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NOTES

LcrG is Required for Efficient Translocation of *Yersinia* Yop Effector Proteins into Eukaryotic Cells

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Extracellular *Yersinia* disables the immune system of its host by injecting effector Yop proteins into host cells. We show that a *Yersinia enterocolitica* nonpolar *lcrG* mutant is severely impaired in the translocation of YopE, YopH, YopM, YpkA/YopO, and YopP into eukaryotic cells. LcrG is thus required for efficient internalization of all the known Yop effectors.

The capacity of *Yersinia* species (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) to resist the immune systems of their hosts depends on the Yop virulon, which is encoded by the 70-kb pYV plasmid. This virulon allows extracellular bacteria adhering to the surfaces of eukaryotic cells to inject bacterial proteins into the cytosol in order to disable these cells (9). Translocation of the intracellular effectors (YopE, YopH, YpkA/YopO, YopM, and YopP) across the eukaryotic cell membrane requires at least two other secreted proteins, namely, YopB and YopD (5, 12–14, 19, 22, 28, 33–35). The Yop proteins are secreted outside the bacterial cell by a contact (type III) secretion apparatus called Ysc (1, 2, 4, 10, 16, 18, 23, 24, 37). The translocators YopB and YopD are encoded by a large operon that also encodes LcrV and LcrG (3, 20, 26). LcrG is a 96-amino-acid (11-kDa) protein that appears to be involved in the control of Yop release (31). In addition, LcrG has been shown to bind LcrV, a protein required for the secretion of YopB and YopD (21, 29). Yop secretion occurs only when bacteria are in contact with eukaryotic cells or deprived of Ca^{2+} ions. A nonpolar *lcrG* mutant of *Y. pestis* is Ca^{2+} blind, secreting large amounts of Yops in the absence as well as in the presence of eukaryotic cells or Ca^{2+} ions, like the *yopN* mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* and the *tyeA* mutant of *Y. enterocolitica* (5, 11, 14, 28, 31). In spite of their deregulated phenotype, *yopN$_{45}$* mutant bacteria (i.e., bacteria in which the *yopN* gene is interrupted after codon 45) can efficiently deliver Yop effectors into the cytosol of eukaryotic cells (5). YopN is thus thought to act at the level of Yop release as the stop valve of the secretion apparatus. TyeA is required for the translocation of a subset of Yop effectors (14). We have recently shown that LcrG can bind to HeLa cells via heparan sulfate proteoglycans and that addition of exogenous heparin can interfere with the translocation of Yops into HeLa cells (7). We inferred that LcrG could have an important role to play in translocation and that interaction with heparan sulfate could affect the activity of LcrG. In this work, we present evidence that LcrG is indeed essential for efficient translocation to occur.

Construction and characterization of an *lcrG* mutant. To investigate the role of LcrG in the secretion of Yops and their subsequent translocation into eukaryotic cells, we constructed an *lcrG* nonpolar mutant. First, we inactivated the chromosomal gene encoding β-lactamase A of *Y. enterocolitica* E40(pYV40) (34) with the mutator plasmid pKNG105 (15) to produce strain MRS40(pYV40). Next, 147 bp (bp 22 to 169) of *lcrG* were deleted from pMRS22 (Table 1) by site-directed mutagenesis (17) with oligonucleotide MIPA310 (5′-AGTCTTCCCATTTTGATAAG CTAGCGGAGCGCGAG-3′), which is identical to nucleotides 5 to 21 and nucleotides 170 to 187 of *lcrG* but which changes Pro_{58} to Leu. The mutated allele of *lcrG*, called *lcrG*$_{\Delta 8–57}$, was verified by sequencing, cloned in a suicide vector, and introduced into MRS40(pYV40) to create strain MRS40(pMRS4043) (Table 1). The *lcrG* mutant strain was tested for Ca^{2+} dependency and in vitro Yop secretion (2, 8). The mutant was unable to grow at 37°C in the presence or absence of Ca^{2+} (data not shown) and as such was defined as growth thermosensitive. The *Y. enterocolitica lcrG* mutant secreted all the Yops in the presence and absence of Ca^{2+} (Fig. 1) and was thus Ca^{2+} blind, as was previously described for *Y. pestis* (31). The translocators YopB and YopD, whose genes are situated downstream of LcrG, are efficiently secreted, demonstrating the nonpolarity of the *lcrG* mutation. Yop secretion was prevented by Ca^{2+} ions after the introduction of plasmid pMSK23, containing *lcrG* alone transcribed from the *yopE* promoter, into MRS40(pMRS4043) (Table 1; Fig. 1). This confirmed the nonpolarity of the *lcrG* mutation.

LcrG is involved in the translocation of the YopE cytotoxin. Wild-type *Y. enterocolitica* induces a cytotoxic response on HeLa cells that is characterized by the rounding up and detachment of the target cells due to the disruption of actin microfilaments (25, 27). After 2 h of infection, the *lcrG* mutant bacteria were unable to induce this cytotoxicity (data not shown). This observation suggested that the *lcrG* mutant was impaired in its ability to internalize YopE, the major cytotoxin, inside HeLa cells. To investigate this further, we introduced plasmid pMS111, encoding $YopE_{130}$-Cya (i.e., a hybrid protein made of 130 residues of YopE fused to Cya), into wild-type *Y. enterocolitica*, a *yscN* secretion mutant, the *lcrG* mutant, a *yopN* mutant, and a *yopB* translocation mutant (Table 1). Cultured PU5-1.8 macrophages were infected

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TABLE 1. Plasmids used in this work

Plasmid	Genotype and/or description	Reference or origin
pAB409	pYV40 *yopH*$_{\Delta 1-352}$ *yopE*$_{21}$ *yopO*$_{\Delta 65-558}$ *yopP*$_{23}$ *yopM*$_{23}$ *yopB*$_{\Delta 89-217}$	6, 19, 30, 36
pABL403	pYV40 *yopH*$_{\Delta 1-352}$ *yopE*$_{21}$ *yopO*$_{\Delta 65-558}$ *yopP*$_{23}$ *yopM*$_{23}$	6, 19, 36
pAB6	pTM100 P$_{yopM}$ *yopM*$_{100}$-*cyaA'*$^{+}$; encodes YopM$_{100}$-Cya	5
pCD10	pTM100 P$_{sycE}$ *yopO*$_{143}$-*cyaA'*$^{+}$; encodes YopO$_{143}$-Cya	14
pIM41	pYV40 *yopN*$_{45}$	5
pMRS20	pBluescriptII SK^{+} + PCR-amplified fragment (using MIPA271 [CCGGAATTCACTTTCATACCAAGAGCTGA] and MIPA64 [ATGTCGACCTGTCGTCTCTTGTTG]) of pYV227 (8) cloned in *Eco*RI and *Sal*I sites; contains *lcrRGV*	This work
pMRS22	pBluescriptII SK^{+} + *Hin*dIII-*Xmn*I fragment of pMRS20; contains *lcrR' lcrG lcrV'*	This work
pMRS42	pMRS22 *lcrG*$_{\Delta 8-57}$; contains *lcrR' lcrG*$_{\Delta 8-57}$ *lcrV'*	This work
pMRS43	pKNG101 (15) + *Xba*I-*Sal*I fragment of pMRS42; encoding *lcrG*$_{\Delta 8-57}$	This work
pMRS99	pYV40 *yopN*$_{45}$ *lcrG*$_{\Delta 8-57}$	5; this work
pMRS4043	pYV40 *lcrG*$_{\Delta 8-57}$	This work
pMS3	pACYC184 + oriT$_{RK2}$ + *yopE sycE*	32
pMS111	pTM100 *sycE*$^{+}$, P$_{yopE}$ *yopE*$_{130}$-*cyaA'*$^{+}$; encodes SycE and YopE$_{130}$-Cya	33
pMSK3	pTM100 P$_{yopE}$ *yopP*$_{99}$-*cyaA'*$^{+}$; encodes YopP$_{99}$-Cya	35
pMSK23	*Xba*I-*Hin*dIII deletion of pMRS72 (29); pBC19R; P$_{yopE}$ *lcrG*	This work
pMSK48	pYV40 *yopH*$_{\Delta 1-352}$ *yopE*$_{21}$ *yopO*$_{\Delta 65-558}$ *yopP*$_{23}$ *yopM*$_{23}$ *lcrG*$_{\Delta 8-57}$	6, 19, 36; this work
pMSL41	pYV40 *yscN*$_{\Delta 169-177}$ (secretion mutant)	34, 37
pMSLH99	pTM100 P$_{yopH}$ *yopH*$_{99}$-*cyaA'*$^{+}$; encodes YopH$_{99}$-Cya	34
pPW401	pYV40 *yopB*$_{\Delta 89-217}$ (translocation mutant)	5, 30

with each of these strains in the presence of cytochalasin D. We monitored both the release of hybrid adenylate cyclase into the culture medium and the accumulation of cyclic AMP (cAMP) inside the eukaryotic cells. In good agreement with the Ca^{2+} blind phenotype, the *lcrG* and *yopN* mutant bacteria secreted much more YopE$_{130}$-Cya into the culture medium than the wild-type strain (5) (Table 2). Hence, the *lcrG* mutant strain was able to efficiently secrete Yops in the presence of eukaryotic cells, but this Yop secretion was deregulated and probably independent of eukaryotic cell contact. Unlike the *yopN* mutant bacteria but like the *yopB* mutant bacteria, the *lcrG* mutant bacteria were unable to induce high levels of cAMP accumulation in the cytosol of PU5-1.8 macrophages (Table 2). LcrG was thus involved in the delivery of YopE into eukaryotic cells. Introduction of *lcrG* on plasmid pMSK23 into the *lcrG* mutant strain resulted in the recovery of the translocation ability of YopE, thus showing that the translocation phenotype was due solely to the defect in the *lcrG* gene (Table 2). To visualize directly the internalization of YopE inside eukaryotic cells, macrophages infected with wild-type and mutant *lcrG* isogenic *Y. enterocolitica* overproducing YopE from plasmid pMS3 (32) were subjected to immunostaining and examined by confocal microscopy. YopE appeared dispersed in the cytosol of macrophages infected with the wild-type bacteria but not in the cytosol of cells infected with the mutant *lcrG* bacteria (Fig. 2). Taken together, these results led us to conclude that LcrG is essential for the efficient translocation of YopE across the eukaryotic cell membrane.

We also tested the secretion and translocation phenotypes of a *Yersinia lcrG yopN* double mutant strain (pMRS99) (Table 1). This strain was Ca^{2+} blind for Yop secretion like the *lcrG* and *yopN* individual mutant strains (data not shown). However, the *lcrG yopN* mutant strain did not significantly translo-

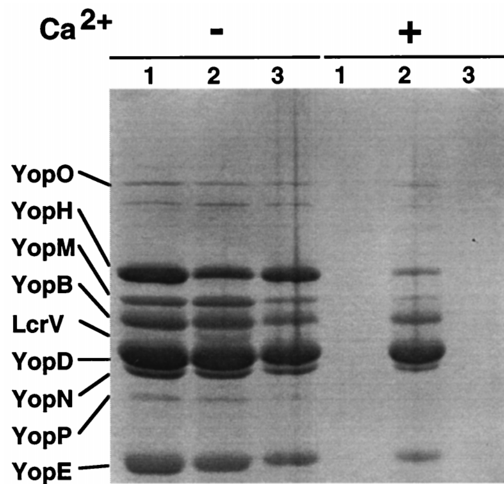

FIG. 1. The *lcrG* mutant strain MRS40(pMRS4043) is Ca^{2+} blind. Yop secretion by the *Y. enterocolitica* wild-type strain MRS40(pYV40) (lane 1), the *lcrG* mutant strain MRS40(pMRS4043) (lane 2), and the complemented strain MRS40 (pMRS4043)(pMSK23) (lane 3) in the absence (−) and in the presence (+) of Ca^{2+} was analyzed. Bacteria were grown in brain heart infusion-oxalate or brain heart infusion-Ca^{2+}, and Yop secretion was induced for 4 h at 37°C. Purified Yops were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue.

TABLE 2. Role of LcrG in the translocation of YopE-Cya, YopH-Cya, and YopM-Cya into PU5-1.8 macrophages

Hybrid protein	Plasmid(s)	Characteristic(s)	cAMP concn[a]	AC activity in RPMI medium[b]
YopE$_{130}$-Cya	pYV40	Wild type	5.7 ± 2.5	12.6 ± 7.5
	pMSL41	*yscN*	0.36 ± 0.14	0.30
	pIM41	*yopN*$_{45}$	9.2 ± 2.5	125
	pPW401	*yopB*$_{\Delta 89-217}$	0.09 ± 0.03	12.3
	pMRS4043	*lcrG*$_{\Delta 8-57}$	0.24 ± 0.21	69.2 ± 15.8
	pMRS99	*yopN*$_{45}$, *lcrG*$_{\Delta 8-57}$	0.09 ± 0.02	ND
	pMRS4043, pMSK23	*lcrG*$_{\Delta 8-57}$, *lcrG*$^{+}$	9.6	ND
	pYV40, pMSK23	Wild type, *lcrG*$^{+}$	8.5	ND
YopH$_{99}$-Cya	pYV40	Wild type	2.0 ± 0.8	2.3
	pPW401	*yopB*$_{\Delta 89-217}$	0.27 ± 0.14	2.8
	pMRS4043	*lcrG*$_{\Delta 8-57}$	0.17 ± 0.06	9.4
YopM$_{100}$-Cya	pYV40	Wild type	1.2 ± 0.7	2.7
	pPW401	*yopB*$_{\Delta 89-217}$	0.03 ± 0.03	7.0
	pMRS4043	*lcrG*$_{\Delta 8-57}$	0.38 ± 0.24	10.6

[a] Data are means of two experiments (in nanomoles per milligram of protein) or three experiments (in nanomoles per milligram of protein ± standard deviation), each carried out in duplicate. Cells were infected with bacteria for 2 h in the presence of cytochalasin D.

[b] Adenylate cyclase (AC) units per milliliter of supernatant from RPMI medium of macrophages. ND, not determined.

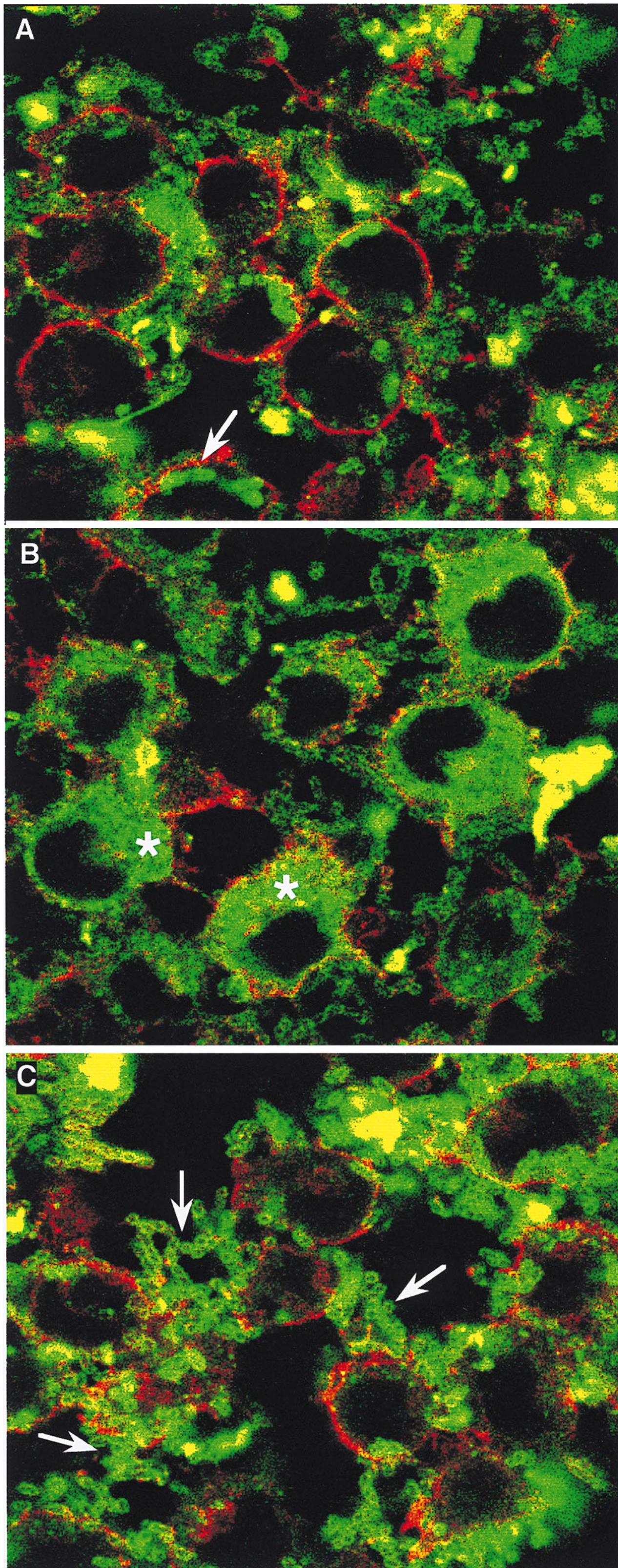

FIG. 2. Delivery of YopE into macrophages. PU5-1.8 macrophages grown on coverslips were infected for 2 h with *Y. enterocolitica* MRS40(pPW401)(pMS3), a *yopB* mutant bacterium overproducing YopE (A); MRS40(pYV40)(pMS3), a wild-type bacterium overproducing YopE (B); or MRS40(pMRS4043)(pMS3), an *lcrG* mutant bacterium also overproducing YopE (C). The asterisk indicates YopE inside the cytosol of the macrophages. The arrows indicate bacteria. After infection, the cells were fixed, incubated with purified anti-YopE antibodies, stained with fluorescein isothiocyanate-labelled anti-rabbit antiserum, and examined by confocal microscopy. The eukaryotic cell membranes were labelled with wheat germ agglutinin-Texas red. Each panel shows a single optical plane at the level of the nucleus. Note that in panel C, bacteria are heavily stained because of deregulated and depolarized Yop secretion.

TABLE 3. Role of LcrG in the translocation of YopO-Cya and YopP-Cya into PU5-1.8 macrophages

Hybrid protein	pYV	Genotype	cAMP concn[a]
$YopE_{130}$-Cya	pABL403	*yopH yopE yopO yopP yopM*	7.8
	pAB409	*yopH yopE yopO yopP yopM yopB*	0.35
	pMSK48	*yopH yopE yopO yopP yopM lcrG*	0.58
$YopH_{99}$-Cya	pABL403	*yopH yopE yopO yopP yopM*	9.5
	pAB409	*yopH yopE yopO yopP yopM yopB*	0.71
	pMSK48	*yopH yopE yopO yopP yopM lcrG*	1.4
$YopM_{100}$-Cya	pABL403	*yopH yopE yopO yopP yopM*	12.8
	pAB409	*yopH yopE yopO yopP yopM yopB*	0.09
	pMSK48	*yopH yopE yopO yopP yopM lcrG*	0.28
$YopO_{143}$-Cya	pABL403	*yopH yopE yopO yopP yopM*	10.7
	pAB409	*yopH yopE yopO yopP yopM yopB*	0.03
	pMSK48	*yopH yopE yopO yopP yopM lcrG*	0.25
$YopP_{99}$-Cya	pABL403	*yopH yopE yopO yopP yopM*	1.4
	pAB409	*yopH yopE yopO yopP yopM yopB*	0.08
	pMSK48	*yopH yopE yopO yopP yopM lcrG*	0.10

[a] Data are means of two experiments (in nanomoles per milligram of protein) carried out in duplicate. Cells were infected for 2 h in the presence of cytochalasin D.

cate $YopE_{130}$-Cya into macrophages (Table 2). Thus, the function of LcrG is not solely to control the opening of the Yop secretion pore by YopN to allow Yop release and subsequent translocation. Rather, LcrG is itself independently required for optimal translocation of YopE.

LcrG is involved in the internalization of YopH, YopM, YopO, and YopP. We then investigated whether translocation of $YopH_{99}$-Cya and $YopM_{100}$-Cya was also dependent on LcrG (Table 1). Although the *lcrG* mutant bacteria secreted more $YopH_{99}$-Cya and $YopM_{100}$-Cya into the culture medium than the wild-type bacteria, they did not induce significant accumulation of cAMP in infected macrophages (Table 2). Thus, the efficient internalization of YopH and YopM was also dependent on the presence of LcrG.

We also wanted to look at the translocation of $YopO_{143}$-Cya and $YopP_{99}$-Cya into eukaryotic cells (Table 3). Because these Yops are not translocated as efficiently as YopE, YopH, and YopM, this must be studied in a *Y. enterocolitica* strain lacking the Yop effectors YopE, YopH, YopO, YopP, and YopM (12, 14, 35). Due to the lack of competition for the secretion and translocation apparatuses, the translocation of the Yop-Cya hybrid is optimized. We thus introduced the $lcrG_{\Delta 8-57}$ allele into the Yop effector polymutant strain MRS40(pABL403) (Table 1). As can be seen in Table 3, the translocation of $YopO_{143}$-Cya, $YopP_{99}$-Cya, and the other Yop-Cya hybrid proteins was greatly reduced in the *lcrG* mutant strain compared to that in the parental strain. The level of translocation of each of the hybrid Cya proteins by the polymutant *lcrG* was almost similar to that of the polymutant *yopB* strain. Thus, LcrG is involved in the translocation of all the known effector Yops.

Conclusions. The phenotype of the newly constructed *Y. enterocolitica lcrG* mutant is unique. Not only is it Ca^{2+} blind like the *yopN* and *tyeA* mutants (5, 11, 14, 28, 31), but it is also a

weak Yop translocator like the *yopB* and *yopD* mutants (5, 22, 28). This phenotype clearly shows that LcrG is involved in translocation of all the Yop effectors. This is in contrast to the *yopN* mutant, which translocates all the Yops efficiently, and the *tyeA* mutant, which is required for the translocation of only a subset of Yop effectors, namely, YopE and YopH (5, 18).

There are several possibilities regarding the role of LcrG in translocation. LcrG could be an essential element of the translocation machinery along with YopB and YopD. It is also possible that LcrG is an element regulating the deployment of the translocation apparatus or the action of the translocation process itself. We have recently shown that LcrG can bind to HeLa cells via heparan sulfate proteoglycans and that heparin can interfere with the translocation of Yops inside HeLa cells (7). Thus, LcrG could be a Yop apparatus ligand whose interaction with heparan sulfate proteoglycans augments its function in the translocation of Yops into eukaryotic cells. We plan to investigate these possibilities in greater detail in our future work.

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