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LcrG, a Secreted Protein Involved in Negative Regulation of the Low-Calcium Response in *Yersinia pestis*

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The purpose of this study was to define the function of LcrG, the product of the first gene in the *lcrGVHyopBD* operon of the low-Ca^{2+}-response (LCR) virulence plasmid of *Yersinia pestis*. We created a *Y. pestis* strain having an in-frame deletion in *lcrG*. This nonpolar mutant had an abnormal LCR growth phenotype: it was unable to grow at 37°C in the presence of 2.5 mM Ca^{2+} ("Ca^{2+} blind") but was able to grow at 37°C when 18 mM ATP was present. At 37°C it failed to downregulate the expression and secretion of its truncated product (LcrG), V antigen, and YopM. All of these mutant properties were complemented by plasmids carrying normal *lcrG*. However, a nonpolar *lcrE* mutation and an *lcrH* mutation (both also causing a Ca^{2+}-blind phenotype) were not complemented in this way. The *Y. pestis* parent strain expressed LcrG at 37°C in the presence and absence of Ca^{2+} and transported it to the medium when Ca^{2+} was absent. We identified two LCR-regulated loci, *lcrD* and *yscDEF*, required for this transport. Complementation analysis of the *Y. pestis lcrR* strain previously shown to lack the expression of LcrG showed that the loss of LcrG but not of LcrR caused the Ca^{2+}-blind phenotype of that mutant. Taken together, the results show that LcrG is a negative regulator of the LCR, perhaps functioning in Ca^{2+} sensing along with LcrE.

Three members of the genus *Yersinia* are pathogenic for humans. *Yersinia pestis* is the causative agent of a systemic disease, bubonic plague (12). Infections by *Y. pseudotuberculosis* and *Y. enterocolitica* are manifested as invasive yersiniosis (13).

Virulent yersiniae carry related plasmids (2, 6, 46), which encode a complex virulence determinant called the low-Ca^{2+}-response (LCR). The 75-kilobase-pair pCD1 is the LCR plasmid in *Y. pestis* KIM (19, 23).

Expression of the LCR is manifested in vitro by the requirement for millimolar concentrations of Ca^{2+} or nucleotides (such as ATP) for maximal growth yield of yersiniae at 37°C (8, 10, 63). The effects of calcium and ATP on growth are not identical. Unlike Ca^{2+}, ATP never fully restores the maximal growth yield at 37°C. Both components probably act as environmental stimuli (72). In the absence of Ca^{2+} at 37°C, a metabolic downshift and a cessation of bacterial growth occur; these responses are known as growth restriction (10, 71). Under these conditions, despite the shutdown of net protein synthesis, a set of virulence-associated proteins is expressed and secreted. Those proteins include V antigen and 10 Yop proteins (11, 47, 48, 62). At 37°C in the presence of Ca^{2+} or ATP, the abundance of these proteins is decreased ca. three- to sevenfold in vitro and their secretion is almost completely blocked (15, 64). Operons that are coordinately downregulated in transcription at 37°C by Ca^{2+} are considered to belong to an LCR stimulon (LCRS) (64). In addition to encoding V antigen and Yop proteins, these operons encode proteins that participate in LCR induction in the absence of Ca^{2+}, proteins necessary for LCR downregulation in the presence of Ca^{2+}, and proteins responsible for secreting LCRS products (64). The expression of the LCRS and of the associated growth restriction does not occur at 26°C regardless of the calcium or ATP concentration (23, 61).

Yop proteins are secreted without N-terminal processing by an LCR plasmid-encoded Yop secretion (*ysc*) system (34, 36). The signals directing the transport of Yop proteins have been localized within the first 50 to 100 amino acids of the protein sequences (34). The same mechanism is necessary for the secretion of V antigen (58).

V antigen and Yop proteins have been shown to be important virulence determinants (65). Yop proteins are thought to function in the protection of extracellularly exposed bacteria by disarming natural host defense mechanisms, such as phagocytosis (YopE and YopH) and activation of platelets by thrombin (YopM) (5, 24, 30, 31, 53, 55). V antigen appears to be bifunctional. It is protective in both passive and active immunizations (9, 29), suggesting a role as an antihost factor. It also is a positive modulator of the LCR, being necessary for restriction of growth and maximal Yop protein expression (49, 50).

An approximately 25-kilobase-pair "Ca^{2+} dependence region" of the LCR plasmids has been shown to be responsible for the regulation of the LCR (23, 64). LcrF (VirF in *Y. enterocolitica*) from this region is thought to be thermally activated when yersiniae experience a shift in temperature from 26 to 37°C (14, 69). This product has DNA-binding properties and in turn mediates the thermal induction of a regulon called the *yop* regulon, which contains a subset of the LCRS (17). LcrE (also called YopN) (22, 68, 70) and LcrQ (54) then can participate in a Ca^{2+}-sensing process that results in the partial downregulation of LCRS operon transcription at 37°C when Ca^{2+} is present. The *lcrE* gene product has been found on the bacterial surface as well as in the medium, and it was postulated that it might function in Ca^{2+} sensing at the bacterial surface (21, 22). Genetic evidence indicated that LcrQ, also a secreted protein, functions after LcrE in the regulatory cascade (54). Both are thought to be near the top of the regulatory hierarchy of the signal transduction mechanism. The distalmost negative

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regulatory component known is the product of the *lcrH* gene (3, 51). This putative repressor is necessary for downregulation of the LCRS in response to either Ca^{2+} or ATP. The overexpression of *lcrH* in certain strains leads to the repression of at least one LCRS operon and abolishes the restrictive growth response (3). Mutants defective in *lcrE*, *lcrQ*, or *lcrH* exhibit a "Ca^{2+}-blind" phenotype. At 37°C they undergo the restriction of growth and strong Yop protein and V antigen expression irrespective of the Ca^{2+} concentration.

In the absence of Ca^{2+}, several operons in addition to *lcrF* are necessary for the expression of V antigen and Yop proteins. These include the *lcrB* locus (*virB* in *Y. enterocolitica*) (20, 23, 35) and *lcrDR* (1, 43, 44). *lcrDR* has been shown to be weakly to moderately downregulated in transcription by Ca^{2+} and hence to belong to the LCRS (44). Yersiniae with an insertion mutation in one of these inductive loci exhibit a "Ca^{2+}-independent" phenotype. Such mutants do not undergo growth restriction in the absence of Ca^{2+} following a temperature shift, they express only low levels of LCRS operons, and they do not secrete LCRS proteins.

The V antigen operon, *lcrGVHyopBD*, encodes both putative virulence proteins (V antigen and perhaps YopB and YopD) and regulatory proteins (V antigen and LcrH) (3, 39, 40, 50). The first cistron, *lcrG*, expresses a basic 11-kDa protein of unknown function (3, 40, 50). A previously characterized insertion mutation in *lcrG* had a polar effect on the downstream regulatory genes *lcrV* (encoding V antigen) and *lcrH* and hence did not reveal properties unique to *lcrG*. Subsequent studies of the *lcrDR* operon, which lies immediately upstream of *lcrGVHyopBD*, found that MudI1 (Ap^r *lac*) insertions in *lcrD* or *lcrR* abolished the expression of LcrG, even though V antigen and LcrH, products of the next genes in *lcrGVHyopBD*, were expressed strongly (1, 43). Moreover, primer extension analysis of one of these insertion mutants revealed the same 5′ end for *lcrGVHyopBD* as was previously found in the parent, *Y. pestis* KIM5 (1, 50).

These intriguing data prompted us to investigate the *lcrG* locus in more detail. We constructed and characterized a strain carrying a nonpolar deletion in *lcrG*. Our data indicate that LcrG is a secreted protein involved in the negative regulatory pathway of the LCR. The findings suggest that LcrG may be part of the Ca^{2+}-sensing machinery and hence may play an active role in transducing a Ca^{2+} signal into a regulatory effect on LCRS operons.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *Y. pestis* strains were cultivated in heart infusion broth and on tryptose blood agar base (Difco Laboratories, Detroit, Mich.) medium for DNA manipulations or in TMH defined liquid medium (61) for physiological studies. *Escherichia coli* strains were grown in L broth or on L agar medium (16). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at concentrations of 25 μg/ml for ampicillin and kanamycin, 12.5 μg/ml for tetracycline HCl, and 200 μg/ml for streptomycin unless indicated otherwise.

β-Galactosidase assays. β-Galactosidase activity was assayed colorimetrically as described previously (23, 37, 61). Numbers representing the β-galactosidase specific activities shown in Fig. 4B are average values of two experiments, each consisting of assays carried out in triplicate.

DNA techniques. Transformation of *E. coli* was done by the $CaCl_2$ procedure as described by Maniatis et al. (33). Transformation of *Y. pestis* by electroporation was carried out as described previously (41). Plasmids were screened by the method of Kado and Liu (26) and isolated by a standard alkaline lysis procedure (4) or by use of the Qiagen kit (Qiagen Inc., Studio City, Calif.). Restriction endonuclease analysis and cloning were carried out by standard methods (33). DNA fragments were purified from agarose gels by use of Qiaex kits (Qiagen). Polymerase chain reaction (PCR) techniques described in this paper were carried out by use of the GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR fragment carrying the deletion in *lcrG* (bp 115 to 159; amino acids 39 to 53 of LcrG) was synthesized with primers PΔG and PG (see Fig. 1b). Primer PΔG carried a 45-bp deletion and had the following sequence: 5′-TTATTGCAAGAAATGTGTGCTGATATCGG C//GAAGAGATAAAGCCAGCGGAGCGCGAG-3′, where // indicates the location of the deletion. Primer PG contained a 27-bp complementary-strand sequence from within the sequence downstream of *lcrV* (5′-CTCGCTTGATGCCAT TTTGCAGTTGGT-3′). Detection of the deletion introduced into *Y. pestis* LCR plasmid pCD1 by marker exchange was carried out by the PCR with primer PG (shown above) and primer PG1, containing bp 62 to 82 of *lcrG* (5′-CCGACAGC GATCACCGCGCAA-3′). Reactions were carried out in accordance with the manufacturer's (Perkin-Elmer) protocol, except that when the deletion was created, dimethyl sulfoxide (Sigma) was present in the reaction mixture at a final concentration of 5% (vol/vol). DNA fragments were isolated after 30 cycles of 1-min incubations at 94, 56, and 72°C. Products were purified by use of Centricon 30 microconcentrators in accordance with the manufacturer's (Amicon, Danvers, Mass.) protocol. The PCR product was digested with an appropriate restriction enzyme and used for replacement of the corresponding fragment in the original plasmid. The replaced region was sequenced by the dideoxy chain termination method (56) with double-stranded DNA templates and the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with [α-^{35}S] dATP from New England Nuclear Corp. (Boston, Mass.). Primers for the PCR and sequence-specific oligonucleotides used in sequencing were synthesized at the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington).

Plasmid constructions. Plasmid pES92 was used for the construction of a nonpolar deletion in *lcrG*. It was created by recloning of the *Yersinia* DNA insert of plasmid pSB2 (Table 1) into suicide vector pUK4134 (57; Table 1). *Eco*RI sites in the multiple cloning region of pSB2 were used to excise the cloned *Bgl*II-*Pvu*II insert containing part of *lcrD*, intact *lcrR* and *lcrG*, and part of *lcrV* (1). The ends were filled in with the Klenow enzyme and ligated into the *Eco*RV site of the suicide vector. The pUK4134 suicide vector allows positive, streptomycin-based selection of bacteria that have undergone an allelic exchange. This vector requires a π protein for its stable maintenance; therefore, its derivatives were selected for in *E. coli* SY327 (λ*pir*).

pSB6, used in the complementation studies of *Y. pestis lcrR* mutant KIM5-3142, was constructed by cloning the *lcrR*-containing *Bst*XI-*Eco*RV fragment of pSB2 into the *Sma*I restriction site of the pKK223-3 vector (Pharmacia-LKB) Piscataway, N.J.). In pSB6, *lcrR* expression is driven constitutively by the P_{tac} promoter.

Cellular fractionation. For protein isolations, *Y. pestis* was adapted at 26°C in TMH defined liquid medium containing 2.5 mM Ca^{2+} when required (61). After about 10 generations (total), when the A_{620} of the cultures reached 0.15 to 0.2, the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
E. coli K-12		
SY327 (λ*pir*)	Δ (lac-pro) *argE*(Am) *rif nalA recA56* λ*pir*	38
XL1 Blue	*end-1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96* (Nal^r) *relA1* (Δ*lac*) [F$^+$ *proAB*$^+$ *lacI*^{q}ZΔM15::Tn*10* (Tc^r)]	Stratagene, La Jolla, Calif.
Y. pestis[b]		
KIM5	pCD1 (LCR$^+$), pPCP1, pMT1	R. R. Brubaker
KIM6	pPCP1, pMT1 (LCR$^-$)	R. R. Brubaker
KIM5-3001	Sm^r, pCD1 (LCR$^+$), pPCP1, pMT1	32
KIM5-3001.2	Sm^r, pCD1 *lcrD*(Δ192-343) (LcrD$^-$), pPCP1, pMT1	44
KIM5-3001.3	pCD1 *lcrD*(Δ618-644) (LcrD$^-$), pPCP1, pMT1	44
KIM5-3001.4	pCD1 *lcrD*(Δ159-167) (LcrD$^-$), pPCP1, pMT1	44
KIM5-3001.5	Sm^r, pCD1 *lcrG*(Δ39-53) (LcrG$^-$), pPCP1, pMT1	This work
KIM5-3001.6	Sm^r, pCD1 *lcrE*(Δ48-197) (LcrE$^-$), pPCP1, pMT1	45
KIM5-3042.7	pCD1 *lcrD*::MudI1 (Ap^r *lac*) *b*:Tn*9* (LcrD$^-$ LcrR$^-$), pPCP1, pMT1	43, 23
KIM5-3142	pCD1 *lcrR*::MudI1734 (Km^r Lac$^+$) (LcrR$^-$), pPCP1, pMT1	60
KIM5-3240	pCD1 *lcrH*::*cat yopJ*::MudI1734 (Km^r Lac$^+$) (LcrH$^-$ YopB$^-$ YopD$^-$ YopJ$^-$), pPCP1, pMT1	51
KIM8-3060.9	pCD1 *yscD*::MudI1 (Ap^r *lac*) (YscDEF$^-$), pMT1 (Pla$^-$), pGW600[c] (Tc^r Mu C$^+$)	23, 25
Plasmids		
pBR322	Cloning vector; Ap^r Tc^r	7
pAVA1	Cloning vector; Ap^r Cm^r; pACYC184 lacking the *Ava*I site	49
pKK223-3	Cloning vector; Ap^r	Pharmacia-LKB
pUK4134	Suicide vector; *oriR6K oriT cos rpsL* (Ap^r)	57
pJIT7-2	*Hin*dIII-*Cla*I fragment of *Hin*dIII-G of pCD1 cloned into pBR322; LcrG$^+$ Ap^r	40
pAVA2-1	*Hin*dIII-G fragment of pCD1 with a deletion of the two internal *Ava*I fragments and cloned into pAVA1 (LcrG$^+$ YopB$^+$)	42
pSB2	*Bgl*II-*Pvu*II fragment of pCD1 containing *'lcrD lcrR lcrG lcrV'* in the pIC20R vector (Ap^r)	1
pES92	pCD1 *Bgl*II-*Pvu*II fragment of pSB2 moved into pUK4134	This work
pGS1	*Bgl*II-*Pvu*II fragment of pES92 carrying *lcrG*(Δ39-53) and cloned into pUK4134	This work
pSB6	pCD1 *Bst*I-*Eco*RV fragment of pSB2 cloned into the pKK223-3 vector (LcrR$^+$ Ap^r)	This work
pYPDR (*lcrD*Δ192-343)	*Msc*I-*Eco*RV fragment of pCD1 with a deletion of the internal *Nar*I fragment in *lcrD* and cloned into pBluescript II KS (−) (LcrR$^+$)	44

[a] Numbers in parentheses indicate the amino acids deleted from the gene product.
[b] All *Y. pestis* strains are Pgm$^-$ (66). Native virulence plasmids of *Y. pestis* are LCR plasmid pCD1 (23); pPCP1, encoding the plasminogen activator protease Pla (59); and pMT1, encoding the capsular protein (52).
[c] pGW600 encodes the native Mu repressor (27).

temperature was shifted to 37°C. The absorbance was monitored at hourly intervals. Seven hours after the temperature shift, 20 ml of the culture was centrifuged. Extracellular proteins from supernatants were precipitated overnight at 4°C with trichloroacetic acid (final concentration, 5% [vol/vol]). Proteins were resuspended in cold TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na_2EDTA) at a neutral pH and a volume of 0.5 ml per A_{620} ml of the starting culture. After a wash with TE buffer, the bacterial pellet was resuspended in TE buffer at the same volume as the supernatant proteins (i.e., 0.5 ml per A_{620} ml of the original culture) and lysed by passage through a French press at 20,000 lb/in^2. Nonlysed cells and bacterial debris were removed by centrifugation at 3,440 × *g* for 5 min at 4°C. The protein extracts were stored at −20°C.

SDS-PAGE and Western analysis. Resolution of proteins was carried out by standard 12 or 15% (wt/vol) acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (28). Immunoblotting analysis was performed as previously described (43) with Immobilon-P membranes (Millipore). In the case of LcrG, because of its basic isoelectric point (8.64), carbonate buffer (pH 9.9) (18) was used for electrotransfer from SDS-polyacrylamide gels onto Immobilon-P membranes. Preparation of the purified rabbit anti-LcrV [peptide NH_2-(C)SVMQRLLDDTSGK-COOH] antibody used in this work was described by Skrzypek et al. (58). Polyclonal rabbit anti-YopM [peptide NH_2-(C)ETTDKLEDDVFE-COOH] antibodies were described previously (53). Antipeptide antibodies raised against amino acids 80 to 91 of the predicted LcrG protein [peptide NH_2-(C)DGKRPRKPTMMR-COOH] were made by Clarissa Cowan essentially as described previously (43).

RESULTS

Construction of nonpolar *Y. pestis lcrG* mutant KIM5-3001.5. Plasmid pES92 carried the *Bgl*II-*Pvu*II fragment of

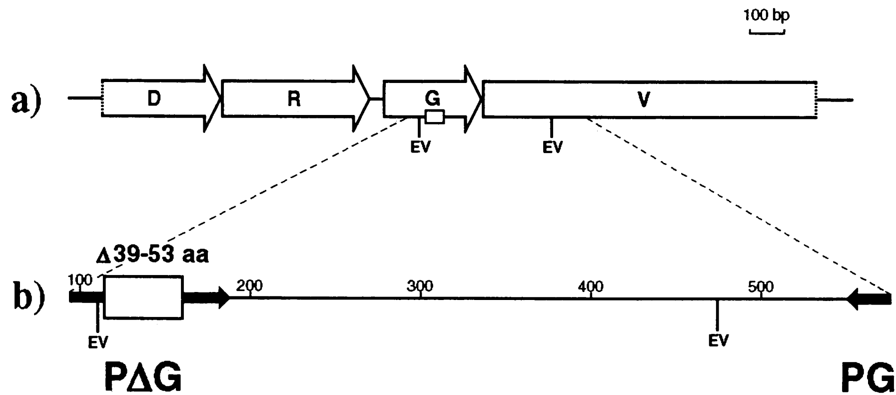

FIG. 1. Construction of a nonpolar deletion in *lcrG*. (a) Portion of the physical map of the *lcrDR* and *lcrGVHyopBD* operons of *Y. pestis* LCR plasmid pCD1. Open arrows delineate partial *lcrD*, complete *lcrR* and *lcrG*, and a fragment of *lcrV*. The direction of transcription of all of these is indicated by the arrowheads. The small box shows the position of the deletion in *lcrG*. (b) PCR primers used in creating an in-frame deletion in *lcrG*. The orientations of the PCR primers are indicated by closed arrows. The open box indicates the deleted region in *lcrG* (bp 115 to 159; amino acids [aa] 39 to 53). Numbers indicate nucleotides within *lcrGVHyopBD* starting with 1 as the first nucleotide in the *lcrG* open reading frame. EV, *Eco*RV restriction site.

pCD1 (1) cloned into the pUK4134 suicide vector (57). Its insert coded for *Y. pestis lcrG*; most of the *lcrDR* operon, located directly upstream from *lcrG*; and part of the downstream *lcrV* (Fig. 1a). To create an in-frame deletion in *lcrG*, we chose the PCR technique of Vallette et al. (67), which involves a PCR fragment synthesized by use of a normal-sized primer (PG; Fig. 1b) and a longer one carrying an in-frame deletion (PΔG; Fig. 1b). The resulting construct, pGS1 (Table 1), carried the expected deletion of bp 115 to 159 in *lcrG*. This construct was then transferred into plasmid pCD1 of Sm^r *Y. pestis* KIM5-3001 by allelic exchange. The nonpolar *Y. pestis lcrG* mutant strain isolated in this way was named KIM5-3001.5 (Table 1).

Growth response of the *lcrG* mutant to Ca^{2+} and ATP. *Y. pestis lcrG* mutant KIM5-3001.5 grew in a manner similar to that of parent strain KIM5-3001 at 26°C in TMH defined medium and following a temperature shift to 37°C in unsupplemented TMH medium or in TMH medium containing 18 mM ATP (Fig. 2A). However, growth restriction of strain KIM5-3001.5 was not prevented by the presence of 2.5 mM Ca^{2+}; hence, this mutant has a Ca^{2+}-blind growth phenotype. To confirm that the Ca^{2+}-blind growth of the mutant was due to the *lcrG* mutation, we tested plasmid pJIT7-2, carrying *lcrG* with its own calcium-regulated promoter, for its ability to complement the *lcrG* mutation. Figure 2B shows the growth phenotypes of the mutant and of the comple-

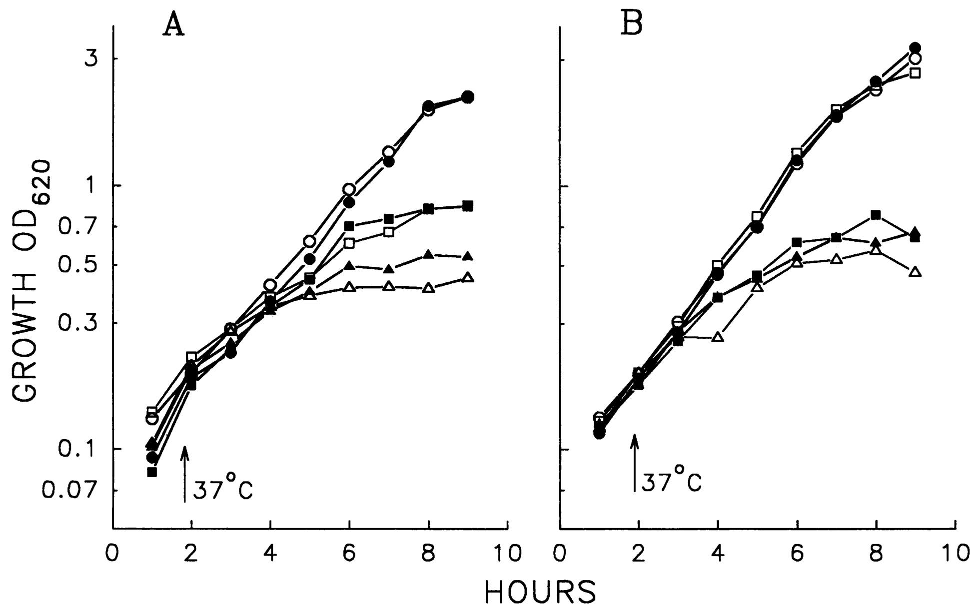

FIG. 2. Growth characteristics of the *lcrG* mutant. (A) Growth in the presence of 18 mM ATP. Open symbols, parent strain KIM5-3001; closed symbols, *lcrG* mutant strain KIM5-3001.5. (B) Growth in the presence of 2.5 mM Ca^{2+}. Open symbols, complemented strain KIM5-3001.5 carrying the LcrG-encoding plasmid pJIT7-2; closed symbols, *lcrG* mutant strain KIM5-3001.5. Symbols for both panels A and B: ○ and ●, 26°C; □ and ■, 37°C, with ATP or Ca^{2+}; △ and ▲, 37°C, without ATP or Ca^{2+}. The arrows below the curves indicate the time of the temperature shift in cultures incubated at 37°C. OD_{620}, optical density at 620 nm.

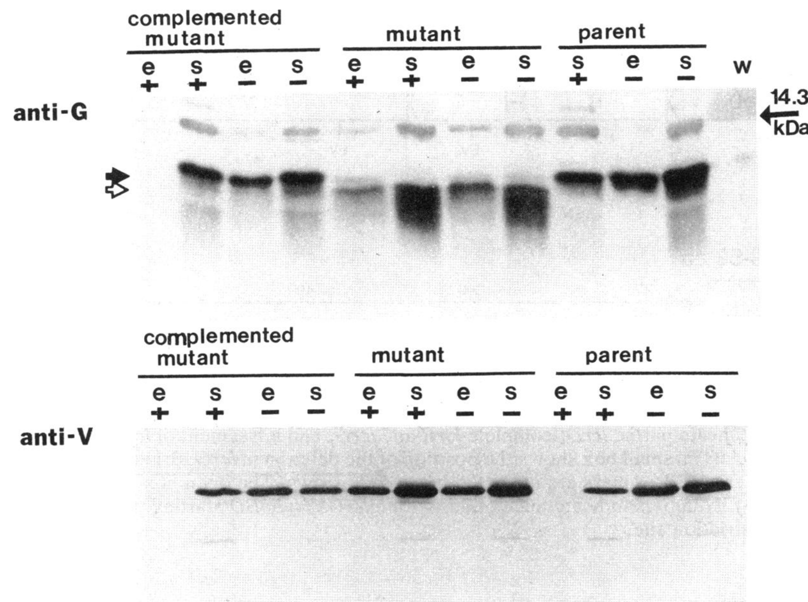

FIG. 3. Expression and secretion of LcrG and V antigen by the *lcrG* mutant strain (KIM5-3001.5), the parent strain (KIM5-3001), and the complemented *Y. pestis lcrG* strain (KIM5-3001.5 carrying pJIT7-2). Proteins were resolved in SDS-polyacrylamide gels containing 12 or 15% (wt/vol) acrylamide for LcrG or LcrV, respectively, and analyzed in immunoblots with anti-LcrG serum or anti-LcrV serum. Each lane contained proteins from the same number of bacteria, as determined by A_{620} measurements. + and −, proteins isolated from bacteria grown in the presence and absence, respectively, of 2.5 mM Ca^{2+}; e, extracellular (secreted) proteins; s, soluble (cellular) proteins. Portions of the immunoblots are shown. The sample containing the extracellular proteins from parent strain KIM5-3001 grown in the presence of Ca^{2+} (upper panel) showed no bands (see Fig. 6), like the sample containing the extracellular proteins from complemented mutant strain KIM5-3001.5(pJIT7-2) grown under the same conditions (upper panel), and was omitted to permit the inclusion of size standards (lane w). The closed arrow indicates LcrG; the open arrow indicates the truncated *lcrG* gene product. More slowly migrating bands are due to the cross-reaction of proteins with the anti-LcrG antiserum. The LcrG band, in addition to having an appropriate apparent molecular mass, was identified by its alteration in size in the *lcrG* mutant. It was also absent in *Y. pestis* KIM6 (Table 1) extracts or in immunoprecipitates made from the same extracts with anti-LcrG serum (Fig. 6 and data not shown).

mented strain in the presence and absence of Ca^{2+}. The plasmid completely restored the ability of the mutant to grow at 37°C in the presence of Ca^{2+}. Similar data were obtained for plasmid pAVA2-1, in which *lcrG* was carried by the pAVA1 derivative of pACYC184 (49; Table 1). Vectors (pBR322 or pAVA1) alone had no effect on the mutant (data not shown). Therefore, we conclude that the growth defect was specifically due to the *lcrG* mutation.

Interestingly, the growth of the *lcrG* mutant strain was calcium dependent, like that of the parent strain, when CFUs were determined at 37°C on complex medium plates (tryptose blood agar base) in the presence or absence of sodium oxalate (calcium-chelating agent) and $MgCl_2$ (data not shown). Results similar to these were previously obtained for another Ca^{2+}-blind mutant, *lcrR Y. pestis* (1). Because of this strong medium dependence, we based our LCR phenotype designations on the growth of yersiniae in TMH defined liquid medium.

Lcr protein expression by the mutant and its complemented strains. To characterize the mutant and complemented strains more precisely, we determined the profiles of cytoplasmic and supernatant proteins in Western blots with antipeptide antibodies (anti-LcrG, anti-YopM, and anti-LcrV). The parent strain, *Y. pestis* KIM5-3001, produced more 11-kDa LcrG at 37°C in the absence than in the presence of Ca^{2+}, but the difference in expression under the two conditions was not very large (Fig. 3, upper panel). Interestingly, LcrG was found to be exported to the medium by the *Y. pestis* parent strain but only when Ca^{2+} was absent. These data show that Ca^{2+} only slightly regulates the expression of *lcrG* but has a strong downregulating effect on LcrG secretion. The *lcrG* mutant expressed its truncated LcrG under both conditions but showed abnormal LCR regulation, indicated by strong expression and a significant level of secretion of the mutant LcrG in the presence of Ca^{2+}. These results showed that the Ca^{2+}-blind phenotype of the mutant included LCRS gene expression as well as the LCR growth property. As with the growth phenotype (Fig. 2B), strain KIM5-3001.5 carrying complementing plasmid pJIT7-2 showed restoration of the pattern of LcrG expression seen in the *Y. pestis* parent strain: LcrG was expressed more in the absence than in the presence of Ca^{2+} and was transported only when Ca^{2+} was absent (Fig. 3, upper panel). We noticed some instability of the truncated LcrG in the soluble extracts in both the presence and the absence of Ca^{2+}, reflected by degradation products, visible in Fig. 3, upper panel. Because the mutant is Ca^{2+} blind, it would be anticipated that the turnover characteristic in the absence of Ca^{2+} would also be seen in the presence of Ca^{2+}. The normal LcrG was more stable, although some degradation did occur in the absence of Ca^{2+}. Surprisingly, we could not detect the mutant LcrG and its degradation products in the soluble and supernatant fractions from the complemented strain.

As with LcrG, V antigen expression and secretion by the *lcrG* mutant were constitutive at 37°C (Fig. 3, lower panel). Similar data were obtained when immunoblots were probed with anti-YopM antibodies (data not shown). The wild-type

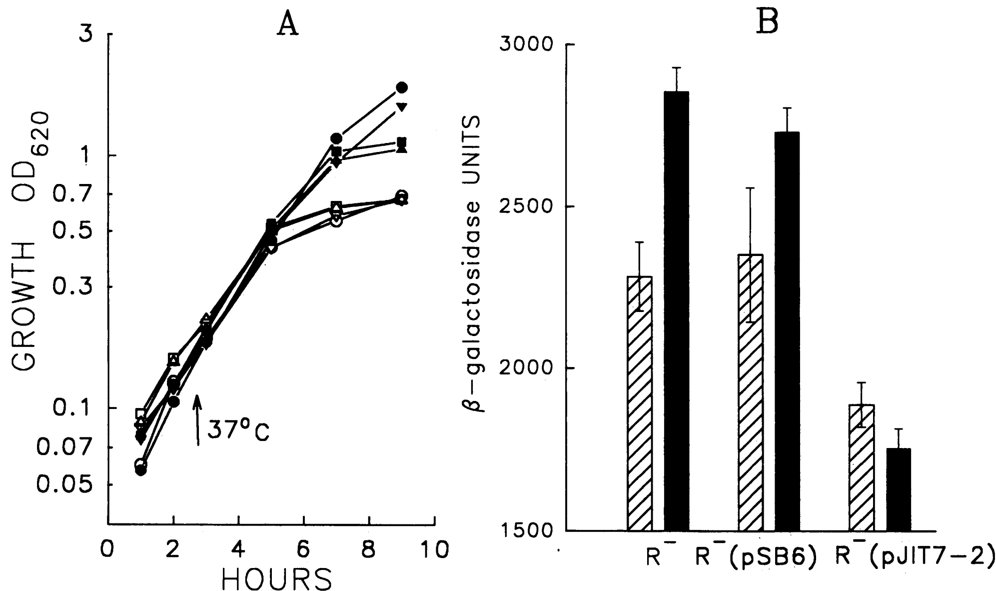

FIG. 4. Complementation of *lcrR Y. pestis*: growth characteristics and β-galactosidase expression from *lcrR*::MudI1734. (A) Growth. Bacteria were grown at 26°C in TMH defined medium lacking or containing 2.5 mM Ca^{2+}. The temperature was shifted to 37°C at the time indicated by the arrow. Symbols: open, Ca^{2+} absent; closed, Ca^{2+} present; ○ and ●, reference Lcr^+ *Y. pestis* KIM5; △ and ▲, *lcrR Y. pestis* KIM5-3142; ▽ and ▼, *lcrR* mutant carrying pJIT7-2 ($LcrG^+$); □ and ■, *lcrR* mutant carrying pSB6 ($LcrR^+$). (B) β-Galactosidase expression. Samples from the cultures described in panel A were assayed for β-galactosidase activity 6 h after a temperature shift. One unit of β-galactosidase is defined as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg of protein. Closed bars, Ca^{2+} present; hatched bars, Ca^{2+} absent. Results are the mean ± standard error of the mean for two separate experiments, each run in triplicate. OD_{620}, optical density at 620 nm.

copies of *lcrG* in the complemented strain restored LCR regulation: the transport of V antigen and YopM, like that of LcrG, was prevented in the presence of Ca^{2+} (Fig. 3, lower panel). These data extended the characterization of the effects of the *lcrG* mutation on LCRS operon expression and indicate that LcrG functions in the pathway that downregulates LCRS gene expression and protein secretion in response to Ca^{2+}.

To gain information about where LcrG acts in the negative regulatory pathway, we tested for alterations of the Ca^{2+}-blind phenotypes of nonpolar *Y. pestis lcrE* mutant KIM5-3001.6 and *Y. pestis lcrH* mutant KIM5-3240 (Table 1) caused by the LcrG-expressing plasmid pJIT7-2 or pJIT7-2 in combination with pAVA2-1 (to provide still more copies of functional *lcrG*). We also included parent strain *Y. pestis* KIM5-3001 in these complementation tests. There was no effect of either the single complementing plasmid or the pair of complementing plasmids on the growth of any of the yersiniae, nor was there any regulatory effect on LcrG expression or secretion in the complemented *lcrH* strain (data not shown).

Effect of LcrG on the growth and *lcrR* expression of the *Y. pestis lcrR* mutant. Another Ca^{2+}-blind mutant of *Y. pestis* is the *lcrR* strain generated by insertion mutagenesis, which created an operon fusion of *lcrR* to *E. coli lacZYA* (1). It was intriguing that this mutant failed to express LcrG, while LcrV and LcrH, the products of the next genes in the same operon, were expressed efficiently. The data from that study suggested that intact *lcrR* was necessary for LcrG synthesis or stability in the absence of Ca^{2+} and that LcrR functioned in the downregulation of the *lcrGVHyopBD* operon in the presence of calcium.

In this work, we sought to determine whether the defect in the *lcrR* mutant was caused by the loss of LcrG or LcrR. Accordingly, we tested whether LcrG supplied by pJIT7-2 or LcrR supplied by pSB6 would alter the phenotype of *Y. pestis* KIM5-3142 carrying the *lcrR* insertion mutation.

Figure 4A shows the growth phenotypes of the reference Lcr^+ strain, the *lcrR* mutant, and the strains for the complementation tests. Only the product of the *lcrG* gene was able to restore the normal LCR growth pattern. Plasmid pSB6 containing *lcrR* behind a *tac* promoter had no effect on the growth of the *lcrR* mutant. This lack of complementation was also observed when the *lcrR* mutant contained pYPDR (*lcrD*Δ192-343) carrying the *lcrDR* operon with its functional native promoter but with an internal nonpolar deletion in *lcrD* (Table 1 and data not shown).

The pattern of *lcrR* transcription (as shown by β-galactosidase activity) of the mutant and its derivatives carrying pJIT7-2 or pSB6 is shown in Fig. 4B. As observed previously (1), a temperature upshift resulted in the enhancement of *lcrR* expression in the *lcrR* mutant in both the presence and the absence of Ca^{2+}. Supplying a normal *lcrR* on pSB6 had little effect on the expression of the mutated *lcrR* copy in pCD1. In contrast, LcrG supplied from pJIT7-2 caused decreased *lcrR* expression at 37°C and restored some downregulation in the presence of Ca^{2+}. Although the results of such complementation experiments with multicopy plasmids must be treated cautiously, our findings indicate that the phenotype of the *lcrR* mutant was caused by the loss of LcrG. In the presence of Ca^{2+}, LcrG functions in downregulating the expression of *lcrR* and most likely of other LCRS genes.

Expression and secretion of V antigen and YopM by the *lcrR* mutant complemented by LcrG. Figure 5, upper panel, shows the abundance of V antigen in soluble cellular and extracel-

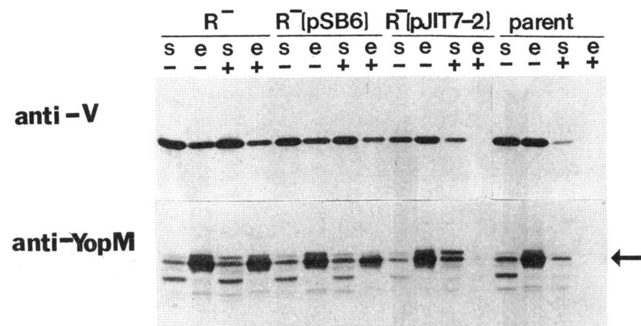

FIG. 5. Complementation of *lcrR Y. pestis* as analyzed by expression and secretion of V antigen and YopM. Proteins from the same number of bacteria of each strain, as determined by A_{620} measurements, were resolved on SDS-polyacrylamide (12% [wt/vol] acrylamide) gels. Immunoblots of cellular and secreted proteins were analyzed with anti-LcrV serum (upper panel) and anti-YopM serum (lower panel). R⁻, *lcrR Y. pestis* KIM5-3142; R⁻(pSB6), *lcrR Y. pestis* carrying pSB6 (LcrR⁺); R⁻(pJIT7-2), *lcrR Y. pestis* carrying pJIT7-2 (LcrG⁺); parent, *Y. pestis* KIM5. + and −, growth in the presence and absence, respectively, of 2.5 mM Ca^{2+}; s, soluble (cellular) proteins; e, extracellular (secreted) proteins. The relevant portions of the immunoblots are shown. The arrow in the lower panel indicates the band corresponding to YopM. Additional bands may be due to YopM degradation products (53).

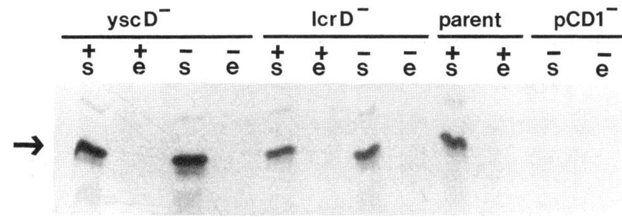

FIG. 6. LcrG expression and secretion in *ysc* and *lcrD Y. pestis*. Proteins were resolved on SDS-polyacrylamide (15% [wt/vol] acrylamide) gels and analyzed in immunoblots with anti-LcrG serum. The relevant portions of the immunoblots are shown. Each lane contained proteins from the same number of bacteria, as determined by A_{620} measurements. yscD⁻, *yscDEF Y. pestis* KIM8-3060.9; lcrD⁻, *lcrD Y. pestis* KIM5-3001.3; parent, *Y. pestis* KIM5-3001; pCD1⁻, *Y. pestis* KIM6. + and −, growth in the presence and absence, respectively, of Ca^{2+} (2.5 mM); s, soluble (cellular) proteins; e, extracellular (secreted) proteins. The arrow indicates LcrG.

lular fractions of the *lcrR* mutant and its plasmid-supplemented derivatives. The results are consistent with the growth characteristics shown in Fig. 4A. The mutant as well as its derivative carrying pSB6 coding for *lcrR* alone showed a Ca^{2+}-blind phenotype, reflected in constitutive expression and transport of V antigen (Fig. 5, upper panel). However, when pJIT7-2 (LcrG⁺) complemented that mutant, the Ca^{2+}-dependent pattern of expression of the *lcrGVHyopBD* operon was restored. Similar results were obtained for expression and secretion of YopM (Fig. 5, lower panel). For LcrG, we noticed very weak reactivity with anti-LcrG serum in Western blots of all cellular and supernatant fractions isolated from *lcrR* as well as pSB6-carrying *lcrR Y. pestis* strains (data not shown). Perhaps this small amount of LcrG expression accounts for the partial Ca^{2+} responsiveness of the *lcrR* mutant (Fig. 4A). When pJIT7-2 was present in the *lcrR* mutant, the bacteria behaved like the parent yersiniae in expressing LcrG strongly in soluble fractions and transporting it only at 37°C in the absence of Ca^{2+} (data not shown).

LcrG transport by *Y. pestis*. Transport of LcrG outside the yersinial cells was an unexpected property. To determine whether this secretion required known LCR membrane components, we tested the *Y. pestis* calcium-independent *yscDEF* mutant (25) and three nonpolar *lcrD* mutants (44) (Table 1) for expression and transport of LcrG. Immunoblots showed that LcrG was expressed by these mutants grown at 37°C in the presence and absence of Ca^{2+} but that it was not secreted under either condition (Fig. 6). These findings show that directly or indirectly, some components of both membrane-associated systems (*ysc* and *lcrD*) participate in the secretion of LcrG in the absence of Ca^{2+}.

DISCUSSION

In an effort to determine the function of LcrG in the LCR of *Y. pestis*, we constructed and characterized a nonpolar mutant carrying an in-frame deletion in *lcrG*. The mutant showed a Ca^{2+}-blind LCR phenotype but responded normally to ATP. Its defect was fully complemented by plasmids supplying LcrG in *trans*, showing that the mutant phenotype was solely due to the loss of functional LcrG. In the course of this work, we found that LcrG is secreted by parental *Y. pestis* when Ca^{2+} is absent and that temperature is the major component affecting the expression of *lcrG*: Ca^{2+} only slightly downregulates *lcrG* expression.

The constitutive LCR expression of the mutant indicates that LcrG participates in the downregulation of LCRS operons in response to Ca^{2+}. Additional evidence for a role in the negative pathway of LCR regulation was the observation that LcrG supplied in *trans* downregulated *lcrR* expression and restored some ability to further downregulate *lcrR* expression in response to Ca^{2+} (Fig. 4B). Negative regulation also was restored for expression and secretion of V antigen and YopM (Fig. 5). This phenotype for the nonpolar *lcrG* mutant is strikingly different from the Ca^{2+}-independent phenotype caused by a polar insertion in *lcrG* (40). However, there is no real conflict between the present results and those findings, as the polar mutation also abolished the expression of downstream *lcrV* (40), and it is known from a nonpolar *lcrV* mutant that LcrV is necessary for restriction and maximal expression of LCRS operons (49). It would be expected that a lesion in induction (LcrV⁻) would have an epistatic effect on a lesion in repression (LcrG⁻ LcrH⁻) in a polar mutant, whereas in the present work, only the lesion in repression (LcrG⁻) was present. Overexpression of LcrG in a nonpolar *Y. pestis lcrE* mutant did not change the Ca^{2+}-blind phenotype; in addition, LcrG expressed in *trans* did not complement an *lcrH* mutant. Hence, we place LcrG alongside LcrE (YopN) in the negative regulatory cascade. Like the other secreted components of the LCR negative regulatory pathway, LcrQ and LcrE (22, 54, 68), LcrG may be part of a Ca^{2+}-sensing and signalling mechanism. In this regard, it is significant that LcrG abundance is not strongly decreased when Ca^{2+} is present; LcrG availability would need to be maintained to transduce the hypothetical Ca^{2+} signal. However, no Ca^{2+}-binding motif has been identified so far for any of these proteins. We cannot exclude the possibility that LcrG has a dual role: as an antihost factor when it is secreted and as a cytoplasmically located negative effector of the LCR in the presence of Ca^{2+}. No DNA-binding motif has been identified in the LcrG sequence, suggesting that if LcrG regulates the transcription of LCRS operons, it may do so in a complex with other proteins. However, in the absence of Ca^{2+}, its

negative regulatory function must be prevented. Possible mechanisms include interaction with other LCR regulatory components, covalent modification, and removal by secretion.

We studied the *Y. pestis* Ca^{2+}-blind *lcrR* mutant further to determine whether its phenotype was due to a loss of LcrR, LcrG, or both. Our results led us to conclude that the phenotype of the *lcrR* mutant was caused by the loss of LcrG. Despite the fact that *lcrR* was provided on high-copy-number plasmids, its product could not restore the parental phenotype to the *lcrR* mutant like pJIT7-2 ($LcrG^+$) could. Primer extension analysis of the *lcrG* locus by Price et al. (50) and Barve and Straley (1) mapped a 5′ end for putative *lcrGVHyopBD* transcripts that were expressed efficiently only in the absence of Ca^{2+}. It was assumed that *lcrG* was encoded by the same transcript as that encoding the entire *lcrGVHyopBD* operon, because of the polar effects of a MudI1 (Ap^r *lac*) insertion in *lcrG*. In the *lcrR* mutant, primer extension of *lcrG* identified the same 5′ end as that in the parent strain (1). Interestingly, two-dimensional gel analysis of proteins from the *lcrR* and *lcrDR* polar mutants showed the disappearance of LcrG (1, 43). Our Western analysis of extracts prepared from those mutants confirmed those results by showing that *lcrG* was only very weakly expressed in those mutants (data not shown). In contrast, nonpolar mutations in *lcrD* strains KIM5-3001.2, KIM5-3001.3, and KIM5-3001.4, isolated by Plano and Straley (44), prevented the secretion but not the expression of LcrG (Fig. 6 and data not shown). Accordingly, LcrR may have a positive role in the expression of *lcrG*, not only in the absence of Ca^{2+} but also in its presence. This role could be at a posttranslational level. Alternatively, these intriguing results cannot rule out the possibility that LcrG may be expressed from two transcriptional units: *lcrDRG* and *lcrGVHyopBD*. Further studies, such as nonpolar mutagenesis of *lcrR* and primer extension mapping of the transcripts of the *lcrDR* locus, are needed to solve this problem.

Secretion of LcrG to the medium by yersiniae was an unexpected property. Western analysis of proteins from strains carrying mutations in LCRS operons encoding proteins required for secretion of V antigen and Yop proteins (*lcrD* and *yscBCDEF*) identified components necessary for LcrG transport. It will be interesting to learn the mechanism of LcrG transport and to determine whether this mechanism differs from that for V antigen and Yop proteins.

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