

YopD of *Yersinia pestis* Plays a Role in Negative Regulation of the Low-Calcium Response in Addition to Its Role in Translocation of Yops

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Yersinia pestis produces a set of virulence proteins (Yops and LcrV) that are expressed at high levels and secreted by a type III secretion system (Ysc) upon bacterium-host cell contact, and four of the Yops are vectorially translocated into eukaryotic cells. YopD, YopB, and YopK are required for the translocation process. In vitro, induction and secretion occur at 37°C in the absence of calcium. LcrH (also called SycD), a protein required for the stability and secretion of YopD, had initially been identified as a negative regulator of Yop expression. In this study, we constructed a *yopD* mutation in both wild-type and secretion-defective (*ysc*) *Y. pestis* to determine if the *lcrH* phenotype could be attributed to the decreased stability of YopD. These mutants were constitutively induced for expression of Yops and LcrV, despite the presence of the secreted negative regulator LcrQ, demonstrating that YopD is involved in negative regulation, regardless of a functioning Ysc system. Normally, secretion of Yops and LcrV is blocked in the presence of calcium. The single *yopD* mutant was not completely effective in blocking secretion: LcrV was secreted equally well in the presence and absence of calcium, while there was partial secretion of Yops in the presence of calcium. YopD is probably not rate limiting for negative regulation, as increasing levels of YopD did not result in decreased Yop expression. Overexpression of LcrQ in the *yopD* mutant had no significant effect on Yop expression, whereas increased levels of LcrQ in the parent resulted in decreased levels of Yops. These results indicate that LcrQ requires YopD to function as a negative regulator.

Yersinia pestis, the causative agent of plague, has a plasmid-encoded low-calcium response (LCR) regulatory mechanism that governs the expression and secretion of a set of virulence proteins in response to environmental conditions (40). Contact with a eukaryotic cell triggers an increase in LCR gene expression and the vectorial translocation of four of the secreted Yops (*Yersinia* outer proteins) into the host cell where they disrupt cellular signaling and other biochemistry necessary for phagocytosis and the mobilization of an effective immune response (8, 40). The maximal Yop expression and secretion induced by bacterium-host cell contact is mimicked in vitro by the absence of Ca²⁺ at 37°C; in the presence of millimolar concentrations of Ca²⁺ Yop expression is down-regulated and secretion is completely blocked.

The Yops and LcrV are secreted from the bacterial cell without processing by a specific Yop secretion system (Ysc) that is encoded by the LCR virulence plasmid (8, 40). The Ysc system belongs to the recently identified class of type III, or contact-dependent, secretion systems of both animal and plant gram-negative pathogens. These systems are involved in direct delivery of bacterial proteins into the target cell (30, 52) and are extremely complex: at least 19 gene products are required for the Ysc (8, 40). Translocation into the eukaryotic cell involves YopB, YopK, and YopD (18, 19, 21, 22, 25, 41, 51, 58). YopB may form a pore in the target cell membrane, and YopK may modulate the size of this pore (19, 25). YopD's function in translocation has not been determined.

There have been several gene products identified that play a

role in LCR regulation. Thermal induction of LCR gene expression is mediated by the transcriptional activator LcrF (6, 24). LcrF exhibits sequence similarity to the AraC activator of *Escherichia coli* and has been shown to interact with several *yop* promoters (7, 29). The Ysc system has been shown to have a positive role on the LCR: expression of Yops in a *ysc* mutant is down-regulated, even under conditions that normally promote maximal expression (5, 10, 43–45). It is believed that this connection between contact-triggered secretion and transcriptional up-regulation is the secretion of a repressor (or antiactivator) component through the Ysc (37, 42). This is analogous to the export of an anti-sigma factor through the flagellar export apparatus (which has homology to inner membrane components of type III secretion systems) as a control of late-gene expression in flagellar biosynthesis (26, 27). Two secreted proteins, LcrE and LcrG, appear to regulate secretion of Yops and LcrV in response to environmental Ca²⁺: mutations inactivating either protein cause secretion at 37°C irrespective of Ca²⁺ levels, with a concomitant loss of negative control over Yop and LcrV expression (13, 55). LcrE (also called YopN) is thought to act at the bacterial surface, presumably as the Ca²⁺ sensor, although that activity has not been directly demonstrated (13). The majority of LcrG is located in the cytosol and is thought to block secretion from the cytoplasmic side (37). LcrV, in addition to functioning as an antihost protein, serves as a positive regulatory protein (4, 46, 56). LcrV is able to directly interact with LcrG and is thought to titrate LcrG away from the Ysc system, thus counteracting LcrG's secretion block when elevated levels of LcrV are produced upon induction of the LCR (37).

The secreted protein LcrQ has previously been proposed to be the negative regulator whose secretion allows up-regulation of LCR expression (42). The level of LcrQ in the cell has been

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TABLE 1. Bacterial strains and plasmids used

| Strain or plasmid | Relevant properties ^a | Source or reference |
|---------------------------------------|--|---------------------|
| <i>E. coli</i> strains | | |
| BL21(DE3) | F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) | Novagen |
| SY327(<i>λpir</i>) | $\Delta(lac-pro)$ <i>argE(Am) rif nalA recA56 λpir</i> | 34 |
| DH5 α | <i>end-1 hsdR17(r_K⁻ m_K⁺) supE44 thi-1 recA1 gyrA (Nal^r) relA1 $\Delta(lacIZYA-argF)U169 deoR$</i> [ϕ 80 <i>dlac</i> $\Delta(lacZ)M15$] | 20 |
| XL1-Blue | <i>end-1 hsdR17(r_K⁻ m_K⁺) supE44 thi-1 λ⁻ recA1 gyrA96 (Nal^r) relA1 (Δlac) [F⁺ <i>proAB</i>⁺ <i>lacI^aZ</i>$\Delta M15::Tn10$ (Tc^r)]</i> | Stratagene |
| <i>Y. pestis</i> strains ^b | | |
| KIM5-3001 | Sm ^r pCD1 (Lcr ⁺), pPCP1, pMT1 | 32 |
| KIM5-3001.2 | Sm ^r pCD1 <i>lcrD</i> ($\Delta 192-343$), pPCP1, pMT1 | 44 |
| KIM5-3001.2.1 | Sm ^r pCD1 <i>lcrD</i> ($\Delta 192-343$) <i>yopD</i> ($\Delta 1-305$), pPCP1, pMT1 | This study |
| KIM5-3001.16 | Sm ^r pCD1 <i>yopD</i> ($\Delta 1-305$), pPCP1, pMT1 | This study |
| KIM8-3002 | Sm ^r pCD1 (Lcr ⁺), Pla ⁻ , pMT1 | 38 |
| KIM8-3002.2 | Sm ^r pCD1 <i>yopD</i> ($\Delta 1-305$), Pla ⁻ , pMT1 | This study |
| Plasmids | | |
| pUK4134 | Suicide vector, Ap ^r | 54 |
| pAW102 | 1.64-kb fragment of pCD1 containing $\Delta yopD$ ($\Delta 1-305$) cloned into pUK4134 | This study |
| pTrcH5 | <i>lcrH</i> cloned into pTrc99A (Pharmacia Biotech) | 12 |
| pET-24b | His tag fusion expression vector, Km ^r | Novagen |
| pAW111 | <i>yopD</i> cloned into pET-24b | This study |
| pBAD33 | Expression vector (arabinose inducible), Cm ^r | 16 |
| pAW161 | <i>yopD</i> cloned into pBAD33 | This study |
| pAW162 | <i>lcrQ</i> cloned into pBAD33 | This study |
| pProEX-1 | His tag fusion expression vector, Ap ^r | GibcoBRL |
| pAW171 | <i>yopD</i> cloned into pProEX-1 | This study |
| pGEX-3X | GST fusion expression vector, Ap ^r | Pharmacia Biotech |
| pWQG | <i>lcrQ</i> cloned into pGEX-3X | This study |

^a Numbers in the parentheses in the gene designations represent the amino acids deleted from the encoded protein.

^b All *Y. pestis* strains are Pgm⁻ (60). Native plasmids of *Y. pestis* include the LCR plasmid pCD1 (9, 15), the Pla-encoding pPCP1 (57), and pMT1, which encodes the capsular protein (49).

demonstrated to correlate to the level of Yop expression; i.e., Yop expression is maximal when LcrQ is depleted from the cell, while elevated levels of LcrQ result in down-regulation of Yop expression (42, 50). This is even true in a *ysc lcrQ* double mutant, which exhibits constitutive Yop expression, resulting in the conclusion that LcrQ secretion through the Ysc system is required for full induction of Yop expression (42). LcrQ is secreted from the cell quickly and efficiently both in vitro and after infection of HeLa cells (42). The regulatory function of LcrQ does not require translocation across the target cell membrane, as a *yopB* mutant had the same LCR induction as wild-type cells upon host cell contact (42). LcrQ has not been demonstrated to bind DNA, nor does its deduced amino acid sequence contain any DNA-binding motifs, and therefore, it most likely exerts its regulatory effect in an indirect manner (50).

The intracellular protein LcrH (also called SycD) was originally thought to be involved in the negative regulation of Yop and LcrV expression (4, 48). The phenotype of an *lcrH* mutant is constitutive induction of the LCR, as well as constitutive secretion of LcrV and partial secretion of YopM (4, 45, 48, 56). An *lcrH* mutation also caused a *ysc* strain to become constitutively induced for LCR expression (45). LcrH has subsequently been reported to be required for the stabilization and secretion of YopD (61). This raises the possibility that the *lcrH* phenotype is caused by the decreased stability of YopD. Additional evidence suggesting that YopD may be involved in regulation was the constitutive LCR induction caused by a Genblock insertion into the 3' end of *yopD* (4). However, this effect was attributed to a destabilization of the *lcrGVHyopBD* transcript (4). In this study, we constructed an essentially complete de-

letion of the *yopD* open reading frame and characterized the resulting mutant for LcrV and Yop expression and secretion. Our data indicate that YopD is involved in the negative regulation of the LCR, regardless of a functioning Ysc system. In addition, we demonstrate that LcrQ's negative regulatory function is dependent on the presence of YopD.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. Bacterial strains and plasmids are shown in Table 1. Synthetic oligonucleotides are shown in Table 2. *Y. pestis* strains were grown in heart infusion broth or on tryptose blood agar base medium (Difco Laboratories, Detroit, Mich.) for genetic manipulations. For physiological experiments, *Y. pestis* strains were grown in exponential phase at 26°C in the defined medium TMH (59) with or without Ca²⁺ for 7 to 9 generations and then were diluted to an absorbance at 620 nm (A_{620}) of ca. 0.1 and grown to an A_{620} of ca. 0.2. Cultures were then either held at 26°C or shifted to 37°C for an additional 6 h before harvesting. Unless otherwise indicated, *E. coli* strains were grown in Luria-Bertani (LB) broth (53) or on LB agar plates. Bacteria containing antibiotic resistance markers were grown in the presence of the appropriate antibiotic(s) at a final concentration of 25 μ g/ml (chloramphenicol), 50 μ g/ml (kanamycin), or 100 μ g/ml (ampicillin and streptomycin).

DNA methods. Plasmid DNA was isolated by a standard alkaline lysis procedure or by the use of Qiagen spin or midi-prep columns (Qiagen, Inc., Studio City, Calif.). Cloning methods were essentially as described previously (53). DNA fragments were purified from agarose gels by use of a Qiaquick gel extraction kit (Qiagen, Inc.). Electroporation of DNA into *E. coli* and *Y. pestis* was done as previously described (39). Gene amplification was performed with *Taq* polymerase (either from GibcoBRL, Gaithersburg, Md., or Boehringer Mannheim, Indianapolis, Ind.) or Pfu polymerase (Stratagene, La Jolla, Calif.) in a Perkin-Elmer GeneAmp model 2400 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Unless stated otherwise, 30 cycles of amplification were used, with 1 cycle consisting of denaturing at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 15 s. Total bacterial DNA for gene amplification was prepared as previously described (31). Custom oligonucleotides were obtained either from Genosys Biotechnologies (The Woodlands, Tex.), Integrated DNA Technologies, Inc. (Coralville, Iowa), or the Macromolecular Structure Analysis

TABLE 2. DNA oligonucleotides

| Primer | DNA sequence |
|-----------------|---|
| LCRH1SDA..... | GCT AGA GCT CAG GAG GAA CAT ATG CAA CAA GAG ACG ACA GAC |
| LCRQ1-2..... | CGT GGG ATC CAC ATG AAA ATC AAT ACT CTT CAA TCG |
| LCRQ1SDA..... | GCT AGA GCT CAG GAG GAA CAT ATG AAA ATC AAT ACT CTT CAA TCG |
| LCRQ2-1..... | TTA TGA ATT CCC TCA GCC GTC AGC CGC CGT AT |
| LCRQ348B..... | GCT CTA GAT CAG CCG TCA GCC GCC GTA TC |
| YOPB296A..... | CGC GGA TCC GCT TGC ACC TGA TAC ATT TG |
| YOPBΔ23B..... | GCC GGC CAA ATT ATG CAG CAT GGG TTA TCA ACG CAC |
| YOPBΔ1169A..... | GTG CGT TGA TAA CCC ATG ATG CTG CAT AAT TTG GCC GGC |
| YOPD1SDA..... | CTA GCT AGC GGA GTG AAC ATA TGA CAA TAA ATA TCA AGA CAG AC |
| YOPD921B..... | GCT CTA GAT CAG ACA ACA CCA AAA GCG GC |
| YOPDHTAGB..... | CCG CTC GAG AAC ACC AAA AGC GGC TTT CAT G |
| YOPDOUTDB..... | CGC GGA TCC AGC GGC AAG TCA AAT ACC TG |
| YOPDΔ1A..... | GTT TAA GGA GGA ATA ACC GTC TGA CCA TTG ATG ACC |
| YOPDΔ915B..... | GGT CAT CAA TGG TCA GAC GGT TAT TCC TCC TTA AAC |

Facility (University of Kentucky). DNA sequencing was performed at the Macromolecular Structure Analysis Facility.

Plasmid construction. DNA used for cloning was obtained from pCD1 of *Y. pestis* by amplification with customized oligonucleotide primers. Some of the primers were designed using the *Yersinia pseudotuberculosis* DNA sequence, since portions of the region utilized have not yet been sequenced in *Y. pestis*, and LCR loci typically exhibit at least 95% sequence identity between the two species. Unless stated otherwise, the high-fidelity PFU polymerase was used for amplification of DNA, and the extension time of the cycles was increased to 1 min per kb amplified.

pAW102 (*YopD*) was created by the blunt-end cloning of the DNA sequence flanking, but not including, *yopD* (ca. 950 bases upstream and ca. 700 bases downstream) into the *EcoRV* site of suicide vector pUK4134 (54). The only DNA present from the *yopD* gene was the last 6 nucleotides, coding for the C-terminal amino acid and the stop codon. The DNA fragment for cloning was obtained by recombinant PCR (23) in which internal complementary primers encoded sequence immediately upstream and downstream of the desired deletion of bases 1 through 915 of *yopD*. YOPDΔ915B and YOPDΔ1A were the two internal primers, and YOPB296A (which lay within upstream *yopB*) and YOPDOUTDB (which lay downstream of *yopD*) were the two outside primers; these primers were based on a *Y. pseudotuberculosis* sequence. The resulting fragment was 5' end phosphorylated with T4 kinase (Promega, Madison, Wis.).

Plasmid pAW111 (encoding C-terminally histidine-tagged YopD) was constructed by amplifying *yopD* with YOPD1SDA and YOPDHTAGB, which contained the first 26 bases and bases 893 to 918 (with a G-to-C substitution at base 916 resulting in a conservative missense mutation of leucine to valine at the C-terminal position) of *Y. pseudotuberculosis yopD*, respectively, plus a 5' end with an unencoded *NdeI* (YOPD1SDA) or *XhoI* (YOPDHTAGB) site. The PCR product was cleaved with *NdeI* and *XhoI* and cloned into the *NdeI* and *XhoI* sites of pET-24b (Novagen, Inc, Milwaukee, Wis.).

pAW171 (encoding N-terminally histidine-tagged YopD) was constructed by amplifying *yopD* with YOPD1SDA and YOPD921B (which contained the final 21 bases of *Y. pseudotuberculosis yopD* plus a 5' end with an unencoded *XbaI* site). The PCR product was cleaved with *NdeI* and *XbaI* and cloned into the *NdeI* and *XbaI* sites of pProEX-1 (GibcoBRL).

Plasmid pAW161 (expressing *yopD*) was constructed by cloning *lcrH* and *yopD* into *NheI*- and *XbaI*-cleaved pBAD33 (16). The DNA fragment for cloning was obtained by recombinant PCR amplification (23) to delete bases 23 through 1169 of *yopB*, with YOPBΔ1169A and YOPBΔ23B as the two internal primers based on a *Y. pseudotuberculosis* sequence, and LCRH1SDA and YOPD921B based on *Y. pestis* and *Y. pseudotuberculosis* sequences, respectively, as the two outside primers, followed by digestion with *NheI* and *XbaI* (both sites engineered as nonencoded 5' ends of the outside primer sequences).

Plasmid pAW162 (expressing *lcrQ*) was constructed by amplifying *lcrQ* with primers LCRQ1SDA and LCRQ348B derived from the *Y. pseudotuberculosis lcrQ* sequence and containing unencoded *NheI* and *XbaI* restriction sites in the 5' ends, respectively. The PCR product was cleaved with *NheI* and *XbaI* and cloned into *NheI*- and *XbaI*-cleaved pBAD33.

pWGQ (encoding glutathione S-transferase [GST]-LcrQ) was constructed by amplifying *lcrQ* with primers LCRQ1-2 and LCRQ2-2 based on the *Y. pestis lcrQ* sequence and containing 5' unencoded *EcoRI* and *BamHI* restriction sites, respectively. The PCR product was cleaved with *EcoRI* and *BamHI* and cloned into *EcoRI*- and *BamHI*-cleaved pGEX-3X (Pharmacia Biotech, Piscataway, N.J.). Amplification of the DNA for pWGQ was performed with *Taq* polymerase, and the annealing, extension, and denaturation times for each cycle were 30 s (14).

Construction of mutants. Plasmid pAW102 was used for the deletion of the *yopD* gene. The plasmid was electroporated into Sm^r *Y. pestis*, and the *yopD* deletion was introduced into pCD1 by allelic exchange as previously described

(54). The presence of second crossovers was screened for by PCR, and the presence of mutations was confirmed by DNA sequence analysis. The *yopD* mutation was introduced into *Y. pestis* KIM5-3001, KIM8-3002, and KIM5-3001.2, resulting in *Y. pestis* KIM5-3001.16, KIM8-3002.2, and KIM5-3001.2.1, respectively.

Cell fractionation. Cell pellets and culture supernatants of *Y. pestis* grown in TMH were separated by centrifugation at 4,000 × *g* for 16 min at 4°C. Cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) (135 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.4). Whole-cell fractions were prepared by being resuspended in 1× protein sample buffer (SB) (2). Cellular extracts were made by disintegration of PBS-resuspended cell pellets in the ice-cold cell of a French press (20,000 lb/in²). Low-speed centrifugation (8,000 × *g*, 5 min, 4°C) removed unbroken cells and large debris. The total soluble fraction (cytoplasmic plus periplasmic) was separated from membranes by ultracentrifugation at 513,000 × *g* in a TLA120.2 rotor for 10 min at 4°C, using a Beckman Optima TLX ultracentrifuge (Beckman Instruments, Fullerton, Calif.). Culture supernatant proteins were precipitated either with 5% (wt/vol) trichloroacetic acid (TCA) for 4 h to overnight on ice or with 80% (wt/vol) ammonium sulfate overnight at 4°C. The TCA-precipitated samples were collected by centrifugation at 20,800 × *g* for 30 min at 4°C, neutralized, and resuspended in 1× SB. The ammonium sulfate-precipitated samples were resuspended in PBS, dialyzed against PBS at 4°C by using either a Slide-A-Lyzer 2,000-molecular-weight-cutoff cassette (Pierce, Rockford, Ill.) or Spectra/Por 4 dialysis tubing (Spectrum, Laguna Hills, Calif.), and added to an equal volume of 2× SB.

Antibody preparation. Plasmid pAW111, which encodes a C-terminally polyhistidine-tagged YopD (HT-YopD) behind the T7 promoter of pET-24b (Novagen), and plasmid pTrcH5 (12), which contains the *lcrH* gene behind the *trc* promoter of pTrc99A (Pharmacia Biotech), were introduced into *E. coli* BL21(DE3). Cultures (200 ml) were grown at 37°C in tryptone-phosphate medium (35) to an *A*₆₀₀ of ca. 0.6, at which point high-level expression of the proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1.0 mM followed by incubation at 37°C for an additional 7.5 h. The cells were harvested by centrifugation at 11,300 × *g* for 10 min at 4°C. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl) and lysed by passage through a French pressure cell (20,000 lb/in²). Unlysed cells, large debris, and inclusion bodies were removed by centrifugation at 8,000 × *g* for 5 min at 4°C. The soluble fraction was loaded on a 1-ml Talon resin column (Clontech, Palo Alto, Calif.), washed with 10 column volumes of lysis buffer, and then washed with 10 column volumes of lysis buffer with 6 M guanidinium-HCl to dissociate LcrH bound to HT-YopD (HT-YopD remains bound to the Talon resin in the presence of 6 M guanidinium-HCl). HT-YopD was eluted from the column in lysis buffer with 50 to 200 mM imidazole, with subsequent dialysis against lysis buffer to remove the imidazole. HT-YopD was purified to homogeneity by this procedure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (data not shown).

Plasmid pWGQ, which encodes an N-terminal GST fusion to LcrQ expressed from the promoter of pGEX-3X, was introduced into *E. coli* DH5α. Cultures (100 ml) were grown at 37°C in tryptone-phosphate medium to an *A*₆₂₀ of ca. 0.4, at which point high-level expression was induced by the addition of IPTG to 1.0 mM followed by incubation at 37°C overnight. The cells were harvested by centrifugation at 11,300 × *g* for 20 min at 4°C. Cell pellets were resuspended in PBS and lysed by passage through a chilled French pressure cell (20,000 lb/in²). The soluble fraction, which contained the majority of GST-LcrQ, was obtained by ultracentrifugation at 417,000 × *g* for 15 min in a TLA100.4 rotor at 4°C. GST-LcrQ was purified on a glutathione column (Pharmacia Biotech) as recommended by the manufacturer.

Anti-HT-YopD and anti-GST-LcrQ antibodies were raised in female New Zealand White rabbits as previously described (36). Immunoglobulin G was

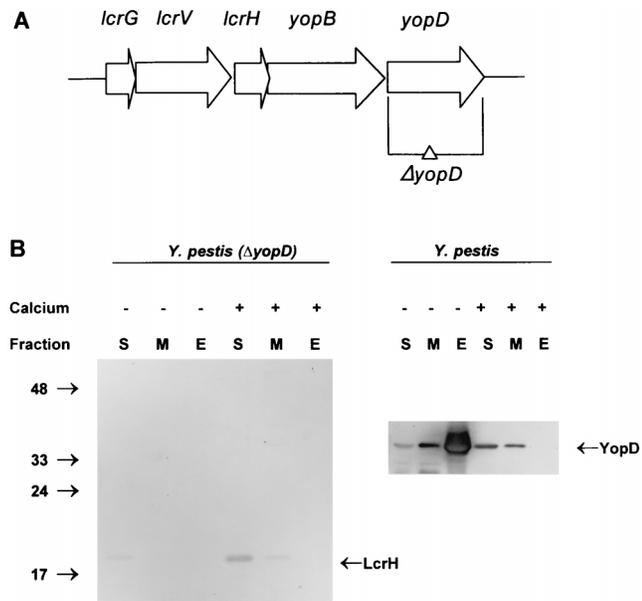


FIG. 1. Construction of a complete $\Delta yopD$ mutation in *Y. pestis*. (A) Schematic representation of the *lcrGVHyopBD* operon of *Y. pestis* LCR plasmid pCD1. The positions and directions of the genes of this operon are shown as horizontal arrows. The DNA region deleted to create the *yopD* mutation is indicated ($\Delta yopD$). (B) Immunoblot analysis of YopD and LcrH expressed in *Y. pestis* KIM8-3002.2 ($\Delta yopD$) and *Y. pestis* KIM8-3002 (parent). Bacteria were grown at 37°C in the absence (-) or presence (+) of 2.5 mM Ca^{2+} . Proteins from equal numbers of cells (0.025 A_{620} unit · ml) were separated by SDS-PAGE in a 15% (wt/vol) polyacrylamide gel. Antibodies were used to detect YopD and LcrH in the soluble (S) (cytoplasmic and periplasmic) fraction, the membrane (M) fraction, and the extracellular (E) fraction. The positions of the molecular mass markers (in kilodaltons) and the LCR-related proteins are indicated.

affinity purified by chromatography on an Affi-Prep-protein A column (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer. Anti-HT-YopD and anti-GST-LcrQ antibody preparations were used for immunoblot analysis at dilutions of 1:40,000 and 1:20,000, respectively.

Protein electrophoresis and immunodetection. Proteins were separated by SDS-PAGE, with 12 or 15% (wt/vol) polyacrylamide gels as indicated, by the method of Laemmli (28). Samples containing whole-cell fractions were boiled for 5 min, while all other fractions were heated to ca. 95°C for 3 to 5 min prior to being loaded on the gels. Samples were loaded so as to contain amounts of the fractions derived from the same volume of original culture. Proteins separated by SDS-PAGE were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) with carbonate buffer (pH 9.9) (37). Specific proteins were visualized on the membranes as previously described (43) by using the polyclonal antibodies specific for the proteins and a secondary goat anti-rabbit antibody (Sigma Chemical, St. Louis, Mo.) conjugated to alkaline phosphatase.

RESULTS

Construction of a *Y. pestis yopD* null mutant. To test the hypothesis that YopD destabilization causes the negative regulatory phenotype originally attributed to *lcrH*, a *yopD* deletion mutant was constructed (Fig. 1A). Recombinant PCR mutagenesis (23) was used to delete essentially the entire open reading frame to ensure that a partial gene product could not be expressed. *yopD* occurs at the end of the *lcrGVHyopBD* operon, so there were no downstream genes to disrupt. However, the potential ρ -independent termination signal (4) was left intact to ensure proper processing of the transcript. The only *yopD* sequence retained was the 6 3'-terminal nucleotides in case that DNA sequence is important for the termination signal. The *yopD* deletion was introduced into pCD1 of *Sm^r Y. pestis* KIM8-3002 by allelic exchange, resulting in the strain KIM8-3002.2.

To test the phenotype of the *yopD* mutant, the mutant and

parent *Y. pestis* strains were grown in the defined medium TMH at 26°C and then shifted to 37°C for 6 h in either the presence or absence of Ca^{2+} . The *yopD* deletion mutant showed a growth phenotype comparable to that of the *lcrH* mutant: it underwent growth restriction when shifted from 26 to 37°C, irrespective of the presence of Ca^{2+} (growth restriction is a phenomenon observed in vitro in which the growth yield of *Y. pestis* is reduced during maximal induction of the LCR; it is not considered to play a role in vivo). As expected, there were no proteins recognized by the anti-YopD antibody in the deletion mutant (Fig. 1B). LcrH served as an internal control: its presence in the mutant demonstrated that the LCR proteins were still being expressed. In addition, the presence of equal levels of LcrH in the parent and *yopD* mutant strains (Fig. 2) showed that the *yopD* mutation was not exerting its phenotype by destabilizing the *lcrGVHyopBD* transcript. This also showed that LcrH does not require YopD to prevent it from being degraded, in contrast to YopD which does require LcrH for stability (61, 62). LcrH expression does not appear to be significantly up-regulated under inductive conditions. This is similar to the findings of a previous report, which noted that there was a 2.3-fold decrease in the level of LcrH expression in the presence of Ca^{2+} from that in the absence of Ca^{2+} , while LcrV expression showed a 3.4-fold decrease (47). This difference was attributed to constitutive expression of LcrH from its own promoter in addition to expression from the Ca^{2+} -regulated promoter. As found in the enteropathogenic *Yersinia* (21, 51, 58), the *Y. pestis yopD* mutant was also unable to efficiently translocate Yops into eukaryotic cells, despite the fact that Yops were effectively secreted (11), further indicating that the *yopD* gene was disrupted.

The *yopD* deletion was also introduced into a *Y. pestis* strain containing the pPCP1 plasmid to ensure that the absence of the Pla protease in the original *yopD* mutant was not affecting the observed results. The suicide vector containing the *yopD* deletion was introduced into pCD1 of *Sm^r Y. pestis* KIM5-3001 by allelic exchange, resulting in the strain KIM5-3001.16. The growth of this strain and its inability to express the YopD protein were characteristics similar to those of *Y. pestis* KIM8-3002.2 (data not shown).

Expression and secretion of LCR gene products. To determine the role YopD plays in the LCR, Yop expression and secretion were examined in both *Y. pestis* KIM8-3002.2 and its parent by immunoblot analysis. The *Y. pestis* strains were grown in TMH with or without Ca^{2+} at 26°C and then shifted to 37°C for 6 h. After 6 h, all sample handling occurred at 4°C to minimize additional protein synthesis. The protein profiles of whole cells (containing the cytoplasmic, periplasmic, and membrane fractions) and culture supernatant (obtained by TCA precipitation) were determined with antibodies raised against YopM (36), LcrV (37), and LcrQ (Fig. 2). Similar results were seen if the culture supernatant fraction was obtained by ammonium sulfate precipitation (data not shown). The expression of YopM and LcrV was up-regulated in the mutant when grown in the presence of Ca^{2+} , indicating that YopD is required for negative regulation. The results were similar when other Yops were analyzed (YopE and YopH) (data not shown). When the samples were examined for the presence of LcrQ, this putative negative regulator was still detected in the cellular fraction of the mutant grown in the presence of Ca^{2+} and in fact appeared to be at a higher concentration than in the parent *Yersinia*. This represents a situation where LcrQ does not cause down-regulation, suggesting that YopD is required for LcrQ to exhibit its negative regulatory activity. The elevated level of LcrQ likely reflects some effect of the LCR on LcrQ expression. There was no

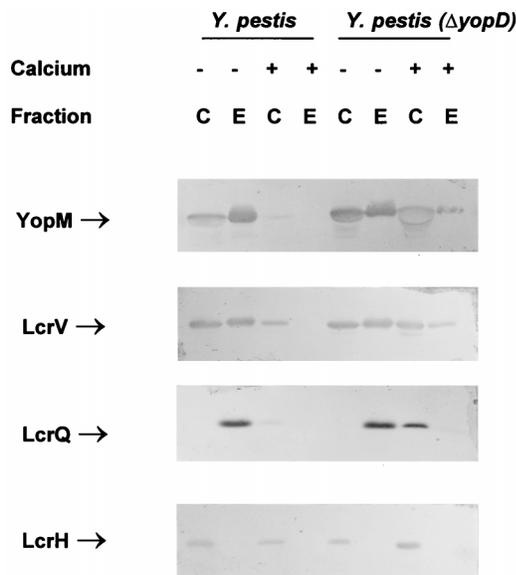


FIG. 2. Immunoblot analysis of YopM, LcrV, LcrQ, and LcrH expressed in *Y. pestis* KIM8-3002 (parent) and *Y. pestis* KIM8-3002.2 ($\Delta yopD$). Bacteria were grown at 37°C in the absence (-) or presence (+) of 2.5 mM Ca^{2+} . Proteins from equal numbers of cells (0.025 A_{620} unit · ml) were separated by SDS-PAGE in a 12 or 15% (wt/vol) polyacrylamide gel. Antibodies were used to detect YopM, LcrV, LcrQ, and LcrH in both the whole-cell (C) (cytoplasmic, periplasmic, and membrane) fraction and the extracellular (E) fraction. The positions of the LCR-related proteins are indicated. The band corresponding to YopM in the whole-cell fraction of *Y. pestis* ($\Delta yopD$) grown in the presence of Ca^{2+} has a hollow appearance, which occurs when a large quantity of protein is analyzed.

difference in Yop expression between wild-type and $\Delta yopD$ *Y. pestis* under normally inductive conditions (the absence of Ca^{2+}).

The absence of YopD had a variable effect on secretion in the presence of Ca^{2+} , when the Ysc system is normally blocked. In the *yopD* mutant, LcrV was not strongly blocked for secretion; i.e., there were similar levels of LcrV present in the whole-cell and culture supernatant fractions, regardless of the presence of Ca^{2+} . However, the secretion of YopM (Fig. 2), as well as other Yops (data not shown), was not completely unblocked, but instead there was partial secretion in the presence of Ca^{2+} . We have observed that YopM overexpression in the wild-type cell can result in partial (i.e., weak) secretion of YopM in the presence of Ca^{2+} (3). Therefore, YopM's partial secretion in the *yopD* mutant could be due to the up-regulation of expression as opposed to YopD playing a direct role in YopM secretion. Importantly, it is evident that secretion of LcrV differs from that of the other Yops, at least as far as YopD is concerned.

Yop protein expression and secretion were also checked for the Pla-containing *Y. pestis* KIM5-3001.16 and its parent by immunoblot analysis (data not shown). The patterns of Yop and LcrV expression in the presence and absence of Ca^{2+} were similar in the two different *yopD* mutants. However, some of the secreted proteins are sensitive to the Pla protease, causing difficulty in the interpretation of protein secretion. This was especially true for LcrQ, which is present in the culture supernatant fraction only as a degradation product in *Y. pestis* KIM5-3001 (data not shown).

Localization of YopD. Soluble (periplasm and cytoplasm), total membrane, and supernatant fractions isolated from *Y. pestis* KIM8-3002 cultures were examined for the presence of YopD by immunoblot analysis (Fig. 1B). As expected, in the

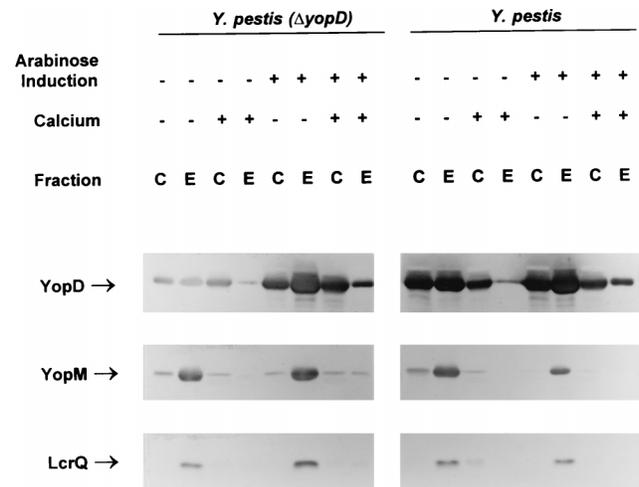


FIG. 3. Immunoblot analysis of YopD, YopM, and LcrQ expressed in the complemented *Y. pestis* $\Delta yopD$ strain (KIM8-3002.2 carrying pAW161) and the parent *Y. pestis* with overexpression of YopD (KIM8-3002 carrying pAW161). Bacteria were grown at 37°C in both the absence (-) and presence (+) of 2.5 mM Ca^{2+} . Expression of YopD from pAW161 was either uninduced (-) or induced (+) by the addition of arabinose to 0.05% (wt/vol). Proteins from equal numbers of cells (0.025 A_{620} unit · ml) were separated by SDS-PAGE in a 12 (YopD and YopM) or 15% (LcrQ) (wt/vol) polyacrylamide gel. Antibodies were used to detect YopD, YopM, and LcrQ in both the whole-cell (C) (cytoplasmic, periplasmic, and membrane) fraction and the extracellular (E) fraction. The positions of the LCR-related proteins are indicated.

presence of Ca^{2+} , when secretion is blocked and the LCR is repressed, no YopD was observed in the culture supernatant. Instead, there appeared to be comparable levels of YopD in both the soluble and membrane fractions. YopD has two predicted transmembrane spans (17), so it is not surprising to find it associated with the membrane. In contrast, in the absence of Ca^{2+} , when secretion is not blocked and the LCR is maximally expressed, the majority of YopD was found in the culture supernatant. However, YopD could still be detected in the soluble fraction. This indicates that it is not necessary to completely deplete the cell of YopD to obtain increased LCR expression.

Expression of YopD in wild-type and *yopD* *Y. pestis*. Complementation was performed to show that the phenotype of the *yopD* mutant was solely due to its mutation. *lcrH* and *yopD* were cloned in tandem behind the arabinose-inducible promoter of pBAD33 (16) by amplifying the region from pCD1 and using recombinant PCR mutagenesis (23) to remove the entire *yopB* gene. The rationale for including *lcrH* was that since the gene product had recently been demonstrated to be a chaperone for YopD (61), *lcrH* may be required for efficient YopD expression. Subsequent sequencing of the plasmid revealed a single-base deletion in the start codon for *lcrH*, resulting in the inability to express the gene. However, YopD was expressed in *E. coli* from the plasmid despite no LcrH expression (data not shown). The plasmid was introduced into wild-type and $\Delta yopD$ *Y. pestis*. Despite the fact that a very low level of expression is supposed to occur in the absence of induction of pBAD vectors (16), YopD was expressed without the addition of arabinose, although induction did result in an increase in YopD levels (Fig. 3).

In the *yopD* mutant, YopD expression from the plasmid without induction with arabinose resulted in lower-than-wild-type levels of YopD, but this amount of YopD was capable of complementing the mutant for Yop expression and secretion

(Fig. 3). In addition, the growth phenotype of the complemented strain was similar to that of the wild type: it underwent growth restriction when shifted from 26 to 37°C only in the absence of Ca²⁺. When the level of YopD was increased, either by inducing the plasmid in the *yopD* mutant or expressing YopD from the plasmid in the wild-type strain without induction, the level of Yop expression did not decrease significantly (Fig. 3). Evidently, YopD is not the limiting factor for down-regulation. pAW171, encoding only a histidine-tagged YopD without the *lcrH* gene, provided similar complementation when expressed in the *yopD* mutant (data not shown). To confirm that additional LcrH could not overcome the *yopD* mutation, a plasmid containing the *lcrH* gene behind an IPTG-inducible promoter (pTrcH5) was used. This plasmid was unable to complement $\Delta yopD$ *Y. pestis* (data not shown).

Overexpressing YopD to significantly higher levels, such as when the plasmid was induced with arabinose in wild-type cells, did result in an apparent decrease in YopM expression. However, the growth rate of these cells significantly decreased over time (data not shown). This decrease in growth rate could be differentiated from the growth restriction normally seen under LCR-inductive conditions, since the level of YopM decreased rather than increased. It is possible that higher levels of YopD are toxic to the bacterial cell. This makes it difficult to interpret the significance of the lower level of YopM observed in these cells containing high levels of YopD.

Effect of the *yopD* mutation in a *ysc* strain. Yop expression in a *ysc* mutant is down-regulated, even under conditions that normally promote maximal expression, presumably through the inability to secrete a negative regulator (37, 42). A *ysc lcrQ* double mutant has previously been reported to show constitutive Yop expression (42). It was concluded that secretion of LcrQ is a prerequisite to achieve maximal induction of Yop expression. Since YopD also plays a role in negative regulation, a *ysc yopD* double mutant was constructed to determine the role of secretion on the action of YopD. The $\Delta yopD$ null mutation was moved into pCD1 of *Y. pestis* KIM5-3001.2 ($\Delta lcrD$) by allelic exchange (LcrD is an essential component of the Ysc system [43, 44]). This strain contains pPCP1; however, the Pla protease does not present difficulties in interpreting results for intracellular proteins.

The $\Delta lcrD$ parent and $\Delta lcrD \Delta yopD$ strains were analyzed for LCR expression and secretion (Fig. 4). As expected, the $\Delta lcrD$ parent strain had a low level of LCR expression and no detectable secretion. In contrast, the double mutant had high levels of cell-associated YopM and LcrV, with neither protein detected in the culture supernatant. These results indicate that the role YopD plays in negative regulation occurs irrespective of the Ysc system. Also, since no secretion was observed, not even the unblocked secretion of LcrV and partial secretion of YopM seen in the $\Delta yopD$ mutant, YopD's observed role in secretion must depend on a functional Ysc. LcrQ was detected in the double mutant, as well as the $\Delta lcrD$ parent, irrespective of the presence of Ca²⁺ (Fig. 4). These results indicate that LcrQ secretion is not required for Yop expression to be up-regulated if YopD is absent, which supports the idea that LcrQ alone cannot down-regulate Yop expression.

LcrQ's regulatory function is dependent on YopD. The level of the negative regulator LcrQ within the cell has been proposed to correlate inversely to the level of Yop expression; i.e., Yop expression is maximal when LcrQ is depleted from the cell, whereas elevated levels of LcrQ result in down-regulation of Yop expression (42, 50). To determine if increased levels of LcrQ could overcome a deletion of essentially the entire *yopD* gene, *lcrQ* was cloned behind the arabinose-inducible promoter of pBAD33 and introduced into both *Y. pestis* and *Y.*

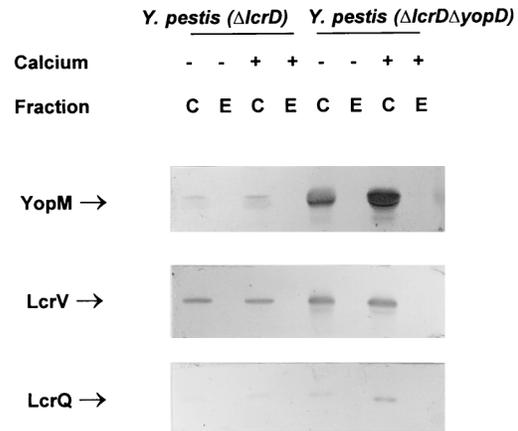


FIG. 4. Immunoblot analysis of YopM, LcrV, and LcrQ expressed in the *ysc* mutant *Y. pestis* KIM5-3001.2 ($\Delta lcrD$) and the double mutant *Y. pestis* KIM5-3001.2 ($\Delta lcrD \Delta yopD$). Bacteria were grown at 37°C in the absence (-) or presence (+) of 2.5 mM Ca²⁺. Proteins from equal numbers of cells (0.025 A₆₂₀ unit · ml) were separated by SDS-PAGE in a 12 (YopM and LcrV) or 15% (LcrQ) (wt/vol) polyacrylamide gel. Antibodies were used to detect YopM, LcrV, and LcrQ in both the whole-cell (C) (cytoplasmic, periplasmic, and membrane) fraction and the extracellular (E) fraction. The positions of the LCR-related proteins are indicated.

pestis ($\Delta yopD$). No increase in LcrQ levels was observed without induction with arabinose (Fig. 5 versus Fig. 2), indicating tight control of expression by the promoter. Yop expression and secretion were monitored by observing YopM levels in whole-cell and culture supernatant fractions of cells either induced or not induced with arabinose (Fig. 5). The level of Yop expression was constitutively down-regulated when LcrQ was overexpressed in wild-type *Y. pestis*, and no secretion was detected. This is in agreement with previously reported accounts for overexpression of LcrQ (50). In contrast, the increased levels of LcrQ were not sufficient to overcome the constitutively induced $\Delta yopD$ phenotype: the level of YopM expression was only slightly reduced upon strong overexpres-

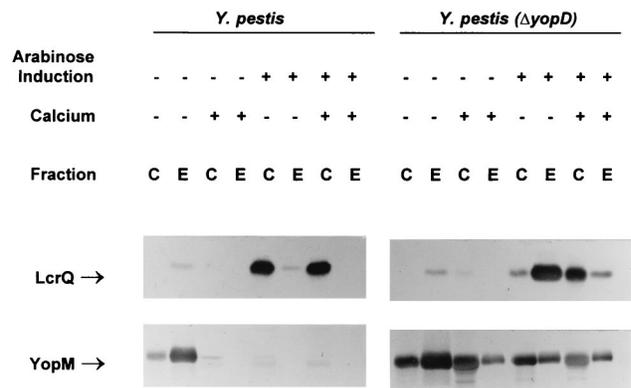


FIG. 5. Immunoblot analysis of LcrQ and YopM expressed in *Y. pestis* KIM8-3002 (parent) and *Y. pestis* KIM8-3002.2 ($\Delta yopD$) with overexpression of LcrQ from plasmid pAW162 in both strains. Bacteria were grown at 37°C in the absence (-) or presence (+) of 2.5 mM Ca²⁺. Expression of LcrQ from pAW161 was either uninduced (-) or induced (+) by the addition of arabinose to 0.2% (wt/vol). Proteins from equal numbers of cells (0.025 A₆₂₀ unit · ml) were separated by SDS-PAGE in a 12 (YopM) or 15% (LcrQ) (wt/vol) polyacrylamide gel. Antibodies were used to detect LcrQ and YopM in both the whole-cell (C) (cytoplasmic, periplasmic, and membrane) fraction and the extracellular (E) fraction. The positions of the LCR-related proteins are indicated.

sion of LcrQ (Fig. 5). This further supports the idea that LcrQ requires YopD to exert its negative regulatory function. However, it is possible that the small decrease in YopM expression associated with LcrQ overexpression could reflect an inefficient or secondary role of LcrQ alone on Yop expression.

Interestingly, it was observed that the large majority of LcrQ was cell associated when the protein was overexpressed in the parent *Y. pestis* (Fig. 5). In fact, the amount of LcrQ seen in the culture supernatant was comparable to the amount that was secreted without the additional LcrQ expression, giving the impression that all additional LcrQ was not being secreted. In contrast, when LcrQ was overexpressed in the $\Delta yopD$ mutant, more was secreted than was observed in the parent strain. This suggests that YopD may function to limit or control the secretion of LcrQ. However, the failure of overexpressed LcrQ to shift the phenotype of the $\Delta yopD$ mutant cannot be attributed to its increased secretion, as there was still more LcrQ in the cellular fraction of the *yopD* strain than is ever present in the wild-type strain.

DISCUSSION

In this study, we tested the hypothesis that YopD, previously shown to be necessary for Yop translocation (21, 41, 51, 58), also is a terminal negative regulator of the LCR. Our *yopD* null mutation resulted in constitutively induced expression of LcrV and the Yops, and provision of YopD in *trans* complemented this regulatory phenotype. The *yopD* mutant exhibited this phenotype regardless of a functioning Ysc system, demonstrating that its regulatory effect is not at the level of secretion.

Our finding that YopD is a negative regulator helps explain the phenotype originally attributed to LcrH (4, 48). A mutation in *lcrH*, whether polar or nonpolar, resulted in constitutive induction of the LCR (4, 48). It is now known that LcrH (also called SycD), binds to YopD and apparently stabilizes it against degradation (61). Accordingly, we can infer that the phenotype of *lcrH* mutants is likely due to their drastically decreased net expression of YopD. There is a previous finding that would appear to be inconsistent with this interpretation: a constitutively repressed phenotype occurred when LcrH was overexpressed in a *Y. pseudotuberculosis* strain with a Genblock insertion in *yopD* (4). However, the insertion was at the end of *yopD*, and perhaps enough of the YopD protein was expressed to retain its regulatory function, provided there also was extra LcrH to ensure its stability.

YopD's negative regulatory function probably operates through the action of LcrQ (also called YscM), which has previously been identified as being required for LCR down-regulation (42, 50). We showed here that YopD must be present for negative regulation to occur: in our *yopD* mutant, Yop expression was up-regulated, despite the fact that LcrQ was still present in the cell. In fact, overexpressing LcrQ in the *yopD* mutant could not overcome the observed phenotype. In addition, the inability to secrete LcrQ in the *yopD ysc* double mutant did not result in down-regulation of Yop expression. Therefore, we conclude that LcrQ is not sufficient for negative LCR regulation. Previous reports describing an *lcrQ Y. pseudotuberculosis* strain (42) indicate that YopD alone also is not sufficient, so we hypothesize that both LcrQ and YopD are necessary for LCR negative regulation.

Our data support the hypothesis that LcrQ is the limiting factor in determining whether negative regulation will occur. Previous studies documented an inverse correlation of intracellular amounts of LcrQ and expression of Yops (42, 50), and LcrQ was demonstrated to be quickly and efficiently secreted from the bacterial cell upon induction by contact with eukary-

otic cells (42). We also found that the level of LcrQ in the cell seemed to directly correlate to the amount of negative LCR regulation, with the new finding that YopD had to be present. However, although YopD was necessary for negative regulation, altering its level had no significant effect on Yop expression. This is consistent with the observation that there still is YopD present in the cell under conditions of maximal LCR induction. Therefore, we hypothesize that LcrQ is rate limiting for LCR negative regulation.

YopD likely influences LcrQ function in some manner. We showed that YopD's role is not due to an indirect effect on Yop secretion, as strong secretion still occurred in the *yopD* mutant, and a *yopD ysc* double mutant also showed constitutive induction. One potential role for YopD in the translocation process is as an extracellular chaperone. In this model, YopD would be expected to bind the translocated Yops, including YopH. Because LcrQ and the amino-terminal end of YopH exhibit 42% sequence identity (50), it may be that YopD also directly binds to LcrQ. Future work will determine whether there is a direct interaction between YopD and LcrQ, as this has not yet been shown.

Interestingly, the existing data suggest that LcrQ and YopD specifically affect each other's secretion to the culture medium. We found that when LcrQ was overexpressed, only a limited amount of it was released, even though the secretion machinery was not blocked by the absence of Ca^{2+} . The amount of overexpressed LcrQ that was secreted increased in the *yopD* mutant, suggesting that LcrQ secretion is affected by YopD's presence. Likewise, LcrQ is implicated in the control of YopD and LcrV secretion: an *lcrQ* mutant of *Y. pseudotuberculosis* secreted LcrV and all Yops normally in the absence of Ca^{2+} , but in the presence of Ca^{2+} , when secretion normally is blocked, LcrV and YopD were secreted (50). These phenomena might be manifestations of a hierarchy in the LCR induction and secretion mechanisms that could be reflected in the order in which the *Yersinia* proteins are secreted. A set of proteins would need to be secreted early to put in place the contact-induced translocation machinery. YopD and YopB would be among these proteins. Early LcrQ release would initiate contact-induced amplification of the antihost response while the existing pool of Yop toxins is being translocated into the host cell. Early LcrV release might be needed on two accounts: this would be one way of removing the inner secretion gate, LcrG, from its secretion-blocking location at the cytoplasmic face of the inner membrane (37); also, LcrV may play a role in translocation of Yops (38). This kind of hierarchy is supported by the phenotype of the partially secretion-defective *Yersinia enterocolitica virG* mutant, which, interestingly, was most severely defective in secretion of LcrV, YopB, and YopD (1). It would be of interest to examine LcrQ secretion in this mutant.

Our findings require a revision of the current model of LCR gene regulation to take into account YopD's role (Fig. 6). In the presence of Ca^{2+} , LcrE and LcrG block the secretory machinery from outside the cell and from the cytoplasmic face, respectively, as previously proposed (13, 37). This retains both LcrQ and YopD in the cell where they can function to down-regulate LcrV and Yop expression. The possibility of additional LCR components playing a role in gene regulation cannot be ruled out with the current data (denoted in Fig. 6 by ?). The secretion block also prevents the secretion of the low levels of LcrV and Yops that are in the cell in the presence of Ca^{2+} . The continued, albeit low-level, expression of YopD along with other LCR gene products in the absence of an inductive signal is consistent with the model, since some YopD must be expressed to function as a negative regulator even

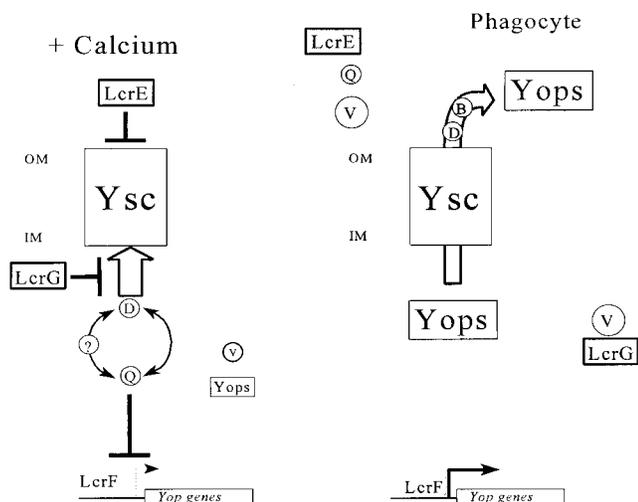


FIG. 6. Model for regulation of the LCR in *Y. pestis*. In the presence of Ca^{2+} (+ Calcium), LcrE and LcrG block the Yop secretion system (Ysc) at the cell surface and in the cytoplasm, respectively. The secretion block prevents the secretion of LcrQ (Q) and YopD (D). These two proteins work in concert to cause a down-regulation of Yop expression. There may be other components (?) required that have not yet been identified. In the absence of Ca^{2+} and in vivo when the bacteria contact a eukaryotic cell, LcrE is released from the cell surface. This relieves the secretion block and allows secretion of both LcrQ and YopD, which results in induction of the *yop* genes, including *lcrV*. The increase in LcrV (V) levels titrates LcrG away from the Ysc, resulting in full LCR induction and secretion. YopD and YopB (B) are then involved in translocating the Yops into the eukaryotic cell. OM, outer membrane; IM, inner membrane.

though it is subject to the same regulation. (LcrQ appears to be more Ca^{2+} independent in its expression [42].) Upon contact with the eukaryotic cell or removal of Ca^{2+} from the medium, the secretion block is relieved. It has been proposed that this occurs by LcrE sensing the change in the environment (13), which then may allow the release of some secretion system-associated LcrG (37), accompanied by the secretion of low levels of Yops, as well as LcrQ and YopD. The secretion of LcrQ occurs very efficiently (42). The decrease in intracellular levels of the negative regulators allows an increase in the expression of the LCR gene products, including LcrV. The increased level of LcrV is then able to bind to LcrG and release it from the secretion system, thereby resulting in a complete unblocking of Yop secretion. This then results in full induction of the LCR until the time when the cellular concentration of LcrQ increases to the point where it can again function as a negative regulator. Since there are still low levels of YopD present in the cell under inductive conditions, its intracellular concentration might not need to be increased for negative regulation to be reestablished.

There are at least two proteins required for negative control of LCR gene expression; however, the precise mechanism of this regulation is unknown. There is an LCR-specific transcriptional activator, LcrF (6, 24), whose activity could potentially be affected by the binding of a negative regulator. However, no direct interaction between LcrF and either LcrQ or YopD has yet been documented. In the flagellar system, where negative regulation of terminal loci in the regulatory cascade is due to the secreted anti-sigma factor FlgM (26, 27), the signal for induction of late-gene expression derives from monitoring the growing flagellar structure and not from an environmental cue (33). In contrast, the LCR regulon has previously been thought to respond to an extracellular signal, whether it be contact with a eukaryotic cell or the absence of Ca^{2+} . However, the fact that YopD is known to be necessary for vectorial translocation of

effector Yops into the eukaryotic cell raises the intriguing possibility that terminal regulation in the LCR is linked to the presence of a functional translocation mechanism at the interface between the bacterial and eukaryotic cells. Accordingly, regulation of Yop expression at the level of transcription could be tied to the bottom-line function of the LCR—i.e., the poisoning of the host cell by translocation of Yops.

One similar feature between the LCR and flagellar biogenesis systems is that the signal that elicits regulation within the bacterium most likely needs to be transmitted across both inner and outer membranes, although the method of transmitting the signal likely will differ, as the structures of the secretion machineries distal to the inner membrane in the two systems do not share homology. Flagellar late-gene expression is decreased as the filament grows, due to a decrease in the rate of export of flagellar products, which is thought to result in an increase in the intracellular concentration of FlgM (26, 27). It is not clear how LCR gene regulation is terminated after induction has occurred. The analogous situation would be that as Yops are translocated into the host cell, there is a point where the rate of secretion is slowed, thereby allowing the intracellular concentration of LcrQ to increase. Alternatively, the secretion channel may again become blocked when the bacterium dissociates from the eukaryotic cell. It could be that *Yersinia* uses two proteins for negative regulation instead of just one to make the system more sensitive to changes in LcrQ levels. In the flagellar system, low levels of FlgM are thought to exist in the cell during flagellar late-gene expression (i.e., under inductive conditions for synthesis of the flagellar filament) (26, 27), while LcrQ is not detected in the cell upon maximal induction of the LCR. This would then require that the intracellular pool of LcrQ accumulate before the system could be shut off. YopD could be responsible for allowing the cell to respond to even very small levels of LcrQ, as under inductive conditions, YopD is the most abundant Yop. The decrease or cessation of translocation may initiate the process of LCR down-regulation by focusing YopD's function on its interaction with LcrQ.

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