

Roles of LcrG and LcrV during Type III Targeting of Effector Yops by *Yersinia enterocolitica*

KRISTIN L. DEBORD,[†] VINCENT T. LEE,[‡] AND OLAF SCHNEEWIND*

Department of Microbiology and Immunology, University of California Los Angeles School of Medicine,
Los Angeles, California 90095

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***Yersinia enterocolitica* target effector Yop proteins into the cytosol of eukaryotic cells by a mechanism requiring the type III machinery. LcrG and LcrV have been suggested to fulfill essential functions during the type III targeting of effector Yops. It is reported here that knockout mutations of *lcrG* caused mutant yersiniae to prematurely secrete Yops into the extracellular medium without abolishing the type III targeting mechanism (Los phenotype [loss of type III targeting specificity]). Knockout mutations in *lcrV* reduced type III targeting of mutant yersiniae but did not promote secretion into the extracellular medium (Not [no type III targeting]). However, knockout mutations in both genes caused Δ *lcrGV* yersiniae to display a Los phenotype similar to that of strains carrying knockout mutations in *lcrG* alone. LcrG binding to LcrV resulted in the formation of soluble LcrGV complexes in the bacterial cytoplasm. Membrane-associated, bacterial-surface-displayed or -secreted LcrG could not be detected. Most of LcrV was located in the bacterial cytoplasm; however, small amounts were secreted into the extracellular medium. These data support a model whereby LcrG may act as a negative regulator of type III targeting in the bacterial cytoplasm, an activity that is modulated by LcrG binding to LcrV. No support could be gathered for the hypothesis whereby LcrG and LcrV may act as a bacterial surface receptor for host cells, allowing effector Yop translocation across the eukaryotic plasma membrane.**

Pathogenic yersiniae, e.g., *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, attach to mammalian cells and inject cytotoxic proteins across the plasma membrane into the eukaryotic cytosol (51). During infection, this mechanism allows yersiniae to escape phagocytic killing and establish residence within the lymphoid tissues of an infected host (42). *Yersinia* type III secretion machines catalyze the transport of cytotoxic proteins across the bacterial double-membrane envelope (36). Once yersiniae enter their host and are exposed to a 37°C environment, type III machinery components are expressed and assembled in the bacterial envelope (19, 22, 23, 62). Secretion can be induced by the removal of calcium ions from the media (11), causing *Y. enterocolitica* to massively export 14 different proteins (YopBDEHMNOPQRT, LcrV, YscM1, and YscM2) into the extracellular medium (36) (*Y. pestis* and *Y. pseudotuberculosis* express only YscM1 [LcrQ] but not YscM2 [49].) During host infection, the high concentration of calcium (1.8 mM) within extracellular fluids prevents the induction of type III secretion (11, 33). Bacterial attachment to host cells provides an inducing signal for type III machines (45), leading to the injection of YopEHMNOPT into the eukaryotic cytosol (type III targeting) (5, 20, 27, 37, 38, 43, 50). Some export substrates (YopBDR) are secreted into the extracellular medium (type III secretion) (30, 32), whereas another substrate, YopQ, is presumably associated with the bacterial envelope

(25). Thus, in response to specific host signals, likely a receptor-ligand interaction between invading pathogens and immune cells, *Yersinia* modify their type III export pathways and direct bacterial virulence factors to several distinct destinations (32).

The genes specifying the type III machinery (*ysc* [Yop secretion]) and export substrates are located on a 70-kb virulence plasmid (11). Knockout mutations in any one of the 22 *ysc* genes (*yscACDEFGHIJKLNOPQRSTUVWXYZ*) abrogate type III export under low-calcium conditions (1, 35), as well as type III targeting and secretion during infection (51). Knockout mutations in genes encoding effector Yops (*yopEHMOPT*) do not affect the type III pathway. In contrast, mutations that prevent the expression of YopD and LcrV abrogate type III targeting without affecting type III secretion (Not phenotype [no type III targeting]) (16, 32, 33, 44, 51). Knockout mutations in *yopN*, specifying another type III export substrate, cause mutant yersiniae to secrete most effector Yops into the extracellular medium (Los phenotype [loss of type III targeting specificity]) (30, 51). When incubated at 37°C in the presence of calcium, *yopN* mutants massively export type III substrates into the extracellular medium, thereby reducing the ability of mutant yersiniae to grow at elevated temperatures (temperature-sensitive or calcium-blind phenotype) (18, 65). Knockout mutations in several other regulatory genes, *syncN*, *tyeA*, and *yscB*, cause a similar phenotype (9, 14, 26, 28).

Previous work identified the *lcrG* gene as a member of the *Yersinia* low-calcium response pathway (4, 41, 48). Knockout mutations in *lcrG* cause mutant yersiniae to secrete Yops even in the presence of calcium (calcium-blind phenotype) (56). LcrG binds another *Yersinia* regulatory protein, LcrV, which is also encoded by the *lcrGVHyopBD* operon (4, 40). LcrG has been reported to be essential for the injection of effector Yops, since mutant *Y. enterocolitica* lacking a functional *lcrG* gene

* Corresponding author. Mailing address: Committee on Microbiology, Department of Molecular Genetics and Cell Biology, The University of Chicago, 920 East 58th St., Chicago, IL 60637. Phone: (773) 834-9060. Fax: (773) 702-3172. E-mail: oschnee@delphi.bsd.uchicago.edu.

[†] Present address: Committee on Microbiology, The University of Chicago, Chicago, IL 60637.

[‡] Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

failed to inject YopE₁₃₀-CyA as well as other reporter proteins into tissue culture cells (52). Fractionation experiments of bacterial cultures suggested that LcrG is located intracellularly and is at least partly associated with the membrane envelope (39, 40). In addition, *Y. pestis* has been reported to secrete small amounts of LcrG in the extracellular medium, and some LcrG is thought to be associated with the surface of *Y. enterocolitica* (7, 56). LcrV is secreted by the *Yersinia* type III pathway (33, 39, 40). *Yersiniae* carrying knockout mutations in *lcrV* did not display defects in calcium regulation since Yop secretion occurred only in the absence and not in the presence of calcium (33, 44, 47, 52, 55). Following induction of the type III pathway by temperature shift and calcium chelation, *lcrV* mutants express significantly less Yop proteins than wild-type *Yersiniae* (33, 55). Overexpression of LcrV in wild-type *Yersinia* strains caused a calcium-blind phenotype similar to that observed for *lcrG* mutant strains (33, 39).

Immunofluorescence microscopy experiments suggested that LcrV may aggregate on the surface of *Yersiniae* that have been induced for type III secretion (16, 44). It is not yet clear whether these aggregates represent the ordered assembly of a filamentous structure composed of LcrV or whether the immunofluorescent signal is caused by LcrV molecules that are secreted by the type III pathway. Antiserum or monoclonal antibody raised against purified LcrV has been reported to disrupt the type III targeting of effector Yops by *Y. pseudotuberculosis* into eukaryotic cells (44). However, the addition of anti-LcrV during tissue culture infection with *Y. pestis* showed no inhibition of type III targeting (16). Using an osmotic disruption of eukaryotic cells and cell fractionation, Straley and coworkers suggested that some LcrV molecules are injected into the cytosol of HeLa cells via a pathway that does not require type III genes (16, 17). The location of LcrV was also investigated by fractionating infected HeLa tissue cultures with the digitonin technique (see below) (33). Some LcrV was found to be secreted into the extracellular medium, but LcrV could not be detected in the cytosol of HeLa cells.

Several models for the function of LcrG and LcrV during infection have been proposed. Straley and coworkers advanced a model whereby LcrG may regulate the activity of the type III machinery by interacting directly with the membrane-embedded secretion apparatus (39). Further, the regulatory function of LcrG could be controlled by its binding to LcrV (titration hypothesis) (39). Wolf-Watz and coworkers proposed that secreted LcrV protein is involved in the assembly or function of an "injectisome" complex that allows translocation of effector Yops beyond the plasma membrane of eukaryotic cells (44). Cornelis and coworkers observed binding of LcrG to proteoglycans on the surface of tissue culture cells (7). Treatment with protease, heparinase, or xyloside, an inhibitor of glycosaminoglycan decorations of eukaryotic surface proteins, reduced LcrG binding to tissue culture cells (7). Further, binding of LcrG, as well as *Yersinia* injection of effector Yops into tissue culture, was competitively inhibited by the addition of heparin, dextran sulfate, and chondroitin sulfate B (7). Cornelis and colleagues proposed that LcrG functions as a bacterial receptor for eukaryotic cells (6, 7).

We wish to understand which *Yersinia* genes are needed for type III secretion and type III targeting (Yop translocation and Yop injection are commonly used terms for the same reac-

tion). To analyze the function of LcrG and LcrV on type III targeting of effector Yops, we have generated *lcrG*, *lcrV*, and *lcrGV* mutant strains. As previously reported (55), *lcrG* and *lcrGV* mutant *Yersiniae* displayed a calcium-blind phenotype for Yop secretion during growth in artificial medium, whereas the *lcrV* strain showed calcium regulation of Yop secretion. In contrast to previous reports (53), fractionation of infected HeLa tissue cultures with the digitonin technique suggests that *lcrG* mutants display a Los phenotype and are capable of injecting effector Yops into HeLa cells. Tissue culture infections with the *lcrV* mutant result in a Not phenotype (33). The *lcrGV* mutant displayed a Los phenotype similar to the *lcrG* mutant strain, suggesting that the role of *lcrG* in type III targeting is epistatic over that of *lcrV*. Biochemical studies suggest that LcrG is required for the type III secretion of LcrV. LcrG, as well as LcrG-LcrV complexes, was observed to be soluble in the bacterial cytoplasm. Membrane-associated LcrG-LcrV, bacterial-surface-exposed LcrG, or secreted LcrG could not be detected. These results support the previously proposed model that LcrG and LcrV function as regulators of the type III targeting pathway in the bacterial cytoplasm (39). The repression of type III targeting that is mediated by LcrG appears to be regulated by LcrG binding to LcrV. The Not phenotype of *lcrV* *Yersiniae* may be caused by LcrG-mediated repression of the type III pathway rather than by a defect in targeting, i.e., the transport of effector Yops across the eukaryotic plasma membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Y. enterocolitica* strains W22703 (wild type) (12), CT1 (*ΔlcrV*) (33), VTL1 (*ΔyopN*) (30), and KUM1 (*ΔyscV*) (8) have been described elsewhere. *Y. enterocolitica* strains MC2 (*ΔlcrG*), NIA6 (*ΔyscD*), and CT130 (*ΔyscN*) were constructed by allelic exchange using the suicide plasmid pLC28 (8). The *lcrG* mutation is comprised of a stop codon, an *EcoRI* site, and a single nucleotide frameshift (TGAATTCA) inserted following codon 9 of *lcrG*. *ΔlcrG* was constructed from two PCR products amplified with the primers LcrG-SacI (5'-AAGAGCTCAGAAGTACAAAAGAATCGTTCC-3') and LcrG-EcoRI-1 (5'-AAGAATTCACATATTCATCAAAAATGGGAAGAT-3'), as well as LcrG-EcoRI-2 (5'-AAGAATTCACAAAACGCTTAAACAGGCAG-3') and LcrG-PstI (5'-AACTGCAGTATCGAGACTATTTTTTTTTC-3'). The PCR products were cut with *SacI-EcoRI* or *EcoRI-PstI*, fused at the *EcoRI* site, and cloned between the *SacI-PstI* sites of pLC28. The *yscD* mutation is comprised of a stop codon, a *BamHI* site, and a single nucleotide frameshift inserted following codon 9 of *yscD*. *ΔyscD* was constructed from two PCR products amplified with the primers Ysc07-XbaI (5'-AATCTAGACTACCTAATAG CAAGAGT-3') and Ysc09-BamI (5'-AAGGATCCTCACCACTCACAATA CGC-3'), as well as Ysc08-XhoI (5'-AACTCGAGGTGTCATCGAGGTTTACC TC-3') and Ysc10-Bam2 (5'-AAGGTACCTCTGTCGTTTTATCAAGGGA-3'). The PCR products were cut with *XbaI-BamHI* or *BamHI-XhoI*, fused at the *BamHI* site, and cloned between the *XbaI-XhoI* sites of pLC28. The *yscN* mutant allele was designed to replace codons 51 to 390 of *yscN* with a unique *BglII* site (AGA TCT). *ΔyscN* was constructed from two PCR products with the primers YscN-delta-XbaI (5'-AATCTAGACACTCCAGATCGCATTAAATCC-3') and YscN-delta-BglII (5'-AAAGATCTTAAGTAAACATAACTCACCGATGC-3'), as well as *BglII-YscN-delta* (5'-AAAGATCTCAAATCGGGGAGTACCAGA A-3'), and YscN-SalI (5'-AAGTCGACATAATACTCTCTACGCGGTC-3'). The PCR products were cut with *XbaI-BglII* or *BglII-SalI*, fused at the *BglII* site, and cloned between the *XbaI-SalI* sites of pLC28. The *ΔlcrGV* mutation was designed to replace codons 10 to 96 of *lcrG* and codons 1 to 141 of *lcrV* with a unique *EcoRI* site (GAATTC). *ΔlcrGV* was constructed from two PCR products amplified with the primers LcrG-SacI (5'-AAGAGCTCAGAAGTACAAAAGA ATCGTTCC-3') and LcrG-EcoRI-1 (5'-AAGAATTCACATATTCATCA AAAATGGGAAGAT-3'), as well as LcrV-EcoRI (5'-AAGAATTCATGAAT CATCATGGTGATGAA-3') and LcrV-PstI (5'-AACTGCAGTGAGTGTCTG TCGTCTCTTG-3'). The PCR products were cut with *SacI-EcoRI* or *EcoRI-PstI*, fused at the *EcoRI* site, and cloned between the *SacI-PstI* sites of pLC28.

TABLE 1. Plasmids used in this work

Plasmid	Encoded protein (promoter) ^a	Vector	Antibiotic resistance ^b	Source or reference
pKD2	LcrV-DHFR _{His6} (T7)*	pET9a	Km	This work
pKD3	LcrV ₁₋₂₀₀ -DHFR _{His6} (T7)*	pET9a	Km	This work
pKD4	LcrV ₁₋₁₀₀ -DHFR _{His6} (T7)*	pET9a	Km	This work
pKD5	LcrV ₁₋₅₀ -DHFR _{His6} (T7)*	pET9a	Km	This work
pKD6	LcrV ₁₋₁₅ -DHFR _{His6} (T7)*	pET9a	Km	This work
pKD7	LcrV _{Δ2-14} -DHFR _{His6} (T7)*	pET9a	Km	This work
pKD8	LcrV-NPT (<i>lcrG</i>)*	pHSG576	Cm	This work
pKD14	LcrV-GST (<i>syncE</i>)*	pHSG576	Cm	This work
pKD15	FLAG-LcrV (<i>tac</i>)*	pHSG576	Cm	This work
pKD16	GST-LcrV ₂₀₁₋₃₂₆ (<i>tac</i>)*	pHSG576	Cm	This work
pKD17	GST-LcrV ₂₀₁₋₂₇₄ (<i>tac</i>)*	pHSG576	Cm	This work
pKD18	GST-LcrV ₂₇₅₋₃₂₆ (<i>tac</i>)*	pHSG576	Cm	This work
pKD19	GST-LcrV ₁₀₁₋₃₂₆ (<i>tac</i>)*	pHSG576	Cm	This work
pKD20	GST-LcrV ₁₀₁₋₂₇₄ (<i>tac</i>)*	pHSG576	Cm	This work
pKD21	GST-LcrV ₁₅₁₋₂₇₄ (<i>tac</i>)*	pHSG576	Cm	This work
pKD22	GST-LcrV ₅₁₋₃₂₆ (<i>tac</i>)*	pHSG576	Cm	This work
pKD23	GST-LcrV ₅₁₋₂₇₄ (<i>tac</i>)*	pHSG576	Cm	This work
pKD24	GST-LcrV ₁₅₁₋₃₂₆ (<i>tac</i>)*	pHSG576	Cm	This work
pCT60	LcrG (<i>tac</i>)	pHSG576	Cm	This work
pVL48	GST-LcrG (<i>tac</i>)	pHSG576	Cm	This work
pGEX-2TK	GST (<i>tac</i>)	pHSG576	Cm	29
pVL47	GST-LcrV (<i>tac</i>)	pHSG576	Cm	33
pVL69	LcrV-Flag (<i>tac</i>)	pHSG576	Cm	This work
pLC15	YopQ-DHFR _{His6} (T7)	pET9a	Km	8
pHSG576	Low-copy cloning vector		Cm	59
pET9a	T7 polymerase expression		Km	58

^a For entries indicated with an asterisk, the primer sequences can be obtained from the authors upon request.

^b Km, resistance to 50 μg of kanamycin per ml; Cm, resistance to 20 μg of chloramphenicol per ml.

Allelic exchange following mating between *Escherichia coli* S17-1(pLC28) (15), and *Y. enterocolitica* W22703 has been previously described (8). The *lcrG* open reading frame was PCR amplified with two primers carrying abutted *NdeI* and *BamHI* restriction sites: LcrG-Nde (5'-AACATATGAAATCTTCCCATTTGATGA-3') and LcrG-Bam (5'-AAGGATCCCTTAAATAATTTGCCCTCGCATCA-3'). The PCR product was digested with *NdeI/BamHI* and cloned between the *NdeI* and *BamHI* sites of the low-copy-number plasmid pDA255 (3) to generate pCT60. pVL48 was generated by inserting a PCR fragment, amplified with the primers LcrG-Kpn (5'-AAGGTACGAAATCTTCCCATTTGATGA-3') and LcrG-Bam between the *KpnI* and *BamHI* sites of pDA255 (3). Expression of *lcrG* and *gst-lcrG* is under the control of the *tac* promoter. The *lacI^q* allele is also cloned on the low-copy-number vector, and *Y. enterocolitica* transformants were induced to express *lcrG* and *gst-lcrG* by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Other plasmids used in this study are listed in Table 1.

Secretion and targeting assays. Measurements for the secretion of Yop proteins during bacterial growth in tryptic soy broth (TSB) supplemented with either 5 mM calcium or 5 mM EGTA have been previously described (8). Targeting of Yop proteins into the cytosol of HeLa cells was determined by fractionating tissue cultures by the digitonin technique (30). Briefly, overnight cultures of yersiniae were diluted 1:20 into 30 ml of fresh Luria broth and grown for 2 h at 26°C with shaking. Bacteria were sedimented at 8,000 × g for 10 min and suspended in phosphate-buffered saline (PBS). HeLa cells were grown to 80% confluence in 75-cm² tissue culture flasks with Dulbecco modified Eagle medium (DMEM) and 10% fetal bovine serum. Prior to infection, cells were washed twice with PBS, covered with 10 ml of DMEM, and warmed to 37°C for 30 min. Aliquots of HeLa cells were counted, and each flask was infected with yersiniae at a multiplicity of infection of 10 and incubated for 3 h at 37°C with 5% CO₂. Culture media were removed and centrifuged at 32,000 × g for 15 min to separate soluble proteins from nonadherent bacteria in the sediment. HeLa cells, as well as adherent bacteria, were scraped off the flasks into 10 ml of digitonin in PBS and placed on a rotary shaker for 20 min. Samples were centrifuged at 32,500 × g for 15 min. A 7-ml aliquot was withdrawn and precipitated with methanol-chloroform, while the remaining supernatant was discarded. The sediment was suspended in 10 ml of PBS, and a 7-ml aliquot was precipitated with methanol-chloroform. Protein precipitates were solubilized in sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with specific antiserum. Immunoreac-

tive species were quantified as chemiluminescent signals on X-ray film by using laser densitometry scanning.

Cell fractionations. Overnight cultures of *Y. enterocolitica* W22703 were diluted 1:50 into 800 ml of fresh TSB media and grown for 2 h at 26°C and then for 3 h at 37°C in the presence or absence of calcium. Cells were harvested at 6,000 × g for 15 min and suspended in 10 ml of HEPES buffer (20 mM HEPES, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol [DTT]; pH 7.5). Bacteria were broken in a French pressure cell at 14,000 lb/in², and intact cells were removed by centrifugation at 6,000 × g for 10 min. A 3-ml aliquot of crude bacterial extract was removed with the supernatant and subjected to ultracentrifugation at 100,000 × g for 30 min. The supernatant (S) was removed, and the membrane pellet was suspended in 3 ml of HEPES buffer (P). At each fractionation step, aliquots were withdrawn and mixed with an equal volume of sample buffer. Samples were separated on by SDS-15% PAGE and analyzed by immunoblotting.

Protease protection. Overnight cultures of *Y. enterocolitica* W22703 were diluted 1:50 into fresh TSB media and grown for 2 h at 26°C and for 3 h at 37°C in the presence of 5 mM calcium or 5 mM EGTA. Four 1-ml aliquots of cultures were incubated with or without 20 μg proteinase K per ml, 1% SDS, or 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 37°C for 30 min. Proteolysis was quenched by the addition of 1 mM PMSF to all reactions. Proteinase K and SDS were added so that each sample contained the same reagents. Proteins were precipitated with chloroform-methanol, dried, solubilized in 100 μl of equal parts buffer B (6 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl; pH 8.0)/sample buffer, and analyzed by immunoblotting.

Purification of glutathione S-transferase (GST)-LcrG. Overnight cultures of *Y. enterocolitica* MC2(pVL48) were diluted 1:50 into fresh TSB media supplemented with 20 μg of chloramphenicol per ml. Bacteria were grown and induced by incubation for 2 h at 26°C and for 3 h at 37°C. Cells from 500 ml of culture were harvested by centrifugation at 6,000 × g for 15 min. The cell pellet was suspended in 10 ml of F buffer (50 mM Tris-HCl, 20% sucrose, 1 mM DTT; pH 7.0), and bacteria were broken by a single passage through a French pressure cell at 14,000 lb/in². Unbroken cells and debris were removed by centrifugation at 32,500 × g for 15 min. The supernatant was subjected to affinity chromatography on glutathione-Sepharose preequilibrated with F buffer. The column was washed with 30 volumes of wash buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.5), and proteins were eluted with 4 ml of wash buffer containing 10 mM glutathione. Eluted proteins were mixed with an equal volume of sample buffer containing 3

M urea and analyzed by immunoblotting. To determine the enrichment of proteins during the purification procedure, equal volumes of load and eluate fractions were subjected to immunoblotting and chemiluminescent signals quantified using an AlphaScanner instrument.

Protein-binding assays. Full-length and truncated sequences of LcrV were fused to either the C terminus of GST or the N terminus of His₆-tagged dihydrofolate reductase (DHFR_{His6}). LcrV sequences for GST fusions were PCR amplified with appended 5'-*Kpn*I and 3'-*Bam*HI sites. The resulting fragments were subcloned into pCR2.1 (Invitrogen) and digested with *Kpn*I and *Bam*HI. Fragments were inserted into a previously described derivative of pHSG575 (59). For DHFR_{His6} proteins, LcrV sequences were PCR amplified with appended 5'-*Nde*I and 3'-*Kpn*I sites. Resulting fragments were inserted into a previously described plasmid (8). To create an insert for the first 15 codons, appropriate primers were annealed and directly inserted into the expression vector containing DHFR_{His6}. Fusion proteins were expressed and affinity purified from the *E. coli* cytoplasm. Fusion proteins were separated by SDS-15% PAGE, in duplicate. One SDS-PAGE gel was Coomassie brilliant blue stained. The second SDS-PAGE gel was electroblotted onto polyvinylidene difluoride (PVDF) membrane and incubated with purified ³²P-labeled GST-LcrG. The filter membranes were then analyzed on a PhosphorImager instrument for radioactive signals, which are reported as arbitrary units per picomoles. Glutathione-Sepharose beads containing immobilized GST-LcrG were used for ³²P-labeling with 3'-5'-cyclic AMP (cAMP)-dependent heart muscle protein kinase (Sigma). GST-LcrG (2.5 to 5 μg immobilized on 50 μl of 50% slurry of glutathione-Sepharose) in 100 μl of 20 mM Tris-HCl-100 mM NaCl-12 mM MgCl₂-1 mM DTT-0.1 mM cAMP was incubated with 100 to 500 μCi of [γ-³²P] ATP (800 μCi mmol⁻¹) and 100 U of kinase (100 U kinase = 1.4 μg of kinase in 20 mM Tris-HCl-100 mM NaCl-12 mM MgCl₂-1 mM DTT-20% [vol/vol] glycerol). After optimal labeling, typically for 1 h at 37°C, the beads were washed three times with 1 ml of 20 mM Tris-HCl-100 mM NaCl-20% glycerol and ³²P-labeled GST-LcrG was eluted with 50 μl of 20 mM Tris-HCl-100 mM NaCl-20% glycerol-10 mM glutathione. Aliquots were analyzed by scintillation counting and SDS-PAGE-autoradiography to determine the specific activity.

RESULTS

***lcrG* mutants are calcium blind and fail to secrete LcrV.** The *lcrG* mutant strain *Y. enterocolitica* MC2 was generated by inserting a stop codon and frameshift mutation after codon 9 of the *Y. enterocolitica* W22703 (wild-type) *lcrG* gene. Wild-type and *lcrG* mutant yersiniae were grown in TSB at 37°C in the presence of 5 mM calcium (Fig. 1, +Ca²⁺) or 5 mM EGTA (-Ca²⁺). Cultures were centrifuged, and the extracellular medium was separated from the bacterial pellet. Proteins in both fractions were precipitated with trichloroacetic acid (TCA), separated by SDS-PAGE, and analyzed by immunoblotting (Fig. 1). When induced by the chelation of calcium, the type III machines of wild-type yersiniae secreted YopBDE into the extracellular medium. In the presence of calcium, yersiniae secreted only small amounts of YopBDE via the type III pathway (11% YopB, 16% YopD, and 3% YopE). In contrast, the *lcrG* mutant strain MC2 exported 73% YopB, 58% YopD, and 53% YopE (percentage of the total amount of Yop protein in the supernatant and sediment) in the presence of calcium. This result corroborates previous observations that *lcrG* mutations abolish the calcium regulation of the *Yersinia* type III pathway (calcium-blind phenotype) (56). Wild-type yersiniae secreted 8% of LcrV in the presence and 23% of LcrV in the absence of calcium. *lcrG* mutant yersiniae failed to secrete LcrV in either the presence or the absence of calcium. Following centrifugation of *Yersinia* cultures at 25,000 × *g*, LcrG sedimented with the bacteria into the pellet fraction, suggesting that LcrG is not secreted by the type III pathway. *lcrGVH* and *yopBD* form a polycistronic operon that is expressed at a low level when calcium is added to the culture medium (4) (Fig. 1). The *lcrG* mutant strain MC2 expressed the *lcrGVHyopBD* operon

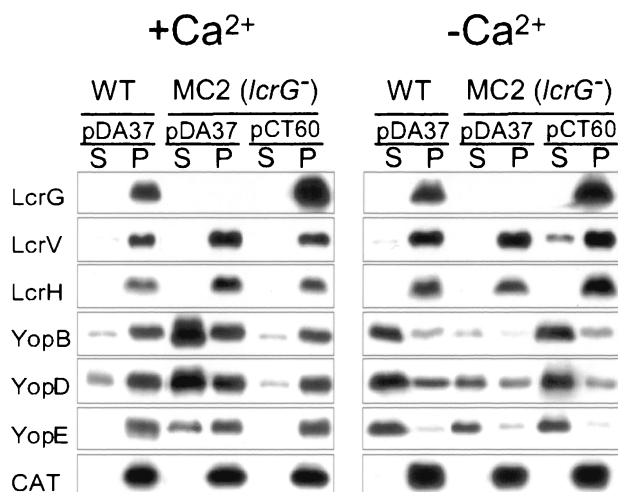


FIG. 1. *lcrG* mutant yersiniae secrete Yops in the presence of calcium. *Y. enterocolitica* W22703 (wild type) and the isogenic *lcrG* mutant, strain MC2, were transformed with pDA37 (vector control) or pCT60 (wild-type *lcrG*) and grown in the presence of 5 mM calcium or 5 mM EGTA for 2 h at 37°C. Cultures were centrifuged, and the supernatant (S) was separated from the cell pellet (P). Proteins in each sample were precipitated with TCA, solubilized in sample buffer, and analyzed by SDS-PAGE, followed by immunoblotting with antisera raised against LcrG, LcrV, LcrH, YopB, YopD, YopE, and CAT. *Y. enterocolitica* W22703 secretes more YopBDE in the absence than in the presence of calcium. In contrast, the *lcrG* mutant strain displays a calcium-blind phenotype and secretes YopBDE in the presence or absence of calcium. Further, the *lcrG* mutant failed to secrete LcrV. Transformation of *lcrG* mutant cells with pCT60 restored the wild-type phenotype.

at a higher level than for the wild-type yersiniae. Chloramphenicol acetyltransferase (CAT) served as a control for proper fractionation of a cytoplasmic protein. Transformation of *Y. enterocolitica* MC2 with pCT60, encoding wild-type *lcrG*, reduced the calcium-blind phenotype, as well as defects in LcrV secretion and *lcrGVH* and *yopBD* regulation.

Upon fractionation of *Yersinia* cultures, Skrzypek et al. as well as Nilles et al., found that *Y. pestis* secretes significant amounts of LcrG into the extracellular medium (40, 55, 56). Skrzypek et al. reported that both in the absence and in the presence of 2.5 mM Ca²⁺ *lcrG* mutant *Y. pestis* strains secrete LcrG into the extracellular medium (56). We do not know the reason for the discrepancy of these data with our results.

***lcrG* mutants display a Los phenotype during the infection of HeLa cells.** To examine the role of *lcrG* in type III targeting, HeLa cells were infected with wild-type *Y. enterocolitica* W22703 or the *lcrG* mutant strain MC2. The medium was decanted and centrifuged, separating nonadherent bacteria (P; pellet) from the extracellular medium (S; supernatant). HeLa cells with adherent yersiniae were extracted with digitonin, a detergent known to disrupt the cholesterol-containing plasma membrane of HeLa cells but not the bacterial envelope (30). Digitonin extracts were centrifuged to sediment bacteria, as well as HeLa cell debris (P), while the soluble contents of the eukaryotic cytosol remained in the supernatant (S). Proteins were precipitated with chloroform-methanol and analyzed by SDS-PAGE and immunoblotting (Fig. 2). As a control, digitonin extraction released farnesyl protein transferase (FPT)

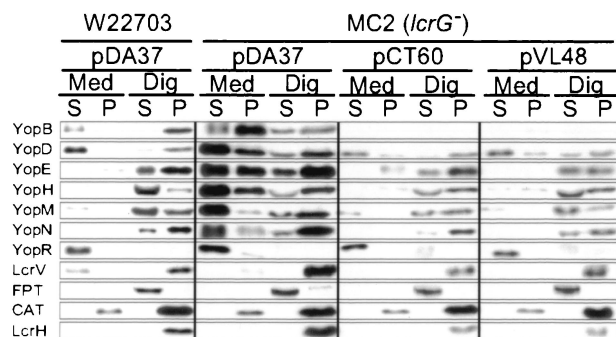


FIG. 2. *lcrG* mutant yersiniae display a Los phenotype and secrete effector Yops into the extracellular medium. HeLa cells were infected with *Y. enterocolitica* W22703 or MC2 ($\Delta lcrG$) harboring plasmids pDA37 (vector control), pCT60 (*lcrG*), or pVL48 (*gst-lcrG*). After incubation for 3 h at 37°C, the tissue culture medium (Med) was decanted and centrifuged to separate secreted proteins from those present within nonadherent bacteria. HeLa cells, as well as adherent yersiniae, were extracted with digitonin (Dig), a detergent that solubilizes the eukaryotic plasma membrane but not the bacterial envelope. Extracts were centrifuged to separate proteins solubilized from the HeLa cytoplasm from those that sediment with the bacteria. Proteins were precipitated with chloroform-methanol and analyzed by immunoblotting. *Y. enterocolitica* MC2 displayed a loss of targeting specificity (Los) and secreted large amounts of YopB, YopD, YopE, YopH, YopM, and YopN into the culture medium. The Los phenotype was complemented by transforming MC2 cells with either pCT60 or pVL48. As a control for proper fractionation, FPT is located in the cytosol of HeLa cells and is solubilized by digitonin extraction. LcrH and CAT reside in the bacterial cytoplasm and are not solubilized by digitonin extraction.

from the eukaryotic cytosol. LcrH and CAT, proteins that are located in the *Yersinia* cytoplasm (10), were not released and sedimented with the bacteria. Wild-type *Yersinia* targeted YopEHMN into the cytosol of HeLa cells and secreted YopBDR and small amounts of LcrV into the extracellular medium (Fig. 2) (30). The *lcrG* mutant appears to inject small amounts of YopBDEHMN into the cytosol of HeLa cells. Moreover, *lcrG* mutant yersiniae secreted large amounts of YopBDEHMR into the extracellular medium, suggesting a Los phenotype (Fig. 2). The *lcrG* mutant strain overexpressed proteins associated with the type III pathway and failed to secrete LcrV into the extracellular medium. Transformation of strain MC2 with plasmids pCT60 or pVL48, encoding wild-type LcrG or GST-LcrG, respectively, restored the type III targeting specificity of effector Yops. The two- to threefold overexpression of LcrG or GST-LcrG from the IPTG-inducible *tac* promoter (see Fig. 1) reduced the expression of the *Yersinia yop* virulon and reduced the synthesis of YopB. The reduced amount of YopB did not interfere with the type III targeting of YopEHMN. This finding is consistent with the earlier report that YopB may be dispensable for the injection of effector Yops into eukaryotic cells (32). Our results differ from those of Sarker et al., who observed no type III targeting for *lcrG* mutant *Y. enterocolitica* using the Cya fusion approach (53). *lcrG* mutants generated 0.24 ± 0.21 nmol of cAMP per mg protein using YopE₁₃₀-Cya as a reporter. *yscN* mutants (type III mutant control) generated 0.36 nmol of cAMP, while wild-type yersiniae generated 5.7 nmol of cAMP. We do not

know the reason for the discrepancy of these data with our results.

***lcrGV* mutants display a Los phenotype during the infection of HeLa cells.** The *lcrGV* mutant strain KLD1 was generated by replacing *lcrG* codons 10 to 96 and *lcrV* codons 1 to 141 with an *EcoRI* site. The double-mutant strain was grown in TSB at 37°C in the presence of 5 mM calcium, and type III secretion was analyzed by immunoblotting (Fig. 3A). As expected, *Y. enterocolitica* KLD1 ($\Delta lcrGV$) did not express LcrG and LcrV, while strains MC2 ($\Delta lcrG$) and CT1 ($\Delta lcrV$) displayed the expected defects in expression of LcrG and LcrV, respectively. Further, the *lcrGV* mutant strain secreted YopE (and several other Yops) in the presence of calcium, a result consistent with the previously reported calcium-blind phenotype of *lcrGV* double-mutant strains (55). Transformation of KLD1 with plasmids expressing either LcrG or LcrV caused the mutant yersiniae to display the same phenotype as *lcrV* or *lcrG* single-mutant strains, respectively (data not shown). During the infection of HeLa cells, the *lcrV* mutant strain CT1 expressed fewer effector Yops than wild-type or *lcrG* mutant yersiniae (33). Strain CT1 injected reduced amounts of YopH into the eukaryotic cytosol and secreted only small amounts of YopH into the extracellular medium (33) (Fig. 3B). Infection of HeLa cells with the *lcrGV* mutant strain KLD1 revealed an increase in YopH expression, an increase in YopH secretion into the extracellular medium, and an increase in YopH injection into the cytosol of eukaryotic cells. Together, these data suggest that the *lcrGV* mutant strain displays a Los phenotype. Further, LcrV may not be required for the injection of effector Yops into HeLa cells, and the Not phenotype of *lcrV* mutant strains can be explained as the LcrG-mediated repression of the *yop* virulon.

LcrG binds LcrV in the cytoplasm of yersiniae. Plasmid pVL48 encodes a fusion between GST and LcrG. Expression of GST-LcrG is controlled by the IPTG-inducible *tac* promoter. Transformation of pVL48 into *Y. enterocolitica* MC2 and induction with IPTG reversed the calcium-blind phenotype, as well as defects in LcrV secretion and *lcrGVH* and *yopBD* repression (Fig. 1). This result suggests that GST-LcrG may function similar to wild-type LcrG in complementing the defects of the *lcrG* mutant strain. *Y. enterocolitica* MC2(pVL48) was grown in the presence or absence of calcium. The bacteria were harvested by centrifugation and disrupted in a French pressure cell. Unbroken cells and insoluble material were removed by centrifugation, and the supernatant was subjected to affinity chromatography on glutathione-Sepharose. Collected fractions were analyzed on silver-stained SDS-PAGE gels (Fig. 4A). To analyze the collected fractions further, samples were subjected to immunoblotting (Fig. 4B). Purification and enrichment of *Yersinia* proteins was monitored by measuring immunoreactive signals in the eluate (E) and dividing them by those present in crude extracts (L; lysate). An E/L quotient 1.46 was observed for GST-LcrG, indicating enrichment of this polypeptide during affinity chromatography (E/L = 1.78 when cells were grown in the absence of calcium). The E/L quotient of 1.29 (1.26) revealed that LcrV had also been enriched and copurified with GST-LcrG. In contrast, YopB and YopD, proteins that have been proposed to interact with LcrV (54), failed to bind GST-LcrG or -LcrV (E/L = 0). CAT did not copurify with GST-LcrG/LcrV. As a control for the specific binding of

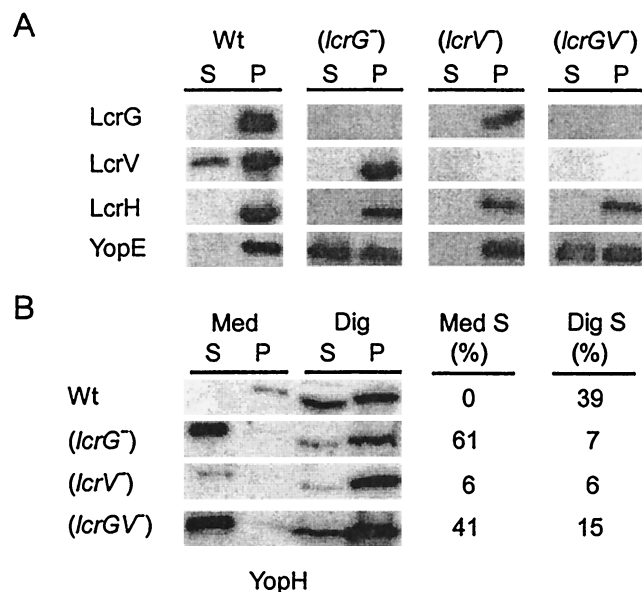


FIG. 3. *lcrGV* mutant yersiniae display a calcium-blind and Los phenotype. (A) *Y. enterocolitica* W22703 (Wt) and the isogenic mutant strains MC2 (*lcrG*), CT1 (*lcrV*), and KLD1 (*lcrGV*) were grown in the presence of calcium for 2 h at 37°C. Cultures were centrifuged, and the supernatant (S) was separated from the cell pellet (P). Protein in each sample was precipitated with TCA, solubilized in sample buffer, and analyzed by SDS-PAGE, followed by immunoblotting with antisera raised against LcrG, LcrV, LcrH, and YopE. The percentage of secreted YopE is indicated. (B) HeLa cells were infected with *Y. enterocolitica* strains MC2 (*lcrG*), CT1 (*lcrV*), and KLD1 (*lcrGV*) and incubated for 3 h at 37°C. The tissue culture medium (Med) was decanted and centrifuged to separate secreted proteins from those present within nonadherent bacteria. HeLa cells, as well as adherent yersiniae, were extracted with digitonin (Dig), a detergent that solubilizes the eukaryotic plasma membrane but not the bacterial envelope. Extracts were centrifuged to separate proteins solubilized from the HeLa cytoplasm from those that sediment with the bacteria. Proteins were precipitated with chloroform-methanol and analyzed by immunoblotting with antiserum raised against purified YopH. The percentage of YopH secreted into the extracellular medium (Med S) or targeted into the cytosol of HeLa cells (Dig S) is indicated.

LcrV to GST-LcrG, *Yersinia* extracts were subjected to affinity chromatography on glutathione-Sepharose containing bound GST. LcrG, LcrV, LcrH, YopB, and YopD did not bind to GST, suggesting that the observed coelution of GST-LcrG and LcrV is caused by the direct interaction between LcrV and LcrG.

To examine the subcellular location of LcrG, wild-type yersiniae were grown in the presence of 5 mM calcium or 5 mM EGTA, harvested by centrifugation, and lysed in a French pressure cell. Unbroken cells were removed by slow-speed centrifugation, and bacterial extracts were analyzed by ultracentrifugation, separating soluble cytoplasmic proteins from the insoluble membrane envelope in the sediment. Proteins in both fractions were precipitated with TCA and analyzed by immunoblotting (Fig. 5A). Cytoplasmic SycH chaperone remained in the supernatant (64), whereas almost all of the inner membrane protein LcrD (YscV) (46) sedimented into the pellet. Both LcrG and LcrV remained in the supernatant, suggesting that these polypeptides do not associate with the membrane envelope of yersiniae. In contrast, 34% of the YopD

sedimented into the pellet fraction during the centrifugation of extracts obtained from low-calcium-induced yersiniae but not during the centrifugation of extracts from uninduced bacteria.

We sought to determine whether LcrG and LcrV are accessible to protease digestion on the surface of yersiniae. *Y. enterocolitica* W22703 was grown in the presence or absence of calcium and incubated with 20 µg of proteinase K/ml of culture. The addition of proteinase K to the extracellular medium of yersiniae grown in the presence of calcium digested some LcrV and small amounts of YopD, but proteinase K did not digest YopE, LcrG, and LcrH (Fig. 5B). The addition of proteinase K to the extracellular medium of yersiniae grown in the absence of calcium digested all or most of the YopE and YopD, as well as some of the LcrV. In contrast, LcrG and LcrH were not digested by extracellular proteinase K. The addition of SDS dissolved the membrane envelope of yersiniae, allowing access of protease and digestion of all proteins examined. Protease digestion was quenched when proteinase K was added together with PMSF, a known serine protease inhibitor. These results suggest that LcrG and LcrH are located intracellularly, protected from extracellular protease by the membrane envelope of yersiniae, whereas secreted LcrV, YopD, and YopE molecules are accessible to extracellular protease. As reported in Fig. 1, some LcrV is secreted and protease accessible when the yersiniae are grown in the presence of calcium.

LcrG and the type III machinery are required for the secretion of LcrV. Previous work suggested that LcrV may be exported and injected into HeLa cells in a manner that does not depend on the type III secretion machinery (16). Fractionation of *Yersinia*-infected HeLa cells revealed that small amounts of LcrV were secreted into the extracellular medium, while most immunoreactive species sedimented with the bacteria after digitonin extraction (33). To test whether LcrV secretion in the presence or absence of calcium required the type III pathway, we tested various mutant backgrounds. As expected, wild-type yersiniae secreted LcrV in the presence or absence of calcium, whereas YopE secretion occurred only in the absence of calcium (Fig. 6). As a control, cytoplasmic LcrH was not secreted and sedimented with yersiniae into the pellet fraction. The *yopN* mutant strain VTL1 was defective in calcium regulation and secreted large amounts of YopE even in the presence of calcium. Secretion of YopE and LcrV was abolished in *Yersinia* mutants that failed to express the type III machinery components LcrD, YscD, and YscN. These results suggest that the secretion of LcrV requires LcrG, as well as expression of type III machinery components.

LcrG binds to the C-terminal region of LcrV. To identify the LcrV binding site of LcrG, GST and DHFR_{His6} fusions to LcrV were expressed in *E. coli* and purified. After separation by SDS-PAGE and electrotransfer to a PVDF membrane, LcrV fusions were incubated with ³²P-labeled GST-LcrG, and binding was quantified by use of a PhosphorImager (Fig. 7). Fusion of full-length LcrV (amino acids 1 to 326) to the N terminus of DHFR or the C terminus of GST did not interfere with the binding to GST-LcrG, suggesting that a free N or C terminus of LcrV is not required for binding. Truncating the C terminus of LcrV generated the hybrids LcrV₁₋₂₀₀, LcrV₁₋₁₀₀, LcrV₁₋₅₀, and LcrV₁₋₁₅-DHFR_{His6}, all of which failed to bind GST-LcrG. In contrast, truncation at the N terminus of LcrV,

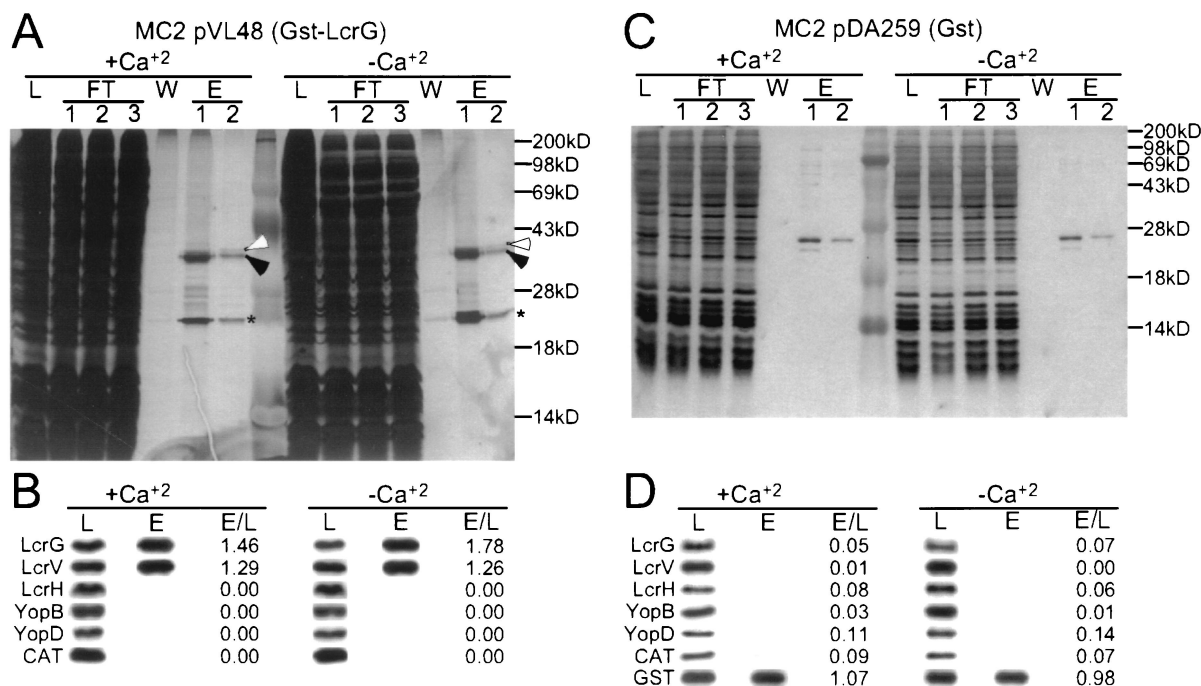


FIG. 4. GST-LcrG binds to LcrV. (A) *Y. enterocolitica* MC2 (*lcrG*)/pVL48 (*gst-lcrG*) was grown at 37°C and induced for type III secretion by the chelation of calcium ions. Expression of GST-LcrG was induced by the addition of 1 mM IPTG to the culture medium. Cells (10^{12} CFU) were harvested by centrifugation and lysed in a French pressure cell, insoluble material was removed by centrifugation at $100,000 \times g$, and the supernatant (L, load) was subjected to affinity chromatography on glutathione-Sepharose. Flowthrough (FT), wash (W), and eluate (E) fractions of 1 ml were collected and analyzed by silver-stained SDS-PAGE. Arrowheads indicate the positions of GST-LcrG (Black) and LcrV (white). The asterisk indicates the migration of an unknown *Yersinia* protein that binds to glutathione-Sepharose. (B) The load (L) and eluate (E; fraction 1) of the affinity chromatography analyses were analyzed by immunoblotting with antisera (LcrG, LcrV, LcrH, YopB, YopD, and CAT) and quantified. Results are reported as the ratio of signal intensity observed for the eluate fraction divided by that of the load fraction (E/L). The migration of molecular mass markers (M) is indicated in kilodaltons. The positions of GST-LcrG (filled arrowhead) and LcrV (open arrowhead) are indicated. As a control for the specific binding of LcrV to GST-LcrG, *Y. enterocolitica* expressing GST alone was subjected to affinity chromatography and analyzed by silver-stained SDS-PAGE (D) and immunoblotting (C).

deleting residues 1 to 100 (GST-LcrV₁₀₁₋₃₂₆), did not interfere with the binding of GST-LcrG. Additional truncations at the N and C termini of GST-LcrV₁₀₁₋₃₂₆ abolished binding. Thus, LcrV residues 100 to 326 are necessary and sufficient for the binding of LcrV to GST-LcrG.

LcrV secretion signals. Fusion of LcrV to the N terminus of neomycin phosphotransferase (NPT) prevented type III secretion of the hybrid proteins (Fig. 8). Similar results were observed when LcrV was fused to the N terminus of GST. Appending amino acids (FLAG sequence) to the C terminus of LcrV also abrogated type III secretion of the epitope tagged protein. In contrast, when the FLAG sequence was appended to the N terminus of LcrV, 21% of the epitope-tagged protein was secreted in the presence of calcium, whereas no secretion occurred in the absence of calcium. All truncations of the N- or C-terminal sequences abrogated LcrV secretion via the low-calcium-induced type III pathway (data not shown).

DISCUSSION

Straley and colleagues characterized LcrG as a regulator of the type III pathway, controlling the synthesis of Yops when yersiniae are grown in the presence of calcium (56). The same group also showed that the regulatory activity of LcrG is controlled via the binding to LcrV (39, 40). Since the amount of

LcrV in the bacterial cytoplasm is dependent on the expression of the *lcrGVH*YopBD operon, as well as on the type III export of LcrV, LcrG may act as a sensor for the assembly and function of the type III pathway. The data reported here corroborate this model. LcrV secretion appears to require the presence of LcrG. Although it is tempting to speculate that LcrG binding to the C-terminal end of LcrV may allow initiation of the polypeptide into the type III pathway, none of our data provide definitive proof for this notion. Once LcrV is exported by the type III pathway, the liberated LcrG may bind to other LcrV molecules or, if intracellular LcrV concentrations drop below a certain threshold, unbound LcrG may somehow control the expression of *yop* genes. In this model LcrG and LcrV appear to function as a regulatory switch for the expression of Yops prior to the attachment of yersiniae to host cells and the formation of a type III injection device.

There are several unusual features in the recognition of LcrV secretion substrate. (i) LcrV secretion via the type III pathway is not tightly regulated by calcium as is observed for YopE. Consistent with this notion is the observation that yersiniae secrete LcrV into the extracellular medium during tissue culture infections, i.e., when the concentration of extracellular calcium is 1.8 mM (33). (ii) Unlike typical secretion chaperones (SycE and SycH) (64), LcrG binds to the C-termi-

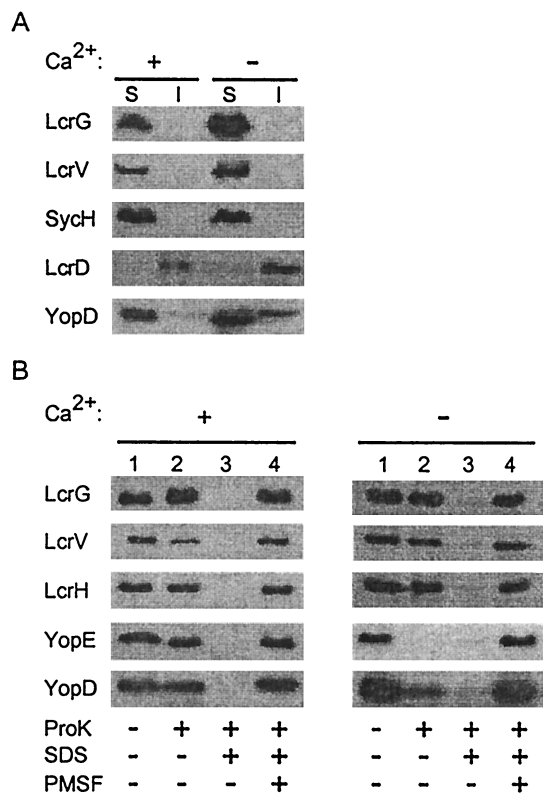


FIG. 5. LcrG is located intracellularly. (A) Cell fractionation of *Y. enterocolitica* W22703 (wild type) was grown in the presence of 5 mM calcium (+Ca²⁺) or 5 mM EGTA (-Ca²⁺) for 3 h at 37°C. Bacteria were lysed in a French pressure cell. Unbroken cells were removed by low-speed centrifugation, and crude extracts were subjected to ultracentrifugation at 100,000 × g. The supernatant (S), containing soluble cytoplasmic contents, was separated from the insoluble (membrane) sediment (I). Samples were analyzed by SDS-PAGE and immunoblotting with antisera raised against LcrG, LcrV, LcrH, YopE, and YopD. (B) Protease protection assay. *Y. enterocolitica* W22703 was grown in the presence or absence of calcium. Four 6-ml culture aliquots (10⁸ CFU/ml) were incubated at 37°C for 30 min with or without 20 μg of proteinase K per ml, 1% SDS, or 1 mM PMSF as indicated. Samples were precipitated with chloroform-methanol, dried, solubilized in sample buffer, and analyzed by SDS-PAGE and immunoblotting.

nal domain of the secretion substrate (53; also, our results). (iii) LcrV does not harbor the secretion signals that have been identified in all other Yops examined thus far (2, 3, 8, 34, 54, 57). (iv) In contrast to other Yops, LcrV signal recognition does not tolerate C-terminal fusions (16, 44; also, our results); however, some insertions at the N-terminal end are permitted. Straley and coworkers proposed that LcrG may physically block the type III pathway (16). In this scenario, one could expect LcrG to somehow be associated with the type III machinery or perhaps to sediment with membranes. A membrane association could not be observed in the fractionation experiments reported here (Fig. 5A). We do not know the mechanism of LcrG-mediated regulation. Since LcrG appears to be a soluble protein, it is conceivable that LcrG may regulate gene expression directly, for example, at the level of transcriptional or posttranscriptional control. We believe that LcrG binding to surface proteoglycans of eukaryotic cells (7) may not play a

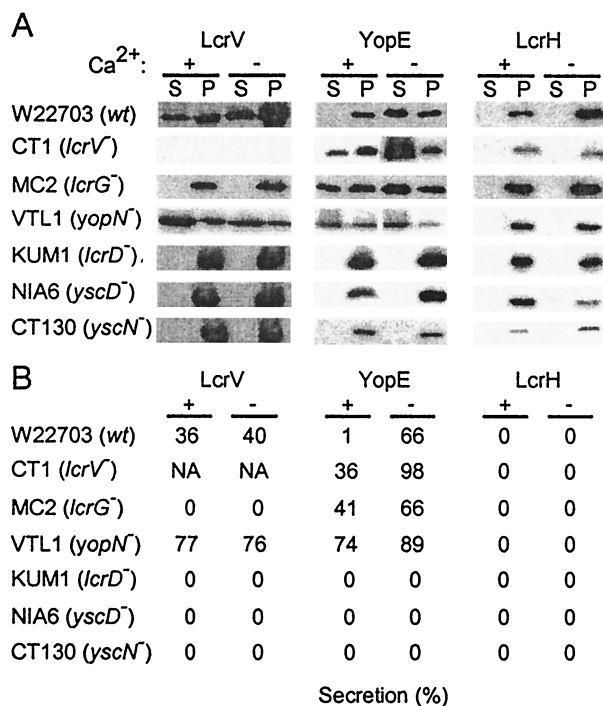


FIG. 6. LcrV is secreted via the type III pathway. (A) *Y. enterocolitica* wild-type strain W22703 (wt) and isogenic mutant strains CT1 (*lcrV*), MC2 (*lcrG*⁻), VTL1 (*yopN*⁻), KUM1 (*yscD*⁻), NIA6 (*yscD*⁻), and CT130 (*yscN*⁻) were grown in presence of 5 mM calcium (+Ca²⁺) or 5 mM EGTA (-Ca²⁺) at 37°C. Cultures were centrifuged, and the supernatant (S) was separated from the cell pellet (P). Protein in each sample was precipitated with TCA, solubilized in sample buffer, and analyzed by immunoblotting with antisera raised against LcrV, YopE, and LcrH. (B) Immunoblots were quantified, and the amount of secreted polypeptide is recorded as a percentage.

role during infection, since LcrG does not appear to be positioned on the surface of *Y. enterocolitica* W22703. LcrG binding to heparin can also be thought of as an affinity for negative charge, a phenomenon that is often observed for nucleotide-binding proteins.

It is reported here that *lcrG* mutant *Yersinia* are capable of injecting effector Yops into HeLa cells but are defective for the specificity of the type III targeting reaction (Los phenotype). In contrast to the Not phenotype of *lcrV* mutants (16, 33, 44), *lcrGV* double mutants display a Los phenotype, suggesting that LcrV may not be essential for the type III targeting mechanism. Earlier work reported that *yopD* mutants display a Not phenotype. However, this defect of *yopD* mutants can be complemented by *gst-yopD* fusions, encoding a hybrid protein that cannot be secreted by the type III pathway (32). The same study suggested also that *yopB* may not be essential for the type III targeting mechanism. Taken together with the results presented here, these observations lead us to assume that the protein products encoded by the *lcrGVHyopBD* operon are not directly involved in effector Yop translocation (type III targeting) (30a, 32, 33; D. M. Anderson, K. Ramamurthi, C. Tam, and O. Schneewind, unpublished results). This view is currently under debate. Some of the above mentioned results are controversial as other groups have observed *yopB* to be essential for effector Yop translocation (5, 21). Further, YopB,

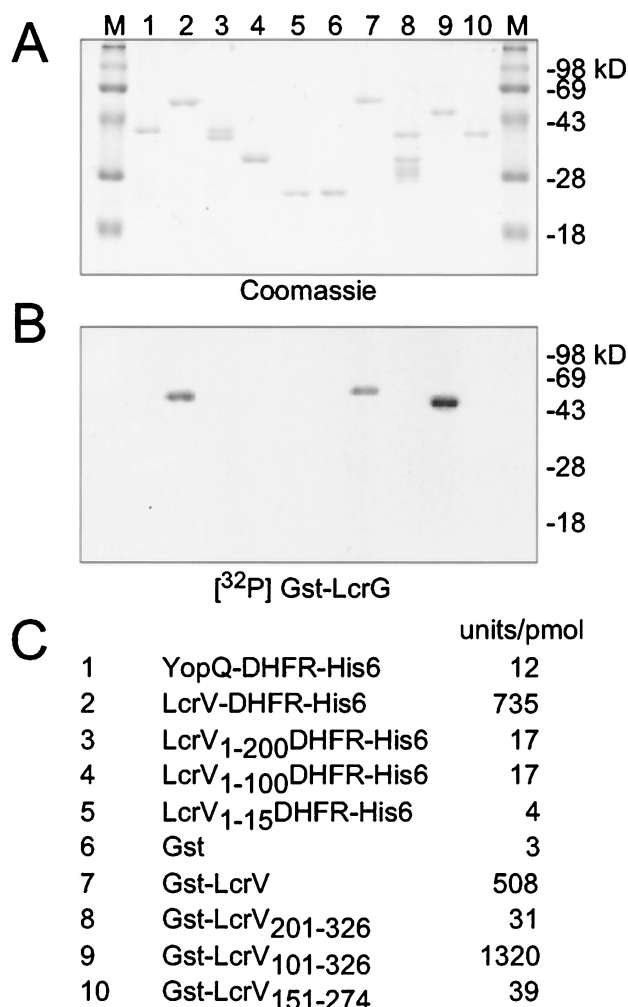


FIG. 7. Binding of GSt-LcrG to LcrV sequences. LcrV gene sequences were fused to the 3'-GST gene sequences or to the 5' end of a gene encoding DHFR_{His6}. Recombinant proteins were purified, separated by SDS-PAGE, and electroblotted onto PVDF membrane in duplicate. One membrane was stained with Coomassie brilliant blue (A), whereas the other was incubated with ³²P-labeled GST-LcrG. Binding was quantified on a PhosphorImager instrument (B) and is reported in arbitrary units per picomoles of LcrV fusion protein (C). The migration of molecular mass markers (M) is indicated in kilodaltons.

YopD, and presumably LcrV form a complex that inserts into lipid bilayers, generating a voltage-conducting pore (21, 60). Cornelis and Wolf-Watz advanced a model whereby YopB, YopD, and LcrV represent a translocation pore through which effector Yops must be transported into the cytosol of eukaryotic cells (13). If so, what is the mechanism that couples type III secretion and effector Yop translocation? Earlier work from the same group suggested that effector Yops may first be secreted by the type III machinery to subsequently engage the translocator complex in a second membrane transport step (54, 57). We have thought of the two-step model as an unlikely scenario given that extracellular transport intermediates (effector Yops) have not yet been detected (30, 51). As an alternative model, Lee et al. proposed that the type III secretion machinery may form a continuous protein conduit through

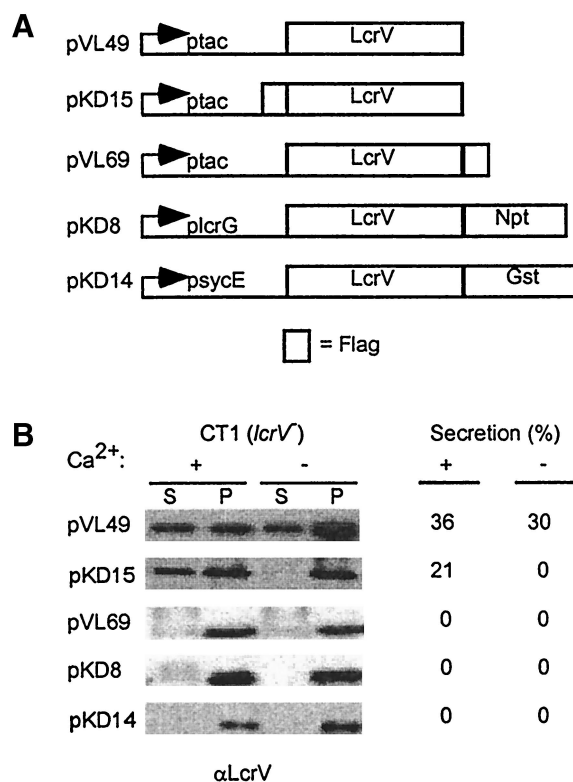


FIG. 8. Fusion of reporter sequences to LcrV. (A) The coding sequences of the NPT gene (*npt*), GST (*gst*), or the FLAG epitope tag (empty box) were fused to coding sequences for either the N or the C terminus of LcrV. Recombinant genes were cloned on a low-copy-number plasmid and expressed from the IPTG-inducible promoter in *Y. enterocolitica* CT1 (*lcrV*). (B) *Yersinia* strains were grown in presence of 5 mM calcium (+Ca²⁺) or 5 mM EGTA (-Ca²⁺) for 3 h at 37°C. Cultures were centrifuged, and the supernatant (S) was separated from the cell pellet (P). Protein in each sample was precipitated with TCA, solubilized in sample buffer, and analyzed by SDS-PAGE and immunoblotting with antiserum raised against LcrV.

which effector Yops travel (30). Hoiczky and Blobel advanced a similar model as YscF-containing needle complexes of *Y. enterocolitica* appear responsible for the formation of a type III targeting conduit between bacteria and eukaryotic cells (24). *yopD* knockout mutations did not interfere with the formation of the YscF polymer or with the penetration of these needle structures into eukaryotic cells (24).

What might be the role of the *lcrGVHypBD*-encoded protein products on type III targeting? Earlier work and this study advance a model whereby intrabacterial LcrG, LcrV, LcrH, and YopD exert a regulatory function for the type III targeting mechanism (4, 33, 39, 40, 55, 56, 63). Data presented here and elsewhere suggest that LcrG may act as a repressor of type III targeting and that this activity can be regulated by LcrG binding to LcrV (16, 33, 39, 40, 53). Further, LcrG is required for LcrV secretion via the type III pathway. YopB and YopD follow the same pathway, and LcrH (SycD) appears to function as a secretion chaperone for these polypeptides (61). The secretion of YopB, YopD, and LcrV may serve a regulatory function, priming *Yersinia* for the type III targeting reaction (35). Furthermore, secreted YopB-YopD-LcrV complexes

could exert a pathogenic role, damaging the plasma membranes of host cells. It has been reported that yersiniae respond to three host signals with type III targeting: glutamate, serum proteins, and the intracellular calcium concentration of eukaryotic cells (Lee et al., unpublished). Knockout mutations in *yopD*, *lcrH*, and *yscM1* and -2 abrogate the *Yersinia* requirement for the glutamate signal (Lee et al., unpublished). Knockout mutations in *lcrG*, but not in *lcrV*, promote effector Yop secretion in the presence but not in the absence of glutamate and serum protein signals (55; Lee et al., unpublished). These studies, together with the data reported here, lead us to assume that the *lcrGVHyopBD* operon may function as a regulator of the type III pathway. In the absence of specific host signals *yopD* and *lcrH* may control the synthesis of secretion substrates, presumably by binding to *yop* mRNA (Anderson et al., unpublished), while *lcrG* and *lcrV* control the activity of the type III machinery by a hitherto-unknown mechanism. We are well aware of the speculative nature of this proposal.

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REFERENCES

- Allaoui, A., R. Schulte, and G. R. Cornelis. 1995. Mutational analysis of the *Yersinia enterocolitica* *virC* operon: characterization of *yscE*, *F*, *G*, *I*, *J*, *K* required for Yop secretion and *yscH* encoding YopR. *Mol. Microbiol.* **18**: 343–355.
- Anderson, D. M., and O. Schneewind. 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* **278**:1140–1143.
- Anderson, D. M., and O. Schneewind. 1999. *Yersinia enterocolitica* type III secretion: an mRNA signal that couples translation and secretion of YopQ. *Mol. Microbiol.* **31**:1139–1148.
- Bergmann, T., S. Hakansson, A. Forsberg, L. Norlander, A. Macellaro, A. Backman, I. Bolin, and H. Wolf-Watz. 1991. Analysis of V antigen *lcrGVHyopBD* operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of *lcrH* and *lcrV*. *J. Bacteriol.* **173**:1607–1616.
- Boland, A., M.-P. Sory, M. Iriarte, C. Kerbouch, P. Wattiau, and G. R. Cornelis. 1996. Status of YopM and YopN in the *Yersinia yop* virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5–1.8 macrophages by the YopB, D, N delivery apparatus. *EMBO J.* **15**:5191–5201.
- Boyd, A. P., N. Grosdent, S. Totemeyer, C. Geuijen, S. Bleves, M. Iriarte, I. Lambermont, J.-N. Octave, and G. R. Cornelis. 2000. *Yersinia enterocolitica* can deliver Yop proteins into a wide range of cell types: development of a delivery system for heterologous proteins. *Eur. J. Cell Biol.* **79**:659–671.
- Boyd, A. P., M.-P. Sory, M. I. Iriarte, and G. R. Cornelis. 1998. Heparin interferes with translocation of Yop proteins into HeLa cells and binds to LcrG, a regulatory component of the *Yersinia* Yop apparatus. *Mol. Microbiol.* **27**:425–436.
- Cheng, L. W., D. M. Anderson, and O. Schneewind. 1997. Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. *Mol. Microbiol.* **24**:757–765.
- Cheng, L. W., and O. Schneewind. 2000. *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for the specific targeting of YopT, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J. Bacteriol.* **182**:3183–3190.
- Cheng, L. W., and O. Schneewind. 1999. *Yersinia enterocolitica* type III secretion: on the role of SycE in targeting YopE into HeLa cells. *J. Biol. Chem.* **274**:22102–22108.
- Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M.-P. Sory, and I. Stainier. 1998. The virulence plasmid of *Yersinia*, an antihist genome. *Microbiol. Mol. Biol. Rev.* **62**:1315–1352.
- Cornelis, G. R., and C. Colson. 1975. Restriction of DNA in *Yersinia enterocolitica* detected by the recipient ability for a derepressed R factor from *Escherichia coli*. *J. Gen. Microbiol.* **87**:285–291.
- Cornelis, G. R., and H. Wolf-Watz. 1997. The *Yersinia yop* virulon: a bacterial system for subverting eukaryotic cells. *Mol. Microbiol.* **23**:861–867.
- Day, J. B., and G. V. Plano. 1998. A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol. Microbiol.* **30**:777–789.
- de Lorenzo, V., E. Eltis, B. Kessler, and K. N. Timmis. 1993. Analysis of *Pseudomonas* gene products using *lacI^q/P_{trp}-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123**:17–24.
- Fields, K. A., M. L. Nilles, C. Cowan, and S. C. Straley. 1999. Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. *Infect. Immun.* **67**: 5395–5408.
- Fields, K. A., and S. C. Straley. 1999. LcrV of *Yersinia pestis* enters infected eukaryotic cells by a virulence plasmid-independent mechanism. *Infect. Immun.* **67**:4801–4813.
- Forsberg, A., A.-M. Viitanen, M. Skunik, and H. Wolf-Watz. 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol. Microbiol.* **5**:977–986.
- Goguen, J. D., J. Yother, and S. C. Straley. 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mud1(*Ap lac*) insertion mutants. *J. Bacteriol.* **160**:842–848.
- Hakansson, S., E. Gaylov, R. Rosqvist, and H. Wolf-Watz. 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. *Mol. Microbiol.* **20**:593–603.
- Hakansson, S., K. Schesser, C. Persson, E. E. Galyov, R. Rosqvist, F. Hombles, and H. Wolf-Watz. 1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J.* **15**:5812–5823.
- Hoe, N. P., and J. D. Goguen. 1993. Temperature sensing in *Yersinia pestis*: translation of the LcrF activator protein is thermally regulated. *J. Bacteriol.* **175**:7901–7909.
- Hoe, N. P., F. C. Minion, and J. D. Goguen. 1992. Temperature sensing in *Yersinia pestis*: regulation of *yopE* transcription by *lcrF*. *J. Bacteriol.* **174**: 4275–4286.
- Hoiczky, E., and G. Blobel. 2001. Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **98**:4669–4674.
- Holmstrom, A., J. Petterson, R. Rosqvist, S. Hakansson, F. Tafazolli, M. Fallman, K.-E. Magnusson, H. Wolf-Watz, and A. Forsberg. 1997. YopK of *Yersinia pseudotuberculosis* controls translocation of Yop effectors across the eukaryotic cell membrane. *Mol. Microbiol.* **24**:73–91.
- Iriarte, M., and G. R. Cornelis. 1999. Identification of SycN, YscX, and YscY, three new elements of the *Yersinia yop* virulon. *J. Bacteriol.* **181**:675–680.
- Iriarte, M., and G. R. Cornelis. 1998. YopT, a new *Yersinia* effector protein, affects the cytoskeleton of host cells. *Mol. Microbiol.* **29**:915–929.
- Iriarte, M., M.-P. Sory, A. Boland, A. P. Boyd, S. D. Mills, I. Lambermont, and G. R. Cornelis. 1998. TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *EMBO J.* **17**:1907–1918.
- Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blannar, D. M. Livingston, and E. K. Flemington. 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with EF2-like properties. *Cell* **70**:351–364.
- Lee, V. T., D. M. Anderson, and O. Schneewind. 1998. Targeting of *Yersinia* Yop proteins into the cytosol of HeLa cells: one-step translocation of YopE across bacterial and eukaryotic membranes is dependent on SycE chaperone. *Mol. Microbiol.* **28**:593–601.
- Lee, V. T., S. K. Mazmanian, and O. Schneewind. A program of *Yersinia enterocolitica* type III secretion reactions is activated by specific signals. *J. Bacteriol.*, in press.
- Lee, V. T., and O. Schneewind. 1999. Type III machines and the pathogenesis of enteric infections caused by *Yersinia* and *Salmonella* spp. *Immunol. Rev.* **168**:241–255.
- Lee, V. T., and O. Schneewind. 1999. Type III machines of pathogenic yersiniae secrete virulence factors into the extracellular milieu. *Mol. Microbiol.* **31**:1619–1629.
- Lee, V. T., C. Tam, and O. Schneewind. 2000. *Yersinia enterocolitica* type III secretion. LcrV, a substrate for type III secretion, is required for toxin-targeting into the cytosol of HeLa cells. *J. Biol. Chem.* **275**:36869–36875.
- Michiels, T., and G. R. Cornelis. 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. *J. Bacteriol.* **173**:1677–1685.
- Michiels, T., J.-C. Vanootehem, C. Lambert de Rouvroit, B. China, A. Gustin, P. Boudry, and G. R. Cornelis. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J. Bacteriol.* **173**:4994–5009.
- Michiels, T., P. Wattiau, R. Brasseur, J.-M. Ruyschaert, and G. Cornelis. 1990. Secretion of Yop proteins by yersiniae. *Infect. Immun.* **58**:2840–2849.
- Mills, S. D., A. Boland, M.-P. Sory, P. van der Smissen, C. Kerbouch, B. B. Finlay, and G. R. Cornelis. 1997. *Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector

- protein. Proc. Natl. Acad. Sci. USA **94**:12638–12643.
38. Monack, D. M., J. Meccas, N. Gori, and S. Falkow. 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. Proc. Natl. Acad. Sci. USA **94**:10385–10390.
 39. Nilles, M. L., K. A. Fields, and S. C. Straley. 1998. The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. J. Bacteriol. **180**:3410–3420.
 40. Nilles, M. L., A. W. Williams, E. Skrzypek, and S. C. Straley. 1997. *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca²⁺ response. J. Bacteriol. **179**:1307–1316.
 41. Perry, R. D., P. A. Harmon, W. S. Bowmer, and S. C. Straley. 1986. A low-Ca²⁺ response operon encodes the V antigen of *Yersinia pestis*. Infect. Immun. **54**:428–434.
 42. Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fallman. 1997. The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of p130^{cas} and FAK, and the associated accumulation of these proteins in peripheral focal adhesion. EMBO J. **16**:2307–2318.
 43. Persson, C., R. Nordfelth, A. Holmstrom, S. Hakansson, R. Rosqvist, and H. Wolf-Watz. 1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18**:135–150.
 44. Petterson, J., A. Holmstrom, J. Hill, E. Frithz-Lindsten, A. von Euler-Matell, E. Carlsson, R. Titball, A. Forsberg, and H. Wolf-Watz. 1999. The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. Mol. Microbiol. **32**:961–976.
 45. Petterson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson, and H. Wolf-Watz. 1996. Modulation of virulence factor expression by pathogen target cell contact. Science **273**:1231–1233.
 46. Plano, G. V., S. S. Barve, and S. C. Straley. 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173**:7293–7303.
 47. Price, S. B., C. Cowan, R. D. Perry, and S. C. Straley. 1991. The *Yersinia pestis* V antigen is a regulatory protein necessary for Ca²⁺-dependent growth and maximal expression of low-Ca²⁺ response virulence genes. J. Bacteriol. **173**:2649–2657.
 48. Price, S. B., K. Y. Leung, S. S. Barve, and S. C. Straley. 1989. Molecular analysis of *lcrGVH*, the V antigen operon of *Yersinia pestis*. J. Bacteriol. **171**:5646–5653.
 49. Rimpilainen, M., A. Forsberg, and H. Wolf-Watz. 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. **174**:3355–3363.
 50. Rosqvist, R., A. Forsberg, and H. Wolf-Watz. 1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect. Immun. **59**:4562–4569.
 51. Rosqvist, R., K.-E. Magnusson, and H. Wolf-Watz. 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. **13**:964–972.
 52. Sarker, M. R., C. Neyt, I. Stainier, and G. R. Cornelis. 1998. The *Yersinia yop* virulon: LcrV is required for extrusion of the translocators YopB and YopD. J. Bacteriol. **180**:1207–1214.
 53. Sarker, M. R., M.-P. Sory, A. P. Boyd, M. I. Iriarte, and G. R. Cornelis. 1998. LcrG is required for efficient translocation of *Yersinia* Yop effector proteins into eukaryotic cells. Infect. Immun. **66**:2976–2979.
 54. Schesser, K., E. Frithz-Lindsten, and H. Wolf-Watz. 1996. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. J. Bacteriol. **178**:7227–7233.
 55. Skrzypek, E., and S. C. Straley. 1995. Differential effects of deletions in *lcrV* on secretion of V antigen, regulation of the low-Ca²⁺ response, and virulence of *Yersinia pestis*. J. Bacteriol. **177**:2530–2542.
 56. Skrzypek, E., and S. C. Straley. 1993. LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. J. Bacteriol. **175**:3520–3528.
 57. Sory, M.-P., A. Boland, I. Lambermont, and G. R. Cornelis. 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. Proc. Natl. Acad. Sci. USA **92**:11998–12002.
 58. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
 59. Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh. 1987. High-copy-number and low-copy-number plasmid vectors for LacZ alpha-complementation and chloramphenicol- or kanamycin-resistance selection. Gene **61**:63–74.
 60. Tardy, F., F. Homble, C. Neyt, R. Wattiez, G. R. Cornelis, J.-M. Ruyschaert, and V. Cabiliaux. 1999. *Yersinia enterocolitica* type III secretion-translocation system: channel formation by secreted Yops. EMBO J. **18**:6793–6799.
 61. Wattiau, P., B. Bernier, P. Deslee, T. Michiels, and G. R. Cornelis. 1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA **91**:10493–10497.
 62. Wattiau, P., and G. R. Cornelis. 1994. Identification of DNA sequences recognized by VirF, the transcriptional activator of the *Yersinia yop* regulon. J. Bacteriol. **176**:3878–3884.
 63. Williams, A. W., and S. C. Straley. 1998. YopD of *Yersinia pestis* plays a role in negative regulation of the low-calcium response in addition to its role in translocation of Yops. J. Bacteriol. **180**:350–358.
 64. Woestyn, S., M.-P. Sory, A. Boland, O. Lequenne, and G. R. Cornelis. 1996. The cytosolic SycE and SycH chaperones of *Yersinia* protect the region of YopE and YopH involved in translocation across eukaryotic cell membranes. Mol. Microbiol. **20**:1261–1271.
 65. Yother, J., and J. D. Goguen. 1985. Isolation and characterization of Ca²⁺-blind mutants of *Yersinia pestis*. J. Bacteriol. **164**:704–711.