

LcrV of *Yersinia pestis* Enters Infected Eukaryotic Cells by a Virulence Plasmid-Independent Mechanism

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Yersinia pestis is the causative agent of bubonic plague and possesses a set of plasmid-encoded, secretable virulence proteins termed LcrV and Yops which are essential for survival in mammalian hosts. Yops and LcrV are secreted by a type III mechanism (Ysc), and Yops are unidirectionally targeted into the cytosol of associated eukaryotic cells in a tissue culture infection model. LcrV is required for Yops targeting, and recent findings have revealed that it can localize to the bacterial surface; however, its fate in this infection model has not been investigated in detail. In this study, we compared the localization of LcrV to that of the targeted proteins YopE and YopM by immunoblot analysis of fractions of *Yersinia*-infected HeLa cultures or by laser-scanning confocal microscopy of infected monolayers. Both LcrV and YopE were secreted by contact-activated, extracellularly localized yersiniae and were targeted to the HeLa cell cytosol. Although a significant amount of LcrV partitioned to the culture medium (unlike YopE), this extracellular pool of LcrV was not the source of the LcrV that entered HeLa cells. Unlike targeting of YopE and YopM, targeting of LcrV occurred in the absence of a functional Ysc apparatus and other virulence plasmid (pCD1)-expressed proteins. However, the Ysc is necessary for LcrV to be released into the medium, and our recent work has shown that localization of LcrV on the bacterial surface requires the Ysc. These results indicate that two mechanisms exist for the secretion of LcrV by *Y. pestis*, both of which are activated by contact with eukaryotic cells. LcrV secreted by the Ysc reaches the bacterial surface and the surrounding medium, whereas the second is a novel, Ysc-independent pathway which results in localization of LcrV in the cytosol of infected cells but not the surrounding medium.

The genus *Yersinia* contains three medically significant species: *Y. pestis*, the etiologic agent of bubonic plague; and the enteropathogenic species *Y. pseudotuberculosis* and *Y. enterocolitica*. The ability of these facultative intracellular, gram-negative pathogens to establish an infection in a mammalian host requires a set of antihost proteins termed LcrV and Yops (6, 40) which are encoded on a common ca. 70-kb virulence plasmid termed pCD1 in *Y. pestis* (18, 42). LcrV and Yops are secreted by a Yop secretion (Ysc) type III mechanism and function during an infection to prevent or evade a host innate immune response (7). Delivery of these proteins to host targets in vivo is most likely tightly regulated, since strains that deploy excess Yops (17, 26) or LcrV (60) are attenuated for virulence in a mouse model. Therefore, *Yersinia* have evolved an elaborate mechanism to couple secretion to regulation of expression of LcrV and Yops.

Expression of LcrV and Yops is thermally induced by the transcriptional activator LcrF (24). In vitro, in media containing sodium and glutamate, maximal thermal induction is prevented by cultivation of *Yersinia* in the presence of millimolar concentrations of Ca²⁺ (12, 65). Under these conditions, LcrV and Yops are minimally expressed and their secretion is blocked. Growth at 37°C in a Ca²⁺-deficient medium results in secretion of maximally induced LcrV and Yops (29, 67, 73). This in vitro, Ca²⁺-absence signal has been proposed to mimic an in vivo signal manifested by intimate association with eukaryotic cells (45, 53, 66). *Y. pestis* lacks several adhesin proteins present in the enteropathogenic *Yersinia*, and it is not known what promotes binding of *Y. pestis* to the surface of

infected cells (63). Contact induction results in expression and secretion of LcrV and Yops, even when Ca²⁺ is present in the growth medium. According to a currently accepted model, Ca²⁺ in the absence of contact prevents thermal induction by stabilizing a closed conformation of Ysc channels. Blockage of channels mediated by outer-surface-bound LcrE (also called YopN [11]) and TyeA (20) and cytoplasmic LcrG (38) causes accumulation of LcrQ and YopD, two proteins involved in negative feedback control of LcrF-mediated induction (45, 72). The LcrE-, TyeA-, and LcrG-mediated block is displaced during cultivation in the absence of Ca²⁺ or upon contact with eukaryotic cells, resulting in secretion of LcrQ and YopD and dissipation of negative feedback control.

At least six effector Yops (YopE, YopH, YopJ, YopM, YpkA, and YopT) are delivered to the cytoplasm of infected eukaryotic cells, where they function to disrupt cellular processes (reviewed in reference 6). Delivery entails secretion of Yops across both membranes of extracellularly localized (51, 62), cell-associated (44) *Yersinia* and subsequent translocation or targeting across the eukaryotic plasma membrane. Secretion from *Yersinia* is mediated by a Ysc secretion system composed of at least 22 gene products (reviewed in references 6 and 40), including the inner membrane protein YscV (formerly termed LcrD [46, 47]) and the outer membrane secretin YscC (21).

The translocator Yops (YopB and YopD) and LcrV, all of which are encoded within the polycistronic *lcrGVH-yopBD* operon (2, 33, 50), are indispensable for the targeting of Yops (16, 37, 44, 53, 57) and may form a structure which mediates transfer of Yops from bacteria to an associated cell (5, 9). Although a *Yersinia* Yops targeting apparatus has not yet been characterized, Cornelis (5) speculated that it may resemble the needle-like structure discovered in *Salmonella typhimurium* (22). Purified preparations of YopB have membrane-disruptive activity, and YopB expressed by *Yersinia* is hemolytic for

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sheep erythrocytes (16), indicating that YopB probably forms part of a translocation pore in the eukaryotic cell plasma membrane. The size of this pore is modulated by the accessory protein YopK (17). YopD has also been suggested to be a pore component, based on the presence of hydrophobic domains in the peptide sequence (36), but is not essential for the hemolytic activity of YopB (16). Although LcrV can interact with YopB and YopD (57), there is no direct evidence that LcrV is a pore component. Instead, findings described by Nilles et al. (37) and Sarker et al. (57) suggest that LcrV is required for assembly of the YopB-containing pore.

Yop secretion into eukaryotic cells is thought to occur only through Ysc channels that make direct contact with the plasma membrane. Hence, in tissue culture infection assays, secretion is unidirectional or polarized, and effector Yops are targeted directly to eukaryotic cells and not released into the medium in significant amounts. Targeting of Yops has been demonstrated indirectly by assaying enzyme activity of Yop-Cya reporter fusions (62) or by observing cytotoxicity manifested as rounding up of the eukaryotic cells (7). Direct assays for targeting have used confocal microscopy (3, 53, 58) and immunoblot analysis of fractionated cultures (25, 37).

One primary function of intracellular Yops is to impair the phagocytic capabilities of host cells, including professional phagocytes such as macrophages and neutrophils (56, 70). YopE (51, 52) and YopT (19) function independently to disrupt actin microfilaments, whereas YopH dephosphorylates structural components of focal adhesions (1, 43). YopJ interferes with host cell signal cascades, with resulting inhibition of tumor necrosis factor alpha expression (39, 54, 55), and induces apoptosis in infected macrophages (31, 32). Specific intracellular functions of YpkA and YopM have not yet been determined, but they localize to the inner face of the eukaryotic plasma membrane (15) and nucleus (58), respectively.

LcrV is essential to virulence of *Yersinia* (49) and, unlike the effector YopS, functions in multiple ways during infection. Within yersiniae, LcrV indirectly induces Yops expression and secretion by inactivating the inner-membrane, LcrG-mediated Ysc block (37, 38). LcrV is also essential for targeting of secreted Yops (37, 57), and LcrV located on the extracellular surface of *Yersinia* may act as a pilot for assembly of a targeting apparatus or may function as part of a Yop targeting complex (9). Once released from *Yersinia*, LcrV may function as a diffusible modulator of host immune responses. An LcrV-containing fusion protein was able to prevent production of the proinflammatory cytokines tumor necrosis factor alpha and gamma interferon (34). Preparations containing LcrV have also been shown to enhance production of the T helper type 2-inducing cytokine interleukin-10 (35) and inhibit chemotaxis of neutrophils (70). In previous reports, we had noted that LcrV, like Yops, becomes associated with eukaryotic cells (37). Although a tissue culture infection model has been used to characterize localization and function of Yops, partitioning of LcrV among compartments in that model has not previously been studied. In this study we investigated LcrV localization and found that LcrV partitioned differently from the vectorially targeted protein YopE. Unlike Yops, significant levels of LcrV were detected in the medium fraction. We also demonstrate that the HeLa cell-associated LcrV first reported by Nilles et al. (37) corresponded to LcrV localized to the HeLa cell cytoplasm, and LcrV did not require YopB or YopD to attain this intracellular localization. Instead, LcrV enters eukaryotic cells by a novel, virulence plasmid-independent pathway.

MATERIALS AND METHODS

Growth conditions, bacterial strains, and eukaryotic cell lines. Strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, *Escherichia coli* DH5 α was grown in Luria-Bertani (LB) (30) medium or on LB agar at 37°C. *Y. pestis* strains were cultivated in heart infusion broth (Difco Laboratories, Detroit, Mich.) at 26°C prior to infection of eukaryotic cells. *Y. pestis* was also cultivated at 26°C on tryptose blood agar (Difco) during construction of the *Y. pestis* lcrE strain KIM8-3233.1 and on tryptose blood agar supplemented as described previously (28), with modifications by Nilles et al. (37), during construction of the yopB strain KIM8-3002.1. In an experiment not shown, LcrV, HT-VN68, and YopM were expressed from plasmids and tested for their secretion from pCD1⁻ *Y. pestis* KIM8 growing in the defined medium TMH (64) at 37°C without added Ca²⁺. When appropriate, bacteria were grown in the presence of antibiotics used at 15 μ g/ml for tetracycline, 50 μ g/ml for kanamycin, or 100 μ g/ml for ampicillin and streptomycin. The HeLa human epithelial cell line was used in this study and was maintained at 37°C with 5% CO₂ in RPMI 1640 medium (RPMI; GIBCO-BRL, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal bovine serum (FBS; GIBCO-BRL). For experiments examining partitioning of LcrV and Yops within infected eukaryotic cells, RPMI supplemented with FBS was replaced with RPMI lacking FBS.

DNA methods and strain construction. Plasmid DNA was purified by midi or spin-prep columns (Qiagen, Inc., Studio City, Calif.), and cloning was performed essentially as described elsewhere (27). Selected DNA was amplified by PCR in a Perkin-Elmer Cetus (Foster City, Calif.) GeneAmp model 2400 thermocycler using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and oligonucleotide primers synthesized by Genosys Biotechnologies (The Woodlands, Tex.). Typical PCR conditions included a 10-min preincubation at 94°C followed by 30 amplification cycles. Denaturation, annealing, and extension reactions were carried out for 30 s each at 94, 55, and 72°C, respectively. PCR fragments were resolved on agarose gels and extracted by using a Qiaquick gel extraction kit (Qiagen). *E. coli* and *Y. pestis* were transformed by the CaCl₂ method (27) and by electroporation (41), respectively. p Δ yopB was generated by PCR amplification of *Y. pestis* DNA from pAW161, using synthetic oligonucleotides lcrH1SDA (5' GCTAGAGCTCAGGAGGAACATATGCAACAAGAGACGACAGAC 3') and yopD921B (5' GCTCTAGATCAGACAACACCAAAAGCGGC 3') constructed by Williams and Straley (72). The PCR product contained the coding sequence for LcrH (also called SycD), the first 7 and last 11 residues of YopB, and YopD. The purified ca. 1.6-kb PCR fragment and *Sma*I-digested pLD55 were ligated by using T4 DNA ligase (Promega, Madison, Wis.) and used to transform *E. coli* DH5 α pir. p Δ yopB was used to create *Y. pestis* KIM8-3002.1 by allelic exchange of pCD1-encoded yopB as described previously (37), and gene replacement was confirmed by PCR. The resulting strain was confirmed to have normal expression and secretion of YopD, which is encoded by the gene immediately downstream of yopB in the lcrGVH-yopBD operon (data not shown). pUK4134.6, carrying an in-frame deletion within lcrE (codons 48 to 197), was used to replace the pCD1-encoded lcrE by allelic exchange in the yopM insertion mutant KIM8-3233. Selection for homologous recombination and subsequent gene replacement were done as described elsewhere (59). Digestion of pCD1 with *Bam*HI was used to verify the correct deletion.

Infection assays. Protein localization during infection of HeLa cell monolayers was assayed essentially as described previously by immunoblotting (9, 37) or laser-scanning confocal microscopy (58). Briefly, HeLa cells were subcultured either into six-well 35-mm-diameter tissue culture plates or on 12-mm-diameter glass coverslips in 24-well cluster dishes (Costar, Cambridge, Mass.) in RPMI with 10% (vol/vol) FBS. Monolayers were incubated at 37°C in 5% CO₂ for roughly 72 h in 6-well dishes to a density of 5×10^5 to 8×10^5 per well for fractionation or 48 h in 24-well dishes to a density of ca. 10^5 for confocal analysis. *Y. pestis* strains were cultivated at 26°C in heart infusion broth, harvested at an optical density at 620 nm of ca. 1.0, and diluted directly into 37°C RPMI lacking FBS. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to 0.1 mM for treatments with *Y. pestis* strains harboring constructs with inducible promoters. Some wells were treated with 5 μ g of cytochalasin D (Sigma) per ml 30 min before infection and then throughout the infection period (58). This treatment has been shown to have no effect on targeting of YopM or YopE by *Y. pestis* (58) or on viability of the bacteria for at least 1 h (8). It has been confirmed to abolish invasion (to below detection) of HeLa cells (8). Immediately before infection, HeLa cells were washed twice with RPMI lacking FBS, and bacteria were added to wells at a multiplicity of infection (MOI) of 10. Plates were then centrifuged at 200 \times g for 5 min to achieve contact between bacteria and target cells and incubated at 37°C in 5% CO₂ for 4 h. Gentamicin (GIBCO-BRL) was added to wells to 7.5 μ g/ml 30 min postinfection for experiments in which extracellular bacteria were killed. After infection, cultures were either fractionated for immunoblot analysis or fixed and stained for microscopic analysis. For culture fractionation, one replicate per infecting strain was treated for 5 min at 37°C in 5% CO₂ with 100 μ g of trypsin (Sigma) per ml. The trypsin treatment was terminated by addition of a protease inhibitor cocktail (Pefabloc, leupeptin, and aprotinin; all from Boehringer Mannheim Corp., Indianapolis, Ind.) added to each well such that each inhibitor was present at 20 μ g/ml. Media were then removed from wells and passed through 0.2- μ m-pore-size filters into tubes on ice, and proteins were precipitated by treatment with 10% (vol/vol) (final concentration) trichloroacetic acid (TCA) on ice for 2 h to overnight. Infected monolayers were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Source or reference
<i>E. coli</i> K-12		
DH5 α	F ⁻ f80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- r_M^+$) <i>phoA supE44 λ^- thi-1 gyrA96 relA1</i>	GIBCO-BRL
DH5 α λ pir	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- r_M^+$) <i>phoA supE44 thi-1 gyrA96 relA1 λpir</i>	Laboratory stock
<i>Y. pestis</i> ^a		
KIM6	pPCP1 (40), pMT1 (40)	R. R. Brubaker
KIM10	pMT1	Laboratory stock
KIM8-3241	pCD1 (<i>lcrV</i> [LcrV Δ 18–215] ^b <i>yopJ</i> ::MudII734 [Km ^r Lac ⁺]), ^c pMT1, pOS50	9
KIM8-3002	Sm ^r ; pCD1 (Lcr ⁺), pMT1	37
KIM8-3002.1	Sm ^r ; pCD1 (<i>yopB</i> [YopB Δ 8–390]), pMT1	This study
KIM8-3002.2	Sm ^r ; pCD1 (<i>yopD</i> [YopD Δ 1–305]), pMT1	72
KIM8-3233	Sm ^r ; pCD1 (<i>yopM</i> ::lacZYA), pMT1	58
KIM8-3233.1	Sm ^r ; pCD1 (<i>yopM</i> ::lacZYA <i>lcrE</i> [LcrE Δ 48–197]), pMT1	This study
KIM5-3001.2.1	Sm ^r ; pCD1 (<i>yscV</i> [YscV Δ 192–343], <i>yopD</i> [YopD Δ 1–305]), pPCP1, pMT1	72
Plasmids and constructs		
pLD55	Ap ^r Tc ^r ; suicide vector for allelic exchange	28
p Δ yopB	PCR-amplified DNA from pAW161 containing Δ yopB (deleting nucleotides 23–1169) in pLD55	This study
pUK4134.6	<i>Pst</i> I deletion of <i>lcrE</i> cloned into pUK4134	48
pHT-V	PCR-amplified <i>lcrV</i> cloned in pPRoEX-1 in-frame with a 23-residue leader sequence encoding His ₆ (<i>lcrV</i>)	9
pHT-VN68	Subcloned <i>lcrV</i> in pPRoEX-1 with a 29-residue, His ₆ -containing leader sequence fused at the <i>Eco</i> RV site in <i>lcrV</i> (<i>lcrV</i> [LcrV Δ 1–67])	9
pTrcV	<i>Bbv</i> 1/ <i>Nco</i> I fragment of pES6-1 cloned into <i>Sma</i> I-cut pTrc99A (<i>lcrV</i>)	9
TrcM.2	Subcloned <i>yopM</i> in pTrc99A (<i>yopM</i>)	48
pAW161	PCR-amplified <i>lcrH</i> and <i>yopD</i> cloned into pBAD33 (14) expression vector (<i>lcrH</i> , <i>yopB</i> [YopB Δ 8–390], <i>yopD</i>)	72
pOS50	Tc ^r ; competitive plasmid for pPCP1	J. Goguen

^a All strains are Pgm⁻ (69).

^b Numbers in brackets list residues deleted from gene product.

^c Descriptions in parentheses give relevant genes present on construct.

washed twice with phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4]) and lysed by treatment as previously described with ice-cold H₂O containing protease inhibitors at 2 μ g/ml each (58) or by disruption in RPMI with a Kontes homogenizer (B pestle, 100 strokes). Lysed cell fractions were then centrifuged at 4°C at 20,800 \times g for 15 min. The liquid supernatant, corresponding to the eukaryotic cell cytoplasmic and nuclear soluble fraction, was removed, and proteins were precipitated by the addition of TCA to 10% (vol/vol). The 20,800 \times g debris and precipitated proteins from media and cellular soluble fractions were dissolved in polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2.3% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β -mercaptoethanol, 60 mM Tris (pH 6.8), and 25% (vol/vol) glycerol.

To test for the extent of induction of Yops expression when the parent Pla⁻ *Y. pestis* KIM8-3002 infects HeLa cells (contact induction), HeLa cells were infected as described above except that the MOI was 5. Additional wells containing RPMI but no HeLa cells received the same number of bacteria. After 2 h incubation at 37°C in 5% CO₂, the entire contents of each well was harvested and the proteins were precipitated with TCA. Twofold serial dilutions of samples representing equal fractions of the original well contents were subjected to SDS-PAGE and analyzed by immunoblotting. LcrV and YopE were assayed by specific antibodies, and the loading of wells with *Yersinia* was compared by probing lanes with an anti-*Yersinia* antibody.

For confocal analysis, media were removed, and infected monolayers were washed with 1.0-ml volumes of Hanks' balanced salt solution (GIBCO-BRL). Samples were then treated sequentially at room temperature with 2% (wt/vol) paraformaldehyde pH 7.4 in PBS for 30 min to fix proteins, with buffer containing 0.5% Triton X-100 for 20 min (52) to permeabilize membranes, and with PBS supplemented with 10% (vol/vol) FBS and 1% (vol/vol) mouse serum (Sigma) for 60 min to block nonspecific antigenic sites. Where appropriate, LcrV and YopM were detected by probing with LcrV- or YopM-specific polyclonal rabbit antibodies followed by incubation with Oregon green-coupled anti-rabbit immunoglobulin G (IgG; Molecular Probes, Eugene, Oreg.) as described elsewhere (58). Coverslips were mounted using SlowFade mounting medium (Molecular Probes) and analyzed by laser-scanning confocal microscopy using a Leica TSC NT confocal system (Ar-Kr laser) and a 63 \times objective. Differential interference contrast (DIC) images were generated by a Leica DM IRB/E inverted microscope with Nomarski optics.

Protein purification. A purified preparation of a His₆-tagged LcrV (HT-V) was used to test the ability of exogenous LcrV to enter HeLa cells and the

specificity of LcrV-specific antibodies. Purification was performed essentially as described elsewhere (10). Briefly, *E. coli* DH5 α expressing pHT-V was cultivated at 37°C in LB to an optical density at 620 nm of ca. 1.0. IPTG was added to 0.1 mM, and the culture was incubated for an additional 3 h. Bacterial lysates were generated by French press as described previously (10), and HT-V was purified by passage of clarified lysates over a Talon (Clontech Laboratories, Inc., Palo Alto, Calif.) metal affinity resin according to the manufacturer's instructions. Pooled samples containing pure HT-V were dialyzed into PBS, and protein concentration was quantitated by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.).

Protein electrophoresis and immunoblot analysis. Proteins from fractionated, infected HeLa cultures were resolved in 12% (wt/vol) polyacrylamide gels by SDS-PAGE (23). Samples were loaded so that lanes represented equivalent volume fractions of the original cultures. Once resolved, proteins were transferred to Immobilon-P (Millipore, Corp., Bedford, Mass.) in Tris-glycine buffer (25 mM Tris, 192 mM glycine [pH 8.3] containing 10% [vol/vol] methanol) as described previously (67). Specific proteins were detected by using polyclonal rabbit antibodies specific for His-tagged LcrV (α -HTV) or His-tagged YopE (α -YopE; gift of G. Plano, University of Miami). In one experiment, the loading of wells with *Yersinia* was compared by using a polyclonal rabbit antibody raised against *Y. pestis* KIM6 whole cells grown at 26°C (anti-*Yersinia*). Proteins were visualized by treatment of immunoblots with alkaline phosphatase- or horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma), followed by development with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP; GIBCO-BRL) or enhanced chemiluminescence (ECL) substrate (Pierce), respectively.

RESULTS

Contact-activated entry of LcrV into infected HeLa cells. A tissue culture infection model has been used to characterize the localization and function of certain Yop proteins once they are secreted by yersiniae. We have hypothesized that LcrV can function in intimate association with infected eukaryotic cells to help mediate transfer of Yops into the cell cytoplasm. The goal of this study was to investigate the localization of LcrV in

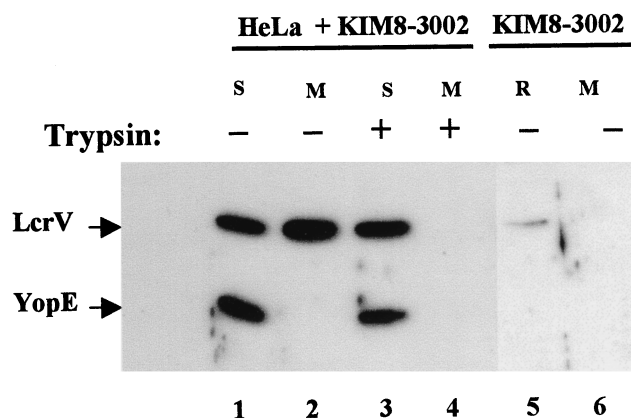


FIG. 1. LcrV partitions to the cell-free medium and HeLa soluble fractions. Duplicate wells containing HeLa monolayers were infected with *Y. pestis* KIM8-3002 at an MOI of 10 (lanes 1 to 4). As a control, an equal dose of *Y. pestis* KIM8-3002 was also added to a well lacking HeLa cells (lanes 5 and 6). After incubation at 37°C-5% CO₂ for 4 h, trypsin was added to 100 µg/ml to one replicate of infected HeLa cells. *Yersinia*-infected HeLa cultures were fractionated, and the cell-free medium (M) and water-lysate soluble (S) fractions were further analyzed. Cell-free medium (M; lane 6) from the *Yersinia*-only well corresponded to the culture supernatant after removal of bacteria by centrifugation. The bacteria were then treated with cold water and pelleted, and the resulting supernatant corresponding to the water-released (R; lane 5) fraction was removed. Samples from each fraction representing 0.3% of the original culture were resolved in a 12% polyacrylamide gel. LcrV and YopE were detected by probing immunoblots with α-HTV and α-YopE followed by secondary antibodies conjugated to horseradish peroxidase and development with ECL reagent.

this model system. We had previously found that unlike the vectorially targeted Yops, LcrV is secreted into the medium and also becomes cell-associated in a tissue culture infection model (37). The cell-associated LcrV was intriguing, since LcrV is required for the targeting of Yops and may function at the interface between a bacterium and associated eukaryotic cell. Furthermore, YopD, an additional protein involved in Yops targeting, may itself be targeted (13), raising the possibility that LcrV localized in a translocation apparatus can also gain access to the eukaryotic cell cytoplasm. We therefore tested whether LcrV could enter infected cells. HeLa cell monolayers were infected with our Pla⁻ parent *Y. pestis* KIM8-3002 for 4 h, and secreted proteins in one replicate culture were tested for accessibility to proteolysis by exogenously added trypsin as an indicator of extracellular localization. Cultures were then fractionated into cell-free medium (containing proteins secreted into the culture medium), cellular soluble (representing HeLa cell cytoplasm and released nuclear proteins), and debris (containing yersinia bound to cell membrane) fractions. We then compared the localization of LcrV to that of the vectorially targeted YopE by immunoblot analysis (Fig. 1). As previously noted (37), LcrV, but not YopE, was released into the culture medium during infection (lane 2) and was completely susceptible to proteolysis by trypsin (lane 4). LcrV, like YopE, was present in HeLa soluble fractions (lanes 1 and 3) and was protected from digestion by trypsin (lane 3), suggesting an intracellular localization for LcrV. Interestingly, quantities of cell-associated LcrV were significant when compared to levels secreted into the medium (lane 2). A similar distribution of LcrV was detected after infection of the macrophage-like cell line J774 (data not shown). The cell-associated LcrV did not arise from yersinia internalized by the HeLa cells, because separate gentamicin protection studies indicate that less than 1% of *Y. pestis* KIM8-3002 enter HeLa cells in a 4-h infection (8). An alternative possibility for cell-associated LcrV was that intracellular LcrV was an artifact of

the fractionation protocol. We have shown that LcrV can localize in deposits on the extracellular surface of *Y. pestis* (9), raising the possibility that the cold water lysis treatment had dissociated surface LcrV, releasing it into the HeLa cytoplasmic fraction. To test this possibility, we incubated equivalent numbers of *Y. pestis* KIM8-3002 in the absence of HeLa cells in a parallel assay. The bacteria were then subjected to the cold water treatment used to lyse eukaryotic cells, and the cell-free medium (lane 6) and water-released fraction (lane 5) were analyzed by immunoblotting with LcrV- and YopE-specific antibodies. As with YopE, LcrV was not secreted by yersiniae in the absence of HeLa cell contact (lane 6). No YopE and only a small amount of LcrV were released by the water treatment (lane 5), indicating that the strong LcrV-specific signal detected in lanes 1 and 3 was not derived from release of surface LcrV into the cytoplasmic fraction.

A concern was whether this control test of yersiniae in the absence of HeLa cells might have been misleading if yersiniae not in the presence of HeLa cells contained only basal amounts of LcrV and YopE. To address this question, we infected HeLa cells or allowed an equal number of *Y. pestis* KIM8-3002 bacteria to incubate in RPMI in a well lacking HeLa cells. After 2 h, the entire contents of each well was recovered, and the TCA-precipitated proteins were compared for their relative contents of LcrV and YopE by analyzing serial dilutions in an immunoblot (Fig. 2). Figure 2A shows that whether HeLa cells were present or not, LcrV was present in comparable amounts, and the same was true for YopE. A similar blot probed with a polyclonal anti-*Yersinia* antibody (Fig. 2B) showed that the samples from wells with and without HeLa cells had contained equal amounts of bacteria (this had also been verified for replicate wells by CFU determinations). These data show that incubation of *Y. pestis* in a cluster dish at 37°C-5% CO₂ in RPMI is sufficient to induce LcrV and YopE expression and that contact with HeLa cells does not cause further induction, unlike the situation for *Y. pseudotuberculosis* (45). However, *Y. pestis* clearly is contact activated for Yops and LcrV secretion and targeting, as these proteins are not secreted without HeLa cells (Fig. 1).

YopE has been demonstrated by multiple experimental techniques to localize to the cytoplasm of infected cells (62). The observation that LcrV, like YopE, was trypsin resistant in our assay suggested that LcrV also was cytoplasmic. We directly analyzed LcrV localization in infected HeLa cells by laser-scanning confocal microscopy to formally test this prediction (Fig. 3). Infection with our parent strain *Y. pestis* KIM8-3002 did not yield a signal detectable by this assay (data not shown). We therefore chose to test localization of LcrV expressed by an *lcrE* mutant of *Y. pestis*, a strain that has been shown to hypersecrete LcrV (60). We replaced wild-type *lcrE* in *yopM* *Y. pestis* KIM8-3233 by allelic exchange with a subcloned *lcrE* lacking codons 48 to 197 to create *Y. pestis* KIM8-3233.1. The *yopM* mutation was present for a control test needed later and was not expected to influence the targeting of LcrV, as there is considerable precedent for use of strains lacking even multiple Yops in targeting studies (e.g., references 15 and 19). HeLa cells were infected in the presence of cytochalasin D with the *lcrE yopM* mutant or the *lcrV yopI* mutant KIM8-3241 expressing HT-VN68 (as a negative control). HT-VN68 is an N-terminally truncated, His-tagged version of LcrV which fails to be secreted by *Y. pestis* (9). LcrV-specific staining of uninfected HeLa cells or HeLa cells infected with *Y. pestis* expressing HT-VN68 did not yield a significant immunofluorescence signal (Fig. 3B and C). Significant staining, however, was detected in the cytoplasm of *lcrE yopM* *Y. pestis*-infected HeLa cells (Fig. 3A; compare immu-

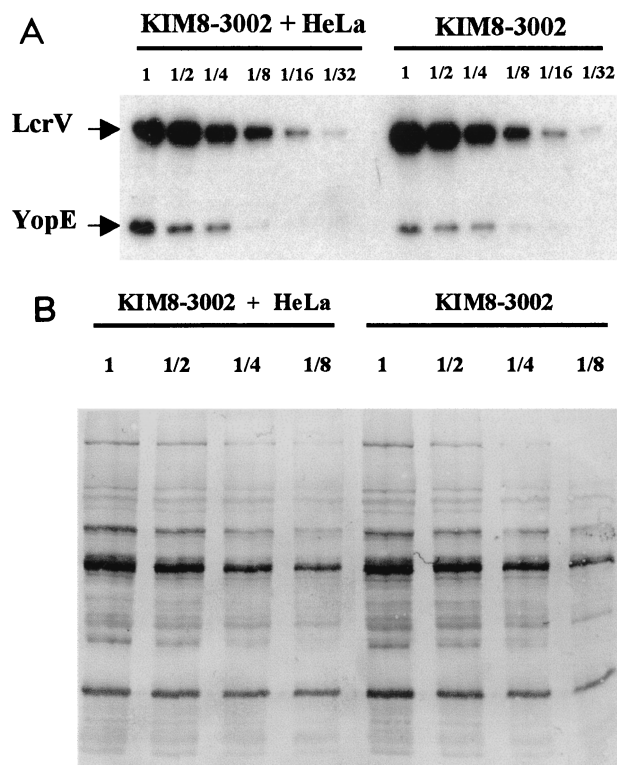


FIG. 2. *Y. pestis* is contact activated for secretion and targeting but not contact induced for expression of LcrV and YopE. HeLa cells were infected with *Y. pestis* KIM8-3002 for 2 h at an MOI of 5, or an equal number of yersiniae were incubated in RPMI without HeLa cells. The total proteins harvested from each well were recovered by TCA precipitation and analyzed in serial twofold dilutions by immunoblotting. In panel A, the blot was probed with a mixture of antibodies to LcrV and YopE; in panel B, a polyclonal anti-*Yersinia* antibody was used. Proteins were visualized by probing immunoblots with IgG coupled to horseradish peroxidase followed by ECL detection (A) or with alkaline phosphatase-coupled IgG followed by development with NBT-BCIP (B). Fold dilutions of the samples are given above the lanes.

no fluorescence and DIC images). LcrV appeared to be localized throughout the cell cytoplasm, and examination of multiple optical planes (data not shown) revealed that intracellular LcrV may also be present in the cell nucleus. The detected signal was LcrV specific, since it could be competed by addition of excess, pure HT-V during staining of monolayers with α -HTV (Fig. 3D). These results confirm that LcrV does gain access to the cytoplasm of infected HeLa cells.

LcrV does not require components of the Yops targeting apparatus to enter HeLa cells. Although our results show that LcrV can enter HeLa cells, it was unclear whether LcrV entry occurred by the Yops targeting mechanism. Targeting of YopE and other Yops requires secretion through a Ysc channel followed by YopB- and YopD-dependent translocation across the eukaryotic plasma membrane (53, 54). Secretion of both LcrV and Yops is Ysc dependent (6, 40), and we predicted that LcrV may also require YopB and YopD to gain access to the HeLa cell cytoplasm. To test this hypothesis, we created *Y. pestis* KIM8-3002.1 (*yopB*) by allelic exchange of parent *yopB* with a subcloned in-frame *yopB* deletion carried on p Δ yopB, which removed essentially all of the 401-residue YopB coding sequence (codons 8 to 390). Consistent with the properties of a nonpolar *yopB* deletion constructed by Håkansson et al. (16), our *yopB* mutation did not affect regulation of Yop expression or secretion and did not have a polar effect on the downstream

gene for YopD (data not shown). We infected HeLa monolayers with *yopB* *Y. pestis* (lanes 1 to 4) or a previously constructed *yopD* (lanes 5 to 8) mutant KIM8-3002.2 (72) and assayed LcrV and YopE localization in fractionated cultures by immunoblot analysis (Fig. 4). In these experiments, infection with the *yopD* strain was carried out in the presence of cytochalasin D to prevent any internalization of the yersiniae which would be unable to target antiphagocytic Yops. This was important here, because *yopD* strains are constitutively strongly induced for Yops and LcrV expression (72), and a few intracellular yersiniae might give an artifactual signal for YopE in the HeLa soluble fraction. As expected, trypsin-inaccessible YopE was not detected in the soluble fractions of HeLa cells infected by *yopB* (lane 3) or *yopD* (lane 7) *Y. pestis*, consistent with the inability of these mutants to target Yops. Instead, YopE was detected in the culture medium (lane 6) or in soluble fractions (lanes 1 and 5) of nontrypsinized cultures. Interestingly, partitioning of LcrV in cultures was unchanged by either the *yopB* or *yopD* mutation in *Y. pestis*: LcrV was detected in the cell-free medium (lanes 2 and 6) as well as soluble fractions from both trypsinized (lanes 3 and 7) and nontrypsinized cultures (lanes 1 and 5). These results indicate that unlike YopE, LcrV does not require YopB or YopD to traverse the plasma membrane of infected HeLa cells.

Extracellular yersiniae directly target LcrV to the HeLa cytoplasm. *Yersinia* can invade eukaryotic cells and survive even within phagolysosomes of professional phagocytes (65). YopE and other Yops, however, are targeted across the eukaryotic cell plasma membrane by extracellular, peripherally associated yersiniae and not by intracellular bacteria (44, 51, 62). Since LcrV did not seem to enter HeLa cells by the Yops targeting pathway, we began to investigate the route of LcrV entry by testing the localization of bacteria from which the intracellular pool of LcrV was derived. We infected HeLa cells with *Y. pestis* KIM8-3002 and either prevented bacterial internalization with cytochalasin D or killed extracellular bacteria with gentamicin to test the source of LcrV detected in these assays (Fig. 5). As expected, YopE was trypsin nonsusceptible (lane 1) and, therefore, targeted when extracellular localization of yersiniae was ensured by the addition of cytochalasin D. In the presence of cytochalasin D (lanes 1 to 4), LcrV was detected in the medium (lane 4), and trypsin-resistant LcrV was detected in the soluble (lane 1) fraction. Neither YopE nor LcrV was detected in samples from gentamicin-treated infections (lanes 5 to 8). As a control, extracellular bacteria were also killed with gentamicin after infection of cytochalasin-treated HeLa cells (lanes 9 to 12) to verify that the amount of cytochalasin D used was sufficient to prevent internalization of the bacteria. Under these conditions, no YopE or LcrV was detected, indicating that sufficient levels of cytochalasin D were used in this assay. Although these results do not rule out low-level secretion of LcrV by intracellular bacteria, these observations do indicate that the LcrV detected in these assays originates from extracellular yersiniae.

Yersiniae are required to mediate entry of LcrV into HeLa cells. The significant amount of YopE detected in the culture medium in Fig. 5 (lane 4) was not seen in repetitions of that experiment. Secretion of LcrV in this tissue culture infection model, however, consistently occurred; more than half of LcrV was released into the culture medium during infection. Given that LcrV-containing fusion proteins have an immunomodulatory effect in the absence of yersiniae, and since entry of LcrV into HeLa cells was YopB and YopD independent, we wondered whether this pool of LcrV in the medium was the source of intracellular LcrV. We have previously demonstrated that HT-V can enter HeLa cells when expressed in *Y. pestis* (9). We

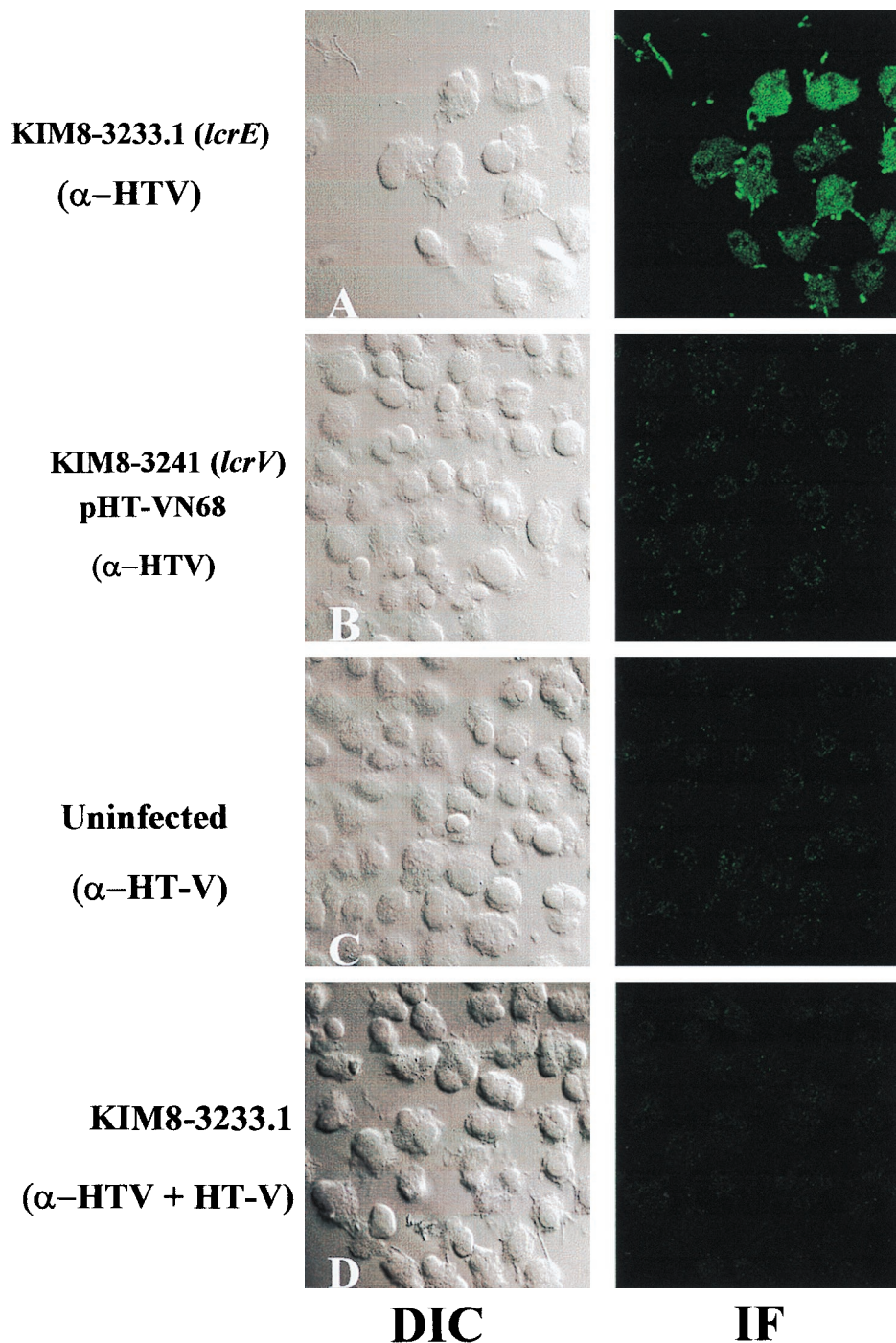


FIG. 3. LcrV can localize to the cytoplasm of infected HeLa cells. HeLa cell monolayers cultivated on glass coverslips in the presence of cytochalasin D were uninfected (C), infected with *Y. pestis* KIM8-3233.1 (*lcrE*) (A), or infected with *Y. pestis* KIM8-3241 expressing HT-VN68 as a negative control (B) for 4 h at 37°C-5% CO₂. Monolayers were then fixed, permeabilized, and stained with α -HTV. During staining of one replicate (D), 10 μ g of pure HT-V (representing a fivefold molar excess over antigen-binding sites of α -HTV) was added during incubation with α -HTV as an antibody specificity control. LcrV was detected by secondary staining with Oregon green-conjugated anti-rabbit IgG followed by visualization by confocal laser-scanning microscopy. A 63 \times objective was used; immunofluorescence (IF) and DIC images are shown.

therefore tested whether HT-V added exogenously to subconfluent monolayers could enter HeLa cells (Fig. 6). Uninfected and *Y. pestis* KIM8-3002-infected HeLa monolayers were incubated for 4 h in the presence of 1 μ g of HT-V (500 ng/ml) purified from *E. coli*. In the test with infected cultures, we wanted to determine whether the interaction between *Yersinia*

and HeLa cells in some way caused or stimulated the internalization of exogenous LcrV. The HT-V migrated more slowly in SDS-PAGE than native, *Yersinia*-derived LcrV (because of the extra mass in the 23-amino-acid His₆-containing leader) and could be distinguished from native LcrV in immunoblots. After incubation, cultures were fractionated and analyzed as before.

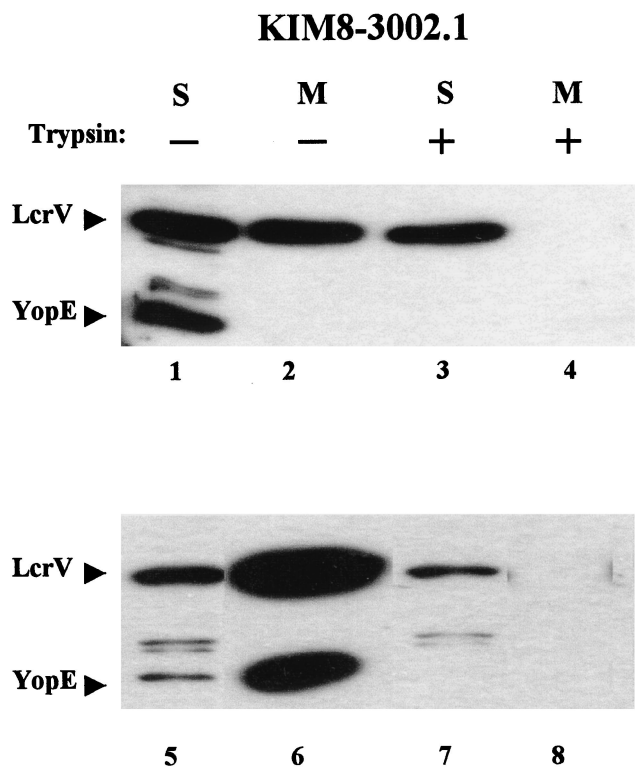


FIG. 4. Entry of LcrV into HeLa cells does not require the translocator Yops, YopB and YopD. HeLa cell monolayers were infected, in duplicate, for 4 h at 37°C-5% CO₂ with *Y. pestis* KIM8-3002.1 (*yopB*) (lanes 1 to 4) or KIM8-3002.2 (*yopD*) (lanes 5 to 8) at an MOI of 10. Infection with *yopD Y. pestis* was done in the presence of cytochalasin D at 5 µg/ml. One replicate of each treatment was treated with trypsin at 100 µg/ml prior to harvest. Fractionation of cultures was done as previously described, and proteins in samples corresponding to 0.3% of original culture volume were resolved in 12% polyacrylamide gels. LcrV and YopE in cell-free medium (M) and soluble (S) HeLa cell water-lysate fractions were detected by immunoblotting with α-HTV and α-YopE. Proteins were visualized by probing with horseradish peroxidase-conjugated anti-rabbit IgG followed by development with ECL reagent.

We did not detect significant amounts of HT-V in HeLa soluble fractions when HT-V was added to uninfected monolayers. Instead, essentially all of the fusion protein was detected in the culture medium. Presence of HT-V during infection of monolayers did not appreciably alter this distribution. Although some HT-V was detected in the soluble fraction, it was susceptible to digestion by trypsin prior to cell lysis, indicating an extracellular localization. In contrast, native, *Yersinia*-derived LcrV in the soluble fraction was not susceptible to trypsin. According to these results, intracellular LcrV must be delivered directly by *Yersinia* and does not result from entry of LcrV that is free in the medium.

A functional Ysc secretion apparatus is not required to mediate entry of LcrV into HeLa cells. Our data suggested that LcrV may enter eukaryotic cells by an entirely novel pathway. It is well established that in vitro secretion of LcrV and Yops requires the Ysc apparatus, and we have recently shown that YscC is required for LcrV to become deposited on the surface of *Y. pestis* (9). We extended our analysis of the LcrV entry pathway by testing whether *Y. pestis* lacking an essential component of the Ysc apparatus could deliver LcrV to the cytoplasm of infected HeLa cells. We used *Y. pestis* KIM5-3001.2.1,

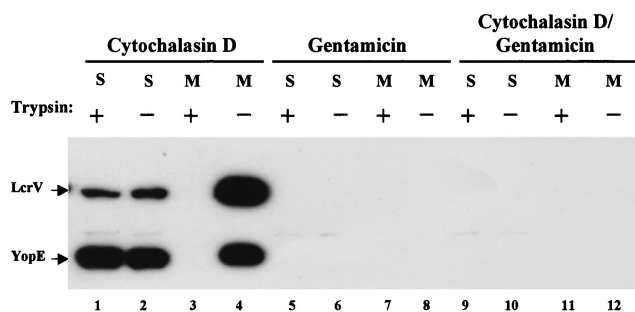


FIG. 5. Extracellular *Y. pestis* targets LcrV. Prior to infection, HeLa monolayers were treated with 5 µg of cytochalasin D per ml for 30 min (lanes 1 to 4 and 9 to 12). These monolayers were then infected with *Y. pestis* KIM8-3002 in the presence of cytochalasin D. Some wells (lanes 5 to 12) received 7.5 µg of gentamicin per ml 30 min after infection to kill extracellular bacteria. After treatment of replicate wells with trypsin, cultures were harvested and fractionated, and samples of cell-free medium (M) and HeLa soluble (S) fractions corresponding to 0.3% of the original culture volume were resolved in 12% polyacrylamide gels. Immunoblots were probed with α-HTV and α-YopE, and LcrV and YopE were detected by incubating immunoblots with horseradish peroxidase-conjugated anti-rabbit IgG followed by development with ECL reagent.

which lacks both *yscV* and *yopD*. The absence of *yopD* served to prevent any downregulation of expression of LcrV and Yops that otherwise could result from the inactivation of the Ysc by the *yscV* mutation. (Recall that YopD is required to prevent maximal thermal induction of the LCR, and *yopD* strains express fully induced levels of both LcrV and Yops [72].) HeLa monolayers were infected with *Y. pestis* KIM5-3001.2.1 as done previously (Fig. 7); as expected, infected cells were not cytotoxic, because Yops could not be secreted and targeted (data not shown). For this experiment, we chose to lyse the infected monolayers by mechanical disruption in a Kontes homogenizer, because as shown in Fig. 1 (lane 5), treatment of yersiniae with cold water released small quantities of LcrV which could contaminate the HeLa soluble fractions. Treatment with a Kontes homogenizer, however, seemed to limit this effect (61). Indeed, immunoblot analysis of proteins released from a *Yersinia*-only control revealed that negligible amounts of LcrV and YopE were released from the bacteria by this method (lane 8), even though easily detectable levels of LcrV and YopE were found in the pelleted bacteria (lane 7). The *Yersinia*-containing low-speed pellets from lysed, infected HeLa cells were analyzed and found to contain readily detectable levels of LcrV and YopE, showing that strong expression of LcrV and Yops had occurred in the *yscV yopD Y. pestis*

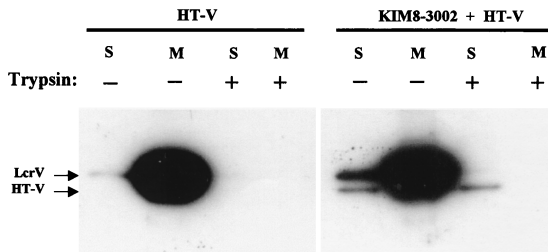


FIG. 6. Delivery by *Y. pestis* is required for LcrV to gain access to the HeLa cell cytoplasm. Purified HT-V was added at 500 ng/ml to HeLa monolayers alone (left) or with *Y. pestis* KIM8-3002 (right), and the cultures were incubated for 4 h at 37°C-5% CO₂. After trypsin treatment and fractionation of cultures, native LcrV and HT-V were detected in cell-free medium (M) and HeLa soluble (S) fractions by immunoblot analysis using α-HTV. Proteins were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and development with ECL reagent.

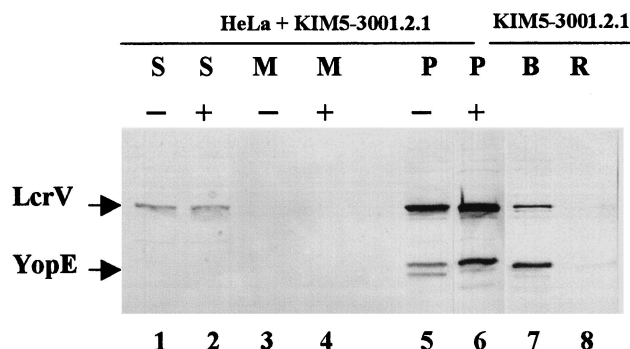


FIG. 7. Entry of LcrV into the HeLa cell cytoplasm does not require a functional Ysc secretion apparatus. *Y. pestis* KIM5-3001.2.1 (*yopD yscV*) was incubated in RPMI for 4 h in the presence (lanes 1 to 6) or absence (lanes 7 and 8) of HeLa cell monolayers. Infected monolayers were treated with trypsin where indicated, and the cell-free medium (M) fraction was harvested as before. Infected HeLa monolayers were scraped, suspended in ice-cold RPMI, and disrupted in a Kontes homogenizer (at least 100 strokes). Disruption was confirmed by analysis of homogenates by phase-contrast microscopy. Subsequent centrifugation resulted in the HeLa soluble (S) fraction and a low-speed pellet (P) containing yersiniae and large HeLa cell debris. Bacteria from the *Yersinia*-only culture were pelleted by centrifugation, suspended in ice-cold RPMI, and subjected to the Kontes homogenizer. Samples were centrifuged to yield the bacterial pellet (B) and supernatant corresponding to Kontes-released (R) proteins. Samples representing 0.5% of original culture volume were resolved in 12% polyacrylamide gels, and immunoblots were incubated with α -HTV and α -YopE. Proteins were visualized by probing immunoblots with alkaline phosphatase-conjugated anti-rabbit IgG and development with NBT-BCIP.

(lanes 5 and 6). As expected, neither LcrV nor YopE was detected in the cell-free medium fraction (lane 3). Surprisingly, LcrV was detected in the HeLa soluble fraction (lanes 1 and 2) and was not susceptible to proteolysis by trypsin (lane 2). This localization was specific for LcrV, since YopE was not detected in either of these fractions. Although these results do not rule out the possibility that other Ysc components are required for LcrV to gain access to the HeLa cell cytoplasm, they do suggest that different pathways mediate release of LcrV into the medium and entry of LcrV into eukaryotic cells.

LcrV enters HeLa cells by a novel, pCD1-independent pathway. All components of the *Y. pestis* Ysc secretion apparatus are encoded, along with LcrV and Yops, on the virulence plasmid pCD1. We tested for an alternative LcrV-specific export pathway by infecting HeLa cells with pCD1⁻ *Y. pestis* KIM10 expressing LcrV under the control of an inducible promoter (Fig. 8A). In preliminary test, *Y. pestis* KIM10 expressing LcrV or YopM did not secrete either protein into culture supernatant when cultivated in defined medium under inductive conditions, even though both proteins were strongly expressed within the bacteria (data not shown). For the experiments with infected HeLa cells, we used YopM, expressed in *Y. pestis* KIM10 under control of the same promoter used for *lcrV*, as a negative control Yop that is dependent on YopB and YopD to be targeted (Fig. 8B). HeLa monolayers were also infected with *Y. pestis* KIM8-3233.1 (*lcrE yopM*) or *Y. pestis* KIM8-3233.1 expressing YopM, as positive controls that cause cellular internalization of LcrV and targeting of YopM, respectively. Uninfected monolayers were used as a specificity control, and HeLa cells infected with *Y. pestis* KIM10 expressing the nonsecretable HT-VN68 served as an additional negative control. All infections were done in the presence of cytochalasin D, since *Y. pestis* KIM10 lacks all Yop-mediated antiphagocytic properties; IPTG was used to induce expression of *trans* copies of *lcrV* and *yopM*. To avoid any possible fractionation artifacts, we directly assayed for cytoplasmic LcrV and YopM by staining fixed, permeabilized monolayers with LcrV-

specific (Fig. 8A) or YopM-specific (Fig. 8B) antibodies, followed by visualization using laser-scanning confocal microscopy. DIC images showed the position of HeLa cells and associated yersiniae. As expected, YopM was detected within HeLa cells infected with *Y. pestis* KIM8-3233.1 expressing YopM (Fig. 8B, column 4) after staining with α -YopM. Conversely, no significant signal was detected in HeLa cells infected with pCD1⁻ *Y. pestis* KIM10 expressing YopM (Fig. 8B, column 3) or the nonsecretable HT-VN68 (Fig. 8B, column 2). Some YopM-specific staining of permeabilized bacteria was detected (Fig. 8B, column 3). When LcrV was expressed in *Y. pestis* KIM10, LcrV-specific staining was detected in the cytoplasm of infected HeLa cells (Fig. 8A, column 3), although the staining was weaker than that detected in some cells of the positive control culture infected with *lcrE yopM Y. pestis*. Although permeabilized bacteria were stained, the signal detected in HeLa cells infected with *Y. pestis* KIM10/pHT-VN68 (Fig. 8A, column 2) was comparable to that in an uninfected negative control (Fig. 8, column 1). Taken together, these results indicate that *Y. pestis* lacking the virulence plasmid pCD1 can target LcrV but not YopM. LcrV, therefore, enters eukaryotic cells by a novel, virulence plasmid-independent mechanism.

DISCUSSION

In this study, we characterized the partitioning of LcrV in a tissue culture infection model. We chose infection of HeLa cells as a model system, since the deployment of Yops by *Yersinia* has been investigated using this model. Previous reports by this lab showed that LcrV, like the Yops, was detected in culture fractions representing the cytoplasm of infected HeLa cells (37), suggesting that LcrV may be targeted into infected eukaryotic cells by the Yops targeting mechanism. In this report, we showed that LcrV does enter infected HeLa cells, but by a mechanism distinct from that which mediates Yops targeting. Our evidence also indicates that at least two pathways exist for the export of LcrV from *Y. pestis*. The first is manifested as Ysc-dependent secretion of LcrV into the tissue culture medium and localization at the bacterial surface (9), while the second, pCD1-independent pathway results in delivery of LcrV directly into the cytoplasm of infected eukaryotic cells.

In vitro in defined medium, both LcrV and YopE are secreted by the Ysc type III mechanism (40). In the tissue culture infection model, Ysc-dependent secretion of YopE and subsequent unidirectional targeting is induced by contact with a target eukaryotic cell (6, 45). In the case of *Y. pseudotuberculosis*, contact also concomitantly induces increased Yops expression (45). This appears not to be the case for *Y. pestis* at 37°C-5% CO₂ in RPMI: thermal induction in the absence of cell contact in this culture condition is sufficient to induce Yops and LcrV expression, and cell contact provides no further induction. However, cell contact clearly causes activation of the Ysc for secretion and targeting. Consistent with this observation, we failed to detect YopE release into tissue culture medium when *Y. pestis* was incubated in tissue culture medium in the absence of HeLa cells. LcrV was also not detected, indicating that its release is probably triggered by the same mechanism that initiates delivery of Yop effectors. Conversely, LcrV was released when *Y. pestis* was incubated in the presence of HeLa cells. Consistent with previous observations (37), significant amounts of LcrV were detected in the tissue culture medium and HeLa soluble fractions, in contrast to YopE, which was unidirectionally targeted into the HeLa cells and not released into the medium in significant amounts. Secretion of

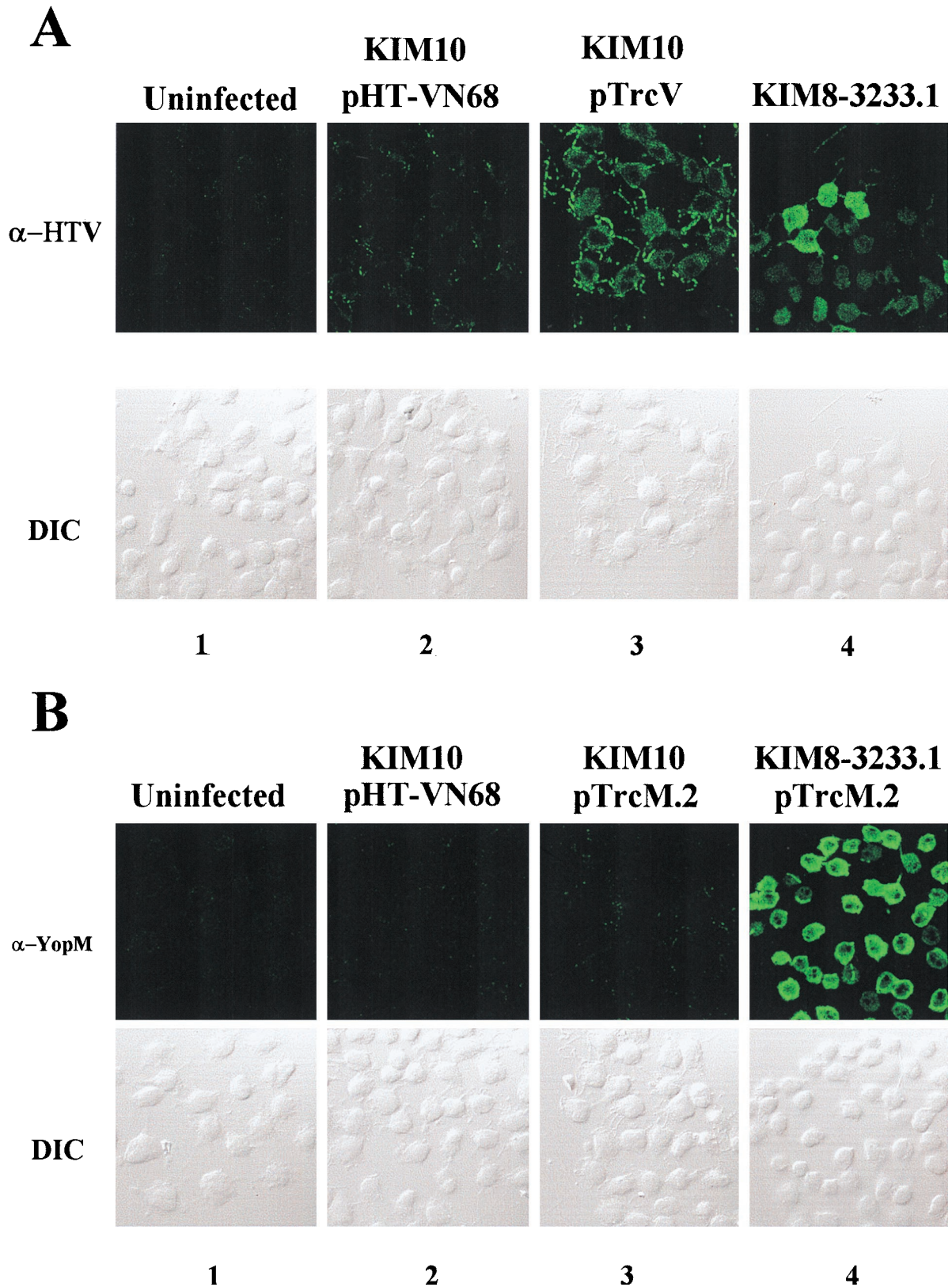


FIG. 8. Entry of LcrV into the HeLa cell cytoplasm does not require pCD1-encoded proteins. Cytochalasin D-treated (5 μ g/ml) HeLa cell monolayers were uninfected (A and B, column 1), infected with *Y. pestis* KIM8-3233.1 (*lcrE yopM*) (A, column 4) or KIM8-3233.1 expressing YopM from pTrcM.2 (B, column 4), and infected with pCD1⁻ *Y. pestis* KIM10 expressing HT-VN68 (A and B, column 2), LcrV (A, column 3), or YopM (B, column 3). IPTG was added to 0.1 mM to cultures for activation of inducible promoters. After 4 h, monolayers were fixed, permeabilized, and stained with α -HTV (A) or α -YopM (B). Proteins were detected by secondary staining with Oregon green-conjugated anti-rabbit IgG followed by visualization by confocal laser-scanning microscopy. A 63 \times objective was used; immunofluorescence (IF) and DIC images are shown.

LcrV into the medium and targeting of YopE both require a functional Ysc apparatus, and LcrV, but not YopE, can localize to the extracellular surface of *Y. pestis* (9). This is true also for the Ysc outer-gate protein LcrE (11), so there already was precedent prior to this study for the surface localization of Ysc accessory proteins that themselves are secreted by the Ysc. Moreover, these proteins are surface localized under conditions that are not permissive for strong Yop expression or secretion (i.e., absence of cell contact). These data suggest that *Yersinia* has the ability to differentially sort proteins secreted by the same pathway. Further investigation is needed to determine how and where this differential sorting occurs.

Although we detected trypsin-resistant LcrV in the HeLa soluble fraction, it was important to rule out several possible sources of artifactual introduction of LcrV into this fraction. For instance, we have shown that LcrV can exist on the extracellular surface of *Y. pestis* and have proposed that LcrV may be a component of a Yops delivery apparatus (9). Accordingly, it is possible that trypsin could not gain access to LcrV incorporated into a structure, and as a result, LcrV would enter the HeLa soluble fraction and appear to be trypsin resistant if some of the structures were released from the bacteria and into the HeLa soluble fraction during the fractionation procedure. Skrzypek et al. (58) have also documented that the treatment with 0.1% Triton X-100 that often is used to lyse eukaryotic cells results in release of Yops from *Y. pestis*. In our hands, even water lysis caused small amounts of LcrV to be released from *Y. pestis* (Fig. 1, lane 5). Similarly, our lab has found that treatment with 10 μ g of digitonin per ml results in nonspecific release of Yops from *Y. pestis* (61). We therefore turned to confocal microscopy to directly demonstrate intracellular LcrV, and we easily detected it when HeLa cells were infected with *Y. pestis* lacking the Ysc outer-gate protein LcrE (Fig. 3 and 8). However, we were unable to detect LcrV targeted from parent *Y. pestis* by this method (data not shown), even though we believe that it was present (Fig. 1 and 5). This difference between parent and *lcrE* yersiniae may arise from a difference in the way LcrV is partitioned within the bacteria for the two strains. Previous studies with *Y. pestis* grown in defined medium lacking Ca^{2+} have shown that essentially all of the LcrV made by *lcrE* yersiniae is released into the medium, whereas in parent *Y. pestis* about half of LcrV stays in the bacterial soluble fraction (60). It may be that LcrE normally exerts a retentive effect on LcrV, which also has a cytoplasmic Ysc-related secretion-regulatory function (38, 60). In the absence of LcrE, this internal pool of LcrV may be freed from its association with the Ysc and be available for targeting by the putative, novel Ysc-independent pathway. In the parent *Y. pestis*, a much smaller amount of LcrV may be available to that pathway, and this might be below the sensitivity of detection by immunofluorescence. We found that the intracellular LcrV has a diffuse distribution. Interestingly, LcrV appeared to be present throughout the cell, including the interior of the nucleus, when Yops also were being targeted (Fig. 3 and 8, KIM8-3233.1), whereas it appeared to be mainly cytoplasmically located when it alone was being targeted (Fig. 8A, KIM10 pTrcV). This could reflect a cellular response to a targeted Yop, that allows LcrV to enter the nucleus, an idea that will be important to test in the future.

We initially thought that LcrV gained access to the HeLa cytoplasm by a mechanism similar to that used by Yops. YopE, for example, lacks the intrinsic ability to enter eukaryotic cells and therefore must be directly delivered by extracellularly associated yersiniae (52). *Y. pestis* used in our experiments was pregrown at 26°C and as a result lacked the thermally inducible, antiphagocytic capsule (4). Such bacteria are readily

phagocytosed by macrophages (65), and in preliminary experiments, there was significantly less LcrV within infected J774 macrophage-like cells unless cytochalasin D was present to prevent phagocytosis (data not shown). A similar observation has previously been reported for YopM targeting by *Y. pestis* (58) as well as for *Y. pseudotuberculosis* internalized into HeLa cells (44, 51). We therefore predicted that the LcrV detected in our experiments was derived from extracellular yersiniae. Indeed, we found that extracellular yersiniae were the source of both cytoplasmic and medium-localized LcrV in infected HeLa cells (Fig. 5). In addition, exogenously added pure LcrV (HT-V) could not enter HeLa cells (Fig. 6). One interpretation of these results is that LcrV must be delivered directly to HeLa cells by associated yersiniae and does not gain access to the cytoplasm by diffusing to HeLa cells in a paracrine fashion from the medium. However, we then found that unlike entry of YopE, entry of LcrV does not require either YopB or YopD. These results indicate that a separate mechanism must exist for the transfer of LcrV from *Yersinia* to infected cells.

Our next test showed that this mechanism does not require a functional Ysc apparatus, because LcrV still entered HeLa cells and became trypsin resistant when the infecting strain was a *yscV yopD* double mutant (Fig. 7). This *Y. pestis* lacked functional YscV, an inner membrane Ysc component that is essential for secretion of LcrV and Yops under inductive conditions in a defined medium (47). The *yopD* mutation counteracted any downregulation that otherwise might have resulted from the inability to secrete the negative regulator LcrQ (45), because YopD is required for LcrQ to have its negative regulatory effect (72). Accordingly, the double mutant expressed LcrV and Yops strongly but failed to secrete either protein into the tissue culture medium and failed to target YopE into the HeLa cells, whereas LcrV was targeted by this strain. We believe that this targeting was real and that it was not complicated by contamination of the HeLa soluble fraction during the fractionation process. Surface localization of LcrV, one potential source of contamination, would not have occurred, because it requires a functional Ysc; second, treatment of bacteria in a Kontes homogenizer did not induce release of LcrV or YopE from the bacteria. Since all Ysc components are encoded on the virulence plasmid pCD1 (18, 42), we tested whether strains lacking pCD1 could mediate targeting of LcrV. LcrV, but not YopM, gained access to the HeLa cytoplasm, indicating that a separate targeting mechanism may be encoded elsewhere in the *Y. pestis* genome. One concern was that because the virulence plasmid pCD1 possesses several insertion sequence elements (42) and has been observed to integrate into the *Yersinia* chromosome (40), it was possible that *Y. pestis* KIM10 was not truly pCD1⁻. We believe that this was not the case, because we were unable to amplify any *ysc*-specific DNA by probing for multiple pCD1-encoded genes by PCR of *Y. pestis* KIM10 DNA (data not shown). Our findings have not proven that no LcrV enters HeLa cells through the Ysc-associated targeting mechanism; in fact, there appeared to be more LcrV targeted by the pCD1⁺ *Y. pestis* KIM8-3233.1 (for some cells in the population) than by the pCD1⁻ *Y. pestis* KIM10 pTrcV (Fig. 8A, columns 4 versus 3). However, our data do show that LcrV is targeted by a Ysc-dependent mechanism that is contact activated (Fig. 4, 7, and 9).

This study raises many fascinating, unanswered questions. However, there is some information available to refine our thinking about two of these: how does this secondary mechanism mediate transfer of LcrV to infected cells, and what is the function of intracellular LcrV. The secondary pathway for LcrV targeting requires cell contact but not secretion by the Ysc type III mechanism. We have recently shown that LcrV

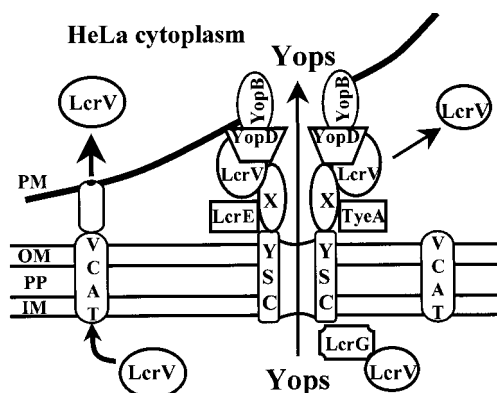


FIG. 9. Model for Ysc-dependent and independent secretion of LcrV. LcrV, YopB, YopD, and possibly other proteins (X) are secreted by contact-activated Ysc secretion channels and assemble into an apparatus that mediates targeting of secreted Yops across the plasma membrane and into the cytoplasm of eukaryotic cells. LcrV shed from these structures diffuses into the surrounding medium. The LcrV-transporting contact-activated translocator (VCAT) can also secrete LcrV from *Yersinia*. Upon contact with the plasma membrane, LcrV not involved in secretion or targeting of Yops is secreted across the inner membrane (IM), periplasm (PP), and outer membrane (OM) of *Y. pestis* through the VCAT mechanism composed of non-pCD1-encoded proteins. LcrV secreted in this manner is delivered directly to associated eukaryotic cells.

truncated at the C terminus gains access to the HeLa cytosol but is not secreted by the Ysc, as it is not detected in the tissue culture medium and does not localize to the surface of *Y. pestis* (9). LcrV's N-terminal 67 residues can be replaced by the first 15 amino acids of YopE, and this protein enters HeLa cells even though it also is not secreted by the Ysc (9). Perhaps the N and C termini are required for Ysc-dependent secretion, while an internal domain is required for the secondary pathway of LcrV targeting. Sequence analysis predicted a Sec signal-like sequence 100 residues from the N terminus (50). However, this sequence is not required for LcrV targeting, since an LcrV lacking this region (LcrV Δ 108–125 [60]) can gain access to the cytoplasm of infected HeLa cells (data not shown). Further investigation is needed to elucidate a novel export and delivery mechanism that recognizes an internal domain in LcrV.

Although we have not identified a specific function for intracellular LcrV, we propose that transfer of LcrV to eukaryotic cells is not a spurious process. In a separate report, we suggest that LcrV may be involved in Yops targeting by acting as an essential component of a Yops targeting apparatus that is assembled at the interface between yersiniae and associated eukaryotic cells (9). We propose that intracellular LcrV is not related to this Yops targeting role, because LcrV entry is independent of the Ysc, whereas Yops targeting, including surface localization of LcrV, requires the Ysc. An immunomodulatory role for free LcrV has also been proposed, based on studies using pure LcrV-containing fusion proteins (34, 35, 71). The target and mechanism by which this occurs remain to be determined. Our purified preparation of LcrV was unable to enter HeLa cells, and although these data do not rule out a role in cytokine regulation, they suggest that intracellular LcrV may not function in the activities defined using LcrV-containing proteins. Finally, we did not notice a cytotoxic effect of LcrV during infections with *yopB* or *yopD* *Y. pestis*; HeLa cells remained flat and adherent to plastic culture dishes. Future work, therefore, will examine whether intracellular LcrV has a specific function in interrupting cellular processes required to eliminate *Yersinia* during an infection.

In this report, we have demonstrated that LcrV partitions differently than the vectorially targeted Yops in a tissue culture infection assay and is able to enter eukaryotic cells by a novel pathway. We propose a model (Fig. 9) in which contact of *Y. pestis* with eukaryotic cells can simultaneously activate two separate pathways in *Y. pestis* for secretion of LcrV. Secretion by the previously characterized Ysc mechanism results in deposition of LcrV on the surface of yersiniae in structures that mediate Yops targeting. These structures may be somewhat dynamic, promoting the release of LcrV into the surrounding medium. We suggest that it is this released pool of LcrV that is free to diffuse away and function as an immunomodulator. The second pathway is a novel, Ysc-independent mechanism that mediates direct targeting of LcrV to the cytosol of associated eukaryotic cells without release into the surrounding medium. Future work will focus on determining the composition of this novel LcrV-transporting contact-activated translocator, assessing whether additional substrates exist, and elucidating a function for intracellular LcrV.

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