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YscB of *Yersinia pestis* Functions as a Specific Chaperone for YopN

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Following contact with a eucaryotic cell, *Yersinia* species pathogenic for humans (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) export and translocate a distinct set of virulence proteins (YopE, YopH, YopJ, YopM, and YpkA) from the bacterium into the eucaryotic cell. During in vitro growth at 37°C in the presence of calcium, Yop secretion is blocked; however, in the absence of calcium, Yop secretion is triggered. Yop secretion occurs via a plasmid-encoded type III, or "contact-dependent," secretion system. The secreted YopN (also known as LcrE), TyeA, and LcrG proteins are necessary to prevent Yop secretion in the presence of calcium and prior to contact with a eucaryotic cell. In this paper we characterize the role of the *yscB* gene product in the regulation of Yop secretion in *Y. pestis*. A *yscB* deletion mutant secreted YopM and V antigen both in the presence and in the absence of calcium; however, the export of YopN was specifically reduced in this strain. Complementation with a functional copy of *yscB* in *trans* completely restored the wild-type secretion phenotype for YopM, YopN, and V antigen. The YscB amino acid sequence showed significant similarities to those of SycE and SycH, the specific Yop chaperones for YopE and YopH, respectively. Protein cross-linking and immunoprecipitation studies demonstrated a specific interaction between YscB and YopN. In-frame deletions in *yopN* eliminating the coding region for amino acids 51 to 85 or 6 to 100 prevented the interaction of YopN with YscB. Taken together, these results indicate that YscB functions as a specific chaperone for YopN in *Y. pestis*.

Yersinia species pathogenic for humans (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) interact directly with the surfaces of eucaryotic cells and translocate a distinct set of virulence proteins (YopE, YopH, YopJ, YopM, and YpkA) from the cytoplasm of the bacterium into the cytoplasm of the eucaryotic cell (11, 12). Once translocated into the eucaryotic cell, Yop proteins disrupt intracellular signaling pathways (YopH [8, 15, 40, 41] and YpkA [25]), prevent specific cytoskeletal rearrangements (YopE [50]), induce apoptotic events in macrophages (YopJ [35, 36, 52]), and eventually kill the target cell. This capability enables the yersiniae to avoid phagocytosis and ensures survival of the bacteria within host tissues (14, 18, 48, 49).

Yops are secreted by a type III, or "contact-dependent," secretion mechanism (12, 33, 61). The secretion of Yops is not a constitutive process; instead, Yop secretion occurs in response to specific signals associated with contacting a eucaryotic target cell (18, 51). Several of the secreted proteins (YopB, YopD, YopK, LcrG, TyeA, and YopN) are not translocated into the eucaryotic cell but function either to regulate Yop export or to target Yops with direct antihost functions into the eucaryotic cell. The secreted LcrG, TyeA, and YopN proteins are thought to function in gating the Yop secretion channel and are required to prevent Yop secretion in the presence of calcium prior to contact with a eucaryotic target cell (19, 28, 56, 63). The secreted V antigen interacts directly with LcrG within the cell and may be required to counteract LcrG's role in blocking Yop secretion (38). In addition, LcrV is specifically required for the secretion of YopB and YopD (53). YopB and YopD are required for translocation of Yops across the eucaryotic cell membrane (9, 26). YopB induces the formation of pores in eukaryotic cell membranes (26), while YopK has been shown to be involved in regulating the translocation of Yops through this channel (27). Recently, Iriarte et al. demonstrated that TyeA was specifically required for the translocation of YopE and YopH (28).

Yersinia species growing in vitro at 37°C in calcium-depleted medium express and secrete large amounts of Yops into the surrounding media. This process requires gene products from the *yscBCDEFGHIJKL* operon (4, 24, 32, 33, 44) and the *yscNOPQRSTU* operon (2, 7, 17, 68), a lipoprotein termed VirG (3), and the *lcrD* gene product (42, 43). In the presence of calcium, virulence plasmid-encoded operons are transcriptionally downregulated (23, 59, 60) and Yop secretion is blocked (11, 33, 61). The downregulation of virulence plasmid operon transcription that occurs in conjunction with the block in Yop secretion requires the participation of YopD (67), LcrH (also called SycD [45, 64]), and LcrQ (also called YscM [47, 58]). Together, these gene products control the production and delivery of Yop proteins in response to specific triggering signals associated either with growth at 37°C in the absence of calcium in vitro or with contact with a eucaryotic cell in vivo.

The sequence elements responsible for targeting YopE and YopH for either secretion into the medium in vitro or translocation into a eucaryotic cell in vivo have been localized to amino-terminal regions of these proteins (54, 57). Sequences encoding the amino-terminal 15 to 17 amino acids of YopE (54, 57, 69), YopN (5), and YopH (57, 69) were shown to be sufficient to direct the export of hybrid reporter proteins; however, translocation of either YopE or YopH hybrid proteins into a eucaryotic cell required a larger amino-terminal region that contained the binding site for a chaperone-like accessory protein termed a specific Yop chaperone (Syc) (65). Recently, Cheng et al. (10) demonstrated that YopE of *Y. enterocolitica* possesses two independent secretion mechanisms, each of which is sufficient but not required for the secretion of YopE

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Construction and properties[b]	Source or reference
E. coli strains		
DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80*dlac*Δ(*lacZ*)M15]	GIBCO-BRL
SY327 (λ*pir*)	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56*	34
XL1-Blue	*end*Δ*1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96* (Nal^r) *relA1* (Δ*lac*) [F′ *proAB*$^+$ *lacI*^{q}*Z*ΔM15::Tn*10* (Tc^r)]	Stratagene
Y. pestis strains[a]		
KIM8	pCD1 pPCP1$^-$ (Pla$^-$) pMT1	23
KIM10	pCD1$^-$ (Lcr$^-$) pPCP1$^-$ (Pla$^-$) pMT1	23
KIM5-3001	Sm^r pCD1 (Lcr$^+$) pPCP1 pMT1	30
KIM5-3001.5	Sm^r pCD1 (Δ*lcrG* [aa 39–53]) pPCP1 pMT1	56
KIM5-3001.6	Sm^r pCD1 (Δ*yopN* [aa 48–197]) pPCP1 pMT1	44
KIM5-3001.P1	Sm^r pCD1 (Δ*yscB* [aa 61–125]) pPCP1 pMT1	This study
KIM8-3002	Sm^r pCD1 (Lcr$^+$) pPCP1$^-$ (Pla$^-$) pMT1	S. C. Straley
KIM8-3002.P2	Sm^r pCD1 (Δ*yopN* [aa 2–10]) pPCP1$^-$ (Pla$^-$) pMT1	This study
KIM8-3002.P3	Sm^r pCD1 (Δ*yopN* [aa 6–25]) pPCP1$^-$ (Pla$^-$) pMT1	This study
KIM8-3002.P4	Sm^r pCD1 (Δ*yopN* [aa 21–40]) pPCP1$^-$ (Pla$^-$) pMT1	This study
KIM8-3002.P5	Sm^r pCD1 (Δ*yopN* [aa 51–85]) pPCP1$^-$ (Pla$^-$) pMT1	This study
KIM8-3002.P6	Sm^r pCD1 (Δ*yopN* [aa 6–100]) pPCP1$^-$ (Pla$^-$) pMT1	This study
KIM8-3002.P7	Sm^r pCD1 (Δ*yopN* [aa 48–197]) pPCP1$^-$ (Pla$^-$) pMT1	This study
Plasmids		
pBluescript II SK(−)	Cloning vector; Ap^r	Stratagene
pBCKS$^-$	Cloning vector; Cm^r	Stratagene
pUK4134	Suicide vector; Ap^r	55
pTRC99a	Expression vector; Ap^r	Pharmacia
pYSCB1	1.7-kb *Xho*I-*Bam*HI fragment of pPH11 (25) cloned into pBluescriptII SK(−)[c]; Ap^r	This study
pΔYSCB1	pYSCB1 digested with *Hpa*I and religated with insertion of an 8-bp *Pst*I linker (5′-GCTGCAGC-3′), resulting in the elimination of a 194-bp Hpa fragment within *yscB*	This study
pUK4134.P1	2.15-kb *Pvu*II fragment of pΔYSCB1 carrying Δ*yscB* (aa 61–125) cloned into the unique *Eco*RV site of pUK4134; Ap^r	This study
pYOPN1	986-bp *Cla*I-*Eco*47III fragment of pGP2 (42) cloned into *Cla*I-*Eco*RV-digested pBCKSII$^-$; Cm^r	This study
pYOPN2	2.1-kb *Bam*HI-*Bst*BI fragment of pGP2 (42) cloned into *Bam*HI-*Cla*I-digested pBluescript SK(−); Ap^r	This study
pUK4134.6	2.2-kb *Eco*RV fragment of pΔYopN.1 carrying Δ*yopN* (aa 48–197) cloned into pUK4134; Ap^r	44
pYSCB6×HIS	440-bp PCR fragment generated with primers YscB1 and YscB2, digested with *Nco*I and *Bam*HI, and cloned into *Nco*I-*Bam*HI-digested pTRC99a	This study

[a] All *Y. pestis* strains are Pgm$^-$ (62). Native plasmids of *Y. pestis* include the LCR plasmid pCD1 (6, 16, 23), the Pla-encoding pPCP1 (59), and pMT1 (46), which encodes the capsular protein.
[b] Numbers in brackets give the amino acids (aa) deleted from the protein.
[c] See Fig. 1 for restriction sites at ends of cloned DNA.

hybrid proteins. One secretion signal is found within the sequence encoding the first 15 amino acids of YopE, and the second is located downstream, between residues 15 and 100. The function of the second secretion signal is dependent upon a functional SycE protein, the specific Yop chaperone for YopE (65). Most recently, Anderson and Schneewind (5) provided evidence that the secretion signal targeting YopE hybrids containing the coding region for only the amino-terminal 15 amino acids of YopE appears to be encoded in the mRNA sequence rather than the peptide sequence, thus suggesting a cotranslational mechanism for Yop secretion.

In addition to the SycE protein, specific Yop chaperones for YopH (SycH [64, 69]) and YopD (SycD [64]) have also been identified and characterized. Syc-like proteins have also been identified in other bacteria equipped with type III secretion pathways (20, 22, 64). Efficient secretion of the protein recognized by the Syc or Syc-like protein is dependent upon the chaperone and the binding site for the chaperone on the secreted protein. The binding site for SycE and SycH has been localized to within amino acids 15 to 70 of YopE and YopH (57, 69), respectively. The presence of SycE has been shown to protect YopE from proteolytic degradation within the bacterial cell; thus, the Syc chaperones may also have a protective function in addition to their specific role in Yop secretion (21).

The present report investigates the role of the *yscB* gene product (24, 32) in Yop secretion and in the regulation of Yop secretion in *Y. pestis*. We constructed and characterized a *Y. pestis* strain carrying a nonpolar deletion in *yscB*. Our data indicate that YscB, like YopN, TyeA, and LcrG, is required to prevent Yop secretion prior to reception of the proper secretion-triggering signal. Specifically, YscB appears to function as a specific chaperone for YopN in *Y. pestis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1 and Fig. 1. *Y. pestis* strains were routinely grown in heart infusion broth or on tryptose blood agar base plates (Difco Laboratories, Detroit, Mich.). For growth curves, *Y. pestis* strains were cultured in the defined liquid medium TMH (23). In this medium, low-Ca^{2+} response (LCR) yersiniae require added Ca^{2+} for full growth yield at 37°C. *Escherichia coli* strains were grown in L broth or on L agar. Bacteria with resistance to antibiotics were grown in the presence of the appropriate antibiotic(s) at a final concentration of 25 μg/ml (chloramphenicol and kanamycin) or 50 μg/ml (ampicillin and streptomycin).

DNA methods. Cloning methods, including the use of restriction endonucleases and T4 DNA ligase, were performed as described by Maniatis et al. (31) with modifications as noted. Plasmid DNA was isolated by the method of Kado and Liu (29), by the PERFECT Prep method (5 Prime→3 Prime, Inc., Boulder, Colo.) or with Qiagen (Chatsworth, Calif.) columns. DNA fragments were isolated and purified from agarose gels by using the Qiaex DNA purification kit (Qiagen). Electroporation of *E. coli* and *Y. pestis* was carried out as previously described (43). PCR (by the technique described in reference 37) was performed by using 21- to 48-nucleotide primers and 30 cycles of amplification. Unless

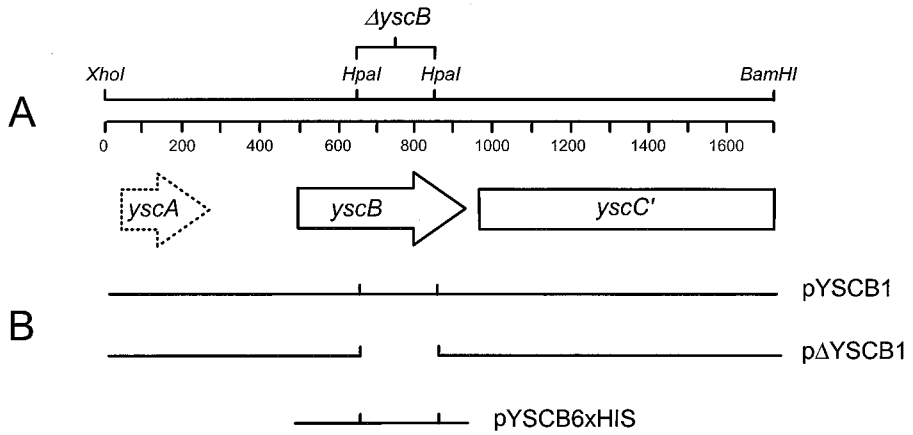

FIG. 1. Physical and genetic maps of the *yscBC′* region of the *Y. pestis* plasmid pCD1. The approximately 1.7-kb *Xho*I-*Bam*HI fragment contains the *yscB* gene and a portion of the upstream *yscC* gene. In *Y. enterocolitica* an additional putative gene, designated *yscA*, was identified upstream of YscB. *Hpa*I restriction endonuclease sites were utilized to create an in-frame deletion in *yscB*. Plasmids pYSCB1, pΔYSCB1, and pYSCB6×HIS were used in complementation studies.

stated otherwise, denaturing, annealing, and extending conditions were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, respectively. Double-stranded DNA was sequenced by the University of Miami School of Medicine DNA sequencing core facility by using a DyeDeoxy Terminator Cycle Sequencing kit and an ABI model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Nucleotide sequences were analyzed with Intelligenetics computer software (IntelliGenetics, Mountain View, Calif.).

Construction of a *yscB* deletion mutant. A YscB-encoding 1.66-kb *Xho*I-*Bam*HI restriction endonuclease fragment of plasmid pPH11 (24) was inserted into *Xho*I-*Bam*HI-digested pBluescript SKII(−) (Stratagene, La Jolla, Calif.) downstream of the vector *lac* promoter, generating plasmid pYSCB1 (Fig. 1). Plasmid pΔYSCB1, carrying a 195-bp in-frame deletion in *yscB*, was constructed by *Hpa*I restriction endonuclease digestion and insertion of an 8-bp *Pst*I linker (New England Biolabs, Beverly, Mass.) to maintain the *yscB* reading frame (Table 1). pBluescript SKII(−) *Pvu*II restriction endonuclease sites flanking the Δ*yscB*-encoding *Xho*I-*Bam*HI fragment were utilized to insert the fragment into the *Eco*RV site of the suicide vector pUK4134, generating plasmid pUK4134.GP1 (Table 1). This plasmid was introduced into *Y. pestis* KIM5-3001 by electroporation, and recipient bacteria that had integrated the clone into pCD1 by homologous recombination were selected for by their resistance to ampicillin, as previously described (55). Following passage under nonselective conditions to allow a second crossover, clones which had resolved the cointegrate by excision of the vector sequences were selected for by their resistance to streptomycin. Streptomycin-resistant resolvants were screened for the correct deletion by PCR and restriction endonuclease digestion. *Y. pestis* KIM5-3001.P1 contained the correct in-frame deletion within pCD1 (Table 1) and was utilized for further study.

Construction of *yopN* deletion mutants. Deletions of *yopN* sequences encoding amino acid residues 2 to 10, 6 to 25, 21 to 40, 51 to 85, and 6 to 100 were constructed by the PCR-ligation-PCR technique (1). Initial PCR amplifications (set A) paired the oligonucleotide primer YopNH1 (5′-CTGGGACAAGCTTATA TTGG-3′) with 2-10A (5′-AATACCCCGCTGCATAAT-3′), 6-25A (5′-ATCG TAAATCAGACTCTG-3′), 21-40A (5′-ATAGTCAGCGGCACTCTG-3′), 51-85A (5′-CAGGTTAATCAATACCTT-3′), and 6-100A (5′-CAGAATGTGAG TGAGCTG-3′). A second set (set B) of PCRs paired the vector T3 primer (5′-AATTAACCCTCACTAAAGGG-3′) with 2-10B (5′-CATAACTACTCCCTG AGA-3′), 6-25B (5′-ATGAAGCGTCGTCATAAC-3′), 21-40B (5′-CTCTGGA CGCTCATTATG-3′), 51-85B (5′-AGCTATAGACTGCAGAGT-3′), and 6-100B (5′-ATGAAGCGTCGTCATAAC-3′). The resultant amplification products were purified from agarose gels and phosphorylated by using T4 polynucleotide kinase, and the respective set A and set B amplification products were ligated together by using T4 DNA ligase (New England Biolabs). Ligated fragments were subsequently used as a template for a second amplification reaction using only the outside primers YopNH1 and T3. The resultant amplification products, which contained defined in-frame deletions within *yopN*, were purified from agarose gels, digested with *Apa*I and *Hin*dIII, and inserted into *Apa*I-*Hin*dIII-digested pYOPN1, creating plasmids pYOPN1.2 (with amino acids 2 to 10 deleted [Δ2-10]), pYOPN1.3 (Δ6-25), pYOPN1.4 (Δ21-40), pYOPN1.5 (Δ51-85) and pYOPN1.6 (Δ6-100). To increase the amount of homologous flanking DNA surrounding each deletion, a *Cla*I-*Bgl*II fragment of each plasmid was cloned into *Cla*I-*Bgl*II-cut pYOPN2 (Table 1), resulting in plasmids pYOPN2.2 (Δ2-10), pYOPN2.3 (Δ6-25), pYOPN2.4 (Δ21-40), pYOPN2.5 (Δ51-85), and pYOPN2.6 (Δ6-100), which provided a minimum of 650 to 1,100 bp of flanking DNA on either side of the deletions. *Pvu*II fragments containing the deletion and flanking DNA were then inserted into *Eco*RV-digested pUK4134. The resulting suicide plasmids pUK4134.P2 (Δ2-10), pUK4134.P3 (Δ6-25), pUK4134.P4 (Δ21-40), pUK4134.P5 (Δ51-85), pUK4134.P6 (Δ6-100), and pUK4134.P7 (Δ48-197) were utilized to move the *yopN* deletions into plasmid pCD1 of *Y. pestis* KIM8-3002 essentially as described for the *yscB* deletion mutant. The resultant *yopN* deletion mutants KIM8-3002.P2 (Δ2-10), KIM8-3002.P3 (Δ6-25), KIM8-3002.P4 (Δ21-40), KIM8-3002.P5 (Δ51-85), KIM8-3002.P6 (Δ6-100), and KIM8-3002.P7 (Δ48-197) were used in protein cross-linking and immunoprecipitation procedures.

Cell fractionation. *Yersinia* strains were grown at 26°C in TMH with or without calcium (2.5 mM $CaCl_2$) to an optical density at 620 nm of 0.15 to 0.20. Cultures were then shifted to 37°C for an additional 5 h. Cell pellets and culture supernatants were separated by centrifugation at 12,200 × *g* for 10 min at 4°C. Cell pellets were washed once with ice-cold buffer A (20 mM Tris-HCl, pH 8.0) and pelleted by centrifugation. Culture supernatant proteins were precipitated with 10% (vol/vol) trichloroacetic acid (TCA) (for 1 h, on ice) and collected by centrifugation at 12,200 × *g* for 10 min at 4°C. Periplasmic fractions were prepared by the osmotic shock procedure as described by Nossal and Heppel (39). Briefly, cell pellets (0.1 g [wet weight]) were resuspended in 4 ml of 33 mM Tris-HCl, pH 7.1, containing 20% (wt/vol) sucrose, and EDTA was added to a 0.1 mM final concentration. After being stirred for 10 min at room temperature (RT), cells were collected by centrifugation at 12,200 × *g* for 10 min at 4°C, and the cell pellet was rapidly dispersed in 8 ml of ice-cold 0.5 mM $MgCl_2$. Following osmotic shock the cell pellets were collected by centrifugation at 12,200 × *g* for 10 min at 4°C, and the supernatants containing the osmotic shock proteins were TCA precipitated. To obtain membrane and cytoplasmic (soluble) fractions, cell pellets were resuspended in buffer A and lysed by a single passage through a chilled French pressure cell at 20,000 lb/in^2. Unlysed whole cells and large debris were removed by centrifugation at 8,800 × *g* for 5 min at 4°C. Total membranes were separated from the soluble fractions of the cleared lysates by ultracentrifugation in a TLA-100.3 rotor at 263,800 × *g* for 20 min at 4°C (Beckman Instruments, Fullerton, Calif.). Membrane preparations were resuspended and stored in buffer A.

YscB overexpression, purification, and anti-YscB antibody preparation. A nucleotide sequence encoding six consecutive histidine residues was added to the portion of *yscB* encoding carboxyl-terminal domain by using PCR methodologies and primers YscB1 (5′-ATATACCATGGAAAATTTACTAAAAAACTTGGC AGCC-3′) and YscB2 (5′-CGGGATCCTTAATGGTGATGGTGATGGTGAT TCCACCCCACGCGAGAC-3′). The resultant PCR product was digested with *Nco*I and *Bam*HI restriction endonucleases and ligated into *Nco*I/*Bam*HI-digested pTrc99a (Pharmacia, Piscataway, N.J.), generating the expression plasmid pYSCB6×HIS (Table 1). Plasmid pYSCB6×HIS was electroporated into *E. coli* DH5α for high-level expression of polyhistidine-tagged YscB (YscB6×HIS). The resultant strain was grown at 37°C in Luria-Bertani medium to an optical density at 620 nm of 0.5, and expression of YscB6×HIS was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.5 mM followed by incubation at 37°C for an additional 4 h. Cultures were harvested by centrifugation at 12,200 × *g* for 10 min at 4°C. Cell pellets were resuspended in 1× binding buffer (Novagen, Inc., Madison, Wis.) and lysed by two passages through a chilled French pressure cell at 20,000 lb/in^2. Unlysed cells, large debris, and inclusion bodies were removed by centrifugation at 8,800 × *g* for 5 min at 4°C. The YscB6×HIS protein was found almost exclusively in the 8,800 × *g* pellet, indicating that the protein was primarily present in inclusion bodies. Pellet fractions containing YscB6×HIS were solubilized with 1× binding buffer containing 6 M urea, insoluble material was removed by ultracentrifugation at 263,800 × *g* for 20 min at 4°C, and the YscB6×HIS protein was purified to homogeneity using the

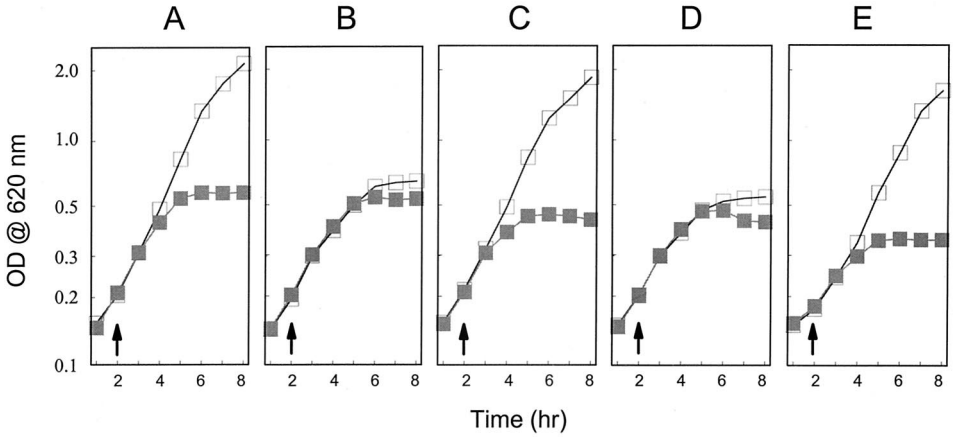

FIG. 2. Growth of the parent strain *Y. pestis* KIM5-3001 (A), the *yscB* deletion strain KIM5-3001.P1 (B), and KIM5-3001.P1 complemented with plasmid pYSCB1 (C), pΔYSCB1 (D), or pYSCB6×HIS (E). *Y. pestis* strains were grown in the presence (open squares) or absence (shaded squares) of calcium in the defined medium TMH. The temperature was shifted from 26 to 37°C when the optical density at 620 nm of the culture reached approximately 0.2 (arrows).

His-Bind Resin and Buffer kit (Novagen). Polyclonal antisera specific for YscB were raised in female New Zealand White rabbits by using the purified YscB6×HIS protein (Animal Pharm Services, Healdsburg, Calif.). Polyclonal antisera specific for YscB were used in immunoprecipitations and to detect YscB in immunoblot analyses at a dilution of 1:10,000.

SDS-PAGE and immunoblotting. Volumes of cellular fractions corresponding to equal numbers of bacteria were mixed 1:1 (vol/vol) with 2× electrophoresis sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis essentially as previously described (43). Those samples to be analyzed with polyclonal antisera specific for YscB were electrophoresed on 14% (wt/vol) acrylamide gels. *Y. pestis* LCR proteins were visualized as previously described (43), by using polyclonal antisera or purified antibodies specific for YopM, V antigen, YopN, YopE, or YscB.

Cross-linking and immunoprecipitation of YscB and YopN. Cultures grown in 25 ml of TMH with or without calcium were harvested by centrifugation at 12,200 × *g* for 10 min at 4°C, washed once with 20 mM HEPES–100 mM NaCl (pH 7.4), resuspended in 3 ml of immunoprecipitation buffer A (20 mM HEPES, 100 mM NaCl, 0.1% Triton X-100 [pH 7.4]), and lysed by two passages through a chilled French pressure cell at 20,000 lb/in^2. Unlysed cells, large debris, and membranes were removed by ultracentrifugation in a TLA-100.3 rotor at 263,800 × *g* for 20 min at 4°C (Beckman Instruments). Soluble proteins (500 μl) were cross-linked with the thio-cleavable amine reactive cross-linker dithiobis (succinimidyl propionate) (DSP) (Pierce Chemical Co., Rockford, Ill.) at a final concentration of 1 mM (20 mM stock solution in dimethyl sulfoxide) for 20 min at RT. Cross-linking reactions were quenched by addition of Tris-HCl (pH 8.0) to a final concentration of 50 mM. Samples were immunoprecipitated with 10 μl of rabbit polyclonal antisera specific for YopN, YscB, or YopE or with preimmune serum from the corresponding rabbit for 2 h at 4°C. Antigen-antibody complexes were collected by addition of 100 μl of 10% (wt/vol) protein A-Sepharose CL-4B (Pharmacia) in immunoprecipitation buffer B (20 mM HEPES, 250 mM NaCl, 0.5% Triton X-100, 0.1% SDS [pH 7.4]) for 2 h at 4°C. Protein A-Sepharose antigen-antibody complexes were pelleted by centrifugation in a microcentrifuge for 15 s at RT, washed three times with 1 ml of immunoprecipitation buffer B, eluted with 100 μl of electrophoresis sample buffer containing 5% β-mercaptoethanol (cleaves DSP cross-links), boiled for 3 min, and subjected to SDS-PAGE and immunoblot analysis.

RESULTS

Construction of a nonpolar *yscB* deletion mutant. To investigate the role of YscB in the expression and delivery of plasmid pCD1-encoded virulence factors, we constructed a 195-bp in-frame internal deletion within *yscB* that was designed to specifically disrupt the structure of the *yscB* gene product without having polar effects on the expression of downstream genes (Fig. 1). The mutation was constructed in plasmid pΔYSCB1 (Fig. 1 and Table 1) and introduced into pCD1 by allelic exchange (55). The resultant strain, KIM5-3001.P1 (Table 1), should express a *yscB* gene product lacking amino acids 61 to 125, but containing three additional alanine residues (amino acid residues 61 to 63 of the deleted gene product) due to the insertion of an 8-bp *Pst*I linker. Plasmids pYSCB1, pΔYSCB1, and pYSCB6×HIS were used in complementation studies.

Growth phenotype of the *yscB* deletion mutant. Figure 2 shows growth curves for the parent strain *Y. pestis* KIM5-3001, the *yscB* deletion mutant *Y. pestis* KIM5-3001.P1, and the *yscB* deletion mutant complemented with plasmid pYSCB1, pΔYSCB1, or pYSCB6×HIS. All strains exhibited full growth yield at 26°C in the presence or absence of calcium (data not shown). The parent strain KIM5-3001 showed a calcium-dependent growth pattern typical for the TMH growth medium (23). In contrast, the *yscB* deletion strain KIM5-3001.P1 underwent growth restriction at 37°C both in the presence and in the absence of calcium (calcium-blind or temperature-sensitive growth phenotype [23]). These data suggest that the wild-type LCR growth phenotype is dependent upon a functional *yscB* gene product. Providing plasmid pYSCB1 (*yscBC′*) or pYSCB6× HIS (*yscB*) in *trans* resulted in complete restoration of the wild-type growth phenotype, while providing plasmid pΔYSCB1 (*yscΔBC′*) in *trans* had no effect on the growth phenotype. These results indicate that the growth defects associated with the *yscB* deletion strain were due solely to disruption of *yscB* and not to polar effects on downstream genes or to spontaneous mutations in other *ysc* or *lcr* loci.

Secretion of YopM, YopN, and V antigen by the *yscB* deletion mutant. Secretion of YopM, YopN, and V antigen was analyzed by immunoblot analysis of TCA-precipitated culture supernatant proteins from the parent strain, *Y. pestis* KIM5-3001, the *yscB* deletion mutant *Y. pestis* KIM5-3001.P1, and the *yscB* deletion mutant complemented with pYSCB1, pΔYSCB1, or pYSCB6×HIS in *trans*. *Y. pestis* strains were grown in TMH with or without calcium at 26°C and then shifted to 37°C for 5 h. The parent strain, KIM5-3001, secreted YopM, YopN, and V antigen into the culture supernatant when grown at 37°C in the absence of calcium (Fig. 3); however, as expected, no secretion of Yops or V antigen was detected in the presence of calcium. The *yscB* deletion mutant grown at 37°C in the presence or absence of calcium secreted YopM and V antigen at levels comparable to those of the parent strain grown at 37°C in the absence of calcium. These results demonstrate that secretion of Yops and V antigen by the *yscB* deletion mutant was no longer blocked in the presence of calcium (calcium-blind secretion phenotype). Interestingly, secretion of YopN by the *yscB* deletion mutant grown at 37°C in the presence or absence of calcium was specifically reduced compared to that by the

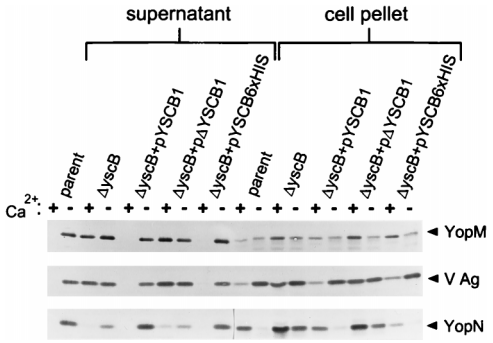

FIG. 3. Immunoblot analysis of YopM, V antigen, and YopN in the supernatant and cell pellet fractions from *Y. pestis* KIM5-3001 (parent), the *yscB* deletion strain KIM5-3001.P1, (Δ*yscB*) and KIM5-3001.P1 complemented with plasmid pYSCB1, pΔYSCB1, or pYSCB6×HIS. Strains were grown in either the presence (+) or in the absence (−) of calcium at 37°C in the defined medium TMH. Polyclonal antisera specific for YopM, V antigen, and YopN were used to detect the presence of these proteins in the supernatant and cell pellet fractions.

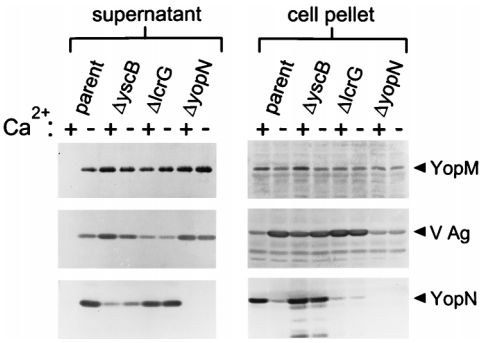

FIG. 4. Immunoblot analysis of YopM, V antigen, and YopN in the supernatant and cell pellet fractions from *Y. pestis* KIM5-3001 (parent), the *yscB* deletion strain KIM5-3001.P1 (Δ*yscB*), the *lcrG* deletion strain KIM5-3001.5 (Δ*lcrG*), and the *yopN* deletion strain KIM5-3001.6 (Δ*yopN*). Strains were grown in either the presence (+) or in the absence (−) of calcium at 37°C in the defined medium TMH. Polyclonal antisera specific for YopM, V antigen, and YopN were used to detect the presence of these proteins in the supernatant and cell pellet fractions.

parent strain grown in the absence of calcium. In contrast, the amount of YopN found associated with the cell pellet was increased in the *yscB* deletion mutant, indicating that the reduction in YopN found in the culture supernatant was most likely due to a reduction in YopN export and not to a defect in YopN expression.

Providing the *yscB*-complementing plasmid pYSCB1 or pYSCB6×HIS in *trans* to the *yscB* deletion mutant completely restored calcium-regulated expression and secretion of YopM, YopN, and V antigen; however, plasmid pΔYSCB1, which does not express a functional *yscB* gene product, failed to complement the expression and secretion defects associated with this strain. These data indicate that the reduction in YopN secretion, as well as the defects in the regulation of Yop and V antigen secretion, was due solely to disruption of *yscB* and not to polar effects on downstream genes or to spontaneous mutations in other *ysc* or *lcr* loci. Furthermore, the ability of pYSCB6×HIS to complement the *yscB* deletion mutant indicates that the addition of six histidine residues to the carboxyl terminus of YscB does not affect its ability to complement YscB function.

Secretion of YopM, YopN, and V antigen by the *yscB* deletion mutant and other mutants defective in the regulation of Yop secretion. To determine whether the reduction of YopN secretion was a unique property of the *yscB* deletion mutant or a property common to mutants defective in calcium-regulated Yop secretion, the secretion of YopM, YopN, and V antigen was analyzed in the parent strain (KIM5-3001) and in the *yscB* (KIM5-3001.P1), *lcrG* (KIM5-3001.5), and *yopN* (KIM5-3001.6) deletion mutants (Fig. 4). The parent strain secreted YopM, YopN, and V antigen only in the absence of calcium; however, as expected, the three calcium-blind mutants secreted these proteins both in the presence and in the absence of calcium. The *yscB* deletion mutant secreted reduced levels of YopN in comparison to the parent strain and the *lcrG* deletion mutant, while the *yopN* deletion mutant failed to express a stable *yopN* gene product. The amount of YopN found in the cell pellet of the *yscB* deletion mutant was similar to the amount found in that of the parent strain grown under conditions in which Yop secretion is blocked (at 37°C in the presence of calcium). These data demonstrate that YscB was specifically required for the efficient secretion of YopN in *Y. pestis*. In addition, YscB was required directly or indirectly to prevent Yop secretion in the presence of calcium.

Identification and localization of the *yscB* gene product. Polyclonal antiserum raised against a polyhistidine-tagged *yscB* gene product was utilized to identify YscB in immunoblots prepared from fractionated *Y. pestis* cultures. Cytoplasmic, membrane, periplasmic, and culture supernatant fractions prepared from both the parent strain and the *yscB* deletion mutant were analyzed for the presence of YscB (Fig. 5). The approximately 15.5-kDa *yscB* gene product localized equally in the cytoplasmic and membrane fractions of the parent strain; however, no YscB was found in the periplasmic fraction or the culture supernatant fraction of the parent strain or in any fraction of the *yscB* deletion mutant. These results, in conjunction with the lack of a classical Sec-dependent secretion signal and no predicted transmembrane domains, indicate that YscB is a cytoplasmic or peripheral membrane protein that is not exported across the inner membrane.

YscB shows extensive similarities with PscB of *Pseudomonas aeruginosa* and SycE and SycH of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*. The specific effect of the *yscB* deletion on the secretion of YopN was similar to effects reported for *sycE* (*yerA*), *sycH*, and *sycD* mutant strains on their cognate Yops. Therefore, we attempted to align the amino acid sequence of YscB with those of the other reported Syc and Syc-like proteins (Fig. 6A). YscB showed extensive similarities with SycE and SycH, the specific Yop chaperones for YopE and YopH, respectively, of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*. The similarity to SycH was highest in the amino-terminal region (33% identity in region A; Fig. 6A),

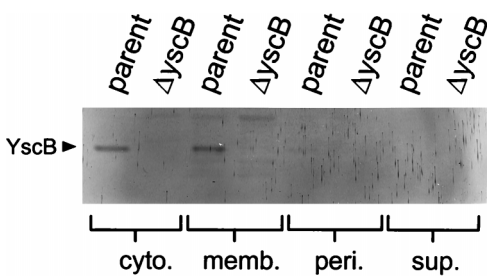

FIG. 5. Cellular localization of YscB. Fractionation was performed on *Y. pestis* KIM5-3001 (parent) and the *yscB* deletion strain KIM5-3001.P1 (Δ*yscB*), which had been grown for 5 h at 37°C in the absence of calcium. Cytoplasmic (cyto.), membrane (memb.), periplasmic (peri.), and culture supernatant (sup.) fractions were analyzed by SDS-PAGE and immunoblot analysis.

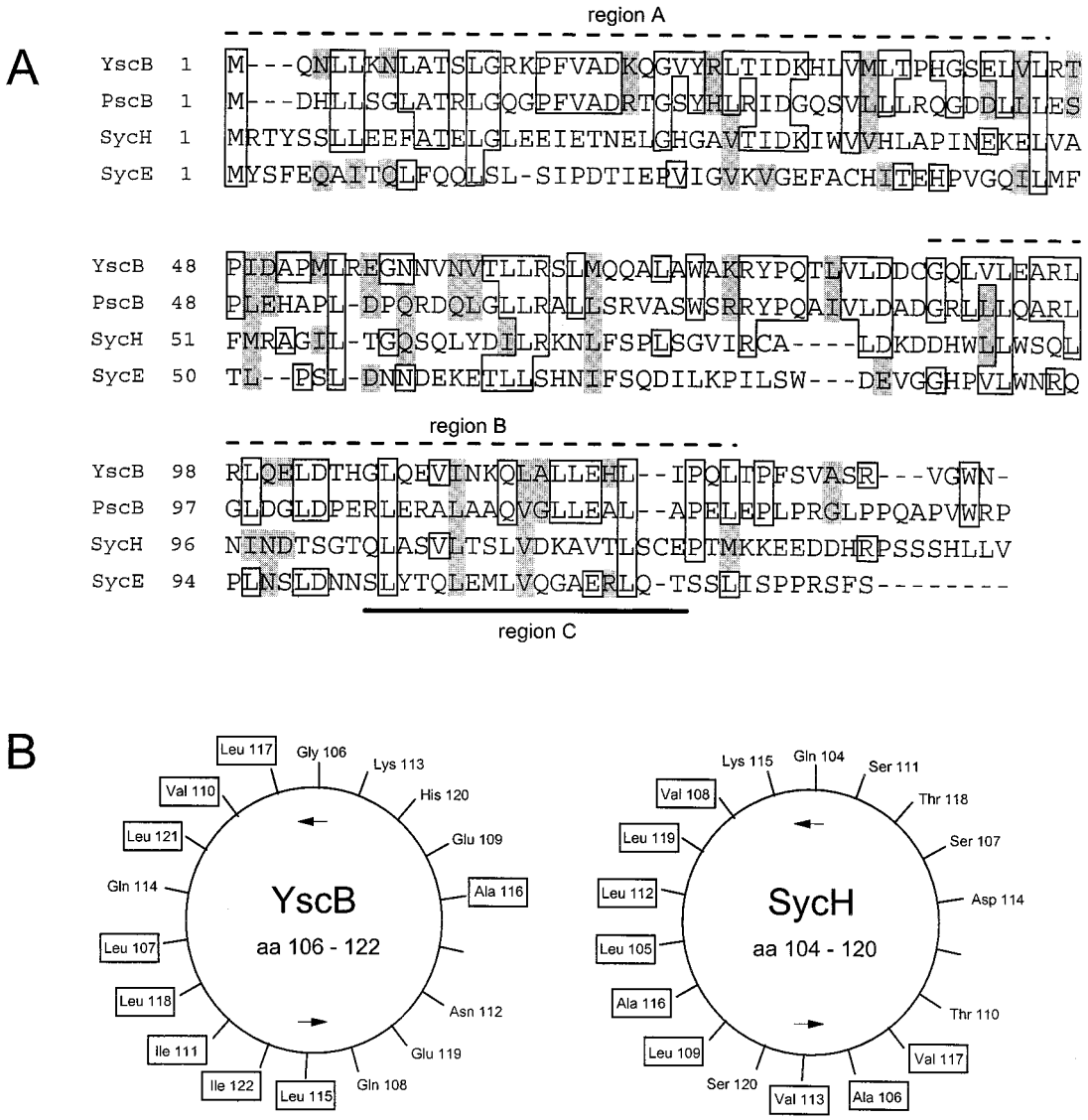

FIG. 6. (A) Alignment of *Y. pestis* YscB with PscB of *P. aeruginosa*, SycH of *Y. pseudotuberculosis*, and SycE of *Y. pestis*. Boxed residues, sequence identity; shaded residues, related or similar amino acids residues; overlined sequences (dashed lines), regions of YscB with the highest (≥30%) identity to SycH (region A) and SycE (region B); underlined sequence, region predicted to form an amphipathic helix (region C); dashes within sequences, gaps introduced to optimize alignment. (B) Helical wheel representation of region C of YscB and SycH. Hydrophobic amino acid residues are boxed.

while the greatest similarity to SycE was in the carboxyl-terminal region (30% identity in region B). YscB showed the greatest overall similarity to PscB of *P. aeruginosa* (41% identity), whose gene is closely linked to gene products which are involved in the secretion of exoenzyme S through a recently discovered type III secretion system (20, 70). Interestingly, a homolog of YopN has also been identified in *P. aeruginosa* (PopN) and is believed to be associated with the same secretion pathway (20).

YscB also shares many of the common structural and functional features of the Syc and Syc-like family of proteins. Like the other Syc and Syc-like proteins YscB is a small (15.5-kDa) cytoplasmic (or peripheral) protein with a conserved carboxyl-terminal leucine repeat motif and a putative amphipathic alpha-helix (Fig. 6B); however, unlike the previously characterized Syc and Syc-like proteins, YscB is a basic protein (pI 9.3). These data, in conjunction with the unique effect of the *yscB* deletion on the export of YopN, indicate that YscB may function as a specific chaperone for YopN in *Y. pestis*.

Interaction of YscB and YopN. In order to confirm the role of YscB as a chaperone for YopN, we attempted to identify an interaction between YscB and YopN using protein cross-linking reagents and immunoprecipitation. Soluble proteins (cytoplasmic and periplasmic proteins) from the parent strain, *Y. pestis* KIM5-3001, the *yscB* deletion mutant (KIM5-3001.P1), and the *yopN* deletion mutant (KIM5-3001.6) grown at 37°C in the presence or absence of calcium were cross-linked by the addition of the thio-cleavable amine reactive cross-linker DSP to a final concentration of 1 mM for 30 min at RT. Polyclonal antisera specific for YopN, YscB, or YopE were used to immunoprecipitate the corresponding protein and proteins cross-linked with the immunoprecipitated protein (coprecipitating proteins). Cross-linked immunoprecipitates were collected by using protein A-sepharose CL-4B, washed, cleaved by the ad-

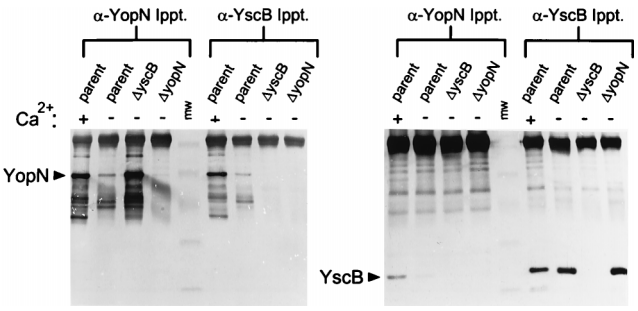

FIG. 7. Coimmunoprecipitation of YopN and YscB following cross-linking with DSP. Soluble extracts from the parent strain, *Y. pestis* KIM5-3001, grown in the presence (+) or absence (−) of calcium, the *yscB* deletion strain KIM5-3001.P1 (Δ*yscB*) grown in the absence of calcium, and the *yopN* deletion strain KIM5-3001.6 (Δ*yopN*) grown in the absence of calcium were cross-linked with DSP (1 mM) for 20 min at RT. Samples were processed as described in Materials and Methods, immunoprecipitated with antisera specific for YopN or YscB, and analyzed by SDS-PAGE and immunoblotting with antisera specific for YopN or YscB. DSP cross-links were broken prior to SDS-PAGE analysis by boiling samples in the presence of 5% β-mercaptoethanol. The locations of YopN and YscB are shown by arrows. MW, molecular size standards (45, 32, 25, 16, and 6.5 kDa). Ippt., immunoprecipitation.

dition of solubilization buffer containing 5% β-mercaptoethanol, and analyzed by SDS-PAGE and immunoblot analysis (Fig. 7).

Antisera specific for YopN immunoprecipitated YopN from the parent strain and the *yscB* deletion strain (Fig. 7). The amount of YopN immunoprecipitated from the soluble fraction of the parent strain grown in the absence of calcium was significantly reduced due to the efficient export of YopN under these conditions. Antisera specific for YscB immunoprecipitated approximately equal amounts of YscB from both the parent strain and the *yopN* deletion strain grown either in the presence or in the absence of calcium. YscB coprecipitated with the immunoprecipitated YopN in the parent strain; however, no YscB was found in the corresponding immunoprecipitate from the *yopN* deletion strain. Similarly, YopN coprecipitated with the immunoprecipitated YscB in the parent strain but not in the *yscB* deletion strain. No YopN or YscB was immunoprecipitated with preimmune sera or coprecipitated with control antisera specific for YopE (data not shown). In addition, no coprecipitation of YscB and YopN was observed in the absence of DSP cross-linking. These data indicate that protein cross-linking with DSP stabilized a complex of YopN and YscB that could be efficiently immunoprecipitated with antisera specific for either YscB or YopN, suggesting that these proteins are closely associated or directly interact with one another in *Y. pestis*.

Localization of the YscB binding region of YopN. Previous studies with SycE and SycH in *Y. enterocolitica* and *Y. pseudotuberculosis* localized the Syc binding region to within amino acids 15 to 100 of YopE and YopH (57, 69), respectively. To begin to map the YscB binding region of YopN, a series of in-frame amino-terminal deletions within *yopN* were constructed and moved into plasmid pCD1 of *Y. pestis* KIM8-3002 (pPCP1$^-$) by allelic exchange. Deletions eliminating the coding region for amino acids 2 to 10, 6 to 25, 21 to 40, 51 to 85, and 6 to 100 of *yopN* were constructed and used in cross-linking and immunoprecipitation studies to localize the YscB binding region of YopN (Fig. 8).

Cross-linking and immunoprecipitation experiments with *Y. pestis* KIM10 (pCD1$^-$ pPCP1$^-$), *Y. pestis* KIM8 (pCD1$^+$ pPCP1$^-$), the *yopN* deletion strain KIM8-3002.P7 (pPCP1$^-$), and pPCP1$^-$ strains containing in-frame internal deletions within the first 100 amino acids of YopN (Fig. 8 and Table 1) were carried out essentially as described for Fig. 7. Antisera specific for YopN immunoprecipitated YopN or the corresponding deleted derivative of YopN from each of the strains expressing a stable *yopN* gene product. *Y. pestis* KIM8 and the *yopN* deletion strain did not express a stable *yopN* gene product. YscB coprecipitated with the *yopN* gene products from *Y. pestis* KIM8 and the corresponding strains expressing *yopN* gene products lacking amino acids 2 to 10, 6 to 25, and 21 to 40 following cross-linking with DSP. No YscB coprecipitated with the *yopN* gene products lacking amino acids 51 to 85 or 6 to 100. Antisera specific for YscB immunoprecipitated approximately equal amounts of YscB from each of the pCD1$^+$ strains of *Y. pestis* (Fig. 8). YopN or the corresponding deleted derivative of YopN coprecipitated with YscB in *Y. pestis* KIM8 and the corresponding strains expressing *yopN* gene products lacking amino acids 2 to 10, 6 to 25, and 21 to 40. A small amount

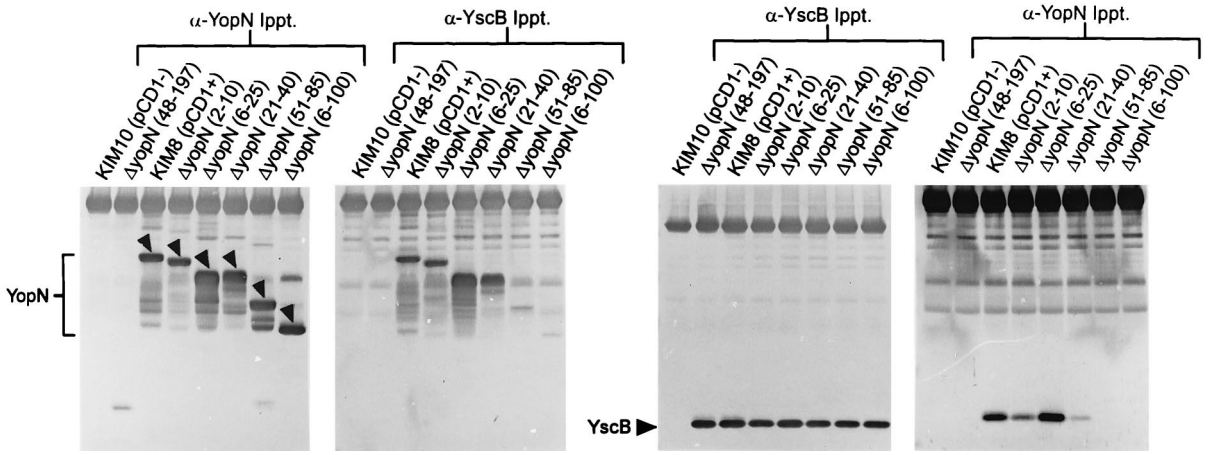

FIG. 8. Localization of a region of YopN required for YscB binding. Soluble extracts from *Y. pestis* KIM10 (pPCP1$^-$ pCD1$^-$), the *yopN* deletion strain *Y. pestis* KIM8-3002.P7 (pPCP1$^-$), *Y. pestis* KIM8 (pPCP1$^-$ pCD1$^+$), and derivatives of *Y. pestis* KIM8-3002 containing in-frame deletions in *yopN* eliminating the coding regions for amino acid residues 2 to 10 [Δ*yopN* (2-10)], 6 to 25 [Δ*yopN* (6-25)], 21 to 40 [Δ*yopN* (21-40)], 51 to 85 [Δ*yopN* (51-85)], and 6 to 100 [Δ*yopN* (6-100)] were cross-linked with DSP (1 mM) for 20 min at RT. Samples were processed as described in Materials and Methods, immunoprecipitated with antisera specific for YopN or YscB, and analyzed by SDS-PAGE and immunoblotting with antisera specific for YopN or YscB. DSP cross-links were broken prior to SDS-PAGE analysis by boiling samples in the presence of 5% β-mercaptoethanol. The locations of YopN and YscB are shown by arrows. Ippt., immunoprecipitation.

of *yopN* gene product was also found associated with *Y. pestis* strains expressing a *yopN* gene product lacking amino acids 51 to 85 and 6 to 100; however, a similar background level of YopN was also found in control immunoprecipitations using either preimmune serum or antisera specific for YopE (data not shown). These results indicate that the region between amino acids 50 and 86 of YopN is required for the interaction of YopN and YscB.

DISCUSSION

Previous studies have suggested a role for the *yscCDEFGI JKL* gene products in the secretion of Yops in *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (4, 24, 32, 33, 44), while the *yscH* locus was recently shown to encode YopR (4). The present study examined the role of the *yscB* gene product in Yop secretion and in the regulation of Yop secretion in *Y. pestis*. A nonpolar *yscB* deletion mutant exhibited calcium-blind growth, secreted V antigen and YopM both in the presence and in the absence of calcium, and specifically secreted reduced amounts of YopN. The amino acid sequence and predicted secondary structure of YscB showed similarities to those of SycE (21, 65) and SycH (64), the specific Yop chaperones for YopE and YopH, respectively. Furthermore, it was demonstrated that YscB could be efficiently cross-linked to YopN and that this association could be prevented by deletions eliminating the regions encoding amino acids 51 to 85 or 6 to 100 of YopN. Together these results suggest that YscB binds to YopN within the cell and functions as a specific chaperone for YopN in *Y. pestis*.

Members of the Syc and Syc-like family of protein chaperones are generally small (15 to 20 kDa), acidic, cytoplasmic proteins that specifically bind to an amino-terminal region of their specific target Yop and share a conserved carboxyl-terminal motif consisting of a leucine-rich amphipathic helix (64, 66). YscB shares numerous characteristics with this family of proteins, including size (15.5 kDa), location (cytoplasmic or peripheral membrane protein), a carboxyl-terminal amphipathic helix (Fig. 6B), and a specific interaction with an amino-terminal region of its specific Yop (YopN); however, YscB is a basic protein (pI 9.3) that is required directly or indirectly for the calcium-dependent regulation of Yop secretion in addition to its role in the secretion of YopN. An additional characteristic of the Syc chaperones is that the genes encoding individual Syc chaperones are generally located in close proximity to the gene encoding their cognate Yop (66). In contrast, the *yscB* locus is the first gene in a multicistronic operon that predominately encodes essential components of the Yop secretion apparatus and is located approximately 8 kb from the *yopN* locus.

The exact function is not completely understood for any of the individual Syc proteins. Insertional or deletional inactivation of an individual Syc protein results in reduced secretion of the respective Yop in vitro (64, 65). In addition, deletion of the Syc binding region from YopE or YopH hybrid reporter proteins prevented translocation of the reporter proteins into eucaryotic target cells (21, 54, 57, 69). Recent evidence reported by Cheng et al. (10) indicates that the Syc binding region of YopE can function to target YopE for secretion in a SycE-dependent manner, independently of a previously identified SycE-independent secretion signal located at the extreme amino terminus of YopE (57, 69). Deletional mutagenesis of *yerA* (*sycE*) reduced YopE export approximately 88%, with the remaining YopE export presumably resulting from the Syc-independent amino-terminal secretion signal (5).

Deletional inactivation of the *Y. pestis yscB* locus did not prevent export of YopN; however, the amount of YopN exported was specifically reduced. These results mirror those found for YopE export in a *sycE* (*yerA*) mutant of *Y. enterocolitica* (10). In addition, the *yscB* deletion mutant lost its ability to block Yop secretion in the presence of calcium, a phenotype similar to that of a *yopN* deletion mutant (19, 63). This phenotype may be a direct effect of YscB loss or, more likely, may be due to the effect of YscB loss on the secretion and/or presentation of YopN. Interestingly, deletional inactivation of *yscB* only reduced the secretion of YopN; however, the ability of the *yscB* deletion strain to block Yop secretion in the presence of calcium was essentially lost, with the exception of a small degree of calcium-dependent secretion regulation for YopN itself (Fig. 3). This indicates either that YscB is necessary for presenting YopN in a conformation capable of blocking Yop secretion in the presence of calcium, that YscB is a direct participant in blocking Yop secretion, or that, in order to block Yop secretion, YopN must be targeted efficiently to the secretion apparatus.

It is noteworthy that Boland et al. (9) recently presented data indicating that YopN, unlike YopE, YopH, and YopM, is not translocated into the eucaryotic target cell. This conclusion was based upon the activity of truncated YopN-CyaA fusion proteins. If these data are confirmed by studies utilizing full-length YopN, it would suggest either that YscB differs in function from SycE and SycH or that YscB, SycE, and SycH share a common function and that the role of SycE and SycH in YopE and YopH translocation is indirect. Woestyn et al. (69) suggest that the Syc chaperones may function to prevent nonspecific interactions within the bacterial cell between Yops destined for translocation across the eucaryotic cell membrane and Yops that facilitate this translocation process, namely YopB and/or YopD. Thus, in the absence of the Syc chaperone, premature interaction of the Yop to be secreted with these or other proteins within the bacterial cell renders the interacting Yops incompetent for secretion. In a similar manner, YscB may prevent premature interactions of YopN with LcrG, TyeA, or other components involved in the regulation of Yop secretion. Interestingly, TyeA has recently been shown to bind to a carboxyl-terminal region of YopN (28). In addition, we have recently identified another protein (Orf2 [19, 63]) that also interacts with YopN within the cell (13). Analysis of these interactions will further our understanding of the mechanism and protein-protein interactions involved in the regulation of Yop secretion.

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