LcrD, a Membrane-Bound Regulator of the Yersinia pestis Low-Calcium Response

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Yersinia pestis, the etiologic agent of bubonic plague, contains a 75-kb virulence plasmid, called pCD1 in Y. pestis KIM. The low-Ca²⁺-response genes of Y. pestis regulate both bacterial growth and the expression of pCD1-encoded virulence determinants in response to temperature and the presence of Ca²⁺ or nucleotides. This study characterizes the nucleotide sequence and protein product of the lcrD locus. An lcrD mutant, in contrast to the parent Y. pestis, did not undergo growth restriction or induce strong expression of the V antigen when grown under conditions (37°C, no Ca²⁺) expected to elicit maximal expression of pCD1 genes. DNA sequence analysis of the cloned lcrD locus showed a single open reading frame that could encode a protein with a molecular weight of 77,804 and a pI of 4.88. LcrD was identified as a 70-kDa inner membrane protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis. LcrD membrane topology was investigated by using lcrD-phoA translational fusions generated with the transposon TnphoA. The alkaline phosphatase activities of the resultant hybrid proteins were consistent with a model predicting eight amino-terminal transmembrane segments that anchor a large cytoplasmic carboxy-terminal domain to the inner membrane.

Yersinia pestis, the etiologic agent of bubonic plague, exhibits a virulence property called the low-Ca²⁺ response (Lcr) (19). The Lcr is manifested in vitro by the requirement for millimolar quantities of Ca²⁺ for maximal growth yield at temperatures above 34°C (80) and by the temperature- and Ca²⁺-regulated expression of virulence-associated proteins. These proteins include the V antigen (12, 56) and a set of coordinately regulated surface proteins (Yops) (7, 52, 53, 65, 66). The Lcr does not occur at 26°C; however, when cultures of Y. pestis are shifted from 26 to 37°C in Ca²⁺-free media, a metabolic shutdown and cessation of growth occur within two generations, a phenomenon known as growth restriction (80). Interestingly, it is during growth restriction that the V antigen and Yops are maximally expressed (66). A number of specific nucleotides, such as ATP, can at least partially substitute for Ca²⁺ at 37°C, preventing growth restriction and downregulating expression of V antigen and Yops in Y. pestis (79). Nucleotides and Ca²⁺ are not transported into the yersiniae (47, 79); therefore, these compounds are thought to act at the cytoplasmic membrane as environmental regulatory signals.

The Lcr is encoded by a ca. 75-kb plasmid called pCD1 in Y. pestis KIM5 (2, 17, 19). This plasmid, which is essential for virulence, carries the regulatory genes for the Lcr and the genes that encode the virulence-associated effector proteins of the Lcr (the V antigen and Yops). Other human pathogenic yersiniae (Yersinia enterocolitica and Yersinia pseudotuberculosis) carry closely related Lcr plasmids that are also necessary for virulence (10).

The Yops are a diverse group of proteins with regard to molecular weight and isoelectric point (6, 37, 65, 66). Genes encoding these proteins are organized into at least five operons dispersed around pCD1 (37, 66). Eleven Yops have been described in Y. pestis (48, 65, 66). Several of these have been shown to be necessary for full virulence of Y. pestis in mice (30, 68). In addition, three of the Yops expressed by Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica have been characterized by function. YopE is antiphagocytic and is a cytotoxin (60); YopH is antiphagocytic and possesses protein tyrosine phosphatase activity (5, 20); and YopM inhibits thrombin-induced platelet aggregation in vitro (29, 30).

The V antigen is a 38-kDa protein that is protective in both active and passive immunization (28). It appears to be bifunctional, serving both as an anti-host factor and as a positive regulator of the Lcr (56, 57, 72, 73).

The regulatory genes for the Lcr consist of at least eight loci that are located in a ca. 20-kb "Ca²⁺ dependence" region on pCD1 (6, 15, 19, 52, 77, 78). The lcrF gene product mediates the thermal induction of pCD1 gene transcription (77). Other loci known to participate in the induction of transcription in the absence of Ca²⁺ include lcrB, lcrC, lcrD, and lcrV (which encodes the V antigen) (15, 19, 56). Y. pestis strains with an insertion mutation in one of these positive regulatory loci exhibit a Ca²⁺-independent phenotype. These mutants do not undergo growth restriction after a temperature shift from 26 to 37°C in the presence or absence of Ca²⁺. In addition, these mutants are unable to induce strong expression of V antigen and Yops.

The lcrE and lcrR loci function in the downregulation of transcription in response to Ca²⁺ (1, 78). Mutants obtained in these loci exhibit a restrictive growth response and maximal V antigen and Yop expression in both the presence and absence of Ca²⁺ at 37°C but are still able to respond normally to the presence of ATP (1, 78). This type of mutant is termed a Ca²⁺-blind mutant. lcrH is necessary for downregulation in response to both Ca²⁺ and ATP. An lcrH mutant of Y. pestis, in addition to being partially Ca²⁺ blind, is ATP blind, as the presence of ATP in the growth medium fails to relieve growth restriction or depress V antigen and Yops expression at 37°C (58).

In this paper we begin a detailed study of the Y. pestis lcrD locus with the long-term goal of determining its function in...
TABLE 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptiona</th>
<th>Reference or source</th>
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<tr>
<td>pCD1</td>
<td>Lcr plasmid of <em>Y. pestis</em> KIM</td>
<td>19</td>
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<td>Derivatives of pCD1</td>
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<td>pCD1::(Mu d11::Tn9)-304.7</td>
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<td>Ap' Km' pGP2::TnphoA</td>
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</table>

a See Fig. 1.

b Numbers indicate the amino acid residue of LcrD in which TnphoA inserted. Numbers preceded by a U indicate the number of base pairs upstream of the LcrD initiation codon where TnphoA inserted.

d The Lcr and in the pathogenesis of plague. We provide further characterization of an lcrD insertion mutant and analyses of the lcrD sequence and of the localization and membrane topology of LcrD.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. *Escherichia coli* K-12 strains HB101 (8), JM107 (76), and XL1-Blue [endA1 hsdR17 (rK- mK+) supE44 thi-1 lambda- recA1 gyrA96 lac [F'proAB lacZAM15 Tn10 (Tet')]] (Stratagene, La Jolla, Calif.) were used as hosts for the M13 vectors. XL1-Blue was also used as the host strain in the T7 polymerase expression system. JM107 was grown in M9 minimal medium (31) to select for F' cells and in 2× YT (34) containing tetracycline (25 μg/ml) both for selection of F' cells and for isolation of single-stranded DNA. All TnphoA fusion plasmids were isolated and maintained in *E. coli* CC118 [Δ[ara-leu]7697 araD139 ΔlacX74 galE galK ΔphoA20 thi rpsE rpoB argE[am] recA1 (32)].

Three strains of *Y. pestis*, which lack the pigmentation virulence determinant (Pgm−), rendering them avirulent except by intravenous injection (71), were used in this study. *Y. pestis* KIM5 contains pCD1 (Lcr+) and *Y. pestis* KIM6 lacks pCD1 (Lcr−), and *Y. pestis* KIM5-3042.7[pCD1 lcrD::Mu d11(Ap' lac::Tn9)] was obtained in a previous study (19, 26) and contains bacteriophage Mu d11(Ap' lac::Tn9) hereafter called Mu d11) inserted into lcrD of pCD1 (Lcr−).

*Y. pestis* strains were grown as previously described in heart infusion broth (Difco Laboratories, Detroit, Mich.) or in defined medium TMH (66). *E. coli* strains were grown in LB broth (LB) (16). Unless indicated otherwise, all bacteria carrying antibiotic resistance markers were grown in the presence of the appropriate antibiotic(s) at a concentration of 25 μg/ml (i.e., ampicillin, tetracycline, kanamycin, and chloramphenicol).

Bacteriophages M13mp18 and M13mp19 (34, 41, 76) were used as vectors for DNA sequencing. A derivative of bacteriophage M13, mGP1-2 (1), that contains the T7 RNA polymerase gene under the control of the lac promoter, was used for the exclusive expression of genes cloned downstream of the T7 promoter of pBluescript KS− (pKS−) (Stratagene, La Jolla, Calif.).

The recombinant plasmids used in this study are described in Table 1. Plasmid pGP2 was constructed by inserting the 4.2-kb *BamHI*-H fragment of pCD1 (19) into the *BamHI* site of the lcrR-containing plasmid pSB3-1 (1). This construct contains the entire lcrD locus and 3 kb of upstream flanking DNA. pGP2 and plasmids pGP2-1 and pGP2-2, which contain the 2.5-kb *ClaI* fragment and the 1.6-kb *HindIII* fragment of pGP2, respectively (Fig. 1), were used in T7 RNA polymerase expression studies.

Mapping of the lcrD− *Y. pestis* mutant. The location of the Mu d11 insertion in *Y. pestis* KIM5-3042.7 was confirmed and refined by Southern blot analysis. Plasmid pCD1 and the pCD1::Mu d11 cointegrate were digested with *HindIII*, resolved on a 0.7% (wt/vol) agarose gel, and probed with the nick-translated *HindIII* P fragment of pCD1.

DNA restriction analysis, cloning, and nucleotide sequencing. Restriction endonuclease analysis and cloning were carried out by standard methods (31). The 3.0-kb *PvuII*-BglII fragment of pCD1 that contains the 3′ end of lcrD and the 2.1-kb *HindIII*-I fragment of pCD1 that encodes the 5′ portion of lcrD were cloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain termination procedure (61) by using the Sequenase sequencing kit from U.S. Biochemical Corp. (Cleveland, Ohio) and [α-35S]dATP from NEN Research Products (Boston, Mass.).

DNA and predicted protein sequences were analyzed by using the PC Gene (IntelliGenetics, Inc., Mountain View, Calif.) computer program. The algorithm of Pearson and Lipman (46) was used to search the nucleic acid and protein sequence data bases for similarities to lcrD and LcrD, respectively. Full-length alignment with FlbF was done by the method of Myers and Miller (38). Potential transmembrane segments were evaluated by the method of Klein et al. (25).

Preparation of antibodies. Peptides corresponding to amino acids 4 to 17 (peptide A, NH2-HDLEWLNRIGERKD [C]-COOH) and 168 to 179 (peptide B, NH2-DYNEARRERR ATI[C]-COOH) of the predicted LcrD protein were synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington). An additional carboxyl-terminal cysteine residue ([C]) was added to each peptide to provide a site for hapten conjugation. The peptides were coupled to the protein carrier bovine serum albumin (BSA) by the addition of glutaraldehyde to 0.25% for 2 h at room temperature or by the Pierce Immon conjugation kit (Rockford, Ill.) by using sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC)-activated BSA. Excess glutaraldehyde was removed by dialysis against phosphate-buffered saline (PBS) containing 135 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 (pH 7.2). Sulfo-SMCC-
conjugated peptides were purified by gel filtration chromatography.

Two pairs of female New Zealand White rabbits were injected subcutaneously, along with the back, with 1 mg of BSA-peptide conjugate emulsified in Freund incomplete adjuvant. At 4 and 8 weeks, the rabbits were boosted each with 1 mg of the same BSA-peptide conjugate emulsified in Freund incomplete adjuvant. Rabbits were bled at 1-week intervals to obtain serum. Antipeptide antibodies were detected by an enzyme-linked immunosorbent assay (ELISA). Antibodies were purified from the serum on Sepharose 6B (Pharmacia-LKB, Piscataway, N.J.) peptide A or B affinity columns prepared by the manufacturer’s protocol. Antiserum (5 to 10 ml) possessing antipeptide antibody activity (as determined by ELISA) was applied to the affinity columns and washed with PBS. Antipeptide antibodies were eluted with 0.2 M glycine-HCl (pH 2.5). The eluents were neutralized with 1 M Tris-HCl (pH 8.0), dialyzed against PBS, and stored in the presence of 0.05% sodium azide at 4°C.

T7 promoter-polymerase expression system. E. coli XL-1 Blue was transformed with pGP2, pGP2-1, pGP2-2, and pKS+ (vector-only control). The clone-specific and control (pKS+) proteins were expressed by using bacteriophage mGP1-2 as previously described (1). Cells were grown at 37°C with gentle shaking (60 rpm) in M9 medium containing 100 µg of ampicillin per ml and supplemented with 20 µg of thiamine per ml and 19 amino acids at 0.01% (wt/vol) each, excluding methionine. When cells reached an A620 of approximately 0.25, they were infected with mGP1-2 at a multiplicity of infection of 10. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 2 mM 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 at 37°C. A 1-ml volume of cells was incubated for 10 min with 50 µCi of [35S]methionine (NEN Research Products). The cells were pelleted in a microfuge at 4°C and solubilized in 100 µl of electrophoresis sample buffer containing 1% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β-mercaptoethanol, 60 mM Tris-HCl (pH 6.8), and 25% (wt/vol) glycerol.

SDS-PAGE and Immunoblot analysis. Samples for SDS-PAGE were prepared from bacteria cultured as described above. Washed cells of E. coli or Y. pestis were lysed by two passages through a French press at 20,000 lb/in². Unlysed whole cells and large debris were removed by centrifugation at 12,200 × g for 10 min at 4°C. The cleared lysate was ultracentrifuged at 263,800 × g for 30 min in a Beckman TL-100 ultracentrifuge. The membrane pellet was resuspended in 10 mM Tris-HCl (pH 8.0), and the ultracentrifugation step was repeated. Separation of Y. pestis inner and outer membrane fractions was accomplished by a modification of the Osborn procedure as described previously (45, 67). Membrane fractions (3 µg of protein per lane) were separated by using Phastsystem 10 to 15% (wt/vol) gradient SDS-polyacrylamide gels (Pharmacia-LKB). Gels were equilibrated in transfer buffer (25 mM Tris–192 mM glycine [pH 8.3] containing 20% [vol/vol] methanol) and electrotransferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, Mass.) in a Bio-Rad Trans-Blot unit (Bio-Rad Laboratories, Richmond, Calif.) at 40 V for 30 min at 4°C. All subsequent procedures were carried out at room
temperature. The membranes were blocked with TBS (20 mM Tris-HCl [pH 7.4], 0.85% [wt/vol] NaCl) containing 5% (wt/vol) BSA for 1 h, and then incubated for 2 h with anti-peptide B antibodies diluted in TBS containing 1% (wt/vol) BSA and 0.05% (vol/vol) Tween 20. After several washes with 0.1% (wt/vol) BSA in TBS, the membranes were incubated for 2 h with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). Membranes were then washed three times with 0.1% (wt/vol) BSA in TBS, and alkaline phosphatase activity was visualized with the Protoblots Western blot kit (immunoblot) AP substrate system (Promega Corp., Madison, Wis.).

Two-dimensional gel electrophoresis. Comparison of two-dimensional protein profiles was carried out for Lcr+/Y. pestis KIM5 and lcrD Y. pestis KIM5-3042.7. Yersinia proteins were radiolabeled by growth of Y. pestis in the presence of 35S]methionine at 37°C in TMM without Ca2+ or ATP (49). 35S-labeled proteins were separated by nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and by SDS-PAGE in the second dimension as described previously (42, 43, 46, 56). The Visage 2000 image analyzer was utilized to quantitate and compare spots on the autoradiograms (Bio Image, Ann Arbor, Mich.).

Isolation of lcrD-phoA fusions. The phoA mutant E. coli strain, CC118, harboring the lcrD-expressing plasmid pGP2, was mutagenized with TnphoA by infection with a lysate of λ::TnphoA (21). Infected cultures were incubated for 2 h at 37°C and plated on LB medium containing 25 μg of ampicillin, 300 μg of kanamycin, and 40 μg of 5-bromo-4-chloro-3-indolyl-phosphate (XP), each per ml. Plasmid DNA was prepared from these colonies by the method of Birnboim and Doly (4) and used to transform CC118. Transformant colonies were selected on LB agar containing ampicillin (25 μg/ml), kanamycin (25 μg/ml), and XP (40 μg/ml). Alkaline phosphatase-positive transformants (blue colonies) were selected from these plates, and their plasmid DNA was purified. DNA from fusion plasmids was analyzed by restriction endonuclease mapping and then by DNA sequence analysis on the double-stranded template to identify fusion junction sites. The synthetic oligonucleotide primer (5’-GAGCGGCA GTCTGATCAC), kindly provided by David O. Wood, University of South Alabama, Mobile, hybridized to the 5’ end of the phoA gene and provided sequence readings of the lcrD-phoA fusion junctions.

Alkaline phosphatase assay. Plasmids with in-frame insertions in lcrD were electrophoretically into Y. pestis KIM5 (14, 50) for the determination of alkaline phosphatase activity. Cells were grown in heart infusion broth containing the appropriate antibiotics at 30°C. Enzyme activity was measured by determining the rate of p-nitrophenyl phosphate hydrolysis by permeabilized cells (36). A 200-μl volume of exponential-phase culture (A600 = 0.5 to 1.0) was mixed with 1.8 ml of 1 M Tris-HCl (pH 8.0), 50 μl of 0.1% SDS, and 50 μl of chloroform. Reactions were initiated by the addition of 0.4 ml of p-nitrophenyl phosphate and terminated by the addition of 1 ml of 1 M sodium carbonate. After centrifugation in a microfuge, the A405 values of the reaction supernatants were measured. Enzyme activities, reported as the change in the A405 min−1 ml−1 A600−1 of the culture, are the average values of duplicate samples.

Nucleotide sequence accession number. The DNA sequence reported here for lcrD is held in GenBank under accession number M77014.

RESULTS

Mapping of the insert in the lcrD mutant. The location of the Mu d1 insertion mutation (19) that initially defined the lcrD locus was mapped by Southern blot analysis to 0.25 kb upstream from the HindIII-G fragment in HindIII-P, at 42.7 kb on the pCD1 map (data not shown; Fig. 1). Analysis of the insert position in relation to sequence data (presented below) showed that the insert was located toward the 5’ end of lcrD. lcrD lies immediately upstream of lcrR and probably is part of the same transcriptional unit (1); accordingly, this mutant is expected to express an approximately 72,000-molecular-weight LcrD protein lacking ca. 5,000 molecular weight from the carboxyl terminus and to lack LcrR expression altogether because of the polarity of the Mu d11 insertion. Yersiniae carrying this insertion mutation in pCD1 are now referred to as Y. pestis KIM5-3042.7. This mutant exhibited a Ca2+-independent phenotype characterized by the lack of growth restriction and by the failure to induce strong V antigen expression at 37°C in the absence of Ca2+ (19).

NEPHGE analysis of Y. pestis KIM5-3042.7 and the parent, Y. pestis KIM5. Two-dimensional protein profiles of 35S-labeled proteins from Y. pestis KIM5-3042.7 and the parent, Y. pestis KIM5, grown under conditions expected to elicit maximal expression of pCD1 genes (37°C, no Ca2+) were compared to determine the effect of insertional inactivation of LcrD (and LcrR) on pCD1-encoded gene products (Fig. 2). No spot corresponding to the LcrD protein was detected in the parent or mutant profile, even when 80 μg of protein from the parent was subjected to immunoblot analysis after separation by NEPHGE (data not shown). However, we did detect LcrD in immunoblots after one-dimensional SDS-PAGE (see below). LcrD, like LcrR (1), evidently fails to enter one of the NEPHGE gel systems. The lcrDR mutant lacked expression of the LcrG protein of the V antigen operon. This could be the result of the predicted polar effect of the Mu d11 insert in lcrD on downstream lcrR: an lcrR mutant also fails to express LcrG (1). Expression of the Yops and V antigen was altered in the LcrD− mutant. The expression of the V antigen was dramatically reduced, and YopD expression was decreased. In contrast, the expression of YopH and YopM was enhanced in the mutant strain as compared with the parent strain.

DNA sequence analysis of lcrD. The coding sequence of lcrD was determined by dideoxy sequence analysis by using both strands of the PvuII-BglII and HindIII-I fragments of pCD1 (Fig. 3). Computer analysis showed a single large open reading frame (ORF) of 2,112 nucleotides. The sequence exhibited no significant homologies to nucleotide sequences in the EMBL and GenBank data bases. No sequences with a good fit to the E. coli promoter consensus (22, 54, 55) were detected within 130 bases upstream of the ORF, even though LcrD is expressed well enough in E. coli to allow screening for in-frame TnphoA insertions (see below). A potential rRNA binding sequence (Shine-Dalgarno [62] sequence) (GGAGTTT) was found 11 nucleotides upstream of the first ATG in the ORF. The termination codon (TGA) of the ORF overlapped with the initiation codon (ATG) of the lcrR gene, as noted previously (1). Computer analysis predicted that the ORF could encode a 704-amino-acid protein having a molecular weight of 77,804 and an isoelectric point (pI) of 4.88. Hydrophathy analysis of the amino acid (aa) sequence (Fig. 4) predicted LcrD to be an integral membrane protein possessing a hydrophobic amino terminus (amino acid [aa] 1 to 347) and a hydrophilic carboxyl terminus (aa 348 to 704). Further
analysis by use of an algorithm developed by Klein et al. (25) detected eight possible membrane-spanning segments in the LcrD protein, all located in the hydrophobic amino-terminal region.

**Sequence homology analysis.** No significant amino acid sequence homologies were found between LcrD and the sequences in the Swiss-Prot or National Biomedical Research Foundation Protein Identification Resource databases. Identity of 100% was found between the amino-terminal 159 aa of LcrD and the reported 159 aa of ORF5 of the pYV plasmid of *Y. enterocolitica* O:3 (74) (partial sequence of the *Y. enterocolitica* analog which maps at the same locus as lcrD of *Y. pestis*). The 159 aa of *Y. enterocolitica* O:3 ORF5 were found by Austin Newton and associates to have homology with FlbF of *Caulobacter crescentus* (40, 59). Newton’s group kindly made their sequence available to our laboratory, and it is presented in comparison with LcrD in Fig. 5. Over the full-length alignment, 32% of the amino acids were identical and a total of 51% were identical or conservative replacements. The amino-terminal 260 aa of LcrD showed the greatest homology with FlbF, exhibiting 49% identity and an additional 26% similarity.

**Identification of LcrD by immunoblot analysis with antipeptide antibodies.** Peptides corresponding to aa 4 to 17 (peptide A) and 168 to 179 (peptide B) of the predicted LcrD protein were synthesized and used as haptons to raise site-directed polyclonal antibodies in rabbits. These peptides were selected as regions of LcrD which were likely to be exposed to the aqueous environment as predicted by the method of Hopp and Woods (23). Antipeptide antibodies were used for Western blot analysis of SDS-PAGE-separated membrane proteins from *Y. pestis* and *E. coli* clones to determine if the antibody preparations recognized the intact LcrD protein expressed from the endogenous promoter for lcrD (Fig. 6). XL1-Blue/pKS+ (Fig. 6, lane 1) lacked a *Y. pestis* insert and therefore did not express LcrD. XL1-Blue/pGP2 (Fig. 6, lane 2) carried a 5.6-kb *Y. pestis* insert containing the entire lcrD gene and expressed a unique 70-kDa protein recognized by both antipeptide antibody preparations (anti-peptide B used for Fig. 6). CC118/pGP2-phoA320 (Fig. 6, lane 3) contained an in-frame *lcrD-phoA* gene fusion and expressed an LcrD-PhoA hybrid protein of ca. 80,000 molecular weight (predicted molecular weight, ca. 78,500). LcrD and the LcrD-PhoA hybrid protein were not detected when samples were heated for 3 min at 100°C before SDS-PAGE and immunoblot analysis (Fig. 6, lanes 4 to 6). *Y. pestis* KIM5 (Fig. 6, lane 8) expressed an immunoreactive protein of the same apparent molecular weight as observed with the LcrD-expressing XL-1 Blue/pGP2. The LcrD mutant *Y. pestis* KIM5-3042.7 (Fig. 6, lane 7) expressed a truncated version of this protein, as expected. LcrD was localized to the inner membrane of *Y. pestis* KIM5 (Fig. 6, lane 11). Small amounts of LcrD were also detected in the outer membrane fraction of *Y. pestis* KIM5 (Fig. 6, lane 12) and probably represent inner membrane contamination of the outer membrane fraction analyzed. No corresponding protein was detected in the inner or outer membrane fractions of the pCD1* Y. pestis* KIM6 (Fig. 6, lanes 9 and 10).

**Isolation of LcrD-alkaline phosphatase (lcrD-phoA) gene fusions.** The transmembrane topology of LcrD was analyzed.
FIG. 3. Nucleotide sequence of lcrD. The sequence of the coding strand is shown. The amino acid residues corresponding to synthetic peptides A and B are shown in boldface type. A short ORF oriented in the opposite direction of lcrD transcription is underlined. The initiation codon of the ORF encoding LcrR is labeled as lcrR START. This sequence has been assigned GenBank accession number M77014.

by using a set of 12 LcrD-alkaline phosphatase (lcrD-phoA) gene fusions. Fusions of the phoA gene in frame with the coding region for a periplasmic-facing segment of a cytoplasmic membrane protein allow export of the PhoA portion of the hybrid protein, resulting in high phosphatase activity which can be detected on XP indicator agar plates by the blue color of the colonies (33). Fusions of phoA to segments of the target protein normally located on the cytoplasmic side of the membrane exhibit low enzymatic activity (9, 33). Transcription of TnphoA into plasmid pGP2 gave rise to a range of pale to dark blue colonies on XP indicator agar. Insertions in the lcrD gene were located by restriction endonuclease mapping with BglII, ClaI, and KpnI. The location of the gene fusion junction was determined by dideoxy sequence analysis by utilizing a primer that hybridized to the 5' end of the phoA gene. Plasmids containing in-frame lcrD-phoA gene fusions were transformed into Y. pestis KIM5.

Enzymatic activities of LcrD-alkaline phosphatase hybrid proteins. The alkaline phosphatase activities of Y. pestis KIM5 expressing the various lcrD-phoA fusions (Fig. 7) corresponded with the intensity of blue color found with these strains and with the corresponding E. coli clones on XP indicator plates. Y. pestis KIM5 had no detectable background level of alkaline phosphatase activity. Fusions of alkaline phosphatase to LcrD at aa 40, 219, 223, 236, 240,
Values indicate clusters of noncharged polar and terminal transmembrane helices model of transmembrane topology

IcrD-phoA (13 U) of alkaline phosphatase activity. The additional TnphoA protein.

upstream of the upstream of the tion of the

regulatory regions required for efficient (Fig. 1). The pattern of activities correlates well with the model of transmembrane topology predicting eight aminoterminal transmembrane helices and a large carboxyl-terminal cytoplasmic domain. The distribution of charged residues relative to potential membrane-spanning segments agrees with the rule for preferential location of positively charged amino acids on the inside of the membrane and clusters of noncharged polar and negatively charged residues on the outside or periplasmic side of the membrane (75). The proposed topology places both the amino and the carboxyl termini on the cytoplasmic side of the membrane.

Analysis of TnphoA insertions upstream of the lcrD ORF. Additional TnphoA insertion sites in pG2 were identified upstream of the LcrD ORF, oriented opposite to the direction of lcrD transcription and located 26, 41, 65, and 101 bp upstream of the lcrD initiation codon. The phoA portions of these inserts were in frame with the coding region of an ORF found on the opposite strand from the lcrD ORF. This ORF initiates 149 bp within the lcrD ORF and extends 103 bp upstream of the lcrD initiation codon (Fig. 3). It was found to have the coding potential for a 9,116-molecular-weight protein. Examination of the sequences upstream of this ORF did not reveal any E. coli-like promoter sequences or ribosome-binding sites. In addition, analysis in E. coli of plasmids pG2-1 and pG2-2, which contain this ORF downstream of the pKs" T7 promoter, utilizing the bacteriophage T7 RNA polymerase expression system (69, 70), revealed no corresponding protein (data not shown). The significance of this ORF in Y. pestis, if any, is currently unknown.

The expression of LcrD from pG2 containing TnphoA insertions upstream of the lcrD ORF was examined by SDS-PAGE and immunoblot analysis to delineate upstream regulatory regions required for efficient expression of LcrD (Fig. 8). Insertions located 26, 41, and 65 bp upstream of the lcrD ORF eliminated the overexpression of LcrD observed with pG2 in Y. pestis KIM5 (the residual expression

presumably is due to the intact lcrD promoter on pCD1). The TnphoA insertion located 101 bp upstream of the lcrD ORF had no effect on the overexpression of LcrD. These data indicate that nucleotide sequences required for efficient expression of LcrD in Y. pestis KIM5 are located within 101 bp of the lcrD initiation codon.

**DISCUSSION**

This study initiated the characterization of the lcrD locus at the molecular level and investigated its role in the Lcr of Y. pestis. The location of lcrD was determined by mapping of the Mu d11 insert that defined the lcrd locus (19) and from DNA sequence analysis. These data showed that lcrD was located immediately upstream of lcrR and, in fact, that the translational stop of lcrD and the translational start of lcrR overlap.

The Mu d11 insertion mutant of lcrD, which is probably also defective for lcrR expression because of transcriptional polarity, exhibits the Ca++-independent growth phenotype (19). In contrast, Y. pestis defective only in lcrR expression (1) shows the Ca++-blind phenotype and constitutive expression of the V antigen and Yops at 37°C. Therefore, lcrD is hypothesized to function in the induction of the virulence operons (19), while lcrR functions in their repression when Ca++ is present at 37°C (1). These distinct phenotypes support the assumption that the Ca++-independent pheno-
Immunoblot analysis for the detection of LcrD. Samples were solubilized at room temperature (lanes 1 to 3 and 7 to 12) or at 100°C for 3 min (lanes 4 to 6). The position of LcrD is indicated by the arrow. Lanes: 1 and 4; E. coli XL1-Blue/pKS⁻ (vector only); 2 and 5, E. coli XL1-Blue/pGP2; 3 and 6, E. coli CC118/pGP2-pho320; 7, Y. pestis KIM5-3042.7; 8, Y. pestis KIM5; 9, Y. pestis KIM6 (inner membrane fraction); 10, Y. pestis KIM6 (outer membrane fraction); 11, Y. pestis KIM5 (inner membrane fraction); 12, Y. pestis KIM5 (outer membrane fraction). LcrD is visualized in lanes 2, 8, 11, and 12. An LcrD-PhoA hybrid protein is visible in lane 3. A truncated version of LcrD is visible in lane 7. The reactive bands migrating below LcrD in lane 8 and the corresponding band in lane 7 represent proteins that cross-react with the anti-peptide B antibodies.

Type of the Mu d11 insertion in lcrD is due to the disruption of lcrD and not to polar effects on lcrR.

Analysis of proteins from mutant (Y. pestis KIM5-3042.7) and parent (Y. pestis KIM5) strains grown under restrictive conditions (37°C, no Ca²⁺) showed an overall downregulation in V antigen and YopD expression in the lcrDR mutant (Fig. 2). The expression of Yops H and M appeared to be enhanced in the mutant. In Y. enterocolitica and Y. pseudotuberculosis, the genes encoding Yops B and D were reported to be part of the same transcriptional unit as lcrGVH (3, 37). This same operon structure probably also exists in Y. pestis, accounting for the concurrent downregulation of V

FIG. 6. Immunoblot analysis for the detection of LcrD. Samples were solubilized at room temperature (lanes 1 to 3 and 7 to 12) or at 100°C for 3 min (lanes 4 to 6). The position of LcrD is indicated by the arrow. Lanes: 1 and 4; E. coli XL1-Blue/pKS⁻ (vector only); 2 and 5, E. coli XL1-Blue/pGP2; 3 and 6, E. coli CC118/pGP2-pho320; 7, Y. pestis KIM5-3042.7; 8, Y. pestis KIM5; 9, Y. pestis KIM6 (inner membrane fraction); 10, Y. pestis KIM6 (outer membrane fraction); 11, Y. pestis KIM5 (inner membrane fraction); 12, Y. pestis KIM5 (outer membrane fraction). LcrD is visualized in lanes 2, 8, 11, and 12. An LcrD-PhoA hybrid protein is visible in lane 3. A truncated version of LcrD is visible in lane 7. The reactive bands migrating below LcrD in lane 8 and the corresponding band in lane 7 represent proteins that cross-react with the anti-peptide B antibodies.

FIG. 7. Schematic two-dimensional model of LcrD and location of lcrD-phoA fusions. The eight predicted membrane-spanning segments are represented by rectangles. Numbers within the rectangles indicate the amino acid residues at each end. Solid circles correspond to fusions with high alkaline phosphatase activity (>120 U); open circles correspond to low-activity fusions (<13 U). Numbers above or below each circle indicate the amino acid residue in which the fusion joint is located.
antigen and YopD in Y. pestis KIM5-3042.7. The differential expression observed at the protein level between the V antigen and YopD and between YopH and YopM indicates that there are differences in the posttranscriptional regulation of the lcrGVHyopBD and the yopH and yopM gene products. In contrast, at the transcriptional level, lcrGVHyopBD and the yop operons appear to be regulated in the same manner (66). A similar phenomenon has recently been reported for Y. enterocolitica W22703 carrying a mutation in the virA locus. Western blot analysis of YopE, YopB, and YopD in the virA mutant revealed high levels of YopE and extremely low levels of YopB and YopD (35).

Yops accumulate in the outer membranes of Y. enterocolitica and Y. pseudotuberculosis when grown at 37°C in the absence of Ca²⁺ but not in Y. pestis because of their degradation via a proteolytic enzyme present only in the outer membrane of Y. pestis (63). However, when Y. pseudotuberculosis is used as a recipient for pCD1, the Y. pestis pCD1-encoded Yops can be observed in the outer membranes. Interestingly, when pCD1::Mu d11-44.5, which contains an insert in lcrD, was moved into an Lcr⁻ Y. pseudotuberculosis recipient strain, no Yops were found in the outer membrane (48), indicating that LcrD may be involved as a regulator or a direct participant in the secretion or exportation of Yops to the outer membrane. This could explain why Yops H and M were not seen in the outer membranes of Y. pseudotuberculosis containing pCD1::Mu d11-44.5 even though the two-dimensional gel analysis of Y. pestis KIM5-3042.7 whole cells indicated that they were being overexpressed. A secretion defect could account for the increased levels of YopH and YopM found in the two-dimensional protein profiles of Y. pestis KIM-3042.7 but cannot account for the reduced levels of V antigen and YopD.

DNA sequence analysis of the cloned lcrD gene revealed a single large ORF predicted to code for a protein with a molecular mass of 77,804 Da and a pI of 4.88. A 70-kDa insert-specific protein was identified by Western blot analysis by utilizing antibodies raised against synthetic peptides corresponding to specific regions of the deduced LcrD amino acid sequence. Insertional mutagenesis of the cloned lcrD gene with the transposon TnphoA eliminated the 70-kDa protein, confirming that it was the lcrD gene product. The resultant LcrD-PhoA fusion product was identified by Western blot analysis. However, neither LcrD nor the LcrD-PhoA fusion protein could be detected when solubilization was performed at 100°C. This presumably was due to aggregation of the proteins, which prevented them from entering the polyacrylamide gel. Indeed, a large aggregate was visualized at the top of the gel under these same conditions in samples obtained from a T7-RNA polymerase expression system (data not shown). The irreversible aggregation of hydrophobic membrane proteins after boiling in SDS sample buffer has been reported previously. For example, the E. coli lactose permease (11), the E. coli SecY protein (24), and the Rickettsia prowazekii ATP/ADP translocase (51) all aggregate upon heating at 100°C in SDS sample buffer.

LcrD was localized to the inner membrane fraction of Y. pestis KIM5 by immunoblot analysis of purified inner and outer membrane fractions. Hydropathy analysis of the predicted amino acid sequence of LcrD revealed two distinctive domains. The amino-terminal half was very hydrophobic, possessing eight potential transmembrane domains; in contrast, the carboxyl-terminal portion was quite hydrophilic. Transcriptional control systems which respond to extracellular environmental stimuli often require transmembrane signaling to stimulate transcription of target genes (64). In the case of Y. pestis, data to date have indicated that Ca²⁺ and nucleotides are the major chemical components that are sensed by the yersinia to regulate the Lcr. These observations suggest that Y. pestis expresses regulatory systems capable of responding to these signals. Evidence that LcrD is an integral membrane protein introduces the possibility that it is involved in sensing and/or transmembrane signaling of the environmental cues of Ca²⁺ and/or nucleotides. Previous studies on the transcriptional regulation of lcrDR are consistent with the idea that lcrDR is primarily regulated by temperature and not by Ca²⁺ or nucleotides. In light of these findings, the topological orientation of LcrD was of interest.

We proposed a topological model for LcrD based on the hydrophilic profile and the distribution of charged amino acids on the polypeptide chain. TnphoA mutagenesis was utilized to test the proposed topological model. The activities of the various LcrD-PhoA fusion proteins support the proposed model (Fig. 7). PhoA fusions expressing high enzymatic activity were obtained in three of the four periplasmic loops predicted by the structural model. Hybrid proteins with low activity were obtained in two of the three predicted cytoplasmic loops. The two LcrD-PhoA fusions (pGP2-pho249 and pGP2-pho297) with junctions in putative membrane-spanning segments lie near enough to the periplasmic space to have significant enzymatic activity (13). No lcrD-phaa fusions with detectable alkaline phosphatase activity were obtained in either the amino- or carboxyl-terminal segments. The model predicts a cytoplasmic location for both the amino- and carboxyl-terminal regions.

The recent sequencing and analysis of the lbf gene of C. crescentus by Austin Newton’s laboratory at Princeton University, Princeton, N.J. (40, 59) revealed significant amino acid homology between the predicted lbf gene product and that predicted for LcrD of Y. pestis (Fig. 5). The amino-terminal regions demonstrated the greatest homology, showing 49% identity with an additional 26% similarity between the two predicted sequences. Five smaller (16 to 23
aa) regions of homology exhibiting from 47 to 56% identity were found spread over the remainder of the predicted amino acid sequences. The flbF gene product of C. crescentus is required for the cell cycle-specific expression of flagellar gene products (39). The regulation of gene expression in both systems involves a complex cascade of regulatory proteins that function together to coordinate the expression of numerous target genes (44). Recent communication with C. Ginocchio and J. Galan (18) has revealed an additional protein which shares homology with LcrD and FlbF. The predicted amino acid sequence of the invA gene product of Salmonella typhimurium (18) demonstrated significant homology with LcrD and FlbF. The identification of homologous regulatory genes from such diverse systems may indicate that lcrD and flbF are members of a new regulatory gene family that has yet to be investigated.

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