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 *yersinia* 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 *yersinia* 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 (10, 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 *yersinia* 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 *yersinia* 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 d11 (Ap lac) in the first cistron caused the *yersinia* 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 nonpolymur 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\textsuperscript{+} response in both causing restriction and promoting virulence gene expression.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. Escherichia coli K-12 strains HB101 (5), x1553 (F\textsuperscript{−} minAl purE2 supE42 pxdc3 minB2 his-s5 nala28 metC65 T3\textsuperscript{r} ilv-277 cycB2 cycA1 hsdR2), and y\textsubscript{2}3388 (22) were obtained from Roy Curtiss III, Washington University, St. Louis, Mo. E. coli K-12 strain SY327 \textsuperscript{\textregistered} was obtained from Virginia Miller, University of California, Los Angeles, Calif. E. coli K-12 strain GM2163 (F\textsuperscript{−} ara-14 leuB6 tonA13 lacY1 tsx78 supE44 galK2 lac\textsuperscript{−} dcm-6 hisG4 rpsL136 dam-13::Tn9 yxl-5 mut-1 thi-1 hsdR2) was obtained from the E. coli Genetic Stock Center (Yale University, New Haven, Conn.). E. coli K-12 strain DH5\textalpha{} was obtained from Bethesda Research Laboratories (Bethesda, Md.). Their cultivation has been described previously (19, 34, 42); chloramphenicol was used at 10 \mu{}g/ml to select for retention of the Tn9 insertion in strain GM2163.

Y. pestis KIM strains lack the pigment-encoding 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\textsuperscript{−}, 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\textsuperscript{+} but YopJ\textsuperscript{−} because of an insertion of Mu d111734 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\textsubscript{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\textsuperscript{−} mutant originally designated mutant 44.6 (for pCD1::Mu d111[Ap lac]44.6) (19). It has a Mu d111[Ap lac] insert in lcrD (2a, 19), conferring resistance to ampicillin, (Ap\textsuperscript{+}), 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\textsubscript{2} (20 mM) was always included, and 2.5 mM CaCl\textsubscript{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 \mu{}g/ml (kanamycin, tetracycline, chloramphenicol) or 100 \mu{}g/ml (ampicillin). Y. pestis KIM5-3173 and V antigen-negative derivatives described below were unable to grow in TMH containing kanamycin at 25 \mu{}g/ml if the concentration of MgCl\textsubscript{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\textsuperscript{+} response, we either used a lower concentration of kanamycin (10 \mu{}g/ml) or omitted it altogether, even though we did not use a low MgCl\textsubscript{2} concentration in the experiments reported herein. This had no detrimental effect, because the pCD1::Mu d111734 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 \mu{}g/ml in experiments where low-Ca\textsuperscript{+} response properties were being characterized for Y. pestis strains that were Ap\textsuperscript{+}.

Previously described plasmids used in this study were pCD1 (19), pCD1 yopJ::Mu d111734 (38), pBR322 (6), pJJT7 (34), pGW600 (23), pIC20R (28), and pJM703.1 (21, 30). pJJT7 has the lcrGVH-containing HindIII G fragment of pCD1 cloned into the HindIII site of pBR322, and pJM703.1 is a suicide vector, able to replicate only in bacteria, such as E. coli SY327 \textsuperscript{\textregistered} kip, that supply the pir gene product needed for its replication. pAVA3 has the HindIII G fragment of pCD1 cloned into the HindIII site of pAVA1, a version of pACYC184 (13) which had been modified by nuclease S1 digestion to eliminate the unique AvaI 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 pJJT7\textsuperscript{+}, pJJT7-8, pVS1, and pCD1 lcrV(51-645)::MCR yopJ::Mu d111734.

Bacteriophage PIL4, which infects but does not lysogenize or lytically infect Y. pestis (19), was used to transduce pCD1 lcrV(51-645)::MCR yopJ::Mu d111734 from E. coli x1553 into Y. pestis KIM6.

\textbf{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). pJJT7 produced in the dam dcm E. coli GM2163 was cleaved with XbaI at a unique site 45 bases downstream from the ATG of lcrV and with XhoI 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 pJJT7\textsuperscript{−}, this deletion derivative of pJJT7 was blunt-end ligated. After transformation into E. coli DH5\textalpha{}, plasmid DNA was isolated and confirmed to have the anticipated structure by restriction analysis. The lcrV in pJJT7\textsuperscript{−} was predicted from the lcrV DNA sequence (37) to have undergone an in-frame deletion.

\textbf{In-frame deletion-insertion in lcrV.} An 81-base Clal-EcoRI fragment was excised from the multiple cloning region (MCR) of pIC20R and ligated by electrophoretic separation on a polyacylamide gel (12% [wt/vol] acrylamide) and then eluted by diffusion (37°C overnight) from mined 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 pJJT7 by XbaI-XhoI digestion (pJJT7\textsuperscript{−}). After transformation into an E. coli DH5\textalpha{}, the resulting construct, pJJT7-8, 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 pJJT7-8, lcrV had undergone in-frame deletion-insertion events. During
the construction, several bases had been lost from both ends of the Clal-EcoRI MCR: 1 base from the EcoRI end and 17 from the Clal end, giving an insert of 63 bases, present in the deleted lcrV in the orientation 5' EcoRI end–Clal 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 GAG GCG TTT CGC GAG cTC GAG

New England Nuclear Corp., Boston, Mass.) and boiled in water near one HindIII end (the site within the insert with the native sequence. The original reading frame was retained.

This construct, pVS1, was isolated, blunt ended with T4 DNA ligase, and ligated into EcoRV-cut and Aps ClaI-EcoRI restriction fragments of the plasmid profile for determining the effects of the lcrV mutation was Y. pestis KIM5-3173, in which the pCD1 derivative contained the deleted and inserted lcrV and an insertion of Mu dII11734 in yopJ. Accordingly, the appropriate reference strain for determining the effects of the lcrV mutation was Y. pestis KIM5-3173. Its pCD1 derivative contained the wild-type lcrV and the same insertion of Mu dII11734 in yopJ. That strain will be used as 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 2.5 mM CaCl2 and having 10× lower methionine (final concentration, 0.1 mM) to permit labeling to high specific activity with 35S-methionine. After ca. six generations of pregrowth in exponential phase at 26°C, the temperature was shifted to 37°C when the A620 of the culture reached ca. 0.1 for the lcrV mutant and for the reference strain growing in the presence of Ca2+. When the A620 reached ca. 0.2 for the reference strain in the absence of Ca2+. Five hours later, at an A620 of ca. 1.0, 1-ml amounts of all cultures were labeled with 35S-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 MgCl2, 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 MgCl2) per ca. 6 × 106 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 MgCl2, 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 microtube and stored at -70°C. All samples had more than 2 × 106 trichloroacetic acid-precipitable cpm per μl. Volumes containing 5 × 106 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, a minimal amount of SDS-containing agarose glue was used to stick the first-dimensional 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 d11734 or Mu d11(Ap lac) operon fusions were measured as described previously (19, 29, 34, 42). LcrH, Yops, and V antigen proteins were quantified 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. $10^6$ to $5 \times 10^7$, 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 yop J:Mu d11734.

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 HindIII 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 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 pl 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 yop J:Mu d11734 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).
V ANTIGEN OF Y. PESTIS

FIG. 2. Nonequilibrium two-dimensional protein profiles for the lcrV mutant and the reference Y. pestis strain. The yersinia were grown at 37°C in defined medium containing or lacking Ca²⁺ and were labeled with [³⁵S]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% (w/vol) polyacrylamide gel electrophoresis in the second dimension. Panels A and B, reference Y. pestis KIMS-3173; panels C and D, lcrV Y. pestis KIMS-3241.2. Panels A and C, no Ca²⁺; panels B and D, 2.5 mM Ca²⁺. 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-Ca²⁺-response proteins are also indicated.

For example, integrated optical density values (optical density × mm) from the autoradiograms of Fig. 2 were as follows: for LcrH, 3.93 (+Ca²⁺) and 3.08 (−Ca²⁺) in the reference strain versus 3.63 (+Ca²⁺) and 6.48 (−Ca²⁺) in the mutant; for YopH, 2.92 (+Ca²⁺) and 29.06 (−Ca²⁺) in the reference strain versus 1.62 (+Ca²⁺) and 5.14 (−Ca²⁺) in the mutant.

Low-Ca²⁺ response growth properties of the lcrV Y. pestis. The lcrV mutant was Ca²⁺ independent: it grew to full yield at 37°C whether or not Ca²⁺ was present (data not shown).

In contrast, the reference strain Y. pestis KIMS-3173 demonstrated the Ca²⁺-dependent growth typical of LcrV Y. pestis, as it showed full growth yield at 37°C in the presence of Ca²⁺ but entered restriction when Ca²⁺ was absent. The mutant and reference strains grew the same at 26°C (data not shown). This surprising Ca²⁺-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-Ca²⁺ response.

Effect of the lcrV mutation on yopJ transcription. The lcrV mutation was constructed in a pCDI 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-Ca²⁺ 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 the yopJ gene as indexed by β-galactosidase activity. We have previously shown that yop expression is regulated at the transcriptional level by Ca²⁺ (26, 42). Panel A of Fig. 3 shows a typical set of β-galactosidase measurements. Both the reference and lcrV mutant yersinia showed a low basal level of yopJ expression at 26°C, maximal expression at 37°C in the absence of Ca²⁺, and downregulation by Ca²⁺. This shows that the Ca²⁺ regulation of transcription was still functional in the mutant, even though its growth was Ca²⁺ independent. However, under all growth conditions, the β-galactosidase activities shown by the mutant were lower than those of the parent. The small difference in the expression between the reference strain and the mutant at 37°C in the presence of Ca²⁺ 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 Ca²⁺-regulated low-Ca²⁺ response products (Fig. 2) and indicated that the V antigen might play a role in the Ca²⁺-related regulation of transcription in the low-Ca²⁺ 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 Ca²⁺-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 Ca²⁺-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 HindIII G fragment of pCD1, which contains the native Ca²⁺-regulated lcrGVH promoter as well as the
FIG. 4. Growth of the lcrV and reference Y. pestis strains containing complementing plasmids. The Yersinia 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 Ca²⁺ was absent; closed symbols indicate that 2.5 mM CaCl₂ was present. Refer to Methods for strain designation. (A) Growth of Y. pestis KIM5-3173 (pJIT7); ○ and ●, lcrV Y. pestis KIMS-3241.2 (pJIT7). (B) ○ and ●, Reference Y. pestis KIM5-3173 (pAVA3); ○ and ●, the lcrV Y. pestis KIMS-3241.2 (pAVA3).

non-Ca²⁺-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-Ca²⁺ 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 Ca²⁺. 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 Ca²⁺ 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 Ca²⁺-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 Ca²⁺ independent because of a Mu dII(Ap lac) insert in the Ca²⁺-dependence gene lcrD. Its growth at 37°C in the presence or absence of Ca²⁺ was unaffected by pAVA3 (data not shown). This was further evidence that our complementation test was valid: had the lcrV mutant been Ca²⁺ independent because of an adventitious defect in a Ca²⁺-dependence gene, that defect would not have been complemented by the cloned HindIII 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 Ca²⁺) 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 Ca²⁺ (Fig. 3C). However, when Ca²⁺ was present, the yopJ transcription was lower in the mutant than in the reference strain.

The distinct phenotypes of the yersinia having pJIT7 or pAVA3 can be accounted for by hypothesizing that the higher-copy-number pJIT7, in supplying multiple copies of the Ca²⁺-regulated lcrGVH promoter, was competing with other Ca²⁺-regulated promoters, such as that of yopJ, for activator components encoded by the Ca²⁺-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 Ca²⁺ 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 LD₅₀ of more than 5 × 10⁶. When this mutant contained the low-copy-number complementing clone pAVA3, it was substantially restored in virulence, with an LD₅₀ of 3.8 × 10⁵. (The intravenous LD₅₀ of the reference strain is 1.2 × 10⁶ [42].) Interestingly, however, pAVA3 caused the reference strain to be decreased in virulence, with an LD₅₀ of 2.0 × 10⁶.

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-Ca²⁺ 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 Ca²⁺ or nucleotides (41). It is not accumulated in the outer membrane fractions of such bacteria, and indeed, only trace amounts can be detected by ¹²⁵I-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-Ca²⁺ 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 Ca²⁺ 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-Ca²⁺ response and is consistent with our previous finding that a Mu dIl(Ap lac) insertion in lcrG, the first gene in the V antigen operon, caused a Ca²⁺- 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 Ca²⁺-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 Ca²⁺- and nucleotide-independent growth phenotype (38). The observations in the present study suggest that the Ca²⁺-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 Ca²⁺-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 Ca²⁺- 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 Ca²⁺ (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 Ca²⁺, consistent with a negative effector role of LcrH on Yop expression. LcrH also functions in the Ca²⁺- and nucleotide-mediated negative regulation of its own V antigen operon, presumably acting at the Ca²⁺-regulated promoter upstream of lcrG (37, 38). Hence, at 37°C when Ca²⁺ is present, LcrH acts like a repressor for the low-Ca²⁺ response operons that are regulated at the transcriptional level by Ca²⁺ and nucleotides. Consistent with this idea, the mutant with a Mu dIl(Ap lac) insert in lcrG, which was unable to express LcrH from the strong, Ca²⁺-regulated lcrGHV 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 Ca²⁺, conditions that are strongly inductive for expression of the Ca²⁺- 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.

Figure 5 diagrams these ideas. For the sake of simplicity, only regulation by temperature and Ca²⁺ are considered. Temperature acts through Ca²⁺-dependence genes, including lcrF, to induce strong expression of Ca²⁺-regulated low-Ca²⁺ response operons, including the V antigen operon and yop operons (19, 47). LcrH is hypothesized to be expressed both from the Ca²⁺- and nucleotide-regulated promoter upstream of the entire V antigen operon and from its own non-Ca²⁺-regulated promoter that lies within the 3′ end of lcrV (37). In the presence of millimolar Ca²⁺, at least
three genes, lcrE, lcrR, and lcrH, participate in bringing about downregulation of Ca\textsuperscript{2+}-regulated operons (2, 38, 48). Under these conditions, LcrH, like the V antigen, is thought to be expressed only weakly from the Ca\textsuperscript{2+}-regulated promoter; however, its continued function is thought to be ensured by its expression from the non-Ca\textsuperscript{2+}-regulated lcrH transcriptional unit (37).

In the reference Y. pestis strain (Fig. 5, top), strong LcrH expression from the Ca\textsuperscript{2+}-regulated promoter is accompanied by strong V antigen expression. This is hypothesized to neutralize the negative regulatory function of LcrH under these conditions and permit strong net expression of the Ca\textsuperscript{2+}-regulated genes, such as those encoding V antigen and Yops. Hence, at 37°C in the absence of Ca\textsuperscript{2+}, there was strong β-galactosidase expression from the yopJ::Mu dII(Ap lac) operon fusion and strong V antigen and Yop expression as seen in two-dimensional protein profiles. In the presence of Ca\textsuperscript{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\textsuperscript{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 dII(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\textsuperscript{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\textsuperscript{2+}-regulated operons. Further, the mutant lacked the low amount of V antigen expression that would occur from the Ca\textsuperscript{2+}-downregulated transcript in the reference strain and accordingly lacked a small counteracting effect on LcrH even in the presence of Ca\textsuperscript{2+}. Hence, the mutant expressed slightly, but reproducibly, lower β-galactosidase activity from the operon fusion than did the reference strain in the absence of Ca\textsuperscript{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 pJT7, 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 pJT7’ or pJT7-8 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


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