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Yersinia pestis LcrV Forms a Stable Complex with LcrG and May Have a Secretion-Related Regulatory Role in the Low-Ca^{2+} Response

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Yersinia pestis **contains a virulence plasmid, pCD1, that encodes many virulence-associated traits, such as the Yops (*Yersinia* outer proteins) and the bifunctional LcrV, which has both regulatory and antihost functions. In addition to LcrV and the Yops, pCD1 encodes a type III secretion system that is responsible for Yop and LcrV secretion. The Yop-LcrV secretion mechanism is believed to regulate transcription of *lcrV* and *yop* operons indirectly by controlling the intracellular concentration of a secreted repressor. The activity of the secretion mechanism and consequently the expression of LcrV and Yops are negatively regulated in response to environmental conditions such as Ca^{2+} concentration by LcrE and, additionally, by LcrG, both of which have been proposed to block the secretion mechanism. This block is removed by the absence of Ca^{2+} or by contact with eukaryotic cells, and some Yops are then translocated into the cells. Regulation of LcrV and Yop expression also is positively affected by LcrV. Previously, LcrG was shown to be secreted from bacterial cells when the growth medium lacks added Ca^{2+}, although most of the LcrG remains cell associated. In the present study, we showed that the cell-associated LcrG is cytoplasmically localized. We demonstrated that LcrG interacts with LcrV to form a heterodimeric complex by using chemical cross-linking and copurification of LcrG and LcrV. Additionally, we found that small amounts of LcrV and YopE can be detected in periplasmic fractions isolated by cold osmotic shock and spheroplast formation, indicating that their secretion pathway is accessible to the periplasm or to these procedures for obtaining periplasmic fractions. We propose that the cytoplasmically localized LcrG blocks the Yop secretion apparatus from the cytoplasmic side and that LcrV is required to remove the LcrG secretion block to yield full induction of Yop and LcrV secretion and expression.**

Yersinia pestis, the causative agent of plague, has a plasmid-encoded low-calcium response (LCR) regulatory mechanism that governs the expression and secretion of a set of virulence proteins in response to environmental conditions (9, 47). In vitro, the secreted Yops (*Yersinia* outer proteins) and V antigen (also called LcrV) are expressed maximally and secreted at 37°C in the absence of Ca^{2+}. In the presence of millimolar concentrations of Ca^{2+}, their expression is downregulated and their secretion is completely blocked. It has become apparent from tissue culture infection models that the absence of Ca^{2+} in vitro mimics an unidentified signal that the yersiniae receive when they are adherent to a eukaryotic cell (36, 47). This signal triggers vectorial translocation of at least some of the Yops into the eukaryotic cell, where they derange cellular signalling and other biochemistry necessary for phagocytosis and the mobilization of an effective immune response (15, 29, 38, 47). Concomitantly, expression of Yops and LcrV becomes maximally induced. A popular model for the connection between the contact-triggered secretion and the transcriptional upregulation is the secretion of a repressor component by the Yop secretion system (3, 8, 31, 47). Accordingly, secretion and its regulation are central to the LCR.

Yops and LcrV are secreted without processing by a proprietary Yop secretion system that is encoded by the LCR virulence plasmid (9, 42). This mechanism, encoded by at least 19 genes, represents a recently recognized class of secretion system termed type III (39) or contact dependent, whose members appear to function for virulence protein secretion in a number of gram-negative animal and plant pathogens (9). Secretion of all Yops and LcrV appears to be regulated negatively in response to environmental Ca^{2+} by two proteins, LcrE and LcrG. The secreted protein LcrE (also called YopN) acts at the bacterial surface, presumably as a Ca^{2+} sensor, although that activity has not been directly demonstrated (13). LcrG, also secreted, is believed to act in conjunction with LcrE (42) to block secretion of Yops and LcrV in the presence of Ca^{2+}. Mutations inactivating either LcrE or LcrG cause strongly induced secretion and expression of all Yops and LcrV in vitro at 37°C whether Ca^{2+} is present or absent (13, 36, 42, 43). Additional proteins, such as LcrQ, YscO, or YscP, appear to modulate the secretion of only a subset of the Yops and LcrV (1, 26, 35).

LcrV is both an LCR regulatory protein and an antihost protein with strong immunomodulatory effects (3, 23, 43). LcrV has a quantitative effect on induction of LCR-regulated gene expression, being necessary for full transcriptional induction of the LCR stimulon: in the absence of LcrV, only partial induction occurs in vitro at 37°C in the absence of Ca^{2+} (32, 43). Analysis of double mutants having defects in *lcrV* and one of the negative regulatory LCR loci (*lcrE* or *lcrG*) led to the conclusion that LcrV does not function directly as an activator, but instead counteracts negative regulation. *lcrV Y. pestis* mutants are capable of strongly secreting YopM when the protein is expressed independently of LCR regulation, indicating that LcrV is not essential for secretion (43). This finding, coupled with evidence that it is cytoplasmic rather than secreted LcrV that functions in regulation, prompted the hypothesis that LcrV might act at the level of the LCR repressor (43). Interestingly, *lcrE*, but not *lcrG*, *Y. pestis* hypersecretes V antigen,

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leaving little LcrV inside the bacteria, whereas in parental *Y. pestis* cultures, almost half of the LcrV is located in the soluble cellular fraction (43). Moreover, mutations in either *lcrE* or *lcrG* are epistatic over mutations in *lcrV*, indicating that LcrV's function is somehow bypassed or irrelevant in *lcrE* or *lcrG* backgrounds (43) and suggesting that LcrV, LcrE, and LcrG function in the same pathway. These findings suggest that LcrV interacts in some way with LcrE and LcrG.

The present study was undertaken initially as separate investigations of the regulatory roles of LcrG and LcrV in the LCR. We report evidence that these two proteins directly interact to form a stable complex and describe localization experiments that determine the likely sites of action of LcrG and its complex with LcrV.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. pestis* strains used were KIM5 (from R. R. Brubaker, Michigan State University), KIM5-3001 (Sm^r; 19), KIM5-3241 [Δ*lcrV* (aa 18–215) *yopJ*::Mu dI1734 ($LcrV^-$ $YopJ^-$ Km^r Lac^+); 32], and KIM5-3001.6 [Δ*lcrE*(aa 48–197); 31]. For genetic manipulations (e.g., transformation, isolation of plasmid DNA), *Y. pestis* strains were grown in heart infusion broth or on tryptose blood agar base medium (Difco Laboratories, Detroit, Mich.) at 26°C. Growth of *Y. pestis* for physiological studies was conducted in a defined medium, TMH, as previously described (45). Briefly, *Y. pestis* cultures were grown in exponential phase at 26°C with shaking at 200 rpm for about eight generations, and final cultures to be harvested were initiated at 26°C at an A_{620} of ca. 0.1. When the A_{620} reached ca. 0.2, the temperature was shifted to 37°C, and incubation was continued for 6 h before cells were harvested. *Escherichia coli* strains used were GM2163 (51) and DH5α (Gibco BRL, Grand Island, N.Y.). *E. coli* strains were grown in LB broth or on LB agar plates (10). Streptomycin and ampicillin, both at 100 μg/ml (Sigma Chemical, St. Louis, Mo.), were used to supplement the various media as appropriate. pHTV is a plasmid that was constructed to overexpress a polyhistidine-tagged LcrV fusion protein (HTV) having 19 residues (including 6 His residues) fused to the N terminus of LcrV (12). HTV is expressed from the pProEX-1 vector (Gibco BRL) under control of the strong *trc* promoter and was induced during growth at 37°C with 0.1 to 0.5 mM isopropyl-β-D-galactopyranoside (IPTG; Sigma) in both *E. coli* and *Y. pestis*. Plasmid pTL61T (20) was used to provide expression of β-galactosidase and β-lactamase, used for quality control in studies to localize LcrG. pES6-1 (43) contains the *Hin*dIII G fragment of the *Y. pestis* KIM5 LCR plasmid pCD1 and encodes intact LcrG, LcrV, LcrH, and YopB. Plasmid pGST-G (see below) in *E. coli* DH5α grown at 37°C was used to overexpress a glutathione *S*-transferase-LcrG fusion protein (GST-G) for purification of LcrG. GST-G expression was induced by addition of 1 mM IPTG when the culture A_{620} had reached ca. 0.8. Incubation of the culture was continued for an additional ca. 3 h before cells were harvested. Plasmids pAraG18K (expressing LcrG), pAraV18K (expressing LcrV), and pAraGV18K (expressing both LcrG and LcrV) were constructed (see below) to express LcrG and LcrV under control of the *araBAD* promoter. pTT4, containing *lcrE* under control of the *tac* promoter (12) (see below), was used to overexpress LcrE in the $YopD^-$ *Yersinia pseudotuberculosis* III (pIB15) (from Hans Wolf-Watz, University of Umeå, Umeå, Sweden). Cultures in TMH lacking added Ca^{2+} were grown at 26°C to A_{620} of 0.4. The temperature was shifted to 37°C, and incubation was continued to allow the culture A_{620} to reach 0.8. IPTG was added to 0.1 mM, and incubation was continued for 5 or 6 h before the culture supernatant was harvested.

DNA methods. Plasmid DNA was isolated by an alkaline lysis method (4), by the method of Kado and Liu (17), or by the use of Qiagen columns (Qiagen, Inc., Studio City, Calif.). Cloning methods were essentially as described previously (40). DNA fragments were isolated from agarose gels by using the Qiaex DNA purification kit (Qiagen). Electroporation of DNA into *E. coli*, *Y. pestis*, and *Y. pseudotuberculosis* was done as described previously (28). To construct pTT4 (12), *lcrE* was obtained from pGP2-1 (30) by cutting at the nonmethylated *Cla*I site upstream of *lcrE*, filling in with Klenow fragment, and cutting downstream (in the multiple cloning site of the pBluescript II SK− vector [Stratagene, La Jolla, Calif.]) with *Bam*HI. This fragment was cloned into *Sma*I- and *Bam*HI-cut pTTQ8 (44) and electroporated into *E. coli* DH5α for preliminary characterization and then into *Y. pseudotuberculosis* III(pIB15). pGST-G was constructed by cloning a *Bam*HI- and *Eco*RI-cleaved PCR fragment containing *lcrG* into *Bam*HI- and *Eco*RI-cleaved pGEX-3X (Pharmacia Biotech, Piscataway, N.J.). Primers for the PCR were as follows: upstream, 5′-CTA TGG ATC CTA ATG AAG TCT TCC CAT TTT-3′; downstream, 5′-AGC GTG AAT TCA TTA AAT AAT TTG CCC TCG CAT-3′. The template was pES6-1. The PCR reaction used the GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, Conn.) according to the manufacturer's protocol, and the product was generated with 30 cycles at 94°C for 1 min, 37°C for 2 min, and 68°C for 2 min in a Perkin Elmer Model 480 thermocycler. Correctness of the fusion junction was verified by sequencing of double-stranded DNA (with sequence-specific primers) by the dideoxy-chain termination method (41) using the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP from New England Nuclear Corp. (Boston, Mass.). Plasmids pAraG18K, pAraV18K, and pAraGV18K were constructed by cloning a *Bam*HI- and *Eco*RI-cleaved PCR product into pBAD18-Kan (14). Primers used were AraG-Start (5′-GGA ATT CAG GAG GAA ACG ATG AAG TCT TCC CAT TTT GAT-3′) and AraG-Stop (5′-CGC GGA TCC TTA AAT AAT TTG CCC TCG-3′) to make pAraG18K, AraV-Start (5′-GGA ATT CAG GAG GAA ACG ATG ATT AGA GCC TAC GAA-3′) and AraV-Stop (5′-CGC GGA TCC TTA TCA TTT ACC AGA CGT GTC-3′) to make pAraV18K, and AraG-Start and AraV-Stop to make pAraGV18K. The PCR was performed as above except for the cycling profile, which was 30 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min in a Perkin Elmer GeneAmp Model 2400 thermocycler.

Cell fractionation and protein purification. Bacterial cells were harvested by centrifugation and suspended in phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.4]) or *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; Research Organics Inc., Cleveland, Ohio)-buffered saline (HBS; 135 mM NaCl in 0.1 M HEPES [pH 7.4]). Cellular extracts were made by disintegration of bacteria in a French press (20,000 psi). Low-speed centrifugation (8,000 × *g*, 10 min) removed unbroken cells and large debris. Total soluble proteins (cytoplasmic plus periplasmic) were separated from membranes by ultracentrifugation at 417,000 × *g* for 15 min in a TLA100.4 rotor or at 513,000 × *g* in a TLA120.2 rotor for 10 min, using a Beckman Optima TLX ultracentrifuge. The top 10 ml of ca. 20 ml of culture supernatant from *Y. pestis* grown in TMH medium at 37°C was removed without disturbing the cell pellet, and total proteins were precipitated with 5% (vol/vol) trichloroacetic acid (TCA) for 2 h to overnight on ice. After centrifugation (14,000 × *g* for 30 min) to pellet the precipitated proteins, the pellet was neutralized with 15 μl of 1 M Tris-Cl, pH 8.0, resuspended to a concentration corresponding to an A_{620} of 20 in 2× sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris [pH 6.8] containing 20% [vol/vol] glycerol, 4% [wt/vol] SDS, and 2% [vol/vol] β-mercaptoethanol) (2), and stored at −20°C.

Periplasmic fractions were isolated from *Y. pestis* by two methods: conversion to spheroplasts (46) and cold osmotic shock (25). (i) For formation of spheroplasts, cells corresponding to 10 A_{620} units · ml were suspended in 1 ml of ice-cold Tris-sucrose buffer (25% [wt/vol] sucrose, 10 mM Tris [pH 7.8]). Spheroplasts were made by incubation for 5 min at room temperature with lysozyme (0.5 mg/ml) followed by rapid addition of 2 ml of 15 mM Na_2EDTA (pH 7.5). Incubation was continued at room temperature until spheroplasts were evident by light microscopy (~5 min). The spheroplasts were recovered by centrifugation (8,000 × *g* for 10 min), and the supernatant containing periplasmic proteins was retained. Periplasmic proteins released from the formation of spheroplasts, secreted proteins in the original culture supernatant, and proteins from the spheroplasts were concentrated with TCA as described above. (ii) For cold osmotic shock, *Y. pestis* cells corresponding to 10 A_{620} units · ml were harvested and resuspended in 1 ml of Tris-sucrose-EDTA buffer (30 mM Tris-Cl, 20% [wt/vol] sucrose, 1 mM Na_2 EDTA [pH 8.0]) and incubated for 10 min at room temperature with gentle agitation on a rocker platform. The cells were harvested by centrifugation (8,000 × *g* for 10 min), rapidly resuspended in 1 ml of ice-cold 5 mM $MgSO_4$, and then incubated on ice with shaking for 10 min. The cellular fraction was separated from the periplasmic fraction by centrifugation (8,000 × *g* for 10 min), and the periplasmic, culture supernatant, and shocked-cell fractions were concentrated with TCA as described above.

HTV was purified from *E. coli* and *Y. pestis* soluble protein extracts by metal chelation chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) and following the manufacturer's protocol. GST-G from *E. coli* overexpressing the fusion protein was purified on a glutathione column (Pharmacia). GST-G was then digested with factor Xa protease (Boehringer Mannheim Biochemicals, King of Prussia, Pa.) and passed over the glutathione column a second time to obtain LcrG. Although this procedure removed most of the free GST, some nonetheless was not removable by the glutathione column, despite repeated passages, and remained in the final LcrG preparation. LcrG was obtained from *Y. pestis* by antibody affinity chromatography. An anti-LcrG (α-LcrG) antibody column was made by cross-linking a rabbit polyclonal antibody specific for LcrG (see below) to protein A-Sepharose (Sigma) (~2 mg of antibody to 1 ml of wet beads) as previously described (16). Antibodies were allowed to bind to protein A by incubation in PBS overnight with shaking at 4°C. Next, cross-linking was done after washing the beads twice with 10 volumes of 0.2 M Na borate buffer (pH 9.0) by incubation for 30 min at room temperature with 20 mM dimethylpimelimidate (Sigma) in 0.2 M Na borate (pH 9.0). The cross-linking reaction was stopped by washing the beads in 0.2 M ethanolamine (pH 8.0) followed by incubation for 2 h at room temperature as described previously (16). The beads were washed once with PBS, and noncovalently bound antibody was eluted with 100 mM glycine (pH 3.0) followed by final equilibration with PBS. *Y. pestis* soluble protein extracts were passed over this column, followed by 20 column volumes of PBS and a preelution wash with 10 column volumes of 10 mM sodium phosphate buffer (pH 6.8) prior to elution of the bound protein. Bound LcrG was eluted with acidic 100 mM glycine (pH 3.0) into tubes containing 1 M Na_2HPO_4 (pH 8.0) to neutralize the acidic glycine. All protein concentrations were measured by using the bicinchoninic acid assay (Pierce Chemical, Rockford, Ill.).

Antibody reagents. Two α-LcrV reagents were used: one with reactivity against both LcrG and LcrV (α-VG) and one specific only for LcrV (α-HTV) (12). The

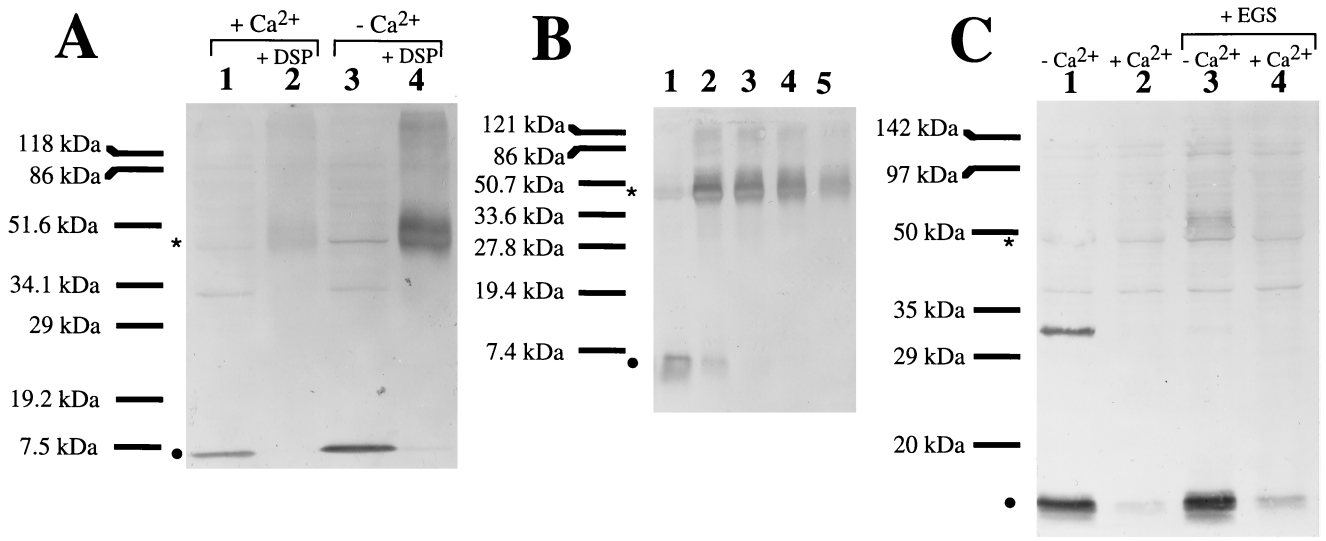

FIG. 1. LcrG forms a stable complex in *Y. pestis*. *Y. pestis* KIM5 was grown at 37°C in the presence or absence of Ca^{2+}. A homobifunctional amine-reactive cross-linker was added to cell-free soluble protein extracts (A and B) or growing cultures (C). Cross-linked protein samples corresponding to 0.05 A_{620} unit · ml of culture were separated by SDS-PAGE in a 12% gel and analyzed in duplicate immunoblots with α-GST-G or α-HTV. Data are shown for detection with α-GST-G. (A) Lanes 1 and 2, the yersiniae were grown in medium containing Ca^{2+}; lanes 3 and 4, the yersiniae were grown in medium without Ca^{2+}; lanes 1 and 3, the samples were not cross-linked; lanes 2 and 4, the samples were cross-linked with 3 mM DSP. (B) Lanes 1 to 5, the samples were cross-linked with 0, 1, 3, 6, and 10 mM DSP, respectively. (C) Lanes 1 and 3, the yersiniae were grown in medium lacking Ca^{2+}; lanes 2 and 4, the yersiniae were grown in medium containing Ca^{2+}; a cross-linker (1 mM EGS) was added to the cultures of lanes 3 and 4 before the soluble cellular fraction was prepared. The position of LcrG is indicated with a dot, and the position of the 48- to 50-kDa complex is indicated with an asterisk. The locations of molecular mass standards are indicated at the left of each panel.

entire procedure for generating α-GV was described in detail previously (48). Briefly, a 10-liter culture of *Y. pestis* KIM5 was grown at 37°C in Ca^{2+}-deficient complex medium (6, 7). LcrV was semipurified from the cell extract of the harvested bacteria by sequential ammonium sulfate precipitation (20% saturation and then 50%), dialyzed, and fractionated by anion-exchange chromatography. Female New Zealand White rabbits were immunized with the semipurified LcrV. Serum samples having reactivity against LcrV were pooled and extensively adsorbed with broken cells of *Y. pestis* KIM6 (lacking the LCR plasmid) and *Y. pestis* KIM5-3042 ($LcrV^-$ due to an insert of Mu dI1 [Ap^r *lac*] in upstream *lcrG*, but overexpressing Yops [27]). Immunoglobulin G (IgG) was partially purified by ammonium sulfate fractionation followed by dialysis against PBS. α-LcrV prepared in this way also had reactivity against LcrG because of the phenomenon reported in this paper: in the cytoplasm LcrV forms a tight complex with LcrG. The adsorption failed to remove the α-LcrG reactivity because the bacteria used for adsorption did not express LcrG.

To obtain α-LcrE antibody, the proteins in the supernatant from 1-liter cultures of *Y. pseudotuberculosis* III(pIB15, pTT4) were TCA precipitated and separated in preparative SDS–12% polyacrylamide gels. LcrE was electroeluted from bands that were well separated from other proteins and not contaminated by the similar-sized YopD due to the absence of YopD in the *Y. pseudotuberculosis* host strain. Antibody was raised in female New Zealand White rabbits as previously described (30), except that booster doses used LcrE-containing gel pieces that had been ground to a slurry in PBS.

α-HTV (12) and α-GST-G were raised in female New Zealand White rabbits as previously described (30). Rabbit α-β-galactosidase was obtained from Cooper Biomedical (West Chester, Pa.), and rabbit α-β-lactamase was the generous gift of Kathleen Postle (Washington State University, Pullman, Wash.). IgG was purified on protein A-Sepharose (Sigma) and dialyzed against PBS. Mouse α-YopE was the generous gift of Gerard P. Andrews and Arthur M. Friedlander (USAMRIID, Ft. Dietrick, Md.).

Protein electrophoresis and immunodetection. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 12 or 15% (wt/vol) polyacrylamide gels as indicated, according to the method of Laemmli (18). Samples containing whole cells or membrane fractions were boiled or heated at ca. 95°C for 3 to 5 min prior to being loaded on the gels. β-Mercaptoethanol was omitted from protein samples that were cross-linked with dithiobis(succinimidyl propionate) (DSP) to preserve the cross-linking. Lanes in which subcellular fractions (soluble, membrane, and secreted proteins) were compared were loaded so as to contain amounts of the fractions derived from the same volume of original culture. Proteins separated by SDS-PAGE were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) using carbonate transfer buffer (pH 9.9) (42). Specific proteins (LcrG, LcrV, YopE, LcrE, β-galactosidase, and β-lactamase) were visualized on the membranes as previously described (30) by using the polyclonal antibodies specific for the proteins and a secondary antibody (goat anti-rabbit or goat anti-mouse; Sigma) conjugated to alkaline phosphatase.

Chemical cross-linking of proteins. Proteins in whole-cell extracts, soluble protein fractions, or cell-free culture supernatants were cross-linked in either PBS or HBS, using ethylene glycolbis(succinimidylsuccinate) (EGS), ethylene glycolbis(sulfosuccinimidyl-succinate) (sulfo-EGS), DSP, bismaleimidohexane (BMH), or disuccinimidyl suberate (DSS) (all purchased from Pierce) as cross-linkers at indicated concentrations for 20 to 30 min at room temperature according to the manufacturer's instructions. After cross-linking, the reactions were quenched by supplying excess primary amines (e.g., Tris-Cl [pH 8.0] to a 50 to 100 mM final concentration); in the case of BMH, excess sulfhydryl groups (e.g., mercaptoethanol) were added by addition of an equal volume of 2× SDS sample buffer. Optimal concentrations of cross-linkers had to be determined empirically by testing a series of concentrations for each cross-linker and sample type (e.g., whole cultures, cellular extracts or fractions, purified proteins), as the different samples presented different amounts of interfering components (such as primary amino groups) to the cross-linking reaction. For cross-linking cultures of *Y. pestis* growing in TMH, a sample of culture corresponding to 20 A_{620} units · ml was transferred after ca. 5 h of growth at 37°C to a prewarmed 250-ml flask in a shaking water bath and EGS was added to a final concentration of 1, 5, or 10 mM. Cross-linking was allowed to occur for 30 min at 37°C before quenching with Tris, added to a final concentration of 50 mM, for 15 min. Cultures were removed to ice and processed with a French press to obtain soluble fractions as described above. All cross-linking experiments using organic-soluble cross-linkers included samples that were mock treated with dimethyl sulfoxide (Sigma) to 10%, which was used as the cross-linker vehicle.

RESULTS

LcrG and LcrV interact with each other. To further elucidate the regulatory roles of LcrG and LcrV in the LCR, we determined if LcrG and LcrV interacted with other proteins. Soluble extracts from *Y. pestis* KIM5 or KIM5-3001 grown in the presence or absence of Ca^{2+} were chemically cross-linked using a variety of commercially available reagents (EGS, sulfo-EGS, DSP, BMH, and DSS). Immunoblots of cross-linked samples that were probed in duplicate blots with α-VG (directed against both LcrV and LcrG), α-HTV (specific for LcrV), or α-GST-G (specific for LcrG) showed a novel band of ~48 to 50 kDa in each case (Fig. 1A, visualized for α-GST-G in lanes 2 and 4; Fig. 3 shows this band visualized in duplicate blots with α-HTV and α-GST-G). This band was weakly visible in non-cross-linked samples (Fig. 1A, lanes 1 and 3, Fig. 1B, lane 1, and Fig. 1C, lanes 1 and 2) and had been noticed

previously in many immunoblots as a "cross-reactive band" (24). After cross-linking, this band became broadened, perhaps due to having variable numbers of attached cross-linker molecules, and often exhibited weak staining of the part that was densest. Its size of 48 to 50 kDa was consistent with LcrG (~11 kDa) and LcrV (~37 kDa) interacting to form a complex. This band was greatly intensified upon cross-linking (Fig. 1A, lanes 2 and 4, and Fig. 1B) and was more prominent in yersiniae grown in the absence of Ca^{2+} than in its presence, suggesting that the appearance of the complex was linked to the LCR. A similar band, similarly Ca^{2+} regulated in amount, was obtained when growing cultures of *Y. pestis* KIM5 were subjected to cross-linking (Fig. 1C). The in vivo result showed that the putative interaction represented by the new band likely reflected an interaction that was occurring in live bacteria and was not an artifact of performing the cross-linking procedure after the bacteria had been taken from LCR-inductive conditions and handled in the cold to obtain cell extracts. Its presence after SDS-PAGE of whole-cell samples or extracts that had not been cross-linked indicated that the interaction was very strong.

Because LcrV-specific and LcrG-specific antibodies detected a complex of similar size, we determined if LcrG and LcrV were interacting. We cross-linked soluble-protein samples from an *E. coli* strain carrying plasmid pES6-1, which encodes LcrG, LcrV, LcrH, and YopB. Immunoblots of the cross-linked samples from *E. coli* probed with a mixture of α-HTV and α-GST-G showed that we could detect the 48- to 50-kDa complex in *E. coli* (Fig. 2A), suggesting that the interacting proteins were encoded on pES6-1. As a control, we introduced pHTV into *E. coli* to see if HTV could cross-link to any *E. coli* proteins. HTV did not form a complex in *E. coli* (Fig. 2A), even though HTV can be cross-linked to form the complex in the $LcrV^-$ *Y. pestis* KIM5-3421 containing pHTV (Fig. 2A). This indicates that HTV does not interact with *E. coli* proteins but does bind to a *Y. pestis* protein. These results, combined with the size of the complex (48 to 50 kDa) and its visualization with antibodies against LcrG (α-GST-G) and LcrV (α-HTV) but not with antibody to LcrH (data not shown) suggest that LcrG (~11 kDa) and LcrV (~37 kDa) can form a heterodimeric complex. To further test the requirement of LcrV to form the 48- to 50-kDa complex, LcrG, LcrV, or both LcrG and LcrV were expressed in *E. coli* under the control of the arabinose-inducible P_{BAD} promoter. Soluble protein extracts were prepared from *E. coli* containing the cloning vector (pBAD18-Kan), pAraG18K, pAraV18K, or pAraGV18K and cross-linked with 3 mM DSP. The resulting un-cross-linked and cross-linked samples were analyzed in duplicate immunoblots for visualization of LcrG, using α-GST-G (Fig. 2B), or visualization of LcrV, using α-HTV (data not shown). This experiment showed that only when both LcrG and LcrV were coexpressed did the 48- to 50-kDa complex appear when probed with α-GST-G (Fig. 2B, lane 8) or α-HTV (data not shown). This experiment also allowed us to rule out the possibility that the 48- to 50-kDa complex was composed of only LcrG, as a band of an appropriate size was not seen in cross-linked extracts containing only LcrG (Fig. 2B, lane 4).

LcrG and LcrV can be copurified. To further substantiate that LcrG and LcrV interact, we determined if they could be copurified. LcrG was purified on resin containing α-GST-G: DSP-cross-linked or untreated soluble protein extracts from *Y. pestis* KIM5 grown in the absence of Ca^{2+} were applied to the column, and the resulting fractions were analyzed on duplicate immunoblots probed with α-GST-G or α-HTV (Fig. 3). LcrG and the 48- to 50-kDa complex were recovered from the column as expected (Fig. 3B, lanes 3 and 6 [no cross-linker], and lane 9 [with cross-linker], respectively). All or most of the LcrG present in the protein samples was bound by the column, with none appearing in the column flowthrough (Fig. 3B, lanes 2, 5, 8, and 11). In non-cross-linked samples, LcrV appeared in both the flowthrough (Fig. 3A, lanes 2 and 5) and eluate fractions (Fig. 3A, lanes 3 and 6), demonstrating that some LcrV could be retained by the α-GST-G column, even though α-GST-G has no reactivity for LcrV (e.g., see Fig. 3B). We hypothesized that this LcrV was binding to the LcrG bound on the column. All or most of the LcrG in the *Y. pestis* extracts, but not all of the LcrV that was present, was retained by the α-LcrG column. This shows that LcrV is present in excess when compared to LcrG and raises the possibility that all the LcrG present is capable of interacting with LcrV. Additionally, most or all of the free LcrG chases into the complex after cross-linking with 3 mM or greater concentrations of DSP (Fig. 1 and 3). However, the inability to visualize free LcrG could partially be due to blocking of epitopes by DSP and not to the complexing of all

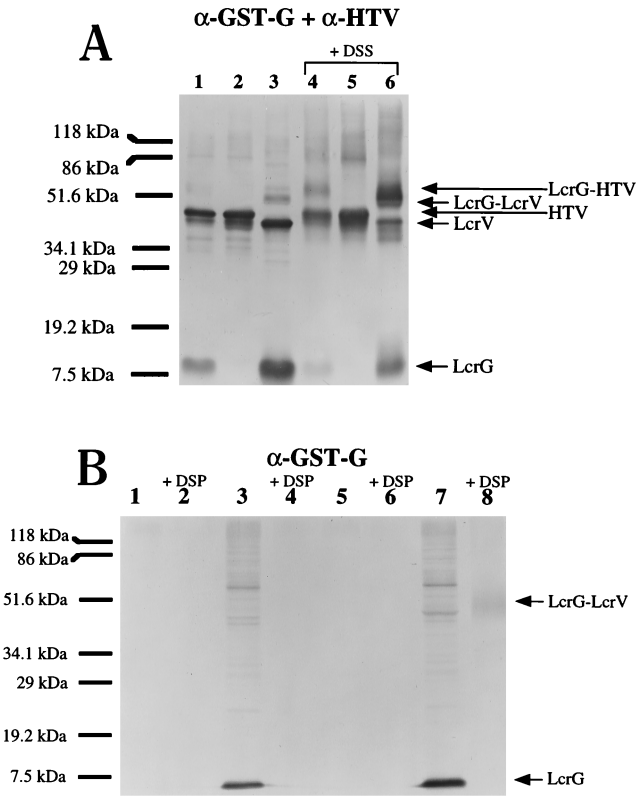

FIG. 2. LcrG interacts with a protein encoded by pES6-1. (A) Soluble protein extracts were isolated from *Y. pestis* KIM5-3241 ($LcrV^-$ $YopJ^-$) or *E. coli* GM2163 or DH5α harboring plasmid pHTV or pES6-1. Some samples were cross-linked with DSS used at 1 or 5 mM. Samples corresponding to 0.05 A_{620} unit · ml of original culture were separated by SDS-PAGE in a 12% gel and analyzed in an immunoblot probed with a mixture of α-GST-G and α-HTV. Lane 1, *Y. pestis* KIM5-3241(pHTV); lane 2, DH5α(pHTV); lane 3, GM2163(pES6-1); lane 4, *Y. pestis* KIM5-3241(pHTV) cross-linked with 5 mM DSS; lane 5, DH5α(pHTV) cross-linked with 1 mM DSS; lane 6, GM2163(pES6-1) cross-linked with 1 mM DSS. (B) Soluble-protein extracts corresponding to 0.1 A_{620} unit · ml of original culture from *E. coli* DH5α grown in the presence of 0.1% (wt/vol) arabinose were cross-linked with 3 mM DSP (lanes 2, 4, 6, and 8) or mock treated with dimethylsulfoxide (lanes 1, 3, 5, and 7), separated by SDS-PAGE in a 12% gel, and analyzed in duplicate immunoblots with α-GST-G or α-HTV antibody (data shown for α-GST-G). The *E. coli* contained plasmids pBAD18-Kan (vector control, lanes 1 and 2), pAraG18K ($LcrG^+$, lanes 3 and 4), pAraV18K ($LcrV^+$, lanes 5 and 6), and pAraGV18K ($LcrGV^+$, lanes 7 and 8). The locations of molecular mass standards are indicated at the left.

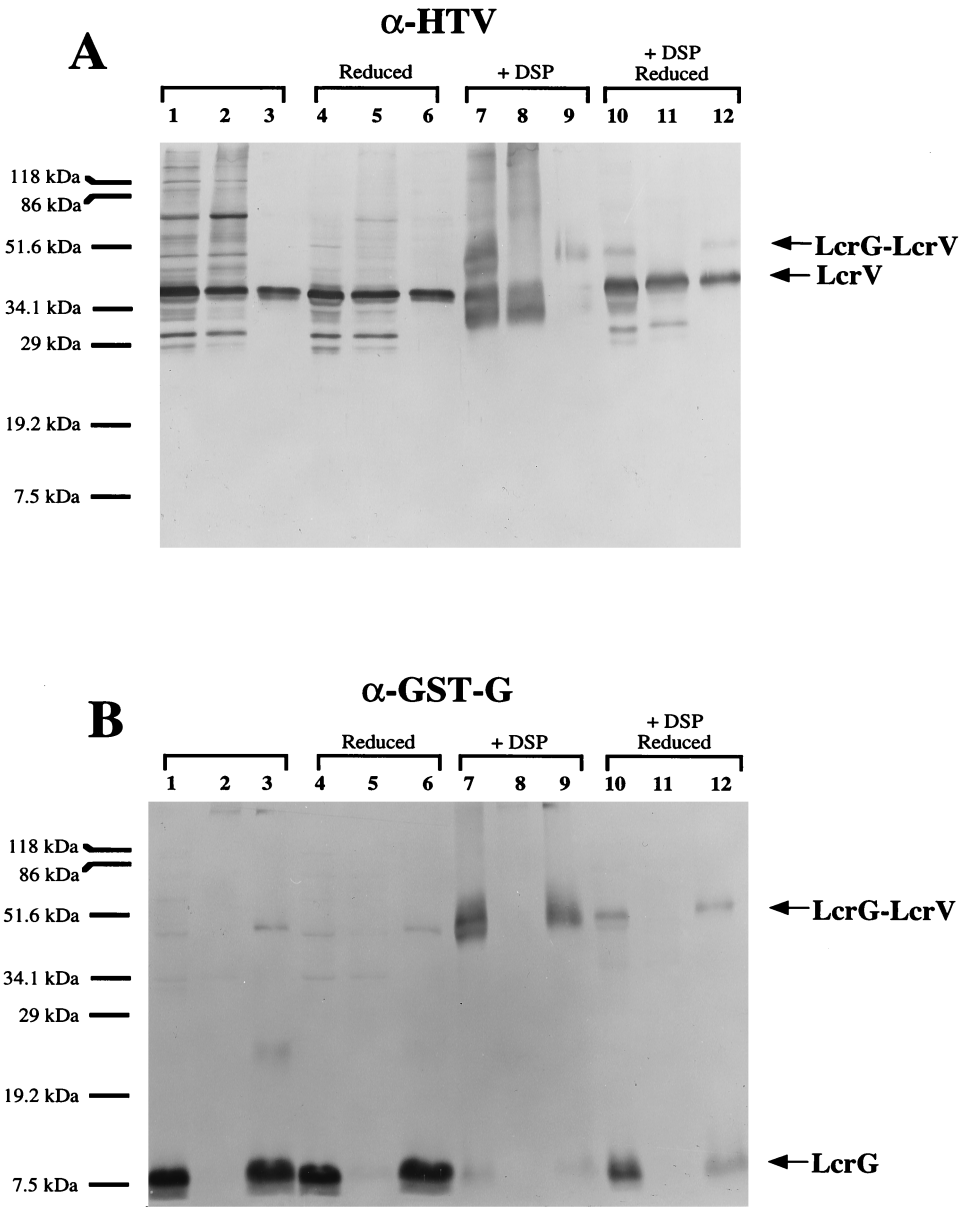

FIG. 3. LcrV copurifies with LcrG by α-GST-G affinity chromatography, and the isolated complex contains LcrG and LcrV. Soluble-protein extracts, either cross-linked with 3 mM DSP or untreated, from *Y. pestis* KIM5 grown at 37°C without calcium were applied to an α-GST-G antibody column. Samples removed from the extracts before application to the column were loaded in lanes 1, 4, 7, and 10, and samples of the flowthrough material were loaded in lanes 2, 5, 8, and 11. After washing, the proteins bound to the column were eluted with 100 mM glycine (pH 3.0) (lanes 3, 6, 9, and 12). Before-column samples corresponded to 0.05 A_{620} unit · ml of the original culture. Samples of flowthrough and elution fractions corresponded to 0.1 A_{620} unit/ml of original culture. The samples were separated by SDS–12% PAGE and analyzed in duplicate immunoblots with α-HTV (A) or α-GST-G (B). Lanes are otherwise identical in panels A and B. Lane 1, before-column sample; lane 2, flowthrough fraction; lane 3, elution fraction; lanes 4 to 6, same as lanes 1 to 3 plus 5% β-mercaptoethanol; lanes 7 to 9, same as lanes 1 to 3 plus 3 mM DSP; lanes 10 to 12, same as lanes 7 to 9 plus 5% β-mercaptoethanol. The locations of molecular mass standards are indicated at the left of each panel.

of the LcrG with LcrV. To assess the contribution of the cross-linker to visualization of LcrG, we cross-linked *Y. pestis* soluble-protein fractions from cultures grown in the absence or presence of Ca^{2+} with various amounts of cross-linker and visualized the complex and the free LcrG with α-GST-G (data for absence of Ca^{2+} illustrated in Fig. 1B). This experiment showed that our ability to detect the complex was unaffected by concentrations of cross-linker as high as 3 mM, at which concentration all of the free LcrG had disappeared. At 6 mM crosslinker, the band due to the complex appeared slightly more diffuse (more strikingly visible in samples with lower amounts of complex than in Fig. 1B), and at 10 mM DSP, the amount of complex appeared to decrease, probably due to blockage of epitopes by DSP. Similar results were obtained with another amine-reactive cross-linker, EGS; however, the sulfhydryl-reactive BMH could not chase all free LcrG into the complex even at concentrations as high as 10 mM (data not shown). In the presence of Ca^{2+}, when there are only small amounts of LcrG and LcrV in the cells, all of the LcrG was bound in the LcrG-LcrV complex at DSP concentrations as low as 1 mM (data not shown). These findings suggest that most or all of the LcrG in the soluble cellular fraction can be cross-linked to LcrV, whereas the reverse is not true (Fig. 3).

Using the α-LcrG affinity column to purify LcrG and the 48-

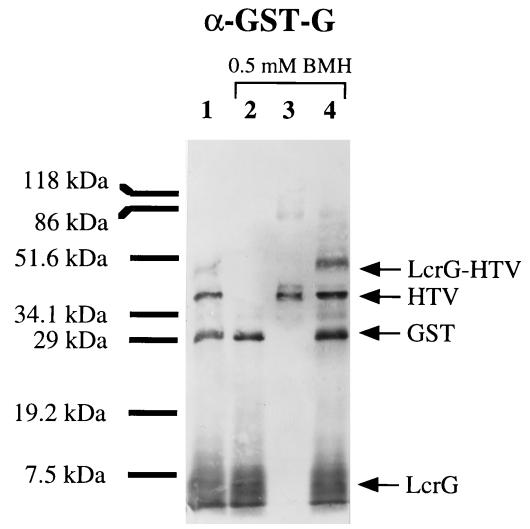

FIG. 4. Purified LcrG and LcrV interact in vitro. LcrG obtained from affinity-purified and protease-cleaved GST-G and affinity-purified HTV (both produced in and isolated from *E. coli*) were mixed in vitro and cross-linked with the homobifunctional sulfhydryl-reactive cross-linker BMH. Protein samples containing 25 pmol of each protein were separated by SDS–12% PAGE and analyzed by immunoblot with α-GST-G. Lane 1, HTV and LcrG, not cross-linked; lane 2, LcrG plus 0.5 mM BMH; lane 3, HTV plus 0.5 mM BMH; lane 4, HTV and LcrG plus 0.5 mM BMH. The locations of molecular mass standards are indicated at the left. Note that the α-GST-G antibody recognized HTV in this experiment (lanes 1, 3, and 4), whereas in all other experiments it did not.

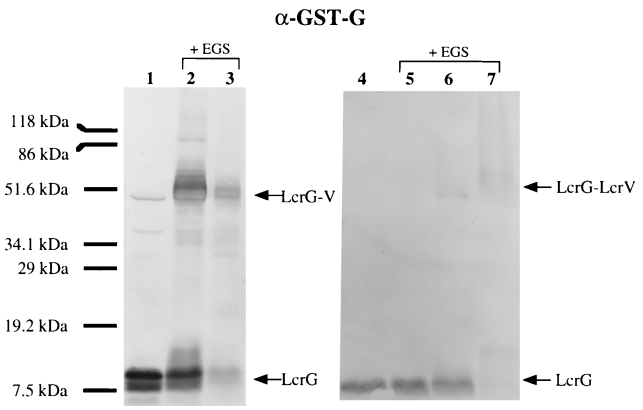

FIG. 5. The LcrG-LcrV complex is secreted, but the majority is cell associated. *Y. pestis* KIM5 cultures grown at 37°C in the absence of Ca^{2+} were separated into cell and culture supernatant fractions. The culture supernatant was cross-linked with 1, 5, or 10 mM EGS and subsequently concentrated with TCA. The cells were fractionated into soluble and membrane fractions and cross-linked with 1 mM EGS. The resulting samples were separated by SDS–12% PAGE and analyzed in duplicate immunoblots with α-GST-G or α-HTV (data shown for α-GST-G). Lanes were loaded with fractions corresponding to $0.2 A_{620}$ unit · ml of original culture. Lane 1, soluble fraction; lane 2, soluble fraction plus 1 mM EGS; lane 3, membrane fraction plus 1 mM EGS; lane 4, culture supernatant; lane 5, culture supernatant plus 1 mM EGS; lane 6, culture supernatant plus 5 mM EGS; lane 7, culture supernatant plus 10 mM EGS. The locations of molecular mass standards are indicated at the left.

to 50-kDa complex allowed us to show that the complex recovered from the column contained LcrG and LcrV. We used the cross-linker DSP in these experiments because it dissociates under reducing conditions, such as the addition of β-mercaptoethanol, allowing analysis of the components of the cross-linked complex. Upon reduction of the 48- to 50-kDa complex purified on the α-LcrG column (Fig. 3A and B, lanes 9) we were able to recover LcrV (Fig. 3A, lane 12) and LcrG (Fig. 3B, lane 12), showing that the complex recognized by α-GST-G and α-HTV contains LcrG and LcrV. We also determined whether the LcrG-LcrV complex would be obtained when cross-linked samples from $LcrV^-$ *Y. pestis* KIM5-3241 containing pHTV were purified on an Ni-NTA column. As predicted, the LcrG-HTV complex and LcrG copurified along with HTV, even though LcrG alone does not bind to this column (data not shown).

Purified LcrG and LcrV interact in vitro. As a final test for interaction between LcrG and LcrV, we purified HTV and GST-G fusion proteins from *E. coli* to test whether the purified proteins could interact in vitro. After purification of GST-G, the GST portion was cleaved from the protein with factor Xa and passed over a glutathione column to remove the GST. This removed all but a recalcitrant amount of GST from the preparation. When we mixed these proteins and subsequently cross-linked them with DSS or BMH, we obtained the LcrG-LcrV complex (Fig. 4). This showed that LcrG and LcrV by themselves could interact to form the stable complex, without the involvement of another bacterial protein. For some unknown reason when the purified HTV used in this experiment was probed with α-GST-G, the HTV was visualized on a Western blot; however, other batches of purified HTV and HTV in extracts of *E. coli* or *Y. pestis* do not cross-react with the antibody (data not shown).

Localization of LcrG and LcrG-LcrV complex. Previous studies had shown that about half of the LcrV and a portion of the LcrG synthesized are secreted by the Yop secretion system (e.g., see references 42 and 43). This suggested the possibility that the complex might be secreted. As the localization of the complex directs our thinking about its possible function, we carried out localization studies for LcrG and its complex with LcrV. We cross-linked cell-free culture supernatants from *Y. pestis* grown with or without Ca^{2+}, using concentrations of the amine-reactive EGS or sulfhydryl-reactive BMH up to 10 mM. The proteins were then recovered by precipitation with TCA and analyzed in immunoblots. We saw LcrG in non-cross-linked (Fig. 5, lane 4) and cross-linked samples and the LcrG-LcrV complex in cross-linked samples (Fig. 5, lanes 5 to 7, for LcrG visualization and data not shown for LcrV). These results show that the LcrG-LcrV complex is secreted or can form upon release of LcrG and LcrV into the culture medium. A comparison of the amounts of LcrG in soluble and extracellular fractions showed that the majority of the LcrG and of the complex was in the soluble fraction. Previous work (42) also showed more LcrG in the soluble fraction than in culture supernatants, but the difference did not appear as great as that shown in Fig. 5 (lane 1 versus lane 4) because supernatants were more concentrated when analyzed in the earlier studies. In several investigators' hands in our lab and in different experiments done by single individuals, the amount of extracellular LcrG has varied from that shown in Fig. 3 of reference 42 to that shown in Fig. 6 in this study (where LcrG was not detectable in the culture supernatant); however, in general, Fig. 5 best represents the vast majority of findings: i.e., that most of LcrG is in the soluble cellular fraction, and some is secreted. We do not know what causes the variability. Interestingly, some LcrG and complex were present in the total membrane fraction of *Y. pestis* KIM5 cells grown in the absence of Ca^{2+} (Fig. 5, lane 3; also true for inner membranes isolated by sucrose density gradient centrifugation [data not shown]). In other experiments examining the localization of LcrG without using cross-linking, more LcrG was associated

with the membrane fraction in the presence than in the absence of Ca^{2+} (data not shown).

We tested whether the amount or distribution of LcrG or the LcrG-LcrV complex obtained after cross-linking was affected by an *lcrE* mutation. *Y. pestis* KIM5-3001.6 *lcrE*, which constitutively expresses and secretes Yops irrespective of the presence of Ca^{2+} at 37°C, resembled the induced parent *Y. pestis* in expression and localization of LcrG and the complex.

All data implicated the soluble cellular fraction as the location for most LcrG and suggested the soluble cellular fraction as a candidate for LcrG's site of action. However, this fraction in all of our work to date combined proteins from two cellular compartments, the periplasm and the cytoplasm. To determine the source of LcrG in the soluble cellular fraction, we used two different methods of separating cytoplasm from periplasm, cold osmotic shock and the formation of spheroplasts. We used *Y. pestis* KIM5 carrying the plasmid pTL61T to provide non-LCR regulated control proteins for the cytoplasm (β-galactosidase) and the periplasm (β-lactamase). As expected, the bulk of the β-lactamase was localized to the periplasm (Fig. 6), and not unexpectedly, a small amount was present in the cytoplasm. Also as expected, β-galactosidase (Fig. 6) and the cytoplasmic protein LcrH (also called SycD; data not shown) were found only in the cytoplasm. Regardless of whether we used cold osmotic shock (Fig. 6) or spheroplast formation (data not shown), we saw LcrG in the cytoplasmic fraction of the cells and not the periplasm; in these experiments, very little LcrG was in the culture supernatant (not visible in Fig. 6). These results show that the soluble cell-associated LcrG is cytoplasmic. Surprisingly, small amounts of LcrV (Fig. 6A) and at least two other Yops, YopE (Fig. 6B) and LcrE (YopN) (Fig. 6C), were reproducibly found in the periplasmic fraction (although not in the periplasm obtained by spheroplast formation in the case of LcrE [data not shown]); however, YopM and YopH could not be detected in periplasmic fractions (data not shown).

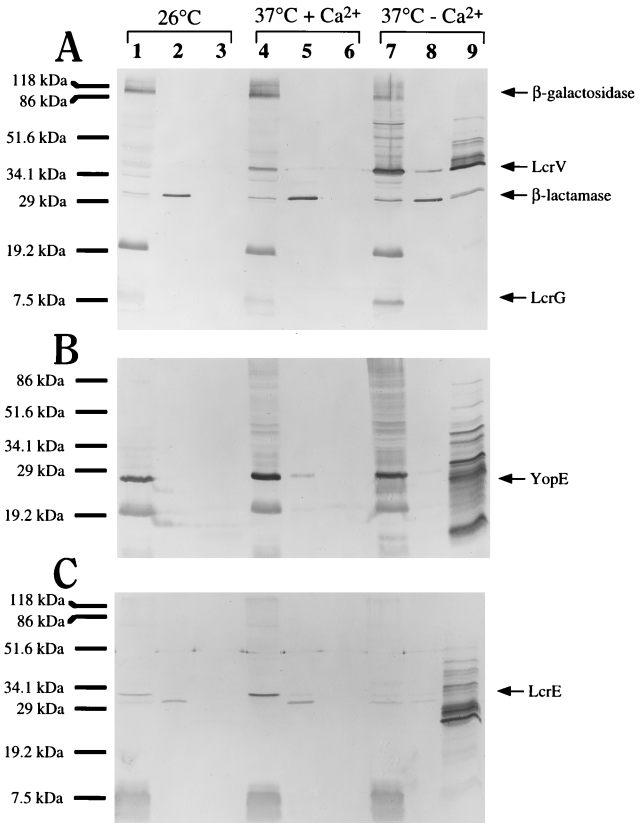

FIG. 6. Soluble cellular LcrG is cytoplasmic; soluble cellular LcrV is mainly cytoplasmic but is also detected in the periplasm. *Y. pestis* KIM5 containing pTL61T to provide non-LCR-regulated control proteins for the cytoplasm (β-galactosidase) and periplasm (β-lactamase) was grown at 26 or 37°C (plus or minus Ca^{2+}) and subjected to cold osmotic shock. Proteins from the shocked cells, the osmotic shock fluid (periplasmic proteins), and the culture supernatant were concentrated by TCA precipitation. Samples corresponding to 0.1 A_{620} unit · ml of original culture were separated by SDS-PAGE and analyzed in replicate immunoblots with various antibodies. (A) Immunoblot probed with an antibody cocktail containing α-GST-G, α-HTV, α-β-galactosidase, and α-β-lactamase. (B) Immunoblot probed with α-YopE antibody. (C) Immunoblot probed with α-LcrE antibody. Lanes 1 to 3, cultures were grown at 26°C; lanes 4 to 6, cultures were grown at 37°C with Ca^{2+}; lanes 7 to 9, cultures were grown at 37°C without Ca^{2+}. Lanes 1, 4, and 7, shocked cells; lanes 2, 5, and 8, periplasm (shock fluid); lanes 3, 6, and 9, culture supernatants. The locations of molecular mass standards are indicated at the left.

DISCUSSION

In this study, we discovered and characterized an interaction between two LCR regulatory proteins in *Y. pestis*, the negative regulatory protein LcrG and the bifunctional, positive regulatory and virulence protein LcrV. LcrG and LcrV were found to form a complex of a size likely to contain one molecule of each, and purified complexes could be dissociated to yield LcrG and LcrV. The LcrG-LcrV complex was sufficiently stable to withstand heating in SDS and β-mercaptoethanol and subsequent SDS-PAGE, and presumably formed in native protein extracts, as immunoprecipitation with α-GST-G of whole-cell extracts not subjected to cross-linking also brought down LcrV (Fig. 3 and reference 50). However, the LcrG-LcrV complex did mostly dissociate upon SDS-PAGE, and its study was facilitated by cross-linking. A range of homobifunctional cross-linker types was tested and found to work: both aqueous and nonaqueous, with spacer arms ranging from 11.4 to 16.1 Å and having reactivities either for free amino groups (EGS, sulfo-EGS, DSP, and DSS) or sulfhydryl groups (BMH). As LcrG and LcrV each have only one cysteine, stabilization of the complex with BMH implies that these unique cysteines come within ca. 16 Å of each other when LcrG and LcrV interact.

Recently, it was reported that LcrV and LcrH (34) overexpressed from one transcript in *E. coli* can be copurified and that these proteins interact over a period of many hours in an enzyme-linked immunosorbent assay (21). Possible sources of copurification are the large-scale preparative protocol used (21) or a fusion protein which we have found is made between LcrV and LcrH when expressed from a single clone in *E. coli* (12). *lcrV* and *lcrH* are in the same translational frame on their native transcript, and *lcrV*'s termination codon is inefficient in *E. coli*, resulting in an LcrV-LcrH chimera. We did not find LcrH copurifying with LcrV when the proteins were expressed from separate transcripts in *E. coli* (12). Additionally we saw no evidence of an interaction between LcrV and LcrH in the studies reported herein.

The LcrG-LcrV complex was obtained from growing *Y. pestis* cultures or cellular extracts subjected to cross-linking and after cross-linking cellular extracts from *E. coli* containing a plasmid encoding LcrG, LcrV, LcrH, and YopB. The LcrG-LcrV complex was generated in vitro by combining purified LcrG and HTV and subjecting the mixture to cross-linking. Formation of the complex in vitro showed that the complex could form posttranslationally, without the intervention of other bacterial proteins, but this result does not rule out the possibility that the complex may interact with other *Yersinia* components. Chemical cross-linking experiments revealed two

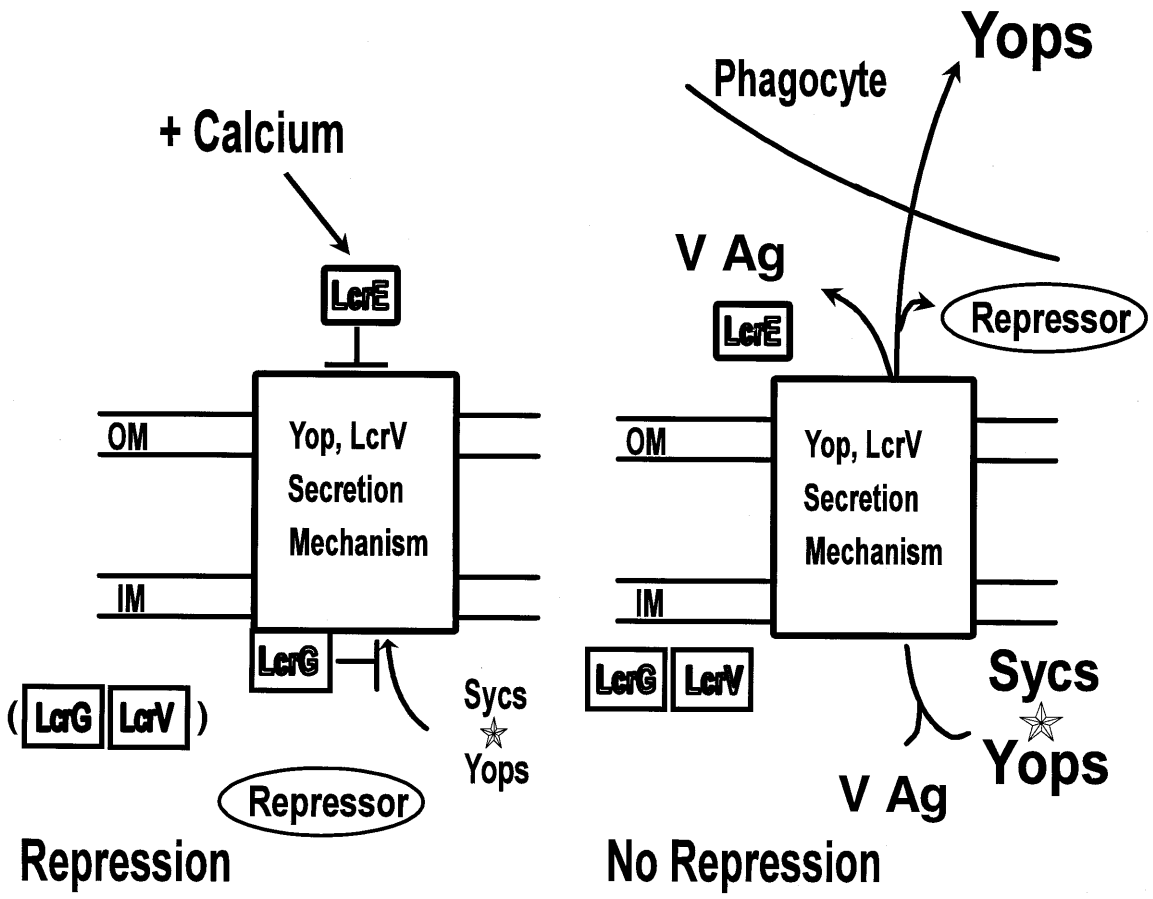

FIG. 7. Model for regulation of LCR in *Y. pestis*. In the presence of Ca^{2+}, LcrG serves to block the Yop secretion mechanism in the cytoplasm and LcrE blocks secretion at the cell surface. The secretion blockage keeps the putative LCR repressor in the cell, resulting in repression of LCR-regulated operons. In the absence of Ca^{2+} and in vivo when the bacteria contact a eukaryotic cell, LcrE is released and relieves the secretion block at the cell surface; this allows secretion of some LCR repressor, which allows induction of LCR operons, including *lcrV*. The increase in LcrV levels titrates LcrG away from the secretion mechanism by forming the LcrG-LcrV complex in the cytoplasm. This removal of the inner secretion block results in full LCR induction.

or three minor higher-molecular-weight species (Fig. 1, 3, and 5), but these did not copurify with the LcrG-LcrV complex in affinity chromatography, and we do not yet know their significance.

The results presented in this paper have allowed the formulation of a revised model for how LcrV and LcrG function in the regulation of the *Y. pestis* LCR at 37°C (Fig. 7). In the presence of Ca^{2+} LcrG may directly or indirectly block the secretory machinery from the cytoplasm, a role consistent with data presented in this report. LcrE blocks the secretory machinery from the outside as previously proposed (13, 47). It is more stringently required for secretion blockage than is LcrG, as an $LcrE^-$ mutant is strongly constitutive for secretion (31, 42), whereas an $LcrG^-$ mutant varies from being strongly constitutive (42) to leaky (having a partial blockage of secretion in the presence of Ca^{2+} [31]). The blocking of secretion by LcrG and LcrE maintains the putative LCR repressor (as well as Yops and LcrV) inside the cell and thereby prevents maximal induction of the LCR. Our results suggest that even in the presence of Ca^{2+} the majority of LcrG can associate with LcrV, and we postulate that noncomplexed LcrG is critical for determining whether secretion will be fully blocked. Removal of Ca^{2+} from the medium or contact with eukaryotic cells is sensed by LcrE, perhaps allowing release of the secretion system-associated LcrG and some secretion of Yops and of the LCR repressor. A decrease in intracellular concentration of the LCR repressor then allows increased expression of LCR-regulated genes, which include *lcrV*. The increased levels of LcrV then cause a stronger unblocking of secretion by titrating the remaining LcrG. Hence, LcrV counteracts LcrG's negative regulatory role. The net effect of removing the secretion blocks by both LcrE and LcrG leads to the full induction of the LCR seen in vitro and provides LcrV in excess of LcrG to be secreted and function as an antihost protein. We realize that this model presents only part of the mechanism for regulating the activity of the Yop secretion system. We do not yet have the complete picture for how the signalling through LcrE affects LcrG.

This model incorporates a better picture for the localization and likely site of action for LcrG and LcrV that developed from our localization experiments. Our findings show that in the absence of Ca^{2+}, LcrG is primarily located in the cytosol, with smaller amounts being found associated with membranes and the culture supernatant. We previously showed that the release of LcrG into the supernatant required the Yop secretion system (42). It is possible that some LcrG is transported as the LcrG-LcrV complex, perhaps as part of the mechanism for unblocking the secretion system, and our data do not rule this out (Fig. 5); however, as this appears to be the minority of the LcrG or cross-linkable LcrG-LcrV complex, we are reluctant

to focus on the supernatant as being the major site for the function of either LcrG or the complex. A previous model for LCR regulation placed LcrG at the cell surface along with LcrE because, like LcrE, LcrG was secreted in the absence of Ca^{2+}, and transcomplementation experiments showed that multicopy *lcrG* did not relieve the effect of (i.e., did not bypass) an *lcrE* mutation (42). Moreover, the similar phenotypes of *lcrE* and *lcrG* mutants suggested that they may have similar functions. We still believe that LcrG and LcrE have a similar function; i.e., they both block secretion in the absence of an unblocking signal (e.g., removal of Ca^{2+} or contact with cells) and they are both necessary (but neither is sufficient) for this function. Evidence and cellular localization implicate LcrE as an environmental sensor, responding to Ca^{2+} concentration or cell contact (5, 13), whereas LcrG's localization makes this role improbable for LcrG. LcrG may respond to an internal signal, such as an increase in LcrV levels, to unblock secretion from the inner face of the inner membrane. LcrG's interaction with LcrV is compatible with the major site of LcrG's function being in the cytoplasm, because we previously showed that LcrV does not need to be secreted to have its regulatory function (43).

LcrG's interaction with LcrV provides an attractive model for LcrV's regulatory function and represents another new aspect of our updated model. Previously a role for LcrV in secretion was discounted because overexpression of YopM under control of a *trc* promoter in an *lcrV* mutant resulted in YopM secretion in medium lacking Ca^{2+}, showing that LcrV is not necessary for secretion. Our present results showing an interaction of LcrV and LcrG suggest that LcrV does function in the control of secretion: i.e., LcrV removes the LcrG secretion block. The model is consistent with LcrV's previously postulated regulatory role in counteracting negative regulation rather than functioning directly in a positive capacity to increase expression of LCR-regulated proteins. Our model is also consistent with the epistasis of *lcrE* and *lcrG* mutations over an *lcrV* mutation, which indicated that *lcrE*, *lcrG*, and *lcrV* act in a common pathway. It is clear that if LcrV acts to counteract LcrG, then LcrV's function would be irrelevant in an $LcrG^-$ mutant. Similarly, if LcrV is necessary for unblocking secretion, then its function would be irrelevant in an $LcrE^-$ mutant, which is constitutively unblocked for secretion. However, we cannot yet explain why YopM was secreted in an *lcrV* mutant that we would predict to be blocked in secretion. Possibly, overexpression of Yops (such as YopM) by a heterologous promoter coupled to destabilization of the secretion machinery via loss of LcrE's blocking function in the absence of Ca^{2+} results in displacement of LcrG from the secretion machinery by the high levels of secretion substrate (i.e., YopM).

For our model to work, the levels of LcrG and LcrV would have to be independently controlled under some circumstances either through transcriptional or posttranscriptional mechanisms. Primer extension data show that the *lcrV* transcript begins immediately upstream of *lcrG* (33), and Northern analysis of a series of *Yersinia* strains having insertion mutations indicated that the V-antigen operon continues downstream through *lcrH* and *yopBD*, comprising the *lcrGVHyopBD* operon (3, 22). However, it has not been rigorously proven that LcrG and LcrV are translated from a single transcript. The 5′ end found for *lcrV* occurs downstream of the proposed ribosome-binding site for *lcrG*, raising the possibility that LcrG is not made from this transcript. Perhaps LcrG is made from a transcript that starts upstream of *lcrD* and encompasses *lcrDRG*. This would result in overlapping transcripts, *lcrDRG* and *lcrGVHyopBD*. Our original Mu *d*I1 (Ap^r *lac*) insertion in *lcrG* that we found to be polar on *lcrV* and *lcrH* (27) could have exerted its polarity because of this overlap. These observations support the idea that *lcrG* and *lcrV* levels could be regulated separately at the transcriptional level, and a posttranscriptional mechanism of controlling LcrG and LcrV levels does not need to be invoked.

While determining the localization of LcrG, we found easily detectable amounts of LcrV and at least two Yops in the periplasmic fraction in both the presence and absence of Ca^{2+}. One primary antibody we used to detect Yops in some of our immunoblots recognizes most of the *Yersinia* secreted proteins, and it revealed that only a subset of LCR-associated proteins appeared to be released into the periplasm (data not shown). In other immunoblots where we examined periplasmic fractions with antibodies specific to YopE, YopH, YopM, or LcrE (YopN), we could detect YopE and LcrE but not YopH or YopM (however, LcrE was not released by the spheroplasting procedure; data not shown). Evidence shows that a subset of *E. coli* cytoplasmic proteins is released into periplasmic fractions by osmotic shock but not by formation of spheroplasts (11); this behavior is interpreted as indicative of a peripheral membrane association for the released proteins. In contrast, we can release some LCR proteins by both osmotic shock and formation of spheroplasts; this behavior has been interpreted as indicative of periplasmic localization. Finding some LCR proteins but not all in periplasmic fractions (prepared by two different methods) is interesting, as Yops and LcrV are secreted by a type III secretion mechanism (39) for which there is no evidence of a periplasmic intermediate in the secretion process. Our results must be interpreted with caution: the data show that either the cytosolic compartment or the secretion pathway used by these LCR proteins is accessible by osmotic shock and spheroplast formation. Future study will be required to determine the basis for this release and the apparent selectivity of it.

This study has enlarged our picture of the type III secretion mechanism that functions to secrete Yops in *Yersinia*. This secretion mechanism was already known to be environmentally regulated in its activity by proteins, LcrG and LcrE, that are themselves secreted by this mechanism. The *Yersinia* type III mechanism is apparently regulated both from within and without by specialized secretion substrates. These may represent a novel class of environmentally responsive mobile regulator that modulates secretion system activity. Moreover, by placing LcrV's regulatory role at the level of secretion, we can now attribute a role in Yop secretion to every member of the complex *lcrGVHyopBD* operon: LcrG and LcrV as discussed above, LcrH as the specific Yop chaperone (SycD) for YopD and perhaps also YopB (49), and YopB and YopD as postsecretion partitioning proteins that function in the translocation of Yops into a eukaryotic cell (5, 37). *lcrGVHyopBD* is now a fifth large operon necessary for the fully functional, environmentally modulated *Yersinia* Yops secretion mechanism. The *lcrGVHyopBD* operon specifies secretion-regulatory proteins and ones that direct the final destination of the secreted proteins. It will be interesting to find out how conserved these functions are in the type III secretion systems of other pathogens.

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REFERENCES

1. **Allaoui, A., R. Scheen, C. Lambert de Rouvroit, and G. R. Cornelis.** 1995. VirG, a *Yersinia enterocolitica* lipoprotein involved in Ca^{2+} dependency, is related to ExsB of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**4230–4237.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Bergman, T., S. Häkansson, Å. Forsberg, L. Norlander, A. Macellaro, A. Bäckman, I. Bölin, and H. Wolf-Watz.** 1991. Analysis of the V antigen *lcrGVH-yopBD* operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. J. Bacteriol. **173:**1607–1616.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Boland, A., M.-P. Sory, M. Iriarte, C. Kerbourch, P. Wattiau, and G. R. Cornelis.** 1996. Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. EMBO J. **15:**5191–5201.
6. **Brubaker, R. R., A. K. Sample, D. Z. Yu, R. J. Zachorchak, P. C. Hu, and J. M. Fowler.** 1987. Proteolysis of V antigen from *Yersinia pestis*. Microb. Pathog. **2:**49–62.
7. **Brubaker, R. R., and M. J. Surgalla.** 1964. The effect of Ca^{++} and Mg^{++} on lysis, growth, and production of virulence antigens by *Pasteurella pestis*. J. Infect. Dis. **114:**13–25.
8. **Cornelis, G., J.-C. Vanootegem, and C. Sluiters.** 1987. Transcription of the *yop* regulon from *Y. enterocolitica* requires trans acting pYV and chromosomal genes. Microb. Pathog. **2:**367–379.
9. **Cornelis, G. R.** 1994. *Yersinia* pathogenicity factors. Curr. Top. Microbiol. Immunol. **192:**243–263.
10. **Davis, R. H., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **El Yaagoubi, A., M. Kohiyama, and G. Richarme.** 1994. Localization of DnaK (chaperone 70) from *Escherichia coli* in an osmotic-shock-sensitive compartment of the cytoplasm. J. Bacteriol. **176:**7074–7078.
12. **Fields, K. A., and S. C. Straley.** Unpublished data.
13. **Forsberg, Å., A.-M. Viitanen, M. Skurnik, and H. Wolf-Watz.** 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol. Microbiol. **5:**977–986.
14. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
15. **Häkansson, S., E. E. Galyov, R. Rosqvist, and H. Wolf-Watz.** 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. Mol. Microbiol. **20:** 593–603.
16. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
19. **Lindler, L. E., M. S. Klempner, and S. C. Straley.** 1990. *Yersinia pestis* pH 6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague. Infect. Immun. **58:**2569–2577.
20. **Linn, T., and R. S. Pierre.** 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. J. Bacteriol. **172:**1077–1084.
21. **Motin, V. I., Y. A. Nedialkov, and R. R. Brubaker.** 1996. V antigen-polyhistidine fusion peptide: binding to LcrH and active immunity against plague. Infect. Immun. **64:**4313–4318.
22. **Mulder, B., T. Michiels, M. Simonet, M.-P. Sory, and G. Cornelis.** 1989. Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. Infect. Immun. **57:**2534–2546.
23. **Nakajima, R., V. L. Motin, and R. R. Brubaker.** 1995. Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. Infect. Immun. **63:**3021–3029.
24. **Nilles, M. L., and S. C. Straley.** Unpublished data.
25. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241:**3055–3062.
26. **Payne, P., and S. C. Straley.** Unpublished data.
27. **Perry, R. D., P. A. Harmon, W. S. Bowmer, and S. C. Straley.** 1986. A low-Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. Infect. Immun. **54:**428–434.
28. **Perry, R. D., M. Pendrak, and P. Schuetze.** 1990. Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. J. Bacteriol. **172:**5929–5937.
29. **Persson, C., R. Nordfelth, N. Holmström, S. Häkansson, R. Rosqvist, and H. Wolf-Watz.** 1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18:**135–150.
30. **Plano, G. V., S. S. Barve, and S. C. Straley.** 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173:**7293–7303.
31. **Plano, G. V., and S. C. Straley.** 1995. Mutations in *yscC*, *yscD*, and *yscG* prevent high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. J. Bacteriol. **177:**3843–3854.
32. **Price, S. B., C. Cowan, R. D. Perry, and S. C. Straley.** 1991. The *Yersinia pestis* V antigen is a regulatory protein necessary for Ca^{2+}-dependent growth and maximal expression of low-Ca^{2+} response virulence genes. J. Bacteriol. **173:**2649–2657.
33. **Price, S. B., K. Y. Leung, S. S. Barve, and S. C. Straley.** 1989. Molecular analysis of *lcrGVH*, the V antigen operon of *Yersinia pestis*. J. Bacteriol. **171:**5646–5653.
34. **Price, S. B., and S. C. Straley.** 1989. *lcrH*, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. Infect. Immun. **57:**1491–1498.
35. **Rimpiläinen, M., Å. Forsberg, and H. Wolf-Watz.** 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis*, shows extensive homology to YopH. J. Bacteriol. **174:**3355–3363.
36. **Rosqvist, R., Å. Forsberg, M. Rimpiläinen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic protein YopE of *Yersinia* obstructs the primary host defence. Mol. Microbiol. **4:**657–667.
37. **Rosqvist, R., Å. Forsberg, and H. Wolf-Watz.** 1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect. Immun. **59:**4562–4569.
38. **Rosqvist, R., K.-E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. **13:**964–972.
39. **Salmond, G. P. C., and P. J. Reeves.** 1993. Membrane traffic wardens and protein secretion in Gram-negative bacteria. Trends Biochem. Sci. **18:**7–12.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Skrzypek, E., and S. C. Straley.** 1993. LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. J. Bacteriol. **175:**3520–3528.
43. **Skrzypek, E., and S. C. Straley.** 1995. Differential effects of deletions in *lcrV* on secretion of V antigen, regulation of the low-Ca^{2+} response, and virulence of *Yersinia pestis*. J. Bacteriol. **177:**2530–2542.
44. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene. **51:**255–267.
45. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51:**445–454.
46. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. USA **78:**1224–1228.
47. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol. Microbiol. **8:**1005–1010.
48. **Une, T., and R. R. Brubaker.** 1984. Roles of V antigen in promoting virulence and immunity in yersiniae. J. Immunol. **133:**2226–2230.
49. **Wattiau, P., B. Bernier, P. Deslée, T. Michiels, and G. R. Cornelis.** 1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA **91:**10493–10497.
50. **Williams, A. W., and S. C. Straley.** Unpublished data.
51. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. Decruz, M. Noyer-Weidner, S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. **17:** 3469–3478.