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Isolation and Characterization of Ca^{2+}-Blind Mutants of *Yersinia pestis*

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The plasmid pCD1 is required for expression of the low-calcium response (LCR), virulence, and production of V antigen in *Yersinia pestis* KIM. Five independent mutants constitutive for the LCR at 37°C (Lcr^c) were obtained through ethyl methanesulfonate mutagenesis followed by ampicillin enrichment. A sixth, spontaneous mutant was obtained directly through ampicillin enrichment. These mutants failed to grow at 37°C regardless of calcium concentration and produced V antigen constitutively at this temperature. All six mutations were located on pCD1. One mutation was mapped to a 1-kilobase region of *lcrA*. Based on complementation mapping of this mutation, the *lcrA* locus was divided into two new loci, *lcrD* and *lcrE*. This mutation, *lcrE1*, did not alter the transcription of other genes in the LCR region and was *cis*-recessive to *lcr* mutations. Several lower-molecular-weight outer membrane proteins which were observed in the parent strain grown at 37°C in the presence of 2.5 mM calcium were reduced in quantity or absent from the mutant strain.

When cultured at 37°C in the absence of Ca^{2+}, virulent strains of *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* display an unusual phenotype which we refer to as the low-calcium response (LCR) (6, 8–10, 20, 24). This response is characterized by cessation of growth within two generations after a shift from 26 to 37°C in Ca^{2+}-free medium and by the coordinate expression of the virulence-associated antigens V and W. The effect is enhanced by 20 mM $MgCl_2$. It has been suggested that these conditions, which simulate the mammalian intracellular environment with respect to Ca^{2+} and Mg^{2+} concentrations, provide signals necessary for adaptation to intracellular survival (7). The LCR can be repressed by the addition of 2.5 mM Ca^{2+} and is not observed at 26°C regardless of Ca^{2+} concentration.

In all three *Yersinia* species, the LCR is encoded by one of a family of closely related plasmids that are also required for expression of virulence (1, 16–18). Recently, we described Mu dl(Ap *lac*) insertions clustered within a 17-kilobase (kb) region of the 75.4-kb plasmid pCD1 of *Y. pestis* KIM5 (19). These mutations eliminated expression of the LCR and simultaneously reduced virulence of the strains which harbored them. Similar results with other *Y. pestis* strains have been reported by Portnoy et al. (31, 32). Based on the analysis of a large number of mutants, these results indicate that in *Y. pestis*, genes in common are required for expression of both the LCR and virulence. (Recently, Wolf-Watz et al. [39] isolated a single *Y. pseudotuberculosis* mutant which did not exhibit Ca^{2+}-dependent growth at 37°C but which remained virulent. Thus, this aspect of the LCR may not be an absolute requirement for the expression of virulence in all yersiniae species.) We have also demonstrated that in *Y. pestis* (i) the LCR region of pCD1 contains at least three genetic loci, *lcrA*, *lcrB*, and *lcrC*, as determined by directions and levels of transcription; (ii) transcription of *lcrB* and *lcrC* is regulated by temperature, increasing 4- and 11-fold respectively, after a shift from 26 to 37°C; and (iii) transcription is not regulated by Ca^{2+} within the genes affected by these Mu dl insertions (19).

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The Lcr$^-$ insertion mutants isolated in our initial study did not allow us to assign specific functions to any of the *lcr* loci since the Lcr phenotypes of all mutants were similar. In an effort to identify loci specifically involved in regulation by Ca^{2+}, we isolated a second class of mutants in which the response to Ca^{2+} is altered rather than abolished. These mutants, which we refer to as LCR constitutive (Lcr^c), exhibit the LCR at 37°C regardless of Ca^{2+} concentration, i.e., they are Ca^{2+} blind. Analysis of these mutants allowed us to describe further the regulation of the LCR and to define in greater detail the genetic loci which comprise the LCR region of pCD1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. Bacterial strains, plasmids, and bacteriophage are listed in Tables 1 and 2.

Media, growth conditions, and buffers. The defined medium (DM) is that of Zahorchak and Brubaker (40) supplemented with 20 mM $MgCl_2$ and, where indicated, 2.5 mM $CaCl_2$. Conditions for determination of the Lcr phenotype in DM have been described previously (19). Tryptose blood agar base (TB; Difco Laboratories, Detroit, Mich.) supplemented with 2.5 mM $CaCl_2$ was used for growth of *Y. pestis* on solid medium and was used to prepare the Ca^{2+}-deficient magnesium oxalate medium of Higuchi and Smith (21). Lcr$^+$ strains are unable to grow on this medium at 37°C. Determination of the Lcr phenotype on solid medium was done either by serial dilution and plating for quantitation or by patching of isolated colonies for screening. Heart infusion broth (HIB; Difco) was used for liquid culture of *Y. pestis*. *Escherichia coli* strains were cultured in Luria broth or on LB agar (28). Ampicillin, tetracycline, kanamycin, and chloramphenicol were used at concentrations of 50, 25, 25, and 25 μg/ml, respectively (Sigma Chemical Co., St. Louis, Mo.). P buffer is 0.033 M sodium phosphate (pH 7.0).

Plasmid DNA manipulations. Plasmid sizes were screened by the method of Kado and Liu (22). Isolation of plasmid DNA was by the method of Birnboim and Doly (2) followed by centrifugation in cesium chloride-ethidium bromide equilibrium gradients (34) when necessary. Transformation was

TABLE 1. Bacterial strains

Strain[a]	Relevant genotype and properties	Source or reference
Y. pestis		
KIM5	pCD1$^+$ Lcr$^+$	R. Brubaker
KIM6	pCD1$^-$ Lcr$^-$	Spontaneous pCD1 segregant of KIM5
KIM7	pCD1$^-$ pGW600$^+$ Lcr$^-$ Tc^r	19
UTP1000	pUT1000$^+$ Lcr$^+$ Km^r	Mutagenesis of KIM5 with Mu pf7701
UTP1001	pUT1001$^+$ Lcr^c Km^r	EMS mutagenesis of UTP1000
UTP1002	pUT1002$^+$ Lcr^c Km^r	EMS mutagenesis of UTP1000
UTP1003	pUT1003$^+$ Lcr^c Km^r	EMS mutagenesis of UTP1000
UTP1004	pUT1004$^+$ Lcr^c Km^r	EMS mutagenesis of UTP1000
UTP1005	pUT1005$^+$ Lcr^c Km^r	EMS mutagenesis of UTP1000
UTP1006	pUT1006$^+$ Lcr^c Km^r	Spontaneous mutant of UTP1000
UTP1007	pUT1007$^+$ Lcr^c	pCD1-pUT1001 recombinant
UTP1604	pUT1008$^+$ Lcr^c Km^r	Tn*5* mutagenesis of UTE1018, transduction of KIM6
E. coli		
χ1553	K-12 F$^-$ *minA1 purE42 supE42 pdxC3 minB2 his-53 nalA28 metC65* T3^r *ilv-277 cycB2 cycA1 hsdR2*	R. Goldschmidt
LE392	K-12 F$^-$ *hsdR514 supE44 supF58* Δ *(lacIZY)6 galK2 galT22 metB1 trpR55* λ$^-$	37
UTE1017	pUT1001$^+$ Kan^r	χ1553 transformant
UTE1018	pACYC184$^+$ pUT1007$^+$ Cm^r Tet^r	LE392 transformant

[a] UTP indicates a *Y. pestis* strain and UTE indicates an *E. coli* strain.

by the $CaCl_2$ method of Dagert and Ehrlich (13). Transduction techniques for introducing large plasmids into *Y. pestis* with bacteriophage P1 have been described previously (19, 41). Plasmids lacking antibiotic resistance markers were introduced into *E. coli* LE392 by cotransformation with either pBR322 or pACYC184. Isolates were screened for the presence of the unmarked plasmid by the Kado and Liu method. When necessary, strains were cured of pBR322 or pACYC184 by fusaric acid selection (3). Restriction enzyme digestions, cloning, and agarose gel electrophoresis were performed essentially as described previously (27).

Isolation of Lcr^c mutants. (i) EMS mutagenesis. Isolated colonies of UTP1000 were inoculated into HIB-kanamycin for five independent mutagenesis experiments. Overnight cultures were diluted 100-fold in HIB-kanamycin and grown to a density of approximately 2×10^8 cells per ml at 30°C. To 1 ml, either 30 or 0 μl of ethyl methanesulfonate (EMS; Sigma) was added. The cultures were shaken at 250 rpm for 2 h at 30°C, centrifuged, washed two times in P buffer plus 6% sodium thiosulfate to inactivate EMS (26), and resuspended in 2 ml of HIB-kanamycin. Samples were diluted and plated on TB-kanamycin and grown at 30°C to determine percent survival (approximately 8% for each mutagenesis). The remainder was grown overnight at 30°C, diluted 100-fold in HIB-kanamycin, grown to full density, and stocked (40% glycerol, −20°C).

(ii) Ampicillin enrichment. Overnight cultures grown in DM-kanamycin at 26°C were diluted to an optical density at 620 nm (model 240 spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) of 0.1 in DM-kanamycin plus 2.5 mM $CaCl_2$, grown to an optical density at 620 nm of 0.25 at 26°C, and then shifted to 37°C. After 3 h, ampicillin was added to a final concentration of 50 μg/ml. Four hours later, cells were harvested, washed twice in P buffer, and suspended in DM-kanamycin at 1/4th the original volume. Samples were diluted and plated on TB-kanamycin to determine percent survival, and the remainder was grown overnight at 26°C. The overnight growths were used to inoculate DM-kanamycin cultures for a second round of enrichment identical to the first. After two cycles of enrichment, survivors were present at a frequency of 2.7×10^{-5} among EMS-treated cultures and 6.7×10^{-7} for an unmutagenized culture.

(iii) Screening for Lcr^c phenotype. After the second ampicillin enrichment, cells were plated on TB-kanamycin and grown at 30°C. One hundred colonies were patched to duplicate TB-kanamycin plates and grown at 30 and 37°C. Presumptive Lcr^c mutants, which grew poorly at 37°C, were purified, and the phenotype was confirmed by growth in DM-kanamycin and plating efficiencies on TB-kanamycin and magnesium oxalate at 30 and 37°C. Approximately 45% of the isolates recovered from EMS-treated cultures and 4% of the isolates from the unmutagenized culture were Lcr^c.

Mutagenesis of pCD1 derivatives with Mu d1(Ap *lac*), Mu pf7701, and Tn*5*. Techniques for the preparation of Mu lysates and infection of recipient bacteria have been described previously (19). pCD1 derivatives lacking antibiotic resistance markers were mutagenized with Mu d1(Ap *lac*) in *E. coli* LE392 and transduced to *Y. pestis* KIM7. Using this technique, greater than 80% of the ampicillin-resistant isolates carried pCD1::Mu d1 cointegrates. UTP1000 was derived by mutagenesis of KIM5 with Mu pf7701 followed by screening for isolates which gave rise to pCD1 segregants at a reduced frequency on magnesium oxalate-kanamycin at 37°C. Such growth is expected of Lcr$^+$ strains carrying Mu pf7701 in pCD1 since loss of the plasmid results in killing by kanamycin. For mutagenesis of pUT1007 with Tn*5*, a P1 lysate grown on a pool of LE392 derivatives containing

TABLE 2. Plasmids and bacteriophage

Plasmids and phage	Description	Reference[a]
Plasmids		
pBR322	Ap^r Tc^r	4
pACYC184	Cm^r Tc^r	12
pGW600	Tc^r; encodes high levels of Mu repressor protein	23
pCD1	Native plasmid; Lcr^+	19
pUT1000	pCD1::Mu pf7701; Mu located between 29 and 32 kb; Lcr^+ Km^r	
pUT1001	*lcrE1* mutant of pUT1000; Lcr^c Km^r	
pUT1002	Lcr^c mutant of pUT1000; Km^r	
pUT1003	Lcr^c mutant of pUT1000; Km^r	
pUT1004	Lcr^c mutant of pUT1000; Km^r	
pUT1005	Lcr^c mutant of pUT1000; Km^r	
pUT1006	Lcr^c mutant of pUT1000; Km^r	
pUT1007	pUT1001 lacking Mu pf7701; *lcrE1*; Lcr^c	
pUT1008	pUT1007::Tn*5*; Tn*5* located between 32 and 38 kb; *lcrE1*; Lcr^c Km^r	
pUT1009	pCD1::Mu dl(Ap *lac*); Mu located between 2 and 13 kb; Lcr^+ Ap^r	
pUT1010	pUT1009 deleted for *Xba*I fragment B Δ(25–52 kb); Lcr^- Ap^r	
pUT1011	pUT1009 deleted for *Xba*I fragment C Δ(52–71 kb); Lcr^- Ap^r	
pUT1101	pBR222 carrying *Bam*HI fragment H of pCD1	
pUT1102	pBR322 carrying *Hin*dIII fragment I of pCD1	
pUT1103	pBR322 carrying *Hin*dIII fragment N of pCD1	
pUT1104	pBR322 carrying *Hin*dIII fragment J of pCD1	
Mu dl(Ap *lac*)	Mu *c*ts62 d(Ap^r trp'B^+A'-Δ W209-*lacZYA*)	11
Mu pf7701	Mu *c*ts62::Tn*5* (Km^r) (Δ*IS50* right) Δ(445-3)	M. Howe

[a] Except where noted, plasmids were constructed for this study.

random Tn*5* insertions was used to transduce UTE1018 to kanamycin resistance. Due to the instability of Tn*5* insertions during transduction, many of the Kan^r recipients result from transposition of Tn*5* to pUT1007 rather than from integration of transduced chromosomal fragments. A second P1 lysate grown on the pool of resistant isolates was used to introduce pUT1007::Tn*5* derivatives into KIM6. Isolated colonies were screened for the Lcr^c phenotype conferred by pUT1007.

Recombination between plasmids in vivo. To transfer the *lcrE1* mutation carried by pUT1001 to an unmarked pCD1, pUT1001 was transduced into KIM5, and kanamycin-resistant isolates were selected. After initial selection, colonies were screened for the Lcr^c phenotype, and plasmid content was determined. Isolates that were both Lcr^c and $pUT1001^-$, resulting from recombination to yield the unmarked mutant plasmid and loss of pUT1001 due to incompatibility with pCD1, were obtained at a frequency of approximately 0.8%. For mapping the *lcrE1* mutation with deleted derivatives of pCD1, UTP1604 was transduced with either pUT1010 or pUT1011, and isolates were selected at 30°C that were both kanamycin and ampicillin resistant. Isolated colonies were screened for reversion to wild type on TB at 30 and 37°C and on magnesium oxalate at 37°C under antibiotic selection for pUT1008 only. Strains harboring only pUT1010 or pUT1011 were recovered owing to loss of pUT1008 by incompatibility and transposition of Tn*5* during the initial selection period. To map the *lcrE1* mutation with cloned fragments of pCD1, UTE1017 was transformed with the desired clone, and a P1 lysate was prepared on the resulting strain. This lysate was used to transduce KIM7. Kanamycin-resistant isolates were selected at 30°C and screened for the Lcr phenotype as described above. The Lcr^- isolates obtained by this method were $pUT1001^-$ or harbored a deleted pUT1001.

Protein analysis. Cultures (500 ml) were grown at 26 and 37°C in HIB or HIB-magnesium oxalate in the manner described for testing Lcr phenotypes in DM (19). Cells were fractionated by a procedure similar to that described by Schnaitman (35). Briefly, cultures were centrifuged, washed in 0.01 M Tris hydrochloride (pH 8.0), suspended in 0.01 volume of 0.01 M Tris hydrochloride (pH 8.0)–2.0 mM EDTA, and passed through a French pressure cell four times at 10,000 to 12,000 lb/in^2. Unbroken cells were removed by centrifugation at 3,000 × *g* for 5 min, and samples were layered on a 30 to 65% sucrose discontinuous gradient. After centrifugation at 186,000 × *g* for 17 h at 4°C, 25 fractions of 0.5 ml each were collected. Protein concentrations of each fraction were determined as described by Bradford (5). Inner and outer membrane fractions were confirmed by assaying for $NADH_2$ oxidase (29) and 2-ketodeoxyoctanoic acid (30, 38), respectively. Samples containing approximately 15 μg of protein were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gels as described by Laemmli (25). Lower-molecular-weight proteins were resolved by electrophoresis through 15% polyacrylamide-sodium dodecyl sulfate-urea gels as recommended by Bethesda Research Laboratories (Gaithersburg, Md., 1981–1982 Catalog, p. 89–90) (36). Molecular weight size standards were obtained from Sigma.

Other techniques. V antigen was measured by fused rocket immunoelectrophoresis as previously described (19). β-Galactosidase levels were determined for cells grown at 30

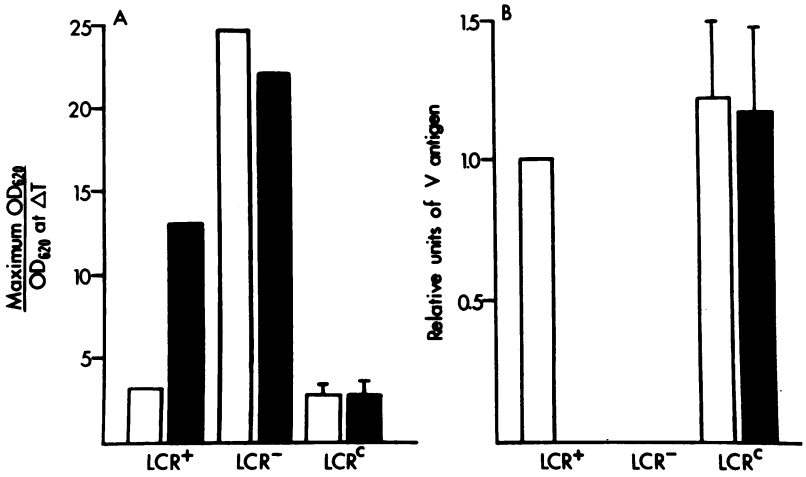

FIG. 1. Growth and V antigen production at 37°C. Levels were determined in DM with (solid bars) and without (open bars) 2.5 mM $CaCl_2$. Lcr^+ is UTP1000; Lcr^- is the pUT1000 segregant of UTP1000. Values for these strains are the average of two independent experiments. Lcr^c represents the combined and averaged totals of UTP strains 1001 to 1006 from two independent experiments. Bars indicate standard deviations. (A) Growth is expressed as the ratio of the maximum optical density at 620 nm (OD_{620}) obtained to the optical density at 620 nm at the time of the shift from 26 to 37°C (ΔT). (B) V antigen values are normalized for culture density and are expressed relative to the wild-type level.

and 37°C in DM by the sodium dodecyl sulfate-chloroform method described by Miller (28).

RESULTS

Growth and V antigen production of Lcr^c mutants. Five independent Lcr^c mutants of *Y. pestis* UTP1000 were obtained by EMS mutagenesis followed by ampicillin enrichment for cells unable to grow at 37°C in DM plus 2.5 mM $CaCl_2$. A sixth, spontaneous Lcr^c mutant was obtained directly through ampicillin enrichment of an unmutagenized culture. All six mutants failed to grow at 37°C regardless of Ca^{2+} concentration (Fig. 1). Plating efficiencies reflected this same pattern, with the exception of UTP1002, which was similar to the Lcr^+ UTP1000 (equal efficiency at 26 and 37°C with Ca^{2+}; 5.6×10^{-5} at 37°C without Ca^{2+}). Isolated colonies of the Lcr^c mutants obtained at 37°C on Ca^{2+}-supplemented medium were shown to be segregants cured of pUT1000 which grew normally at 37°C. Thus, these were not merely temperature-sensitive mutants but were specifically altered in LCR expression. Lcr^+ strains produced V antigen at 37°C only in the absence of Ca^{2+}. In contrast, all six Lcr^c mutants produced V antigen constitutively at 37°C (Fig. 1). As determined by fused rocket immunoelectrophoresis, the V antigens made by the mutants were immunologically identical to that of the wild type. Like Lcr^+ strains, the mutants did not make detectable V antigen at 26°C.

Localization of the mutations to pUT1000. All six mutations were shown to be located on pUT1000 in two ways. First, mutants cured of pUT1000 were restored to Lcr^+ by transduction with the parent pUT1000. Second, the Lcr^- KIM7 strain acquired the Lcr^c phenotype when transduced with pUT1000 derivatives from any of the mutant strains. Grown in DM and plating efficiencies were identical to those of the original mutants (data not shown).

Mapping of *lcrE1*. The location of the EMS-induced mutation carried by pUT1001 (strain UTP1001) was mapped by both complementation and recombination techniques.

(i) Complementation by Lcr^- pCD1::Mu d1 plasmids. Previously, we described 1 Lcr^+ and 16 Lcr^- insertion mutants of pCD1 obtained with bacteriophage Mu d1(Ap *lac*) (19). The locations of these insertions are shown in Fig. 2. For complementation mapping, UTP1001 was transduced with each of these plasmids, and plating efficiencies were determined at both 30 and 37°C in the presence of antibiotic selection for both pUT1001 and pCD1::Mu d1. The Lcr^+ UTP1000 strain plated with equal efficiency at 30 and 37°C in the presence of Ca^{2+}, whereas UTP1001 was reduced approximately 10^3-fold at 37°C (Fig. 3). The merodiploid pUT1001-pCD1::Mu d1 28.4 (Lcr^c/Lcr^+) was identical to UTP1000 both in the presence and absence of Ca^{2+} (data shown for the presence of Ca^{2+} only), indicating that the mutation causing Lcr^c is recessive to the wild type in *trans*. Complementation of Lcr^c was observed with all Lcr^- pCD1::Mu d1 plasmids with insertions in *lcrB* and *lcrC* and to the left of map position 45.5 kb in *lcrA*. Only those pCD1::Mu d1 plasmids with insertions between 45.5 and 48.6 kb in *lcrA* did not complement the mutation. On the basis of this, we concluded that the mutation must lie in the

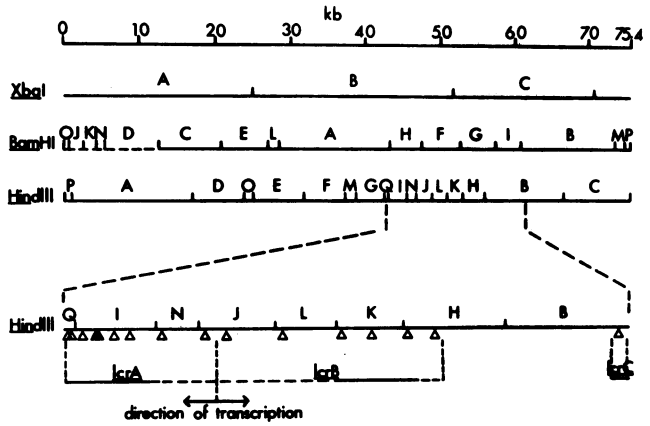

FIG. 2. Restriction maps of pCD1. The enlarged section represents the LCR region. Arrows indicate the positions of Mu d1(Ap *lac*) insertions resulting in the Lcr^- phenotype (19).

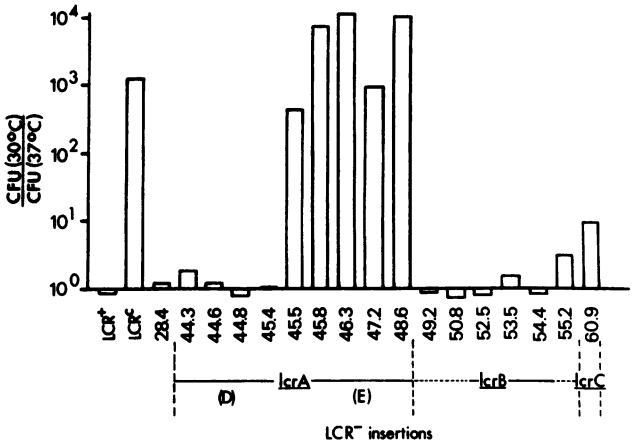

FIG. 3. Complementation of an Lcr^c mutation by Lcr$^-$ pCD1 derivatives. The Lcr$^-$ pCD1::Mu dl plasmids shown in Fig. 2 were used in complementation experiments to map the Lcr^c mutation of pUT1001. Lcr$^+$ is UTP1000. Lcr^c is UTP1001. Numbers represent map positions, in kilobases, of the Mu dl insertions. pCD1::Mu dl 28.4 lies outside the LCR region and confers the Lcr$^+$ phenotype. Results are expressed as the ratio of the plating efficiency obtained at 30°C to that obtained at 37°C on Ca^{2+}-supplemented medium (TB).

3.8-kb region between 45.4 and 49.2 kb. In addition, because of the distinctive complementation pattern observed in *lcrA*, we divided this region into two separate loci, *lcrD* and *lcrE*, and designated the mutation causing the Lcr^c phenotype and carried by pUT1001 as *lcrE1*. In contrast to the Lcr^c-Lcr$^+$ strain, plating efficiencies of the Lcr^c-Lcr$^-$ merodiploid strains on magneisum oxalate were variable and, in general, similar to that obtained in the presence of Ca^{2+}. Thus, the complementation observed was incomplete and did not result in true reversion to wild type. Because true reversion to the Lcr$^+$ phenotype was not observed, the location of *lcrE1* was confirmed by recombination mapping.

(ii) Recombination mapping. Two strategies were employed for the mapping of *lcrE1* by recombination. In the first series of experiments, the ability of deleted derivatives of pCD1 to restore the Lcr$^+$ phenotype was determined. The plasmids pUT1010 and pUT1011 are deleted for the 27-kb and 19-kb *Xba*I B and C fragments, respectively. To avoid complications due to recombination between Mu pf7701 of pUT1001 and Mu dl of pUT1010 and pUT1011, the Tn*5*-marked derivative of pUT1001, pUT1008, was used in these experiments. Of 500 isolates, 5 (1.0%) were restored to Lcr$^+$ after recombination with the undeleted pUT1009 (Table 3). Of 500 isolates, 3 (0.6%) were restored to wild type by recombination with pUT1011, indicating that the mutation was not located in the 19-kb region deleted from this plasmid. No Lcr$^+$ isolates were recovered from UTP1604 transduced with pUT1010 (0 of 676), strongly suggesting that the mutation was located in the 27-kb region which had been deleted from this plasmid. This region, between map positions 25 and 52 kb, includes the 3.8-kb *lcrE* locus defined in the complementation experiments described above. To more precisely determine the location of *lcrE1*, we next used cloned fragments from the *lcrE* region in recombination experiments with pUT1001. Lcr$^+$ recombinants were recovered by using both the *Bam*HI H and *Hin*dIII J cloned fragments but not the *Hin*dIII N or I cloned fragments (Table 3). As determined by restriction enzyme digestion, the *Bam*HI H fragment contains only 1 kb of the leftmost end of *Hin*dIII-J, and, therefore, *lcrE1* must lie in the region between 47.8 and 48.8 kb on pCD1. Plasmids isolated from the Lcr$^+$ recombinants obtained with the *Hin*dIII J cloned fragment were unaltered as determined by restriction enzyme digestion. However, the plasmid recovered from the Lcr$^+$ recombinant obtained with the *Bam*HI H cloned fragment was increased in size (Fig. 4). Restriction enzyme digestion with *Bam*HI showed that pBR322 was present and that there were two copies of the *Bam*HI H fragment. In addition, this plasmid conferred ampicillin resistance, confirming the integration of pUT1101 into pUT1001. These results confirm that recombination between pUT1101 and pUT1001 did occur, although at low frequency. Spontaneous isolates of UTP1001 able to grow at 37°C on TB-kanamycin arose at a frequency of approximately 6.6×10^{-4}. Three hundred of these colonies were screened and found to be Lcr$^-$. Thus, the spontaneous reversion rate is less than 2.2×10^{-6} and would not be expected to affect the outcome of the above experiments.

Functions of *lcrB*, *lcrD*, and *lcrE* are required for expression of the Ca^{2+}-blind phenotype. Isolates of KIM7 harboring pUT1007::Mu dl(Ap *lac*) cointegrates were selected for the ability to grow at 37°C on TB-ampicillin. Analysis of 13 of these isolates in which the Lcr^c phenotype had been suppressed showed that all had Mu dl insertions located in the LCR region (Table 4). In addition, the insertions were

TABLE 3. Recombination mapping of *lcrE1* with deleted derivatives and cloned fragments of pCD1[a]

Plasmid	Restriction fragment	Frequency of phenotypes recovered[b]		
		Lcr^c	Lcr$^-$	Lcr$^+$
	Region deleted			
pUT1009[c]	None	270/500	225/500	5/500 (1.0%)
pUT1010[c]	25–52 kb (*Xba*I-B)	558/676	118/676	0/676
pUT1011[c]	52–71 kb (*Xba*I-C)	290/500	207/500	3/500 (0.6%)
	Region cloned			
pUT1101[d]	44.6–48.8 kb (*Bam*HI-H)	1,019/1756	736/1,756	1/1,756 (0.06%)
pUT1102[d]	44.1–46.5 kb (*Hin*dIII-I)	1,521/3105	1,584/3,105	0/3,105
pUT1103[d]	46.5–47.8 kb (*Hin*dIII N)	277/530	253/530	0/530
pUT1104[d]	47.8–49.1 kb (*Hin*dIII-J)	1,275/2,286	1,009/2,286	2/2,286 (0.09%)

[a] Isolates were selected at 30°C before screening for Lcr phenotypes. See Materials and Methods for complete descriptions of techniques used.
[b] Lcr$^+$ phenotypes were confirmed by quantitative plating. Plasmids from Lcr$^+$ recombinant strains were unaltered as determined by restriction enzyme digestion except as noted in the text. Number found/number tested.
[c] Recombination was between pUT1008 and the plasmid listed.
[d] Recombination was between pUT1001 and the plasmid listed.

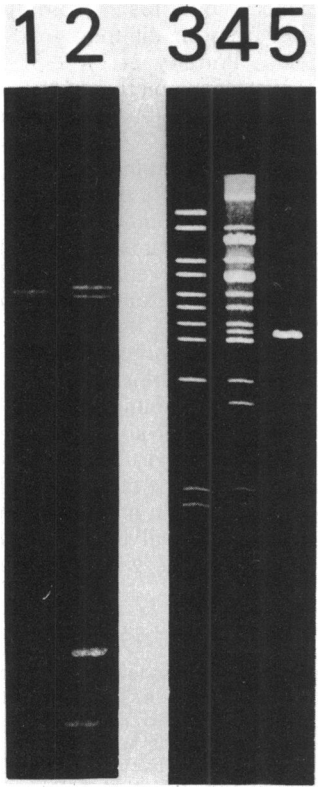

FIG. 4. pUT1001::pUT1101 cointegrate. Agarose gel electrophoresis of plasmid DNA from UTP1001 (lane 1) and KIM7(pUT1001::pUT1101) (lane 2). In lane 1, pUT1001 migrates to approximately the same position as the cryptic plasmid of *Y. pestis*. In lane 2, pUT1001 is increased in size by the integration of pUT1101 and migrates more slowly than the cryptic plasmid. The smaller pesticin plasmid is present in both strains (16). KIM7(pUT1001::pUT1101) also harbors pGW600. *Bam*HI restriction digests are shown for pCD1 (lane 3), for pBR322 (lane 5), and for plasmid DNA from KIM7(pUT1001::pUT1101) (lane 4). Note that in lane 4, pBR322 is present and the relative intensity of restriction fragment H is increased, confirming the integration of pUT1101 into pUT1001. Additional bands observed in lane 4 are due to the pesticin plasmid pGW600 and Mu-specific fragments. Fragment A is missing from pUT1001::pUT1101 due to the integration of Mu pf7701.

distributed throughout the region at approximately the same frequency as the previously described Lcr$^-$ insertion mutants (19). As expected, isolates selected at 30°C which retained the Lcr^c phenotype (169 of 170) were found to have insertions located outside the LCR region (Table 4). The effect of *lcrE1* on the transcription of other genes in the LCR region was determined by measuring β-galactosidase levels of the 13 strains harboring pUT1007::Mu d1 cointegrates in the LCR region. As observed above for Lcr$^-$ pCD1::Mu d1 cointegrates, transcription was not affected by Ca^{2+} concentration but was increased in response to temperature by approximately five-, three-, and threefold in *lcrB*, *lcrD*, and *lcrE*, respectively. The levels of β-galactosidase were approximately the same as reported previously (19; data not shown).

Protein analysis. Protein profiles were determined for KIM5 and UTP1007 grown at 30 and 37°C in Ca^{2+}-supplemented and Ca^{2+}-depleted media. The Lcr$^+$ KIM5 strain produced several proteins at 37°C in the presence of Ca^{2+} that either were produced in reduced quantity or were absent from outer membrane-enriched fractions of cells grown in the absence of Ca^{2+}. The major differences were observed with proteins having molecular weights of less than 36,000 (Fig. 5a and b). In contrast to the Lcr$^+$ strain, the protein profile of Lcr^c UTP1007 grown at 37°C was the same regardless of Ca^{2+} concentration and was identical to that of KIM5 grown at 37°C without Ca^{2+} (Fig. 5c and d). No differences between the wild type and mutant were observed at 30°C.

DISCUSSION

Previous work identified three genetic loci of pCD1 required for both the LCR and virulence (19). Because their phenotypes with respect to the LCR were essentially identical, the analysis of Lcr$^-$ insertion mutants did not allow us to determine which genes in the LCR region, if any, are directly involved in the response to calcium. The analysis of Lcr^c mutants described here demonstrates that at least one gene involved in regulation by Ca^{2+} is located in the newly defined *lcrE* locus of pCD1. This locus was identified by complementation mapping of an EMS-induced mutation and is contained within the locus previously designated *lcrA*. The mutants examined failed to grow at 37°C regardless of calcium concentration and produced V antigen constitutively at this temperature. Thus, *lcrE* is involved in regulating both of these phenotypes.

There are three mechanisms which might lead to the Lcr^c phenotype. (i) The mutation may result in an alteration of gene regulation. However, the constitutive phenotype resulting from the *lcrE1* mutation cannot be due to an alteration in *lcrE* regulation because *lcrE::lacZ* operon fusions are regulated identically in the presence and absence of the *lcrE1* mutation (19; this study). (ii) The phenotype may be the result of destruction of the *lcrE* product. Although our observation that insertion mutations in *lcrE* result in the Lcr$^-$ phenotype (19; this study) would appear to rule out this possibility, it does not because these mutations may be polar on *lcrD*. The loss of only *lcrE* may be sufficient to cause the Lcr^c phenotype. (iii) If the *lcrE* product is activated or inactivated by Ca^{2+}, the Lcr^c phenotype could result from an altered *lcrE* protein product that is no longer affected by Ca^{2+}. The data available at present are not sufficient to test this hypothesis.

TABLE 4. Mu d1(Ap *lac*) insertions in pUT1007[a]

Selection temp (°C)	Phenotype	Location of Mu d1		No. of isolates
		*Bam*HI fragment	*Hin*dIII fragment	
37	Lcr$^-$	A	I	1
		H	I	3
		H	J	2
		F	—[b]	6
		G	H	1
30	Lcr^c	A	E	1
		E	—[b]	3
		C	—[b]	5
		B	—[b]	2

[a] pUT1007 confers the Lcr^c phenotype. pUT1007::Mu d1 cointegrates transduced to KIM7 were selected at 30 or 37°C on TB-ampicillin and the Lcr phenotypes and plasmid profiles were examined.

[b] —, Not determined. Sites of Mu d1 integration in *Hin*dIII fragments were determined only when it was unclear from the *Bam*HI data whether the insertion was located in the LCR region.

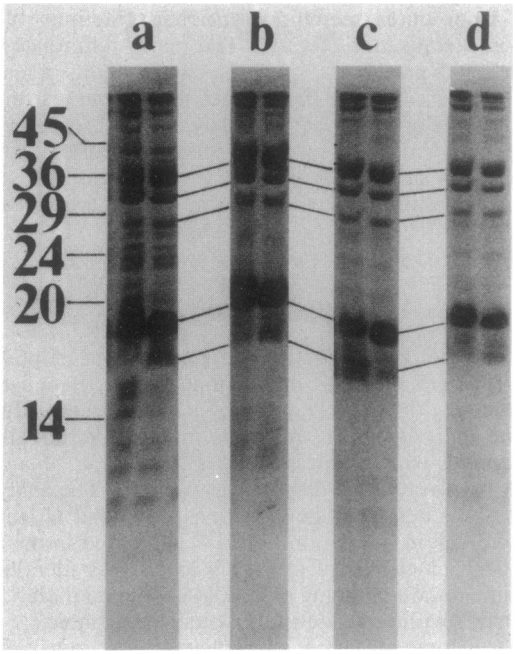

FIG. 5. Protein profiles of the Lcr$^+$ KIM5 and the Lcr^c UTP1007 grown at 37°C. Outer membrane-enriched fractions obtained from sucrose gradients were run on 15% polyacrylamide-sodium dodecyl sulfate-urea gels to resolve lower-molecular-weight proteins. (a) KIM5 grown in HIB plus 2.5 mM Ca^{2+}. (b) KIM5 grown in HIB-magnesium oxalate. (c) UTP1007 grown in HIB plus 2.5 mM Ca^{2+}. (d) UTP1007 grown in HIB-magnesium oxalate. All gels were stained with Coomassie blue R. Molecular weight standards are shown on the left ($\times 10^3$). Note that there are several species present in lane a that are absent or reduced in quantity in lanes b through d.

The loss of genes from the *lcrB*, *lcrD*, or *lcrE* locus was sufficient to destroy the LCR in the presence of a mutation otherwise causing the Lcr^c phenotype. This indicates that either all components derived from these loci must be intact for the LCR to occur, or, if the LCR results from a series of events, the gene product affected by the *lcrE1* mutation is required before the products from the *lcrB*, *lcrD*, or *lcrE* locus. The presence of the *lcrE1* mutation also had no effect on transcription in the *lcrB*, *lcrD*, or *lcrE* locus. Because no Mu dl insertions were obtained in *lcrC*, we could not assess the effect of the *lcrE1* mutation on transcription in this locus or determine whether an insertion in *lcrC* suppresses the Lcr^c phenotype.

The observation that protein profiles of the mutant strain grown at 37°C were not affected by Ca^{2+} concentration and were identical to those of the wild type grown in the absence of Ca^{2+} is consistent with the hypothesis that Lcr^c mutants are blind to the presence of Ca^{2+}. The reduction in relative amounts of some protein species from outer membrane-enriched fractions of Lcr$^+$ strains grown in the absence of Ca^{2+} at 37°C is a new observation. Although it is possible that pCD1 causes production of these proteins only in the presence of Ca^{2+}, there is no firm evidence of Ca^{2+}-regulated transcription in this system. We think it likely that these changes result from the degradation of protein species, their release from the membrane, or some other posttranscriptional event which occurs in the absence of Ca^{2+}. In contrast to our observations, others have reported either no differences in *Y. pestis* outer membrane proteins or the appearance of unique proteins in the absence of Ca^{2+} (14, 15). These differences could result from differences in the strains examined or from the different growth medium used in our experiments. Portnoy et al. (33) noted medium-dependent effects in the examination of *Y. enterocolitica* and *Y. pseudotuberculosis* plasmid-associated outer membrane proteins.

Of the six mutants examined in this study, the location of the mutation was determined for only one. Because the LCR appears to result from a complex series of events, the mapping of other mutations that result in the Lcr^c phenotype may reveal additional genes required for the response to calcium. *lcrE* is the first locus from the LCR region for which a function has been at least partially defined. We now have evidence that at least one other locus in this region is involved in regulation by temperature (J. Yother, T. W. Chamness, J. D. Goguen, submitted for publication). It is possible that much of the 17-kb LCR region of pCD1 is involved in the regulation of virulence genes which may be located outside the region, as is the structural gene for the V antigen (19, 31). Determination of whether loci such as *lcrE* produce products directly involved in virulence or whether they regulate virulence genes will require more complete analysis.

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