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lcrR, a Low-Ca^{2+}-Response Locus with Dual Ca^{2+}-Dependent Functions in *Yersinia pestis*

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The low-Ca^{2+} response (Lcr) of *Yersinia* includes a regulatory cascade and a set of virulence-related proteins, one of which is the V antigen. The regulatory genes modulate both bacterial growth and expression of the virulence-related proteins in response to temperature and the presence of Ca^{2+} and nucleotides. In this study we defined a new Lcr locus, *lcrR*, in *Yersinia pestis* KIM. An *lcrR* mutant, obtained by insertion mutagenesis, failed to grow at 37°C whether Ca^{2+} was present or not. However, it grew normally in the presence of ATP, showing that the Ca^{2+}- and nucleotide-responsive mechanisms are separate in *Y. pestis*. The *lcrR* mutant was avirulent in mice, probably due to its compromised growth at 37°C. β-Galactosidase measurements and Northern (RNA blot) analysis revealed that *lcrR* transcription was regulated primarily by temperature. The DNA sequence of the *lcrR* locus contained a single open reading frame of 441 bases that could encode a protein with a molecular weight of 16,470 and a pI of 10.73. Expression of an *lcrR*-containing clone in *Escherichia coli* yielded a 16,000-molecular-weight protein. At 37°C, the *lcrR* mutant strongly expressed V antigen and initiated *lcrGVH* transcription whether Ca^{2+} was present or not, indicating that this mutant had lost the transcriptional downregulation of *lcrGVH* shown by the parent in the presence of Ca^{2+}. In the absence of Ca^{2+}, the mutant failed to express LcrG, even though *lcrGVH* mRNA initiated upstream of *lcrG* at the normal sites. These data suggest that the *lcrR* locus is necessary for the regulation of LcrG expression in the absence of Ca^{2+}. Therefore, this locus has a dual regulatory role in the low-Ca^{2+} response.

Yersinia pestis, the causative agent of bubonic plague, exhibits a virulence property called the low-calcium response (Lcr) that is unique to the *Yersinia* species pathogenic for humans. The Lcr is encoded by highly homologous Lcr plasmids in these yersiniae (39), exemplified by the 75-kilobase (kb) plasmid pCD1 in *Yersinia pestis* KIM (2, 15, 19), and is manifested in vitro as two coordinately expressed properties: the requirement for millimolar quantities of Ca^{2+} for maximal growth at 37°C and the temperature- and Ca^{2+}-regulated expression of virulence-associated proteins, including the V and W antigens and Yops (yersinial plasmid-encoded outer membrane proteins) (4, 8, 10, 38, 39, 47). As a consequence of the Lcr, *Y. pestis*, when shifted from 26 to 37°C, ceases growth within two generations (56), a phenomenon known as growth restriction. Reinitiation of growth occurs if the cultures are returned to 26°C; at 37°C, growth restriction is prevented if a variety of nucleotides, including ATP (57), or one of the divalent cations Ca^{2+}, Sr^{2+}, and Zn^{2+} (56) is present in the growth medium. Zahorchak and Brubaker (57) showed that nucleotides are neither hydrolyzed exogenously nor transported into the bacterium and suggested that they might function by chelating Mg^{2+}; however, their mechanism of action has not been clarified further. Mg^{2+} potentiates the Lcr, with the most rapid growth cessation occurring at a concentration of 20 mM Mg^{2+} (56).

The effects of Ca^{2+} and ATP on growth are not identical, as ATP never fully restores maximal growth at 37°C, whereas Ca^{2+} does (57). Accordingly, it is significant that the regulatory effects of temperature, Ca^{2+}, and ATP are genetically distinct. Three types of mutants have been obtained that show an aberrant response to Ca^{2+}. Ca^{2+}-independent mutants are ones that have lost the Ca^{2+} requirement and do not undergo growth restriction following a temperature shift from 26 to 37°C in the absence of Ca^{2+} (and ATP) (13, 19, 36, 39, 56). They are also unable to induce strong expression of V antigen and Yops. Ca^{2+}-blind mutants exhibit a restrictive growth response and maximal V antigen and Yop expression in both the presence and absence of Ca^{2+} following a temperature shift (55). A third type of mutant, in addition to being partially Ca^{2+} blind, is ATP blind, as the presence of ATP in the growth medium fails to relieve growth restriction or depress V antigen and Yop expression at 37°C (41). Currently, the mechanisms by which ATP and Ca^{2+} regulate the Lcr are unknown, although the data presented in this paper and work done by Price and Straley (41) show that these compounds act through independent pathways. The thermal enhancement of expression of V antigen and Yops requires a separate locus from those affected in the Ca^{2+} and nucleotide regulatory mutants (54).

That locus, *lcrF* (*virF* in *Y. enterocolitica* [14]), and genes involved in the regulation by Ca^{2+} are contained in a ca. 18-kb region on the Lcr plasmids called the Ca^{2+}-dependence region (3, 13, 14, 19, 37, 38, 54, 55). In *Y. pestis* this region contains at least five *lcr* genes (called *vir* in *Y. enterocolitica*) that are thermally induced to different degrees. They function in the regulation of Yop and V antigen expression (5, 14, 16, 19, 36, 40, 54, 55). Insertion mutations in this region all caused Ca^{2+} independence (5, 13, 19), but these mutations may have affected multiple *lcr* genes through polar effects. In contrast, *lcrE* mutations, induced within the Ca^{2+}-dependence region by ethyl methanesulfonate, caused the Ca^{2+}-blind phenotype and defined a locus involved in the regulation of pCD1 genes in response to Ca^{2+} (55).

Perry et al. (36) identified the V antigen-encoding *lcrGVH* operon adjacent to the Ca^{2+}-dependence region by using insertion mutagenesis. This operon is regulated at the tran-

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scriptional level by temperature and Ca^{2+} or nucleotides, being expressed maximally at 37°C and in the absence of Ca^{2+} and nucleotides (36, 40). The *lcrGVH* mutant fails to express the V antigen and two additional proteins, LcrG and LcrH; is avirulent; and exhibits the Ca^{2+}- and ATP-independent growth phenotype (36). These observations indicated that this operon codes for at least one virulence gene and at least one Ca^{2+}-dependence gene. Further study of the *lcrH* gene showed that it is involved in the *Y. pestis* responses to both ATP and Ca^{2+}, as it was an *lcrH* insertion mutant that was ATP blind and partially Ca^{2+} blind. *lcrH* is also involved in the regulation of at least one *yop* gene (41).

The Yops are a heterogeneous group of proteins encoded by genes that are arranged in multiple operons dispersed over the Lcr plasmids (3, 5, 31, 46, 47). In *Y. pestis*, *yop* genes have been found so far in five operons scattered around pCD1 (26, 47). The *yop* genes have been shown to be regulated at the transcriptional level by temperature and Ca^{2+}, and they themselves are not involved in the regulation of the Lcr, but they encode virulence proteins (5, 31, 43, 47; K. Leung and S. C. Straley, unpublished data). The temperature- and Ca^{2+}-regulated Yops and presumably also the V antigen constitute the antihost effector component of the Lcr virulence regulon.

Molecular and functional analysis of the genes that mediate Ca^{2+} dependence is vital for understanding the functioning of the Lcr and its role in pathogenesis. In the present study we have defined a new locus, *lcrR*, which has a dual regulatory role governed by the presence or absence of Ca^{2+}. It is involved in the downregulation of *lcrGVH* transcription in the presence of Ca^{2+} and is necessary for LcrG protein expression in the absence of Ca^{2+}. An *lcrR* mutant was Ca^{2+} blind but completely normal in its ATP response, indicating that the Ca^{2+} and ATP sensory/regulatory mechanisms are separate. Even though this mutant expressed the V antigen strongly whether Ca^{2+} was present or not, it was avirulent. Our findings show that *lcrR* plays an important role in the regulation of the low-Ca^{2+} response.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. *Escherichia coli* K-12 strains HB101 (6), χ2338 (20), JM107 (53), and XL1-Blue {*endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1* lambda$^-$ *recA1 gyrA96 relA1* [*lac*] [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]} (Stratagene, La Jolla, Calif.) were used as hosts for the M13 vectors. XL1-Blue was also used as a host for the mGP1-2 vector in the T7 promoter-polymerase expression system (S. Tabor, personal communication). JM107 was grown in M9 minimal medium (28) to select for F^+ cells and 2× YT medium (30) for the isolation of single-stranded DNA for sequencing. XL1-Blue was grown in 2× YT containing tetracycline (25 μg/ml) both for selection of F^+ cells and isolation of single-stranded DNA.

The two strains of *Y. pestis* KIM used in this study lacked the pigmentation virulence determinant (Pgm$^-$), a property genetically and biochemically unrelated to the Lcr. Lack of this determinant renders these strains avirulent except by intravenous injection (50). *Y. pestis* KIM5 contains pCD1 and is Lcr$^+$. *Y. pestis* KIM5-3042.3[pCD1 *lcrR*::Mu dI1(Ap^r *lac*)*b*::Tn*9*] contains bacteriophage Mu dI1(Ap^r *lac*)*b*::Tn*9* inserted into *lcrR* of pCD1 and is called Ca^{2+} blind.

Bacteriophages M13mp18 and M13mp19 (53) were used as vectors for sequencing. A derivative of bacteriophage M13 called mGP1-2 (Tabor, personal communication), which contains the T7 RNA polymerase gene under the control of the *lac* promoter, was used to infect the appropriate host in the T7 RNA polymerase-promoter expression system.

Mutant isolation and preparation for characterization. The *lcrR Y. pestis* KIM was identified on lactose indicator medium as containing a Mu dI1(Ap^r *lac*) (hereafter called Mu dI1) insert in a gene regulated by temperature and Ca^{2+} (47), but its properties have not previously been described. The plasmid profile showed that Mu dI1 was inserted in pCD1. For initial phenotypic characterization, the pCD1 *lcrR*::Mu dI1 was transformed into a clean background not containing the helper phage used for the Mu dI1 infection but having the cloned native Mu repressor (pGW600 [22]) to inhibit transposition (19, 47). pGW600 was cured from the strain, and the Mu dI1 insert was stabilized by a Tn*9* insertion in the *b* gene of Mu dI1 (19, 47) before animal studies and further characterization were done.

Growth of cultures for the determination of Lcr phenotype and for the measurement of β-galactosidase and V antigen. Bacteria were acclimated and grown as described previously in the defined medium TMH (47). The medium, containing $MgCl_2$ (20 mM), was supplemented with either $CaCl_2$ (2.5 mM) or disodium ATP (18 mM) as indicated. Ampicillin (25 μg/ml) was included for growth of *Y. pestis* KIM5 3042.3. Growth was monitored by measurement of A_{620}. For growth at 37°C, the temperature was shifted from 26 to 37°C when the A_{620} reached 0.2. Samples were withdrawn at 4 h after the temperature shift, and V antigen, β-lactamase and β-galactosidase determinations were made as described before (47). A unit of V activity is 1 mm of rocket height per mg of protein electrophoresed by rocket immunoelectrophoresis. β-Lactamase values were used to correct for any possible nonspecific effects of various incubation conditions on transcription or plasmid copy number (47). Protein was measured by the method of Lowry et al. (27).

Identification of the *lcrR* gene product by using the T7 promoter-polymerase expression system. *E. coli* XL1-Blue was transformed with the *lcrR*-containing pSB3, and the clone-specific proteins were expressed by using mGP1-2 as suggested by S. Tabor (personal communication). A single colony was grown overnight at 37°C in 10 ml of M9 defined medium containing ampicillin (100 μg/ml) and supplemented with 19 amino acids at 0.01% (wt/vol) each, except methionine, and thiamine (20 μg/ml) (49). Cells from the overnight culture were diluted to an A_{620} of 0.08 in the supplemented M9 medium and grown for several hours at 37°C with gentle shaking (60 rpm). At an A_{620} of approximately 0.25, the cells were infected with mGP1-2 at a multiplicity of infection of 20, and IPTG (isopropylthiogalactopyranoside) was added to a 2 mM final concentration to induce expression of the T7 RNA polymerase gene present on mGP1-2. After 30 min at 37°C, rifampin was added to a final concentration of 200 μg/ml, and the culture was incubated for 30 min. Then, 1 ml of cells was pulsed with [^{35}S]methionine (51 μCi; New England Nuclear Corp., Boston, Mass.) for 5 min. The cells were centrifuged and solubilized in 150 μl of electrophoresis sample buffer containing 1% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β-mercaptoethanol, 60 mM Tris (pH 6.8), and 25% (wt/vol) glycerol.

Protein analysis. Resolution of proteins obtained from the T7 RNA polymerase-promoter expression system was done by one-dimensional polyacrylamide gel electrophoresis (PAGE) with 16% (wt/vol) SDS–polyacrylamide gels as described by Laemmli (24). Comparison of two-dimensional protein profiles was carried out for Lcr$^+$ *Y. pestis* KIM5 and *lcrR Y. pestis* KIM5-3042.3 whole cells. The yersiniae were grown at 37°C in TMH without Ca^{2+} or ATP (47). Methio-

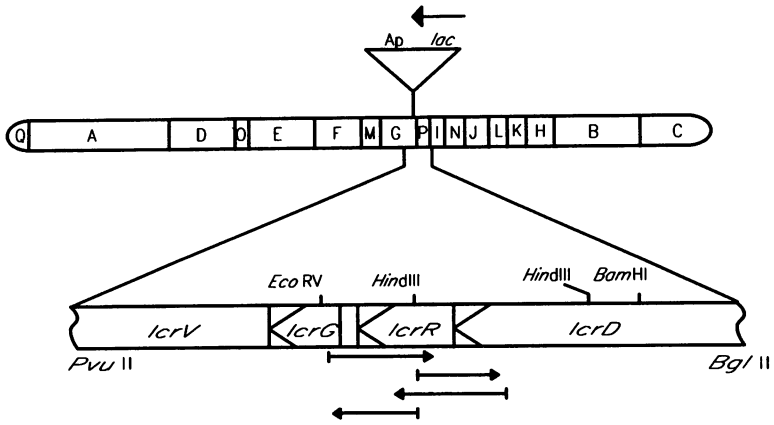

FIG. 1. Physical map of pCD1 showing the *Hin*dIII fragments A through Q. The inverted triangle shows the Mu dI1 insert location in *Y. pestis* KIM5-3042.3. The arrow above the inverted triangle shows the direction of transcription of *lac* as well as of *lcrR*. The expanded diagram below shows the region that includes the partial *Hin*dIII G and I fragments and the complete *Hin*dIII P fragment. The complete *lcrG* and *lcrR* and the truncated *lcrV* and *lcrD* contained within this region are indicated. The restriction endonuclease sites for *Pvu*II, *Bgl*II, *Eco*RV, and *Bam*HI were used to generate DNA fragments from this region for cloning. The strategy for sequencing the *lcrR* gene within the *Hin*dIII G and P fragments is shown. Arrows with smaller arrowheads denote the sequenced templates.

nine was reduced to a concentration of 0.1 mM (instead of the usual 1.0 mM) in the medium to permit labeling of proteins to high specific activity. At 5 h after the temperature shift to 37°C, both strains had entered growth restriction. [^{35}S]methionine was then added (to 1.0 mCi/ml), and the cells were incubated for an additional 30 min, centrifuged, and boiled for 2 min in electrophoresis sample buffer. Proteins in samples containing 10^6 cpm were separated by two-dimensional electrophoresis by employing nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE as described previously (33, 34). Prestained protein molecular weight standards and ^{14}C-methylated protein molecular weight standards were purchased from Amersham Corp. (Arlington, Ill.). The gels were treated with En^{3}Hance (New England Nuclear), and the labeled spots were visualized by fluorography at −70°C by using Kodak X-O-Mat AR film (Eastman Kodak Co., Rochester, N.Y.).

Virulence testing in mice. Virulence testing of the *lcrR* mutant was carried out as described by Straley and Bowmer (47). The 50% lethal dose values were calculated by the method of Reed and Muench (42).

DNA restriction analysis, cloning, and nucleotide sequencing. Restriction endonuclease analysis and cloning were carried out by standard methods (28). Fragments of pCD1 generated by complete digestion with the restriction enzymes *Bgl*II, *Hin*dIII, *Pvu*II, *Eco*RV, and *Bam*HI, used alone or in pairs, were cloned into pBR322, pKS(−) (Stratagene, La Jolla, Calif.), and pIC-20R (29). The recombinant products were transformed into *E. coli* HB101, χ2338, and XL1-Blue. The approximate location of Mu dI1 and direction of transcription of *lacZ* for the insert in pCD1 *lcrR*::Mu dI1 *b*::Tn*9* were determined by comparison of *Bam*HI and *Hin*dIII digests of this plasmid as described by Straley and Bowmer (47) (refer to Fig. 3). Further mapping of the insert was carried out by subjecting the chimeric *Hin*dIII G-Mu dI1 fragments from pCD1 *lcrR*::Mu dI1 *b*::Tn*9* to Southern blotting (28), with nick-translated *Hin*dIII fragment G as the probe. The insert was located in the *Hin*dIII G fragment of pCD1, within 100 nucleotides of the junction between *Hin*dIII-G and *Hin*dIII-P (Fig. 1). The *lcrR*-containing 3.0-kb *Pvu*II-*Bgl*II fragment of pCD1 was cloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain termination procedure (44) with the T7 DNA polymerase Sequenase from United States Biochemical Corporation (Cleveland, Ohio) and [^{35}S]dATP from New England Nuclear Corp. (Boston, Mass.). The 17-base universal primer provided in the Sequenase kit and a set of *lcrR*-specific synthetic 15- to 21-base oligonucleotides synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington) were used as sequencing primers. Both strands were sequenced to confirm the *lcrR* sequence.

DNA analysis. DNA and predicted protein sequences were analyzed with the PC Gene (IntelliGenetics, Inc., Mountainview, Calif.) and Bionet (IntelliGenetics, Inc.) computer programs. The algorithm of Pearson and Lipman (35) was used to search the nucleic acid and protein sequence data bases for similarities to *lcrR* and LcrR, respectively.

RNA isolation and primer extension analysis. RNA was isolated by a procedure developed by Shirish S. Barve (Ph.D. thesis, University of Kentucky, Lexington, 1990) and described by K. Y. Leung and S. C. Straley (26). Samples (20 μg) of RNA were hybridized to an end-labeled complementary DNA oligonucleotide located from 45 to 80 or 33 to 56 bases downstream from the first ATG of *lcrG* or *lcrR*, respectively. Primer extension of the DNA-RNA hybrid transcripts was carried out with reverse transcriptase (Moloney murine leukemia virus reverse transcriptase; Bethesda Research Laboratories, Gaithersburg, Md.). The extension products were resolved by PAGE. These products were compared with the extension products derived from DNA sequencing reactions with the same *lcrG*-specific oligonucleotide as primer to determine the +1 start site of the *lcrGVH* mRNA transcript.

RESULTS

Mutant isolation and insert mapping. Our lab has been using insertion mutagenesis to identify the pCD1-encoded genes that are regulated by temperature and/or Ca^{2+} and ATP. Mutants of *Y. pestis* KIM containing random insertions in pCD1 genes were generated by using the transposing operon fusion phage Mu dI1 (12). This phage, when oriented properly, generates transcriptional fusions of *lacZ* to the promoter of the gene disrupted by the insertion. We exploited this property to screen for mutants that showed

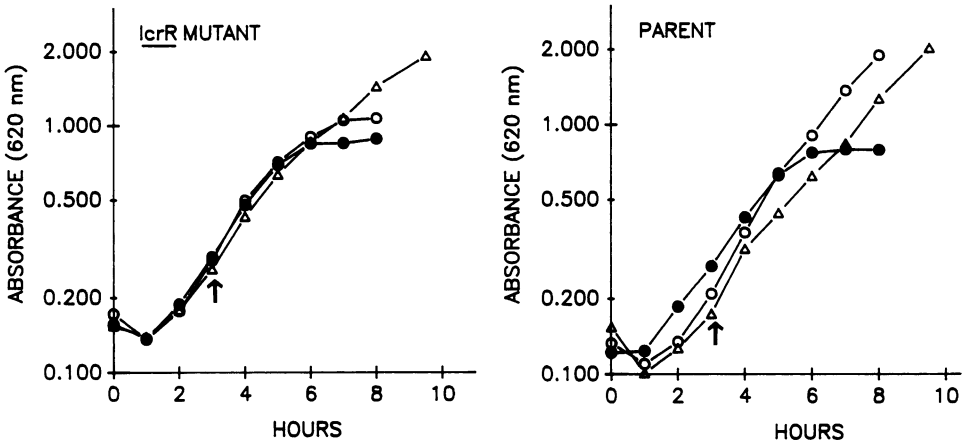

FIG. 2. Growth of the *lcrR* mutant *Y. pestis* KIM5-3042.3 (left panel) and the parent *Y. pestis* KIM5 in TMH containing 20 mM Mg^{2+}. Following adaptation to the medium for ca. seven generations at 26°C, bacteria were grown at 26°C in TMH containing 2.5 mM Ca^{2+} (○), 18 mM ATP (△), or no addition (●), and the temperature was shifted to 37°C following one generation of growth (arrow).

effects of temperature and Ca^{2+} on transcription by measuring the expressed β-galactosidase activity (47). One isolate showed *lac* expression at 37°C in both the presence and absence of Ca^{2+}. This isolate also made V antigen (detected by rocket immunoelectrophoresis) under similar conditions (37°C, with or without Ca^{2+}).

Analysis of the pCD1::Mu dI1 plasmid from this isolate by restriction endonuclease digestion revealed the direction of transcription and approximate location of the insert (47) (Fig. 1). Higher-resolution mapping performed by Southern analysis located the insert at position 42.3 kb on the pCD1 map, within 100 nucleotides of the end of the *Hin*dIII G fragment. This fell just outside of the previously defined Ca^{2+}-dependence region of pCD1 (19), and several pieces of evidence indicated that it represented a new *lcr* locus, which we designated *lcrR*. Sequence analysis (this work) demonstrated that out of the total 441 nucleotides of *lcrR*, 114 nucleotides (the 5′ end) were located in the *Hin*dIII P fragment and the remaining 327 nucleotides were located in the *Hin*dIII G fragment. Furthermore, sequence analysis of *lcrR* in conjunction with that of *lcrGVH* (40) showed that the *lcrR* translational stop was separated from the *lcrG* translational start by 41 nucleotides and that the transcriptional starts (+1 sites) of the *lcrGVH* operon were located upstream of *lcrG* in this region, separating *lcrR* and *lcrG*. Analysis of the mutant phenotype of *Y. pestis* KIM5 3042.3 (this work) revealed that the Mu dI1 insert did not abolish V antigen expression or affect the transcriptional startpoints of the *lcrGVH* operon (whereas a Mu dI1 insert within *lcrG* abolishes V antigen expression [36, 40]). These facts taken together show that the Mu dI1 insertion is located within a transcriptional unit distinct from *lcrGVH*.

Effect of Ca^{2+}, ATP, and temperature on the growth of the *lcrR* mutant. At 26°C, the *lcrR* mutant *Y. pestis* KIM5-3042.3 showed no difference in growth compared with the parent strain *Y. pestis* KIM5 and showed normal growth restriction at 37°C in the absence of Ca^{2+} (Fig. 2). However, the growth restriction of the *lcrR* mutant was not prevented by the presence of 2.5 mM Ca^{2+}. In contrast to this aberrant Ca^{2+} response, the mutant showed a normal growth response (relief from restriction at 37°C) to the addition of 18 mM ATP.

Interestingly, the growth of the *lcrR* mutant was Ca^{2+} dependent like that of the parent when CFUs were determined at 37°C on complex plating medium (Tryptose Blood Agar Base, Difco) with and without 25 mM each of sodium oxalate (to chelate calcium) and $MgCl_2$ (data not shown). This is an example of the strong medium dependence that sometimes is seen for the Lcr growth phenotype. Differences were found previously in the growth of *lcrC* mutant *Y. pestis* in defined liquid medium with or without added Ca^{2+} versus growth on complex plating medium with or without chelation by oxalate (19). In that study and in all of our subsequent work, we based our Lcr phenotype designations on the growth in defined liquid medium, as it is more reproducible physiologically than is culture on complex plating medium. Accordingly, the *lcrR* mutant growth phenotype will be designated Ca^{2+} blind, as seen in TMH.

Effect of Ca^{2+}, ATP, and temperature on V antigen expression of the LcrR$^-$ mutant. The *lcrR* mutant *Y. pestis* KIM5-3042.3 and the parent *Y. pestis* KIM5 made basal levels of V antigen at 26°C (Fig. 3). Following the temperature shift to 37°C, both strains showed induction of V antigen expression under all incubation conditions, but to various degrees. Both strains showed equal and maximal induction of V antigen expression in the absence of Ca^{2+} or ATP. However, in the presence of Ca^{2+}, the *lcrR* mutant continued to show strong V antigen expression in contrast to the parent strain, which

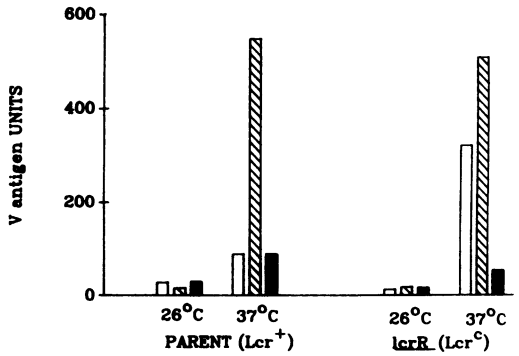

FIG. 3. V antigen expression by the parent *Y. pestis* KIM5 and the *lcrR* mutant *Y. pestis* KIM5-3042.3 grown in TMH containing 20 mM Mg^{2+}. Bacteria were grown as described in the legend to Fig. 2, and samples for assay of V antigen content were taken 4 h after the temperature shift. Different growth conditions: 2.5 mM Ca^{2+} (open bars), no additions (hatched bars), 18 mM ATP (solid bars). One unit of V antigen expression was defined as 1 mm of rocket height per mg of protein electrophoresed (rocket immunoelectrophoresis).

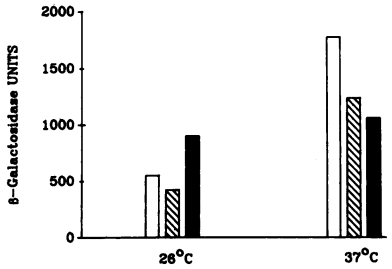

FIG. 4. β-Galactosidase expression by the *lcrR* mutant *Y. pestis* KIM5-3042.3. Duplicate cultures were made at 26°C in TMH containing 2.5 mM Ca^{2+} (open bars), no additions (hatched bars), or 18 mM ATP (solid bars), as described in the legend to Fig. 1, and one was shifted to 37°C following one generation of growth. Samples for assay of β-galactosidase activity were taken 4 h after the temperature shift. One unit of β-galactosidase activity is defined as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolysed per min per mg of protein.

showed only a little increase over the basal level. The presence of ATP had a similar effect on V antigen expression by both strains, which showed only small increases over the basal levels at 26°C. Thus, with respect to Ca^{2+} but not ATP, the mutant expressed V antigen and underwent a restrictive growth response constitutively, showing that it was Ca^{2+} blind.

Effect of Ca^{2+}, ATP, and temperature on *lcrR* transcription: β-galactosidase measurements. Determinations of β-galactosidase levels expressed by the *lcrR* mutant were made at 26°C and at 4 h after the temperature shift to 37°C under various conditions of added Ca^{2+} or ATP and no additions (Fig. 4).

lcrR showed weak basal transcription at 26°C, as indicated by β-galactosidase expression (Fig. 4). A temperature upshift resulted in the enhancement by ca. threefold of β-galactosidase expression in the presence or absence of Ca^{2+} but had little effect when ATP was present. Thus, the major regulation of *lcrR* was due to temperature, but there were also contributions to the regulation from the presence or absence of Ca^{2+} or ATP. These could have resulted indirectly from the unbalanced low-Ca^{2+} response regulation in this mutant.

Northern (RNA blot) analysis of RNA isolated from the parent and the *lcrR* mutant. LcrR protein could be directly or indirectly involved in the autoregulation of its operon. This aspect of regulation would be abolished in the $LcrR^-$ mutant *Y. pestis* KIM5-3042.3. Hence, the information about *lcrR* transcription in the *lcrR* mutant obtained by measurements of β-galactosidase expression may not accurately reflect the transcriptional activity of *lcrR* in response to temperature and Ca^{2+} in the parent. To address this point and also provide information about the transcript(s) encoding the LcrR protein, kinetics of *lcrR* mRNA expression were analyzed in both parent and mutant by Northern analysis. Total cellular RNA was isolated from the parent *Y. pestis* KIM5 and the *lcrR* mutant *Y. pestis* KIM5-3042.3. Yersiniae were grown in a defined medium with or without Ca^{2+} for several generations at 26°C and then shifted to 37°C. Cells were harvested for RNA isolation at 0 h (26°C, prior to temperature shift) and at 0.75, 1.5, 3, 4.5, and 6 h after the shift to 37°C. RNA was resolved by using 1.5% (wt/vol) agarose gels containing formaldehyde, and the resulting blot was probed with the nick-translated *Hin*dIII P fragment (Fig. 1). The analysis identified five mRNA species in the parent which were approximately 1.08, 0.86, 0.83, 0.56, and 0.53 kb

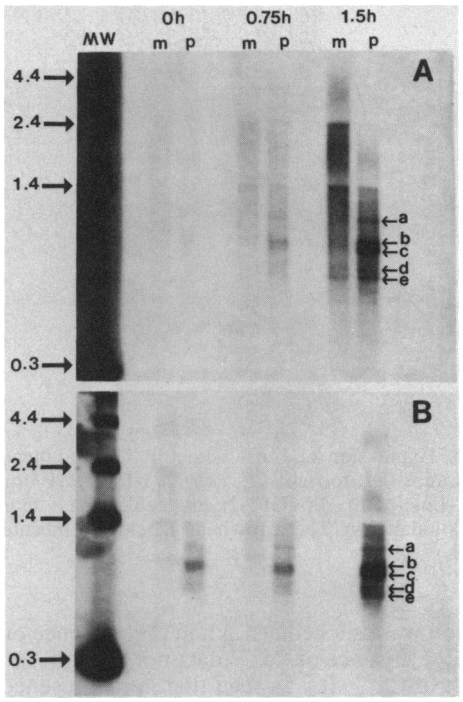

FIG. 5. Northern blot analysis of *Hin*dIII-P-specific mRNA(s): effect of Ca^{2+} and temperature. Total cellular RNA was isolated from the parent *Y. pestis* KIM5 and the *lcrR* mutant *Y. pestis* KIM5-3042.3. Bacteria were grown in defined medium with or without Ca^{2+}, and cells were harvested at appropriate times before (26°C) and after the temperature shift to 37°C. Twenty micrograms of total RNA per strain per time point was loaded in each lane. For a direct comparison at each time point, parent (p) and $LcrR^-$ mutant (m) RNAs were loaded in adjacent lanes. All of the RNAs were probed with the nick-translated *Hin*dIII P fragment. The RNA size ladder (leftmost lane in each panel, with sizes indicated in kilobases) was probed separately with nick-translated *Hin*dIII fragments of lambda DNA (Bethesda Research Laboratories). (A) Ca^{2+} was absent in the growth medium for the bacteria; (B) Ca^{2+} was present. The numbers over the sets of lanes indicate the times in hours after the temperature shift that samples were taken from the cultures for the preparation of RNA. RNA species indicated with a, b, c, d, and e are, respectively, the 1.08-kb, 0.86-kb, 0.83-kb, 0.56-kb, and 0.53-kb species discussed in the text.

in size (Fig. 5). In contrast, the *lcrR* mutant was found to be lacking the 1.08-kb, 0.86-kb, and 0.83-kb mRNA species but showed the presence of slightly larger, less abundant messages (approximately 1.15, 0.88, and 0.85 kb in size). The messages detected in the *lcrR*::Mu dI1 insertion mutant are expected to be hybrid *lcrR*::*lacZYA* messages, as the mutation generates an operon fusion. This would result in messages that have significantly slower electrophoretic mobilities than their parent counterparts. The postulated sizes of the hybrid messages are 6.18, 5.96, and 5.93 kb, respectively, calculated by adding the 5.1-kb size of the *lacZYA* mRNA to those of the yersinial mRNAs. However, the sizes of the messages that appeared to be shifted in the mutant were much smaller than expected. Perhaps this was due to the 5′ processing known to occur in *lacZ* mRNA (11). The 0.56- and 0.53-kb mRNAs were not altered in size by the *lcrR* mutation.

The 0.86- and 0.83-kb messages missing in the *lcrR* mutant showed strong induction by temperature in the parent. The expression increased in response to the temperature shift in both the presence and absence of Ca^{2+}, although maximal

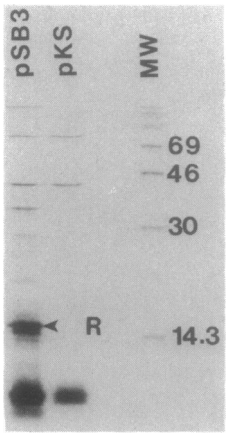

FIG. 6. Expression of LcrR with the T7 promoter-polymerase system. Lanes (left to right): *E. coli* XL1-Blue(PSB3)(mGP1-2); *E. coli* XL1-Blue[pKS(−)](mGP1-2); molecular mass markers (sizes given in kilodaltons). The arrowhead labeled R indicates the putative LcrR.

expression was reached at 1.5 h in the presence of Ca^{2+} and at 3 h in the absence of Ca^{2+} (data not shown). In relation to the effect of Ca^{2+}, it was seen that in its presence the basal level of expression in both strains (0 h, 26°C) was markedly stronger than in its absence. Interestingly, the mutant failed to show increased expression of any of the five messages upon continued incubation at 37°C in the presence of Ca^{2+}. In fact, the messages completely disappeared by 1.5 h following the temperature shift. This suggests that in the presence of Ca^{2+}, the *Hin*dIII-P-containing transcripts require a functional *lcrR* product for their continued expression. These findings corroborated the β-galactosidase expression data by showing that temperature has a major effect on expression of *lcrR*. However, they also revealed details of regulation that may occur posttranscriptionally and which are not fully understood at present.

Virulence testing of the *lcrR* mutant in mice. The intravenous 50% lethal dose of the *lcrR* mutant in outbred mice was greater than 1.2×10^6 cells. This avirulence may have resulted from the strong growth defect in this mutant, as with the previously characterized ATP-blind and partially Ca^{2+}-blind *lcrH* mutant (41).

Cloning of the pCD1 fragment containing *lcrR* and expression of the cloned genes by the T7 RNA polymerase-promoter system in *E. coli*. Initially, a cloned 1.7-kb *Eco*RV-*Bgl*II fragment (Fig. 1) was analyzed by the minicell system to identify LcrR and other possible proteins encoded by this region of pCD1. This attempt was unsuccessful, because the construct was lacking the promoter for the operon that contains *lcrR* (as revealed by subsequent sequence analysis). Hence, we turned to the T7 RNA polymerase-promoter expression system, which provides a very strong promoter (T7), thereby overcoming any transcriptional barrier. The system also avoids possible toxicity to the host caused by the expression of the cloned gene(s).

lcrR was cloned as an *Eco*RV-*Bam*HI fragment of pCD1 (Fig. 1) inserted downstream of the T7 promoter in pKS(−), and the recombinant plasmid was called pSB3. pSB3 was used to transform *E. coli* XL1-Blue. T7 RNA polymerase was supplied by infection with mGP1-2, which contains the T7 RNA polymerase gene under the control of the *lac* promoter, inducible by IPTG. The cells were then treated with rifampin to decrease background *E. coli* message expression without affecting T7 RNA polymerase. Following labeling with [^{35}S]methionine, the expressed proteins were analyzed by SDS-PAGE (Fig. 6). An abundant 16,000-molecular-weight protein was the predominant clone-specific product. Two minor pSB3-specific species of 29,500 and 21,500 molecular weight were also produced. These minor species may represent truncated or processed products originating from a partial open reading frame upstream of *lcrR* in the clone.

DNA sequence analysis. The DNA sequence encompassing the site of the Mu dI1 insert in *lcrR* showed an open reading frame 441 bases in length (Fig. 7). No *E. coli*-like promoter

```
                                                    GCGCAAACTGATTGAGAGCGAATACTATGGCT   32

TGCCGGTACTTTCATACCAAGAGCTGACTCAGCAGATTAATATCCAACCACTTGGACGAATTTGCTT   99

Met Met Ala Asp Leu Leu Ile Pro Trp Leu Thr Glu His Gly Leu Val Cys
[ ] ATG GCA GAC CTT TTA ATT CCG TGG CTT ACC GAA CAT GGC TTG GTT TGC   150

His Pro His Thr Leu Ser Gly Thr Pro Ile Ser Leu Gly Ser Ala Phe Gln
CAC CCT CAT ACT TTG TCT GGC ACC CCC ATT TCT TTA GGT TCG GCC TTT CAA   201

Leu Ala Gly Leu Lys Leu Ala Trp Arg Val Glu Ile Glu Gln Arg Arg Val
TTA GCT GGC CTC AAG CTT GCC TGG CGC GTA GAA ATT GAA CAA AGG CGG GTT   252

Trp Ile Val Leu Ile Gln Arg Val Glu Gln Arg Arg Gly Leu Lys Asn Pro
TGG ATC GTG CTT ATC CAA CGA GTG GAA CAA CGT CGA GGG CTG AAA AAT CCC   303

Phe Ala Ala Leu Tyr Met Leu Ala Asn Ala Ala Arg Ala Val Leu Gly Pro
TTC GCG GCA CTT TAT ATG TTA GCT AAT GCA GCG CGG GCC GTT CTT GGC CCT   354

Asp Tyr Tyr Leu Tyr Gly Asn Val Asp Val Leu Ala Gly Ser Ser Leu Ser
GAC TAT TAT CTG TAT GGC AAT GTC GAT GTA CTG GCG GGG AGT TCT CTC AGT   405

Thr Gln Arg Leu Ala His Phe Tyr Arg Arg Trp Thr Gly Ala Lys Glu Leu
ACG CAA CGG CTC GCT CAT TTT TAT CGG CGT TGG ACC GGG GCC AAA GAA TTA   456

Cys Thr Gly Trp Phe Ser Leu Lys Val Ser Gln Val Ile Thr Leu Ser Asn
TGC ACC GGG TGG TTC TCA CTA AAA GTA TCA CAA GTC ATC ACC TTA TCT AAT   507

Met Lys Lys Arg Gln Asn Asn Gly Phe Ala TER
ATG AAA AAG CGA CAA AAC AAC GGC TTT GCC TGA CAAGCTAAATAAAAATAACGTAA   563

                    lcrG Start
TAGAATAGGAGGTAGATT [ ] AAG TCT TCC CAT TTT                          599
```

FIG. 7. Nucleotide sequence of *lcrR*. The sequence of the antisense strand is shown. Bases comprising the potential ribosome-binding site are represented in larger letters and underlined. Bases complementary to the oligonucleotide used as a primer in the primer extension analysis are in boldface letters. The startpoint of the frame encoding LcrG is indicated.

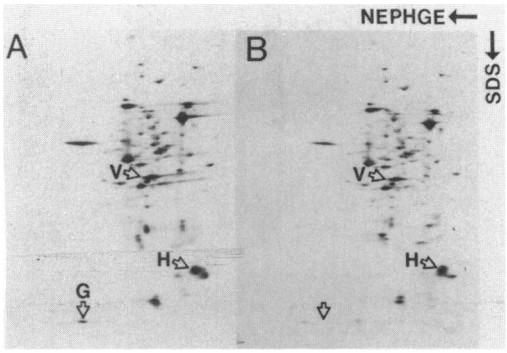

FIG. 8. Two-dimensional protein profiles of the parent, Lcr^+ *Y. pestis* KIM5 (A), and the mutant, $LcrR^-$ *Y. pestis* KIM5-3042.3 (B) grown as described in the legend to Fig. 1 at 37°C in a defined medium without Ca^{2+} and labeled with [^{35}S]methionine. The first-dimension electrophoresis was NEPHGE, and the second-dimension electrophoresis was into 12% (wt/vol) polyacrylamide–SDS gels. The locations of proteins encoded by the *lcrGVH* operon are indicated by arrows.

consensus sequences were detected upstream from *lcrR*. Primer extension analysis was carried out by using an oligonucleotide primer located 33 to 56 bases downstream from the first ATG in *lcrR* (Fig. 7) and total cellular RNA isolated from the parent *Y. pestis* KIM5 grown at 37°C with and without Ca^{2+}. This analysis did not reveal any transcriptional start site (+1 site of mRNA) for *lcrR*, suggesting that this gene may be part of a multicistronic operon. An *E. coli*-like ribosome-binding site (45) was located 7 bases upstream of the first ATG in *lcrR*. No stem-and-loop structures resembling rho-independent transcriptional termination sites (7) were found downstream from the translational termination codon of *lcrR*. The DNA sequence analysis also showed a partial open reading frame which started immediately upstream from *lcrR* and spanned the entire region up to the *Bam*HI site in pSB3 (Fig. 1). This partial open reading frame is the 3′ end of the *lcrD* gene (S. S. Barve and S. C. Straley, unpublished data).

Analysis of the predicted LcrR protein. The computer analysis of the *lcrR* open reading frame showed that *lcrR* could encode a protein of 16,470 molecular weight, having an isoelectric point (pI) of 10.73. The hydropathic index computation showed that LcrR was hydrophobic, with a grand average of hydropathy value of 0.17 (23). The same program (SOAP of PCGene) further classified LcrR as a peripheral protein (21). No significant similarities were found between the predicted LcrR and the sequences in the Swiss-Prot or National Biomedical Research Foundation Protein Identification Resource (NBRF-PIR) data bases.

NEPHGE analysis of the *lcrR* mutant and the parent. To confirm the predicted pI (10.73) of LcrR and locate its electrophoretic position in the whole-cell protein pattern of *Y. pestis*, pSB3-directed proteins obtained by the T7 expression system were analyzed by two-dimensional NEPHGE. The analysis failed to show the representative LcrR protein (data not shown) which was seen in relative abundance in the same sample analyzed by SDS-PAGE (Fig. 6). This discrepancy was apparently due to the failure of LcrR protein to enter the first-dimension gel. Similar problems in the resolution of other hydrophobic proteins in standard two-dimensional electrophoresis have been encountered previously (18).

To determine the effect of insertional inactivation of LcrR on other pCD1-encoded gene products, we compared the two-dimensional protein profiles of ^{35}S-labeled proteins from the *lcrR* mutant and the parent grown under conditions expected to elicit maximal expression of pCD1 genes (37°C, without Ca^{2+}) (Fig. 8). Interestingly, the *lcrR* mutant failed to express LcrG, which is encoded by the V operon *lcrGVH* (36). However, the mutant made normal amounts of both LcrV and LcrH, encoded by the same operon (36).

Primer extension analysis of *lcrGVH* in the *lcrR* mutant. Primer extension analysis was carried out to determine whether the *lcrR* mutation had affected the transcriptional startpoints for *lcrGVH*. Total cellular RNA obtained from the parent *Y. pestis* KIM5 and the *lcrR* mutant *Y. pestis* KIM5-3042.3 grown in a defined medium with and without Ca^{2+} was analyzed by primer extension. The oligonucleotide used as the primer was identical to the one used by Price et al. to identify the +1 mRNA sites of *lcrGVH* (40). The data obtained showed that in the *lcrR* mutant, the *lcrGVH* mRNA initiated at +1 sites identical to those in the parent (Fig. 9) (40). This shows that *lcrGVH* transcription initiation was unaffected in the *lcrR* mutant. The data also showed that the *lcrR* mutant had lost the ability to downregulate transcription of the *lcrGVH* operon in the presence of Ca^{2+}. This finding was fully consistent with the Ca^{2+}-blind growth and expression of the V antigen demonstrated by the *lcrR* mutant.

DISCUSSION

This study identified a new Lcr locus, *lcrR*, characterized it at the molecular level, and investigated its role in the low-Ca^{2+} response of *Y. pestis*. The data indicate that *lcrR* has a dual regulatory function dictated by the presence or absence of Ca^{2+}.

Like other Ca^{2+} dependence genes described previously (19), *lcrR* itself is not appreciably regulated by Ca^{2+}, but an intact *lcrR* locus is necessary for the regulation of other Lcr genes by Ca^{2+}. This was evident from the measurements of β-galactosidase expression from *lcrR*::Mu dI1: *lcrR* is regulated at the transcriptional level mainly by temperature. However, the basal level of *lcrR* expression was enhanced in the presence of Ca^{2+}. These regulatory features are not understood at present.

Northern analyses, performed to extend the β-galactosidase observations, essentially corroborated these findings by showing that temperature is the major component functioning in the regulation of *lcrR*. However, they also revealed other regulatory features which are not completely understood at present. For example, the *lcrR* mutant grown in the presence of Ca^{2+} showed the absence of the *Hin*dIII P-specific messages starting at 1.5 h following the temperature shift, suggesting that *lcrR* may play a role in the enhancement or maintenance of the temperature-induced expression of the operon under these conditions. Our data did not assess whether this is a direct or indirect effect.

The data also raised the possibility of posttranscriptional regulation through processing and/or degradation by endoribonucleolytic activity. *Hin*dIII-P probing consistently detected five messages in the total cellular RNA obtained from the parent *Y. pestis* KIM5 grown in the presence and absence of Ca^{2+}. The five messages could arise from the action of enzymes other than those endoribonucleases known to function primarily in processing of stable RNA (1). Such an enzymatic activity, which has been demonstrated by Nilsson et al. (32) in the case of *bla* and the *ompA* mRNA, can give rise to specific degradation products and even result in the accumulation of some them. The observed high

concentration of the 0.86- and 0.83-kb messages with respect to the other messages could be due to such an accumulation event. Further work will be required to prove how the multiple messages arise and how their generation relates to regulation in the low-Ca^{2+} response.

The *lcrGVH* operon is one target for *lcrR* function. This operon encodes the V antigen and is regulated at the transcriptional level by Ca^{2+} (40). The *lcrR* mutant showed strong expression of V antigen at 37°C whether or not Ca^{2+} was present, indicating that *lcrR* functions in the downregulation of *lcrGVH* in the presence of Ca^{2+}.

However, interestingly, *lcrR* evidently is not necessary for the *Y. pestis* response to ATP, because the *lcrR* mutant responded fully to 18 mM ATP at 37°C by growing normally and downregulating V antigen expression. Presumably, the effect of ATP was mediated by *lcrH* (41). This is strong evidence that there are distinct mechanisms for preventing growth restriction at 37°C in response to Ca^{2+} and ATP. Additional evidence supporting this idea came from the properties of an $LcrH^-$ mutant, which is ATP blind but retains a weak response to Ca^{2+} (41).

Despite constitutive V antigen expression at 37°C, the *lcrR* mutant was avirulent in mice. V antigen is considered a major virulence determinant for *Y. pestis* (7). It is protective (25), and indirect evidence suggests its requirement for survival and prolonged growth of the yersiniae in mice (51, 52). The *lcrR* mutant may be avirulent because of its inability to grow at 37°C in the presence of Ca^{2+}, a condition that would apply to blood and interstitial fluid. It is also possible that the normal regulation of virulence genes in response to temperature and Ca^{2+}, disrupted in the *lcrR* mutant, is essential for virulence.

Five observations indicate that *lcrR* is the last gene in an operon that includes at least one more gene. Primer extension analysis of the *lcrR* locus with total cellular RNA isolated from the parent grown in the presence and absence of Ca^{2+} failed to reveal a +1 site for *lcrR* (data not shown). Sequencing data have shown the existence of a large open reading frame immediately upstream of *lcrR* that contains the *lcrD* gene and that the translational stop of *lcrD* and the translational start of *lcrR* overlap (S. S. Barve and S. C. Straley, unpublished data). Mu dI1 inserts in *lcrD* abolish LcrG expression, perhaps as an indirect result of their polar effect on downstream *lcrR* (S. S. Barve and S. C. Straley, unpublished data). Finally, downstream from *lcrR* is the beginning of the *lcrGVH* operon (36, 40), distinct from the operon containing *lcrR*; the Mu dI1 insert in *Y. pestis* KIM5-3042.3 that defined the *lcrR* locus neither eliminated expression of V antigen nor affected the *lcrGVH* transcriptional startpoints (Fig. 8 and 9). However, until the multiple messages seen in northern analyses probed with *Hin*dIII-P (Fig. 5) and *Hin*dIII-G (S. B. Price, S. S. Barve, and S. C. Straley, unpublished data) are definitively mapped, the operon structure that we have deduced for the *lcrR* region needs to be considered preliminary.

It was surprising that the $LcrR^-$ mutant failed to express LcrG, the product of the first cistron in *lcrGVH*, while V antigen and LcrH, encoded by the same operon (36), were expressed strongly. Furthermore, the primer extension analysis showed that in the *lcrR* mutant, *lcrGVH* transcription initiated at the same +1 site as in the parent and that this initiation was strong in both the presence and absence of Ca^{2+}, as expected from the measurement of V antigen expression in this mutant. Taken together, these findings indicate that *lcrR* is necessary for LcrG protein expression in the absence of Ca^{2+} and support the interpretation that LcrR

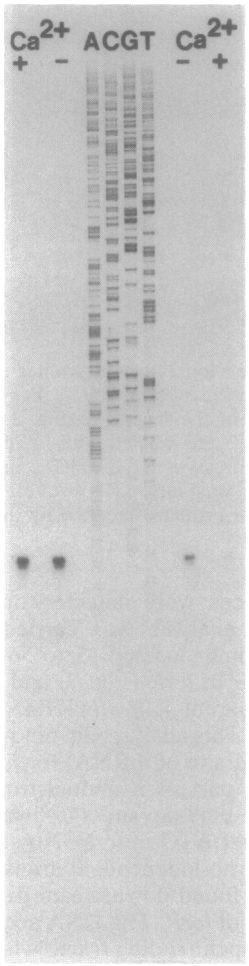

FIG. 9. Primer extension analysis of *lcrG*. The transcriptional start site for this gene was determined by using RNA isolated from *Y. pestis* KIM5-3042.3 (left panel) and *Y. pestis* KIM5 (right panel) grown at 37°C in TMH containing (+) or lacking (−) 2.5 mM Ca^{2+}. The 35-mer oligonucleotide primer used was complementary to bases 45 to 80 after the first ATG of *lcrG*. The sequencing reactions indicated by A, C, G, and T were primed with the same oligonucleotide used for the primer extension.

functions in the downregulation of the *lcrGVH* operon at the transcriptional level in the presence of Ca^{2+}.

However, LcrR is not the only component involved in Ca^{2+} sensing and repression of *lcrGVH*. Yother and Goguen (55) have described mutants of *Y. pestis* obtained by ethyl methanesulfonate mutagenesis. These mutants exhibit a Ca^{2+}-blind phenotype identical to that of the *lcrR* mutant and define the *lcrE* locus. That locus is located at ca. 48.0 kb on the pCD1 map and ca. 5.7 kb upstream of *lcrR*, within the Ca^{2+}-dependence region originally designated *lcrA* (19). A partially Ca^{2+}-blind phenotype was also observed in the case of the $LcrH^-$ mutant (41). Therefore, we speculate that the downregulation of *lcrGVH* at 37°C in the presence of Ca^{2+} involves a network comprising components encoded by *lcrH* and *lcrE* along with *lcrR* (Fig. 10).

Our working model for the role of the *lcrR* locus in the low-Ca^{2+} response assigns dual regulatory functions to LcrR protein that are governed by Ca^{2+} at 37°C. Our data indicate that besides the negative regulatory function, LcrR protein also has a second function as a positive regulator at 37°C in the absence of Ca^{2+}. We found that although the transcrip-

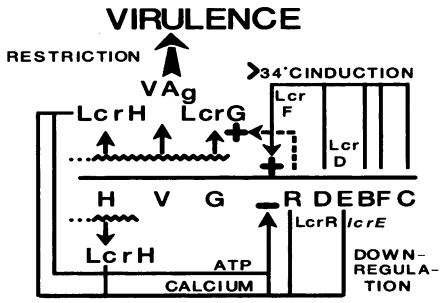

FIG. 10. Model illustrating regulatory interactions in the low-Ca^{2+} response of *Y. pestis*. VAg, V antigen. The letters denote *lcr* loci. Arrows from these indicate positive (+) and negative (−) regulatory effects of their gene products on expression of the V antigen operon *lcrGVH*. This latter operon is depicted in greater detail than are the other *lcr* loci, with wavy lines indicating mRNAs (and dots indicating that the 3′ ends of these messages have not been mapped). The postulated role of *lcrR* in the posttranscriptional regulation of LcrG expression is indicated by the broken arrow.

tional initiation at *lcrG* was not affected, LcrG protein was not expressed in the absence of LcrR protein. This suggests that the positive regulatory function is involved posttranscriptionally, at the mRNA or protein level. However, LcrR protein could also carry out its positive effector function indirectly at the DNA level if it downregulated a component that in turn acts posttranscriptionally as a negative regulator of LcrG. This possibility is suggested by the observation that LcrG protein is expressed by *E. coli* χ2338 harboring a clone containing the *lcrGVH* operon in the absence of the LcrR protein.

We can argue against an alternative interpretation for the loss of LcrG expression in the *lcrR* mutant, namely, that LcrG actually is expressed from an *lcrRG* transcript and not from *lcrGVH*. That idea is suggested by the absence of a Shine-Dalgarno sequence for *lcrG* in *lcrGVH* but the presence of one (GGAGG) immediately upstream of the *lcrGVH* transcriptional startpoints, 7 bases from the ATG of *lcrG* (40). However, a clone containing the *Hin*dIII G fragment of pCD1 and lacking both an intact *lcrR* and a properly oriented exogenous promoter still expresses LcrG in *E. coli* (36). Furthermore, a Mu dI1 insert in *lcrD* at 43.2 kb on the pCD1 map (in *Hin*dIII-P) also abolishes LcrG expression, presumably via a polar effect on *lcrR* (S. S. Barve and S. C. Straley, unpublished data). Finally, it is significant that *Hin*dIII-P-specific messages are not downregulated in expression by Ca^{2+}, whereas all *Hin*dIII-G-specific messages are (S. B. Price and S. C. Straley, unpublished data). Any hypothesis invoking transcriptional readthrough or overlapping transcriptional units in the *lcrR* region to explain the abolishment of LcrG expression in the *lcrR* mutant will also have to account for these observations.

The absence of LcrG in the *lcrR* mutant has a significant implication for our understanding of the restrictive-response property of the low-Ca^{2+} response. The *lcrGVH* operon is known to contain a gene necessary for the restrictive response in the absence of Ca^{2+} or ATP, because a Mu dI1 insert in *lcrG*, which also abolishes LcrV and LcrH expression, eliminates this response, resulting in Ca^{2+}-independent growth (36). The $LcrR^-$ and $LcrH^-$ mutants, which express LcrV, enter growth restriction in both the presence and absence of Ca^{2+}. Because LcrG expression is abolished in the $LcrR^-$ mutant, LcrV is implicated by process of elimination as the *lcrGVH* gene product that is responsible for the restrictive response to Ca^{2+} and nucleotide deprivation. It is the only *lcrGVH* gene product that is always expressed in yersiniae that show the restrictive response. It has long been known that V antigen expression by and growth restriction of *Y. pestis* are closely linked (9). Furthermore, all mutants in which V expression is weak fail to show a restrictive growth response, in contrast to mutants with constitutive V antigen expression at 37°C, which show a restrictive response in both the presence and absence of Ca^{2+} (19, 41, 55). At the same time, the V antigen is a secreted protein that is thought to have a direct role in the pathogenesis of *Y. pestis*. Its role is uncertain because no one has yet constructed a clean V^- mutant and because of the inability to completely purify this labile protein (9). It is possible that V antigen has regulatory functions both within *Y. pestis* and upon mammalian cells. It also is possible that the restrictive growth response is seen only in vitro, where under unnatural physiological conditions, strong expression (and secretion) of V antigen elicits a stress response. In this latter scenario, V antigen is predicted to be a virulence protein, and its role in eliciting restriction has been a serendipitous aid in unraveling the Lcr regulatory cascade. Further investigation is needed to assess the validity of these speculations. Further work will also determine the mechanism whereby the *lcrR* locus affects LcrG expression. This surprising finding revealed a new level of complexity in the low-Ca^{2+} response.

The *lcrR* sequence has been submitted to the GenBank data base with accession number M35740.

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