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The *Yersinia pestis* V Antigen Is a Regulatory Protein Necessary for Ca^{2+}-Dependent Growth and Maximal Expression of Low-Ca^{2+} Response Virulence Genes

STUART B. PRICE,† CLARISSA COWAN, ROBERT D. PERRY,‡ AND SUSAN C. STRALEY*

Department of Microbiology and Immunology, Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40536-0084

Received 4 September 1990/Accepted 4 February 1991

The low-Ca^{2+} response is a multicomponent virulence regulon of the human-pathogenic yersiniae in which 12 known virulence genes are coordinately regulated in response to environmental cues of temperature, Ca^{2+}, and nucleotides such as ATP. Yersinial growth also is regulated, with full growth yield being permitted at 37°C only if Ca^{2+} or a nucleotide is present. In this study, we constructed and characterized a mutant *Yersinia pestis* specifically defective in the gene encoding the V antigen, one of the virulence genes of the low-Ca^{2+} response. An in-frame internal deletion-insertion mutation was made by removing bases 51 through 645 of *lcrV* and inserting 61 new bases. The altered *lcrV* was introduced into the low-Ca^{2+} response plasmid in *Y. pestis* by allelic exchange, and the resulting mutant was characterized for its two-dimensional protein profiles, growth, expression of an operon fusion to another low-Ca^{2+} response virulence operon, and virulence in mice. The mutant had lost its Ca^{2+} and nucleotide requirement for growth, showed diminished expression of Ca^{2+}- and nucleotide-regulated virulence genes, and was avirulent in mice. The mutation could be complemented with respect to the growth property by supplying native V antigen operon sequences in *trans* in high copy number (on pBR322). Partial complementation of the growth defect and almost complete complementation of the virulence defect were seen with a lower-copy-number complementing replicon (a pACYC184 derivative). The data are consistent with the interpretation that V antigen is bifunctional, with a role in regulating growth and expression of low-Ca^{2+} response virulence genes in addition to its putative role as a secreted virulence protein.

The human-pathogenic yersiniae have a multicomponent virulence property called the low-Ca^{2+} response (Lcr phenotype) (8), which contains a set of regulatory genes and 12 identified, coordinately regulated virulence genes. The virulence gene products include a set of 11 surface proteins called Yops, which can be shed into the medium, and a secreted protein called the V antigen (11, 35, 36, 43). The regulatory genes modulate expression of the virulence genes in response to the environmental inputs of temperature, Ca^{2+}, and nucleotides such as ATP (19). The low-Ca^{2+} response is encoded by a ca. 75-kb plasmid (3, 17, 36), the one in *Yersinia pestis* KIM being called pCD1 (19).

A curious manifestation of the low-Ca^{2+} response is the requirement of millimolar Ca^{2+} or ca. 10 to 20 mM nucleotide for the bacteria to show full growth yield at 37°C (20, 49). Ca^{2+} and nucleotides are not utilized nutritionally (33, 49, 50) but may act as signals which initiate global responses to the external environment. In the absence of Ca^{2+} and nucleotides, the yersiniae carry out an orderly metabolic shutdown and cease growth after about two generations, a response called growth restriction (10, 50). Paradoxically, restriction occurs under the very conditions that elicit maximal expression of the virulence genes of the low-Ca^{2+} response (10, 42): these show low basal transcription at 26°C, maximal transcription at 37°C in the absence of Ca^{2+} and nucleotides, and downregulated expression (but ca. two- to threefold higher than at 26°C) at 37°C when Ca^{2+} or a nucleotide is present (19, 26, 42). The significance of the restrictive response in pathogenesis of the yersiniae is not understood. Apparently, it reflects the operation of the global regulatory mechanism in the low-Ca^{2+} response, because all mutations altering the ability to show restriction have also altered the regulation of expression of V antigen and Yops (2, 19, 34, 38, 47, 48).

There is a ca. 20-kb region on pCD1, often called the Ca^{2+}-dependence region, which is necessary for the induction of virulence gene expression by temperature and the downregulation by Ca^{2+} at 37°C (19). Within this region, loci for which functions have been classified include *lcrF*, which encodes a *trans*-acting factor that mediates the response to temperature (47); *lcrK*, which functions in Yop secretion (39); and *lcrE* and *lcrR*, which function in the downregulation by Ca^{2+} (2, 48). The operon which encodes the V antigen, previously called *lcrGVH* for its first three cistrons (34), is also part of the Ca^{2+}-dependence region. Like the *yop* operons, it is subject to regulation by the Ca^{2+}-dependence genes, but unlike *yop* operons, it has a regulatory function in the low-Ca^{2+} response. It contains at least one gene necessary for the restrictive response and at least one virulence gene, because an insertion of Mu dI1(Ap *lac*) in the first cistron caused the yersiniae to be Ca^{2+} and nucleotide independent in their growth and avirulent in mice (34). One regulatory gene in that operon, *lcrH*, is necessary for the downregulation of transcription in response to both Ca^{2+} and nucleotides at 37°C, negatively regulating its own *lcrGVH* operon as well as the operons encoding Yops (38). However, *lcrH* is not the gene in *lcrGVH* responsible for restriction, because a mutant with an insertion in *lcrH* still showed growth restriction (38). The first gene in the operon, *lcrG*, also may not be involved in restriction, because a

* Corresponding author.
† Present address: Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519.
‡ Present address: Department of Microbiology and Immunology, LSU Medical Center, Shreveport, LA 71130-3932.

mutant that did not express LcrG protein still showed restriction (2).

The V antigen operon may contain one or more *yop* genes downstream of *lcrH* by analogy with the operon structure in *Yersinia enterocolitica* (31). These have not yet been characterized in *Y. pestis*, but they, along with the V antigen gene *lcrV*, are candidate virulence genes in the operon. The V antigen is historically important as the first antigen discovered to be associated with virulence in *Y. pestis* (11, 12), and increasingly refined tests continue to show that it is a protective antigen (25, 46). However, its role in virulence has not been elucidated.

This study was undertaken to learn more about the V antigen by constructing and characterizing a *Y. pestis* strain having a nonpolar mutation in *lcrV*, rendering the bacteria specifically defective in expressing the V antigen. The data indicate that the V antigen may be a bifunctional protein: in addition to its proposed role as a virulence protein, it has important regulatory functions in the low-Ca^{2+} response in both causing restriction and promoting virulence gene expression.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. *Escherichia coli* K-12 strains HB101 (5), χ1553 (F⁻ *minA1 purE42 supE42 pdxC3 minB2 his-53 nalA28 metC65* T3ʳ *ilv-277 cycB2 cycA1 hsdR2*), and χ2338 (22) were obtained from Roy Curtiss III, Washington University, St. Louis, Mo. *E. coli* K-12 strain SY327 λ*pir* (30, 40) was obtained from Virginia Miller, University of California, Los Angeles, Calif. *E. coli* K-12 strain GM2163 (F⁻ *ara-14 leuB6 tonA13 lacY1 tsx78 supE44 galK2* λ⁻ *dcm-6 hisG4 rpsL136 dam-13*::Tn*9 xyl-5 mtl-1 thi-1 hsdR2*) was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, Conn.). *E. coli* K-12 strain DH5α was obtained from Bethesda Research Laboratories (Bethesda, Md.). Their cultivation has been described previously (19, 34, 42); chloramphenicol was used at 10 μg/ml to select for retention of the Tn*9* insertion in strain GM2163.

Y. pestis KIM strains lack the pigmentation virulence property, rendering them avirulent in mice by peripheral challenge while still fully virulent by the intravenous route (45). *Y. pestis* KIM6 lacks pCD1, is Lcr⁻, and is avirulent by intravenous challenge (45). *Y. pestis* KIM5-3173 has the same genetic background as the V antigen-negative mutant described in this work. It is Lcr⁺ but YopJ⁻ because of an insertion of Mu dI11734 in *yopJ* of pCD1 (38, 42). This abolishes expression of YopJ and renders the yersiniae resistant to kanamycin (Km) but does not significantly affect the intravenous 50% lethal dose (LD_{50}) or infection kinetics in mice (42, 44). *Y. pestis* KIM5-3240 is an *lcrH* mutant described previously (38). *Y. pestis* KIM5-3043.2 is an Lcr⁻ mutant originally designated mutant 44.6 [for pCD1::Mu dI1(Ap *lac*)44.6] (19). It has a Mu dI1(Ap *lac*) insert in *lcrD* (2a, 19), conferring resistance to ampicillin, (Apʳ), and contains the Mu repressor-encoding plasmid pGW600 (23), which stabilizes the insert against further transposition and confers resistance to tetracycline.

Y. pestis strains were grown as previously described in the defined medium TMH (42). $MgCl_2$ (20 mM) was always included, and 2.5 mM $CaCl_2$ was present as indicated. Unless otherwise noted, bacteria with antibiotic resistances were grown in the presence of the appropriate antibiotic(s) at a final concentration of 25 μg/ml (kanamycin, tetracycline, chloramphenicol) or 100 μg/ml (ampicillin). *Y. pestis* KIM5-3173 and V antigen-negative derivatives described below were unable to grow in TMH containing kanamycin at 25 μg/ml if the concentration of $MgCl_2$ was low (1 mM). We do not know the basis for this effect, but because it represented a significant effect of kanamycin on a phenotype that is central to the low-Ca^{2+} response, we either used a lower concentration of kanamycin (10 μg/ml) or omitted it altogether, even though we did not use a low $MgCl_2$ concentration in the experiments reported herein. This had no detrimental effect, because the pCD1::Mu dI11734 plasmid in these bacteria was stably maintained (data not shown). In the spirit of minimizing concentrations of antibiotics where possible, we used ampicillin at 25 μg/ml in experiments where low-Ca^{2+} response properties were being characterized for *Y. pestis* strains that were Apʳ.

Previously described plasmids used in this study were pCD1 (19), pCD1 *yopJ*::Mu dI11734 (38), pBR322 (6), pJIT7 (34), pGW600 (23), pIC20R (28), and pJM703.1 (21, 30). pJIT7 has the *lcrGVH*-containing *Hin*dIII G fragment of pCD1 cloned into the *Hin*dIII site of pBR322, and pJM703.1 is a suicide vector, able to replicate only in bacteria, such as *E. coli* SY327 λ*pir*, that supply the *pir* gene product needed for its replication. pAVA3 has the *Hin*dIII G fragment of pCD1 cloned into the *Hin*dIII site of pAVA1, a version of pACYC184 (13) which had been modified by nuclease S1 digestion to eliminate the unique *Ava*I site. The fragment was oriented such that the direction of transcription of *lcrGVH* (which has its own promoter in the cloned DNA) was opposite that for the *tet* gene. This work describes the construction of pJIT7′, pJIT7-8, pVS1, and pCD1 *lcrV*(51-645)::MCR *yopJ*::Mu dI11734.

Bacteriophage P1L4, which infects but does not lysogenize or lytically infect *Y. pestis* (19), was used to transduce pCD1 *lcrV*(51-645)::MCR *yopJ*::Mu dI11734 from *E. coli* χ1553 into *Y. pestis* KIM6.

In-frame deletion in *lcrV*. Plasmid DNA was isolated by the method of Birnboim and Doly (4). Restriction endonuclease digestion and cloning were done according to standard methods (1, 27). pJIT7 produced in the *dam dcm E. coli* GM2163 was cleaved with *Xba*I at a unique site 45 bases downstream from the ATG of *lcrV* and with *Xho*I at a unique site 601 bases farther downstream in *lcrV*. The vector-containing fragment was isolated, filled in with the Klenow fragment of DNA polymerase I (New England BioLabs, Inc., Beverly, Mass.), and treated with calf intestine phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). To make pJIT7′, this deletion derivative of pJIT7 was blunt end ligated. After transformation into *E. coli* DH5α, plasmid DNA was isolated and confirmed to have the anticipated structure by restriction analysis. The *lcrV* in pJIT7′ was predicted from the *lcrV* DNA sequence (37) to have undergone an in-frame deletion.

In-frame deletion-insertion in *lcrV*. An 81-base *Cla*I-*Eco*RI fragment was excised from the multiple cloning region (MCR) of pIC20R and isolated by electrophoretic separation on a polyacrylamide gel (12% [wt/vol] acrylamide) and then eluted by diffusion (37°C overnight) from minced gel fragments containing the desired band. The purified DNA was blunt ended with Klenow and ligated to the blunt-ended, phosphatased vector-containing fragment obtained from pJIT7 by *Xba*I-*Xho*I digestion (pJIT7′). After transformation into a *E. coli* DH5α, the resulting construct, pJIT7-8, was characterized by restriction analysis and by sequencing the fusion junctions in the double-stranded plasmid template (1). The sequences were analyzed by the PC/GENE programs (IntelliGenetics, Mountain View, Calif.). In pJIT7-8, *lcrV* had undergone in-frame deletion-insertion events. During

the construction, several bases had been lost from both ends of the *Cla*I-*Eco*RI MCR: 1 base from the *Eco*RI end and 17 from the *Cla*I end, giving an insert of 63 bases, present in the deleted *lcrV* in the orientation 5′ *Eco*RI end–*Cla*I end 3′. The final altered *lcrV* was lacking the native bases 51 through 645 and contained 61 new bases. The sequence in the junction region was *lcrV*′ 5′GAG GAT CTA **GaT TCG AGC TCG GTA CCC GGG GAT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTT CGC GAG** cTC GAG AAA3′, where the boldface indicates bases contributed by the MCR and the lowercase letters indicate two bases, one at each junction of the insert with *lcrV*, that are also present in the native *lcrV* sequence. The original reading frame was retained.

Analysis of proteins expressed in *E. coli* minicells. Minicells were isolated from *E. coli* χ2338 containing pJIT7, pJIT7′, or pJIT7-8 and were labeled with [^{35}S]methionine (0.25 mCi/ml; New England Nuclear Corp., Boston, Mass.) and boiled in electrophoresis sample buffer as previously described (14). Samples containing 40,000 trichloroacetic acid-precipitable cpm were analyzed by one-dimensional electrophoresis in sodium dodecyl sulfate (SDS)–12% (wt/vol) polyacrylamide gels (24), and samples containing ca. 150,000 trichloroacetic acid-precipitable cpm were analyzed by two-dimensional electrophoresis in which the first dimension was nonequilibrium pH gradient electrophoresis (NEPHGE) (32) and the second was an SDS–12% (wt/vol) polyacrylamide gel. For one-dimensional electrophoresis, flanking lanes contained ^{14}C-labeled molecular weight standards (Amersham Corp., Arlington, Ill.). Gels were impregnated with En^{3}Hance (New England Nuclear), dried, and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Isolation of the V antigen-negative *Y. pestis* KIM5-3241.2. pJIT7-8 was cleaved with *Cla*I, which cut within the *Yersinia* DNA near one *Hin*dIII end (the site within *lcrV* [34] had been removed by the internal deletion) and with *Nhe*I, which cut in the flanking pBR322 DNA 200 bases beyond the other *Hin*dIII end of the cloned DNA. The fragment containing the altered *lcrV* was isolated, blunt ended with Klenow, and ligated into *Eco*RV-cut and phosphatased suicide vector pJM703.1. This construct, pVS1, was transformed into *E. coli* SY327 λ*pir* for characterization by restriction analysis.

pVS1 was transformed into *E. coli* χ1553 containing pCD1 *yopJ*::Mu dI11734, with selection for Ap^r encoded by pVS1. Because pVS1 cannot replicate in this *E. coli* strain, Ap^r isolates were the result of integration of pVS1 into pCD1 *yopJ*::Mu dI11734 by a homologous recombination event. The pCD1 derivative in one such isolate was characterized by restriction analysis. Its plasmid was then transduced by P1L4 into *Y. pestis* KIM6 with selection for Km^r (from Mu dI11734) and Ap^r. The transduction and all subsequent growth of *Y. pestis* during the isolation of the V antigen-negative mutant were done at 30°C to avoid accidentally selecting for point mutations in regulatory *lcr* genes.

After characterization of its plasmid profile and restriction patterns, the resulting *Y. pestis*, containing pCD1 with pVS1 integrated in homologous *lcrGVH* sequences, was grown for nine transfers in heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 1 mM $MgCl_2$ and 0.2% (wt/vol) xylose, each transfer allowing seven generations of growth. Kanamycin was present to ensure retention of the pCD1 derivative, but ampicillin was omitted, to permit the survival and accumulation of yersiniae in which a second recombination had resolved the pCD1::pVS1 cointegrate, with subsequent loss of the replication-deficient pJM703.1-derived replicon. The resulting culture was spread onto 50 tryptose blood agar base (Difco) plates containing kanamycin. The ca. 58,200 colonies were replica plated onto tryptose blood agar base containing either kanamycin or ampicillin. Possible Km^r Ap^s resolvants were tested twice for growth on medium containing either kanamycin or ampicillin, and 85 were probed by colony blot with end-labeled ^{32}P-81-base MCR from pIC20R and ^{32}P-pJM703.1 that had been labeled by nick translation with a kit from New England Nuclear. Only one colony was the desired MCR$^+$ pJM703.1$^-$ combination, arising from a recombination event that exchanged the mutated *lcrV* for the native one in pCD1. Two Ap^s colonies were unexpectedly MCR$^+$ pJM703.1$^+$, and the rest were MCR$^-$ pJM703.1$^-$, being the products of recombination events on the same side of the *lcrV* mutation as the original integration event. The one MCR$^+$ pJM703.1$^-$ isolate was characterized for its plasmid profile and restriction pattern and was designated *Y. pestis* KIM5-3241.2. Its pCD1 derivative contained the deleted and inserted *lcrV* and an insertion of Mu dI11734 in *yopJ*. Accordingly, the appropriate reference strain for determining the effects of the *lcrV* mutation was *Y. pestis* KIM5-3173, in which the pCD1 derivative had the wild-type *lcrV* and the same insertion of Mu dI11734 in *yopJ*. That strain will be called the reference strain rather than the parent strain, because the *lcrV* mutant was not derived directly from it; however, the genetic backgrounds of the two strains in theory are identical.

Complementation tests. pJIT7, pBR322, and pAVA3 were transformed singly into the *lcrV Y. pestis* KIM5-3241.2 and the reference strain *Y. pestis* KIM5-3173. pAVA3 also was transformed into the *lcrD* mutant strain *Y. pestis* KIM5-3043.2.

Protein profiles of the *lcrV* mutant and reference *Y. pestis*. The reference *Y. pestis* KIM5-3173 and the *lcrV Y. pestis* KIM5-3241.2 were grown at 37°C in TMH containing or lacking 2.5 mM $CaCl_2$ and having 10× lower methionine (final concentration, 0.1 mM) to permit labeling to high specific activity with [^{35}S]methionine. After ca. six generations of pregrowth in exponential phase at 26°C, the temperature was shifted to 37°C when the A_{620} reached ca. 0.1 for the *lcrV* mutant and for the reference strain growing in the presence of Ca^{2+} and when the A_{620} reached ca. 0.2 for the reference strain in the absence of Ca^{2+}. Five hours later, at an A_{620} of ca. 1.0, 1-ml amounts of all cultures were labeled with [^{35}S]methionine as previously described (34).

Samples were prepared for analysis of NEPHGE two-dimensional protein profiles by using the following modification of a described protocol (34). The labeled cultures were chilled by swirling in ice-water for 1 to 3 min, centrifuged for 2 min in a refrigerated microfuge, and placed on ice. The pellets were washed in 1 ml of ice-cold 10 mM Tris HCl (pH 8.0) containing 5 mM $MgCl_2$, and the washed pellets were rapidly mixed with 150 μl of freshly prepared, 100°C SDS–2-mercaptoethanol solution (0.3% [wt/vol] SDS; 5.0% [vol/vol] 2-mercaptoethanol; 10 mM Tris HCl, pH 8.0; 5 mM $MgCl_2$) per ca. 6×10^8 cells. The mixtures were incubated on ice for 2 to 3 min, and then 1/10 vol of 10× nuclease solution (1 mg/ml DNase I; 500 μg/ml RNase A; 0.5 M Tris HCl, pH 7.0; 50 mM $MgCl_2$, aliquoted and stored at −70°C) was added. The mixtures were briefly vortexed and incubated on ice for 2 min or until the samples were no longer viscous. Samples were then adjusted to 0.5% (wt/vol) SDS, boiled for 1 min, incubated on ice for 3 min, and centrifuged for 10 min in a refrigerated microfuge. The supernatant was transferred to a clean microfuge tube and stored at −70°C. All samples had more than 2×10^6 trichloroacetic acid-precipitable cpm per μl. Volumes containing 5×10^6 cpm were analyzed by NEPHGE two-

dimensional electrophoresis. The final samples contained ≤0.071% SDS and 1.57% Nonidet P-40. To prevent fuzziness and artifactual splitting of spots in the lower third of the gel (7), a minimal amount of SDS-containing agarose glue was used to stick the first-dimension tube gel to the second-dimension slab gel (ca. 0.5 ml per 20-cm slab gel). Fluorography was carried out as described above.

Low-Ca^{2+} response properties. Bacterial growth and β-galactosidase activity expressed in Miller units from Mu dI11734 or Mu dI1(Ap *lac*) operon fusions were measured as described previously (19, 29, 34, 42). LcrH, Yops, and V antigen proteins were quantitated from the autoradiograms of the two-dimensional gels by using a Bio Image Visage 2000 Machine Vision densitometer and analytical software (Bio Image, Ann Arbor, Mich.).

Virulence tests in mice. The intravenous LD_{50} in BALB/c mice was determined for *lcrV Y. pestis*, for this mutant containing pAVA3, and for the reference *Y. pestis* KIM5-3173 containing pAVA3 by retro-orbitally injecting 0.1-ml bacterial doses (38, 44). The dosage groups of five mice were caged separately and monitored for 14 days. The doses ranged, in 10-fold increments, from ca. 10^0 to 5×10^7, on the basis of colony counts made from the bacterial suspensions. For the next-to-highest dose, an extra mouse was injected and sacrificed 1 day later for recovery of bacteria from its macerated liver and spleen. These were confirmed to be the challenge strains by antibiotic resistance, colony morphology, and plasmid profile.

RESULTS

Construction of an *lcrV Y. pestis*. To study the role of the V antigen in the low-Ca^{2+} response of *Y. pestis*, we constructed a deletion-insertion mutation that would affect only the structure of the *lcrV* product without altering the reading frame for *lcrV* or the expression of downstream genes. The deleted bases were well upstream of the non-Ca^{2+}-regulated putative transcription initiation sites for *lcrH*, which previously were found near the end of *lcrV* (37); hence, the deletion was not expected to prevent initiation at those sites. We made the mutation in a clone containing *lcrGVH* and then allowed recombination into pCD1 *yopJ*::Mu dI11734.

Figure 1 shows the minicell analysis of the starting clone pJIT7 and of pJIT7-8, the construct containing the deletion-insertion mutation. Also shown are the proteins expressed from pJIT7′, a construct which contained the deletion in *lcrV* but not the MCR insertion. In addition to the β-lactamase precursor and processed products from the pBR322 vector (indicated by Bla in Fig. 1), pJIT7 expressed the 37,230-Da V antigen, the 19,015-Da LcrH product, and the 11,021-Da LcrG protein encoded by the *Y. pestis lcrGVH* operon contained in the cloned *Hin*dIII G fragment of pCD1. The ca. 42,000-Da protein expressed by the clone may be YopB, whose gene also may be part of the *lcrGVH*-containing transcriptional unit (31). The identity of the 26,000-Da *Yersinia*-specific band is not certain; it may be the product of a cistron that was only partially contained in the cloned DNA.

From DNA sequence analysis, pJIT7′ was predicted to encode a 14,452-Da internally truncated LcrV protein. Except for full-length V antigen, this clone expressed all other clone-specific proteins plus a new species migrating at 13,200 Da, probably the internally truncated LcrV (Fig. 1, lane pJIT7′, arrow). We modified *lcrV* beyond the deletion present in pJIT7′ to provide a diagnostic hybridization signal and diagnostic restriction sites as an aid in the identification of the desired final mutation in *Y. pestis*. The inserted

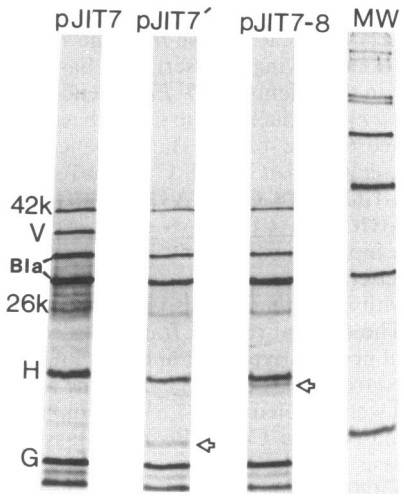

FIG. 1. Minicell analysis of clones used in the construction of the *lcrV* mutation in pCD1. Shown are the proteins expressed in *E. coli* minicells by the *lcrGVH*-containing *Hin*dIII G fragment of pCD1 cloned into pBR322 (pJIT7), by a derivative of pJIT7 lacking bases 51 to 645 of *lcrV* (pJIT7′), and by a further derivative having 61 new bases inserted into the site of the deletion (pJIT7-8). V, H, and G indicate the LcrV (V antigen), LcrH, and LcrG proteins; Bla indicates the precursor and processed β-lactamase proteins expressed by pBR322; and 42k and 26k indicate two additional insert-specific products. The open arrows indicate species thought to be products of the mutated *lcrV* genes. The MW lane contains (in daltons) ^{14}C-methylated lysozyme (14,300), carbonic anhydrase (30,000), ovalbumin (46,000), bovine serum albumin (69,000), phosphorylase *b* (92,500 and 100,000), and myosin (200,000).

portion of the MCR of pIC20R retained the reading frame for *lcrV*, and the internally truncated and inserted LcrV was predicted to be 16,726 Da and to have a pI of 5.86. The new protein species in minicells containing pJIT7-8 migrated as a 17,800-Da protein (Fig. 1, lane pJIT7-8, arrow). In two-dimensional gel electrophoresis, this protein was found in the expected pH region of the gel (data not shown). Importantly, the expression of LcrH and of the 42,000-Da species from cistrons downstream of *lcrV* were not affected by the *lcrV* mutation. The deletion-insertion mutation in pJIT7-8 was introduced into pCD1 *yopJ*::Mu dI11734 by allelic exchange.

Protein profiles. Figure 2 shows NEPHGE two-dimensional protein profiles for the *lcrV* mutant and reference *Y. pestis* strains grown at 37°C in the presence or absence of Ca^{2+}. As expected, the V antigen was absent from its normal position in the profiles for the *lcrV* mutant (circles in panels C and D). However, we were not able to identify the truncated product of the internally deleted and inserted *lcrV* in the gels. Its expression was weaker in *E. coli* minicells than the other *lcrGVH* products (Fig. 1), raising the possibility that it might not be as stable as the native V antigen; perhaps a similar situation existed in *Y. pestis*. Importantly, the mutant retained its ability to express LcrH, indicating that the mutation did not have a strong polar effect on the expression of downstream genes. LcrH and Yops showed decreased abundance in the presence of Ca^{2+} in both the mutant and reference *Y. pestis*. However, in the mutant, the expression of all of these proteins was lower than in the reference strain, both in the presence and absence of Ca^{2+}, an important regulatory consequence of the *lcrV* mutation that was seen in *Y. pestis* but not in *E. coli* (see Discussion).

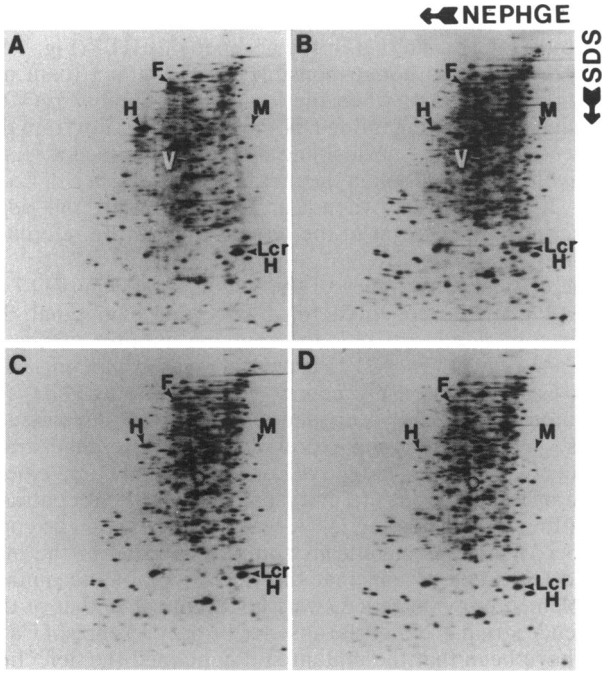

FIG. 2. Nonequilibrium two-dimensional protein profiles for the *lcrV* mutant and the reference *Y. pestis* strain. The yersiniae were grown at 37°C in defined medium containing or lacking Ca^{2+} and were labeled with [^{35}S]methionine 5 h after being placed at 37°C. Two-dimensional electrophoresis was carried out, with NEPHGE in the first dimension and uniform SDS–12% (wt/vol) polyacrylamide gel electrophoresis in the second dimension. Panels A and B, reference *Y. pestis* KIM5-3173; panels C and D, *lcrV Y. pestis* KIM5-3241.2. Panels A and C, no Ca^{2+}; panels B and D, 2.5 mM Ca^{2+}. The location of the V antigen is indicated by the white-backed arrowhead labeled V in panels A and B; its absence in the mutant is seen in the circled comparable locations in panels C and D. The identities of several other low-Ca^{2+} response proteins are also indicated.

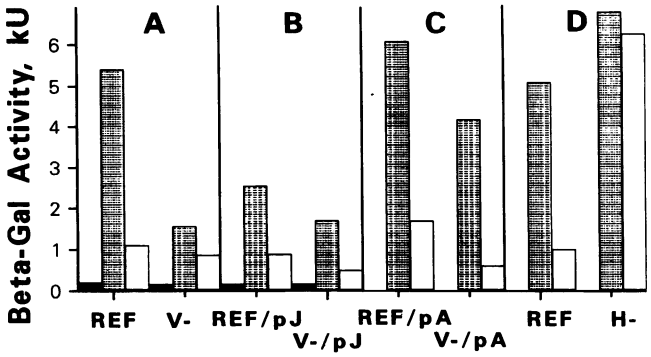

FIG. 3. β-galactosidase activities expressed by a transcriptional fusion to the *yopJ* operon in several *Y. pestis* strains. The yersiniae were grown in the defined medium TMH. Cells were harvested for measurements of β-galactosidase activity 4 h after being placed at 37°C or, for cells at 26°C, 4 h after reaching A_{620} of 0.2. Activities are expressed as Miller units (29). Solid bars (in panels A and B only), growth was at 26°C in TMH with no Ca^{2+}. Stippled bars, growth at 37°C with no Ca^{2+}. Open bars, growth at 37°C with 2.5 mM $CaCl_2$. (A) V−, the *lcrV Y. pestis* KIM5-3241.2; REF, the reference *Y. pestis* KIM5-3173. (B) V−/pJ, *Y. pestis* KIM5-3241.2 (pJIT7); REF/pJ, *Y. pestis* KIM5-3173 (pJIT7). The data of panel B were taken in the same experiment as those for panel A. (C) REF/pA, *Y. pestis* KIM5-3173 (pAVA3); V−/pA, *Y. pestis* KIM5-3241.2 (pAVA3). (D) (H−), the *lcrH Y. pestis* KIM5-3240; REF, the reference *Y. pestis* KIM5-3173.

For example, integrated optical density values (optical density × mm^2) from the autoradiograms of Fig. 2 were as follows: for LcrH, 3.93 (+Ca^{2+}) and 31.08 (−Ca^{2+}) in the reference strain versus 3.63 (+Ca^{2+}) and 6.48 (−Ca^{2+}) in the mutant; for YopH, 2.92 (+Ca^{2+}) and 29.06 (−Ca^{2+}) in the reference strain versus 1.62 (+Ca^{2+}) and 5.14 (−Ca^{2+}) in the mutant.

Low-Ca^{2+} response growth properties of the *lcrV Y. pestis*. The *lcrV* mutant was Ca^{2+} independent: it grew to full yield at 37°C whether or not Ca^{2+} was present (data not shown). In contrast, the reference strain *Y. pestis* KIM5-3173 demonstrated the Ca^{2+}-dependent growth typical of Lcr$^+$ *Y. pestis*, as it showed full growth yield at 37°C in the presence of Ca^{2+} but entered restriction when Ca^{2+} was absent. The mutant and reference strains grew the same at 26°C (data not shown). This surprising Ca^{2+}-independent growth of the *lcrV* mutant was the first indication in this study that the V antigen might play a regulatory role in the low-Ca^{2+} response.

Effect of the *lcrV* mutation on *yopJ* transcription. The *lcrV* mutation was constructed in a pCD1 plasmid that already contained an insertion of Mu dI11734 in *yopJ*, creating an operon fusion of *yopJ* to *E. coli lacZYA*. This insertion has no known effects on other low-Ca^{2+} response properties or on virulence of the bacteria in mice (42), but its presence allowed us to determine the effect of the *lcrV* mutation on transcription of a *yop* gene as indexed by β-galactosidase activity. We have previously shown that *yop* expression is regulated at the transcriptional level by Ca^{2+} (26, 42). Panel A of Fig. 3 shows a typical set of β-galactosidase measurements. Both the reference and *lcrV* mutant yersiniae showed a low basal level of *yopJ* expression at 26°C, maximal expression at 37°C in the absence of Ca^{2+}, and downregulation by Ca^{2+}. This shows that the Ca^{2+} regulation of transcription was still functional in the mutant, even though its growth was Ca^{2+} independent. However, under all growth conditions, the β-galactosidase activities shown by the mutant were lower than those of the parent. The small difference between the reference strain and the mutant at 37°C in the presence of Ca^{2+} was consistently seen: 939 ± 124 U for the reference strain (six experiments) versus 701 ± 119 U for the mutant (four experiments). These findings were consistent with the observations made at the protein level for expression of Ca^{2+}-regulated low-Ca^{2+} response products (Fig. 2) and indicated that the V antigen might play a role in the Ca^{2+}-related regulation of transcription in the low-Ca^{2+} response.

Growth restriction in complemented strains. Because the mutant phenotype was surprisingly complicated, we thought it imperative to determine whether its defects could be complemented by the native DNA in *trans*. We wanted to determine whether the Ca^{2+}-independent growth and decreased LcrH and Yop expression were due specifically to the *lcrV* mutation or whether, despite our precautions (see Materials and Methods), the final pCD1 derivative had acquired a point mutation elsewhere, perhaps in one of the Ca^{2+}-dependence genes. We tested two plasmids, the pBR322-derived pJIT7 and the pACYC184-derived pAVA3, for their ability to complement the *lcrV* mutation. Both contained the *Hin*dIII G fragment of pCD1, which contains the native Ca^{2+}-regulated *lcrGVH* promoter as well as the

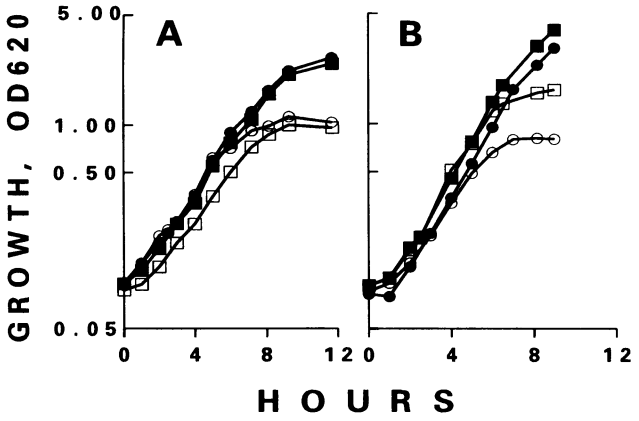

FIG. 4. Growth of the *lcrV* and reference *Y. pestis* strains containing complementing plasmids. The yersiniae were grown at 37°C as described in the legend for Fig. 3 (and the data for panel A were taken during that same experiment). In both panels, open symbols indicate that Ca^{2+} was absent; closed symbols indicate that 2.5 mM $CaCl_2$ was present. (A) ○ and ●, Reference *Y. pestis* KIM5-3173 (pJIT7); □ and ■, *lcrV Y. pestis* KIM5-3241.2 (pJIT7). (B) ○ and ●, Reference *Y. pestis* KIM5-3173 (pAVA3); □ and ■, the *lcrV Y. pestis* KIM5-3241.2 (pAVA3).

non-Ca^{2+}-regulated putative promoter upstream of *lcrH*. In *Yersinia pseudotuberculosis*, pBR322- and pACYC184-derived plasmids have relative copy numbers of ca. 10 and 1, respectively (18). Accordingly, it was possible that our two constructs might have different effects due to different copy numbers and that this might be informative about the regulation operating in the low-Ca^{2+} response.

Figure 4A shows the growth of the reference strain and of the *lcrV* mutant, both containing pJIT7. The presence of the complementing DNA in the mutant completely restored the normal restrictive response to incubation at 37°C in the absence of Ca^{2+}. In both the reference and the *lcrV* mutant *Y. pestis* containing pJIT7, there was a small but reproducible decrease in the final cell yield at 37°C in the presence of Ca^{2+} compared with their growth without the complementing plasmid. pBR322 alone had no significant effect on the growth of either strain (data not shown). These data show that the complementing DNA was sufficient to counteract the growth defect in the *lcrV* mutant and indicate that the growth defect was specifically due to the *lcrV* mutation and not to an adventitious mutation in another regulatory gene.

The lower-copy-number pAVA3 also caused restoration of Ca^{2+}-dependent growth to the mutant but did not have as complete an effect as pJIT7 did (Fig. 4B). We also tested the effect of pAVA3 on the growth of a mutant which was Ca^{2+} independent because of a Mu dI1(Ap *lac*) insert in the Ca^{2+}-dependence gene *lcrD*. Its growth at 37°C in the presence or absence of Ca^{2+} was unaffected by pAVA3 (data not shown). This was further evidence that our complementation test was valid: had the *lcrV* mutant been Ca^{2+} independent because of an adventitious defect in a Ca^{2+}-dependence gene, that defect would not have been complemented by the cloned *Hin*dIII G fragment of pCD1.

***yopJ* expression by the complemented strains.** Figure 3B shows the β-galactosidase activities expressed from the operon fusion to *yopJ* in the *lcrV* mutant and the reference *Y. pestis* complemented with pJIT7. The effect of the *lcrV* mutation on *yopJ* transcription was not counteracted by the high-copy-number clone, and in the reference strain, the maximally induced expression (at 37°C, no Ca^{2+}) was significantly lower than that in the absence of pJIT7 (Fig. 3A). pBR322 itself was not responsible for this effect (data not shown). In contrast, when the lower-copy-number pAVA3 supplied the complementing DNA, *yopJ* expression from the reference strain was normal and there was significant restoration of *yopJ* induction in the mutant in the absence of Ca^{2+} (Fig. 3C). However, when Ca^{2+} was present, the *yopJ* transcription was lower in the mutant than in the reference strain.

The distinct phenotypes of the yersiniae having pJIT7 or pAVA3 can be accounted for by hypothesizing that the higher-copy-number pJIT7, in supplying multiple copies of the Ca^{2+}-regulated *lcrGVH* promoter, was competing with other Ca^{2+}-regulated promoters, such as that of *yopJ*, for activator components encoded by the Ca^{2+}-dependence genes. Accordingly, *yopJ* expression in the reference strain was decreased under inductive conditions, and complementation of the defect in the mutant was masked. In contrast, when the complementing DNA was supplied in lower copy number, there was significant complementation of the mutant defect. The reason that the phenotype of the mutant complemented with pAVA3 was not identical to that of the reference strain in either the absence or the presence of Ca^{2+} may have been that the mutant still contained the defective copy of *lcrV*, whereas all copies of *lcrGVH* in the reference strain were normal (see Discussion).

Virulence tests. The *lcrV* mutant was avirulent in mice, with an LD_{50} of more than 5×10^6. When this mutant contained the low-copy-number complementing clone pAVA3, it was substantially restored in virulence, with an LD_{50} of 3.8×10^2. (The intravenous LD_{50} of the reference strain is 1.2×10^2 [42].) Interestingly, however, pAVA3 caused the reference strain to be decreased in virulence, with an LD_{50} of 2.0×10^4.

DISCUSSION

In this study, we constructed and characterized a specifically V antigen-negative *Y. pestis* to determine the role of V antigen in the low-Ca^{2+} response. Our findings are consistent with the surprising conclusion that the V antigen is a bifunctional protein with a role in regulation as well as virulence.

The V antigen originally was discovered as a diffusible protein present in cultures of virulent *Y. pestis* (11, 12). Its release into the medium apparently is specific, as it appears along with two Yops in cell-free supernatants containing no detectable cytoplasmic proteins when *Y. pestis* is grown at 37°C in the absence of Ca^{2+} or nucleotides (41). It is not accumulated in the outer membrane fractions of such bacteria, and indeed only trace amounts can be detected by ^{125}I-surface-labeling experiments that easily demonstrate the presence of Yops in outer membranes (41a, 43). Accordingly, it is significant that V antigen is protective in both active and passive immunization (25, 46): this protein apparently functions as an antihost effector component of the low-Ca^{2+} response. Further, Une and Brubaker (46) concluded that V antigen per se was a virulence factor, because injection of an extract containing V antigen into mice given Lcr$^-$, plasmid-cured derivatives of *Y. pestis* KIM, *Y. pseudotuberculosis* PB1, or *Y. enterocolitica* WA increased retention of the bacteria in spleens. Our finding that the *lcrV Y. pestis* mutant was avirulent in mice is consistent with this conclusion. However, the complex phenotype of this mutant makes our data difficult to interpret. It is possible that the

mutant was avirulent because of its diminished Yop expression as well as its inability to express normal V antigen. Overall, the experimental evidence favors the interpretation that V antigen is a virulence determinant with antihost properties.

In addition to the extracellular pool of V antigen, there is a significantly larger cytoplasmic pool of the protein (43, 46, 49), making intact cells the material of choice for its isolation (9). Our data indicate that this intracellular V antigen affects the growth of the bacteria and modulates Yop expression. The *lcrV Y. pestis* had lost the Ca^{2+} and nucleotide requirements for growth, but supplying the normal V gene in high copy number in *trans* restored the restrictive response. This indicates that the expression of V antigen is involved in the growth manifestation of the low-Ca^{2+} response and is consistent with our previous finding that a Mu dI1(Ap *lac*) insertion in *lcrG*, the first gene in the V antigen operon, caused a Ca^{2+}- and nucleotide-independent growth phenotype (34). In addition to abolishing expression of LcrG, that insertion eliminated V antigen expression by a polar effect (34). At that time, we hypothesized that the V antigen operon contains at least one Ca^{2+}-dependence gene. The LcrH protein is eliminated as necessary for growth restriction, because a specifically engineered $LcrH^-$ mutant of *Y. pestis* does not display a Ca^{2+}- and nucleotide-independent growth phenotype (38). The observations in the present study suggest that the Ca^{2+}-independent growth phenotype can be generated by specifically abolishing expression of normal-sized V antigen. Even though Yop expression was diminished in our mutant, we have previously shown that expression of individual Yops can be completely abolished by transposon insertions with no effect on the Ca^{2+}-dependent growth of the bacteria (42). Accordingly, we hypothesize that strong expression of V antigen is necessary for the restrictive response.

The significant decrease in Yop and LcrH expression in the *lcrV* mutant indicates that V antigen also plays a positive regulatory role in the expression of Ca^{2+}- and nucleotide-regulated operons. The data are consistent with the interpretation that V antigen functions to counteract the negative regulatory function of LcrH. Our previous work had shown that LcrH mediates the downregulating effect of ATP and contributes to the downregulation by Ca^{2+} (38). Since the $LcrH^-$ *Y. pestis* mutant has the same genetic background (38) as strains used in the present study, we repeated the measurement of *yopJ* transcription as indexed by β-galactosidase expression and included the data in Fig. 3D. As previously shown, the *lcrH* mutant failed to downregulate *yopJ* transcription in the presence of Ca^{2+}, consistent with a negative effector role of LcrH on Yop expression. LcrH also functions in the Ca^{2+}- and nucleotide-mediated negative regulation of its own V antigen operon, presumably acting at the Ca^{2+}-regulated promoter upstream of *lcrG* (37, 38). Hence, at 37°C when Ca^{2+} is present, LcrH acts like a repressor for the low-Ca^{2+} response operons that are regulated at the transcriptional level by Ca^{2+} and nucleotides. Consistent with this idea, the mutant with a Mu dI1(Ap *lac*) insert in *lcrG*, which was unable to express LcrH from the strong, Ca^{2+}-regulated *lcrGVH* promoter, showed remarkable overexpression of a set of proteins which we now know are the Yops (Fig. 5 in reference 34).

A major question left unanswered by the previous work was how the negative function of LcrH is eliminated at 37°C in the absence of Ca^{2+}, conditions that are strongly inductive for expression of the Ca^{2+}- and nucleotide-regulated genes, including *lcrH*. We hypothesize that the intracellular pool of V antigen serves to counteract LcrH's negative function under inductive conditions.

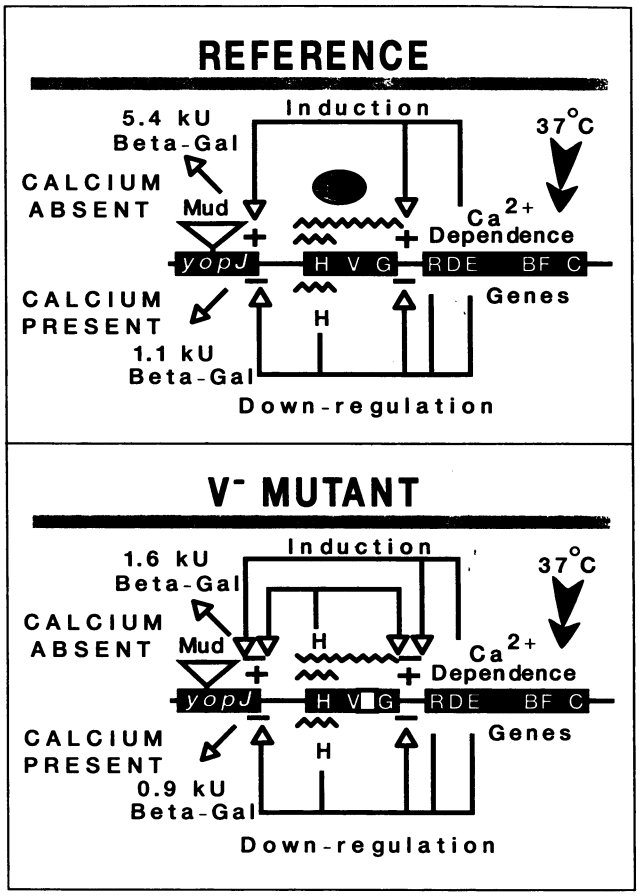

FIG. 5. Models for low-Ca^{2+} response regulation, incorporating findings from this study. Temperature is indicated as an initiating stimulus; however, pathways for thermal regulation are not otherwise shown. The shaded boxes indicate genetic loci, and the letters within these indicate low-Ca^{2+} response genes. A box labeled *yopJ* represents the *yopJ* operon, which is coordinately regulated along with the several other *yop* operons (not shown). The triangle over *yopJ* indicates an insertion of Mu dI1(Ap *lac*) in *yopJ*, fusing this operon to *lacZYA*. Beta-Gal, β-galactosidase. Arrows from the genes indicate positive (+) and negative (−) regulatory effects of their gene products on expression of the V antigen operon *lcrGVH* and *yop* operons. The wavy lines indicate mRNAs for *lcrGVH*. These have been mapped for initiation sites but not for their 3′ ends (36). Although *lcrGVH* belongs to the Ca^{2+}-dependence region because of its regulatory functions, it is enlarged and set apart in this diagram for the sake of clarity and emphasis. Top panel, the regulation in the reference strain *Y. pestis* KIM5-3173 used in this study. Bottom panel, model for regulation in the *lcrV* mutant *Y. pestis* strain constructed in this study.

Figure 5 diagrams these ideas. For the sake of simplicity, only regulation by temperature and Ca^{2+} are considered. Temperature acts through Ca^{2+}-dependence genes, including *lcrF*, to induce strong expression of Ca^{2+}-regulated low-Ca^{2+} response operons, including the V antigen operon and *yop* operons (19, 47). LcrH is hypothesized to be expressed both from the Ca^{2+}- and nucleotide-regulated promoter upstream of the entire V antigen operon and from its own non-Ca^{2+}-regulated promoter that lies within the 3′ end of *lcrV* (37). In the presence of millimolar Ca^{2+}, at least

three genes, *lcrE*, *lcrR*, and *lcrH*, participate in bringing about downregulation of Ca^{2+}-regulated operons (2, 38, 48). Under these conditions, LcrH, like the V antigen, is thought to be expressed only weakly from the Ca^{2+}-regulated promoter; however, its continued function is thought to be ensured by its expression from the non-Ca^{2+}-regulated *lcrH* transcriptional unit (37).

In the reference *Y. pestis* strain (Fig. 5, top), strong LcrH expression from the Ca^{2+}-regulated promoter is accompanied by strong V antigen expression. This is hypothesized to neutralize the negative regulatory function of LcrH under those conditions and permit strong net expression of the Ca^{2+}-regulated genes, such as those encoding V antigen and Yops. Hence, at 37°C in the absence of Ca^{2+}, there was strong β-galactosidase expression from the *yopJ*::Mu dI1(Ap *lac*) operon fusion and strong V antigen and Yop expression as seen in two-dimensional protein profiles. In the presence of Ca^{2+}, LcrH acts as a negative regulator. Accordingly, *yopJ* transcription was downregulated ca. fivefold, as determined by β-galactosidase activity, and V antigen and Yops were found to be less abundant in the protein profiles.

In the *lcrV* mutant (Fig. 5, bottom) there is no functional V antigen to counteract the negative action of LcrH under inductive conditions, and the Ca^{2+}-regulated operons (including the one containing *lcrH* itself) are expressed at a lower level in the mutant than in the reference strain. Hence, there was ca. 3.5-fold-lower β-galactosidase activity from the *yopJ*::Mu dI1(Ap *lac*) fusion in the mutant than from the reference strain, and LcrH and Yops were seen at lower levels in two-dimensional protein profiles for the mutant than in those for the reference. Ca^{2+} evidently permits stronger negative regulation by LcrH than can occur in the absence of this cation, because the mutant showed downregulation of its Ca^{2+}-regulated operons. Further, the mutant lacked the low amount of V antigen expression that would occur from the Ca^{2+}-downregulated transcript in the reference strain and accordingly lacked a small counteracting effect on LcrH even in the presence of Ca^{2+}. Hence, the mutant expressed slightly, but reproducibly, lower β-galactosidase activity from the operon fusion than did the reference strain in the presence of Ca^{2+}, and Yop and net LcrH expression also were seen to be lower in the mutant than in the reference in two-dimensional protein profiles.

In the *lcrV* mutant strain containing the low-copy-number pAVA3, the complementation was not complete, perhaps because a large fraction of the total LcrH expression was unaccompanied by expression of V antigen (because of the mutant operon present in pCD1). In contrast, when many copies of the V antigen operon were provided in *trans* by the high-copy-number pJIT7, the multiple normal copies of the operon probably successfully competed with the single mutant operon's promoter for activating components, and the majority of LcrH expression would be accompanied by V antigen expression.

Our data did not reveal the mechanisms of V antigen and LcrH action. At this writing, there is no evidence that either protein directly interacts with DNA or with RNA polymerase. Our searches of the data bases for sequence similarities (37) did find the C-terminal end of the *Bacillus* bacteriophage SP01 sigma factor (GP28) to be the best match for V antigen (residues 128 through 218 of GP28 with residues 11 through 104 of V antigen; 24% identity in a 95-amino acid overlap). However, the match failed the normalized alignment score test of significance (15, 16; unpublished data). It is interesting that there was no significant effect of *lcrV* mutations on expression of LcrG or LcrH from pJIT7′ or pJIT7-8 in *E. coli* (Fig. 1), whereas LcrH expression was diminished in *Y. pestis*. One possible explanation is that V antigen and LcrH have to interact with another *lcr* gene product(s) not present in the cloned DNA to have their effects. Clearly, direct tests for possible mechanisms of these proteins are needed. However, this study has revealed a surprising regulatory role of V antigen and has opened possible directions for future investigation.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Barve, S. S., and S. C. Straley.** 1990. *lcrR*, a low-Ca^{2+}-response locus with dual Ca^{2+}-dependent functions in *Yersinia pestis*. J. Bacteriol. **172**:4661–4671.
2a. **Barve, S. S., and S. C. Straley.** Unpublished data.
3. **Ben-Gurion, R., and A. Shafferman.** 1981. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. Plasmid **5**:183–187.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–267.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
7. **Bravo, R.** 1984. Two-dimensional gel electrophoresis: a guide for the beginner, p. 3–36. *In* J. E. Celis and R. Bravo (ed.), Two-dimensional gel electrophoresis of proteins. Methods and applications. Academic Press, Inc., New York.
8. **Brubaker, R. R.** 1986. Low-calcium response of virulent yersiniae, p. 43–48. *In* L. Lieve, P. F. Bonventre, J. A. Morello, S. D. Silver, and H. C. Wu (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
9. **Brubaker, R. R., A. K. Sample, D. Z. Yu, R. J. Zahorchak, P. C. Hu, and J. M. Fowler.** 1987. Proteolysis of V antigen from *Yersinia pestis*. Microb. Pathog. **2**:49–62.
10. **Brubaker, R. R., and M. J. Surgalla.** 1964. The effect of Ca^{++} and Mg^{++} on lysis, growth, and production of virulence antigens by *Pasteurella pestis*. J. Infect. Dis. **114**:13–25.
11. **Burrows, T. W.** 1956. An antigen determining virulence in *Pasteurella pestis*. Nature (London) **177**:426–427.
12. **Burrows, T. W., and G. A. Bacon.** 1956. The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. Br. J. Exp. Pathol. **37**:481–493.
13. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
14. **Clark-Curtiss, J. E., and R. Curtiss III.** 1983. Analysis of recombinant DNA using *Escherichia coli* minicells. Methods Enzymol. **101**:347–362.
15. **Doolittle, R. F.** 1981. Similar amino acid sequences: chance or common ancestry? Science **214**:149–159.
16. **Doolittle, R. F., D. F. Feng, M. S. Johnson, and M. A. McClure.** 1986. Relationships of human protein sequences to those of other organisms. Cold Spring Harbor Symp. Quant. Biol. **51**:

447–455.
17. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **27**:839–841.
18. **Forsberg, A., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-pIB1-encoded *trans*-acting elements controlled by temperature and calcium. Mol. Microbiol. **2**:237–245.
19. **Goguen, J. D., J. Yother, and S. C. Straley.** 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu d1(Ap *lac*) insertion mutants. J. Bacteriol. **160**:842–848.
20. **Higuchi, K., L. L. Kupferberg, and J. L. Smith.** 1959. Studies on the nutrition and physiology of *Pasteurella pestis*. III. Effects of calcium ions on the growth of virulent and avirulent strains of *Pasteurella pestis*. J. Bacteriol. **77**:317–321.
21. **Isberg, R. R., J. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50**:769–778.
22. **Jacobs, W. R., J. F. Barrett, J. E. Clark-Curtiss, and R. Curtiss III.** 1986. In vivo repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium*, *Streptococcus mutans*, and mycobacterial genomic libraries. Infect. Immun. **52**: 101–109.
23. **Krueger, J. H., and G. C. Walker.** 1983. Mud(Ap *lac*)-generated gene fusions in studies of gene expression. Methods Enzymol. **100**:501–509.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **Lawton, W. D., R. L. Erdman, and M. J. Surgalla.** 1963. Biosynthesis and purification of V and W antigen in *Pasteurella pestis*. J. Immunol. **91**:179–184.
26. **Leung, K. Y., B. S. Reisner, and S. C. Straley.** 1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. Infect. Immun. **58**:3262–3271.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
29. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
31. **Mulder, B., T. Michiels, M. Simonet, M.-P. Sory, and G. Cornelis.** 1989. Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. Infect. Immun. **57**:2534–2541.
32. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.
33. **Perry, R. D., and R. R. Brubaker.** 1987. Transport of Ca^{2+} by *Yersinia pestis*. J. Bacteriol. **169**:4861–4864.
34. **Perry, R. D., P. A. Harmon, W. S. Bowmer, and S. C. Straley.** 1986. A low-Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. Infect. Immun. **54**:428–434.
35. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
36. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
37. **Price, S. B., K. Y. Leung, S. S. Barve, and S. C. Straley.** 1989. Molecular analysis of *lcrGVH*, the V antigen operon of *Yersinia pestis*. J. Bacteriol. **171**:5646–5653.
38. **Price, S. B., and S. C. Straley.** 1989. *lcrH*, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. Infect. Immun. **57**:1491–1498.
39. **Rosqvist, R., A. Forsberg, M. Rimpilainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic YopE of *yersinia* obstructs the primary host defense. Mol. Microbiol. **4**:657–667.
40. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
41. **Straley, S. C.** 1988. The plasmid-encoded outer-membrane proteins of *Yersinia pestis*. Rev. Infect. Dis. **10**:S323–S326.
41a. **Straley, S. C.** Unpublished data.
42. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51**:445–454.
43. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. USA **78**:1224–1228.
44. **Straley, S. C., and M. L. Cibull.** 1989. Differential clearance and host-pathogen interactions of $YopE^-$ and $YopK^-$ $YopL^-$ *Yersinia pestis* in BALB/c mice. Infect. Immun. **57**:1200–1210.
45. **Une, T., and R. R. Brubaker.** 1984. In vivo comparison of arivulent Vwa^- and Pgm^- or Pst^r phenotypes of yersiniae. Infect. Immun. **43**:895–900.
46. **Une, T., and R. R. Brubaker.** 1984. Roles of V antigen in promoting virulence and immunity in yersiniae. J. Immunol. **133**:2226–2230.
47. **Yother, J., T. W. Chamness, and J. D. Goguen.** 1986. Temperature-controlled plasmid regulon associated with low-calcium response in *Yersinia pestis*. J. Bacteriol. **165**:443–447.
48. **Yother, J., and J. D. Goguen.** 1985. Isolation and characterization of Ca^{2+}-blind mutants of *Yersinia pestis*. J. Bacteriol. **164**:704–711.
49. **Zahorchak, R. J., and R. R. Brubaker.** 1982. Effect of exogenous nucleotides on Ca^{2+} dependence and V antigen synthesis in *Yersinia pestis*. Infect. Immun. **38**:953–959.
50. **Zahorchak, R. J., W. T. Charnetzky, R. V. Little, and R. R. Brubaker.** 1979. Consequences of Ca^{2+} deficiency on macromolecular synthesis and adenylate energy charge in *Yersinia pestis*. J. Bacteriol. **139**:792–799.