Comparative Analysis of Type III Effector Translocation by *Yersinia pseudotuberculosis* Expressing Native LcrV or PcrV from *Pseudomonas aeruginosa*

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The homologues LcrV of *Yersinia* species and PcrV of *Pseudomonas aeruginosa* are pore-forming components. When expressed in a *Yersinia lcrV* background, PcrV formed smaller pores in infected erythrocyte membranes, correlating to a lowered translocation of *Yersinia* effectors. To understand this phenomenon, cytotoxins exoenzyme S of *P. aeruginosa* and YopE of *Yersinia* were introduced into a *Yersinia* background without Yop effectors but expressing LcrV or PcrV. Comparable translocation of each substrate indicated that substrate recognition by LcrV/PcrV is not a regulator of translocation. *Yersinia* harboring *pcrV* coexpressed with its native operon efficiently translocated effectors into HeLa cell monolayers and formed large LcrV-like pores in erythrocyte membranes. Thus, a PcrV complex with native *P. aeruginosa* translocon components is required to form fully functional pores for complete complementation of effector translocation in *Yersinia*.

A number of gram-negative animal- and plant-interacting bacteria use a common type III secretion system (TTSS) to secrete proteins across the bacterial envelope and to translocate antihost virulence effectors into host cells [1, 2]. Translocation of antihost effectors into target cells is facilitated by a collection of translocon components [1, 2]. Presumably, this apparatus is a multiprotein complex that forms a channel in the eukaryotic membrane through which effector proteins pass into the cytosol of the infected cell. In pathogenic *Yersinia* species, channel formation is implied by the organism's ability to generate contact-dependent, bacteria-induced lytic activity toward erythrocytes and macrophages [3–5]. The fact that *Yersinia*-induced erythrocyte lysis depends on *Yersinia* outer protein (Yop) B, YopD, and LcrV corroborates their essential role in Yop effector translocation [6]. Both YopB and YopD could also be secreted directly into liposomes, and, when these were fused with planar lipid bilayers, current fluxes that indicated transmembrane channel formation were induced [7]. However, this was contradicted recently by the lack of current fluctuations after the addition of purified YopB or YopD to lipid bilayers [5]. In fact, this latter assay was used to highlight LcrV as the core component of a translocation channel that might be stabilized by an interaction with membrane-associated YopB and YopD [5]. Reflecting this likely association, LcrV, YopB, and YopD are all encoded within the *lcrGVHyopBD* operon (figure 1) [8], and antibodies directed against certain epitopes of LcrV can provide passive protection against *Yersinia* infections by blocking the translocation of Yop effector proteins [9–11].

Of interest, the opportunistic pathogen *Pseudomonas aeruginosa* encodes a homologous operon, denoted *pcrGVHpopBD* (figure 1), which suggests that this pathogen uses a similar strategy for effector translocation [12]. Indeed, *P. aeruginosa*–mediated lysis of erythrocytes depends on PopB, PopD, and PcrV [13], which,

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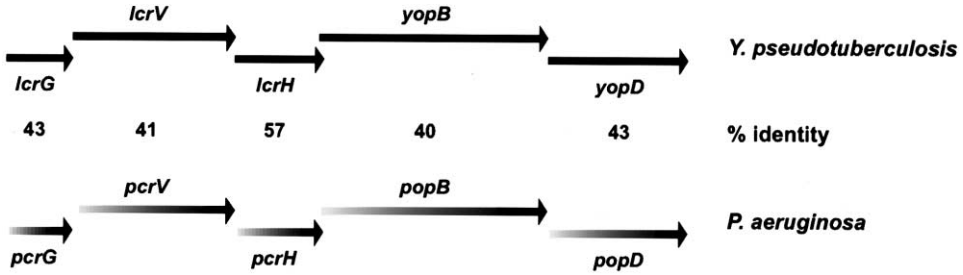

Figure 1. Schematic representation of the translocase operons of pathogenic *Yersinia* species *(lcrGVHyopBD)* and *Pseudomonas aeruginosa (pcrGVHpopBD).* The amino acid identities shared by each corresponding product are indicated [12].

like their *Yersinia* counterparts—YopB, YopD, and LcrV, respectively—are essential for the translocation of antihost effector proteins into target cells [14, 15]. Like LcrV, purified PcrV induces pore formation when it is applied to planar lipid bilayers [5], and polyclonal antibodies directed against PcrV perturb the translocation of type III toxins during *P. aeruginosa* infection [14, 16]. Significantly, components of the *Yersinia* and *P. aeruginosa* translocon operons are functionally interchangeable [10, 17, 18].

Although PcrV can functionally complement an *lcrV* null mutant of *Y. pseudotuberculosis* with respect to Yop synthesis and secretion [10], the *Yersinia* effectors YopE and YopH were poorly translocated [5]. This phenomenon correlated with PcrV, inducing smaller pores in the membranes of infected erythrocytes and causing smaller stepwise conductivity changes when purified protein was applied to planar lipid bilayers [5]. However, other possibilities governing the translocation efficiencies could not be excluded. It is possible that PcrV from *P. aeruginosa* might be less efficient at recognizing and translocating the *Yersinia* substrates (Yops), compared with its ability to recognize and translocate native *P. aeruginosa* substrates (exoenzymes). To investigate the contribution of substrate recognition in LcrV- or PcrV-dependent translocation, we measured the extent of the cytotoxins exoenzyme S (ExoS) of *P. aeruginosa* or YopE of *Yersinia,* after they had been translocated inside HeLa cell monolayers by a specially engineered *Y. pseudotuberculosis* strain devoid of Yop effectors but expressing either LcrV or PcrV. In addition, we considered that fully functional PcrV produced from *Yersinia* might require coproduction with native *P. aeruginosa* translocon components. To this end, we extensively characterized a full-operon deletion of *lcrGVHyopBD* in *Y. pseudotuberculosis* complemented with the corresponding *pcrGVHpopBD* operon of *P. aeruginosa.* Our results ruled out a role for LcrV/PcrV-mediated substrate recognition during translocation by *Yersinia* and also highlighted the need for species-specific LcrV- or PcrV-protein interactions for the optimal function of this virulence strategy during *Yersinia* or *Pseudomonas* infections.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in table 1. *Escherichia coli* strains were grown in Luria Bertini broth (LB) or on Luria agar plates. Unless otherwise stated, *Yersinia* strains were routinely grown in LB. For growth on solid media, *Yersinia*-selective agar base (Difco) or blood agar base (Merck) was used. Where appropriate, the antibiotics ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), and gentamicin (Gm; 20 μg/mL) were used.

DNA methods. Isolation of plasmid DNA from *E. coli* strains was done using the High Purification Plasmid Miniprep kit (Roche Applied Science). Standard DNA manipulation techniques, including the use of restriction enzymes, in vitro DNA cloning, and transformations were done essentially as described elsewhere [26]. Genelute minus EtBr spin columns (Sigma) were used to recover DNA fragments from agarose. DNA sequencing reactions were done using the DYEnamic ET terminator cycle-sequencing kit (Amersham Biosciences), and products were analyzed with an ABI Prism 377 DNA sequencer (Amersham Pharmacia Biotech).

Construction of plasmids and in-frame deletion mutants. Generation of the mutagenesis vector pAH70, which was used to construct the in-frame Δ*lcrV* mutation in the parental strains YPIII/pIB29ME and YPIII/pIB29MEK, has been described elsewhere [10]. The mutagenesis vector used to construct the in-frame Δ*yopB* mutation in the same parental strains was developed as follows: polymerase chain reaction (PCR)–amplified DNA fragments were generated by overlap PCR [27], using wild-type *Yersinia* YPIII/pIB102 as the template and PCR primers A, 5′-AAG TGA CAC TAG TGA GCA ACT CTA CTC T-3′ (*Spe*I); B, 5′-TGG CGT TGA GCG GTC ATG G-3′; C, 5′-GAC CGC TCA ACG CCA ACT GTT TAA GTT TAA GGA GGA A-3′; and D, 5′-TCA TCA GTC TAG AAC CGC TGA CCA TCT C-3′ (*Xba*I). The resulting 780-bp fragment contained sequences flanking the *yopB* gene, with an in-frame deletion of codons 13–399. This fragment was cloned into *Spe*I-*Xba*I–digested pDM4 [22], which gave rise to pMF463.

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Phenotype/genotype	Source/reference
Strains		
Yersinia pseudotuberculosis		
YPIII/pIB102	*yadA*::Tn*5,* Km^R (wild type)	[19]
YPIII/pIB29ME	pIB102, *yopH, yopM, yopE,* Km^R	[20]
YPIII/pIB29MEV	pIB29ME, *lcrV* in-frame full-length deletion of codons 10–313, Km^R	Present study
YPIII/pIB29MEB	pIB29ME, *yopB* in-frame full-length deletion of codons 13–399, Km^R	Present study
YPIII/pIB29MEK	pIB29ME, *yopK* in-frame full-length deletion of codons 1–182, Km^R	[20]
YPIII/pIB29MEKV	pIB29MEK, *lcrV* in-frame full-length deletion of codons 10–313, Km^R	Present study
YPIII/pIB29MEKB	pIB29MEK, *yopB* in-frame full-length deletion of codons 13–399, Km^R	Present study
YPIII/pIB799	pIB102, in-frame full-length deletion of entire *lcrGVHyopBD* operon, Km^R	Present study
YPIII/pIB799-29MEK	pIB29MEK, in-frame full-length deletion of entire *lcrGVHyopBD* operon, Km^R	Present study
Escherichia coli		
ΔH5α	ϕ80d*lacZ*Δ*M*15, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_K^-, m_K^+), *supE44, relA1, deoR,* Δ(*lacZYA-argF*)U169	Stratagene
S17-1λ*pir*	*recA, thi, pro, hsdR*$^-$*M*+,<RP4:2-Tc:Mu:Km:Tn*7*>Tp^R, Sm^R	[21]
Plasmids		
pDM4	Suicide plasmid carrying *sacBR,* Cm^R	[22]
pAH70	582 bp *Xba*I-*Sac*I PCR fragment of Δ$lcrV_{10–313}$ in pDM4, Cm^R	[10]
pMF463	780 bp *Spe*I-*Xba*I PCR fragment of Δ$yopB_{13–399}$ in pDM4, Cm^R	Present study
pJEB35	1115 bp *Xho*I-*Xba*I PCR fragment of Δ*lcrGVHyopBD* in pDM4, Cm^R	Present study
pTS103	*orf1* and *exoS* cloned into pUC19, Amp^R	[23]
pTS103-Gm	Derivative of pTS103 with a Gm cassette introduced into the Amp resistance gene, Gm^R, Amp^S	Present study
pAF19	*Bam*HI -*Sph*I fragment encoding *yerA* and *yopE* cloned into pUC19, Amp^R	[24]
pAF19-Gm	Derivative of pAF19 with a Gm cassette introduced into the Amp resistance gene, Gm^R, Amp^S	Present study
pTB7	*lcrV* cloned under control of the *tac* promoter of pMMB66HE, Amp^R	[8]
pLJ33	*pcrV* cloned under control of the *tac* promoter of pMMB66EH, Amp^R	[10]
p34S-Gm	Cloning vector carrying a Gm resistance cassette, Gm^R	[25]
pMS9	*pcrGVHpopBD* cloned into pUC19, Amp^R	[17]

NOTE. Amp, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; PCR, polymerase chain reaction; R, resistant; S, sensitive.

Construction of the mutagenesis vector pJEB35, which was used to create the in-frame Δ*lcrGVHyopBD* operon null-mutant YPIII/pIB799 and the Δ*yopHMEKlcrGVHyopBD* mutant YPIII/pIB799-29MEK, was similarly developed using the primer combination A, 5′-GCA TGT CTC GAG GCT TAC CGA ACA TGG CTT GG-3′ (*Xho*I); B, 5′-ATG GGA AGA TTT CAT AAT TAC CT-3′; C, 5′-TTA TGA AAT CTT CCC AT GGT GTT GTC TGA CCA-3′; and D, 5′-AAT ACC TCT AGA ATC GTA TCG AGT C-3′ (*Xba*I). The resulting 1115-bp fragment contained sequence flanking upstream of *lcrG* and downstream of *yopD* with an in-frame deletion of *lcrGVHyopBD*. This fragment was cloned into *Xho*I-*Xba*I–digested pDM4 generating pJEB35. All mutagenesis vectors were introduced into *E. coli* S17-1λ*pir* for conjugal mating experiments with *Y. pseudotuberculosis*. Selection for appropriate allelic exchange events to generate the deletion mutants has been described elsewhere [22]. All engineered mutant strains were verified by PCR, sequencing, and Western blot analysis.

To facilitate the maintenance of the *orf1*$^+$/*exoS*$^+$ (pTS103) and *yerA*$^+$/*yopE*$^+$ (pAF19) plasmids in *Yersinia*, a gentamicin resistance marker was introduced in the following way. After digestion of p34S-Gm [25] with *Ecl*136II/*Sst*I (MBI Fermentas), a DNA fragment corresponding to the intact gentamicin cassette was isolated and subsequently cloned into the *Sca*I site of the pUC19 derivates, generating pTS103-Gm and pAF19-Gm, respectively.

Magnesium oxalate (MOX) test. The plating frequencies of the different mutant strains grown under high- and low-Ca^{2+} conditions at 37°C were determined as described elsewhere [8].

Analysis of Yop synthesis. Induction of Yop synthesis and secretion by *Y. pseudotuberculosis* was done using the method of Bergman et al. [8], with the exception that, to induce ex-

pression from alleles under the control of the P_{tac} promoter, 0.4 mmol isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) was added at the time of temperature up-shift. Total and secreted protein samples were collected as described elsewhere [28] and analyzed using Western blotting coupled to detection with the enhanced chemiluminescence (ECL) system, as directed by the manufacturer (Amersham).

Cultivation of HeLa cells and the cytotoxicity assay. The human epithelial cell line HeLa was used in all in vitro infection experiments. Culture maintenance and infections with *Yersinia* essentially followed the guidelines of Sundin et al. [29], except that IPTG (0.4 mmol) was added to both bacteria and cell monolayers prior to infection. The cytotoxicity of infected HeLa cells was monitored by light microscopy, and images were collected at successive time points.

Ras modification assay. *Y. pseudotuberculosis* strains expressing ExoS from pTS103-Gm were grown as described in the cytotoxicity assay and tested for their ability to induce the ExoS-mediated modification of eukaryotic Ras in infected HeLa cells, using the method of Sundin et al. [15]. Modification of Ras was visualized by ECL Western blot using an anti-Ras monoclonal antibody (Transduction Laboratories).

Contact hemolysis of sheep erythrocytes. *Y. pseudotuberculosis* strains and sheep erythrocytes (SVA) were treated as described elsewhere [5], with the exception that IPTG (0.4 mmol) was present throughout the infection. To initiate the hemolysis assay in a round-bottomed 96-well tissue culture plate (Sarstedt), 100 μL of the erythrocyte suspension was mixed with 100 μL of bacterial culture adjusted to an OD_{600} of 0.9, followed by centrifugation at 411 *g* for 5 min. After a 3-h incubation at 37°C, the pellets were carefully resuspended prior to centrifugation at 411 *g* for 20 min, and then 100 μL of each supernatant was transferred to a flat-bottomed 96-well tissue culture plate (Sarstedt). The release of hemoglobin was measured at λ_{540} nm using an iEMS reader MF (Labsystems) and expressed as lytic activity $\times 10^{-2}$. Contact hemolytic assays in the presence of carbohydrates was done using 30 mmol of raffinose, dextrin 15, or dextran 4 [30, 31].

RESULTS

Construction and characterization of a* Yersinia *multiple Yop effector mutant devoid of LcrV or YopB. We were interested in whether substrate specificity could account for a PcrV-mediated delay in translocation by *Yersinia* [5]. In this scenario, PcrV would be more efficient in translocating *P. aeruginosa* substrates (exoenzymes) than *Yersinia*-derived substrates (Yops), and vice versa. We engineered a full-length deletion of codons 10–313 of LcrV in *Y. pseudotuberculosis* that was defective for several translocated Yop effectors (YopH, YopM, and YopE; YPIII/pIB29ME) [20]. This ensured a clean system for our translocation studies. Loss of LcrV production and secretion in YPIII/pIB29MEV was confirmed by the analysis of protein content in total and cleared supernatant fractions by Western blot analysis that used anti-LcrV antiserum (data not shown). Not surprisingly, the *lcrV* mutant was also typically down-regulated for the synthesis and secretion of other Yops (data not shown) [6]. Functionally complemented strains of the *lcrV* null mutant were established by introducing plasmids harboring genes encoding either LcrV (pTB7) or PcrV (pLJ33) under control of the IPTG-inducible P_{tac} promoter, generating YPIII/pIB29MEV, pTB7 and YPIII/pIB29MEV, pLJ33, respectively.

Because YopB is also an essential component of the translocase apparatus [6], for control purposes we engineered a full-length deletion of codons 13–399 of YopB in *Y. pseudotuberculosis* YPIII/pIB29ME. This $\Delta YopB_{13-399}$ strain was termed YPIII/pIB29MEB. Significantly, the *yopB* null mutant lacked YopB production but was comparable to parental bacteria with respect to the production and secretion of other Yops (data not shown). This phenotype is consistent with YopB not being involved in maintaining *yop* regulatory control [3, 18, 32].

LcrV and PcrV form different-sized pores in erythrocyte membranes. Pathogenic *Yersinia* species induce channel formation in target cell membranes, which were conveniently visualized by contact-dependent, bacteria-induced lytic activity toward erythrocytes and macrophages [3–5, 33]. Because this activity is dependent on the translocon components LcrV, YopB, and YopD, these proteins are assumed to form a pore in the target cell membrane through which the Yop effector proteins are delivered into the eukaryotic cell. We used an osmoprotectant assay [3, 5, 33] to measure the size of LcrV and PcrV pores formed after infection with *Y. pseudotuberculosis*. Suitably sized osmoprotectants will block a pore to prevent osmotic lysis of the erythrocytes, which can be quantified by measuring the release of hemoglobin at OD_{540} [5, 33]. Because wild-type *Y. pseudotuberculosis* normally induces low lytic activity on erythrocytes, the assay sensitivity was increased by incorporating a *yopK* deletion into our bacterial strains. Of importance, a loss of YopK is known to increase lytic levels without altering the pattern of Yop synthesis (data not shown) [5, 33]. Therefore, the resulting parental strains YPIII/pIB29MEK, YPIII/pIB29MEKV, and YPIII/pIB29MEKB were used in our osmoprotection assay. After the infection of sheep erythrocytes, a pronounced lytic activity was induced by strains expressing LcrV in cis (figure 2*A,* YPIII/pIB29MEK) and in trans (figure 2*B;* YPIII/pIB29MEKV, pTB7). Moreover, only the largest carbohydrate, dextran 4 (3–3.5 nm diameter [ϕ]), could significantly inhibit this activity ($P < .05$, 58% reduction). Neither raffinose (1.2–1.4 nm ϕ) nor dextrin 15 (2.2 nm ϕ) significantly blocked the lysis through LcrV-mediated pores. In contrast, the lytic activity associated with *Yersinia* expressing PcrV in trans (figure 2*C;* YPIII/pIB29MEKV, pLJ33) was dramatically reduced, compared

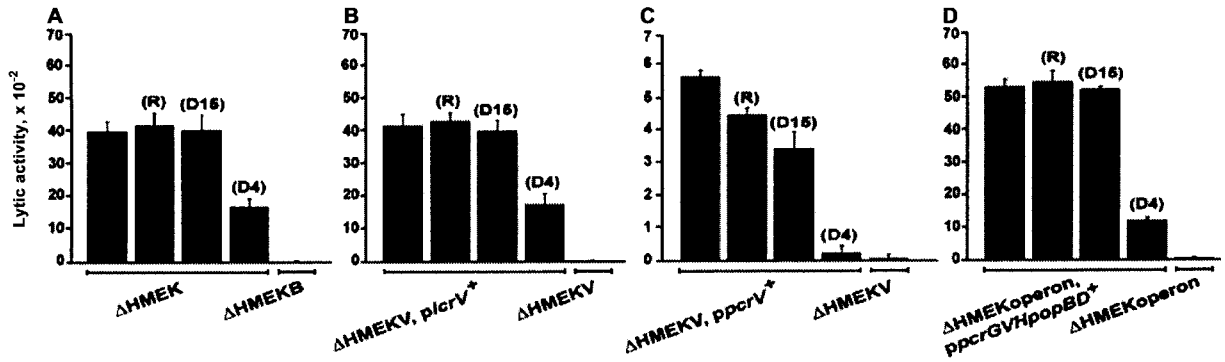

Figure 2. Lytic activity on erythrocytes by *Yersinia pseudotuberculosis* expressing endogenous LcrV (YPIII/pIB29MEK) *(A);* LcrV in trans (YPIII/pIB29MEKV, pTB7) *(B);* PcrV in trans (YPIII/pIB29MEKV, pLJ33) *(C);* or PcrG, PcrV, PcrH, PopB, and PopD in trans (YPIII/pIB799-29MEK, pMS9) *(D).* Sheep erythrocytes were infected with the different strains of *Y. pseudotuberculosis* in the absence or presence of carbohydrates of different diameters (ϕ). The amount of released hemoglobin was quantified spectrophotometrically and expressed as lytic activity $\times 10^{-2}$. Note the different scale on the *Y*-axis in panel *C.* All experiments were done at least 5 times, with quadruplicate data sets. Each bar represents the mean of all values, and the error bar indicates the SD. *C,* Each stepwise reduction in hemoglobin release observed in the presence of a given carbohydrate is statistically significant ($P < .05$, Student's 2-tailed *t* test with unequal variance). R, raffinose (1.2–1.4 nm ϕ); D15, dextrin 15 (2.2 nm ϕ); D4, dextran 4 (3–3.5 nm ϕ).

with that of an isogenic strain expressing LcrV (~7-fold reduction). Furthermore, the smaller carbohydrates raffinose and dextrin 15 significantly ($P < .05$) blocked the PcrV-mediated pores by 21% and 39%, respectively. In the presence of dextran 4, almost complete blockage (96% inhibition) was obtained. These results are consistent with the idea that PcrV formed smaller pores. Analogous with their requirement for effector translocation, the YopB-defective strain YPIII/pIB29MEKB (figure 2*A*) and the LcrV-defective strain YPIII/pIB29MEKV (figure 2*B* and 2*C*) did not induce erythrocyte lysis.

***Neither LcrV nor PcrV exhibits a preference for YopE or ExoS translocation by* Y. pseudotuberculosis.** Having established that the parental strain YPIII/pIB29MEV harboring either LcrV (pTB7) or PcrV (pLJ33) in trans did induce pores of different sizes, we wanted to determine whether LcrV and PcrV exhibited a preference for native substrates during the translocation process. If this were true, it would offer an alternative explanation as to why PcrV poorly translocated the *Yersinia* effectors YopE and YopH [5]. We selected the ExoS effector of *P. aeruginosa* and YopE of *Yersinia,* because ExoS is efficiently secreted by the TTSS of *Y. pseudotuberculosis* and shares similar biological features to YopE [23]. *yopE* and *exoS,* cloned on the high-copy plasmid pUC19 to give pAF19-Gm (*yerA*$^+$/*yopE*$^+$) or pTS103-Gm (*orf1*$^+$/*exoS*$^+$), respectively, were introduced into YPIII/pIB29MEV, pTB7 and YPIII/pIB29MEV, pLJ33. We used immunoblotting techniques to confirm YopE and ExoS synthesis and secretion in *Yersinia* strains grown in Yop-inducing media (data not shown).

To evaluate how efficient YPIII/pIB29MEV, pTB7 and YPIII/pIB29MEV, pLJ33 were at translocating the YopE and ExoS cytotoxins, we utilized the fact that these translocated substrates both induce a characteristic rounding up of the infected target cells [23, 34]. This is due to actin cytoskeleton disruption, which is caused by their GTPase-activating protein (GAP) activity for the Rho family of GTP-binding proteins [35]. HeLa cell monolayers were infected with YPIII/pIB29MEV, pTB7 or YPIII/pIB29MEV, pLJ33, synthesizing either YopE (pAF19-Gm) or ExoS (pTS103-Gm) in trans. Phase-contrast light microscopy was used to monitor the cytotoxic response induced by translocated ExoS (figure 3, *top*) or YopE (figure 3, *bottom*) at different time points after infection. Already at 2 h after infection, *Y. pseudotuberculosis* producing endogenous LcrV or LcrV in trans in combination with either ExoS (figure 3*A* and 3*D*) or YopE (figure 3*F* and 3*I*) induced a complete cytotoxic response on infected HeLa cells. At the same time, however, the morphology of HeLa cell monolayers infected with *Y. pseudotuberculosis* producing PcrV more closely resembled a mock infection (figure 3*B* and 3*C* vs. 3*E* and 3*G* and 3*H* vs. 3*J*). Moreover, full cytotoxicity was markedly delayed, being only observed at 4 h after infection. Of importance, however, we could not detect any difference between the rate of YopE- or ExoS-dependent cytotoxicity induced by either the LcrV- or PcrV-producing strain. This suggests that LcrV and PcrV do not display specificity toward native substrates during the translocation process. As we had expected, strains lacking the essential translocon components YopB [3, 32] and LcrV [10, 36] were unable to translocate either ExoS (figure 3*B* and 3*C*) or YopE (figure 3*G* and 3*H*), even after extended infection periods and the use of higher multiplicities of infection (data not shown).

Efficient in vivo modification of Ras by ExoS translocated from* Y. pseudotuberculosis *producing LcrV. Because the lack of substrate specificity displayed by both LcrV and PcrV is an important finding, we clarified this using an independent second enzymatic activity of ExoS involving ADP ribosylation

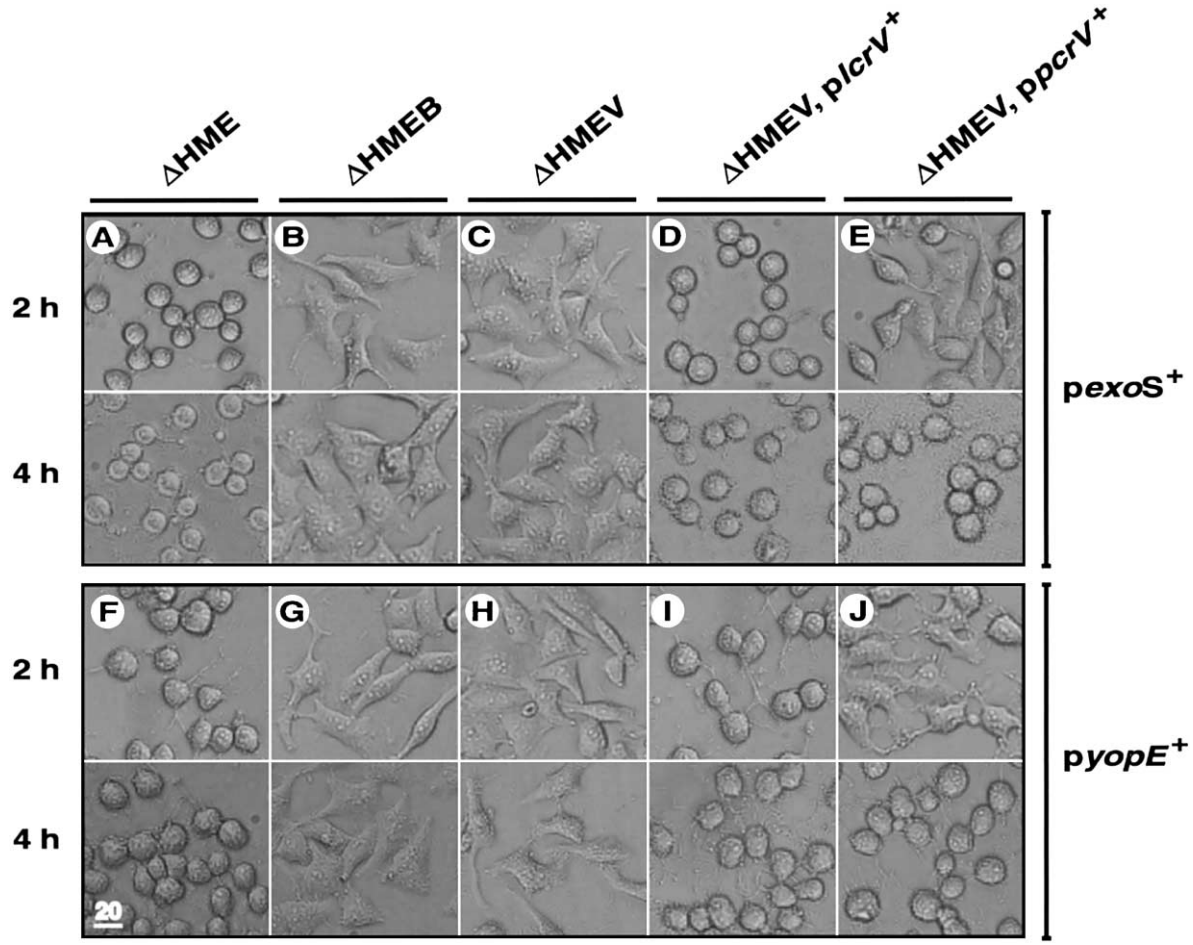

Figure 3. Infection of HeLa cells by different strains of *Yersinia pseudotuberculosis*. At the indicated time points, the effect of the bacteria on the HeLa cells was recorded by phase-contrast microscopy. *Top,* Strains expressing the *Pseudomonas aeruginosa*–derived cytotoxin exoenzyme S (ExoS) in trans from plasmid pTS103–gentamicin (Gm). *Bottom,* Strains expressing the *Yersinia* outer protein (Yop) E cytotoxin in trans from pAF19-Gm. Note the extensive rounding up of the ExoS- and YopE-dependent, cytotoxically affected HeLa cells. Shown are phase-contrast images of YPIII/pIB29ME, pTS103-Gm *(A);* YPIII/pIB29MEB, pTS103-Gm *(B);* YPIII/pIB29MEV, pTS103-Gm *(C);* YPIII/pIB29MEV, pTB7, pTS103-Gm *(D);* YPIII/pIB29MEV, pLJ33, pTS103-Gm *(E);* YPIII/pIB29ME, pAF19-Gm *(F);* YPIII/pIB29MEB, pAF19-Gm *(G);* YPIII/pIB29MEV, pAF19-Gm *(H);* YPIII/pIB29MEV, pTB7, pAF19-Gm *(I);* and YPIII/pIB29MEV, pLJ33, pAF19-Gm *(J).* Scale bar, 20 μm.

toward members of the Ras superfamily of GTPases [37]. Because this activity relies on the eukaryotic 14-3-3 protein family, ExoS-dependent ADP ribosylation of Ras provides a sensitive method for assaying the direct translocation of ExoS into target cells. To assess the level of Ras modification in vivo, HeLa cell monolayers were infected for various times with relevant *Yersinia* strains. Infected cells were directly harvested in sample buffer on ice, and the lysates were separated by SDS-PAGE, followed by immunoblotting with monoclonal anti-Ras antibody. *Yersinia* producing endogenous (figure 4, *lane a*) or in trans (figure 4, *lane d*) LcrV required as few as 60 min to translocate sufficient ExoS to ADP ribosylate the total cellular Ras population, as was indicated by a slower mobility on SDS-PAGE. In contrast, at least 2 h were required for *Yersinia* producing PcrV to induce an ExoS-dependent complete ADP ribosylation of Ras (figure 4, *lane e*). This represents a significant delay in PcrV-mediated translocation, compared with LcrV. As had been expected, bacteria that were defective for either LcrV or YopB were unable to mediate ExoS translocation, and Ras proteins remained unmodified (figure 4, *lanes b* and *c*). The fact that LcrV could efficiently translocate ExoS confirms that LcrV and PcrV do not exhibit specificity toward native translocated substrates.

***Construction and phenotypic analysis of an* lcrGVHyopBD *operon deletion mutant of* Y. pseudotuberculosis.** Having established that substrate specificity does not account for the differences in the translocation efficiencies seen for LcrV or PcrV in *Yersinia,* we decided to investigate whether PcrV requires native proteins encoded by the *pcrGVHpopBD* translocon operon of *P. aeruginosa* to form fully functional pores in *Yersinia.* This possibility was examined by exchanging the entire translocase operon of *Yersinia (lcrGVHyopBD)* with that of *P.*

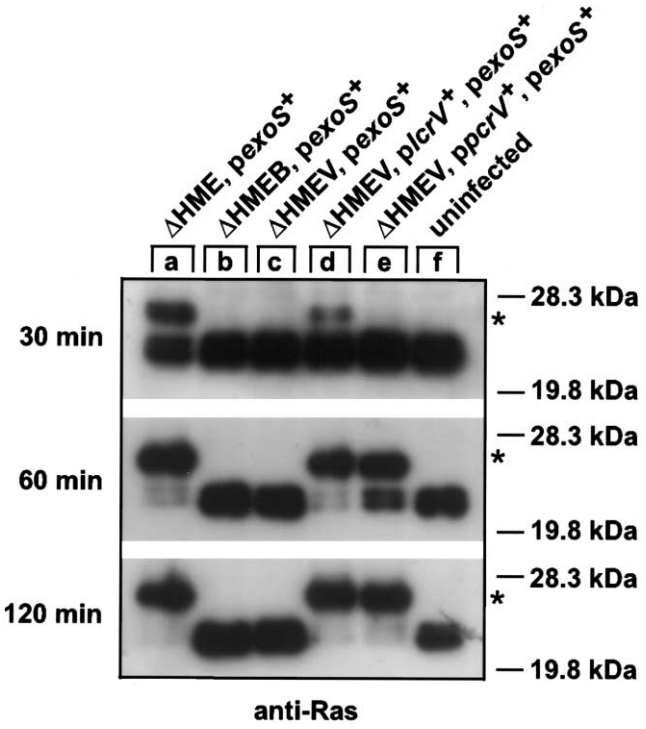

Figure 4. Ras modification in HeLa cells infected with bacteria expressing exoenzyme S (ExoS). HeLa cells were harvested after infection at 30, 60, or 120 min and dissolved in sample buffer. Proteins were separated by SDS-PAGE, followed by immunoblotting with anti-Ras monoclonal antibody. *Lane a,* YPIII/pIB29ME, pTS103–gentamicin (Gm); *lane b,* YPIII/pIB29MEB, pTS103-Gm; *lane c,* YPIII/pIB29MEV, pTS103-Gm; *lane d,* YPIII/pIB29MEV, pTB7, pTS103-Gm; *lane e,* YPIII/pIB29MEV, pLJ33, pTS103-Gm; and *lane f,* uninfected. *, ADP-ribosylated Ras. Bio-Rad prestained low-range protein molecular-weight standards were used in the analysis (bovine serum albumin [80 kDa], ovalbumin [46.9 kDa], carbonic anhydrase [33.5 kDa], soybean trypsin inhibitor [28.3 kDa], and lysosyme [19.8 kDa]).

aeruginosa (pcrGVHpopBD). We engineered a complete deletion of the *Yersinia lcrGVHyopBD* operon in which only the first 6 residues of LcrG and the last 3 residues of YopD remained, giving rise to the mutant YPIII/pIB799. Using immunoblotting, we confirmed the loss of YopB, LcrV, YopD, and LcrH in both total and secreted protein fractions derived from the operon mutant (figure 5*A*, *lanes c* and *d*). Poor-quality antiserum prevented the confirmation of the expected loss of LcrG. In addition, constitutive synthesis and the secretion of YopH and YopE were observed in the operon mutant (YPIII/pIB799) regardless of inducing ($-Ca^{2+}$) or noninducing ($+Ca^{2+}$) growth conditions, which reflects a loss of *yop* regulatory control (figure 5*A*, *lane c* vs. *lane d*). In contrast, protein production and secretion in the wild-type strain were only induced when grown in medium without Ca^{2+}, reflecting normal *yop* regulatory control (figure 5*A*, *lane a* vs. *lane b*). This important regulatory phenotype was further examined by a MOX plating assay. Unlike wild-type *Yersinia,* the operon null mutant displayed a temperature-sensitive (TS) growth phenotype [8], consistent with the requirement for LcrG, LcrH, and YopD in the negative regulation of Yop synthesis [28, 38–41].

To complement the full-length operon mutant, a pMS9 construct encoding the *pcrGVHpopBD* operon from *P. aeruginosa* [17] under control of its native promoter located upstream of *pcrG* was introduced into YPIII/pIB799. At inducing conditions (without Ca^{2+}), we could confirm the presence of PopB, PcrV, and PopD in both total lysates and the culture supernatant fraction of YPIII/pIB799, pMS9 (figure 5*B, lanes b* and *d*). The absence of specific antibodies to PcrG and PcrH prevented their visualization. Of interest, high levels of PopB, PcrV, PopD, YopH, and YopE were even observed in cell lysates prepared from bacteria grown in noninducing conditions (with Ca^{2+}) (figure 5*B, lane a* vs. *lane b*). This result indicates that expression of *pcrGVHpopBD* is unable to restore the regulatory defect associated with an *lcrGVHyopBD* mutant of *Yersinia,* corroborating the TS growth phenotype observed by MOX analysis.

Y. pseudotuberculosis *expressing* pcrGVHpopBD *efficiently translocates Yop effector proteins and forms LcrV-like pores.* Having established the coproduction of PcrV with native translocon components in a suitably engineered strain of *Yersinia,* we wanted to investigate how efficient the complemented strain YPIII/pIB799, pMS9 was at translocating Yop effector proteins. We used the HeLa cell cytotoxicity assay as a means to study the translocation of the YopE cytotoxin into target cells. As expected, the operon deletion mutant YPIII/pIB799 was unable to translocate YopE into the cytosol of infected HeLa cells (figure 6*C* vs. 6*A*), even though secretion was confirmed. In contrast, the wild-type strain induced a dramatic morphological change of the infected HeLa cells (figure 6*B*). Surprisingly, the trans-complemented strain YPIII/pIB799, pMS9 was similar to the wild type in being able to efficiently translocate Yop effectors, as evidenced by the rapid YopE-mediated cytotoxic response (figure 6*D* vs. 6*B*). This result contrasted with the delayed translocation observed when PcrV alone was expressed in the context of *Yersinia* translocator proteins (see figures 3 and 4) [5].

To evaluate whether this efficient translocation was due to an increase in pore size, we examined the trans-complemented operon mutant (YPIII/pIB799, pMS9) in an osmoprotection assay. In so doing, the full-length operon deletion was introduced into the virulence plasmid of YPIII/pIB29MEK. This created an isogenic strain, YPIII/pIB799-29MEK, to enable direct comparisons with the pores formed by other strains used in the present study. The noncomplemented operon mutant was unable to induce pores in the membranes of erythrocytes, which reflects an essential pore-forming function for YopB, YopD, and LcrV (figure 2*D*). In contrast, the operon mutant harboring *pcrGVHpopBD* (YPIII/pIB799-29MEK, pMS9) showed a strong lytic activity that was an additional 34% higher than that induced

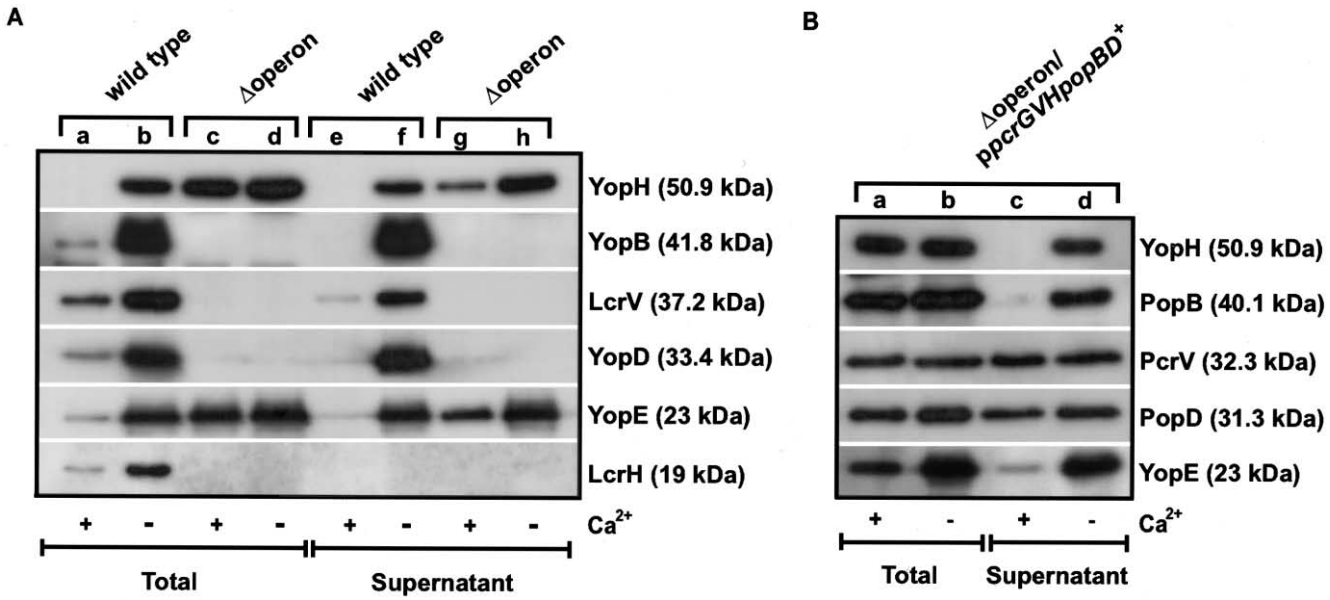

Figure 5. Analysis of protein synthesis and secretion by a translocase operon mutant of *Yersinia pseudotuberculosis* grown either with (+) or without (−) Ca^{2+}. Proteins were separated by SDS-PAGE and identified by immunoblot analysis using polyclonal rabbit anti-YopH, anti-YopB, anti-LcrV, anti-YopD, anti-YopE, anti-LcrH, anti-PopD, or anti-PcrV antisera. Detection of PopB was made using the cross-reactive anti-YopB antisera [15]. Total samples refer to a mixture of proteins secreted into the culture medium and contained within intact bacteria, whereas supernatant samples only contain secreted proteins. *A, Lanes a, b, e,* and *f,* wild-type YPIII/pIB102; *lanes c, d, g,* and *h,* operon deletion mutant YPIII/pIB799; *B, lanes a–d,* complemented YPIII/pIB799, pMS9. Molecular weights shown in parentheses are deduced from the primary sequence.

by YPIII/pIB29MEK (figure 2*D* vs. 2*A*). This is not likely to be a consequence of excessive protein pools with which to form more pores, given that Coomassie staining of secreted proteins fractionated by SDS-PAGE indicated that at least PopB and PopD were secreted at lower levels than native YopB and YopD (data not shown). Of importance, the pores induced by this trans-complemented strain were not blocked by the smaller carbohydrates raffinose (1.2–1.4 nm ϕ) or dextrin 15 (2.2 nm ϕ). Only the largest carbohydrate, dextran 4 (3–3.5 nm ϕ), could block these pores ($P < .05$, 77% reduction) (figure 2*D*). Hence, we conclude that the efficient translocation observed for YPIII/pIB799-29MEK, pMS9 correlated to the formation of larger pores. This strongly suggests that the pores induced by *Yersinia* producing PcrV together with YopB and YopD are not completely functional pores. In fact, when PcrV is co-produced together with its native translocase partners, PopB and PopD, the physical characteristics of the pores formed in erythrocyte membranes clearly resembled those formed by a complex of LcrV, YopB, and YopD. This suggests that PcrV requires native translocator proteins to fully complement defects in *Yersinia* Yop effector translocation.

DISCUSSION

The formation of bacterial-induced pores in biological membranes is an essential means for pathogenic bacteria to translocate toxic molecules into host cells [42]. In the present study, we set out to address whether the pore-forming proteins LcrV of *Yersinia* and PcrV of *P. aeruginosa* determine translocation efficiencies of antihost effectors into the target cell. We confirmed that the cytotoxins YopE of *Yersinia* and ExoS of *P. aeruginosa* were translocated equally well relative to *Yersinia* producing either LcrV or PcrV. We interpret these data to indicate that neither PcrV nor LcrV favors the translocation of native substrates. As had been seen earlier [5], we noted that the rate of effector translocation by *Yersinia* producing PcrV was dramatically delayed. On further investigation of the different pore sizes induced by LcrV and PcrV, we found that a full-translocation operon-deletion mutant (Δ*lcrGVHyopBD*) of *Y. pseudotuberculosis,* when complemented with the corresponding operon *(pcrGVHpopBD)* of *P. aeruginosa,* induced larger LcrV-like pores and concomitantly was able to rapidly translocate Yop effectors. Although this confirmed the absence of substrate recognition by LcrV and PcrV, it also illustrated that PcrV requires additional protein(s) from its native translocase operon to efficiently function in *Yersinia* translocation. Therefore, the reduced PcrV-dependent lysis of infected erythrocytes and the delayed translocation phenotype reported elsewhere [5] was likely due to an inability to form fully functional pores.

The translocation of antihost effectors by TTSS is thought to require the recognition of a signal that predestines a secreted

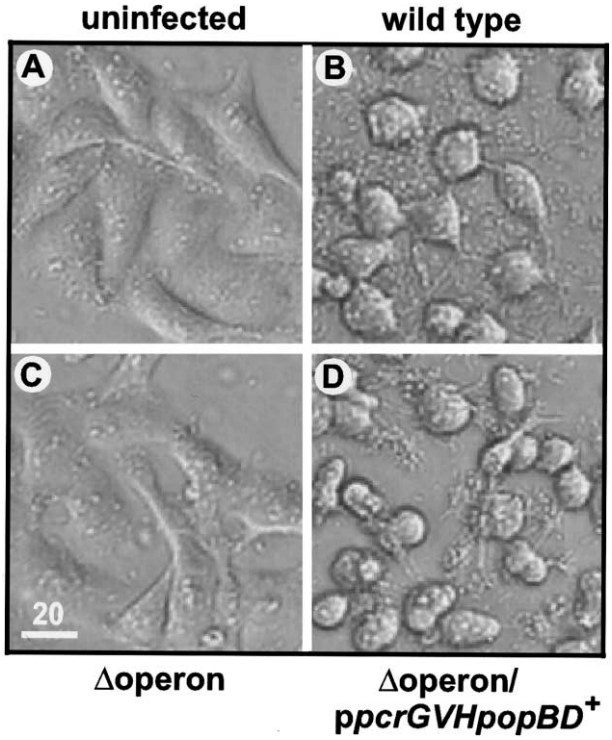

Figure 6. Infection of HeLa cells by different strains of *Yersinia pseudotuberculosis.* At 2 h after infection, the effect of bacteria on the HeLa cells was recorded by phase-contrast microscopy. Note the extensive rounding up of the *Yersinia* outer protein (Yop) E–dependent, cytotoxically affected HeLa cells *(B* and *D).* HeLa cells infected with the full-translocase operon-deletion mutant YPIII/pIB799 show normal cell morphology *(A* vs. *C).* Shown are phase-contrast images of uninfected *(A),* wild-type YPIII/pIB102 *(B),* null-mutant YPIII/pIB799 *(C),* and complemented YPIII/pIB799, pMS9 *(D).* Scale bar, 20 μm.

protein for translocation. It follows that all effectors have a modular secondary structure, which includes an N-terminal domain essential for translocation [1, 2]. Even though no consensus sequence within this domain has been demonstrated, a putative recognition mechanism could conceivably occur through either the core secretion components located at the inner face of the cytoplasmic membrane, of which homologues are found in all known TTSS [1], or after secretion, at the level of the translocon pore. In light of the general promiscuity displayed by TTSS, we considered that the recognition motif might confer a level of substrate specificity that enables endogenous effectors to be translocated more efficiently by their native TTSS. In our hands, however, LcrV and PcrV did not show a preference for translocating either native *Yersinia* effectors or exogenous ExoS from *P. aeruginosa.* Although we cannot rule out the presence of an effector specificity signal, the recognition mechanism is clearly not through the LcrV/PcrV component of the translocation pore.

Significantly, the translocation domain also overlaps with a chaperone-binding domain [43]. Bound chaperone is a requirement for the efficient secretion of cognate effector proteins [44–46] and may impart a specificity signal for translocation. In our study, however, ExoS was coproduced with its native chaperone, Orf1 [23], which did not alter translocation specificity. However, we note that this domain may simply necessitate the requirement for chaperone binding to prevent premature interactions with other proteins prior to secretion. This was elegantly demonstrated for a YopE mutant lacking the translocation domain, because it was only translocated by a strain of *Yersinia enterocolitica* that is unable to produce other Yop effectors [47]. Nevertheless, this does not explain why the YopM effector also contains a modular structure that includes a region necessary for translocation but does not require a known chaperone for efficient secretion [48].

It is noteworthy that specific recognition sequences and/or folding events that enable vast numbers of proteins to be secreted across the bacterial envelope by 1 of 5 secretion pathways (types I–V) have been defined [2, 49, 50]. Some of these secreted proteins also contain varied translocation signals to traverse the eukaryotic membrane. For example, the type I secreted family of RTX repeat toxins contain repetitive motifs thought to insert directly into the host cell plasma membrane [51, 52], whereas toxins secreted by the type II pathway specifically interact with a target cell receptor to facilitate uptake [53, 54]. Therefore, whether the translocation of type III secreted effectors involves a bona fide translocation motif, as was initially proposed [55], remains a focus of our research.

Several reasons made a comparative analysis of translocation efficiencies of the YopE and ExoS effectors meaningful. ExoS is readily secreted and translocated by *Yersinia* [23]. Moreover, both proteins share similar N-terminal domains that exhibit GAP activity toward Rho GTPases. This activity is responsible for actin depolymerization that is a basis for the virulence strategy termed antiphagocytosis [2]. In addition, a modular organization characteristic of effector proteins is also possessed by YopE and ExoS [2, 6], and their efficient secretion requires a homologous cognate chaperone termed YerA/SycE and Orf1, respectively [23]. We have extended these observations to show that *Yersinia* is also able to efficiently translocate ExoS at a rate comparable with native effectors such as YopE. This feature, together with the easily quantifiable ADP ribosylating activity of ExoS that is dependent on the cytosolic eukaryotic protein 14-3-3 [36], makes this protein a sensitive probe for investigating the role of individual proteins in the translocation process.

When PcrV was initially introduced in an *lcrV* null mutant of *Yersinia,* significantly smaller pores were formed in infected erythrocytes [5], which was consistent with findings in the artificial lipid bilayer system [5]. At odds with this was the observation that a PcrV-dependent pore that formed during *P. aeruginosa* infection of erythrocytes was comparable in size to the pores observed for *Yersinia* producing LcrV [5, 13]. To

resolve this issue, we generated a full-translocase operon-deletion mutant of *Yersinia* complemented with the *pcrGVHpopBD* operon of *P. aeruginosa.* When it was used to infect erythrocytes, this strain induced increased lysis with larger pores. In addition, the efficient translocation of Yop effectors was restored, reminiscent to that observed for *Yersinia* expressing LcrV. This is a significant finding that suggests that a PcrV complex with other native *P. aeruginosa* components encoded within the *pcrGVHpopBD* translocase operon are required to form a fully functional pore. Therefore, it would be interesting to analyze whether PcrV purified as a complex with other native components forms larger pores in an artificial bilayer system. Nevertheless, clearly the smaller pores formed when PcrV is produced together with YopB and YopD in *Yersinia* are likely to be the result of partly nonfunctional interactions between the heterologous translocators.

An analysis of hybrids between LcrV and PcrV revealed a central domain that altered translocation efficiencies in *Yersinia* [5]. We envisage that such an approach could well identify key LcrV/PcrV domains that promote functional interactions with native translocator components. At least in *Yersinia,* there is a precedence for LcrV, YopB, and YopD interactions in vitro [56, 57]. Indeed, our preliminary observations analyzing the functional interchangeability between components of the *Yersinia* and *P. aeruginosa* translocase operons clearly indicated that some components are only able to promote translocation in the presence of their native partners [18]. In view of this, the translocation process is highly regulated, being fine tuned by the specificity of the interaction partners.

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