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A Low-Ca^{2+} Response Operon Encodes the V Antigen of *Yersinia pestis*

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***Yersinia pestis* has a virulence regulon called the low-Ca^{2+} response that is mediated by the plasmid pCD and manifested as regulation of growth and of expression of several virulence-associated properties by Ca^{2+} and temperature. We used Mu dI(Ap *lac*) to obtain a mutation in pCD1 of *Y. pestis* KIM that rendered the bacteria unable to express one of these properties, the V antigen. This mutant also had lost the Ca^{2+} requirement for growth at 37°C and was avirulent in mice. Two-dimensional protein gel electrophoresis showed that the Mu dI(Ap *lac*) insertion had eliminated 13,000- and 18,000-molecular-weight proteins in addition to the V antigen. We mapped the Mu dI(Ap *lac*) insertion within pCD1, cloned the *Hin*dIII fragment spanning the insertion location, prepared two subclones of this fragment, and identified the proteins these clones expressed in *Escherichia coli* minicells. The data indicated that the V gene lies within an operon containing three genes; *lcrG* (encoding the 13,000-molecular-weight protein), *lcrV* (encoding the 38,000-molecular-weight V antigen), and *lcrH* (encoding the 18,000-molecular-weight protein). Therefore, the V operon contains the structural gene for V antigen, at least one virulence gene, and at least one Ca^{2+}-dependence gene.**

The low-Ca^{2+} response (Lcr^+ or Vwa^+ phenotype) is a set of Ca^{2+}- and temperature-regulated properties in *Yersinia pestis* (plague) and the related species *Y. pseudotuberculosis* and *Y. enterocolitica* (5). All three species contain Lcr plasmids (1, 10–12) that specify the two components of the Lcr^+ phenotype: (i) a growth requirement for millimolar concentrations of Ca^{2+} at temperatures above 34°C (6, 14, 26, 43) and (ii) expression of several virulence-related proteins, the V and W antigens (7) and several outer membrane proteins called Yops (2, 28, 30, 31, 33, 34, 39). A variety of nucleotides (e.g., ATP) can relieve the growth requirement for Ca^{2+}; however, their effect may be indirect (43). The V antigen and Yops are expressed maximally at temperatures above 34°C in the absence of Ca^{2+} (30, 33, 43).

A large region on the Lcr plasmids contains genes necessary for the growth dependence on Ca^{2+} and for the temperature-enhanced expression of some plasmid genes (13, 28, 29, 41, 42). On pCD1 of *Y. pestis* KIM5, this Ca^{2+}-dependence region is 17 kilobases (kb) long and contains at least five low-Ca^{2+}-response (*lcr*) genes (13, 41, 42). These *lcr* genes seem to serve as regulators of Yop and V antigen expression (2, 13, 33). Insertion mutations made in these Ca^{2+}-dependence *lcr* genes cause the loss of regulated Yop and V antigen expression as well as the loss of Ca^{2+} dependence for growth (2, 13; R. D. Perry, P. Haddix, E. B. Atkins, T. K. Soughers, and S. C. Straley, Contrib. Microbiol. Immunol., in press). The normal consequence of *lcr* gene expression is thought to be the maximal synthesis of these virulence properties in the appropriate mammalian environment during an infection (33, 36).

V antigen is a protective antigen (20), and indirect evidence has indicated that it might promote long-term proliferation of yersiniae in mice (37, 38). However, there is no definitive evidence of a pathogenic role for V antigen, because no mutants exist that lack only this antigen.

Consequently, we sought a mutant of *Y. pestis* lacking the V antigen to open a new approach to the questions of its pathogenic function and its regulation of expression. Our characterization of the V^- isolate showed that the V antigen is encoded by the central gene of three in an operon that contains at least one virulence gene and at least one Ca^{2+}-dependence gene.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. With the exception of the *Escherichia coli* strains mentioned below, the bacteria and bacteriophages and their cultivation were as previously described (33). *Y. pestis* strains all lacked the pigmentation virulence determinant (Pgm^-), a property genetically and biochemically unrelated to the low-Ca^{2+} response. *Y. pestis* KIM5 contains pCD1 (and is Lcr^+), KIM6 lacks pCD1 (and is Lcr^-), and KIM7 lacks pCD1 (and is Lcr^-) but contains pGW600, which encodes the native Mu repressor (18). *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-73) is Lcr^+ and $YopJ^-$ (33) [from here on, we use Mu dI1 to denote Mu dI1(Ap *lac*)]. *E. coli* K-12 strains HB101 (3), χ2001 (9), and χ2338 (16) were obtained from Roy Curtiss III (Washington University). The plasmids used in this work are described in Table 1.

Cultivation of bacteria. *Y. pestis* cells were grown in a defined medium (TMH) which contained 20 mM $MgCl_2$ and, if specifically noted, 2.5 mM $CaCl_2$ or 18 mM disodium ATP (33). For growth at 37°C, the temperature was shifted from 26°C to 37°C when the A_{620} reached 0.2. The appropriate antibiotics were included at 25 μg each per ml during growth of all bacteria that carried drug resistance markers. In some experiments, *Y. pestis* was grown in oxalated tryptose blood agar base broth (Difco Laboratories, Detroit, Mich.) or oxalated heart infusion broth (Difco) containing 0.2% (wt/vol) xylose. The broths had been rendered Ca^{2+} deficient by addition of sodium oxalate and $MgCl_2$ (20 mM) (15).

Mutant isolation and preparation for characterization. *Y. pestis* KIM6 (pCD1::Mu dI1-42) was identified on lactose indicator medium as containing Mu dI1 inserted in a gene strongly regulated by temperature and Ca^{2+} (33). Mu dI1 was inserted in pCD1. For initial phenotypic characterization, the pCD1::Mu dI1 was transferred to a clean *Y. pestis* KIM7 background (not containing helper phage used for the Mu dI1

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TABLE 1. Plasmids used in this study

Plasmid	Description[a]	Reference
pGW600	Tc^r; encodes native Mu repressor	18
pCD1	Lcr plasmid of *Y. pestis* KIM5	13
Derivatives of pCD1		
pCD1::(Mu dI1::Tn*9*)-73	Ap^r Cm^r; *yopJ*	33
pCD1::Mu dI1-42	Ap^r; *lcrGVH*	This study
pCD1::(Mu dI1::Tn*9*)-42	Ap^r Cm^r; *lcrGVH*	This study
pBR322	Ap^r Tc^r	4
Derivatives of pBR322		
pLOX4	*Bgl*II-D of pCD1	This study
pJIT7	*Hin*dIII-G of pCD1	This study
pJIT7-1	Left[b] *Hin*dIII-*Cla*I fragment of *Hin*dIII-G of pCD1	This study
pJIT7-2	Right[b] *Cla*I-*Hin*dIII fragment of *Hin*dIII-G of pCD1	This study

[a] Tc, Tetracycline; Ap, ampicillin; Cm, chloramphenicol.
[b] See Fig. 4.

infection) (13, 33). Before animal studies and further characterization were done, 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 (13, 33).

Low-Ca^{2+} response properties of *Y. pestis* KIM7 (pCD1::Mu dI1-42). Growth of the bacteria and V antigen, β-galactosidase, and β-lactamase expression were measured as previously described (33). A unit of V activity is 1 mm of rocket height per mg of protein electrophoresed by rocket immunoelectrophoresis. β-Lactamase data were used to correct for any possible nonspecific effects of various incubation conditions on transcription or plasmid copy number (33). Protein was measured by the method of Lowry et al. (21).

Virulence testing in mice. Female 8-week-old outbred mice (HSD: [ICR] BR) (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were inoculated retro-orbitally, five mice per dose, and monitored (33). The 50% lethal dose values were computed by the method of Reed and Muench (32). An extra mouse received a dose of 10^6 bacteria; at 24 h after infection, it was sacrificed, and a tryptose blood agar plate (with no antibiotics) was streaked from the suspension generated from the macerated mouse liver and spleen (33). The resulting colonies all resembled typical *Y. pestis* colonies; five colonies were further tested and were found to be indistinguishable from the challenge strain in colony morphology, plasmid profile, and drug resistances (Ap^r Cm^r).

DNA restriction analysis and cloning. Restriction endonuclease digestion, physical mapping of restriction sites, and cloning were done by standard methods (22). Fragments of pCD1 generated by complete digestion with *Bgl*II and *Hin*dIII were cloned into pBR322, and the products were transformed into *E. coli* χ2001. The cloned *Bgl*II D and *Hin*dIII G fragments (pLOX4 and pJIT7, respectively) were obtained by screening clones for their plasmid size (17) and restriction pattern. The orientation of the *Hin*dIII G fragment within pCD1 was determined by comparing the restriction patterns of pJIT7 and pLOX4 from digests with *Hin*dIII, *Pvu*II, and *Xho*I. *Cla*I-*Hin*dIII subclones of pJIT7 were prepared and transformed into *E. coli* K-12 strains HB101 and χ2338 (pJIT7-1 and pJIT7-2, Table 1). The approximate location of Mu dI1 and the direction of transcription of *lacZ* of the insert in *Hin*dIII-G of pCD1::Mu dI1-42 were determined by comparison of *Bam*HI and *Hin*dIII digests of this plasmid (see reference 33, Fig. 3, which illustrates this method). For higher-resolution mapping, the two chimeric *Hin*dIII G-Mu dI1 fragments from pCD1::Mu dI1-42 were isolated, digested with restriction enzymes (*Nco*I, *Pvu*II, and *Cla*I), and electrophoresed alongside uncut chimeric fragments and size standards consisting of a mixture of restriction fragments of *Hin*dIII-G. The DNA was then subjected to Southern blotting and probed with nick-translated *Hin*dIII fragment G (22).

Minicell analysis of cloned gene products. Minicells were isolated from *E. coli* K-12 χ2338 and labeled with [^{35}S]methionine (0.2 mCi/ml, New England Nuclear Corp., Boston, Mass.) as previously described (9). The incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was determined (24, 25), and volumes containing 10,000 to 40,000 cpm were analyzed by one- (19) or two-dimensional (23) electrophoresis. All sodium dodecyl sulfate slab gels contained a uniform 12% (wt/vol) concentration of acrylamide. The gels were impregnated with En^{3}Hance (New England Nuclear), dried, and used to expose X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for approximately 10^8 disintegrations. ^{14}C-labeled molecular-weight standards were obtained from Amersham Corp. (Arlington Heights, Ill.).

Peptides affected by the Mu dI1 insert in pCD1::[Mu dI1::Tn*9*]-42. Two-dimensional gels were prepared and compared for *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-42), which carries Mu dI1::Tn*9* in the *Hin*dIII G fragment of pCD1; for *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-73), which carries Mu dI1::Tn*9* in the *Hin*dIII C fragment of pCD1 (disrupting expression of a Yop but having no effect on growth or V antigen expression [33]); and for minicells of *E. coli* K-12 χ2338 (pJIT7). The yersiniae were grown at 37°C in TMH without Ca^{2+} or ATP as in the measurement of low-Ca^{2+} response properties (33), except that the temperature was shifted to 37°C when the culture absorbance reached 0.15 (instead of 0.2), to avoid methionine starvation of the non-restricting mutant *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-42). Methionine was present at 0.1 mM (instead of the usual 1.0 mM) to permit labeling to high specific activity. At 5 h after the temperature shift to 37°C, *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-73) had entered growth restriction, whereas *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-42) was still growing exponentially. [^{35}S]methionine was then added (to give 1.3 mCi/ml); the cells were incubated for an additional 30 min, centrifuged, and boiled for 2 min in electrophoresis sample buffer containing 1% (wt/vol) sodium dodecyl sulfate. The *E. coli* minicells were grown and radiolabeled as described above. For electrophoresis, samples contained 10^6 cpm (*Y. pestis*) or 5×10^4 cpm (*E. coli*); first-dimension gels were prepared both by isoelectric focusing (IEF) (23) and by nonequilibrium-pH-gradient electrophoresis (NEPHGE) (25). Fluorography was carried out for approximately 10^9 (*Y. pestis*) or 10^8 disintegrations (minicells).

RESULTS

Mutant isolation and mapping of the insert. We used the operon fusion phage Mu dI1 (8) to obtain random insertion

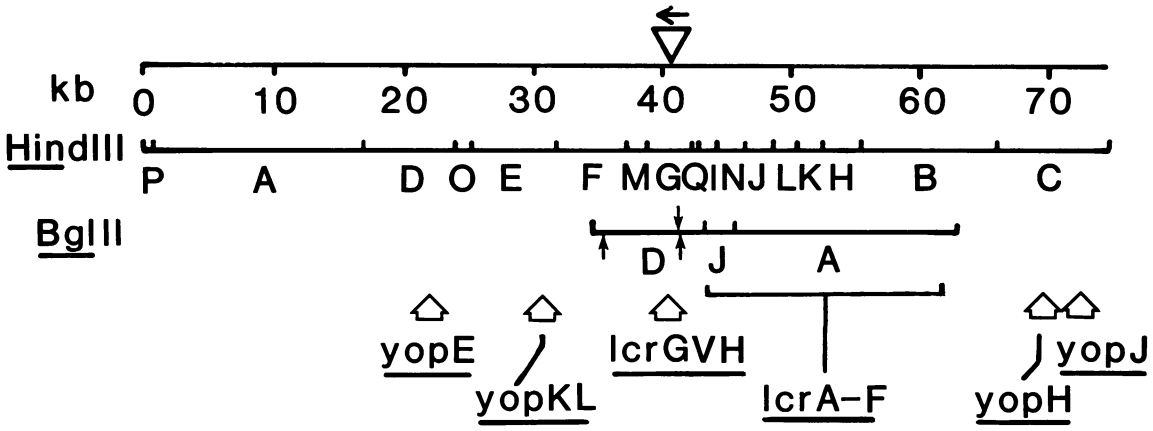

FIG. 1. Restriction map of pCD1 of *Y. pestis* KIM, showing locations of *lcr* and *yop* genes. The *Hin*dIII map and a portion of the *Bgl*II map are shown. The point of the triangle on the kilobase scale indicates the position of the Mu dI1 insert within *lcrG*, the first gene in the V operon. The direction of transcription of *lcrG* is indicated by the arrow overhead. The downward-pointing arrow on the *Bgl*II map indicates the position of the unique, asymmetric *Xho*I site within *Bgl*II-D and *Hin*dIII-G. The two upward-pointing arrows on the *Bgl*II map indicate the *Pvu*II sites within *Bgl*II-D. These sites were used to establish the orientation within pCD1 of a higher-resolution restriction map for the *Hin*dIII G fragment (see the text and Fig. 4). The locations of Mu dI1 inserts that define *lcr* and *yop* loci are indicated below the map (13, 33).

mutations in pCD1 genes. For Mu dI1 inserts oriented properly, expression of β-galactosidase from the *lacZ* gene of Mu dI1 is under the control of the promoter of the disrupted gene (8). We used this property to screen for mutants that showed strong effects of temperature and Ca^{2+} on transcription (measured as β-galactosidase activity) (33). One isolate made no detectable V antigen (by rocket immunoelectrophoresis, which would detect as little as 1% of the parental level of expression) and therefore potentially contained an insert in the V gene.

Restriction endonuclease digestion of the pCD::Mu dI1 plasmid revealed the direction of transcription and approximate location of the insert (Fig. 1). Higher-resolution mapping placed the insert at 41.8 kb on the pCD1 map. This is about 0.5 kb from the previously described distal-most insert in *lcrA* (13).

Low-Ca^{2+} response properties of the mutant. The mutant, *Y. pestis* KIM5 (pCD1::[Mu dI1::Tn*9*]-42), grew normally at 26°C but did not show growth restriction (Ca^{2+} dependence) at 37°C in the absence of added Ca^{2+}. Instead, under all three tested conditions (no addition; $+Ca^{2+}$; and +ATP), it exhibited slightly slower growth than the maximal rate shown by the parent strain (data not shown). Therefore, although this mutant was Ca^{2+} independent, it differed from previously described Ca^{2+}-independent mutants (13) by failing to show the maximal growth rate. The mutant also failed to respond to Ca^{2+} deprivation at 37°C in complex media (oxalated heart infusion broth and oxalated tryptose blood agar base broth). This did not reflect a generalized derangement in cellular shutdown responses, because the mutant did restrict normally in response to methionine starvation (data not shown).

The mutant did not express any V antigen, judging by both rocket immunoelectrophoresis (data not shown) and two-dimensional electrophoresis of radiolabeled cellular extracts with specific activities of at least 10^5 cpm/μg of protein (e.g., see Fig. 5). There also was no detectable V antigen in the cell-free spent medium after growth of the bacteria at 37°C in TMH or complex medium (tested both by rocket immunoelectrophoresis and two-dimensional gel electrophoresis; data not shown). Since this Mu dI1 insert eliminated expression of both V and growth restriction, the simplest interpretation would be that V antigen itself mediates the restrictive response to Ca^{2+} deprivation. However, we show below that V antigen is only one of three proteins eliminated as a result of the insertion within an operon that contains the V gene. Accordingly, we designate this locus *lcrGVH*.

β-Galactosidase expression by the LcrGVH⁻ mutant. β-Galactosidase expression by the LcrGVH⁻ mutant and V antigen expression by the parent *Y. pestis* KIM5 are compared in Fig. 2. We anticipated that temperature, Ca^{2+}, and ATP would have the same effects on transcription of the V gene (measured as β-galactosidase) as they have on V antigen activity. The data of Fig. 2 indicate that *lcrGVH* expression (transcription) by the mutant and V expression by the parent differed mainly by a constant scaling factor under all tested growth conditions except restrictive conditions. Under this one condition (37°C in the absence of Ca^{2+}), *lcrGVH* expression was suppressed, whereas V antigen expression was enhanced. It is significant that this is the one condition in which the parent and LcrGVH⁻ bacteria differed strongly in their growth state: the LcrGVH⁻ bacte-

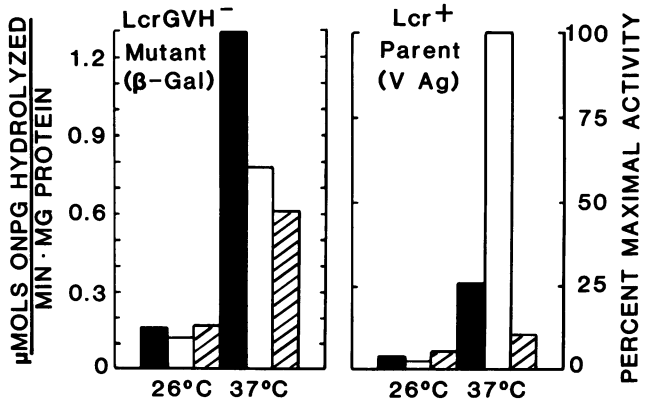

FIG. 2. β-Galactosidase expression by the LcrGVH⁻ mutant and V antigen expression by the parent *Y. pestis* KIM5 grown in TMH containing 2.5 mM Ca^{2+}, 18 mM ATP, or no addition. The scale was chosen to fully present the large enhancement of V expression under restrictive conditions (37°C, no additions). Additions: ■, 2.5 mM Ca^{2+}; ▨, 18 mM ATP; □, no addition.

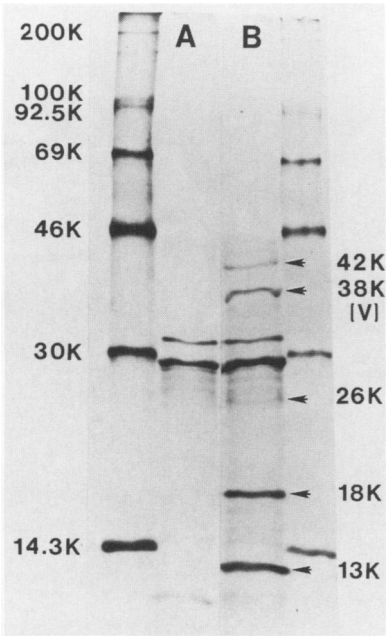

FIG. 3. Expression of the *lcrGVH*-containing cloned *Hin*dIII G fragment of pCD1 in *E. coli* minicells. ^{35}S-labeled minicells were prepared from *E. coli* K-12 χ2338 containing either the cloned *Hin*dIII G fragment of pCD1 (pJIT7) or the cloning vector pBR322. Outside lanes: molecular-weight markers. Lanes: A, pBR322; B, pJIT7.

ria were actively growing, whereas the parent strain was undergoing growth restriction.

Avirulence of the LcrGVH$^-$ mutant in mice. The intravenous 50% lethal dose of the LcrGVH$^-$ mutant in outbred mice was greater than 1.4×10^6. This is similar to that for *Y. pestis* KIM6 (lacking pCD1 altogether), whereas *Y. pestis* KIM5 (Lcr$^+$) has an intravenous 50% lethal dose of 80. This avirulence cannot be attributed to instability of the pCD1::Mu dI1 plasmid in vivo, because all five tested colonies, recovered from infected mice 24 h after injection, had retained the pCD1::[Mu dI1::Tn*9*]-42 plasmid (data not shown). Therefore, the *lcrGVH* locus contains at least one virulence gene for *Y. pestis*.

Cloning the pCD1 fragment containing *lcrGVH* and expression of the cloned genes in *E. coli* minicells. Because Mu dI1 has a polar effect within an operon, eliminating transcription of all genes downstream from an insert, it was possible that the V gene lay within an operon that contained at least one other gene downstream from the Mu dI1 insertion at 41.8 kb. We therefore cloned the 3.6-kb *Hin*dIII fragment containing *lcrGVH* to identify the proteins encoded by this fragment and to facilitate our comparison of two-dimensional electrophoregrams of LcrGVH$^-$ and Lcr$^+$ bacteria. The cloned fragment encoded five polypeptides with molecular weights of 42,000, 38,000 (V antigen), 26,000, 18,000, and 13,000 (Fig. 3).

We established the approximate locations for some of the corresponding genes in pCD1 by finding the orientation of the *Hin*dIII G fragment within pCD1, subcloning the two *Hin*dIII-*Cla*I fragments from the original pJIT7 clone, and determining which of the five proteins were expressed in minicells by the resulting subclones. The results of these experiments are shown in Fig. 4.

Neither subclone expressed detectable V antigen in rocket immunoelectrophoresis, and neither expressed the 38,000-molecular-weight V protein. However, pJIT7-2 expressed a 15,000-molecular-weight protein in amounts similar to those typically seen for V antigen in minicells. The other four proteins were accounted for between the two subclones (Fig. 4). We conclude that the internal *Cla*I site lies within the V structural gene. pJIT7-2 also expressed the 13,000-molecular-weight protein, presumably encoded by a gene to the right of the V gene in the map of the fragment. This raised the possibility that this protein is disrupted by the Mu dI1 insert defining *lcrGVH*, because this insert (triangle on

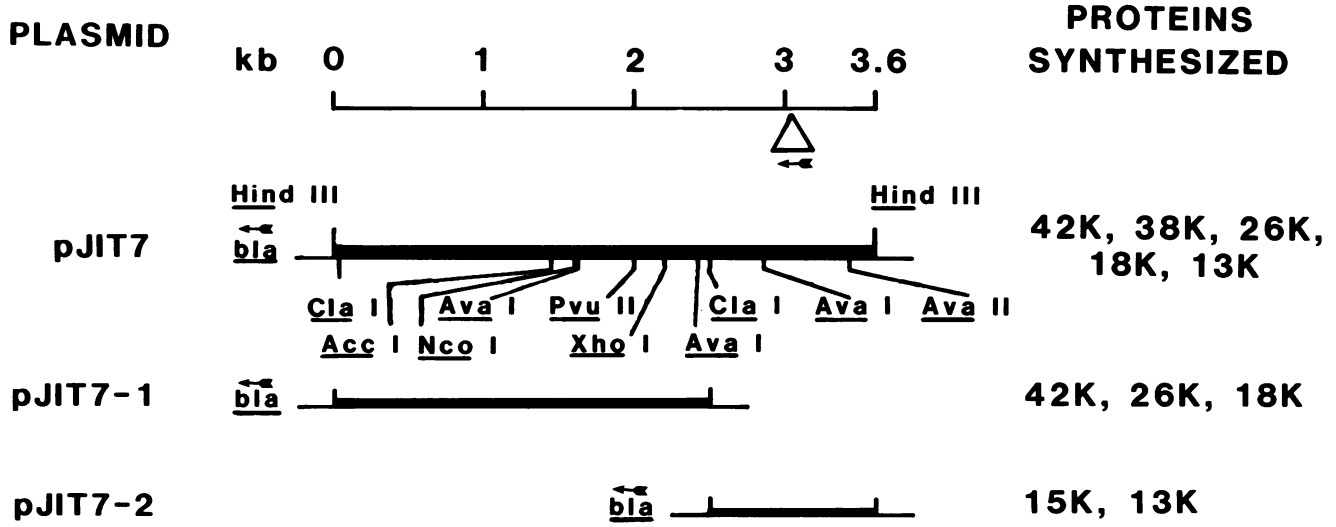

FIG. 4. Partial ordering of coding regions within *Hin*dIII-G of pCD1. The *Hin*dIII G fragment of pCD1 is represented in the orientation it has within the pCD1 map of Fig. 1. Cleavage sites for several restriction endonucleases are indicated. On the kilobase scale, the Mu dI1 insert in *lcrG* is indicated by a triangle which points at the site of the insertion. The direction of transcription for *lcrG* is shown by the arrow under the triangle. Two subclones containing the *Hin*dIII-*Cla*I fragments of *Hin*dIII-G are diagrammed below the *Hin*dIII-G map to show their relationship to the original clone. The thick lines in these maps represent pCD1 DNA; thin lines represent part of the vector (pBR322) DNA. The orientations of the cloned fragments within the vector are shown by the location and direction of transcription of the *bla* gene of pBR322. To the right of each map are listed the proteins expressed by the clones in *E. coli* minicells.

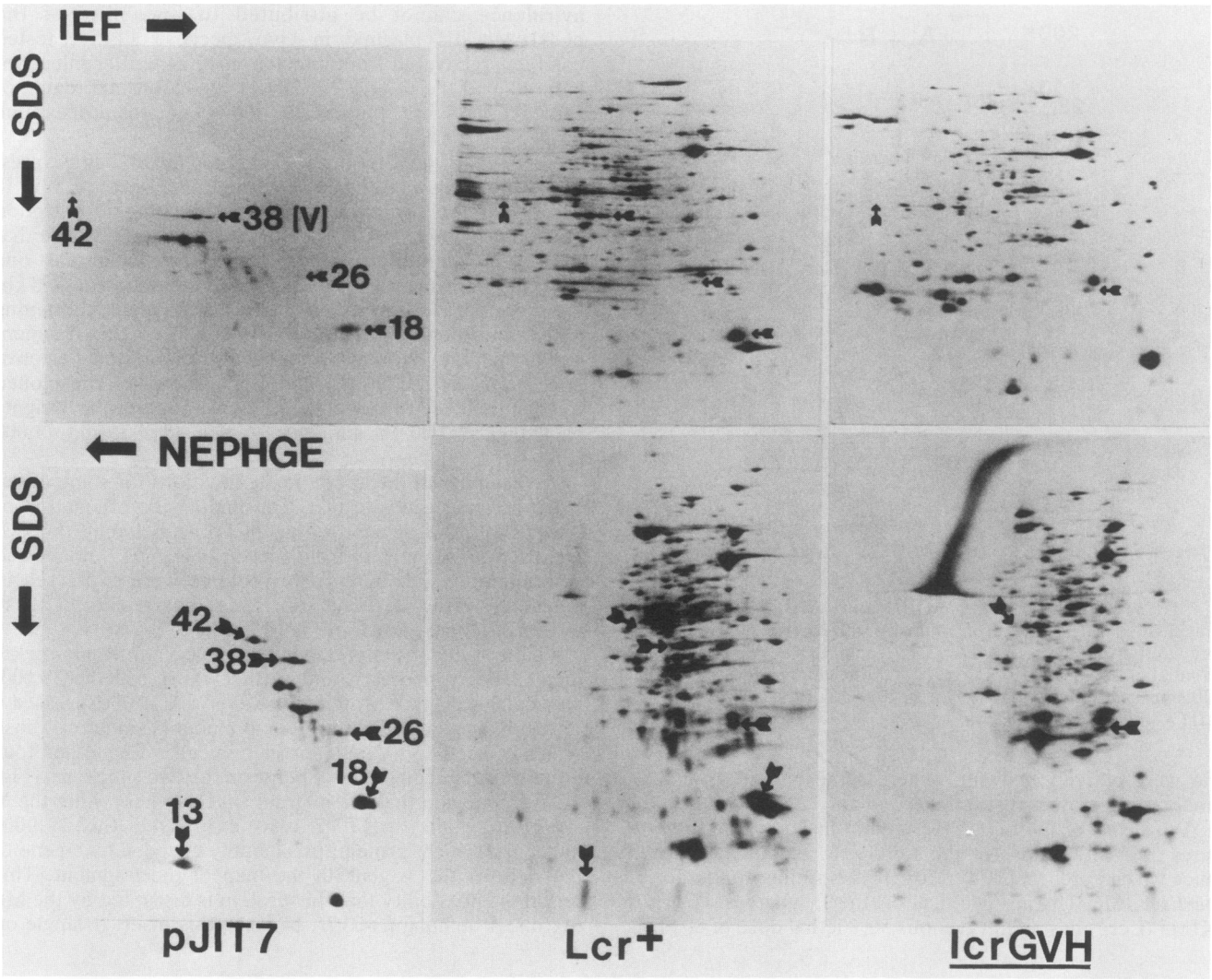

FIG. 5. Two-dimensional protein profiles of LcrGVH$^-$ and Lcr$^+$ *Y. pestis* KIM and of *E. coli* minicells containing the cloned *Hin*dIII G fragment of pCD1 (pJIT7). *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-42) (*lcrGVH*), *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-73) (Lcr$^+$), and *E. coli* minicells were grown and labeled with [^{35}S]methionine. The first-dimension electrophoresis was either equilibrium IEF (top three panels) or NEPHGE (bottom three panels). The second dimensions were 12% acrylamide-sodium dodecyl sulfate gels. The proteins expressed by the cloned *Hin*dIII G fragment in pJIT7 were identified by comparing the protein profiles of minicells containing pJIT7 with those of minicells containing pBR322 (data not shown). Arrows identify proteins encoded by the *Hin*dIII G fragment.

the kilobase scale of Fig. 4) is located far enough to the right of the *Cla*I site to be beyond the start of the V gene. The other three proteins were expressed by pJIT7-1 and cannot be ordered on the map from these data.

Proteins eliminated by the Mu dI1 insertion defining *lcrGVH*. We compared two-dimensional protein profiles for ^{35}S-labeled *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-42) (*lcrGVH*) and an Lcr$^+$ positive control strain, *Y. pestis* KIM6 (pCD1::[Mu dI1::Tn*9*]-73), grown under conditions expected to elicit maximal expression of V (and therefore of other proteins encoded by a hypothetical V operon). Because the protein profile for the growing LcrGVH$^-$ bacteria was anticipated to differ from that of the growth-restricted Lcr$^+$ bacteria, we used the two-dimensional protein profiles from [^{35}S]methionine-labeled minicells containing pJIT7 for reference. For the first dimension of electrophoresis, IEF and NEPHGE gels were used to identify all of the cloned proteins and to demonstrate convincingly their presence or absence in the LcrGVH$^-$ mutant.

The IEF gels, shown in the top three panels of Fig. 5, reveal that the LcrGVH$^-$ mutant did not express the 18,000-molecular-weight protein as well as the V antigen, but did express the 26,000- and 42,000-molecular-weight proteins. The 13,000-molecular-weight protein evidently was too basic to be retained on IEF gels; to see it, NEPHGE gels had to be prepared for the first dimension (Fig. 5, lower panels). These gels show that the LcrGVH$^-$ mutant also did not express the 13,000-molecular-weight protein. The Lcr$^+$ strain expressed all five *Hin*dIII-G-encoded proteins. A striking feature of the NEPHGE profile for the LcrGVH$^-$ mutant was the tremendous increase in abundance of a basic species that shows some streaking (aggregation?) in both dimensions of electrophoresis. We have tentatively identified this protein as YopH (Perry et al., in press). At present, we do not understand why this Yop (and not others) is expressed so strongly by this mutant. The IEF and NEPHGE gels were prepared from the same batches of *Y. pestis* but from different batches of minicells containing pJIT7. The two

spots that are much more strongly labeled in minicells in the lower than in the upper panel of Fig. 5 are *E. coli* proteins that vary in their intensity of labeling from one minicell preparation to another.

DISCUSSION

The aim of this study was to examine the expression and function of the V antigen of *Y. pestis*. The data presented here indicate that the single Mu dI1 insertion defining *lcrGVH* eliminated expression of three proteins having molecular weights of 13,000, 18,000, and 38,000. Taken with the data of Fig. 4, the simplest interpretation is that the V antigen is encoded by an operon, the V operon, containing three genes. The first gene in the transcriptional unit encodes the 13,000-molecular-weight protein, the V gene is in the center, and the distal-most gene encodes the 18,000-molecular-weight protein. We assigned these genes the respective designations of *lcrG*, *lcrV*, and *lcrH*. The Mu dI1 insert defining this operon evidently lies within *lcrG*. The genes encoding the 26,000- and 42,000-molecular-weight proteins of *Hin*dIII-G of pCD1 evidently are contained in a separate transcriptional unit(s): they lie on the downstream side of the Mu dI1 insert, and their expression would also have been eliminated had they been contained within the V operon. All genes in the operon lie within *Hin*dIII-G and accordingly are accounted for by *lcrG*, *lcrV*, and *lcrH*, because a distinct transcriptional unit is defined by a Mu dI1 insert in *Hin*dIII-G just 0.5 kb upstream from the site of the insertion in pCD1::Mu dI1-42 (unpublished data).

When comparing β-galactosidase expression by the LcrGVH$^-$ mutant (V operon transcription) with expression of V antigen activity by the parent (Fig. 2), we found a striking difference for bacteria grown at 37°C without Ca^{2+}. This may have been caused by the continued active growth of LcrGVH$^-$ bacteria, whereas the parental strain had stopped growing because of Ca^{2+} deprivation. During restriction, net protein synthesis ceases as a result of a significant decrease in total protein synthesis (40, 44). In contrast, V-specific activity strongly increases, starting at the time of temperature shift to 37°C and not reaching steady state until the cells have stopped growing (43; Perry et al., in press). Accordingly, V antigen should constitute a smaller proportion of total protein in actively growing bacteria than in bacteria in growth restriction. It is significant that V activity and Yop expression are affected similarly by Ca^{2+} and temperature, whether *yop* gene expression is measured at the transcriptional level by β-galactosidase expression from Mu dI1 inserts (33) or at the Yop protein level by Coomassie brilliant blue staining of outer membrane protein profiles (Perry et al., in press). The inserts defining the *yop* operons did not cause the bacteria to be Ca^{2+} independent (33), and thus for the comparison of *yop* gene expression with V activity, the mutant and parent bacteria always were in the same physiological state. The simplest model to account for these observations is that the same regulatory mechanism controls expression of the V and Yop operons.

Definitive tests have not been made for the role of the V antigen in pathogenesis, because mutant yersiniae lacking only this antigen have not yet been obtained. Nonetheless, there is evidence consistent with the idea that V functions to undermine or prevent a host defense mechanism, resulting in unchecked proliferation of the yersiniae in mice (38). Consistent with this, we view the V antigen as part of the end result of the low-Ca^{2+} response: it may be an anti-host molecule whose expression is adjusted to the appropriate mammalian environment by the gene products encoded by the Ca^{2+}-dependence region of pCD1. In this model, the Yops are other virulence properties regulated by those genes, and maximal expression occurs under conditions thought to exist in the phagolysosome of macrophages (27) where *Y. pestis* has been shown to grow (35, 36). We speculate that the 13,000- and 18,000-molecular-weight LcrG and LcrH proteins also are end products of the low-Ca^{2+}-response pathway.

Because the LcrGVH$^-$ mutant failed to show the restrictive response to Ca^{2+} deprivation, at least one of the three proteins must itself be necessary for Ca^{2+} dependence. The *lcr* genes previously identified in pCD1 (*lcrA* to *F*) may be required for Ca^{2+} dependence because they are necessary for strong expression of the Ca^{2+} dependence gene(s) in the V operon. Until localized mutagenesis is completed, affecting single genes in the operon, we will not know which genes in the V operon are responsible for Ca^{2+} dependence and which are virulence genes.

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