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Differential Effects of Deletions in *lcrV* on Secretion of V Antigen, Regulation of the Low-Ca^{2+} Response, and Virulence of *Yersinia pestis*

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The *Yersinia pestis* V antigen is necessary for full induction of low-calcium response (LCR) stimulon virulence gene transcription, and it also is a secreted protein believed to have a direct antihost function. We made four nonpolar deletions in *lcrV* of *Y. pestis* to determine if secretion, regulation, and virulence functions could be localized within the V antigen (LcrV). Deletion of amino acids 25 to 40 caused secretion of LcrV to be decreased in efficiency; however, removal of residues 108 to 125 essentially abolished LcrV secretion. Neither mutation had a significant effect on LCR regulation. This showed that LcrV does not have to be secreted to have its regulatory effect and that the internal structure of V antigen is necessary for its secretion. Both mutants were avirulent in mice, showing that the regulatory effect of LcrV could be separated genetically from its virulence role and raising the possibility that residues 25 to 40 are essential for the virulence function. This study provides the best genetic evidence available that LcrV per se is necessary for the virulence of *Y. pestis*. The repressed LCR phenotype of a mutant lacking amino acids 188 to 207 of LcrV raised the possibility that the deleted region is necessary for regulation of LCR induction; however, this mutant LcrV was weakly expressed and may not have been present in sufficient amounts to have its regulatory effect. In double mutants containing this mutant *lcrV* and also lacking expression of known LCR negative regulators (LcrG, LcrE, and LcrH), full induction of the LCR occurred in the absence of functional LcrV, indicating that LcrV promotes induction not as an activator per se but rather by inhibiting negative regulators.

The genus *Yersinia* is composed of three pathogenic species, *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. As facultative intracellular parasites, they cause invasive disease in humans and other mammals, ranging from bubonic plague to gastroenteritis (9, 12). All three species have been shown to have a common virulence-regulatory response called the low-calcium response (LCR) (6, 63). The LCR is due to related virulence plasmids of ca. 75 kbp (5, 15) and has two manifestations in vitro. (i) At 37°C, millimolar amounts of Ca^{2+} downregulate the expression of the virulence proteins V antigen (LcrV) and *Yersinia* outer proteins (Yops) and abolish V-antigen and Yop secretion (8, 26, 41, 59, 60). (ii) In certain media (19), the bacteria require millimolar amounts of calcium for maximal growth yield at 37°C (22, 74). In the absence of Ca^{2+} at this temperature, a metabolic downshift takes place and bacterial growth stops within two generations. This phenomenon is called growth restriction (75). During restriction, V antigen and Yops are maximally expressed and secreted, even though net protein synthesis has ceased (2, 5, 6). We do not know what causes restriction, but in our work this property serves as a convenient marker for strong induction of LCR-regulated virulence genes. When Ca^{2+} is present, there is only partial induction of those virulence genes and no secretion of LcrV and Yops.

Evidence is accumulating that during systemic infection, the pathogen is located mainly extracellularly, where abundant Ca^{2+} would be present (63). Yersiniae may be able to attach to the surface of host cells and vectorially target functional Yops into those cells, causing paralysis of phagocytosis (17, 48). Therefore, it is apparent that in our in vitro studies, the presence of Ca^{2+} may simulate the condition that yersiniae experience when growing free in blood or interstitial fluid, and the absence of Ca^{2+} in vitro may simulate the relevant in vivo signals encountered at the host cell surface (17, 63, 64).

The Ca^{2+} dependence region, encompassing ca. 25 kbp of the LCR plasmids, regulates the expression and secretion of LcrV and Yops in response to environmental stimuli (63). Thermal induction of LcrV and Yops expression is mediated by the LcrF product of this region (11, 14, 23, 72); operons subject to regulation by LcrF belong to the *yop* regulon. Operons that are coordinately downregulated at 37°C by Ca^{2+} are considered to belong to an LCR stimulon (LCRS) (63). The putative regulator that mediates the Ca^{2+}-elicited repression has not been identified, hence the stimulon designation.

LcrE (also called YopN) (18, 68, 73), LcrG (52), and LcrQ (47) are secreted by yersiniae and have been suggested to be part of a Ca^{2+}-sensing mechanism (18, 47, 63). Together with the cytoplasmically located protein LcrH (2, 44), they participate in downregulation of the LCRS in response to Ca^{2+}. Mutational inactivation of their genes leads to the Ca^{2+}-blind, constitutively induced phenotype at 37°C (44, 52, 73), characterized by growth restriction and maximal V-antigen and Yops expression at 37°C irrespective of the Ca^{2+} concentration.

In the absence of Ca^{2+}, four loci are necessary for achievement of full thermal induction of LcrV and Yops expression: these encode the Yop secretion mechanism (*lcrD* and the two large *ysc* operons [16, 20, 31, 38, 69]) and LcrV itself. Mutations in these genes abolish the Ca^{2+} requirement for growth (Ca^{2+}-independent phenotype). Such mutants show only partial induction of LCRS operons at 37°C. Secretion mechanism (*lcrD ysc*) mutants also do not secrete LCRS proteins.

Yops are thought to have direct antihost functions that protect extracellularly exposed bacteria by disarming natural host

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defense mechanisms (e.g., YopE, YopH, and YopM) (4, 17, 21, 27, 46, 48). Their secretion involves an N-terminal (50- to 100-amino-acid [aa]) determinant that directs transport without processing (30). In *Y. pestis*, Yops are attacked by a plasminogen activator protease (Pla), encoded on a species-specific ca. 9.5-kbp pPCP1 plasmid and, as a result, do not accumulate on the bacterial surface in vitro (49, 54–56).

The V antigen (LcrV) has intrigued investigators ever since its discovery as a serological reactivity present only in fully virulent strains of *Y. pestis* (8). Polyclonal and monoclonal antibodies specific for the V antigen of *Y. pestis* passively protect against experimental plague (7, 26, 65, 66). A protective epitope responsible for this immunity resides within the central portion of the V antigen (aa 176 to 276) (34). Limited evidence suggests that anti-V antibodies may neutralize an antihost function of LcrV in the extracellular environment. LcrV is secreted from yersiniae (49) and accumulates outside the cells even when Pla is present (7, 57). Moreover, the V antigen has never been found to associate with the bacterial surface as do Yops in enteropathogenic yersiniae or in Pla$^-$ *Y. pestis* (58).

The target and mechanism of LcrV's action against host defenses remain obscure. Une and Brubaker (65) showed that a V-antigen-containing extract could prolong the persistence in mice of *Y. pestis* lacking its LCR plasmid. Brubaker has proposed that the V antigen functions to delay the onset of cell-mediated immunity (65, 66). In contrast to mice infected with *Y. pestis* lacking its LCR plasmid, mice infected with Lcr$^+$ yersiniae fail to develop granulomas, to produce interferon γ and tumor necrosis factor α, and to rapidly eliminate the bacteria (35, 61, 66). This indicates that the *Y. pestis* infection imposes LCR-mediated immunosuppression that compromises the host cell-mediated immune response but does not identify which LCR plasmid-encoded product(s) is responsible.

The V antigen is encoded by the *lcrGVH-yopBD* operon situated in the Ca^{2+} dependence region of the LCR plasmids (2, 36, 43). The characterization of nonpolar mutants with in-frame deletions in *lcrV* has shown that the V antigen is necessary for full induction of the LCRS (2, 42). LcrV$^-$ yersiniae are avirulent in mice, but because their mutations affected the expression of many LCRS-encoded products, those mutants did not provide genetic proof of the V antigen's role as a virulence protein.

To learn more about the V antigen and localize regions of this protein involved in regulation of the LCR and in the virulence of *Y. pestis*, we created and characterized a set of *lcrV* mutants carrying nonpolar deletions that were smaller than previously described (Δ9 to 18 aa versus Δ83 or Δ198 aa). Further characterization of one of our *lcrV* deletions in various LCR mutant backgrounds (LcrH$^-$, LcrE$^-$, and LcrG$^-$) supported a model for LcrV's regulatory role in which LcrV counteracts a negative regulatory component in the bacterial cytoplasm.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Table 2 summarizes the phenotypes of all of the *Y. pestis* strains used lacking complementing plasmids.

Media and growth conditions. *Escherichia coli* strains were grown in L broth or on L agar medium (13). Heart infusion broth and Tryptose blood agar base media (Difco Laboratories, Detroit, Mich.) were used for routine cultivation of *Y. pestis* strains. For growth experiments, protein isolations, and β-galactosidase assays, *Yersinia* strains were grown in TMH defined liquid medium (59) supplemented with 2.5 mM $CaCl_2$ as indicated in the figure legends. This medium is one that permits expression of the LCR restrictive growth response at 37°C in the absence of Ca^{2+}. *Y. pestis* strains were pregrown at 26°C as described by Straley and Bowmer (59) for about eight generations in the exponential phase. Final cultures were initiated by appropriate dilutions, and after about two generations of growth, when the A_{620} reached 0.2 to 0.3, the temperature was shifted to 37°C. Absorbance was monitored at hourly intervals. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations unless indicated otherwise: 50 μg/ml for ampicillin, 25 μg/ml for kanamycin, 12.5 μg/ml for tetracycline-HCl, and 200 μg/ml for streptomycin.

β-Galactosidase assays. β-Galactosidase activity was assayed colorimetrically (32). The data presented here (see Fig. 2B) are average values of two experiments, each consisting of assays carried out in duplicate.

DNA techniques. Transformation of *E. coli* was done by a standard $CaCl_2$ procedure (29). Transformation of *Y. pestis* or *E. coli* by electroporation was carried out as described by Perry et al. (37). Plasmid DNA was isolated on a small scale by the method of Kado and Liu (24) or Birnboim and Doly (3) and on a large scale with the Qiagen kit (Qiagen Inc., Studio City, Calif.). Restriction endonuclease analysis and cloning were accomplished by standard methods (29). DNA fragments were resolved in 40 mM Tris-HCl (pH 8.0)–20 mM sodium acetate–2 mM Na_2-EDTA in 0.8 to 2.0% [wt/vol] agarose gels (Sigma) or in 4% (wt/vol) gels consisting of a mixture of 3% (wt/vol) NuSieve GTG and 1% (wt/vol) SeaKem GTG agarose (FMC BioProducts, Rockland, Maine). DNA fragments were purified from agarose gels with Qiaex kits (Qiagen). PCRs used to create three of the four deletions presented in this report were performed with the GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, Conn.). PCRs were performed in accordance with the manufacturer's protocol, except that for reactions with long primers (PΔ1, PΔ2, and PΔ3 [described below]), glycerol and nucleotides were present in final concentrations of 5% (vol/vol) and 100 mM, respectively. PCR products were isolated after 25 to 30 cycles of three 1-min reactions performed at 94, 55, and 72°C. PCR fragments were purified by isolation from agarose gels with the Qiaex kit (Qiagen) or with Centricon 100 microconcentrators (Amicon, Danvers, Mass.) in accordance with the manufacturer's protocols. Confirmatory sequencing of the DNA regions carrying in vitro-made mutations was performed by the dideoxy-chain termination method (50) on double-stranded DNA templates with the Sequenase 2.0 Sequencing Kit (United States Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP from New England Nuclear Corp. (Boston, Mass.). Oligonucleotides for sequencing were sequence-specific primers. All of the primers used in this work were synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington).

Construction of nonpolar mutations in *lcrV*. We used the PCR method of Vallette et al. (67) to introduce three nonpolar deletions into *lcrV* in pES6-1, which contains most of the *Y. pestis lcrGVH-yopBD* operon cloned into pUC19* (Table 1). This method employs two primers for the synthesis of a PCR fragment, one carrying an extensive internal deletion and another, regular one. The primers and deletions were planned in the vicinity of restriction sites to simplify the exchange of appropriate DNA fragments. PCR fragments 315 bp long carrying the *lcrV* deletion (bp 73 to 120; aa 25 to 40), 309 bp long carrying the *lcrV* deletion (bp 322 to 375; aa 108 to 125), and 216 bp long carrying the *lcrV* deletion (bp 562 to 621; aa 188 to 207) were synthesized with the primer pairs PΔ1-P1, PΔ2-P2, and PΔ3-P3, respectively (see Fig. 1). The primers carrying deletions PΔ1 (60 bp), PΔ2 (57 bp), and PΔ3 (63 bp) were long enough to ensure the proper pairing of DNA strands on both sides of the deletion. The nucleotide sequences of the primers carrying deletions were as follows: PΔ1, 5′-GAG GATCTAGAAAAAGTTAGGGTGGAACAACTT//GATAAAAATATAGAT ATTTCCATTAAA-3′; PΔ2, 5′-ATCATCATCGATACGATCGGCGGTTAA// TGATTCAAGGAACTCTTTTACTCGCTTGAT-3′; PΔ3, 5′-CATTTTCTCG AGAATTTTGTACTCTGCGCTGGCTAT//ATGGATATTTATGGTGCCAC TACTAGA-3′. The symbol // indicates the location of the deletion. Accidentally, primer PΔ3 carried a 1-bp substitution located immediately 5′ to the site of the deletion. As a result, in the *lcrV*(aa 188–207) mutant, the correct sequence 5′-..TTT//ATG..-3′ was replaced by the sequence 5′-..TAT//ATG..-3′. Primers P1 (5′-ATCATCATCGATACGATCGGCGGT-3′), P2 (5′-GAGGATCTAGAAA AAGTTAGGGTG-3′), and P3 (5′-GCCGATCGTATCGATGATGATATT-3′) contain sequences on the strands opposite those of the primers carrying deletions.

The deletion removing bp 73 to 120 (aa 25 to 40 of LcrV) was situated 3′ to the *Xba*I restriction site (Fig. 1, part 1). The second deletion (aa 108 to 125), positioned 5′ to the *Cla*I site, started at bp 322 of *lcrV* and was 54 bp long (Fig. 1, part 2). The third deletion removed bp 562 to 621 (aa 188 to 207) of LcrV and was situated 5′ to the *Xho*I restriction site (Fig. 1, part 3). We replaced the 356-bp *Xba*I-*Cla*I or the 258-bp *Cla*I-*Xho*I restriction fragment of the *Hin*dIII-G insert in pES6-1 with smaller ones carrying the PCR-generated deletions (described in Fig. 1 and below). For each deletion, several prospective clones were isolated in *E. coli* GM2163 and analyzed by sequencing the first 650 bp of *lcrV*. Three plasmids carrying the proper Δ*lcrV*(aa 25–40), Δ*lcrV*(aa 108–125), and Δ*lcrV*(aa 188–207) sequences, named pV25, pV108, and pV188, respectively (Table 1), were chosen for further study.

The fourth mutation in *lcrV* (bp 651 to 671; aa 217 to 225) (Fig. 1, part 4) was created in pJIT769 (Table 1) by removing the internal *Xho*I-*Bst*XI restriction fragment and then filling the 5′ protruding ends of the *Xho*I restriction site and removing the 3′ protruding ends of the *Bst*XI site with T4 polymerase (Gibco-BRL, Gaithersburg, Md.). To restore the *lcrV* reading frame, an intermediate construct carrying the deletion of 25 bp (not in frame) was digested once again with the *Xho*I enzyme and this was followed by filling in with Klenow and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
E. coli		
SY327 (λ *pir*)	Δ(*lac pro*) *argE*(Am) *rif nalA recA56* λ *pir*	33
GM2163	F$^-$ *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 supE44 galK2 galT22* λ$^-$ *dcm-6 hisG4 rpsL136* (Sm^r) *dam-13*::Tn*9* (Cm^r) *xyl-5 mtl-1 thi-1 hsdR2* ($r_K^-m_K^-$) *mcrB1 mcrA*	70
XL1-Blue	Δ*end-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, La Jolla, Calif.
M2141	*minA minB* Δ(*lac pro*) *argE*(Am) *rif nalA recA56* λ *pir*	F. Neidhardt
Y. pestis[a]		
KIM5-3001	Sm^r pCD1 (Lcr$^+$) pPCP1 pMT1	28
KIM5-3001.2	Sm^r pCD1 (Δ*lcrD* [aa 192–343][b] [LcrD$^-$]) pPCP1 pMT1	39
KIM5-3001.5	Sm^r pCD1 (Δ*lcrG* [aa 39–53][b] [LcrG$^-$]) pPCP1 pMT1	52
KIM5-3001.6	Sm^r pCD1 (Δ*lcrE* [aa 48–197][b] [LcrE$^-$]) pPCP1 pMT1	40
KIM5-3001.9 (Δ25–40)	Sm^r pCD1 (Δ*lcrV* [aa 25–40][b]) [LcrV$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.10 (Δ108–125)	Sm^r pCD1 (Δ*lcrV* [aa 108–125][b]) [LcrV$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.10.1	Sm^r pCD1 (Δ*lcrV* [aa 108–125][b] Δ*lcrE* [aa 48–197][b] [LcrV$^-$ LcrE$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.11 (Δ188–207)	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b]) [LcrV$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.11.1	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] Δ*lcrE* [aa 48–197][b] [LcrV$^-$ LcrE$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.11.2	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] Δ*lcrG* [aa 39–53][b] [LcrV$^-$ LcrG$^-$][c]) pPCP1 pMT1	This work
KIM5-3001.11.3	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] *lcrH*::*cat yopJ*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$][c]) pPCP1 pMT1	This work
KIM5-3301	Sm^r pCD1 (*yopJ*::MudI1734 [Km^r Lac$^+$] [Lcr$^+$ YopJ$^-$]) pPCP1 pMT1	Laboratory stock
KIM5-3301.1 (YopJ$^-$Δ25–40)	Sm^r pCD1 (Δ*lcrV* [aa 25–40][b] *yopJ*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopJ$^-$][c]) pPCP1 pMT1	This work
KIM5-3301.2 (YopJ$^-$Δ108–125)	Sm^r pCD1 (Δ*lcrV* [aa 108–125][b] *yopJ*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopJ$^-$][c]) pPCP1 pMT1	This work
KIM5-3301.3 (YopJ$^-$Δ188–207)	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] *yopJ*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopJ$^-$][c]) pPCP1 pMT1	This work
KIM5-3301.4 (YopJ$^-$Δ217–225)	Sm^r pCD1 (Δ*lcrV* [aa 217–225][b] *yopJ*::MudI1734 [Km^r Lac$^+$] [Lcr$^+$ YopJ$^-$]) pPCP1 pMT1	This work
KIM5-3241[d]	pCD1 (Δ*lcrV* [aa 18–215][b] *yopJ*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopJ$^-$][c]) pPCP1 pMT1	42
KIM5-3401	Sm^r pCD1 (*lcrH*::*cat yopJ*::MudI1734 [Km^r Lac$^+$] [LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$]) pPCP1 pMT1	40
KIM5-3311	Sm^r pCD1 (*yopKL*::MudI1734 [Km^r Lac$^+$] [YopKL$^-$]) pPCP1 pMT1	This work
KIM5-3311.1	Sm^r pCD1 (Δ*lcrV* [aa 25–40][b] *yopKL*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopKL$^-$][c]) pPCP1 pMT1	This work
KIM5-3311.3	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] *yopKL*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopKL$^-$][c]) pPCP1 pMT1	This work
KIM5-3691	Sm^r pCD1 (*yopH*::MudI1734 [Km^r Lac$^+$] [YopH$^-$]) pPCP1 pMT1	This work
KIM5-3691.1	Sm^r pCD1 (Δ*lcrV* [aa 25–40][b] *yopH*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopH$^-$][c]) pPCP1 pMT1	This work
KIM5-3691.3	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] *yopH*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopH$^-$][c]) pPCP1 pMT1	This work
KIM5-3221	Sm^r pCD1 (*yopE*::MudI1734 [Km^r Lac$^+$] [YopE$^-$]) pPCP1 pMT1	This work
KIM5-3221.1	Sm^r pCD1 (Δ*lcrV* [aa 25–40][b] *yopE*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopE$^-$][c]) pPCP1 pMT1	This work
KIM5-3221.3	Sm^r pCD1 (Δ*lcrV* [aa 188–207][b] *yopE*::MudI1734 [Km^r Lac$^+$] [LcrV$^-$ YopE$^-$][c]) pPCP1 pMT1	This work
Plasmids		
pUC19	Cloning vector; Ap^r Tc^r	71
pUC19*	pUC19 with *Xba*I site removed by *Xba*I digestion followed by filling in with Klenow and religation	This work
pUK4134	Suicide vector; *oriR6K oriT cos rpsL* (Ap^r)	51
pTrcM.1	*Taq*I fragment of pBS10 (46) carrying *yopM* filled in with Klenow and recloned into *Sma*I site of pTrc99 (1)	40
pUK4134-5	*Eco*RV fragment of pGP2 (39) carrying Δ*lcrE*(aa 48–197)[b] cloned into pUK4134	40
pJIT7	*Hin*dIII G fragment of pCD1 cloned into vector pBR322 (*lcrGVH-yopBD′*)	36
pJIT76	Derivative of pJIT7 carrying *cat* GenBlock inserted into *Nco*I site of *lcrH* (*lcrGVH*::*cat yopBD′*)	43
pJIT76(Δ188–207)	Derivative of pJIT76 carrying Δ*lcrV*(aa 188–207)[b]	This work
pJIT769	pJIT76 plasmid with deletion of internal *Eco*RI fragment Δ*lcrH-yopBD′* (*lcrGVH′*)	This work
pJIT7694 (pV)	pJIT769 derivative with Δ*lcrG* (aa 39–53)[b] (Δ*lcrG lcrVH′*)	This work
pJIT7694(Δ25–40) [pV(Δ25–40)]	pJIT7694 derivative with Δ*lcrV* (aa 25–40)[b] (Δ*lcrG* Δ*lcrV* [aa 108–125][b] *lcrH′*)	This work
pJIT7694(Δ108–125) [pV(Δ108–125)]	pJIT7694 derivative with Δ*lcrV*(aa 108–125)[b] (Δ*lcrG* Δ*lcrV* [aa 108–125][b] *lcrH′*)	This work

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Properties	Reference or source
pJIT7694(Δ188–207) [pV(Δ188–207)]	pJIT7694 derivative with Δ*lcrV* (aa 188–207)[b] (Δ*lcrG* Δ*lcrV* [aa 188–207][b] *lcrH′*)	This work
pJIT769(Δ217–225) [pV(Δ217–225)]	Derivative of pJIT769 with Δ*lcrV* (aa 217–225)[b] (*lcrG*Δ*lcrV* [aa 217–225][b] *lcrH′*)	This work
pJIT7692-3 (pVH)	Derivative of pJIT7694 with *lcrH* restored (Δ*lcrG lcrVH yopBD′*)	This work
pES6-1	*Hin*dIII-G of pCD1 from pJIT7 recloned into pUC19∗ (*lcrGVH yopBD′*)	This work
pV25	pES6-1 with Δ*lcrV* (aa 25–40)[b] in cloned *Hin*dIII-G of pCD1	This work
pV108	pES6-1 with Δ*lcrV* (aa 108–125)[b] in cloned *Hin*dIII-G of pCD1	This work
pV188	pES6-1 with Δ*lcrV* (aa 188–207)[b] in cloned *Hin*dIII-G of pCD1	This work
pVS2	*Hin*dIII-G of pV25 recloned into pUK4134	This work
pVS3	*Hin*dIII-G of pV108 recloned into pUK4134	This work
pVS4	*Hin*dIII-G of pV188 recloned into pUK4134	This work
pVS5	*Eco*RV-*Ssp*I fragment of pJIT769(Δ217–225) with Δ*lcrV* (aa 217–225)[b] recloned into pUK4134	This work
pGVS1	Derivative of pGS1 (52) and pVS4 carrying Δ*lcrG* (aa 39–53)[b] and Δ*lcrV* (aa 188–207)[b] in pUK4134 vector	This work
pVHS1	Derivative of pES92 (52) and pJIT76(Δ188–207) carrying Δ*lcrV* (aa 188–207)[b] and *lcrH*::*cat*	This work

[a] All *Y. pestis* strains are Pgm$^-$ (65). Native virulence plasmids of *Y. pestis* are LCR plasmid pCD1 (15, 20), Pla-encoding plasmid pPCP1 (54, 55), and pMT1, which encodes the capsular protein (45).

[b] Numbers in brackets or parentheses are the amino acids deleted from the protein product.

[c] All *Y. pestis lcrV* mutants presented have been marked similarly (LcrV$^-$), even though the various mutations had different effects on LCR regulation and on secretion of LCR products (see text and Table 2).

[d] KIM5-3241 was previously designated KIM5-3241.2 (42).

religation. Several prospective clones were isolated in *E. coli* GM2163, and their DNA sequences were determined. The final construct was named pJIT 769(Δ217–225) or pV(Δ217–225) for simplicity (Table 1).

Plasmid constructions. Plasmid pES6-1, used for construction of the *lcrV* deletions, was made by recloning the *Hin*dIII G restriction fragment of pCD1 from pJIT7 into the *Hin*dIII restriction site of the pUC19∗ vector (Table 1). The resulting derivative was selected in *E. coli* XL1-Blue (Table 1). pES6-1 therefore carried only one *Xba*I restriction site (in the cloned *Yersinia* DNA), a property important for later manipulations. Plasmids pVS2, pVS3, pVS4, and pVS5 were constructed in suicide vector pUK4134 (Table 1). The ends of the *Hin*dIII restriction fragments from plasmids pV25, pV108, and pV188 or the *Eco*RV-*Ssp*I fragment of pJIT769(Δ217–225) (Table 1) that carried the predicted LcrV deletions were filled in with the Klenow enzyme and ligated into the *Eco*RV restriction site of the suicide vector. The pUK4134 vector carries the *rpsL* gene coding for the *E. coli* ribosomal protein which confers streptomycin sensitivity on resistant strains (51). It requires the product of a *pir* gene for its replication. Therefore, recombinant plasmids were selected in *E. coli* SY327 (λ *pir*) for stable maintenance (Table 1). Plasmid pJIT7694 (abbreviated pV), used in complementation studies of all *lcrV* mutants, was a derivative of pJIT76, which carries the *lcrGVH-yopBD′* operon with the *cat* (Tn*9* chloramphenicol acetyltransferase) GenBlock (Pharmacia, Uppsala, Sweden) inserted into the *lcrH* locus (Table 1). pJIT7694 was constructed in two steps: first, the internal *Eco*RI restriction fragment of pJIT76 (coding for the 3′ end of the *cat* gene, the 3′ end of *lcrH*, the *yopBD′* locus, and a sequence from pBR322) was removed. The resulting intermediate plasmid was named pJIT769 (Table 1). In the next step, *lcrG* was inactivated by replacing the *Hin*dIII-*Xho*I fragment with the *Hin*dIII-*Xho*I fragment of pGS1 (52) carrying the in-frame deletion (aa 39 to 53) in *lcrG*. pV expressed *lcrV* from a native promoter, whereas *lcrG* and *lcrH-yopBD′* were inactivated. Plasmids pJIT7694(Δ25–40), pJIT7694(Δ108–125), and pJIT7694(Δ188–207), abbreviated pV(Δ25–40), pV(Δ108–125), and pV(Δ188–207) (Table 1) and used in complementation studies, were derivatives of pJIT7694 and carried nonpolar deletions in *lcrV* that were created in this work. These plasmids were constructed by replacing the original *Xba*I-*Cla*I or *Cla*I-*Xho*I restriction fragment of pJIT7694 with the smaller one isolated from pV25, pV108, or pV188 (Fig. 1 and Table 1). Plasmid pJIT7692-3 (abbreviated pVH) (Table 1), used in complementation studies, was constructed by replacing the smaller of the two *Xho*I-*Eco*RI restriction fragments (carrying truncated *lcrH*) of pJIT7694 with the original one from pJIT7 (Table 1). In the resulting construct, *lcrV* and *lcrH-yopBD′* were expressed from a native promoter whereas *lcrG* was inactivated by the nonpolar deletion (aa 39 to 53).

Isolation of *Y. pestis lcrV* deletion mutants. To analyze the effect of deletions in *lcrV* in the original *Y. pestis* background, the *lcrV* deletions were transferred from pVS2, pVS3, pVS4, and pVS5 by marker exchange (51) into Sm^r strain *Y. pestis* KIM5-3001, as well as into Sm^r Km^r strain *Y. pestis* KIM5-3301 containing a MudI1734 insertion in *yopJ* (Table 1; the *yopJ* mutation was made earlier [59, 61]). The Sm^r and Sm^r Km^r clones of *Y. pestis* which had undergone the *lcrV* gene replacement were identified in PCRs with standard sequencing primers or the primer pair P1 and P2 or P3 (described earlier) and P4 (5′-TTCTCGCTCC CATCCACCTGA-3′ [Fig. 1]). The Sm^r *Y. pestis* strains carrying Δ*lcrV*(aa 25–40), Δ*lcrV*(aa 108–125), and Δ*lcrV*(aa 188–207) were designated KIM5-3001.9, KIM5-3001.10, and KIM5-3001.11, respectively (Table 1). For simplicity, these strains will hereafter be referred to by the shorter designations Δ25–40, Δ108–125, and Δ188–207. The Sm^r Km^r derivatives were named KIM5-3301.1, KIM5-3301.2, and KIM5-3301.3 and will be referred to hereafter as YopJ$^-$Δ25–40, YopJ$^-$Δ108–125, and YopJ$^-$Δ188–207, respectively (Table 1). The Sm^r Km^r *Y. pestis* strain carrying the deletion *lcrV*(aa 217–225) was named KIM5-3301.4 (referred as YopJ$^-$Δ217–225) (Table 1). The first three deletions were also moved to the following additional Sm^r *Y. pestis* hosts: YopH$^-$ KIM5-3691, YopE$^-$ KIM5-3221, YopKL$^-$ KIM5-3311 (Table 1).

Construction of double mutants. For construction of double mutants, plasmid pUK4134-5 with the in-frame deletion Δ*lcrE*(aa 48–197) (Table 1) was moved into the Sm^r Δ*lcrV*(aa 108–125) or Sm^r Δ*lcrV*(aa 188–207) background and marker exchange was carried out (51). The resulting two double mutants (KIM5-3001.10.1 and KIM5-3001.11.1) were identified by restriction digestion and PCR analysis of the pCD1 plasmid with the primer pair P1 and P2 or P3 and P4. To obtain the LcrV$^-$ LcrG$^-$ double mutant *Y. pestis* KIM5-3001.11.2 (Table 1), the *lcrV*-containing *Cla*I restriction fragment of pGS1 (52) was replaced with the corresponding *Cla*I fragment of pVS4 (Table 1) carrying Δ*lcrV*(aa 188–207). The construct with Δ*lcrG*(aa 39–53) and Δ*lcrV*(aa 188–207) in the suicide vector was named pGVS1 (Table 1). To isolate the LcrV$^-$ LcrH$^-$ double mutant *Y. pestis* KIM5-3001.11.3 (Table 1), the *Xba*I-*Xho*I restriction fragment of pV188 (Table 1) carrying Δ*lcrV*(aa 188–207) replaced the original *Xba*I-*Xho*I fragment of pJIT76 (Table 1). The intermediate plasmid construct was named pJIT76(Δ188–207). In the next step, a *Cla*I restriction fragment of pJIT76(Δ188–207) carrying Δ*lcrV*(aa 188–207) and *lcrH* with the *cat* (Tn*9* chloramphenicol acetyltransferase) GenBlock (Pharmacia) was used to replace the original *Cla*I restriction fragment of pES92 (52). The final construct, carrying Δ*lcrV*(aa 188–207) and *lcrH*::*cat* in the suicide vector, was designated pVHS1 (Table 1). Plasmids pGVS1 and pVHS1 were transferred into Sm^r *Y. pestis* KIM5-3001, and marker exchange was carried out. Sm^r clones of *Y. pestis* which had undergone gene replacement were identified by restriction digests of plasmid pCD1 and from the PCR products obtained with primers PG and PG-1 to detect the LcrG$^-$ mutation (52) or P3 and P4 to detect the LcrV$^-$ mutation (see above).

Protein isolation and Western (immunoblot) analysis. *Y. pestis* cultures were initiated at 26°C in TMH defined medium supplemented or not with Ca^{2+} and shifted to 37°C as described earlier. Seven hours after the temperature shift, 20-ml samples were removed and the cells were pelleted. Supernatant proteins were recovered after overnight precipitation at 4°C with trichloroacetic acid at a final concentration of 5% (vol/vol). The bacteria were washed and resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na_2-EDTA) in a volume equal to 0.5 ml × A_{620} of the original culture and lysed by passage through a French press at 20,000 lb/in^2. Unlysed cells were removed by centrifugation at 3,440 × *g* for 5 min at 4°C. Membranes (inner plus outer) were separated from the soluble cellular extract by centrifugation in a Beckman TL-100 ultracentrifuge at 263,800 × *g* for 20 min at 4°C and resuspended in TE buffer in a volume of 0.1 ml × A_{620} of the original harvested culture. Supernatant proteins were resuspended in the

TABLE 2. LCR phenotypes of *Y. pestis* strains used in this study[a]

Y. pestis strain (key properties)[b]	Restriction		Expression[c]				Secretion[c]			
			−Ca^{2+}		+Ca^{2+}		−Ca^{2+}		+Ca^{2+}	
	−Ca^{2+}	+Ca^{2+}	LcrV	YopM	LcrV	YopM	LcrV	YopM	LcrV	YopM
KIM5-3001 (parent)	2+	−	2+	2+	±	±	2+	2+	−	−
KIM5-3301 (YopJ$^-$)	2+	−	2+	2+	±	±	2+	2+	−	−
KIM5-3311 (YopKL$^-$)	2+	−	NT[d]	NT	NT	NT	NT	NT	NT	NT
KIM5-3691 (YopH$^-$)	2+	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3221 (YopE$^-$)	2+	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3301.4 (Δ*lcrV* [aa 217–225])	2+	−	2+	2+	±	±	2+	2+	−	−
KIM5-3001.9 (Δ*lcrV* [aa 25–40])[e]	+	−	2+	2+	±	±	+	2+	−	−
KIM5-3301.1 (Δ*lcrV* [aa 25–40] YopJ$^-$)	2+	−	2+	2+	±	±	2+	2+	−	−
KIM5-3311.1 (Δ*lcrV* [aa 25–40] YopKL$^-$)	+	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3691.1 (Δ*lcrV* [aa 25–40] YopH$^-$)	+	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3221.1 (Δ*lcrV* [aa 25–40] YopE$^-$)	+	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3001.10 (Δ*lcrV* [aa 108–125])[e,f]	+	−	2+	2+	±	±	−	2+	−	−
KIM5-3301.2 (Δ*lcrV* [aa 108–125] YopJ$^-$)	2+	−	2+	2+	±	±	±	2+	−	−
KIM5-3001.2 (Yop secretion$^-$)	−	−	±	±	±	±	−	−	−	−
KIM5-3241 (Δ*lcrV* [aa 18–215]	−	−	−	+	−	±	−	+	−	−
KIM5-3001.11 (Δ*lcrV* [aa 188–207])[e]	±	−	±	+	−	±	±	+	−	−
KIM5-3301.3 (Δ*lcrV* [aa 188–207] YopJ$^-$)	+	−	±	2+	−	±	±	2+	−	−
KIM5-3311.3 (Δ*lcrV* [aa 188–207] YopKL$^-$)	±	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3691.3 (Δ*lcrV* [aa 188–207] YopH$^-$)	±	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3221.3 (Δ*lcrV* [aa 188–207] YopE$^-$)	±	−	NT	NT	NT	NT	NT	NT	NT	NT
KIM5-3001.5 (LcrG$^-$)	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
KIM5-3001.6 (LcrE$^-$)	2+	2+	2+	2+	2+	2+	3+	2+	3+	2+
KIM5-3401 (LcrH$^-$)	2+	+	2+	2+	2+	2+	2+	2+	2+	+
KIM5-3001.10.1 (Δ*lcrV* [aa 108–125] LcrE$^-$)	+	+	2+	NT	NT	NT	±	NT	NT	NT
KIM5-3001.11.1 (Δ*lcrV* [aa 188–207] LcrE$^-$)	+	+	±	2+	+	2+	+	2+	±	2+
KIM5-3001.11.2 (Δ*lcrV* [aa 188–207] LcrG$^-$)	+	+	+	2+	+	2+	±	2+	±	2+
KIM5-3001.11.3 (Δ*lcrV* [aa 188–207] LcrH$^-$)	2+	+	+	2+	+	2+	+	2+	±	+

[a] Strains with complementing plasmids are not included in this listing.
[b] See Table 1 for more complete strain descriptions.
[c] The listing gives relative levels of expression and secretion as determined from immunoblot analysis only.
[d] NT, not tested.
[e] The same phenotype was shown by Pla$^-$ strains (lacking pPCP1) carrying the *lcrV* mutation (data not shown and strains not listed in Table 1 [53]).
[f] This same phenotype was shown by strains carrying the Δ*lcrV*(aa 108–125) mutation and also an insertion mutation in the *yopK*, *yopH*, or *yopE* gene (data not shown and strains not listed in Table 1 [53]).

same volume of TE buffer as proteins from the soluble cellular (cytoplasm plus periplasm) fraction.

Proteins were separated by standard sodium dodecyl sulfate (SDS)–12 or 15% (wt/vol) acrylamide polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (25). Immunoblotting analysis of polyacrylamide gels was performed as previously described (38), with Immobilon-P membranes (Millipore, Bedford, Mass.). With LcrG, because of its basic isoelectric point (8.64), carbonate buffer (pH 9.9) was used for electrotransfer from SDS-polyacrylamide gels into Immobilon-P membranes (52).

Preparation of antibodies. LcrV-specific antipeptide antibody was raised against a peptide corresponding to C-terminal amino acids 314 to 326 (NH_2-[C]-SVMQRLLDDTSGK-COOH) of the predicted LcrV sequence (synthesized by the Macromolecular Structure Analysis Facility, University of Kentucky, Lexington). An additional amino-terminal cysteine residue ([C]) was added to each peptide to provide a site for hapten conjugation. The peptides were coupled to the protein carrier bovine serum albumin (BSA) with 0.25% (vol/vol) glutaraldehyde or with the Imject immunogen conjugation kit (Pierce, Rockford, Ill.) with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate-activated BSA. New Zealand White rabbits were injected subcutaneously at multiple sites with a total of 1 mg of BSA-peptide conjugate emulsified with Freund's complete adjuvant and boosted 2 weeks later with 1 mg of the BSA-peptide conjugate emulsified in Freund's incomplete adjuvant. Antipeptide antibodies were detected by using an enzyme-linked immunosorbent assay. Antibodies were purified from the serum on Sepharose 6B (Pharmacia LKB, Piscataway, N.J.) peptide affinity columns prepared and used in accordance with the manufacturer's protocol. The affinity-purified antibodies were stored in phosphate-buffered saline at −20°C.

Polyclonal rabbit anti-YopM antibodies against the peptide NH_2-[C]-ETTDKLEDDVFE-COOH have been described previously (46). Polyclonal rabbit antipeptide antibodies anti-LcrD and anti-YscR were described by Plano et al. (38) and Fields et al. (16), respectively. Antipeptide antibodies raised against amino acids 80 to 91 of the predicted LcrG protein sequence were described previously (52). Antipeptide antibodies raised against amino acids 153 to 163 of the predicted LcrH protein (peptide NH_2-[C]-AIKLKKEMEHE-COOH) sequence were made by Clarissa Cowan in our laboratory essentially as described previously (38).

Analysis of proteins in *E. coli* minicells. Minicells were isolated from *E. coli* M2142 containing pUC19*, pES6-1, pV25, or pV108 and labeled with [^{35}S]methionine (0.25 mCi/ml) from New England Nuclear Corp. (10). Samples containing 40,000 trichloroacetic acid-precipitable cpm were analyzed by one-dimensional electrophoresis in SDS–12% (wt/vol) acrylamide PAGE gels (25). The gels were impregnated with En^{3}Hance (New England Nuclear), dried, and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 5×10^7 disintegrations. ^{14}C-labeled molecular weight standards were obtained from Amersham Corp.

Virulence testing in mice. To test the virulence of the *lcrV* mutants, female 6- to 8-week-old BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine) were inoculated retroorbitally with 0.1 ml of bacterial suspensions grown at 26°C in heart infusion broth and diluted in phosphate-saline buffer as described previously (44, 62). Groups of five mice per dose were caged separately and monitored for 14 days. Two doses of bacteria, 10^3 (5.7×10^2 to 3.4×10^3 CFU) and 10^5 (4.7×10^4 to 5.8×10^5 CFU) bacteria were used for injection of animals. The actual doses (in parentheses) were determined from colony counts from the culture suspensions. A single dose of 10^3 (7×10^2 to 3.7×10^3 CFU) bacteria was used for the reference (parental) strain as a positive control. An extra mouse for each strain, injected with the highest dose, was sacrificed for recovery of bacteria from the macerated liver and spleen. Colonies obtained from these mice were confirmed by antibiotic resistance, colony morphology, and plasmid profile to be the challenge strain.

RESULTS

Preliminary characterization of *lcrV* mutations. We constructed four small in-frame deletion mutations in the *Y. pestis* *lcrV* gene (Fig. 1). Before moving the *Hin*dIII G fragments

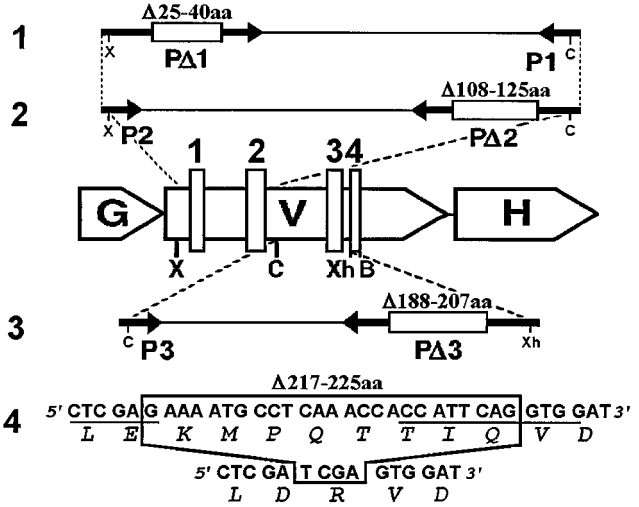

FIG. 1. Construction of nonpolar deletions in *lcrV*. The central diagram is a schematic map of part of the *lcrGVH-yopBD* operon. The positions and direction of transcription of the first three genes of this operon are shown as horizontal arrow-styled boxes. The letters G, V, and H inside the boxes represent *lcrG*, *lcrV*, and *lcrH*, respectively. The deleted regions in the vicinity of *Xba*I (X), *Cla*I (C), *Xho*I (Xh), and *Bst*XI (B) sites are marked as vertical bars and numbered 1, 2, 3, and 4. Parts 1, 2, and 3 show primers PΔ1 and P1, which were used to construct nonpolar deletion *lcrV*(aa 25–40); primers PΔ2 and P2, used to construct deletion *lcrV*(aa 108–125); and primers PΔ3 and P3, used for deletion *lcrV*(aa 188–207). Orientations of the PCR primers are shown as black arrows. Open boxes under amino acid numbers indicate the deleted regions. Part 4 shows the DNA sequence representing the fourth mutation, Δ(aa 217–225), in *lcrV*, which was a deletion-replacement construct. The upper sequence shows the region before deletion; the bottom sequence represents the same region after replacement of nine aa (E to Q) with two aa (D and R). The 5′ and 3′ underlined sequences represent the *Xho*I and *Bst*XI restriction sites, respectively. The corresponding amino acids are in italics.

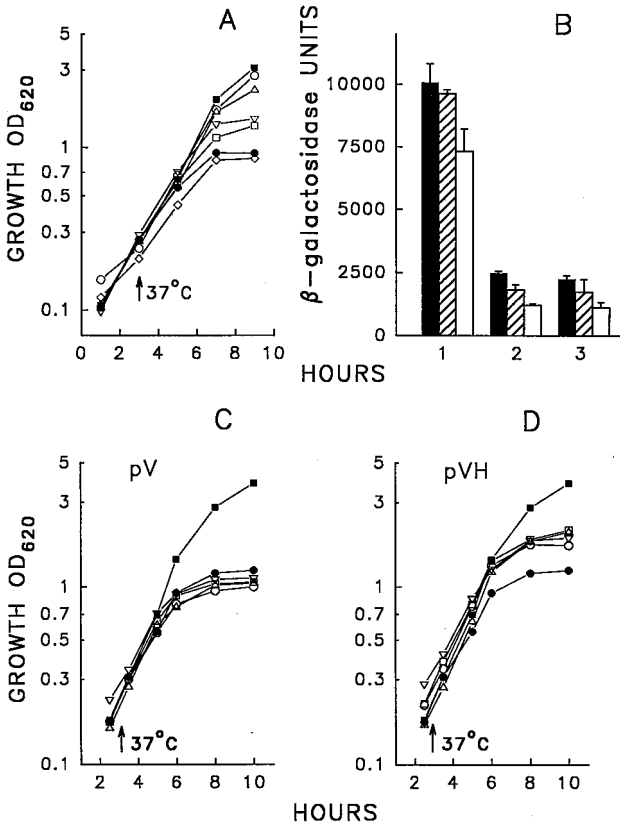

FIG. 2. Growth and *yopH*, *yopKL*, and *yopE* expression of *Y. pestis lcrV* mutants. (A) Growth. Bacteria were preadapted by growth at 26°C in TMH defined medium containing or lacking 2.5 mM Ca^{2+}, and the temperature was shifted to 37°C following ca. two generations of growth (arrow). Symbols: ● and ■, parent strain *Y. pestis* KIM5-3001; ▽, Δ25–40; □, Δ108–125; △, Δ188–207; ○, LcrV$^-$ *Y. pestis* KIM5-3241; ◇, YopJ$^-$Δ217–225; ●, for the parent and open symbols for the rest of strains, Ca^{2+} absent; ■, 2.5 mM Ca^{2+} present (the parent). In the presence of 2.5 mM Ca^{2+}, all mutants grew like the parent strain. OD_{620}, optical density at 620 nm. (B) β-Galactosidase expression from *yopH*::MudI1734, *yopKL*::MudI1734, and *yopE*::MudI1734. Bacteria were grown in TMH medium not supplemented with Ca^{2+} as in the experiment of panel A. Samples for β-galactosidase assay were taken at 6 h after the temperature shift. One unit of β-galactosidase activity is defined as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg of protein. Columns: 1, *lcrV* mutations in the YopH$^-$ *Y. pestis* KIM5-3691 host; 2, *lcrV* mutations in the YopE$^-$ KIM5-3221 host; 3, *lcrV* mutations in the YopKL$^-$ KIM5-3311 host. ■, host strains; ▨, Δ*lcrV*(aa 25–40) derivatives; □, Δ*lcrV*(aa 188–207) derivatives. Results like those for the strains containing Δ*lcrV*(aa 25–40) were obtained for the mutants having Δ*lcrV*(aa 108–125) (data not presented). Each bar is the mean value of two experiments, each consisting of assays carried out in duplicate; error bars indicate ±1 standard deviation. (C and D). Complementation of *Y. pestis lcrV* mutants by pV and pVH. Bacteria were preadapted as described for panel A. Symbols are as in panel A, except that open circles show the growth of the complemented parent *Y. pestis* KIM5-3001 and the YopJ$^-$Δ217–225 mutant is not included.

carrying the deletions into *Y. pestis* LCR plasmid pCD1, we checked their expression of the *lcrGVH-yopBD′* operon in *E. coli* M2141 minicells. Proteins expressed by pV25 carrying Δ*lcrV*(aa 25–40), pV108 carrying Δ*lcrV*(aa 108–125), pES6-1 carrying *lcrGVH-yopBD′*, and the pUC19* vector as a control were compared by SDS–12% (wt/vol) acrylamide PAGE analysis. As expected, minicells containing pES6-1, pV25, and pV108 expressed three proteins known to be encoded by the *Y. pestis* V operon, LcrG, LcrV, and LcrH, as well as a fourth protein of 42 kDa (probably YopB) (36). The gels also revealed the slightly faster migration of the V antigen encoded by pV25 and pV108 than that of the full-sized LcrV protein expressed from pES6-1. There was no effect of the mutations in *lcrV* on expression of the downstream *lcrH* and *yopB* products in *E. coli* (data not presented).

The mutation deleting aa 217 to 225 in *lcrV* was an in-frame deletion-replacement of seven aa (nine aa replaced with two aa) in *lcrV* (Fig. 1, part 4). Before being moved into the *Y. pestis* background, this mutation in plasmid pV(Δ217–225) was tested in complementation studies of the previously constructed LcrV$^-$ Δ*lcrV*(aa 18–215) mutant strain *Y. pestis* KIM5-3241 (Table 1 and reference 42). Surprisingly, pV(Δ217–225) provided full complementation, indicating that its mutant LcrV protein might be functionally normal.

Growth characteristics and β-galactosidase expression. The *Y. pestis lcrV* mutants grew at the same rate as the parent strains *Y. pestis* KIM5-3001 and KIM5-3301 at 26 and 37°C in the presence of Ca^{2+} (data not presented). As shown in Fig. 2A, our mutants differed in growth restriction in the absence of Ca^{2+} following a temperature shift to 37°C. Mutants Δ25–40 and Δ108–125 showed an intermediate level of restriction, while Δ188–207 showed essentially no growth restriction in the absence of Ca^{2+}. The growth properties of Δ188–207 resembled those of previously described Δ*lcrV*(aa 18–215) mutant strain KIM5-3241, which fails to make stable LcrV (Table 1 and reference 42). We included the latter strain in our experiments as a reference for LCR properties when *lcrV* was completely absent (Fig. 2A). Because growth restriction is a marker for LCR induction, these findings indicated that the mutants varied in LCR regulatory defects from weak [Δ*lcrV*(aa 25–40) and Δ*lcrV*(aa 108–125)] to strong [Δ*lcrV*(aa 188–207)].

In the KIM5-3301 background, where pCD1 carries a tran-

scriptional fusion of *yopJ* to the *E. coli lacZYA* operon, we noticed stronger growth restriction. The mutant YopJ$^-$Δ217–225 showed a normal LCR growth response, the YopJ$^-$ strains carrying Δ*lcrV*(aa 25–40) and Δ*lcrV*(aa 108–125) showed a nearly wild-type Ca^{2+}-dependent phenotype, whereas mutant YopJ$^-$Δ188–207 exhibited an intermediate level of restriction (data not shown). The MudI1734 insertion in *yopJ* has previously been shown to have no effect on the LCR of the KIM5-3301 host strain. Apparently, the differences in restriction of our Δ*lcrV* mutants in the KIM5-3001 and KIM5-3301 hosts reflect an unknown interaction between LcrV and the product(s) of the *yopJ* locus that is manifested only when LcrV is mutated.

lcrV mutations Δ(aa 25–40), Δ(aa 108–125), and Δ(aa 188–207) were also introduced into derivatives of pCD1 carrying a MudI1734 insertion that created a fusion of *yopH*, *yopKL*, or *yopE* to the *E. coli lacZYA* operon. Those insertions were also known to have no effect on the LCR (59). These Yop$^-$ backgrounds, in contrast to the YopJ$^-$ host, caused no changes in the growth phenotype of our *lcrV* mutants (data not presented). Therefore, by measuring β-galactosidase specific activity, we were able to determine whether there were effects of the *lcrV* nonpolar mutations on the Ca^{2+}-regulated transcription of different *yop* operons. The intermediate type of growth restriction of mutant Δ25–40 shown in Fig. 2A was reflected in slightly lower levels of *yopH*, *yopKL*, and *yopE* transcription (measured by β-galactosidase specific activity), but the small decrease was not statistically significant. Similar data were obtained for the Δ108–125 mutation (data not presented). However, the Ca^{2+}-independent growth of mutant Δ188–207 correlated with still lower transcription of *yopH*, *yopKL*, and *yopE* (Fig. 2B). That effect was more visible for the YopE$^-$ and YopKL$^-$ yersiniae than for the YopH$^-$ host. These results raised the possibility that the aa 188 to 207 region of LcrV is important for activation of growth restriction and maximal expression of Yops in yersiniae.

Complementation studies of the *lcrV* mutants. To verify that the growth phenotypes of mutants Δ25–40, Δ108–125, and Δ188–207 were due only to their *lcrV* mutations, we tested plasmid pV, carrying *lcrV* with its own calcium-regulated promoter (Table 1), for the ability to complement the *lcrV* deletions. pV restored the growth restriction at 37°C in the absence of Ca^{2+} in all three mutants (Fig. 2C), as well as in LcrV$^-$ reference mutant *Y. pestis* KIM5-3241 (data not presented). The extra copies of LcrV made all of the strains, including the parent strain, show a slightly but consistently stronger restrictive response than did the parent strain lacking pV. The vector alone had no effect on the growth phenotype of the parent strain (data not presented).

In contrast, pV(Δ188–207) (Table 1) did not restore growth restriction to the Δ188–207 mutant or to LcrV$^-$ *Y. pestis* KIM5-3241, whereas pV(Δ25–40) and pV(Δ108–125) (Table 1) only partially complemented the growth defects of Δ188–207 or LcrV$^-$ *Y. pestis* KIM5-3241 (data not shown). As noted earlier, plasmid pV(Δ217–225) (Table 1) fully complemented both strains (data not presented). These results and the data of Fig. 2 indicate that the mutated forms of V antigen expressed from the complementing plasmids differ in functionality. The LcrV product of YopJ$^-$Δ217–225 resembles the native V antigen. The mutant LcrV(Δ188–207) produced by Δ188–207 yersiniae is defective in the ability to promote induction of the low-Ca^{2+} response, whereas the LcrV(Δ25–40) and LcrV (Δ108–125) products of the Δ25–40 and Δ108–125 mutants respectively, are partially functional.

Plasmid pVH (Table 1), expressing *lcrVH* from native promoters, was not able to restore normal restriction to mutants

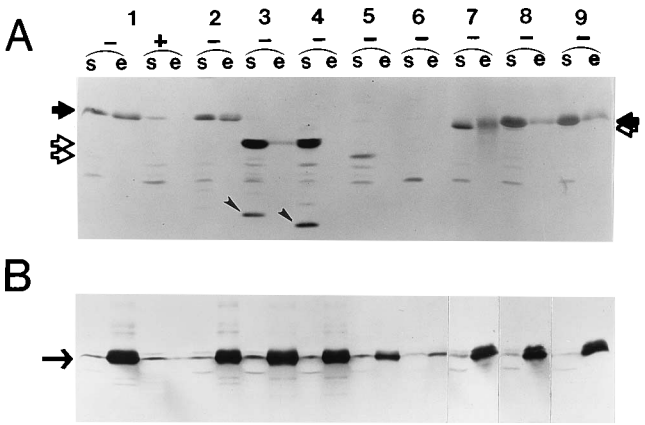

FIG. 3. Immunoblot analysis of V antigen (A) and YopM (B) expressed and secreted by parent strains *Y. pestis* KIM5-3001 and KIM5-3301 and their Δ*lcrV* derivatives. Proteins from equal numbers of cells loaded in each lane were separated by SDS–12% (wt/vol) acrylamide PAGE. Lanes: s, soluble cellular proteins; e, extracellular proteins; −, or +, absence or presence, respectively, of 2.5 mM Ca^{2+} in TMH medium; 1, *Y. pestis* KIM5-3001; 2, *Y. pestis* KIM5-3301; 3, Δ25–40; 4, Δ108–125; 5, Δ188–207; 6, LcrV$^-$ *Y. pestis* KIM5-3241; 7, YopJ$^-$Δ217–225; 8, Δ188–207 complemented by pV; 9, LcrV$^-$ *Y. pestis* KIM5-3241 complemented by pV. (A) Proteins detected by anti-LcrV antibodies raised against aa 314 to 326 of LcrV. The position of the V antigen is indicated by the solid black arrow; the positions of truncated forms of LcrV [LcrV(Δ25–40), LcrV(Δ108–125), LcrV(Δ188–207), and LcrV(Δ217–225)] are marked as open arrows. Arrowheads at the bottom indicate bands (lanes 3 and 4) thought to be LcrV degradation products. In other experiments, the secreted LcrV(Δ217–225) gave a tight band resembling that of native LcrV. The antipeptide antibodies reacted more strongly with the LcrV products of mutants Δ25–40 and Δ108–125 than with the other LcrV proteins on this immunoblot. (B) Proteins detected by anti-YopM antibodies. The position of YopM is indicated by the arrow. The additional bands represent products (probably degradation products) cross-reacting with anti-YopM serum.

Δ25–40 and Δ108–125 and only partially complemented Δ188–207 (Fig. 2D) or LcrV$^-$ *Y. pestis* KIM5-3241 (data not presented). Moreover, pVH partially inhibited the growth restriction of the parent strain KIM5-3001, allowing yersiniae to grow to an optical density at 620 nm of 2 instead of 1.2 (Fig. 2D). These abnormal growth responses of the complemented mutants and the parent strain likely were caused by the presence of extra copies of the LcrV or LcrH product. We speculate that extra amounts of V antigen (from pV) caused hyperinduction of the LCR in our strains (Fig. 2C), whereas LcrH (in pVH) counteracted that effect (Fig. 2D).

Expression and secretion of V antigen and YopM. Antipeptide (anti-LcrV and anti-YopM) antibodies were used for immunoblot analysis of SDS-PAGE-separated soluble cellular and supernatant proteins to characterize expression and secretion of V antigen and YopM by the *Y. pestis* mutants (Fig. 3). Western blot analysis confirmed the Ca^{2+}-regulated *yopM* expression in the parent strains as well as in all of the *lcrV* mutants (data presented only for the parent strain [Fig. 3B, lanes 1]). At 37°C in the absence of Ca^{2+}, mutants Δ25–40, Δ108–125, and YopJ$^-$Δ217–225 expressed and secreted amounts of YopM protein comparable to those produced by the parent strains (Fig. 3B, lanes 3, 4, and 7 versus lanes 1 and 2), despite small differences in their growth restriction phenotypes and *yop* operon transcription as measured from operon fusions. Under the same conditions, there were much smaller amounts of YopM in the culture supernatants of Ca^{2+}-independent mutants Δ188–125 and LcrV$^-$ *Y. pestis* KIM5-3241 (Fig. 3B, lanes 5 and 6). Both strains were fully complemented in expression of YopM (Fig. 3B, lanes 8 and 9), as well as in

growth restriction (Fig. 2C and data not presented) by pV carrying wild-type *lcrV*.

Although the V-antigen expression in our mutants was Ca^{2+} dependent, as in the parent *Y. pestis* strain (Fig. 3A, lanes 1; data presented only for parent strain KIM5-3001), there were differences in the amounts of expressed and/or transported LcrV between individual mutants and the parent strain. At 37°C in the absence of Ca^{2+}, the V antigen was present in nearly equal amounts inside and outside the parental KIM5-3001 and KIM5-3301 yersiniae (Fig. 3A, lanes 1 and 2). The normally restricting YopJ$^-$Δ217–225 mutant showed the parental type of V-antigen expression and secretion (Fig. 3A, lanes 7). It produced a slightly smaller (only seven aa deleted) form of LcrV. Mutants Δ25–40 and Δ108–125 (Fig. 3A, lanes 3 and 4) also produced smaller forms of the V antigen. However, they behaved differently from each other and from the parent strain in efficiency of secretion of LcrV(Δ25–40) and LcrV(Δ108–125). Both mutants contained more intracellular LcrV than the parent strain, and they failed to transport LcrV(Δ25–40) and LcrV(Δ108–125) efficiently from the cells. The secretion defect was much stronger in the Δ108–125 mutant than in the Δ25–40 mutant. Similar results were obtained when the same deletions were analyzed in the KIM5-3301 background (mutants YopJ$^-$Δ25–40 and YopJ$^-$Δ108–125). In that case, the stronger LCR induction was reflected by greater secretion of LcrV(Δ25–40). Nonetheless, *Y. pestis* YopJ$^-$ Δ108–125 still poorly transported the LcrV(Δ108–125) product into the medium (data not presented). These data indicate the potential importance of the aa 108 to 125 region of the V antigen for its secretion.

As shown in Fig. 3A, lanes 5, mutant Δ188–207 poorly expressed the truncated form of the V antigen compared with the parent and secreted this protein in very small amounts (band not visible in this figure). Even though the LCR response was stronger in the corresponding YopJ$^-$Δ188–207 derivative, LcrV(Δ188–207) expression and secretion did not increase appreciably (data not presented). The amount of LcrV(Δ188–207) was not increased even when Δ188–207 contained the pV plasmid carrying extra copies of *lcrV* (Fig. 3A, lanes 8), even though this plasmid complemented the growth defect of this mutant (Fig. 2C). When pV(Δ188–207) (Table 1) was introduced into parent strain KIM5-3001, neither the Δ*lcrV*(aa 188–207) nor the Δ*lcrG* complementing copy was fully expressed (data not presented). However, both Δ*lcrV*(aa 25–40) and Δ*lcrG* on pV(Δ25–40) (Table 1) were strongly expressed in KIM5-3001 (data not presented). These data implicate a *cis* effect of the Δ*lcrV*(aa 188–207) region on the expression of its own operon. These Western analyses also confirmed the absence of truncated forms of the V antigen in protein extracts from reference LcrV$^-$ mutant KIM5-3241 (Fig. 3A, lanes 6).

Additional bands were present in immunoblots of the soluble cellular fractions isolated from mutants Δ25–40 and Δ108–125 (Fig. 3A, lanes 3 and 4, arrowheads), as well as their YopJ$^-$ derivatives (data not presented). No similar bands were seen when soluble extracts from parent strain *Y. pestis* KIM5-3001 (or the YopJ$^-$ parent) were analyzed with the same antibodies (Fig. 3A, lanes 1 and 2). No degradation products were visible in protein extracts isolated from strain Δ188–207 or its YopJ$^-$ derivative (Fig. 3A, lanes 5 and data not presented). However, in this case degradation products might not be visible because of the small amounts of LcrV(Δ188–207) itself. When we moved the Δ*lcrV*(aa 25–40) and Δ*lcrV*(aa 108–125) deletions into pPCP1$^-$ *Y. pestis*, the degradation products were not present (data not shown). Hence, these products reflect a greater susceptibility of the LcrV(Δ25–40) and LcrV(Δ188–207) V-antigen forms than native LcrV to the plasminogen activator protease Pla encoded by the pPCP1 plasmid present in *Y. pestis*. However, we think that this degradation has a small overall effect, as the amounts of degradation products were small compared with those of the full-size mutant V antigens, and the total amount of full-sized V antigen from both mutants was not noticeably decreased below that in the parent. Moreover, the absence of Pla did not alter the amounts of LcrV(Δ25–40) and LcrV(Δ108–125) in culture supernatants (nor was wild-type LcrV affected in expression or secretion by the absence of Pla; data not shown). A comparison of the amounts of LcrV(Δ188–207) in the soluble cellular extracts from the Pla$^+$ and Pla$^-$ yersiniae revealed that the abundance of LcrV(Δ188–207) also was not increased in the absence of the Pla protease. Therefore, we concluded that the low level of this protein in the Δ188–207 cells is not a result of extensive degradation by Pla.

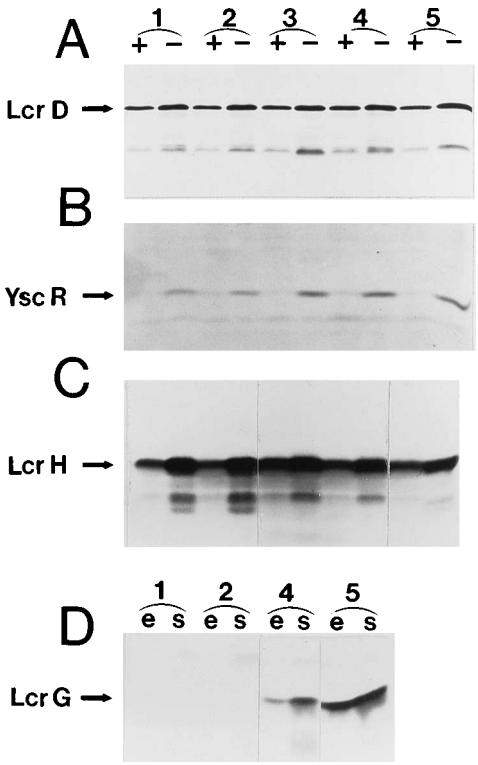

FIG. 4. Immunoblot analysis of LcrD, YscR, LcrH, and LcrG expressed by the parent *Y. pestis* KIM5-3001 and its Δ*lcrV* derivatives. Proteins from equal numbers of cells loaded in each lane were separated by SDS–12 or 15% (wt/vol) acrylamide PAGE. Panels: A and B, membrane proteins that reacted with antibodies against LcrD and YscR, respectively; C, soluble cellular proteins visualized with anti-LcrH; D, soluble cellular (s) and extracellular (e) proteins detected by anti-LcrG. Lanes: −, or +, bacteria grown in the absence or presence, respectively, of 2.5 mM Ca^{2+} (panel D, absence of Ca^{2+} only); 1, LcrV$^-$ *Y. pestis* KIM5-3241; 2, Δ188–207; 3, Δ108–125; 4, Δ25–40; 5, parent *Y. pestis* KIM5-3001. The positions of the LCR-related proteins are indicated. The additional bands are due to proteins (including likely degradation products) that cross-react with the antisera.

Expression of other LCRS proteins. We used Western analysis with antipeptide antibodies to characterize the expression and secretion of other LCRS products by the *lcrV* mutants (Fig. 4). Expression of membrane proteins LcrD and YscR and cytoplasmic protein LcrH was not decreased in any of the *lcrV* mutants (Fig. 4A, B, and C). If anything, greater amounts of LcrH were expressed by the *lcrV* mutants than the parent strain (Fig. 4C, lanes 1 to 4 versus lanes 5). All strains expressed these proteins more strongly in the absence of Ca^{2+} than in its

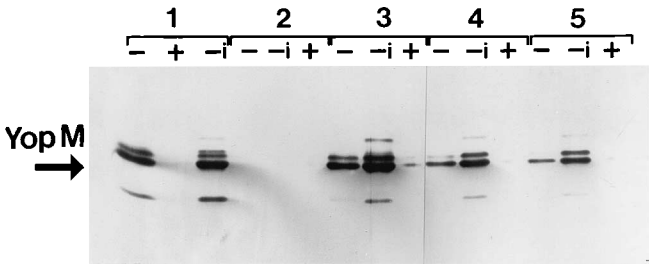

FIG. 5. Immunoblot analysis of YopM secreted by *Y. pestis* strains carrying pTrcM.1. Lanes: − or −i, bacteria grown in TMH defined medium in the absence of Ca^{2+} when IPTG was absent or present, respectively; +, Ca^{2+} present in the medium (note that the regular order of −i and + is reversed in lanes 1). Strains carrying pTrcM.1: lanes 1, parent *Y. pestis* KIM5-3001; lanes 2, LcrD$^-$ KIM5-3001.2; lanes 3, Δ25–40; lanes 4, Δ188–207; lanes 5, LcrV$^-$ KIM5-3241. The position of YopM is indicated. The additional bands represent products (probably YopM degradation products) cross-reacting with anti-YopM serum.

presence. In contrast to that of LcrH, LcrG expression was decreased in the mutants. Isolates Δ25–40 and Δ108–125 expressed lower levels of LcrG than did the parent (Fig. 4D, lanes 4 versus lanes 5; data presented only for Δ25–40), and LcrG expression was essentially abolished in mutant Δ188–207 and in LcrV$^-$ reference mutant KIM5-3241.2 (Fig. 4D, lanes 1 and 2). These data point to a hierarchy of LcrV's effects in the LCR: there was no effect of the *lcrV* mutations on *lcrD* and *yscR*, which do not belong to the *yop* regulon (14); however, expression of YopM and LcrG roughly followed the levels of induction predicted from the growth phenotypes, with the stronger effect on LcrG than on YopM from the Δ*lcrV*(aa 188–207) mutation possibly reflecting a *cis* effect of *lcrV*. The opposite effect on LcrH levels is not understood. *lcrH* has a non-Ca^{2+}-regulated putative promoter (43), the regulation of which has not been studied in detail.

Overexpression of YopM. We had not anticipated an effect of mutations in *lcrV* on secretion of LCRS products. Accordingly, we wanted to determine why our constructs failed to transport the LCR products (YopM, LcrV, and LcrG) to different degrees (Fig. 3 and 4), despite having normal amounts of components (LcrD and YscR) of the secretion mechanism (Fig. 4). We expressed YopM from the independent (non Ca^{2+}-regulated), isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter of the pTrcM.1 plasmid (Table 1) in the Δ188–207 mutant and in LcrV$^-$ *Y. pestis* KIM5-3241. As positive controls, we used the parent *Y. pestis* KIM5-3001 and mutant Δ25–40, and as a negative control, we used LcrD$^-$ *Y. pestis* KIM5-3001.2 (39) carrying pTrcM.1 (Table 1). Figure 5 shows YopM in immunoblots of extracellular fractions from these yersiniae grown at 37°C. In the absence of Ca^{2+}, the positive control strains secreted abundant YopM and the presence of IPTG only increased the amounts of YopM inside and outside the cells (Fig. 5, lanes 1 and 3 and data not presented). Under the same conditions, the secretion-defective LcrD$^-$ mutant was not able to secrete YopM, even though abundant YopM was expressed within the cells (Fig. 5, lane 2; data not shown; reference 40). The Δ188–207 mutant and LcrV$^-$ *Y. pestis* KIM5-3241 secreted small amounts of YopM expressed in small amounts from their LCR plasmids plus uninduced

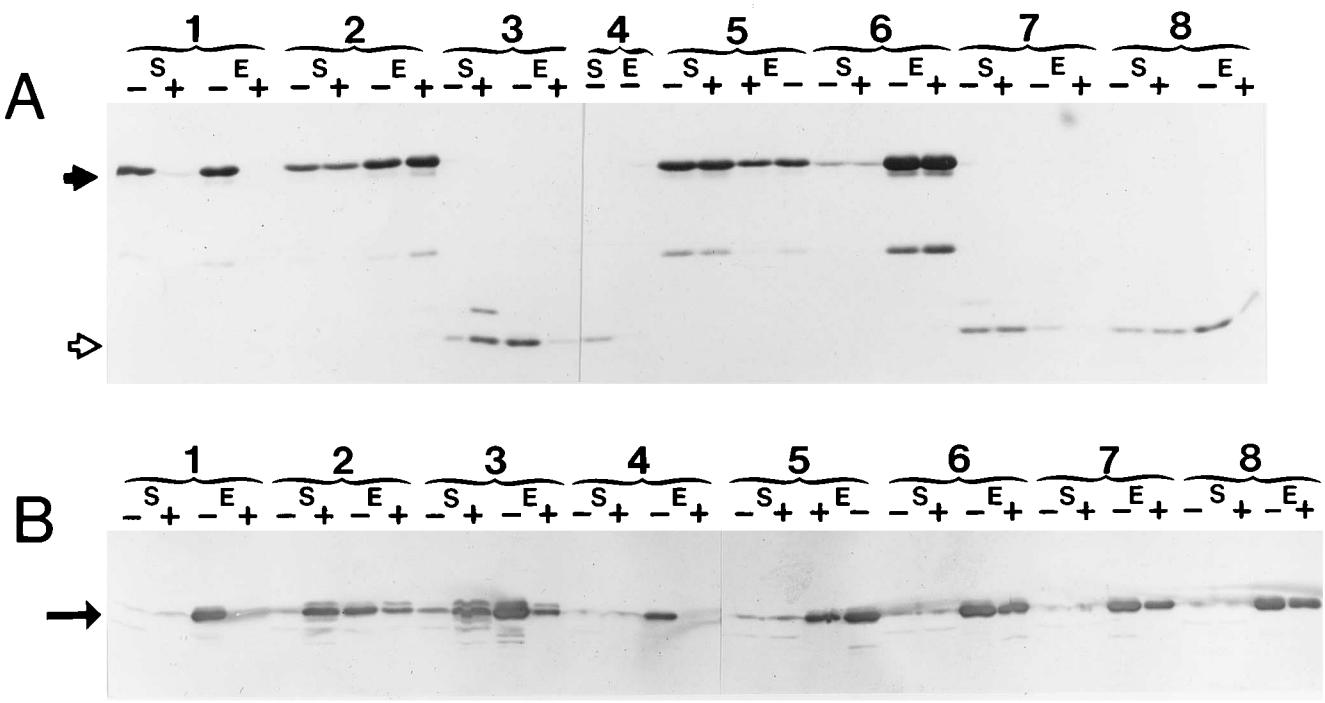

FIG. 6. Immunoblot analysis of LcrV and YopM expressed by double mutants. Proteins from equal numbers of cells loaded in each lane were separated by SDS–12% (wt/vol) acrylamide PAGE. Lanes: s, soluble cellular proteins; e, extracellular proteins; − and +, absence and presence, respectively, of 2.5 mM Ca^{2+} (note that the regular order of + and − is reversed in lanes 5E); 1, parent *Y. pestis* KIM5-3001; 2, LcrH$^-$ KIM5-3401; 3, Δ*lcrV*(aa 188–207) LcrH$^-$ KIM5-3001.11.3; 4, Δ188–207; 5, LcrG$^-$ KIM5-3001.5; 6, LcrE$^-$ KIM5-3001.6; 7, Δ*lcrV*(aa 188–207) LcrG$^-$ KIM5-3001.11.1; 8, Δ*lcrV*(aa 188–207) LcrE$^-$ KIM5-3001.2. (A) Proteins detected by anti-LcrV antibody. The position of the V antigen is indicated by the solid black arrow; the position of the truncated form of LcrV(Δ188–207) is marked by the open arrow. The greater distance between the different forms of LcrV antigen here than in Fig. 3A is a result of better resolution in this SDS-PAGE gel. (B) Proteins detected by anti-YopM antibody. The position of YopM is indicated by the arrow. The additional bands represent products (probably YopM degradation products) that cross-react with anti-YopM serum.

pTrcM.1; IPTG-mediated induction resulted in more YopM secretion (Fig. 5, lanes 4 and 5). We concluded that both mutants have a functional secretion apparatus. Their *lcrV* mutations probably affect LCRS product secretion only indirectly by affecting the amounts of LCRS proteins available for transport.

Characterization of double mutants. To help place LcrV in the LCR regulatory pathway, we made a set of double mutants carrying pairs of nonpolar mutations: *lcrE-lcrV*, *lcrG-lcrV*, and *lcrH-lcrV* (the *lcrH* mutation was not nonpolar, but the downstream *yopB* and *yopD* mutations are not known to have effects on the expression of LCRS proteins or on the secretion of LcrV or Yops into the medium by yersiniae). We used immunoblot analysis to characterize the expression and secretion of Yops and LcrV in these mutants. The data confirmed the constitutively induced LCR phenotypes of the LcrG$^-$, LcrE$^-$, and LcrH$^-$ single mutants (Fig. 6 and data not presented) and supported the previous findings that yersiniae defective in LcrG or LcrE, which are thought to act at the cell surface, constitutively secrete LCRS products at 37°C, whereas yersiniae defective in the cytoplasmic protein LcrH retain a significant degree of Ca^{2+} regulation of secretion (2, 18, 52). All three single mutants secreted YopM in the presence, as well as in the absence, of 2.5 mM Ca^{2+}; however because LcrH$^-$ *Y. pestis* only inefficiently secretes Yops in the presence of Ca^{2+}, this strain accumulated YopM within the cells under that one condition (Fig. 6B, lanes 2, 5, and 6 versus lanes 1). All three double mutants showed the same YopM secretion pattern as their LcrE$^-$, LcrG$^-$, and LcrH$^-$ progenitors (Fig. 6B, lanes 3, 7, and 8 versus lanes 2, 5, and 6). The low level of YopM in the Δ188–207 mutant (Fig. 6B, lanes 4) was manifested in none of our double mutants carrying the same mutation (Fig. 6B, lanes 3, 7, and 8). These data indicate that full LCR induction may occur in the absence of functional LcrV, thereby arguing against a direct positive role for LcrV in the LCR (42). We assume, therefore, that LcrV affects induction not as an activator per se but rather by inhibiting some negative regulators.

As seen earlier (Fig. 3A and B, lanes 1), the parent strain retained a significant amount of V antigen inside the cells, whereas YopM was fully secreted (Fig. 6A and B, lanes 1). Our LcrH$^-$ and LcrG$^-$ single mutants behaved similarly, except that at 37°C they failed to respond to the Ca^{2+} signal and secreted V antigen constitutively (Fig. 6A, lanes 2 and 5). Unexpectedly, the lack of LcrE resulted in hypersecretion: the LcrE$^-$ mutant, in contrast to the parent strain and other mutants, secreted essentially all of the LcrV that was expressed in either the presence or the absence of Ca^{2+} (Fig. 6A, lanes 6 versus lanes 1, 2, and 5). Therefore, we hypothesize that LcrE, thought to be a Ca^{2+} sensor in the LCR, controls V-antigen secretion not only in the presence of Ca^{2+} but also in its absence. Because hypersecretion was not manifested by the LcrG$^-$ strain (Fig. 6A, lanes 5 versus lanes 6), this suggests that LcrE and LcrG act differently at least in relation to secretion of LcrV.

There was an effect of the LcrH$^-$ mutation on secretion of LcrV. The LcrH$^-$ mutant secreted V antigen constitutively, despite retaining significant Ca^{2+} regulation of YopM secretion (Fig. 6A and B, lanes 2). This effect could be due to LcrH, YopB, or YopD, as all are missing in our LcrH$^-$ strain. The different effects observed for YopM and the V antigen in LcrH$^-$ and Lcr$^+$ yersiniae indicate that these two LCRS products are handled differently by the secretion apparatus in yersiniae.

In light of our observation that LcrV(Δ108–125) was secreted very poorly (Fig. 3A, lanes 4 and data not shown), it was of interest to learn whether the LcrE$^-$ mutation would permit LcrV(Δ108–125) to be secreted efficiently. Therefore, we determined the secretion properties of the Δ*lcrE*(aa 48–197) mutant carrying the additional deletion in *lcrV* (Table 1). This strain accumulated the LcrV(Δ108–125) antigen inside the cells and secreted this protein less efficiently than did the parent strain (data not presented). A similar tendency was observed when extracellular proteins were obtained from cultures of the LcrE$^-$ Δ*lcrV*(aa 108–125) mutant supplemented with the pV(Δ108–125) plasmid (Table 1). Little LcrV(Δ108–125) product was detected outside the cells, whereas LcrE$^-$ *Y. pestis* containing pV(Δ25–40) or pV(Δ217–225) (Table 1) hypersecreted not only the normal V antigen expressed from pCD1 but also the truncated LcrV(Δ25–40) or LcrV(Δ217–225) product. These data indicate that even under conditions ensuring efficient V-antigen transport (lack of LcrE), the LcrV(Δ108–125) form of this protein is not secreted efficiently. This underscores the potential importance of the deleted aa 108 to 125 for V-antigen secretion.

TABLE 3. Virulence of *lcrV* mutant *Y. pestis* in mice

Y. pestis strain	No. of mice dead after 14 days/ no. tested at dose of:	
	10^3 bacteria	10^5 bacteria
KIM5-3001 (parent)	5/5	5/5
KIM5-3301 (YopJ$^-$ parent)	5/5	5/5
KIM5-3301 carrying pV	0/5	NT[a]
KIM5-3301 carrying pVH	2/5	1/3
KIM5-3001.9 (Δ25–40)	0/5	0/5
KIM5-3301.1 (YopJ$^-$Δ25–40)	0/5	0/5
KIM5-3301.1 carrying pV	0/5	0/5
KIM5-3301.1 carrying pVH	0/4	0/4
KIM5-3001.10 (Δ108–125)	0/5	0/5
KIM5-3301.2 (YopJ$^-$Δ108–125)	0/5	0/5
KIM5-3301.2 carrying pV	0/5	0/5
KIM5-3301.2 carrying pVH	0/4	0/4
KIM5-3001.11 (Δ188–207)	0/5	0/5
KIM5-3001.11 carrying pV	0/5	0/5
KIM5-3301.4 (YopJ$^-$Δ217–225)	5/5	5/5

[a] NT, not tested.

Virulence of the *lcrV* mutants in mice. Existing data have indicated that LcrV might be a bifunctional protein: in addition to its proposed role as a regulatory protein, it is a virulence factor. To learn more about the role of the V antigen in the virulence of *Y. pestis*, all *lcrV* mutants were tested for lethality in mice (Table 3). Mutants Δ25–40, Δ108–125, and Δ188–207 were avirulent in mice given intravenous doses of ca. 10^3 and 10^5 CFU. In contrast, ca. 10 50% lethal doses (7×10^2 CFU) of the parent *Y. pestis* KIM5-3001 killed all five mice. Similar results were obtained when the YopJ$^-$ KIM5-3301 parent and its *lcrV* derivatives were analyzed (Table 3). In contrast, the YopJ$^-$Δ217–225 mutant retained its virulence properties, consistent with all other evidence indicating that LcrV(Δ217–225) is fully functional.

We were unable to restore virulence to our three LcrV$^-$ mutants by providing only LcrV in *trans* on plasmid pV (Table 3), even though pV restored their LCR phenotypes to a nearly wild-type pattern (slightly hyperrestricting growth property shown in Fig. 2C and immunoblot data not shown). pV decreased the virulence of the parent KIM5-3301 (Table 3), indicating that overexpression of LcrV is detrimental to the bacteria. However, there was no interference of the multicopy pV(Δ25–40) and pV(Δ188–207) plasmids with the virulence properties of the parental strain, even though LcrV(Δ25–40)

was overexpressed in the cytoplasm and secreted in amounts comparable to those of wild-type LcrV (data not presented). Although pVH had a smaller detrimental effect on the virulence of YopJ$^-$ *Y. pestis* KIM5-3301 than did pV, we could not restore the virulence of our mutants with this plasmid either (Table 3). We attempted the complementation tests by using the first-crossover cointegrant strains carrying two copies of *lcrV* (normal and mutated) in pCD1, that had been obtained in the course of creating the Δ25–40, Δ108–125, and Δ188–207 mutants. However, these were fully virulent, apparently because of high-frequency resolution of the cointegrate plasmids: only yersiniae carrying the parental type of pCD1 were recovered from livers and spleens of dead mice. To be sure that the avirulence of our mutants was not caused by unknown additional mutations in other regulatory regions of the LCR plasmids, the cointegrants used to isolate the *lcrV* mutants were resolved in vitro and screened for isolates which had regenerated the parental *lcrV*. These "secondary parents" were fully virulent in mice (data not shown).

DISCUSSION

We have begun to characterize the dual role of the V antigen in virulence and in regulation of the LCRS in *Y. pestis*. To localize regions of this protein that are functionally meaningful, we constructed and characterized four mutants carrying small nonpolar deletions in *lcrV* (Fig. 1).

The Δ108–125 mutant consistently very poorly transported its LcrV(Δ108–125) product outside the cells in all tested *Yersinia* backgrounds, including the hypersecretory LcrE$^-$ strain. Even when Δ*lcrV*(aa 108–125) was supplied in multiple copies in *trans* in LcrE$^-$ *Y. pestis*, LcrV(Δ108–125) was only very weakly secreted into the medium. We think that degradation by the Pla protease was relatively minor, as the total amount of LcrV in the soluble plus extracellular fractions isolated from both mutants was not significantly decreased below that in the parent (Fig. 3). Therefore, we believe that the degradation process had little effect on the amount of V antigen available for transport. Little is known about the mechanism of V-antigen transport. There is no N-terminal signal sequence, and no secretion-associated N-terminal processing of this protein has been reported; however, it does require a functional *yop* secretion mechanism for its export (39). Price et al. (43) noted the presence of an internal signal-like sequence at bp 298 through 381. Our Δ108–125 mutant carried an in-frame deletion of a large part of this region (bp 322–375) and was defective in the secretion of its LcrV product; however, this correlation could be fortuitous, as there is no evidence that a Sec-like protein secretion system participates in the transport of any secreted LCRS protein. It remains possible that a small alteration in conformation or an abnormal juxtaposition of residues due to the deletion in the Δ108–125 *Y. pestis* mutant renders its LcrV(Δ108–125) incompetent for secretion. Either way, our data indicate that the determinants in LcrV that direct its secretion may be different from those in Yops, where secretion determinants lying within N-terminal residues were identified (30). On the basis of those findings, we anticipated that there might be a secretion defect in our mutant lacking aa 25 to 40, but we could not predict the severity of the secretion lesion due to loss of residues 108 to 125 of LcrV. We speculate that the internal structure of the V antigen, in contrast to that of Yops, is necessary for its secretion.

Price et al. (43) mentioned the homology of two 17-base sequences within the *lcrV* gene to the *araI*$_1$ AraC-like binding site of the *araBAD* operon in *E. coli*. Among the mutations created in our study, the deletion of aa 188 to 207 of *lcrV*, positioned exactly in the same region of *lcrV* as the AraC-binding site, had a severe LCR regulatory defect. The Δ188–207 mutant showed very little LCR induction-associated growth restriction at 37°C in the absence of Ca^{2+} and correspondingly poorly expressed the LCRS products (Fig. 2 and 3). In its LCR properties, it resembled the LcrV$^-$ *Y. pestis* KIM5-3241 mutant, described by Price et al. (42), used in our experiments as a reference strain. However, the Δ188–207 mutant expressed a low level of the expected smaller form of LcrV (Fig. 3), whereas the previous construct resulted in an unstable LcrV product and an effectively totally LcrV$^-$ strain (42). The repressed phenotype of the Δ188–207 mutant presented in this report supports previous findings that LcrV has a regulatory role in the LCR (2, 42) and raises the possibility of a potential role for bases 562 to 621 of *lcrV* or aa 188 to 207 of LcrV in LCR induction. The deletion in mutant Δ217–225 may identify a 3′ or C-terminal boundary for this putative regulatory domain in *lcrV* or V antigen, respectively, as this mutant was wild type for every phenotype tested. However, further studies are required to rigorously distinguish among the following possibilities: (i) the deleted part of Δ*lcrV*(aa 188–207) is an *araI*$_1$-like regulatory domain necessary for full LCR induction, (ii) the regulation defect in the Δ188–207 mutant is due to the loss of a specific domain in LcrV that is involved in regulation, and (iii) the regulation defect is due to the low net expression of LcrV(Δ188–207), such that insufficient amounts are present to accomplish the regulation. Nonetheless, our data have provided some information about the cellular location of LcrV's regulatory target. We believe that this target is not the Yop secretion mechanism, because the Δ*lcrV*(aa 188–207) mutation did not compromise secretion. We favor the working hypothesis that LcrV's regulatory target is located in the cytoplasm, because yersiniae retain ca. half of the V antigen they make in the soluble cellular fraction, and essentially normal LCR regulation occurs in the absence of secretion of LcrV in the Δ108–125 mutant.

In this study, we prepared a set of double mutants carrying the Δ*lcrV*(aa 188–207) mutation and also mutations in *lcrE*, *lcrG*, and *lcrH* loci to determine where LcrV's regulatory role fits in the LCR circuitry. All three double mutants resembled LcrV$^+$ single mutants: they entered restriction at 37°C whether Ca^{2+} was present or not and were correspondingly constitutively induced for *yopM* expression. Nonetheless, only weak expression of the LcrV(Δ188–207) protein was observed (Fig. 6). These data indicate that full LCR induction can occur in the absence of functional LcrV. This argues against a direct role for LcrV as a positive regulator of the LCR (2, 42), and we conclude that LcrV might promote induction not as an activator per se but by inhibiting negative regulation. As previously shown (43), LcrH contributes to downregulation of the LCRS at 37°C in the presence of Ca^{2+} but also is strongly expressed under inductive conditions when Ca^{2+} is absent (Fig. 4 and reference 42). A question not answered is how the negative function of LcrH is eliminated in the absence of Ca^{2+} (2, 42). We hypothesize that LcrV may act to inhibit LcrH's negative regulatory function. Our evidence from complementation studies supports this general idea by showing that the relative concentrations of LcrV and LcrH determine the extent of induction of the LCRS. Therefore, we see the action of the V antigen in the LCR as being a modulatory and quantitative inhibition of downregulation.

The V antigen also is thought to function as an antihost effector component of the low-Ca^{2+} response, because it is a protective antigen in both active and passive immunizations (35, 65, 66). To learn more about the role of the V antigen in the virulence of *Y. pestis*, all of our *lcrV* mutants were tested for

lethality in mice. Only the YopJ$^-$Δ217–225 mutant retained virulence; all other *lcrV* mutant strains were avirulent in mice (Table 2), and we were unable to restore virulence with the pV or pVH plasmid. In addition, both plasmids severely decreased the virulence of the parent strain. It appears that both regulation and virulence are sensitive to the ratio of LcrV and LcrH. Strains carrying Δ*lcrV*(aa 25–40) and Δ*lcrV*(aa 108–125) secreted so much less V antigen than the parent that this could have caused their avirulence. However, boosting the level of extracellular LcrV(Δ25–40) in the parent *Y. pestis* with a multicopy plasmid did not decrease its virulence as did extra copies of normal LcrV, suggesting that LcrV(Δ25–40) lacks the feature that is detrimental to virulence when supplied in excess. Accordingly, our data do not rule out the necessity of residues 25 to 40 or 108 to 125 for the virulence function of the V antigen. Importantly, because both mutants YopJ$^-$Δ25–40 and YopJ$^-$Δ108–125 had essentially normal LCR regulation, these data show that the regulatory effect of LcrV can be separated genetically from its virulence function and that secretion of LcrV is not necessary for its regulatory function. They also provide the best genetic evidence available that LcrV per se is necessary for virulence.

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