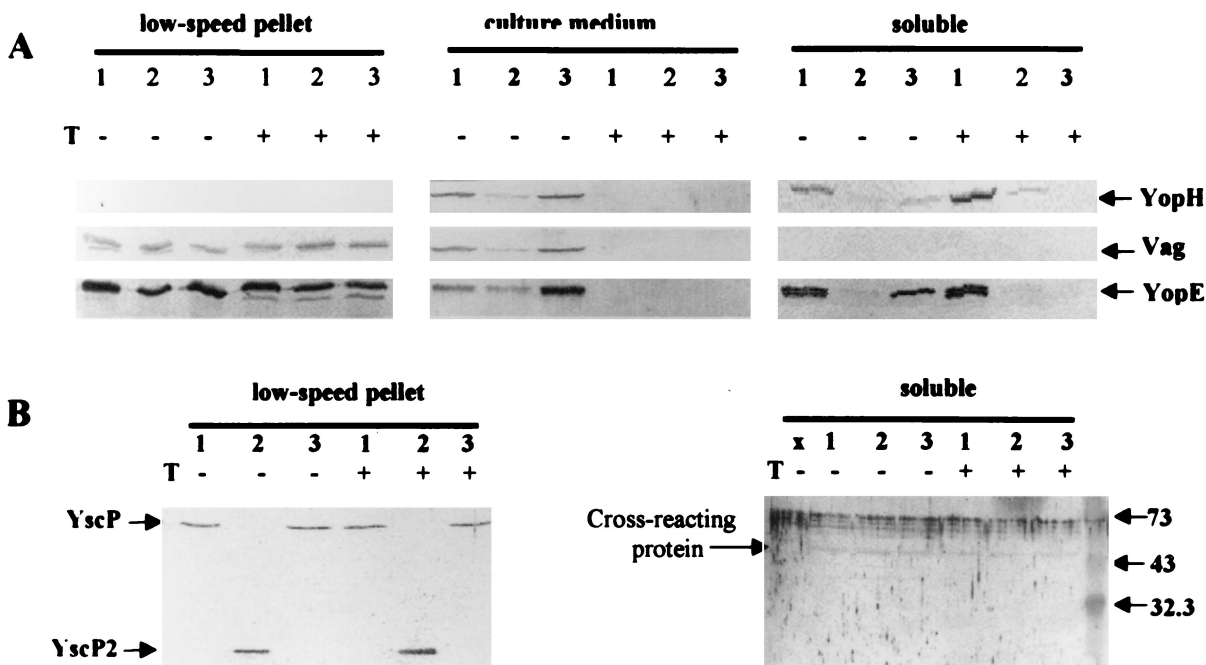


FIG. 12. YscP is not targeted into eukaryotic cells. The Pla⁻ *Y. pestis* strains KIM8-3002 (parent Pla⁻) (lanes 1), KIM8-3002.4 (Δ P2 Pla⁻) (lanes 2), and KIM8-3002.1 (Δ *yopB* Pla⁻) (lanes 3) were used to infect HeLa cells. After 4 h, trypsin (T) was added to a duplicate culture for each strain to assess the protease resistance of proteins in the low-speed pellet (pellet containing yersiniae, obtained after centrifugation of the H₂O-lysed cells), in the culture medium, and in the soluble fraction (Yops that are targeted). Protein samples were separated by SDS-PAGE (12% [wt/vol] acrylamide) and analyzed with primary antibody against YopH, V antigen, YopE, or YscP and secondary antibody conjugated with alkaline phosphatase. (A) Arrows denote YopH, V antigen (Vag), and YopE. (B) Arrows show YscP and YscP2 in the low-speed pellet. They were not detected in the soluble fraction; however, a ~73-kDa artifactual band was detected in all wells including those containing only electrophoresis buffer (lane x), and a cross-reacting band was seen at ca. 45 kDa. Numbers to the right of the gel loaded with soluble fraction represent molecular masses in kilodaltons.

with the parent (data not shown). In contrast, all mice survived infection with *Y. pestis* KIM5-3001.18 ($\Delta P2$) at either dose 10^3 (3×10^3 CFU) or 10^5 (3.7×10^5). Since *Y. pestis* KIM5-3001.17 ($\Delta P1$) expressed and secreted Yops similarly to the parent *Y. pestis* KIM5-3001, the small difference in virulence may suggest that $\Delta P1$ has defects in the secretion of proteins not detected by our analysis. The lack of virulence of *Y. pestis* KIM5-3001.18 ($\Delta P2$) was not surprising, in light of its defects in V antigen and Yop secretion and targeting.

DISCUSSION

The present study found that YscP is a secreted protein and that it is necessary for the normal secretion of Yops and V antigen by *Y. pestis*. The secretion defect in the *yscP* mutant *Y. pestis* KIM8-3002.4 ($\Delta P2$ Pla⁻) also caused decreased targeting of YopE and YopH and probably other Yops. These effects are believed to be due directly or indirectly to a mutation in *yscP* and not to a polar effect on downstream genes.

YscP was expressed under both inductive and noninductive conditions, as are other components of the Ysc, and its expression increased under LCR-inductive conditions, as has been the case for several other *ysc* gene products. When Ca²⁺ was present, YscP itself was present only in the whole-cell fraction of the culture, with most being in the soluble fraction; and in the absence of Ca²⁺, it was mostly secreted, a distribution similar to that of Yops. Additionally, the secretion of YscP was dependent on a functional Ysc, since it was expressed but not secreted in a secretion-negative mutant. However, we do not think that YscP is purely an antihost, LCR effector protein like the six targeted Yops, since YscP itself was not targeted into HeLa or J774 cells. Moreover, a mutation in *yscP* ($\Delta P2$) affected growth and secretion, and these effects have not been attributed to such Yops. Other LCR secreted proteins such as YscO, LcrV, LcrG, LcrE, YopB, YopD, and YopK have the cellular distribution of YscP in yersiniae grown in TMH, even though they function in LCR regulation and Yop targeting. Because YscP itself is secreted by the Ysc, as are YscO (37) and the Ysc-regulatory proteins LcrE (16) and LcrG (49), its own mobility may be an essential part of its function: it is a moving part of the Ysc. The role of YscP in the Ysc may be related to the function of the corresponding type III protein in *Salmonella*, SpaN/InvJ. SpaN/InvJ is a secreted protein that is essential for the secretion of *Salmonella* invasion proteins (Sips) (9). This is in contrast to the corresponding protein in *Shigella*, Spa32, which is associated with the outer membrane and is necessary for the release of Ipas from the bacterial surface but not for their transport to the surface.

Our further characterization of the $\Delta P2$ mutation by complementation led to the finding that too much YscP inactivates the Ysc whereas co-overexpression of YscO with YscP prevented YscP from blocking secretion, as though YscO needs to be present for YscP to function. Possibly, excess YscP forms abnormal complexes with other Ysc proteins and these are unable to promote Yop secretion. In $\Delta P2/P$, most of the YscP and YscP2 is membrane localized (data not shown). In $\Delta P2/P^{17}$, which did not show blocked secretion, the YscP was expressed from a multicopy plasmid but from the weak putative *yscP* promoter (data not shown). The low level of wild-type YscP might alleviate the secretion defect of the $\Delta P2$ strain by displacing YscP2 from any interactions with other Ysc components and reestablishing the normal mechanism for opening the secretion channel.

It is interesting that overexpression of YscP in an *lcrE* mutant had little effect on altering the constitutive secretion of Yops by that mutant. However, secretion was blocked in

strains expressing LcrE and making extra YscP ($\Delta P2/P$, parent/ P , $\Delta P1/P$, and $\Delta lcrG/P$). This may indicate that YscP normally acts at the level of the secretion-modulating mechanism of LcrE: YscP, working in conjunction with YscO, might be necessary for the secretion channel to open under inductive conditions, and this affects the secretion of some Yops more than others.

We hypothesize that YscP may be part of a complex; however, we did not detect YscP interactions with other *Yersinia* proteins by chemical cross-linking, and we did not detect any specific interaction of YscO and YscP in an affinity blot by using the soluble fusion protein GST-YscO as a probe against proteins of the parent *Y. pestis* separated by electrophoresis (data not shown). We are aware that interactions in the Sec system were difficult to detect before means were found to lock in a conformation of this dynamic mechanism, and we have not exhausted all methods to detect protein interactions. Strains with secretion blocked by excess YscP may provide one locked-in secretion conformation and will be useful for future probing of interactions among components of the Ysc. Therefore, we do not rule out the possibility that YscP functions as part of a larger complex, and we speculate that YscP and YscO may participate together as LCR-adapted components of the Ysc type III secretion mechanism.

The phenotype of the $\Delta P2$ strain itself is open to more than one interpretation. First, the finding that Yops are still secreted by the $\Delta P2$ mutant could indicate that YscP is a non-essential component of the secretion machinery under the conditions of our experiments. We cannot rule out the possibility that the secretion defect in this strain was due to the accumulation of YscP2 in membranes, perhaps nonspecifically partially plugging the secretion channel. Second, the phenotype of a strain with a complete deletion of *yscP* might be identical to that of the $\Delta P2$ mutant, and the effect of YscP on secretion might be analogous to that of YscW (VirG). A *yscW* mutant secreted less than wild-type levels of YopB, YopD, and V antigen but a normal level of YopH (1). Third, it is possible that a *yscP* null mutant would be unable to secrete any Yops and that YscP2 has partial function because it is stable and contains 42% of YscP. A similar phenotype was observed for the partially functional protein, YscF^{mod}. An N-terminal truncation of YscF, with its first 12 residues replaced by 8 vector-encoded amino acids, impaired the secretion of YopB and YopD but did not affect the secretion of YopH, which was in contrast to the phenotype of a *yscF* null mutant, which did not secrete any Yops (2). Hence, the partial secretion defect by *yscF*^{mod} *Y. enterocolitica* was due to a partially functional YscF protein. Similarly, YscP2 may have partial function but would lack any functions stemming from its own secretion, since it was not secreted. We presently favor the second hypothesis, because disruption of YscP function by overexpression of wild-type YscP had the same effect on secretion of Yops as did the $\Delta P2$ mutation. However, this must be tested by making a true *yscP* null mutant.

In conclusion, we have shown that YscP is a mobile component of the Ysc that acts at the level of the LcrE modulation of secretion system activity. These data add to our previous evidence that the type III Ysc mechanism has a dynamic moving core, and perhaps YscP is part of this.

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