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The *Yersinia* Yop Virulon: LcrV Is Required for Extrusion of the Translocators YopB and YopD

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LcrV, an essential piece of the Yop virulon, is encoded by the large *lcrGVsycDyopBD* operon. In spite of repeated efforts, the role of LcrV in the Yop virulon remains elusive. In an attempt to clarify this, we engineered a complete deletion of *lcrV* in the pYV plasmid of *Yersinia enterocolitica* E40 and characterized the phenotype of the mutant. Complementation experiments showed that the mutation was not polar with regard to *yopB* and *yopD*. Nevertheless the mutation abolished secretion of YopB and YopD, while secretion of the other Yops was unaffected or even increased. Northern blot analysis showed that transcription of *yopD* was not affected. YopD could be detected inside the bacteria, showing that the lack of its secretion was not due to a lack of translation or to proteolysis. This indicated that LcrV is specifically involved in the process of release of YopB and YopD. We then investigated the possible interactions between LcrV and YopB or YopD. We constructed a glutathione *S*-transferase–LcrV hybrid protein, and we observed that either YopB or YopD could be copurified with it. The same approach showed that LcrV also interacts with LcrG but not with the chaperone SycD. Using deletants of *lcrV*, we then identified a definite LcrG-binding domain in the C terminus of LcrV.

The capacity of yersiniae (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) to resist the immune system of their host depends on the Yop virulon. This system allows extracellular bacteria adhering at the surface of eukaryotic cells to inject bacterial proteins into the cytosol of these cells in order to disarm them or disrupt their communications (for reviews, see references 15, 25, and 52). Translocation of the intracellular effectors (YopE, YopH, YpkA/YopO, YopM) across the eukaryotic cell membrane requires at least two other Yop proteins, namely YopB and YopD (7, 21, 33, 42, 49, 51). Deployment of these translocators at the bacterial surface is triggered by contact with eukaryotic cells and is controlled by proteins of the virulon including YopN, which is supposed to act as a stop valve closing the bacterial secretion channel (7, 18, 42). *yopN* mutant bacteria are deregulated for Yop secretion in the sense that they release most of their Yop effector load outside eukaryotic cells, but they can nevertheless deliver a portion of these effectors inside eukaryotic cells (7).

Yop proteins are transported outside the bacterial cell by a type III secretion apparatus called Ysc, which consists of proteins YscA through YscU, LcrD, and lipoprotein VirG (1–4, 16, 26, 35, 36, 56). Synthesis of the Yops is subject to feedback inhibition: when the secretion apparatus is closed or defective, transcription of the *yop* genes is prevented (14, 34). The proper operation of the system also requires the presence in the bacterial cytosol of small individual chaperones called the Syc proteins (for a review see reference 55). Three such chaperones have been described so far: SycE for YopE, SycH for YopH, and SycD (called LcrH in *Y. pseudotuberculosis* and *Y. pestis*) for YopD. *sycE* mutants secrete much less YopE than the wild type does, and they rapidly degrade their pool of intracellular YopE (9, 19, 54). SycE acts by binding the domain of YopE that is recognized by the translocation apparatus (57). SycH acts in a similar way for YopH (53, 57). SycD binds to YopD and is required for secretion of both YopD and YopB (53). Previous work described SycD as a negative regulator of the transcription of *yop* genes (5, 39). It is not known whether this regulatory effect represents a distinct role of SycD or a consequence of its protective role for YopD.

In vitro, the need for contact with eukaryotic cells can be circumvented by chelating Ca^{2+} ions with agents such as oxalate or EGTA. Under these conditions, effectors and translocators are released in massive amounts in the bacterial culture medium where they form inert aggregates. The *yopN* mutants affected in the contact control are also affected in their response to Ca^{2+} chelation. They secrete Yops, even in the presence of Ca^{2+} ions, and they are said to be "Ca^{2+} blind" (18).

The entire Yop virulon is borne by a 70-kb plasmid called pYV. The genes encoding the Ysc apparatus are arranged as three neighboring operons, while the genes encoding the Yop effectors are scattered around the whole plasmid (Fig. 1). The translocators YopB and YopD as well as LcrG and the chaperone SycD (LcrH) are encoded by the large *lcrGVsycDyopBD* operon, which also encodes LcrV (5, 29, 38). LcrV is a 41-kDa secreted protein that was described in the mid-1950s as a protective antigen of the plague bacillus *Y. pestis* (8, 28, 41). LcrV is one of the major Yops, yet its exact role in the virulon is still unclear. As expected, polar mutations in *lcrV* prevent secretion of LcrV, YopB, and YopD (29, 38). However, nonpolar mutations consisting of small in-frame deletions also impair secretion of other Yops, which suggests that LcrV plays a regulatory role (5, 37, 46). Since the *lcrV* gene is buried in the operon that encodes the translocators, we considered that LcrV may instead be an element of the translocation apparatus which is required either for the operation or deployment of the latter. In this paper, we present data supporting this view.

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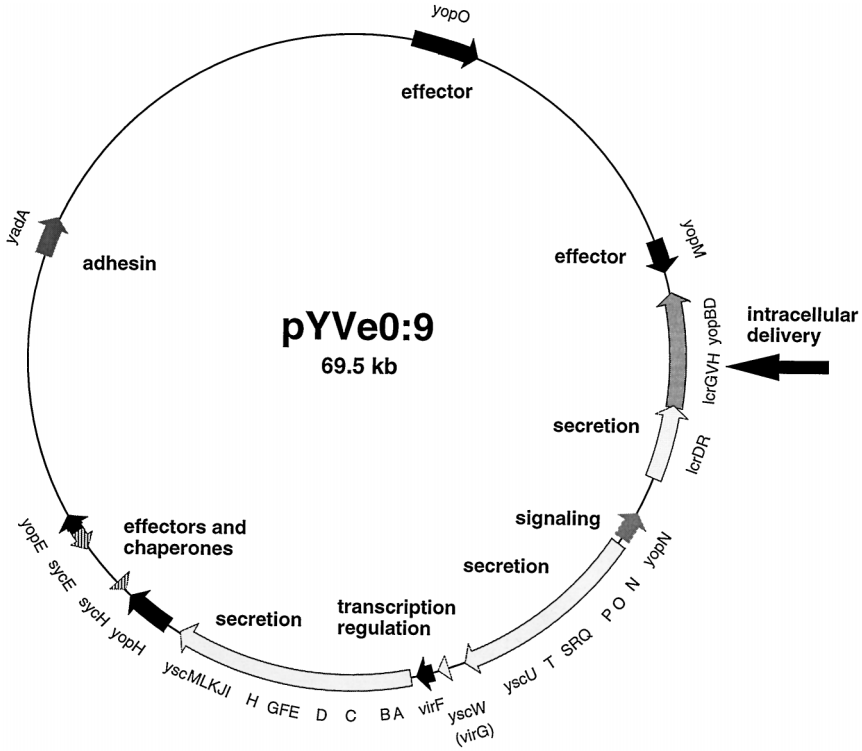

FIG. 1. The pYV plasmid from *Y. enterocolitica* showing the genes and operons discussed in the text. The arrow indicates the *lcrGVHyopBD* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) of serotype O:9 strain W227 (11). *Y. enterocolitica* KNG22703 and MRS40 are the *blaA* mutants of strains W22703 and E40, respectively, in which the gene encoding β-lactamase A was replaced by the *luxAB* genes (22, 45). *Escherichia coli* LK111, received from M. Zabeau (Ghent, Belgium), was used for standard genetic manipulations. *E. coli* CJ236 was used for site-directed mutagenesis (23). *E. coli* XL1 Blue (Stratagene, La Jolla, Calif.) was used to produce the glutathione *S*-transferase (GST) fusion proteins. *E. coli* SM10 lambda *pir*$^+$ constructed by Miller and Mekalanos (27) was used to deliver the mobilizable plasmids in *Y. enterocolitica*. This strain allows replication of *pir* mutants of R6K, and it mobilizes plasmids containing the origin of transfer of RK2. The plasmids used in this study are listed in Table 1.

Bacteria were routinely grown in tryptic soy broth (Oxoid, Basingstoke, England) and plated on tryptic soy agar. For the induction of the *yop* regulon, *Y. enterocolitica* was grown in brain heart infusion broth supplemented with 4 mg of glucose ml^{-1}, 20 mM $MgCl_2$, and 20 mM sodium oxalate. All media were supplemented with the relevant antibiotics. Unless otherwise specified the concentrations were as follows: ampicillin, 200 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; and nalidixic acid, 35 μg ml^{-1}.

Induction of the *yop* regulon, SDS-PAGE analysis of Yops, immunoblotting, and genetic conjugation. Yops were prepared from culture supernatants and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Tricine-SDS-PAGE and Western blotting as described previously (2, 14, 50). For the analysis of the whole-cell protein extract, 8×10^8 bacteria were applied to SDS–14% PAGE. Immunoblotting was carried out with rat monoclonal antibodies 13A4 (anti-YopD), 7C1 (anti-LcrV), 9B7 (anti-YopB), and 6G1 (anti-YopE) as described by Bodeus et al. (6) and polyclonal antibodies against LcrV and LcrG.

To introduce a plasmid into *Y. enterocolitica* by conjugation, the plasmid was first introduced in *E. coli* SM10 lambda *pir*$^+$ by electroporation. This donor strain and the recipient *Y. enterocolitica* were mated during a 2- to 3-h period on a plate at 32°C.

Construction of entire *lcrV* deletion mutant. A 1,800-bp fragment, containing the entire *lcrG*, *lcrV*, and *sycD* (*lcrH*) genes, was amplified by PCR with pYV40 as a template. The upstream primer MIPA384 (5′-ATGAATTCATATGAAGT CTTCCCATT-3′) consisted of nucleotides 1 to 16 of *lcrG* from *Y. pestis* (38) preceded by an *Eco*RI restriction site (underlined). The downstream primer MIPA289 (5′-CATGGATCCTGGGTTATCAACGCACTCATG-3′) consisted of the nucleotides complementary to the last 20 nucleotides (from 483 to 504) of *lcrH* from *Y. pestis* (38) and was preceded by a *Bam*HI restriction site (underlined). The PCR product was then digested with *Eco*RI and *Bam*HI and cloned into the same sites of pBluescriptII SK^+, yielding plasmid pMRS64. An *Nru*I restriction site was then introduced just before the third codon of *lcrV* in pMRS64 by site-directed mutagenesis with oligonucleotide MIPA406 (5′-CAA ATTATTTAATATGTCGCGAGCCTACGAACA-3′), generating plasmid pMRS65. Oligonucleotide MIPA407 (5′-CTGCTAGATGACACGCCCGGGA AATGACACGAGGT-3′) was used to introduce a *Sma*I site after codon 324 of *lcrV* in pMRS65, yielding pMRS67. An in-frame deletion was then generated by digestion of pMRS67 with *Nru*I-*Sma*I followed by religation. This recombinant plasmid containing the mutated allele was called pMRS69. The *Sal*I-*Xba*I fragment of pMRS69 was then cloned into the same sites of the suicide vector precursor pMRS101. This precursor contains two origins of replication: a functional ori_{ColE1} facilitating the production of plasmid DNA and a conditional ori_{R6K} that is only functional in *E. coli* strains producing the π protein (27, 44). The recombinant precursor pMRS70 was then digested by *Not*I and religated to remove ori_{ColE1} and *bla*. The resulting mutator plasmid, unable to replicate in *Y. enterocolitica*, was called pMRS71.

To inactivate the *lcrV* gene on the pYV plasmid, mutator plasmid pMRS71 ($lcrV_{\Delta 3\text{-}324}$) was transferred into *Y. enterocolitica* MRS40(pYV40) by conjugation, and allelic exchange was selected as described by Kaniga et al. (22) except that in the last step, we added 0.4 mM arsenite to the sucrose-containing plate to select for the pYV plasmid (31). The *lcrV* mutant pYV plasmid was designated pMRS4071.

In the course of the experiments (see Results) we observed that the deletion was larger than expected, and sequencing revealed that part of *sycD* was also deleted. This discrepancy presumably resulted from the fact that the design of the oligonucleotides was based on sequence from *Y. pestis* and not from *Y. enterocolitica.*

Construction of $lcrV_{\Delta 2\text{-}32}$ and $lcrV_{\Delta 224\text{-}266}$ alleles. These two alleles were constructed by site-directed mutagenesis as described by Kunkel et al. (23) with single-stranded pMRS20 DNA as a template. A uracil-containing single-stranded pMRS20 was produced from *E. coli* CJ236 *dut ung*. The double-stranded DNAs obtained after in vitro synthesis of the second strand were introduced into *E. coli* LK111, and the mutated plasmids were screened by PCR.

Plasmid pMRS56 containing allele $lcrV_{\Delta 2\text{-}32}$ was constructed by deletion of codons 2 to 32 with MIPA356 (5′-CGAGGGCGCCTTATTTAATATGGAAG AATTGGTTCAGTTAGT-3′), which introduced a *Nar*I restriction site.

Allele $lcrV_{\Delta 224\text{-}266}$ in pMRS52, engineered with MIPA352 (5′-CCTCAAACC ACCATTCACGGCGCCACCACCTGC-3′), lost codons 224 to 266 and gained a *Nar*I restriction site.

Construction of pGEX-derived recombinant plasmids. The *lcrG* gene, amplified from pMRS44 with MIPA382 (5′-ACGTCGACAAGAAGGAGATATAC

TABLE 1. Plasmids used in this study

Plasmids	Relevant characteristics	Reference or source
pYV plasmids		
pGC153	pYVe227 *yopB-GC153*::mini-Mud1 *lac ylpA-YL4*::Tn*3* (does not encode YopB and YopD)	14
pMRS4071	pYV40 $lcrV_{\Delta 3\text{-}324}$ (pYV40 mutated with pMRS71)	This work
pPW2269	pYVe227 *sycD* mutant (out-of-frame deletion between nucleotides 111 and 255 of *sycD*; encodes a 43-residue truncated SycD)	53
pYV40	Wild-type PYV plasmid from *Y. enterocolitica* E40	49
pYVe227	Wild-type pYV plasmid from *Y. enterocolitica* W227	9
Clones and vectors		
pBC18R		10
pBC19R		10
pCNR21	pBC19R P_{yopE} *yopE*	This work
pCNR26	pBC19R P_{yopE} SD T7 *Nde*I *yopE*	This work
pCN29	pGEX-KG *yopD* (encodes GST and YopD)	This work
pCN40	pGEX-KG *yopB sycD* (encodes GST, YopB and SycD)	This work
pCNG42	pGEX-KG *gst-lcrV yopB sycD* (encodes GST-LcrV, YopB and SycD)	This work
pCNG50	pGEX-KG *gst-lcrV sycD* (encodes GST-LcrV and SycD)	This work
pGEX-KG	P_{tac} *gst* ori_{pBR322}	20
pMRS20	pBluescriptII SK^+ and PCR-amplified fragment (by using MIPA271 and MIPA64) containing *lcrRGV*	45
pMRS28	pBC18R and *Eco*RI fragment from pMRS20 (encoding *lcrRGV* under own promoter)	This work
pMRS44	pT7-7 and PCR-amplified *lcrG* (by using MIPA354 and MIPA355) from pMRS20 cloned into the *Nde*I-*Sal*I site	This work
pMRS46	pGEX-KG *gst-lcrV* (encodes GST-LcrV)	45
pMRS50	pGEX-KG *gst-lcrG yopD* (encodes GST-LcrG and YopD)	45
pMRS52	pMRS20 $lcrV_{\Delta 224\text{-}266}$ (created by site-directed mutagenesis with MIPA352)	This work
pMRS56	pMRS20 $lcrV_{\Delta 2\text{-}32}$ (created by site-directed mutagenesis with MIPA356)	This work
pMRS64	pBluescript II SK^+ and PCR-amplified fragment (by using MIPA384 and MIPA289) from pYV40 containing *lcrG*, *lcrV*, and *sycD* genes	This work
pMRS65	pMRS64, *Nru*I site just before codon 3 of *lcrV*	This work
pMRS67	pMRS65, *Sma*I site just after codon 324 of *lcrV*	This work
pMRS69	pMRS67 $lcrV_{\Delta 3\text{-}324}$	This work
pMRS70	pMRS101, *Sal*I-*Xba*I fragment of pMRS69	This work
pMRS72	pCNR26 and PCR-amplified fragment (by using MIPA354 and MIPA122) from pYV40 containing *lcrG*, *lcrV*, and *sycD* genes	This work
pMRS74	pMSL56 with *cyaA* replaced by a PCR-amplified fragment (by using MIPA121 and MIPA317) from pYV40 containing *sycD*, *yopB*, and *yopD* genes	This work
pMRS75	pMRS46 *lcrG* (encodes GST-LcrV and LcrG)	This work
pMRS78	pMRS75 $lcrV_{\Delta 224\text{-}266}$ (encodes GST-$LcrV_{\Delta 224\text{-}266}$ and LcrG)	This work
pMRS83	pMRS75 $lcrV_{\Delta 2\text{-}32}$(encodes GST-$LcrV_{\Delta 2\text{-}32}$ and LcrG)	This work
pMRS84	pMRS50 *lcrV* (*lcrG* replaced by *lcrV*) (encodes GST-LcrV and YopD)	This work
pMSL56	pTM100 P*yopE cyaA*	M.-P. Sory
pPW64	pBC18R *sycD*	53
Suicide vector and mutator		
pMRS101	ori_{R6K} ori_{E1} *sacBR* $oriT_{RK2}$ *strAB*	44
pMRS71	pMRS70 Δori_{E1} (encoding $lcrV_{\Delta 3\text{-}324}$)	This work

ATATG-3′) and MIPA383 (5′-GATGTCGACTTAAATAATTTGCCCT-3′), was cloned into the *Sal*I site of pMRS46, yielding plasmid pMRS75 (encoding GST-LcrV and LcrG). The $lcrV_{\Delta 2\text{-}32}$ and $lcrV_{\Delta 224\text{-}266}$ alleles were amplified from pMRS52 and pMRS56, respectively (with MIPA364 [5′-CGGAATTCTCATGATTAGAGCCTACG-3′] and MIPA64 [5′-ATGTCGACCTGTCGTCTCTTGTTG-3′], and introduced within *Eco*RI-*Sal*I sites of pMRS75, giving pMRS78 (encoding GST-$LcrV_{\Delta 2\text{-}32}$ and LcrG) and pMRS83 (encoding GST-$LcrV_{\Delta 224\text{-}266}$ and LcrG), respectively. The *Bam*HI-*Sal*I fragment, containing the *gst-lcrG* hybrid gene, from pMRS50 (encoding GST-LcrG and YopD) was then replaced by the *Bam*HI-*Sal*I fragment of pMRS75 to generate plasmid pMRS84 (encoding GST-LcrV and YopD). Plasmid pCN29 (encoding GST and YopD) is a pGEX derivative containing a PCR-amplified *Sal*I fragment of *yopD* (obtained with MIPA342 [5′-ATGTCGACTCAGACAACACCAAAAGC-3′] and MIPA343 [5′-ATGTCGACAAGAAGGAGATATACATATGAC-3′]) cloned in the *Xho*I site of the vector. Plasmid pCN40 is a pGEX derivative carrying the *yopB* gene, amplified with MIPA461 (5′-ACGGTCGACCAAAGGAGGATCTAG-3′) and MIPA462 (5′-GGATGAGCTCTTAAACAGTATGGGGTC-3′), cloned in the *Sal*I-*Sac*I sites, and the *sycD* gene, amplified with MIPA287 (5′-CTCAAGCTTAGCGGTCATGGGTTATCAA-3′) and MIPA 293 (5′-CGCAAGCTTAAGAAGGAGATATACATATGCAAC-3′), cloned in the *Hin*dIII site. Plasmid pCNG42 contains the same genes as pCN40 but also encodes a GST-LcrV fusion protein. The *lcrV* gene amplified with MIPA364 (5′-CGGAATTCTCATGATTAGAGCCTACG-3′) and MIPA64 (5′-ATGTCGACCTGTCGTCTCTTGTTG-3′) has been cloned in the *Eco*RI-*Sal*I sites. Plasmid pCNG50 contains *gst-lcrV* as in plasmid pCNG42 and the gene *sycD* as in plasmid pCN40.

Construction of vector pCNR26. pCNR21 is a pBC19R derivative containing the *yopE* gene and the first 33 codons of *sycE*. By directed mutagenesis with MIPA395 (5′-TAAATGATGATATTTTCATATGTATTTCCTCCTTTGGCTATTAAAACAAG-3′), the Shine-Dalgarno (SD) sequence of the *yopE* gene was optimized (AAGGAGG) and a *Nde*I site was introduced, yielding plasmid pCNR26. The ATG within the *Nde*I site is localized nine nucleotides downstream from the SD sequence.

Constructions of the complementing clones. A DNA fragment containing genes *lcrG*, *lcrV*, and *sycD* was amplified from pYV40 with MIPA354 (5′-CCAAAACCATATGAAGTCTTCCCA-3′) and MIPA122 (5′-CACAAGCTTGACCGACTCCAAT-3′). This *Nde*I-*Hin*dIII fragment was cloned into the same sites of pCNR26, yielding pMRS72 (replacement of *yopE* by *lcrGVsycD*).

Plasmid pMRS74 was constructed by cloning a PCR-amplified fragment (using MIPA121 [5′-TTTGGATCCTAATGAATTATCTCAC-3′] and MIPA317 [5′-CGGGGTACCTAAAACTTTCGGTTAATTAA-3′]) containing genes *sycD*,

yopB, and *yopD* from pYV40 into the *Bam*HI-*Asp*718 sites of pMSL56, leading to the replacement of *cyaA* by *sycDyopBD*.

RNA extraction and Northern blot analysis. Total RNA of *Y. enterocolitica* was extracted as described by Lambert de Rouvroit et al. (24), 2 h after induction at 37°C. Electrophoresis, transfer, and hybridization with *yop* DNA used as probes were done as described by Cornelis et al. (12). The *yopD* probe was a PCR-amplified fragment of 500 bp obtained with primers MIPA316 (5′-GGAA GATCTCAAATTCTAAACAGTAACA-3′) and MIPA317 (5′-CGGGGTACC TAAAACTTTCGGTTAATTAA-3′).

Production and purification of hybrid GST fusion proteins. The production and purification of GST fusion proteins were performed as described by Smith and Johnson (47). Briefly, overnight cultures were diluted in 10 ml of broth containing 200 μg of ampicillin/ml to an optical density at 600 nm (OD_{600}) of 0.1. The culture was then incubated vigorously at 37°C until an OD_{600} of 0.8 to 1.0 was reached. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a 1 mM final concentration, and the culture was incubated for an additional 3 h at 37°C. A total of 6×10^9 induced bacteria were resuspended and sonicated for 1 min in 1 ml of cold phosphate-buffered saline (PBS). The cell debris were pelleted by a 5-min centrifugation step in a microcentrifuge, and the supernatant was clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. A total of 800 μl of cleared extract was mixed with 20 μl of glutathione-Sepharose (Pharmacia Biotech) equilibrated with PBS. After 1 to 2 h of incubation with gentle agitation at 4°C, glutathione beads were recovered by centrifugation and washed three times with 100 μl of cold PBS. For SDS-PAGE analysis, proteins were eluted from the glutathione beads by being boiled in sample buffer and were loaded on a gel.

RESULTS

LcrV is necessary for the secretion of YopB and YopD. The hypothesis that LcrV is an element of the translocation apparatus implies that LcrV interacts with other Yops. Hence, small in-frame deletions, such as those previously described (37, 46), are likely to give rise to nonfunctional proteins that might interact with other proteins and lead to a biased phenotype. Therefore we engineered a nonpolar null mutation in *lcrV* by deletion of codons 3 to 324, giving strain MRS40(pMRS4071). The resultant *lcrV* mutant secreted more YopE, YopH, YopM, and YopN (Fig. 2, lane 2) than the wild type, but secretion of LcrV, YopB, and YopD was completely abolished. Surprisingly, the secretion defects for YopB and YopD could not be complemented by the introduction of plasmid pMRS28 (Fig. 2, lane 3) containing *lcrRGV*, which suggested that the mutation in *lcrV* could have a polar effect on the downstream *sycDyopByopD* genes. To address this question, we introduced plasmid pMRS72, containing *lcrGVsycD*, or plasmid pPW64, containing only *sycD*, into the *lcrV* mutant strain MRS40 (pMRS4071). Plasmid pMRS72 restored the secretion of LcrV, YopB, and YopD but plasmid pPW64 could not (Fig. 2, lanes 4 and 5). The *sycD* gene in pPW64 was, however, functional because it could complement a previously characterized *sycD* mutation (Fig. 2, lanes 6 and 7). These results indicated that the *lcrV* mutation had an impact on *sycD* but not on *yopB* and *yopD*. They also showed that the lack of secretion of YopB and YopD was due not only to *sycD* deficiency but also to the loss of the *lcrV* gene. In order to understand the unexpected effect of our mutation on *sycD*, we sequenced the allele $lcrV_{\Delta 3\text{-}324}$ in pMRS69. We found that the deletion extended not to codon 324 of *lcrV* but to nucleotide 109 of *sycD* and thus removed the whole *lcrV* gene and a part of *sycD*. This abnormal extension of the deletion could be explained by the fact that we designed our oligonucleotides based on the sequence of *lcrGVHyopBD* from *Y. pestis* and not from *Y. enterocolitica*. Hence, the genotype of pMRS4071 was quoted as *lcrVsycD*.

To confirm that the lack of secretion of YopB and YopD did not result from a polar effect of the *lcrVsycD* mutation on the downstream *yopB* and *yopD* genes, we constructed a plasmid that contains *sycDyopByopD* genes cloned behind the strong *yopE* promoter. We introduced this plasmid, called pMRS74, into the *lcrVsycD* mutant strain MRS40(pMRS4071) and into the *yopByopD* double-mutant strain W22703(pGC153) (13).

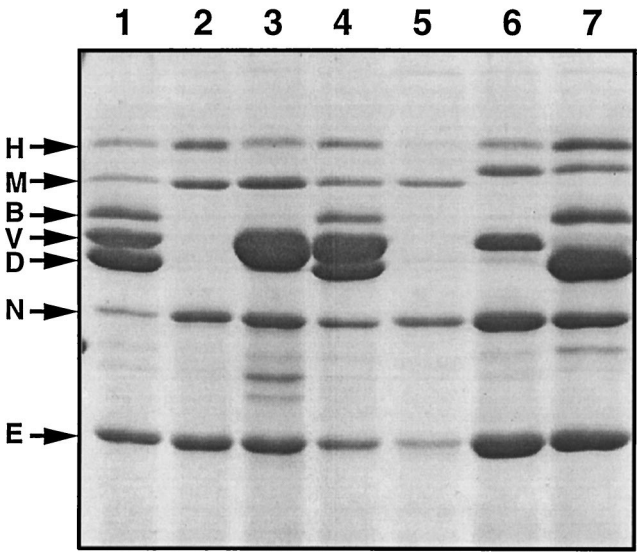

FIG. 2. SDS-PAGE analysis of the Yops (identified at left) secreted by wild-type *Y. enterocolitica* MRS40(pYV40) (lane 1), by MRS40(pMRS4071), an *lcrVsycD* mutant (lane 2), by MRS40(pMRS4071)(pMRS28), an *lcrVsycD* mutant complemented with *lcrRGV* (lane 3; note the overproduction of LcrV), by MRS40(pMRS4071)(pMRS72), an *lcrVsycD* mutant complemented with *lcrGVsycD* (lane 4), by MRS40(pMRS4071)(pPW64), an *lcrVsycD* mutant complemented with *sycD* (lane 5), by KNG22703(pPW2269), a nonpolar *sycD* mutant (lane 6; note that YopD and YopB do not appear in the absence of SycD [53]), and by KNG22703(pPW2269)(pPW64), an *sycD* mutant complemented with *sycD* (lane 7) (Note that YopB and YopD are present. LcrV [V] cannot be distinguished clearly. However, overexpression of SycD does not affect LcrV production and secretion [not shown].) Note that the size of YopM varies between strain E40 and strain W22703.

We observed that YopB and YopD secretion was restored in strain W22703(pGC153) (not shown), but it was not restored in strain MRS40(pMRS4071) (not shown). If we assume that complementing the nonpolar *lcrVsycD* mutation with a multicopy plasmid carrying *sycD* did not deeply modify the system, all these results indicate that LcrV is a factor specifically required for secretion of YopB and YopD.

LcrV is not required for transcription of *yop* genes. Since previous observations suggested that *lcrV* could be a regulator, we analyzed the transcription of *yop* genes in our *lcrVsycD* mutant. We carried out a Northern blot analysis on RNA extracted from the wild-type strain [MRS40(pYV40)], the *lcrVsycD* mutant strain [MRS40(pMRS4071)], and the *lcrVsycD* mutant complemented by *sycD* [MRS40 (pMRS4071)(pPW64)] by using the *yopD* gene as a probe. As seen in Fig. 3, with wild-type bacteria, we observed a major *yopD* transcript of approximately 3,900 nucleotides and a minor transcript of about 1,800 nucleotides (5). The intensities of the transcripts did not differ among the wild type, the *lcrV* mutant, and the *lcrV* mutant complemented by *sycD*, indicating that LcrV is not required for transcription of *yopD*.

LcrV is specifically required for the secretion step. To determine at what level LcrV is required for secretion of YopB and YopD, we monitored the intracellular amounts of YopD and YopE (taken as a control) in wild-type MRS40(pYV40) and in the *lcrVsycD* mutant bacteria complemented by *sycD* [MRS40(pMRS4071)(pPW64)]. After temperature induction of Yop synthesis, total-cell lysates were separated by SDS-PAGE, and YopD and YopE were monitored by immunoblotting with rat monoclonal antibodies. As shown in Fig. 4, equivalent amounts of YopD and YopE could be detected in the total-cell extracts from the *lcrV* mutant MRS40(pMRS4071)

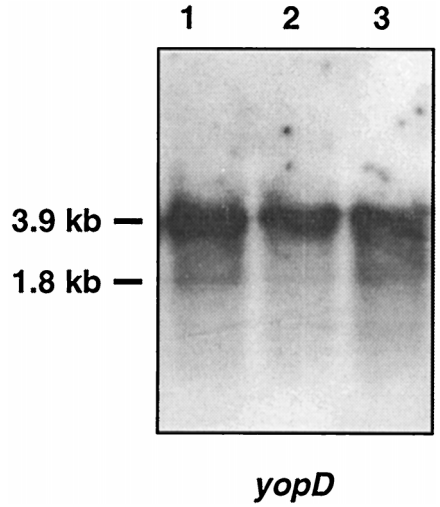

FIG. 3. Northern blot analysis of RNA extracted from wild-type *Y. enterocolitica* MRS40(pYV40) (lane 1), from the *lcrVsycD* mutant MRS40(pMRS4071) (lane 2), and from the *lcrVsycD* mutant complemented by *sycD*, MRS40(pMRS4071)(pPW64) (lane 3). The probe was a PCR-amplified fragment internal to *yopD*.

(pPW64) and wild-type MRS40. From this result we concluded that the main effect of LcrV on YopD is to promote its secretion.

LcrV binds to YopB and YopD. Since LcrV is involved in secretion of YopB and YopD, we tested whether it could interact with these proteins. In this attempt, we took advantage of the GST fusion protein expression and purification system (47). We constructed plasmid pMRS84, which encodes a GST-LcrV hybrid protein and YopD, in order to overproduce the two proteins simultaneously. To serve as a control, we constructed pCN29, which encodes GST and YopD separately. After production in *E. coli* XL1 Blue, GST-LcrV hybrid proteins were purified from the crude extracts with glutathione-

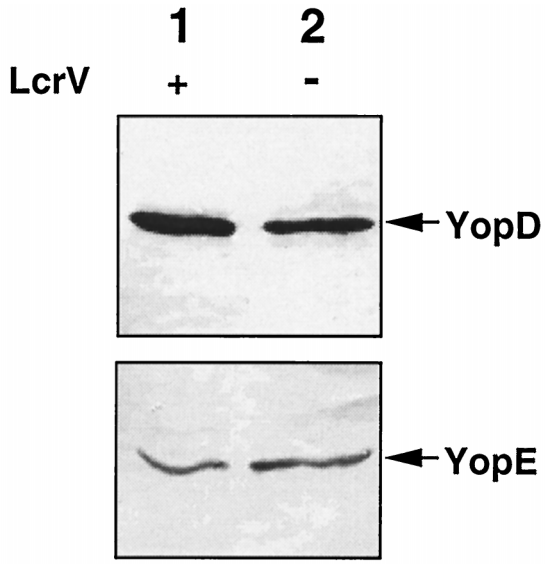

FIG. 4. Western blot analysis of YopD and YopE in total-cell proteins using anti-YopD and anti-YopE monoclonal antibodies. Lane 1, wild-type *Y. enterocolitica* MRS40(pYV40); lane 2, MRS40(pMRS4071)(pPW64), an *lcrVsycD* mutant complemented with the *sycD* gene.

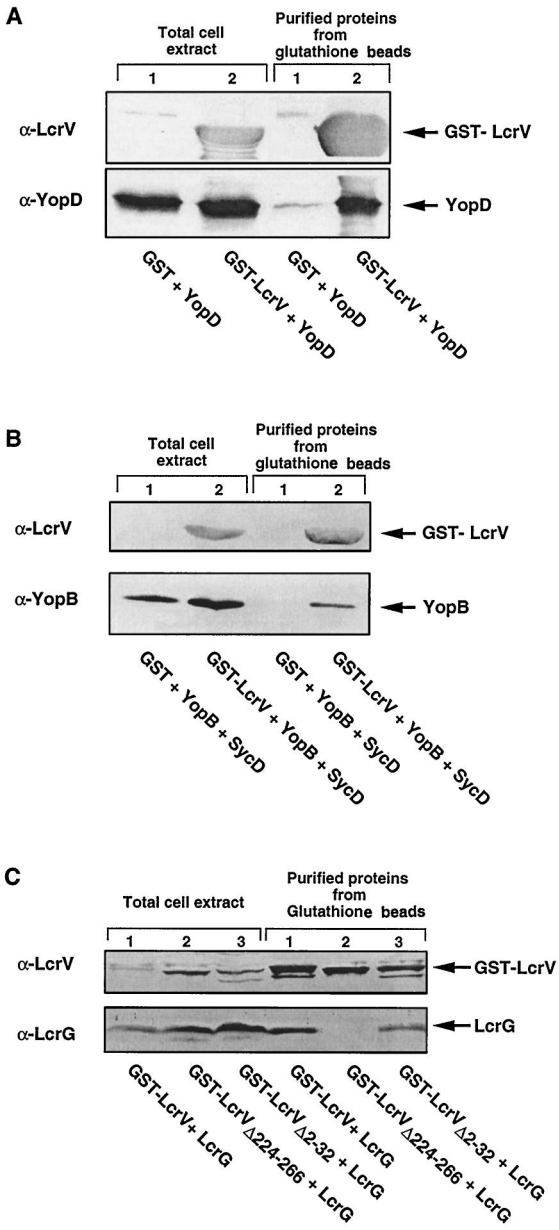

FIG. 5. Binding of YopD, YopB, and LcrG to a GST-LcrV hybrid. (A) Western blot analysis with anti-LcrV and anti-YopD monoclonal antibodies of total-cell extracts and of the soluble extracts purified on glutathione-Sepharose beads from *E. coli* XL1(pCN29) overproducing GST and YopD (lanes 1) and XL1(pMRS84) overproducing GST-LcrV and YopD (lanes 2). (B) Western blot analysis with polyclonal anti-LcrV and monoclonal anti-YopB antibodies of the total-cell extracts and of the soluble extracts purified on glutathione-Sepharose beads from XL1(pCN40) overproducing GST, YopB, and SycD (lanes 1) and XL1(pCNG42) overproducing GST-LcrV, YopB, and SycD (lanes 2). (C) Western blot analysis with anti-LcrV and anti-LcrG polyclonal antibodies of the total-cell extracts and of the soluble extracts purified on glutathione-Sepharose beads from XL1(pMRS75) producing GST-LcrV and LcrG (lanes 1), XL1(pMRS78) producing GST-LcrV$_{\Delta 224\text{-}266}$ and LcrG (lanes 2), and XL1(pMRS83) producing GST-LcrV$_{\Delta 2\text{-}32}$ and LcrG (lanes 3).

Sepharose beads and were analyzed for the recovery of a second protein. As shown in Fig. 5A, when YopD was overproduced together with GST-LcrV, it copurified with GST-LcrV on the glutathione beads. In contrast, when YopD was overproduced together with GST instead of GST-LcrV, no

copurification occurred, which indicated that YopD specifically binds to LcrV. In order to investigate the binding of YopB to GST-LcrV, we then constructed plasmid pCNG42, which encodes the fusion protein GST-LcrV and YopB but also SycD in order to avoid degradation of YopB (30). To serve as a control, we also constructed plasmid pCN40, which encodes GST and YopB separately as well as encoding SycD. After overproduction in *E. coli* XL1 Blue, the soluble extracts were incubated with glutathione-Sepharose beads. After the beads were washed, the purified proteins were analyzed by immunoblotting (Fig. 5B). As shown in Fig. 5B, YopB copurified with GST-LcrV but not with GST alone. To rule out any positive role of SycD in the binding of LcrV to YopB, we tested the capacity of LcrV to bind SycD. We cloned *sycD* downstream of *gst-lcrV*, giving plasmid pCNG50. The GST-LcrV hybrid protein was purified from the crude extract of *E. coli* carrying pCNG50 with glutathione-Sepharose beads and was analyzed for the recovery of SycD by immunoblotting. We could not copurify SycD with GST-LcrV (data not shown), which indicated that SycD does not bind to LcrV, a result that is in good agreement with the observation of Fields et al. (17). From these experiments, we conclude that LcrV binds to YopB and that SycD does not prevent this binding.

LcrV binds to LcrG. Since LcrG is encoded by the same large *lcrGVHyopBD* operon, we tested whether it could also interact with LcrV. To demonstrate such an association, we cloned the *lcrG* gene downstream of *gst-lcrV*, giving plasmid pMRS75. A soluble extract from *E. coli* carrying plasmid pMRS75 was mixed with glutathione-Sepharose beads, and proteins absorbed on the beads were analyzed by Western blotting. As shown in Fig. 5C, LcrG copurified with GST-LcrV. We have previously shown that binding between YopE and SycE and binding between YopH and SycH occur at definite domains of the Yop proteins (57). Therefore, we tested whether the association that we observed between LcrV and LcrG would also involve a specific domain of LcrV. To analyze this, we engineered two in-frame deletion mutations in *lcrV*, namely $lcrV_{\Delta 2\text{-}32}$ and $lcrV_{\Delta 224\text{-}266}$, and we substituted these deletants for *lcrV* in pMRS75, giving plasmids pMRS83 and pMRS78, respectively. Total-cell extracts from *E. coli* carrying either pMRS83 or pMRS78 were mixed with glutathione-Sepharose beads, and proteins absorbed on the beads were analyzed by immunoblotting. As shown in Fig. 5C, LcrG copurified with GST-$LcrV_{\Delta 2\text{-}32}$ but not with GST-$LcrV_{\Delta 224\text{-}266}$, suggesting that there is a unique LcrG-binding site situated in the carboxy-terminal domain of LcrV.

DISCUSSION

In this study, we attempted to construct a complete nonpolar deletion of *lcrV* in the pYV plasmid. Complementation and sequence analysis showed that the deletion also encompassed part of *sycD*. Nevertheless, the mutation did not prevent transcription of the distal *yopB* and *yopD* genes. The mutation abolished secretion of LcrV, YopB, and YopD. When *lcrV* and *sycD* were supplied in *trans*, secretion of YopB and YopD was restored. When *sycD* only was reintroduced in this mutant, secretion of YopB and YopD did not resume but YopD could be detected inside the bacteria, showing that the lack of secretion was not due to a lack of transcription or translation or to proteolysis. These observations strongly suggest that LcrV could be specifically involved in the process of release of YopB and YopD. If this is true, one would expect some interactions between LcrV and YopB or YopD. Using GST-LcrV fusion proteins, we observed that YopB or YopD could indeed be copurified with GST-LcrV. The same approach showed that the region spanning residues 224 to 266 of LcrV also interacts with LcrG. This result confirmed the previous findings of Nilles et al. (32) and suggests that LcrG is another piece of the translocation apparatus. In good agreement with results reported by Fields et al. (17), we did not observe any interaction between GST-LcrV and SycD, indicating that SycD does not act as a chaperone for LcrV as it does for YopD (53). We conclude from all these results that LcrV interacts with YopB and YopD to promote their release from the bacterium. How could this occur? One should point out first that, so far, the secretion domain has been clearly identified only for effector Yops but not for the translocator Yops (see reference 15 for a review). Thus, one cannot exclude the possibility that the Ysc secretion apparatus only recognizes LcrV or a complex involving LcrV, YopB, and YopD rather than YopB and YopD individually. Further work is required to clarify this.

Our results also show that when YopB and YopD are not secreted they do not obstruct the secretion channel. Indeed the *lcrV* mutant does not secrete YopB and YopD but secretes the effectors. This indicates that YopB and YopD without LcrV do not obstruct the secretion channel. In this respect, it would be worthwhile trying to localize YopB and YopD in the bacterium in the presence and in the absence of LcrV.

Finally, does LcrV only play a role in YopB and YopD secretion or does it also play a structural role in the translocation apparatus? We would like to suggest that LcrV is not only required for proper placement (localization) of YopB and YopD but that it also forms some kind of a short pilus below YopB and YopD. However, at this stage, this remains pure speculation.

According to our hypothesis, LcrV is expected to be essential for virulence because it is needed for the deployment of the translocation apparatus. This last conclusion is in perfect agreement with the conclusion drawn by Skrzypek and Straley (46), who showed that LcrV is essential for the virulence of *Y. pestis.*

Finally, our results rule out the idea issued from previous studies that LcrV is a regulator: *yopD* transcription was not affected in a mutant completely lacking LcrV and the YopD protein was clearly present in the extract from these mutant bacteria. Such observations may seem to be contradictory to those of Bergman et al. (5), who observed that a nonpolar deletion of *Y. pseudotuberculosis lcrV*, leaving a truncated gene of 750 bp, is severely downregulated in transcription of the *lcrGVHyopBD* operon and of *yopE*. We think that the discrepancy could be explained by the presence of a truncated LcrV in these previous studies. Indeed, if a truncated LcrV protein associates with YopB and YopD, such a complex might obstruct the secretion channel, which could result in turning on the feedback regulatory mechanism which prevents transcription of *yop* genes when Yops release is compromised (14, 34, 40, 51a). Experiments using *Y. pestis* by Price et al. (37) and by Skrzypek and Straley (46) also led to the conclusion that LcrV plays a regulatory role. However, in *Y. pestis*, this type of analysis is hampered by the fact that the Yops are attacked by the plasminogen activator protease Pla (43, 48) and Yops are detected by immunoblotting. The analysis of the *lcrV* mutants of *Y. pestis* focused on the secretion of YopM rather than YopB and YopD, which may have been misleading. However, in their more recent work showing the interaction between LcrV and LcrG, Nilles et al. (32) suggest that LcrV could function in the control of secretion. Thus, we think that the data presented in this paper can be conciliated with previous observations and promote a reevaluation of the role of LcrV in Yop secretion.

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