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Mutations in *yscC*, *yscD*, and *yscG* Prevent High-Level Expression and Secretion of V Antigen and Yops in *Yersinia pestis*

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The *Yersinia pestis* low-Ca^{2+} response stimulon is responsible for the temperature- and Ca^{2+}-regulated expression and secretion of plasmid pCD1-encoded antihost proteins (V antigen and Yops). We have previously shown that *lcrD* and *yscR* encode proteins that are essential for high-level expression and secretion of V antigen and Yops at 37°C in the absence of Ca^{2+}. In this study, we constructed and characterized mutants with in-frame deletions in *yscC*, *yscD*, and *yscG* of the *ysc* operon that contains *yscA* through *yscM*. All three mutants lost the Ca^{2+} requirement for growth at 37°C, expressed only basal levels of V antigen and YopM in the presence or absence of Ca^{2+}, and failed to secrete these proteins to the culture supernatant. Overproduction of YopM in these mutants failed to restore YopM export, showing that the mutations had a direct effect on secretion. The protein products of *yscC*, *yscD*, and *yscG* were identified and localized by immunoblot analysis. YscC was localized to the outer membrane of *Y. pestis*, while YscD was found in the inner membrane. YscG was distributed equally between the soluble and total membrane fractions. Double mutants were characterized to assess where YscC and YscD act in low-Ca^{2+} response (LCR) regulation. *lcrH*::*cat-yscC* and *lcrH*::*cat-yscD* double mutants were constitutively induced for expression of V antigen and YopM; however, these proteins were not exported. This finding showed that the *ysc* mutations did not directly decrease induction of LCR stimulon genes. In contrast, *lcrE-yscC*, *lcrG-yscC*, *lcrE-yscD*, and *lcrG-yscD* double mutants as well as an *lcrE-lcrD* double mutant expressed only basal levels of V antigen and YopM and also failed to secrete these proteins to the culture supernatant. These results indicated that a functional LCR secretion system was necessary for high-level expression of LCR stimulon proteins in the *lcrE* and *lcrG* mutants but not in an *lcrH*::*cat* mutant. Possible models of regulation which incorporate these results are discussed.

Yersinia pestis, the etiologic agent of plague, responds to specific environmental signals by way of a highly regulated multicomponent virulence property called the low-Ca^{2+} response (LCR) (28). This response is manifested in vitro by a requirement for millimolar concentrations of Ca^{2+} for cultivation in certain media at temperatures above 34°C (24) and by the Ca^{2+}-regulated expression and secretion of virulence-associated proteins. The set of operons regulated by these environmental signals is referred to as the LCR stimulon (LCRS) (76). The LCRS is encoded on the ca. 75-kb plasmid termed pCD1 in *Y. pestis* KIM5 (23) and on closely related plasmids carried by the other human pathogenic yersiniae, *Y. enterocolitica* and *Y. pseudotuberculosis* (3). LCRS operons encode a set of secreted antihost proteins (V antigen and Yops), regulatory proteins and proteins required for the secretion and targeting of antihost proteins.

V antigen is a secreted protein encoded by *lcrV* of the *lcrGVH-yopBD* operon (5, 53, 57, 58). No specific antihost function has been assigned to V antigen; however, it is a protective antigen required for full virulence in mice (12, 41, 74, 78). Yops are secreted proteins encoded by multiple operons scattered over the LCR plasmid (11, 13, 75, 77). They appear to function as antihost proteins by subverting host defense systems, such as by preventing phagocytosis of attached yersiniae (YopE [65] and YopH [64]), disrupting eucaryotic signal transduction (YopH [9, 10] and YpkA [26]), and specifically binding thrombin (YopM [42, 62]). Recently, the polarized transfer of YopE from attached yersiniae into the cytosol of eucaryotic cells has been demonstrated (22, 66, 67). The transfer process required the participation of a multicomponent LCR plasmid-encoded secretion system and at least one other Yop (YopD) (22).

Induction of V antigen and Yop expression is observed at temperatures above 34°C. Thermal induction of *lcrV* and *yop* genes is mediated by the *lcrF* gene product (14, 33). LcrF is a transcriptional activator that shows similarity to the AraC family (15) and has been shown to bind to sequences upstream of the *yopE* and *yopH* promoters (40). The chromosomally encoded *ymoA* gene product modulates the amplitude of thermal induction (14). The product of *lcrV* (V antigen) is also required for full induction of LCRS proteins, suggesting that V antigen has dual functions (57, 74). Finally, induction of the LCRS is also dependent on gene products required for export of V antigen and Yops (4, 21, 55, 56).

Full thermal induction of LCRS operons is possible only when yersiniae are grown without Ca^{2+}. In the presence of Ca^{2+}, LCRS operon transcription is downregulated and the secretion of V antigen and Yops is blocked. Ca^{2+}-mediated downregulation of the LCRS and blockage of secretion is mediated through the *lcrE* (23, 81, 85), *lcrG* (73), *lcrQ* (63), and *lcrH* (5, 59) gene products. Mutants in these loci show constitutive induction of LCRS operons at 37°C. The *lcrE*, *lcrG*, and *lcrQ* gene products are secreted by the yersiniae and have been suggested to be components of a Ca^{2+}-sensing mechanism (23, 63, 73, 76). Genetic evidence indicates that the cytosolic *lcrH* gene product is a distal component of the Ca^{2+}-mediated pathway (5, 63, 76); however, LcrH shows no resemblance to known DNA-binding proteins, and its regulatory effect is likely to be indirect.

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Secretion of LCRS proteins is mediated by a multicomponent LCR plasmid-encoded secretion system (46–48). Proteins secreted by this system lack cleavable signal sequences but require an amino-terminal secretion signal for export (46, 48). Components of the *ysc* operon containing *yscA* through *yscM* (*yscA-M*) (32, 47), the *ysc* operon containing *yscN* through *yscU* (4, 21), and the *lcrDR* (55, 56) operon have been implicated in LCRS protein secretion. In *Y. enterocolitica*, the *yscA-M* (47), *yscN* (84), and *yscU* (1) loci have been shown to be involved in Yop secretion. The *yscL* (63), *yscR*, and *yscS* (4) gene products have been shown to be required for Yop secretion in *Y. pseudotuberculosis*. Finally, in *Y. pestis*, a requirement for the *yscC*, *yscD*, *yscG* (this study), *yscR* (21), and *lcrD* (55, 56) gene products has been demonstrated for V antigen and Yop secretion. In addition, secretion of individual Yops is dependent on specific cytoplasmic chaperone proteins, called Syc (82, 83). These proteins may be important in targeting Yops to the secretion apparatus. Interestingly, mutations in genes encoding components required for secretion of V antigen and Yops also prevent high-level expression of LCRS proteins (4, 21, 55, 56). In addition, these mutants no longer require Ca^{2+} for growth at temperatures above 34°C.

Homologs of specific Ysc proteins and of LcrD have recently been identified in other bacterial systems. These include gene products required for invasion of epithelial cells (*Salmonella typhimurium* [25, 30], *Shigella flexneri* [2, 71, 80], and enteroinvasive *Escherichia coli* [34]), gene products encoded within the *hrp* gene clusters of plant pathogens (*Xanthomonas campestris* [19, 36], *Pseudomonas solanacearum* [29], and *Erwinia amylovora* [79]), and gene products required for secretion and assembly of the bacterial flagella (*Caulobacter crescentus* [61, 69], *S. typhimurium* [38], *Bacillus subtilis* [7, 8], *Campylobacter jejuni* [49], and *E. coli* [44]). These functionally diverse systems all involve secretion, or surface localization, of proteins via a novel pathway (type III secretion system [68]).

YscC of *Y. pestis* and *Y. enterocolitica* is a member of the PulD superfamily of membrane-associated proteins (27). Members of this family are involved in transport of extracellular proteins, phage particles, or DNA across the outer membrane of bacteria (27). Although the sequences of *yscC* from both *Y. enterocolitica* and *Y. pestis* were examined previously (32, 47), a role for YscC in export of LCRS proteins has not been demonstrated. The present report investigates the role of YscC as well as YscD and YscG in expression and secretion of LCRS proteins in *Y. pestis*. Although YscC shows homology to other proteins involved in export processes, the sequences of YscD and YscG are unique to the genus *Yersinia* at this time.

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 (28, 75). In this medium, LCR^+ yersiniae require added Ca^{2+} for full growth yield at 37°C. *E. coli* strains were grown in L broth or on L agar (17). Bacteria with antibiotic resistances were grown in the presence of the appropriate antibiotic(s) at a final concentration of 25 μg/ml (kanamycin) or 50 μg/ml (ampicillin and streptomycin).

DNA methods. Cloning methods, including the use of restriction endonucleases and T4 DNA ligase, were performed essentially as described by Maniatis et al. (45). Plasmid DNA was isolated by an alkaline lysis procedure (6), by the method of Kado and Liu (37), or with Qiagen columns (Qiagen, Inc., Studio City, Calif.). DNA fragments were isolated and purified from agarose gels by using a Qiaex DNA purification kit (Qiagen). Electroporation of *E. coli* and *Y. pestis* was done as previously described (54). The PCR technique (51) was performed by using 18- to 28-nucleotide primers and 30 cycles of amplification. Unless stated otherwise, denaturing, annealing, and extending conditions were 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min, respectively. Double-stranded DNA was sequenced by the method of Sanger et al. (70), using synthetic nucleotide primers, [^{35}S]dATP (ICN, Costa Mesa, Calif.), and a Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Nucleotide sequences were analyzed with PC Gene computer software (Intelligenetics, Mountain View, Calif.).

Construction of mutants. Plasmids pΔYSCC.1, pΔYSCD.1, pΔYSCD.2, pΔYSCG.1, and pΔLCRE.1 carrying in-frame deletions in *yscC*, *yscD*, *yscG*, and *lcrE*, respectively, were constructed by restriction endonuclease digestion as described in Table 1. Restriction endonuclease fragments which contained the deleted sequences flanked by homologous DNA were subcloned into the *Eco*RV site of the suicide vector pUK4134, generating plasmids pUK4134.12, pUK4134.13, pUK4134.14, pUK4134.15, and pUK4134.6 (Table 1). These plasmids were 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 (72). 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/or restriction endonuclease digestion. *Y. pestis* KIM5-3001.12, KIM5-3001.13, KIM5-3001.14, KIM5-3001.15, and KIM5-3001.6 each contained the correct in-frame deletion within pCD1 (Table 1) and were used for further study. Plasmids pUK4134.12 and pUK4134.14 were also used to introduce in-frame deletions in *yscC* or *yscD* into pCD1 of *Y. pestis* KIM5-3001.5 (Δ*lcrG*), KIM5-3001.6 (Δ*lcrE*), and KIM5-3401 (Δ*lcrH*::*cat*) essentially as described above. In addition, pUK4134.3 was used to introduce an in-frame deletion in *lcrD* into pCD1 of *Y. pestis* KIM5-3001.6 (Δ*lcrE*). Double mutants were screened by PCR and/or restriction endonuclease digestion to confirm the presence of each in-frame deletion.

Isolation of point mutations in *lcrD*. Plasmid pYPD was transformed into *E. coli* LE30 (*mutD5*) (18). Transformants were grown at 37°C in L broth to induce mutator activity. Plasmids potentially carrying point mutations in *lcrD* were isolated and used to transform *Y. pestis* KIM5-3001.3 (Δ*lcrD* [aa 618–644]) (gene designations are explained in Table 1, footnote *a*). *Y. pestis* KIM5-3001.3 is Ca^{2+}-independent for growth at 37°C; however, providing plasmid pYPD in *trans* restores Ca^{2+}-dependent growth (55). Strains receiving plasmids carrying mutations in *lcrD* were selected for by their ability to form colonies at 37°C on tryptose blood agar plates containing 20 mM $MgCl_2$ and 20 mM sodium oxalate (sodium oxalate added to chelate Ca^{2+}). Six pYPD derivatives, designated pYPD.PM1 through pYPD.PM6 (Table 1), that failed to restore Ca^{2+}-dependent growth to *Y. pestis* KIM5-3001.3 were selected for further analysis. These plasmids were transformed back into *Y. pestis* KIM5-3001.3, to confirm that the Ca^{2+}-independent phenotype was associated with the plasmid, and into *E. coli* DH5α for mutation detection and DNA sequence analysis.

Detection and mapping of point mutations. Plasmid DNA was isolated from each of the *E. coli* DH5α strains by standard procedures. A 1.6-kb 5′ region of *lcrD* was amplified by using the vector SK primer (primer 1A) and primer 1B (5′-TTTAGTGCATTCGCTTCC-3′). An overlapping 1.4-kb 3′ region of *lcrD* was amplified by using the vector KS primer (primer 2B) and primer 2A (5′-CAAGGGCCGACTAGGGGA-3′). Following amplification, 10 μl of each reaction mixture was electrophoresed in a 1% (wt/vol) agarose gel and stained with ethidium bromide to verify the sizes of the PCR products. PCR products were purified from 1% (wt/vol) agarose gels by using a Qiaex DNA purification kit (Qiagen) and stored at 4°C. PCR primers (30 ng) were 5′ end labeled by using 5 μl of [γ-^{32}P]ATP (>7,000 Ci/mmol, >100 mCi/ml), 3 μl of 10× kinase buffer (Tris-HCl [0.5 M, pH 7.6], $MgCl_2$ [0.1 M], dithiothreitol [0.15 M]), and distilled H_2O to a volume of 30 μl. T4 polynucleotide kinase (10 U) was added, and the mixture was incubated at 37°C for 45 min. The reaction mixture was then brought to 2 ml with distilled H_2O, and the labeled oligonucleotides were purified and concentrated in a Centricon-3 concentrator (Amicon, Corp., Danvers, Mass.). Amplifications from plasmid pYPD were then repeated in four separate reactions using one labeled primer and the corresponding unlabeled primer. Strand-specific radiolabeled PCR probes were purified from 1% (wt/vol) agarose gels by using a Qiaex DNA purification kit (Qiagen).

The chemical mismatch cleavage procedure was performed essentially as described by Cotten (16), using DNA heteroduplexes consisting of a radiolabeled wild-type strand and an unlabeled mutant strand. Briefly, unlabeled mutant PCR product (200 ng) and radiolabeled wild-type PCR product (20 ng, 50,000 cpm) were mixed in 6 μl of 5× annealing buffer (3 M NaCl, 30 mM Tris-HCl [pH 7.7], 35 mM $MgCl_2$), and the volume was brought to 30 μl. The reaction mixture was heated at 100°C for 5 min to denature the DNA and then incubated at 42°C for 2 h to allow heteroduplex formation. Ice-cold ethanol (100 μl) was added, and the heteroduplex DNA was precipitated.

Precipitated heteroduplex DNA was redissolved in 18 μl of distilled H_2O, and 6 μl of this was used for subsequent chemical modification reactions. For the hydroxylamine reaction, 6 μl of heteroduplex DNA was mixed with 20 μl of hydroxylamine solution (1.39 g of hydroxylamine chloride in 1.6 ml of distilled H_2O, buffered to pH 6.0 with diethylamine) and incubated at 37°C for 20 min. In the osmium tetroxide reaction, heteroduplex DNA was mixed with 2.5 μl of 10× osmium buffer (100 mM Tris-HCl [pH 7.7], 10 mM EDTA, 15% [vol/vol] pyridine) and then placed on ice. Osmium tetroxide (15 μl of a 2% [wt/vol] aqueous solution) was added, mixed thoroughly, and incubated at 37°C for 5 min. Both the hydroxylamine and osmium tetroxide reactions were stopped by addition of 200 μl of stop buffer (0.3 M sodium acetate [pH 5.2], 0.5 mM EDTA, 25 μg of baker's yeast tRNA per ml). Reaction mixtures were precipitated with ice-cold ethanol (750 μl), washed once with 80% ethanol, and dried.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Construction and properties[a]	Source or reference
E. coli K-12 strains		
DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	GIBCO-BRL, Gaithersburg, Md.
SY327 (λ*pir*)	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56*	50
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[b]		
KIM5	pCD1 (Lcr^+), pPCP1, pMT1	R. Brubaker
KIM5-3001	Sm^r pCD1 (Lcr^+), pPCP1, pMT1	43
KIM6-3001	Sm^r pCD1$^-$ (Lcr^-), pPCP1, pMT1	43
KIM5-3001.3	Sm^r pCD1 (Δ*lcrD* [aa 618–644]), pPCP1, pMT1	56
KIM5-3001.5	Sm^r pCD1 (Δ*lcrG* [aa 39–53]), pPCP1, pMT1	73
KIM5-3001.6	Sm^r pCD1 (Δ*lcrE* [aa 48–197]), pPCP1, pMT1	This study
KIM5-3001.12	Sm^r pCD1 (Δ*yscC* [aa 141–454]), pPCP1, pMT1	This study
KIM5-3001.13	Sm^r pCD1 (Δ*yscD* [aa 206–232]), pPCP1, pMT1	This study
KIM5-3001.14	Sm^r pCD1 (Δ*yscD* [aa 62–291]), pPCP1, pMT1	This study
KIM5-3001.15	Sm^r pCD1 (Δ*yscG* [aa 73–99]), pPCP1, pMT1	This study
KIM5-3001.6.1	Sm^r pCD1 (Δ*lcrD* [aa 618–644], Δ*lcrE* [aa 48–197]) pPCP1, pMT1	This study
KIM5-3001.12.1	Sm^r pCD1 (Δ*yscC* [aa 141–454], Δ*lcrE* [aa 48–197]), pPCP1, pMT1	This study
KIM5-3001.12.2	Sm^r pCD1 (Δ*yscC* [aa 141–454], Δ*lcrG* [aa 39–53]), pPCP1, pMT1	This study
KIM5-3001.12.3	Sm^r pCD1 (Δ*yscC* [aa 141–454], *lcrH*::*cat yopJ*::MudI1734 [Km^r Lac^+] [YscC$^-$ LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$]), pPCP1, pMT1	This study
KIM5-3001.14.1	Sm^r pCD1 (Δ*yscD* [aa 62–291], Δ*lcrE* [aa 48–197]), pPCP1, pMT1	This study
KIM5-3001.14.2	Sm^r pCD1 (Δ*yscD* [aa 62–291], Δ*lcrG* [aa 39–53]), pPCP1, pMT1	This study
KIM5-3001.14.3	Sm^r pCD1 (Δ*yscD* [aa 62–291], *lcrH*::*cat yopJ*::MudI1734 [Km^r Lac^+] [YscD$^-$ LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$]), pPCP1, pMT1	This study
KIM5-3401	Sm^r pCD1 (*lcrH*::*cat yopJ*::MudI1734 [Km^r Lac^+] [LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$]), pPCP1, pMT1	59
KIM5-3233	Sm^r pCD1 (*yopM*::*lacZYA*), pPCP1, pMT1	42
Plasmids		
pBluescript IIKS$^-$	Cloning vector, Ap^r	Stratagene
pUK4134	Suicide vector, Ap^r	72
pTRC99A	Expression vector, Ap^r	Pharmacia
pGEX-3X	GST gene fusion vector, Ap^r	Pharmacia
pX2X1-T7	5.9-kb *Xho*I fragment of pCD1 carrying *yscABCDEFGHIJ′* cloned into the *Xho*I site of pBluescript IIKS$^-$	32
pB1B2-T7	3.1-kb *Bam*HI fragment of pX2X1-T7 carrying *yscCDEFG′* cloned into the *Bam*HI site of pBluescript IIKS$^-$	32
pΔLCRE.1	pGP2 (55) digested with *Pst*I and religated, resulting in elimination of the 447-bp *Pst*I fragment within *lcrE*	This study
pΔYSCC.1	pX2X1-T7 digested with *Nar*I and *Msc*I, filled in with Klenow enzyme, and religated, resulting in elimination of the 939-bp *Nar*I-*Msc*I fragment within *yscC*	This study
pΔYSCD.1	pX2X1-T7 digested with *Nco*I and religated, resulting in elimination of the 81-bp *Nco*I fragment within *yscD*	31
pΔYSCD.2	A 1.8-kb *Pst*I-*Xho*I fragment of pB1B2-T7 carrying the 3′ end of *yscD* was eliminated by restriction endonuclease digestion and gel purification; a 3-kb *Nsi*I-*Xho*I fragment of pX2X1-T7 containing 690 bp less of the 3′ end of *yscD* was cloned into the *Pst*I-*Xho*I sites of pB1B2-T7, resulting in a 690-bp *Pst*I-*Nsi*I deletion within *yscD.*	This study
pΔYSCG.1	pYSCG1 was digested with *Sty*I, treated with mung bean nuclease, and then digested with *Xho*I; pX2X1-T7 was digested with *Bam*HI, treated with mung bean nuclease, and then digested with *Xho*I; a 1.3-kb *Bam*HI-*Xho*I fragment of pX2X1-T7 was then cloned into the *Sty*I-*Xho*I sites of pYSCG1, resulting in a 81-bp deletion within *yscG.*	This study
pUK4134.6	2.2-kb *Eco*RV fragment of pΔLCRE.1 carrying Δ*lcrE* (aa 48–197) cloned into pUK4134	This study
pUK4134.12	3.3-kb *Eco*RV fragment of pΔYSCC.1 carrying Δ*yscC* (aa 141–454) cloned into pUK4134	This study
pUK4134.13	1.7-kb *Hpa*I-*Eco*RV fragment of pΔYSCD.1 carrying Δ*yscD* (aa 206–232) cloned into pUK4134	This study
pX2B2-T7	1.2-kb *Xho*I-*Bam*HI fragment of pX2X1-T7 carrying *yscGHIJ′* cloned into the *Bam*HI and *Xho*I sites of pBluescript IIKS$^-$	This study
pYSCC1	2.1-kb *Acc*I-*Pst*I fragment of pX2X1-T7 carrying *yscC* cloned into the *Cla*I and *Pst*I sites of pBluescript IIKS$^-$ such that *yscC* is transcribed from the vector *lac* promoter[c]	This study
pYSCD1	1.3-kb *Dde*I fragment of pX2X1-T7 carrying *yscD* filled in with Klenow enzyme and cloned into the *Eco*RV site of pBluescript IIKS$^-$ such that *yscD* is transcribed from the vector *lac* promoter[c]	This study
pYSCG1	0.6-kb *Eco*RV-*Bst*EII fragment of pX2X1-T7 carrying *yscG* filled in with Klenow enzyme and cloned into the *Eco*RV site of pBluescript IIKS$^-$ such that *yscG* is transcribed from the vector *lac* promoter[c]	This study
pUK4134.14	2.4-kb *Bam*HI fragment of pΔYSCD.2 carrying Δ*yscD* (aa 62–291) filled in with Klenow enzyme and cloned into pUK4134	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Construction and properties[a]	Source or reference
pUK4134.15	1.6-kb *Eco*RV fragment of pΔYSCG.1D.2 carrying Δ*yscG* (aa 73–99) cloned into pUK4134	This study
pXYC.1	1.5-kb *Nci*I-*Eco*RI fragment of pB1B2-T7 filled in following *Nci*I digestion and cloned into the *Sma*I and *Eco*RI sites of pGEX-3X (expresses a GST-YscC fusion protein)	This study
pXYD.1	1.1-kb *Eco*RI fragment of pB1B2-T7 cloned into the *Eco*RI site of pGEX-3X (expresses a GST-YscD fusion protein)	This study
pXYG.1	0.4-kb PCR product digested with *Eco*RI and cloned into the *Eco*RI site of pGEX-3X (expresses a GST-YscG fusion protein)	This study
pTRCM.1	1.2-kb *Taq*I fragment of pBS10 (62) carrying *yopM* filled in with Klenow enzyme and cloned into the *Sma*I site of pTRC99A	This study
pTRCM.2	pTRCM.1 with *Nco*I site removed by *Nco*I digestion followed by treatment with mung bean nuclease and religation (destroyed ATG start codon of LacZ-YopM fusion protein)	This study
pYPD	2.7-kb fragment of pCD1 carrying *lcrD* cloned into the *Eco*RV site of pBluescript IIKS$^-$	56
pYPD.PM1	pYPD (base 229 T→C [aa 35 L→P])	This study
pYPD.PM2	pYPD (base 696 G→A) [aa 191 G→S])	This study
pYPD.PM3	pYPD (base 844 A→G [aa 240 D→G])	This study
pYPD.PM4	pYPD (base 906 C→T [aa 261 R→C])	This study
pYPD.PM5	pYPD (base 1845 C→T [aa 574 Q→stop])	This study
pYPD.PM6	pYPD (base 2134 A→G [aa 670 Y→C])	This study

[a] Amino acid (aa) numbers in gene designations represent the amino acids deleted from the encoded protein. Base numbers in plasmid descriptions represent the position of the point mutation in the DNA sequence of the *lcrD* coding strand (55), with the corresponding amino acid change indicated in brackets.

[b] All *Y. pestis* strains are Pgm$^-$ (78). Native plasmids of *Y. pestis* include the LCR plasmid pCD1 (20, 28), the Pla-encoding pPCP1 (75), and pMT1, which encodes the capsular protein (60).

[c] See Fig. 1 for restriction sites at ends of cloned DNA.

Dried pellets were resuspended in 50 μl of 1 M piperidine and incubated at 90°C for 30 min. Reaction mixtures were then precipitated with 50 μl of 0.6 M sodium acetate (pH 5.2) and 300 μl of ice-cold ethanol. Pellets were washed once with 80% ethanol, dried, and resuspended in 15 μl of formamide loading buffer (70). Samples were denatured at 80°C and separated in a 4% (wt/vol) denaturing polyacrylamide gel (45). The approximate sizes of the cleaved products were estimated by using radiolabeled ϕX174 standards. DNA sequencing was used to identify the mutation detected by chemical mismatch cleavage.

Cell fractionation. *Yersinia* strains were grown at 26°C in TMH with or without Ca^{2+} for 7 to 9 generations and then were diluted to an optical density at 620 nm (OD_{620}) of 0.1 and grown to an OD_{260} of 0.2 to 0.3. Cultures were then either held at 26°C or shifted to 37°C for an additional 4 to 7 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 (100 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and pelleted by centrifugation. Washed cells 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 Beckman TLA-100.3 rotor (Beckman Instruments, Fullerton, Calif.) at 263,800 × *g* for 20 min at 4°C. Inner and outer membrane preparations were isolated in separate experiments as previously described (55). Membrane preparations were resuspended and stored in buffer A. Culture supernatant proteins were precipitated with 10% (vol/vol) trichloroacetic acid (1 h, on ice) and collected by centrifugation at 15,200 × *g* for 15 min at 4°C. Supernatants from cultures of double mutants (see Fig. 8) were subjected to ultracentrifugation at 263,800 × *g* for 20 min at 4°C prior to precipitation of proteins with trichloroacetic acid.

Antibody preparation. Plasmids pXYC.1, pXYD.1, and pXYG.1 (Table 1), which encode fusion proteins of glutathione *S*-transferase (GST) and portions of YscC, YscD, and YscG, respectively, were transformed into *E. coli* DH5α. Cultures (500 ml) of DH5α carrying pXYC.1, pXYD.1, or pXYG.1 were grown at 37°C in LB medium to an OD_{620} of 0.5. High-level expression of the respective fusion proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.1 mM followed by incubation at 37°C for an additional 3 h. Cultures

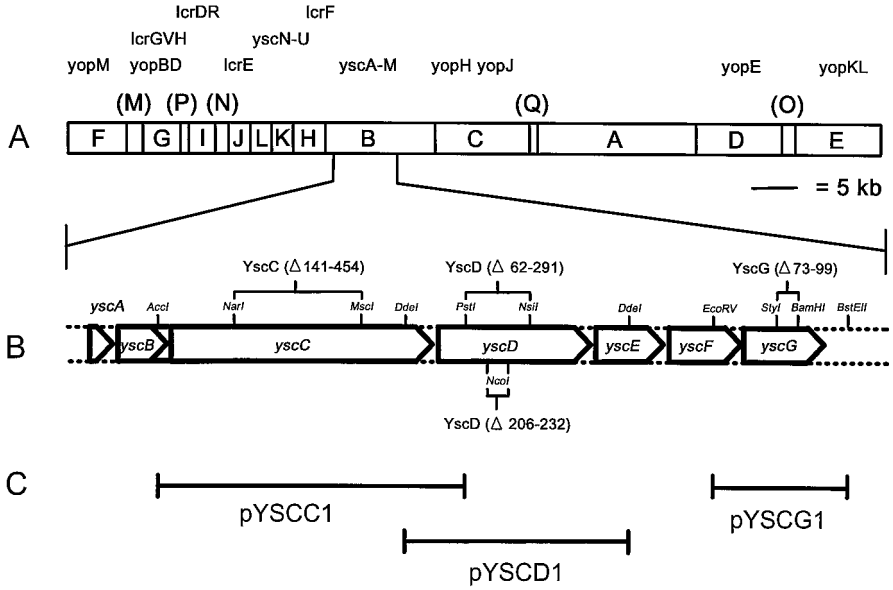

FIG. 1. (A) Physical and genetic maps of *Y. pestis* LCR plasmid pCD1. The approximate locations of known genes are shown above *Hin*dIII fragments A through Q of pCD1. The expanded diagram (B) shows the region from *yscA* to *yscG* and restriction enzyme sites used to create in-frame deletions in *yscC*, *yscD*, and *yscG* (panel B). (C) DNA fragments used in complementation studies.

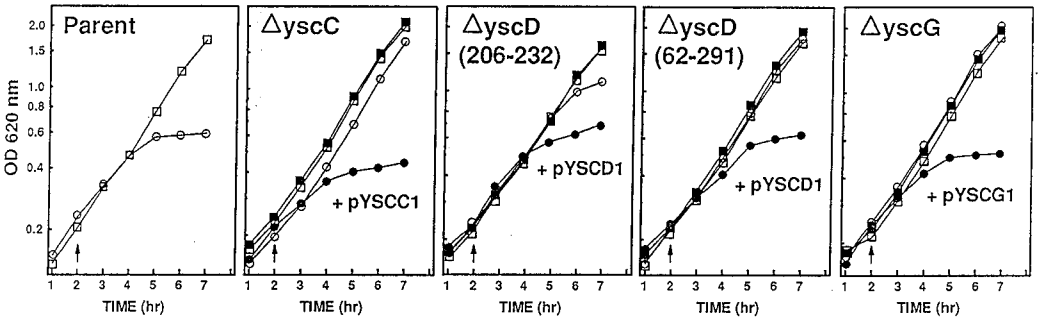

FIG. 2. Growth of *Y. pestis* KIM5-3001 (parent), KIM5-3001.12 (Δ*yscC*), KIM5-3001.13 (Δ*yscD* [aa 206–232]), KIM5-3001.14 (Δ*yscD* [aa 62–291]), and KIM5-3001.15 (Δ*yscG*) with and without complementing plasmids. *Y. pestis* strains were grown in the presence or absence of Ca^{2+} in the defined medium TMH. The temperature was shifted from 26 to 37°C when the OD_{620} of the culture reached approximately 0.2 (arrows). Symbols: open squares, plus Ca^{2+}; open circles, minus Ca^{2+}; closed squares, plus Ca^{2+} with complementing plasmid; closed circles, minus Ca^{2+} with complementing plasmid.

were harvested by centrifugation at 12,200 × *g* for 10 min at 4°C. Cell pellets were resuspended in phosphate-buffered saline (PBS; pH 7.2) 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. GST-YscC and GST-YscG fusion proteins were found almost exclusively in the 8,800 × *g* pellet, indicating that these proteins were in inclusion bodies. The GST-YscD fusion protein was found in the supernatant fraction. The soluble GST-YscD fusion protein was purified by affinity chromatography on a glutathione–Sepharose-4B column (Pharmacia-LKB, Piscataway, N.J.) according to the manufacturer's protocol. GST-YscC and GST-YscG containing inclusion bodies were partially purified by resuspension in PBS (pH 7.2) containing 1% Triton X-100 followed by centrifugation at 8,800 × *g* for 5 min at 4°C. This treatment, which removed contaminating soluble and membrane proteins from the inclusion body preparations, was repeated three times. GST-YscC and GST-YscG fusion proteins were then solubilized from the inclusion bodies in electrophoresis sample buffer (56), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isolated by electroelution with a Centrilutor microelectroeluter (Amicon), and concentrated in a Centricon-30 concentrator (Amicon).

Three pairs of female New Zealand White rabbits were injected subcutaneously, along the back, with 0.5 mg of purified GST-YscC, GST-YscD, or GST-YscG fusion protein emulsified in Freund's complete adjuvant. At 4 and 8 weeks, rabbits were boosted with 0.5 mg of the same fusion protein preparation in Freund's incomplete adjuvant. Antisera were applied to protein A-Sepharose columns (Pharmacia-LKB) to purify the immunoglobulin G fractions. Purified antibody preparations were dialyzed against PBS (pH 7.2) containing 0.05% sodium azide and stored at 4°C. Anti-YscC, anti-YscD, and anti-YscG antibody preparations were used for immunoblot analysis at a dilution of 1:5,000, 1:50,000, and 1:5,000, respectively.

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 SDS-PAGE and immunoblotting essentially as previously described (55). Those samples to be analyzed with the anti-YscG antibody were electrophoresed on 14% (wt/vol) acrylamide gels. *Y. pestis* LCR proteins were visualized as previously described (55), using antipeptide antibodies specific for YopM (62), V antigen (74), YscD (31, 32), and LcrD (55, 56).

Nucleotide sequence accession number. The nucleotide sequence of the *yscB CDEF* region of *Y. pestis* (32) was updated to include the sequence of *yscG* (accession number M83225).

RESULTS

Construction of *yscC*, *yscD*, and *yscG* deletion mutants. The gene products of the 13-cistron *yscA-M* operon have been implicated to be involved in secretion of LCR-specific proteins in *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* on the basis of the phenotype of numerous polar mini-Mud*lac* insertion mutants (32, 47). We are interested in further defining the role of specific membrane-bound components involved in this process in *Y. pestis*. Previously, the region encoding *yscAB CDEFG′*, through the *Bam*HI site within *yscG*, was sequenced in *Y. pestis* (32). We extended this sequence through *yscG* (120 bp) and found only 1-bp difference between our new sequence from *Y. pestis* and that published for the comparable region of *Y. enterocolitica* (47). The predicted amino acid sequences of both YscD and YscG show amino-terminal hydrophobic regions that represent potential membrane-anchoring domains (39, 47). YscC shows significant homology to a family of proteins, which includes PulD (27, 47), that have been implicated in secretion of macromolecules across the outer membrane in a number of diverse bacterial systems.

To further characterize the function of these gene products in the LCR, we constructed in-frame internal deletions in *yscC*, *yscD*, and *yscG* of *Y. pestis* that were designed to specifically affect the structure of the respective gene products without having polar effects on expression of downstream genes (Fig. 1B). Mutations were constructed in cloned fragments of the *yscA/M* operon and then introduced into pCD1 by allelic exchange (72). *Y. pestis* KIM5-3001.12 carries a 942-bp internal deletion in *yscC* and would be expected to express a protein lacking amino acids 141 to 454 of YscC. *Y. pestis* KIM5-3001.13 and KIM5-3001.14 carry 81- and 690-bp internal deletions within *yscD*, respectively. *Y. pestis* KIM5-3001.13 would be expected to express a protein missing amino acids 206 to 232, while KIM5-3001.14 would be expected to express a protein lacking amino acids 62 to 291. *Y. pestis* KIM5-3001.15 has an 81-bp deletion in *yscG* and would be expected to express a protein lacking amino acids 73 to 99 of YscG.

Growth phenotypes of *yscC*, *yscD*, and *yscG* deletion mutants. Figure 2 shows growth curves for the parent strain *Y. pestis* KIM5-3001 and deletion mutants. All strains exhibited full growth yield at 26°C in the presence or absence of Ca^{2+} (not shown) and at 37°C when Ca^{2+} was present. The parent *Y. pestis* KIM5-3001 showed growth restriction typical for media like TMH (24) following a temperature shift from 26 to 37°C in the absence of Ca^{2+}. KIM5-3001.13 (Δ*yscD* [aa 206–232]) showed a delayed growth restriction response following a temperature shift in the absence of Ca^{2+}. *Y. pestis* KIM5-3001.12 (Δ*yscC*), KIM5-3001.14 (Δ*yscD* [aa 62–291]), and KIM5-3001.15 (Δ*yscG*) all failed to show growth restriction in the presence or absence of calcium at 37°C (Ca^{2+}-independent growth phenotype). These data indicated that the wild-type LCR growth phenotype was dependent on the functional gene products of *yscC*, *yscD*, and *yscG*. Providing plasmids pYSCC1 (*yscC*), pYSCD1 (*yscD*), and pYSCG1 (*yscG*) (Table 1 and Fig. 1C) in *trans* to the *yscC*, *yscD*, and *yscG* deletion mutants, respectively, resulted in complete restoration of the wild-type growth response. These results indicated that growth defects associated with *Y. pestis* KIM5-3001.12, KIM5-3001.14, and KIM5-3001.15 were due solely to disruption of *yscC*, *yscD*, and *yscG*, respectively, and not due to polar effects on downstream genes or to spontaneous mutations in other *ysc* or *lcr* loci.

Secretion of V antigen and Yops. Secretion of V antigen and YopM was analyzed by immunoblot analysis of trichloroacetic

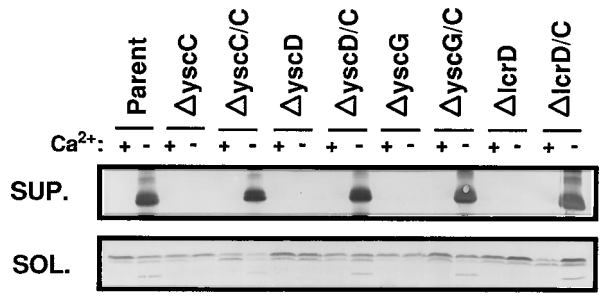

FIG. 3. Immunoblot analysis of YopM in supernatant and soluble fractions from *Y. pestis* KIM5-3001 (parent), KIM5-3001.12 (Δ*yscC*), KIM5-3001.14 (Δ*yscD*), KIM5-3001.15 (Δ*yscG*), and KIM5-3001.3 (Δ*lcrD*) grown at 37°C in both the presence (+) and absence (−) of Ca^{2+}. The *yscC*, *yscD*, *yscG*, and *lcrD* mutants were complemented (/C) with plasmids pYSCC1, pYSCD1, pYSCG1, and pYPD, respectively. Antipeptide antibodies were used to detect YopM in both the culture supernatant fraction (SUP.) and the soluble (cytoplasm plus periplasm) fraction (SOL.).

acid-precipitated culture supernatants from the parent *Y. pestis* KIM5-3001, the *yscC*, *yscD*, and *yscG* deletion mutants, and the mutants complemented with pYSCC1, pYSCD1, and pYSCG1 in trans (Fig. 3). *Y. pestis* KIM5-3001.3 (Δ*lcrD* [aa 618–644]), which had previously been shown to be defective for V antigen and Yop secretion, was used as a secretion negative control strain. *Y. pestis* strains were grown in TMH with or without Ca^{2+} at 26°C and then shifted to 37°C for 6 h. As expected, no secretion of V antigen or YopM was detected at 26 or at 37°C in the presence of Ca^{2+} (data for 26°C and V antigen not shown). The parent strain KIM5-3001 secreted V antigen and YopM into the culture supernatant when grown at 37°C in the absence of Ca^{2+} (inductive conditions for the LCR). As reported previously, the *lcrD* mutant was unable to secrete LCR virulence proteins unless a functional copy of *lcrD* (pYPD) was provided in *trans*. Likewise, *Y. pestis* KIM5-3001.12 (Δ*yscC*), KIM5-3001.14 (Δ*yscD* [aa 62–291]), and KIM5-3001.15 (Δ*yscG*) failed to secrete V antigen and YopM into the culture supernatant whether Ca^{2+} was present or absent. Although no LCR-specific secretion was observed in these strains, detectable levels of both V antigen and YopM were present in the soluble fractions, indicating that there was substrate available for secretion. Providing the complementing plasmids pYSCC1, pYSCD1, and pYSCG1 in *trans* to the respective *yscC*, *yscD*, and *yscG* deletion mutants completely restored Ca^{2+}-regulated expression and secretion of V antigen and YopM. In keeping with its less severe defect in growth response, *Y. pestis* KIM5-3001.13 (Δ*yscD* [aa 206–232]) secreted almost normal levels of V antigen and YopM at 37°C in the absence of Ca^{2+} (data not shown). These data indicate that YscC, YscD, and YscG are all necessary for secretion of V antigen and Yops by *Y. pestis*. Apparently, the small 81-bp deletion in *yscD* carried by *Y. pestis* KIM5-3001.13 (Δ*yscD* [aa 206–232]) eliminated a region of YscD that was not critical for V antigen and YopM expression and secretion.

Secretion of YopM expressed from plasmid pTRCM.2. Plasmid pTRCM.2, which contains the *yopM* gene downstream of the *trc* promoter of plasmid pTRC99A, was used to overex-

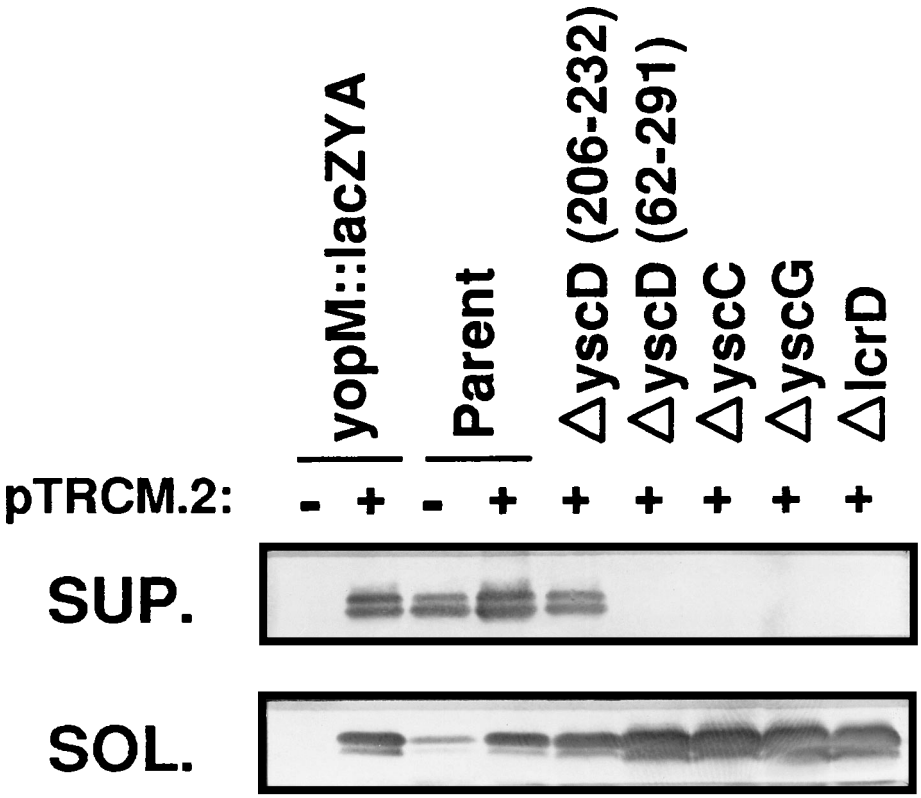

FIG. 4. Immunoblot analysis of YopM in supernatant and soluble fractions from *Y. pestis* KIM5-3233 (*yopM*::*lacZYA*), KIM5-3001 (parent), KIM5-3001.13 (Δ*yscD* [aa 206–232]), KIM5-3001.14 (Δ*yscD* [aa 62–291]), KIM5-3001.12 (Δ*yscC*), KIM5-3001.15 (Δ*yscG*), and KIM5-3001.3 (Δ*lcrD*) with (+) or without (−) plasmid pTRCM.2. *Y. pestis* strains were grown at 37°C in the absence of Ca^{2+}. Expression of YopM from pTRCM.2 was induced by addition of IPTG to 0.1 mM 1 h prior to harvest. Antipeptide antibodies were used to detect YopM in both the culture supernatant fraction (SUP.) and the soluble (cytoplasm plus periplasm) fraction (SOL.).

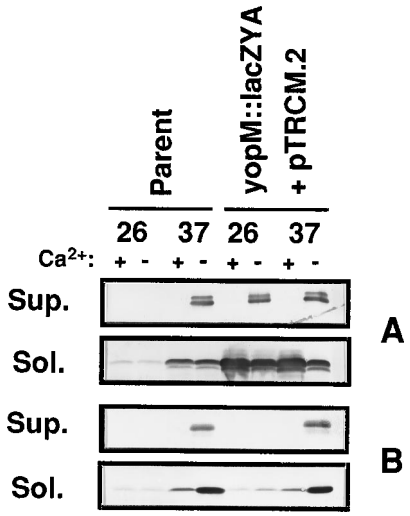

FIG. 5. Immunoblot analysis of YopM and V antigen in supernatant and soluble fractions from *Y. pestis* KIM5-3001 (parent) and KIM5-3233 (*yopM*::*lacZYA*) containing plasmid pTRCM.2. Bacteria were grown at 26°C in the presence (+) or absence (−) of Ca^{2+} for 2 h and then for an additional 4 h at either 26 or 37°C. Expression of YopM from pTRCM.2 was induced by addition of IPTG to 0.1 mM 1 h prior to harvest. Antibodies were used to detect YopM (A) and V antigen (B) in both the culture supernatant fraction (Sup.) and the soluble (cytoplasm plus periplasm) fraction (Sol.).

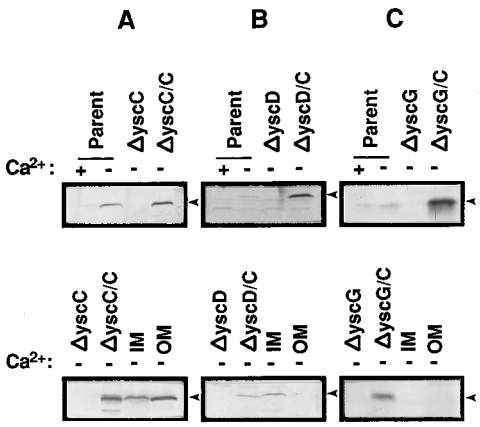

FIG. 6. Localization of YscC, YscD, and YscG by immunoblot analysis. *Y. pestis* KIM5-3001 (parent), KIM5-3001.12 (Δ*yscC*), KIM5-3001.14 (Δ*yscD*), and KIM5-3001.15 (Δ*yscG*) were grown for 6 h following a temperature shift to 37°C in the presence (+) or absence (−) of Ca^{2+}. The *yscC*, *yscD*, and *yscG* mutants were complemented (/C) with plasmids pYSCC1, pYSCD1, and pYSCG1, respectively. Total membrane fractions from these strains and isolated inner membrane (IM) and outer membrane (OM) fractions from *Y. pestis* KIM5 were analyzed for YscC (A), YscD (B), and YscG (C). Antibodies raised against GST-YscC and GST-YscG fusion proteins were used to detect YscC and YscG, respectively. Antipeptide antibodies were used to detect YscD.

press YopM in *Y. pestis* KIM5-3233 (*yopM*::*lacZYA*) and KIM5-3001 and in the *yscC*, *yscD*, *yscG*, and *lcrD* deletion strains. *Y. pestis* strains were grown in TMH without Ca^{2+} at 26°C and then shifted to 37°C for 4 h. Expression of YopM from pTRCM.2 was induced by addition of IPTG to 0.1 mM 1 h prior to harvest. Expression and secretion of YopM were monitored by immunoblot analysis (Fig. 4). As expected, expression of YopM from *Y. pestis* KIM5-3233 (*yopM*::*lacZYA*) could not be detected; however, KIM5-3233 carrying pTRCM.2 expressed and secreted YopM. The parent strain *Y. pestis* KIM5-3001 without pTRCM.2 and KIM5-3001.13 (Δ*yscD* [aa 206–232]) carrying pTRCM.2 expressed and secreted approximately wild-type levels of YopM. *Y. pestis* KIM5-3001 carrying pTRCM.2 expressed and secreted increased amounts YopM. *Y. pestis* KIM5-3001.12 (Δ*yscC*), KIM5-3001.14 (Δ*yscD* [aa 62–291]), KIM5-3001.15 (Δ*yscG*), and KIM5-3001.3 (Δ*lcrD*) carrying pTRCM.2 all expressed high levels of YopM; however, all of the YopM expressed remained in the soluble fraction. These data indicated that YopM expressed from plasmid pTRCM.2 was competent for secretion; however, *yscC*, *yscD*, *yscG*, and *lcrD* deletion mutants were defective in some aspect of the secretion process. These results support a direct role for YscC, YscD, YscG, and LcrD in the secretion of LCR virulence proteins by *Y. pestis*. Apparently, low LCRS protein expression in the *ysc* and *lcrD* deletion mutants was a repercussion secondary to, but not responsible for, the secretion defects found in these mutants. In contrast, it appears that mutations that block LCRS protein secretion also prevent high-level expression of LCRS proteins at 37°C in the absence of Ca^{2+}.

Effect of Ca^{2+} and temperature on secretion of YopM. In the medium TMH, *Y. pestis* requires Ca^{2+} for full in vitro growth yield at 37°C. In addition, the presence of Ca^{2+} in the growth medium at 37°C prevents full expression and secretion of LCRS proteins. Previously, no effects of Ca^{2+} have been documented at temperatures below 34°C. We measured expression and secretion of V antigen (Fig. 5B) and YopM (Fig. 5A) at both 26 and at 37°C by both the parent strain *Y. pestis* KIM5-3001 and *Y. pestis* KIM5-3233 (*yopM*::*lacZYA*) carrying plasmid pTRCM.2. *Yersinia* strains were grown at 26°C in TMH with or without Ca^{2+}. Cultures were then either held at 26°C or shifted to 37°C for an additional 4 h. Expression of YopM from plasmid pTRCM.2 was induced by addition of IPTG to 0.1 mM 1 h prior to harvest. No expression of V antigen or YopM by *Y. pestis* KIM5-3001 was detected at 26°C; however, at 37°C, thermal induction of V antigen and YopM expression was detected in both the presence and absence of Ca^{2+}. As expected, secretion of V antigen and YopM was observed only at 37°C in the absence of Ca^{2+}. Addition of IPTG to cultures of *Y. pestis* KIM5-3233 (*yopM*::*lacZYA*) carrying pTRCM.2 induced high levels of YopM expression at both 26 and 37°C regardless of the presence or absence of Ca^{2+}. Interestingly, YopM expressed from plasmid pTRCM.2 was secreted into the culture supernatant at both 26 and 37°C in the absence of Ca^{2+}; however, the presence of Ca^{2+} prevented secretion of YopM at both temperatures. No significant differences in the expression and secretion of V antigen were found between the parent strain and YopM$^-$ KIM5-3233 carrying pTRCM.2. These data indicate that a functional secretion apparatus is present in *Y. pestis* at 26°C and that Ca^{2+} acts to block the secretion process at both 26 and 37°C. The inability to detect secretion of the V antigen and Yops at 26°C in the parent strain may be due to the low expression of these proteins at this temperature.

Identification of the *yscC*, *yscD*, and *yscG* gene products. Polyclonal antisera directed against a synthetic peptide corresponding to amino acids 396 to 408 of the predicted YscD amino acid sequence (32) or against the GST-YscC, GST-YscD, or GST-YscG fusion protein were used to identify the *yscC*, *yscD*, and *yscG* gene products in immunoblots prepared from fractionated *Y. pestis* cultures. Soluble (periplasm and cytoplasm), total membrane, and culture supernatant fractions isolated from the *yscC*, *yscD*, and *yscG* deletion mutants and from the mutants complemented with plasmids pYSCC1, pYSCD1, and pYSCG1 in *trans* were analyzed for the presence of YscC, YscD, and YscG, respectively. YscC, YscD, and

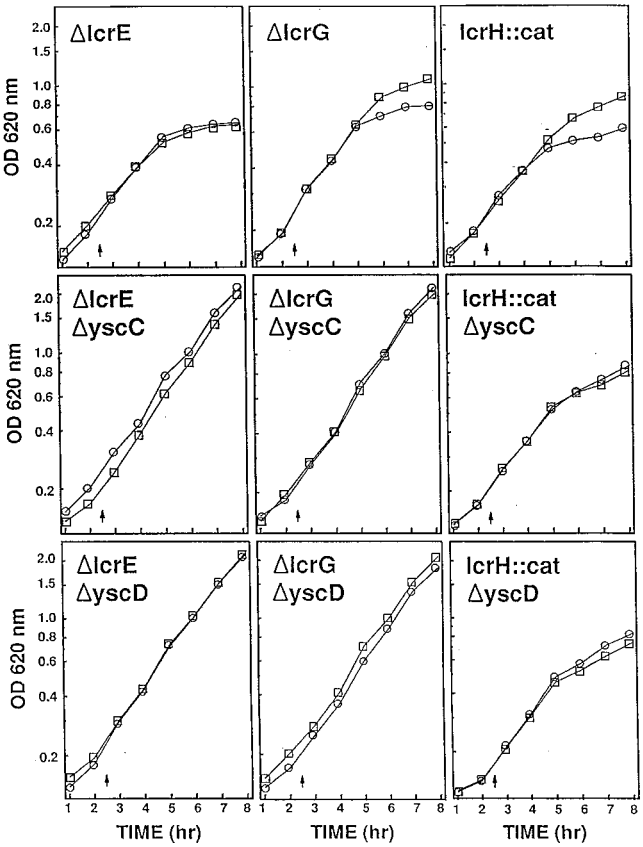

FIG. 7. Growth of *Y. pestis* KIM5-3001.6 (Δ*lcrE*), KIM5-3001.5 (Δ*lcrG*), KIM5-3401 (*lcrH*::*cat*), KIM5-3001.12.1 (Δ*lcrE* Δ*yscC*), KIM5-3001.12.2 (Δ*lcrG* Δ*yscC*), KIM5-3001.12.3 (*lcrH*::*cat* Δ*yscC*), KIM5-3001.14.1 (Δ*lcrE* Δ*yscD*), KIM5-3001.14.2 (Δ*lcrG* Δ*yscD*), and KIM5-3001.14.3 (*lcrH*::*cat* Δ*yscD*). *Y. pestis* strains were grown in the presence or absence of Ca^{2+} in the defined medium TMH. The temperature was shifted from 26 to 37°C when the OD_{620} of the culture reached approximately 0.2 (arrows). Symbols: open squares, plus Ca^{2+}; open circles, minus Ca^{2+}.

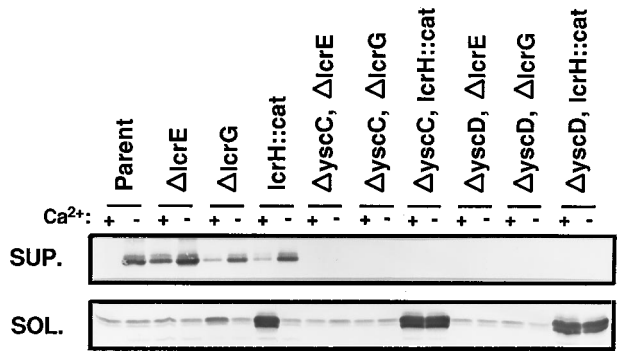

FIG. 8. Immunoblot analysis of YopM in supernatant and soluble fractions from *Y. pestis* KIM5-3001 (parent), KIM5-3001.6 (Δ*lcrE*), KIM5-3001.5 (Δ*lcrG*), KIM5-3401 (*lcrH*::*cat*), KIM5-3001.12.1 (Δ*lcrE*, Δ*yscC*), KIM5-3001.12.2 (Δ*lcrG* Δ*yscC*), KIM5-3001.12.3 (*lcrH*::*cat* Δ*yscC*), KIM5-3001.14.1 (Δ*lcrE* Δ*yscD*), KIM5-3001.14.2 (Δ*lcrG* Δ*yscD*), and KIM5-3001.14.3 (*lcrH*::*cat* Δ*yscD*) grown at 37°C in both the presence (+) and absence (−) of Ca^{2+}. Antipeptide antibodies were used to detect YopM in both the culture supernatant fraction (SUP.) and the soluble (cytoplasm plus periplasm) fraction (SOL.).

YscG were localized to the membrane fraction, with the exception that a small amount of YscC expressed from pYSCC1 was found in the culture supernatant fraction, and approximately half of the YscG expressed from pYSCG1 was found in the soluble fraction (data not shown).

Total membranes isolated from the parent strain *Y. pestis* KIM5 grown at 37°C in the presence or absence of Ca^{2+} were analyzed for the presence of YscC (Fig. 6A), YscD (Fig. 6B), and YscG (Fig. 6C). Total membranes isolated from the *yscC*, *yscD*, and *yscG* deletion mutants and from the mutants complemented with plasmids pYSCC1, pYSCD1, and pYSCG1 in *trans* were used as controls for the presence or absence of YscC, YscD, and YscG, respectively.

The antiserum directed against GST-YscC (Fig. 6A) identified a Ca^{2+}-regulated ca. 63-kDa protein present in the membrane fraction of *Y. pestis* KIM5-3001. The molecular mass predicted from translation of *yscC* is 67.1 kDa before signal peptide cleavage and 64.2 kDa following proteolytic processing (32, 47). The immunoreactive protein was missing from the membranes of the *yscC* deletion mutant KIM5-3001.12 (Δ*yscC*). Plasmid pYSCC1 provided in *trans* restored expression of the *yscC* gene product. A small amount of ca. 64-kDa unprocessed YscC was also visible in YscC-expressing strains. Interestingly, a large proportion of the immunoreactive material identified by the antiserum directed against GST-YscC remained as an insoluble complex at the top of the stacking gel (data not shown).

Antisera raised against the YscD synthetic peptide (Fig. 6B) and GST-YscG (Fig. 6C) identified a ca. 46-kDa protein and a ca. 13-kDa protein as the gene products of *yscD* and *yscG*, respectively. The molecular masses of the proteins identified correspond to the approximate sizes expected from translation of *yscD* and *yscG*. The presence of Ca^{2+} in the growth medium decreased expression of these proteins ca. two- to threefold in *Y. pestis* KIM5-3001. The putative *yscD* gene product was missing from the membrane fraction of the *yscD* deletion mutant KIM5-3001.14. Similarly, the putative *yscG* gene product was not expressed by the *yscG* deletion mutant KIM5-3001.15. Plasmids providing *yscD* or *yscG* in *trans* restored expression of the respective gene products.

Isolated inner and outer membrane preparations from *Y. pestis* KIM5 were analyzed for the presence of YscC, YscD, and YscG. YscC was found predominately in the outer membrane fraction (Fig. 6A). This localization was not unexpected, for YscC shares significant amino acid homology with the PulD superfamily of proteins, members of which are involved in secretion of macromolecules across the outer membrane (27). Interestingly, a small amount of YscC was also found in the trichloroacetic acid-precipitated culture supernatant fraction (data not shown). The source of this protein is unknown; however, we speculate that YscC could be associated with membrane vesicles or blebs present in culture supernatants not subjected to ultracentrifugation. Some YscC was also detected in the inner membrane fraction and probably represents outer membrane contamination of the inner membrane preparation. YscD, which has an amino-terminal hydrophobic domain, was found almost exclusively in the inner membrane preparation (Fig. 6B). YscG, which also possesses an amino-terminal hydrophobic domain, was difficult to detect in isolated membrane fractions, and its localization varied between different preparations of inner and outer membranes. Moreover, it also was found in the soluble fraction (data not shown). These findings suggest that YscG is loosely associated with the membrane and is easily lost.

Construction of *Y. pestis* double-mutant strains with deletion mutations in *yscC* or *yscD* and mutations in *lcrE*, *lcrG*, or *lcrH*. In contrast to the Ca^{2+}-independent growth and constitutively repressed phenotype associated with the *Y. pestis yscC*, *yscD*, *yscG*, and *lcrD* mutants (secretion mutants), *Y. pestis* strains

with mutations in *lcrE*, *lcrG*, and *lcrH* exhibited "Ca^{2+}-blind" growth and constitutively induced V antigen and Yop net mRNA and protein expression (76). The effect of having both classes of mutations in the same background was investigated to determine where the secretion mutations affect transcriptional regulation; to this end, strains that carried mutations in *yscC* or *yscD* and mutations in *lcrE*, *lcrG*, or *lcrH* were constructed. The Δ*yscC* [aa 141–454] deletion mutation was moved into pCD1 of *Y. pestis* KIM5-3001.6 (Δ*lcrE*), KIM5-3001.5 (Δ*lcrG*), and KIM5-3401 (*lcrH*::*cat*) by allelic exchange. Similarly, the Δ*yscD* [aa 62–291] deletion mutation was introduced into pCD1 of these same strains. The two sets of double mutants generated from these experiments were analyzed for LCR growth phenotype (Fig. 7) and LCRS protein expression and secretion of V antigen and Yops (Fig. 8). In addition, the Δ*lcrD* [aa 618–644] deletion mutation was moved into pCD1 of *Y. pestis* KIM5-3001.6, creating an *lcrD-lcrE* double mutant designated KIM5-3001.6.1.

Growth phenotype of double mutants. The parent strain *Y. pestis* KIM5-3001 exhibited wild-type Ca^{2+}-dependent growth at 37°C (Fig. 2). The single-mutant strains *Y. pestis* KIM5-3001.12 (Δ*yscC*) and KIM5-3001.14 (Δ*yscD*) failed to enter growth restriction at 37°C in the presence and absence of Ca^{2+} (Ca^{2+}-independent growth phenotype [Fig. 2]). In contrast, the single-mutant strains *Y. pestis* KIM5-3001.6 (Δ*lcrE*), KIM5-3001.5 (Δ*lcrG*), and KIM5-3401 (*lcrH*::*cat*) entered growth restriction at 37°C in the presence and absence of Ca^{2+} (Ca^{2+}-blind growth phenotype [Fig. 7]). The double-mutant strains *Y. pestis* KIM5-3001.12.1 (Δ*yscC* Δ*lcrE*), KIM5-3001.12.2 (Δ*yscC* Δ*lcrG*), KIM5-3001.14.1 (Δ*yscD* Δ*lcrE*), KIM5-3001.14.2 (Δ*yscD* Δ*lcrG*), and KIM5-3001.6.1 (Δ*lcrD* Δ*lcrE*) exhibited the Ca^{2+}-independent growth phenotype (growth data for KIM5-3001.6.1 not shown). In contrast, the $LcrH^-$ double-mutant strains *Y. pestis* KIM5-3001.12.3 (Δ*yscC lcrH*::*cat*) and KIM5-3001.14.3 (Δ*yscD lcrH*::*cat*) retained the Ca^{2+}-blind growth phenotype associated with the *lcrH*::*cat* mutant. These results demonstrated that the Ca^{2+}-independent growth phenotype associated with the *yscC* and *yscD* deletion strains was dominant over the Ca^{2+}-blind growth phenotype associated with the *lcrE* and *lcrG* deletion mutants. This finding indicated that a functioning secretion apparatus was essential for the Ca^{2+}-blind growth phenotype associated with the *lcrE* and *lcrG* mutations. In contrast, *yscC* and *yscD* mutations had no effect on the Ca^{2+}-blind growth phenotype associated with an $LcrH^-$ strain.

Secretion of V antigen and YopM by double mutants. Single mutants *Y. pestis* KIM5-3001.6 (Δ*lcrE*), KIM5-3001.5 (Δ*lcrG*), and KIM5-3401 (*lcrH*::*cat*) expressed and secreted high levels of V antigen (not shown) and YopM at 37°C in the absence of Ca^{2+} (Fig. 8). *Y. pestis* KIM5-3001.6 (Δ*lcrE*) also expressed and secreted high levels of V antigen and YopM in the presence of Ca^{2+}; however, the culture supernatants of *Y. pestis* KIM5-3001.5 (Δ*lcrG*) and KIM5-3401 (*lcrH*::*cat*) grown in the presence of Ca^{2+} contained only low levels of these proteins. No secretion of V antigen or YopM was detected in the culture supernatant of the parent strain KIM5-3001 grown in the presence of Ca^{2+}. The Ca^{2+}-dependent block on V antigen and Yop protein secretion resulted in a buildup of secretion substrate in the soluble fraction of the *lcrH* and *lcrG* mutants. The accumulation of YopM in the soluble fraction of *Y. pestis* KIM5-3401 (*lcrH*::*cat*) grown in the presence of Ca^{2+} was particularly dramatic.

No secretion of V antigen or YopM was detected from any of the double-mutant strains of *Y. pestis*; however, overall expression of these proteins varied dramatically among the strains tested. *Y. pestis* KIM5-3001.12.1 (Δ*yscC* Δ*lcrE*), KIM5-3001.12.2 (Δ*yscC* Δ*lcrG*), KIM5-3001.14.1 (Δ*yscD* Δ*lcrE*), KIM5-3001.14.2 (Δ*yscD* Δ*lcrG*), and KIM5-3001.6.1 (Δ*lcrD* Δ*lcrE*) expressed only basal levels of V antigen and YopM (data for KIM5-3001.6.1 not shown). In contrast, *Y. pestis* KIM5-3001.12.3 (Δ*yscC lcrH*::*cat*) and KIM5-3001.14.3 (Δ*yscD lcrH*::*cat*), both of which exhibited a Ca^{2+}-blind growth phenotype, expressed high levels of these proteins, indicating that these mutants were derepressed for LCRS protein expression and defective in LCRS protein secretion. The accumulation of YopM in both of these strains was similar to that seen in the *lcrH* mutant grown in the presence of Ca^{2+}.

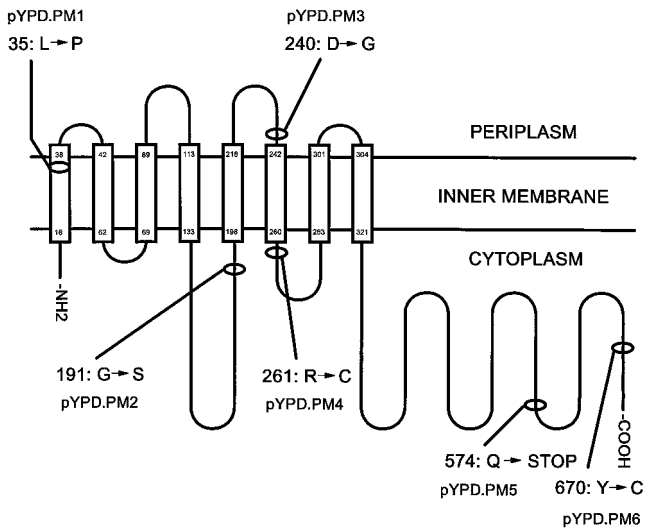

FIG. 9. Locations of point mutations in *lcrD* of plasmids pYPD.PM1 through pYPD.PM6. The number of the amino acid affected by each point mutation and the new amino acid or stop codon encoded by each single-base-pair change are shown on a model of LcrD. The eight membrane-spanning segments are represented by rectangles, with the numbers inside indicating the amino acid residues at each end.

Isolation, identification, and characterization of point mutations in *lcrD*. *Y. pestis* strains carrying insertions or deletions in *lcrD* were unable to secrete LCRS proteins, exhibited a constitutively repressed phenotype (no transcriptional induction of LCRS operons at 37°C regardless of the Ca^{2+} concentration), and showed a Ca^{2+}-independent growth phenotype (56). We decided to investigate whether the growth, transcription, and secretion defects associated with these deletion mutants could be genetically separated. To do so, we generated random point mutations in plasmid pYPD by using *E. coli* LE30 (*mutD5*). Point mutations in *lcrD* were then selected for by their inability to restore Ca^{2+}-dependent growth to *Y. pestis* KIM5-3001.3 (Δ*lcrD* [aa 618–644]). The location of each of the point mutations was determined by the chemical mismatch cleavage method (16), and the actual base pair change was determined by DNA sequencing. Finally, the *lcrD* point mutants were characterized in regard to LcrD expression, transcriptional induction of LCRS operons, and LCRS protein secretion (data not shown).

Figure 9 shows the amino acid changes determined by the six different point mutations isolated in *lcrD* of *Y. pestis*. Point mutations were scattered throughout the *lcrD* coding sequence; however, all of the point mutants exhibited defects in growth, LCRS operon transcription, and LCRS protein secretion. The point mutation in plasmid pYPD.PM5 (aa 574 Q→stop) apparently destabilized the *lcrD* protein product, because no protein was detected by immunoblot analysis. The

severity of the growth, expression, and secretion defects varied among the different point mutants; however, those mutants exhibiting severe defects in secretion also showed severe defects in transcriptional induction and growth response, and vice versa. These results support the hypothesis that LcrD, YscC, YscD, and YscG are directly involved in the secretion of V antigen and Yops. Furthermore, the fact that the extent of defects in expression of V antigen and Yops for each point mutant correlated with the level of defect in secretion for that mutant suggested that the expression defects are secondary to the defects in the secretion process.

DISCUSSION

We have previously shown that deletions within *lcrD* (56) and *yscR* (21) of *Y. pestis* prevent full transcriptional induction of LCRS operons and block secretion of LCRS proteins (see Fig. 1 for the locations of *lcrDR* and the *ysc* operon containing *yscN* through *yscU*). In addition, strains that carried these deletions were no longer dependent upon Ca^{2+} for in vitro growth at 37°C (Ca^{2+}-independent growth phenotype). The present study focused on *yscC*, *yscD*, and *yscG* of the *yscA-M* operon (32, 47). We constructed internal in-frame deletions in each of these loci in pCD1 of *Y. pestis* KIM5-3001.

The *yscC*, *yscD*, and *yscG* deletion mutants failed to show induction of LCRS protein expression at 37°C in the absence of Ca^{2+} and were unable to export LCRS proteins (V antigen and Yops). These results established roles for YscC, YscD, and YscG in secretion and high-level expression of LCRS proteins in *Y. pestis*. YopM was not secreted even when overexpressed from pTRCM.2. This result indicated that the deletions in *yscC*, *yscD*, and *yscG* directly prevented secretion of LCRS proteins in *Y. pestis*. The pleiotropic defects associated with the deletions were analogous to defects described for the Ca^{2+}-independent *lcrD* and *yscR* deletion mutants, suggesting that LcrD, YscR, YscC, YscD, and YscG are all components of the *Y. pestis* LCRS export apparatus.

Interestingly, in the absence of Ca^{2+}, YopM overexpressed from pTRCM.2 was secreted at 26°C as well as at 37°C. The presence of Ca^{2+} blocked the secretion of YopM at both temperatures. The results are the first demonstration that the LCRS secretion system, as well as the mechanism for regulating its activity, is present and fully functional at 26°C, suggesting that expression of components of the secretion apparatus may be regulated less stringently than the anti-host proteins secreted by the system. The ability to independently express and secrete individual Yops at 26°C will provide a system for studying the interactions of individual secreted LCRS proteins with the secretion apparatus in the absence of other secreted LCRS proteins. In addition, selective secretion of an independently expressed LCRS protein at 26°C may provide a simple method for purification of the protein from a culture supernatant devoid of other LCRS proteins. We are currently assessing the feasibility of this method.

The Ca^{2+}-dependent downregulation of transcription and block in LCRS protein secretion at 37°C require the products of at least four *lcr* genes. These components comprise the identified members of a Ca^{2+}-mediated negative regulatory pathway, which includes the *lcrE*, *lcrG*, *lcrQ*, and *lcrH* gene products. *Y. pestis* strains carrying insertions or deletions in these genes exhibit a Ca^{2+}-blind growth phenotype and are derepressed at 37°C in the presence of Ca^{2+} for LCRS protein expression (*lcrE*, *lcrG*, *lcrQ*, and *lcrH* mutants) and secretion (mutants for *lcrE*, *lcrG*, and *lcrQ* [the *lcrQ* mutant was defective only for secretion of V antigen and YopD]) (5, 23, 59, 63, 73, 81). Strains of *Y. pestis* carrying mutations in the secretion genes *yscC*, *yscD*, *yscG*, *yscR*, and *lcrD* express only basal levels of LCRS proteins (constitutively repressed phenotype) and fail to export these proteins to the culture supernatant. We investigated which class of mutations was dominant by introducing *yscC* or *yscD* mutations (constitutively repressed phenotype) into *Y. pestis* strains already carrying mutations in *lcrE*, *lcrG*, and *lcrH* (constitutively induced phenotype). The constitutively repressed phenotype associated with the *yscC* and *yscD* mutants was expressed over the constitutively induced phenotype associated with the *lcrE* and *lcrG* mutants. These results indicated that the constitutively induced phenotype associated with *lcrE* and *lcrG* mutants was dependent on a functional LCR secretion apparatus or that components of the secretion apparatus have independent regulatory functions. We hypothesized that the secretion process itself must be directly involved in the regulation of the LCR, for it was unlikely that secretion system components were all bifunctional, having separate roles in both secretion and regulation. This was particularly unlikely for the outer membrane protein YscC.

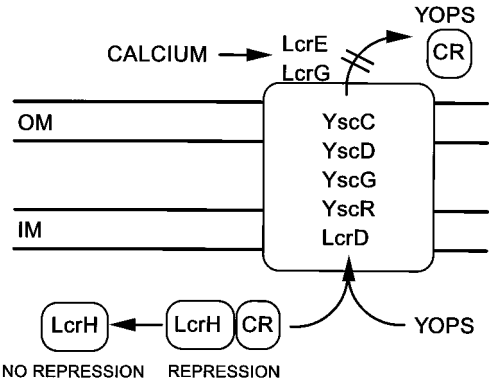

FIG. 10. Model proposed to explain the relationship between the LCR-specific secretion system and Ca^{2+}-dependent regulation of LCRS transcription in vitro. At 37°C in the presence of Ca^{2+}, the secretion of LCRS proteins is blocked. This block requires the presence of Ca^{2+}, LcrE, and possibly LcrG. The secretion block prevents the secretion of a negative regulator, which we have termed a corepressor (CR). Thus, in the presence of Ca^{2+}, the corepressor and LcrH are both inside the cell, which results in a block of LCRS transcription by an unknown mechanism (REPRESSION). Mutations in genes encoding components of the secretion apparatus would prevent secretion in both the presence and absence of Ca^{2+}, resulting in a constitutively repressed phenotype. In the absence of Ca^{2+}, secretion is allowed, resulting in secretion of the corepressor from the cell. The secretion of the corepressor removes the block on transcription (NO REPRESSION) and allows high-level expression and secretion of LCRS proteins. Mutations in *lcrE* or *lcrG* allow secretion in both the presence and absence of Ca^{2+}, resulting in a constitutively induced phenotype.

Further evidence against separate unrelated regulatory functions for components of the secretion apparatus was obtained from strains bearing point mutations of *lcrD*, which showed defects in both expression and secretion of LCRS proteins. Furthermore, mutations which caused severe defects in LCRS protein secretion also caused severe defects in LCRS protein expression, and vice versa. This inability to genetically separate effects of *lcrD* mutations on secretion and gene expression further supports the hypothesis directly linking the secretion process to the regulation of LCRS protein expression.

Interestingly, the Ca^{2+}-blind growth and constitutively induced phenotype associated with the *lcrH*::*cat* mutation was expressed over the Ca^{2+}-independent growth and constitutively repressed LCRS expression associated with the *yscC* and *yscD* mutations. These results indicated that the constitutive induction associated with the *lcrH* mutation was not dependent on a functional LCRS secretion apparatus. Thus, we place

LcrH's negative regulatory effect after that of LcrE and LcrG in the Ca^{2+}-dependent negative regulatory pathway, in agreement with results obtained through overexpression of LcrG (73), LcrQ (63), and LcrH (5) in various mutant backgrounds. Together, these data support the hypothesis that LcrH exerts its negative effect within the cell and close to the level of transcription. However, recent evidence that LcrH may function as a Syc for YopD and perhaps also YopB (82) supports the idea that LcrH's negative regulatory effect may be indirect. The demonstration of growth restriction in strains defective for LCRS protein secretion ruled out the secretion process per se as the cause of this in vitro phenomenon. Apparently, high-level expression of V antigen and Yops is primarily responsible for triggering this growth response.

In contrast to *Y. pestis* KIM5-3001.12.3 and KIM5.3001.14.3, all of the *Y. pestis* strains examined that express functional LcrH required a functional secretion system for induction of LCRS operon transcription, indicating that the negative regulatory effects of LcrH on LCRS transcription normally are counteracted only if a functional secretion system is available. This observation indicated that it was the repressive effect of LcrH that is normally overridden through some action of the secretion apparatus. This hypothesis correlates well with the fact that induction is normally seen only at 37°C in the absence of Ca^{2+}, conditions in which the secretion system is not blocked. We hypothesize that a component (corepressor?) that normally acts with LcrH in the presence of calcium to prevent LCRS transcription is secreted in the absence of Ca^{2+}, allowing high-level expression of LCRS proteins (Fig. 10). Possibly, LcrH, in its role as SycD, promotes a negative regulatory effect in which YopD participates, and YopD would be represented by CR (corepressor) in Fig. 10. This hypothesis would explain why *Y. pestis* secretion mutants have a constitutively repressed phenotype that mimics the situation found in wild-type *Y. pestis* grown in the presence of Ca^{2+}. This would also explain why *lcrE* and *lcrG* mutants, which allow secretion in both the presence and absence of Ca^{2+}, show a constitutively induced phenotype. In summary, we hypothesize that it is the secretion of a negative regulatory component that is ultimately responsible for induction of the LCRS. This hypothesis infers that the role of Ca^{2+} in the LCR is to function with LcrE (and/or LcrG) to prevent LCRS protein secretion, including the secretion of a corepressor. Related ideas of feedback regulation by a Yop and of secretion of a negative regulator have been proposed previously (13, 63).

The sequence of events hypothesized to lead to transcriptional induction of the LCRS (secretion of a corepressor) is similar to the sequence of events involved in coupling flagellin synthesis to flagellar assembly in *S. typhimurium* (secretion of a negative regulator) (35). In this system, the flagellum-specific sigma factor (σ^{28}) is inhibited by an anti-sigma factor (FlgM) (52); however, upon completion of a functional basal body complex, FlgM is secreted, thereby allowing σ^{28} to function. Thus, the secretion of a negative regulator may prove to be a common regulatory theme in bacterial systems.

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