This is the published version of a paper published in *Frontiers in Cellular and Infection Microbiology*. 

Citation for the original published paper (version of record):

Ekestubbe, S., Bröms, J E., Edgren, T., Fällman, M., Francis, M S. et al. (2016)
The amino-terminal part of the needle-tip translocator LcrV of Yersinia pseudotuberculosis is required for early targeting of YopH and in vivo virulence.
*Frontiers in Cellular and Infection Microbiology*, 6: 175
https://doi.org/10.3389/fcimb.2016.00175

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:
http://urn.kb.se/resolve?urn=urn:nbn:se:umu:diva-130149
The Amino-Terminal Part of the Needle-Tip Translocator LcrV of Yersinia pseudotuberculosis Is Required for Early Targeting of YopH and In vivo Virulence

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Type III secretion systems (T3SS) are dedicated to targeting anti-host effector proteins into the cytosol of the host cell to promote bacterial infection. Delivery of the effectors requires three specific translocator proteins, of which the hydrophilic translocator, LcrV, is located at the tip of the T3SS needle and is believed to facilitate insertion of the two hydrophobic translocators into the host cell membrane. Here we used Yersinia as a model to study the role of LcrV in T3SS mediated intracellular effector targeting. Intriguingly, we identified N-terminal lcrV mutants that, similar to the wild-type protein, efficiently promoted expression, secretion and intracellular levels of Yop effectors, yet they were impaired in their ability to inhibit phagocytosis by J774 cells. In line with this, the YopH mediated dephosphorylation of Focal Adhesion Kinase early after infection was compromised when compared to the wild type strain. This suggests that the mutants are unable to promote efficient delivery of effectors to their molecular targets inside the host cell upon host cell contact. The significance of this was borne out by the fact that the mutants were highly attenuated for virulence in the systemic mouse infection model. Our study provides both novel and significant findings that establish a role for LcrV in early targeting of effectors in the host cell.

Keywords: LcrV, type III secretion system, YopH, translocation, pore formation, Yersinia pseudotuberculosis, virulence

INTRODUCTION

Type Three Secretion systems (T3SS) were discovered more than 25 years ago when it was established that Yersinia spp. targeted virulence proteins (effectors) into eukaryotic cells through a delivery mechanism that required close bacteria-host cell contact (Rosqvist et al., 1991, 1994; Sory and Cornelis, 1994). The T3SS require the coordination of more than 20 genes to promote expression and secretion of the virulence proteins from the bacterium (Galán and Wolf-Watz, 2006).

Over the years, T3SS have been described in a wide variety of gram negative pathogens. While some of them, such as Salmonella spp. and Shigella spp., use their T3SS to promote uptake by host
cells (Finlay et al., 1988; Sasakawa et al., 1988, 1989; Elsinghorst et al., 1989), in other pathogens such as *Yersinia* spp. and *Pseudomonas* spp., the T3SS act to block uptake (Rosqvist et al., 1988; Frithz-Lindsten et al., 1997). These opposing outcomes are ascribed to the specific enzymatic activities and corresponding molecular targets of the translocated effectors, rather than the actual T3SS structure and function that is overall well conserved (Rosqvist et al., 1995; Frithz-Lindsten et al., 1997, 1998; Akopyan et al., 2011).

The injection model is widely used to explain the mode of function of the T3SS. It dictates that protein translocation occurs in one step from the bacterial cytosol to the target-cell cytoplasm through a conduit created by the basal body and a needle-like hollow tube that is extended by a tip complex that forms a pore in the host cell membrane (Galan and Wolf-Watz, 2006). While there is evidence from recent elegant studies that the T3SS substrates are indeed secreted through the narrow hollow needle complex (Dohlich et al., 2014; Radics et al., 2014), there is to date no direct experimental evidence that effectors secreted via the needle are subsequently targeted directly into the host cell. On the contrary, a recent study showed that effectors exogenously added to the bacterial surface of both *Yersinia* and *Salmonella* could be translocated in a T3SS-dependent manner, suggesting an alternative mechanism to the one-step injection model (Akopyan et al., 2011).

The secreted substrates can be divided into two functional classes; effectors and translocators. The effectors are delivered into the target cell where they elicit a specific biological response. *Yersinia* is an extracellular pathogen that replicates in the lymphatic tissues of the host (Hanski et al., 1989; Simonet et al., 1990). As such, *Yersinia* needs to be able to block phagocytosis by the host immune cells such as macrophages, and the effector YopH is essential for this event (Rosqvist et al., 1988; Fahlgren et al., 2009). Phagocytosis is preceded by formation of focal adhesion sites, which occurs at the cytoplasmic side of the host cell membrane. Since phagocytosis is a rapid process with an onset immediately after the establishment of bacteria-cell contact, translocation and targeting of the effectors must essentially occur instantly. Consistent with this, studies have shown that YopH is translocated and targeted to the intracellular focal adhesion sites within minutes after target cell contact (Andersson et al., 1996, 1999; Persson et al., 1999), and that the T3SS substrate YopK is involved in YopH targeting to the focal adhesion sites (Thorslund et al., 2011; Dewoody et al., 2013).

The translocators facilitate the delivery of effectors across the plasma membrane (Rosqvist et al., 1994; Sory and Cornelis, 1994; Hakansson et al., 1996; Pettersson et al., 1999). The T3SS encode three functionally conserved translocator proteins, two that are generally hydrophobic and one that is hydrophilic (Mattei et al., 2011). A key signature of the hydrophobic translocators is their putative membrane spanning domain(s), suggesting that they may target host membranes. Indeed, in *Yersinia* spp. the hydrophobic translocators, YopB, and YopD, have been shown to insert into erythrocyte membranes and induce pore formation (Rosqvist et al., 1994; Sory and Cornelis, 1994). LcrV, the hydrophilic translocator, localizes at the tip of the needle complex (Mueller et al., 2005). LcrV facilitates insertion of YopB and YopD in host cell membranes (Broz et al., 2007) and has been proposed to promote translocation by serving as a pore-forming platform. In addition, LcrV also has a role in regulation of yop expression since deletion of *lcrV* results in down-regulated Yop production (Price et al., 1991; Pettersson et al., 1999).

We have previously shown that LcrV secretion levels correlates to *in vivo* virulence of *Yersinia* (Bröms et al., 2007). As for other T3SS substrates, the very N-terminal region of LcrV is somehow recognized by the secretion system and, thus, mutants impaired for secretion have been mapped to the N-terminal region (Bröms et al., 2007). Interestingly, introduction of frame-shift mutations that completely changed the coding sequence of the extreme N-terminal of LcrV, affected neither LcrV secretion nor overall expression/secretion of other Yop proteins. These LcrV mutants also induced YopE mediated cytotoxicity in infected HeLa cells to the same extent as the isogenic wild type strain (Bröms et al., 2007). In this study, we performed a more in depth analysis of the N-terminal mutant strains and could hereby show a novel role for the N-terminal region of LcrV in the early targeting of Yop effectors, and the corresponding ability of *Yersinia* to block phagocytosis by macrophages and promote virulence in mice.

**RESULTS**

To study the role of LcrV in translocation we decided to utilize two previously characterized in-cis N-terminal mutants of LcrV (Bröms et al., 2007). These mutants contain +1 or −1 frame-shift mutations, specifically altering the sequence of the first 15 amino acids, but leaving the remaining protein sequence intact (Figure S1) and will hereafter be referred to as LcrV+1 and LcrV−1 respectively. Importantly, both mutants exhibit intact Yop-regulation, which is a prerequisite to meaningfully study any other role of LcrV.

**The LcrV N-Terminus Is Important for Pore Formation in Erythrocytes**

Previous studies have established a strong correlation between functional T3SS-mediated translocation and the ability to induce pore formation as measured by hemolytic activity in infected erythrocytes (Håkansson et al., 1996; Holmstrom et al., 1997; Neyt and Cornelis, 1999; Olsson et al., 2004; Ryndak et al., 2005). When the LcrV+1 and LcrV−1 strains were used to infect erythrocytes they were essentially unable to induce any significant cytotoxicity in infected HeLa cells to the same extent as the isogenic wild type strain (Bröms et al., 2007). In fact, the levels of hemolysis were found to be similar to that induced by LcrVΔ1 (Figure 1) and will hereafter be referred to as LcrV+1 and LcrV−1 respectively. Importantly, both mutants exhibit intact Yop-regulation, which is a prerequisite to meaningfully study any other role of LcrV.
subjected to SDS-PAGE and Western blot analysis using YopB and YopD antisera. By this approach, we were unable to detect YopB, and the levels of YopD were very low, in the membranes of cells infected with the two LcrV+/−1 strains both in parental background and in the ΔyopK mutant background (Figure 2). As expected (Thorslund et al., 2011), infection with the ΔyopK mutant resulted in increased levels of both YopB and YopD in the membranes (Figure 2). Control experiments verified that during infection the total levels of YopB and YopD were similar for all strains except for the ΔlcrV mutant, for which the expression of the T3SS encoding genes is known to be down-regulated (Bröms et al., 1999) (Figure S2). Overall, the phenotype of the LcrV+/−1 strains was similar to a ΔlcrV mutant with respect to hemolytic activity and membrane insertion of YopB and YopD. Thus, the LcrV+/−1 strains evidently induced YopE mediated cytotoxicity, as reported previously (Bröms et al., 2007), without localizing YopB and YopD to the erythrocyte membrane.

The LcrV+/−1 Mutants Translocate YopE and YopH Despite Lack of Hemolytic Activity

As mentioned above, past studies have shown a strong correlation between lytic activity due to pore formation and the functional translocation of T3SS effectors (Håkansson et al., 1996; Holmström et al., 1997; Neyt and Cornelis, 1999; Olsson et al., 2004; Ryndak et al., 2005). Hence, it was important to verify that the LcrV+/−1 mutants that lacked hemolytic activity really had retained translocation ability. In our previous study we found that these mutants, similar to the wild type strain, induced rapid cytotoxicity in infected HeLa cells (Bröms et al., 2007). In this assay very low levels of translocated YopE are needed for visible cell rounding. Therefore, to detect any possible translocation deficiency, we repeated the assay using different MOIs. At a high MOI (40:1) the wild type strain induced full cytotoxicity within an hour of infection, while at a low MOI (2.5:1), full cytotoxicity was delayed until 3–4 h of infection. Using this approach it was clear that the LcrV+/−1 strains induced a cytotoxic response with similar kinetics as the wild type strain (data not shown), even at MOI 2.5. Since cytotoxic response is an indirect measurement of intracellular targeting of YopE, we decided to confirm more directly that YopE was translocated by the LcrV+/−1 strains. To this end, infected HeLa cells were fixed, permeabilized and incubated with rabbit-anti-YopE antisera followed by analysis using laser scanning confocal microscopy. YopE specific staining was detected in cells infected with the LcrV+/−1 mutants as well as in cells infected with the wild type, thereby confirming the ability of the mutants to translocate YopE (Figure 3A). Expectantly, no YopE specific staining was observed in cells infected with the ΔlcrV mutant. To confirm that this translocation was general and not just specific for YopE, we also monitored translocation of YopH using a beta-lactamase reporter system. HeLa cells were infected with Yersinia variants expressing a YopH-Bla fusion protein. Already after 40 min of infection, translocation of the fusion protein (as visualized by the blue fluorescence) was observed in a majority of cells infected with either the wild type or the LcrV+/−1 mutants (Figure 3B), once again confirming the general ability of the LcrV+/−1 mutants to translocate Yop effectors into the host cell. Quantification of the translocated effectors showed that the LcrV+/−1 mutants translocated YopE and YopH at wild type levels (Figure 4).

Translocation by the LcrV+/−1 Mutants Is Regulated by YopK

As previously mentioned, translocation is up-regulated in absence of YopK, which correlates with larger pores being formed (Holmström et al., 1997). This prompted us to investigate translocation of Yop effectors by the LcrV+1 and LcrV−1 strains in the ΔyopK mutant background, for which no pore formation was seen (Figure 1). To address this, HeLa cells infected with

FIGURE 1 | The LcrV frameshift variants are non-hemolytic. Sheep erythrocytes were infected with Y. pseudotuberculosis strains for 2 h in 96-well plates. The cells were centrifuged and the supernatants collected. The lytic activity was measured as absorbance at 570 nm and is presented as percent of complete lysis (erythrocytes lysed with 1% Triton X-100). The results shown are the mean values ± SD of four independent experiments.

FIGURE 2 | The extreme N-terminal of LcrV is required for membrane insertion of YopB and YopD. Sheep erythrocytes were infected with Y. pseudotuberculosis for 2 h. The cells were lysed and the membranes were isolated by flotation on a sucrose gradient. Of the resulting membrane fraction, 20 μg total protein was subjected to SDS-PAGE and Western blot. The YopB antibody recognizes a non-specific upper band that was used as a loading control, while the lower band is specific for YopB. The experiment was repeated three times and a representative experiment is shown. The image has been cropped to facilitate comparison between samples.
FIGURE 3 | Pore forming activity does not correlate to translocation ability. (A) Intracellular localization of YopE in HeLa cells after 1.5 h infection was analyzed with immunostaining. The samples were viewed using laser scanning confocal microscopy and green staining corresponds to YopE, while the HeLa cell membranes are stained in red. (B) To analyze translocation of YopH, HeLa cells were labeled with the FRET substrate CCF4-AM and infected with the indicated strains expressing YopH-Beta-lactamase fusion protein (YopH-Bla). Images were acquired in a live cell microscope using a longpass filter. Translocation of the Bla-fusion results in a shift of fluorescence from green to blue. Both experiments were repeated at least three times.

the different *Yersinia* strains were fractionated, using a well-established translocation assay (Nordfelth and Wolf-Watz, 2001). The cytosolic proteins were subjected to SDS-PAGE and Western blot using monospecific YopE and YopH antisera. As expected, the Δ*yopK* mutant translocated increased levels of both YopE and YopH compared to the isogenic wild type strain (Figure 4). The LcrV+ and LcrV− strains also translocated increased levels of YopE and YopH in absence of YopK, at the same level as the Δ*yopK* mutant (Figure 4). No intracellular YopE and YopH could be detected in cells infected with the Δ*lcrV* mutant.

Since translocation of effectors occurred although little or no YopB and YopD could be detected in the erythrocyte membranes it was important to establish that effector translocation by the two LcrV+/− strains still required YopB and YopD. To address this, the LcrV+/− strains were also mutated for *yopB* and *yopD*. None of these strains were able to induce cytotoxicity of infected HeLa cells (data not shown) confirming that the effector translocation observed herein still required functional YopB and YopD.

It is puzzling that translocation in the LcrV+/− strains is regulated as in the wild type despite the lack of lytic activity. Contrasting with numerous previous studies, our results show that neither lytic activity nor translocator insertion in erythrocyte membranes have any correlation with levels of Yop effector translocation.

**The Non-hemolytic LcrV+/− Mutants Insert YopD into HeLa Cell Membranes**

Given the observance of translocation in the absence of lytic activity, we decided to examine membrane localization of YopB and YopD in HeLa cells to be consistent with the experimental setup used in the translocation assays described above. We found that the levels of YopB were too low to be detected in cell membranes, regardless of the infecting strain (data not shown). The levels of YopD were very low, yet detectable by Western blot, in HeLa cells infected with the wild type strain (Figure 5). As expected, levels of membrane localized YopD were higher in cells infected with the Δ*yopK* mutant background and, most importantly, when the two LcrV+/− strains were analyzed in this background both mutants were found to insert similar levels of YopD into the membranes as the Δ*yopK* mutant strain (Figure 5). These findings are very important because above all they verify that using the erythrocyte model to study T3SS mediated pore formation and membrane insertion has limitations and can even give contradictory results that mislead conclusions regarding the T3SS mechanism. While the erythrocyte model is a convenient method, the relevance can be questioned, as erythrocytes are not a natural target cell of *Yersinia* during infection of an animal host.

**The LcrV N-Terminus Is Involved in Anti-Phagocytosis and the Intracellular Targeting of YopH**

With the exception of the experiments involving the erythrocyte infection model, the LcrV+/− strains displayed *in vitro* phenotypes that were very similar to the parental strain. Based on these observations, we anticipated that these mutants would be fully virulent. In order to examine this, we decided to use assays that could discriminate the impact of these mutations on *Yersinia* virulence.

One major virulence mechanism mediated by the T3SS of *Yersinia* is the ability to block phagocytosis by professional...
phagocytes (Rosqvist et al., 1988). To analyze if the LcrV+1 and LcrV−1 strains were affected in the ability to block phagocytosis we infected J774 macrophages with the different Yersinia strains for 30 min. The cells were then fixed and a double staining assay was performed to distinguish between extracellular and internalized bacteria. As expected, the wild type strain efficiently blocked phagocytosis with only 24% of infecting bacteria internalized (Figure 6). In contrast, 80% of the infecting ΔlcrV mutant and 85% of the virulence plasmid cured strain were phagocytosed (Figure 6). Interestingly, the LcrV+1 and LcrV−1 strains showed an intermediary phenotype where 41% and 42% of the bacteria were phagocytosed, respectively (Figure 6). Thus, even though the LcrV+/−1 strains were able to translocate both YopE and YopH to levels similar as the wild type strain, some critical aspect of this translocation process must be compromised in a manner that causes a significant reduction in phagocytosis inhibition.

Previous work has established that early targeting of YopH to focal adhesion complexes is essential for phagocytosis inhibition (Andersson et al., 1996; Persson et al., 1997, 1999). One of the YopH targets is Focal Adhesion Kinase (FAK) and it was shown that the initial phosphorylation of FAK in response to Yersinia binding to the host cell is quickly reversed by the powerful phosphatase activity of YopH. By extension, this deactivation of FAK in turn impairs bacterial uptake (Persson et al., 1997). Hence, in order to establish if the N-terminal mutations in LcrV compromised early YopH targeting, we infected HeLa cells for 10 min and then immunoprecipitated FAK. The phosphorylation level of FAK was analyzed by Western blotting of total protein preparations of all samples were repeated at least twice. The signal intensity of the bands was quantified using the Multi Gauge-Image software (Fujifilm) and normalized to the unspecific band. The ratio ± SEM of phosphorylated FAK relative to the ΔyopK mutant, is shown below the Western blot. The results were analyzed using the paired Student’s t-test with the significance set at p ≤ 0.05*. NS, No significant difference.
of YopH to its molecular targets inside the infected host cell.

**Alterations of the LcrV N-Terminal Results in Virulence Attenuation In vivo**

Since the LcrV+1 and LcrV−1 strains were impaired in anti-phagocytosis it was necessary to investigate if this correlated to attenuation in the *in vivo* mouse infection model. To this end, C57/BL6 mice were infected intraperitoneally with various *Yersinia* strains. As expected the ΔlcrV mutant was completely attenuated, and all mice survived a high infection dose (1.6 × 10⁶ CFU) without displaying any signs of disease (fuzzy fur, diarrhea, weight loss, listlessness). Mice infected with the wild type strain showed disease symptoms within a few days even at the lowest dose used (2.1 × 10³ CFU) and all mice had succumbed to the infection by day 8 post infection. Strikingly, the LcrV+1 and LcrV−1 strains were essentially as attenuated as the ΔlcrV mutant. All but one mouse survived infection with the high dose of either LcrV+1 or LcrV−1 (Figure 8). Thus, the LcrV+1 and LcrV−1 strains were indeed highly attenuated for virulence, reflecting that a defect in early targeting of YopH has a dramatic impact on *in vivo* virulence.

**DISCUSSION**

The established role for the translocator LcrV is to facilitate insertion of YopB and YopD in host cell membranes to promote effector translocation (Mattei et al., 2011). Here, we show for the first time that the very N-terminal part of LcrV also has a specific role in early targeting of effectors to promote rapid blockage of phagocytosis in *Yersinia*. We have characterized two lcrV mutants that in contrast to the ΔlcrV mutant, expressed and secreted wild type levels of Yop effectors, but were unique in that they no longer could mediate efficient targeting of YopH to the focal adhesion sites. As the plasmid encoded T3SS is a key element in determining an extracellular pathogenic lifestyle...
of Yersinia the targeting defect of these mutants naturally had a
dramatic effect on their in vivo virulence.

LcrV belongs to the class of hydrophilic translocators that
locates to the tip of the needle complex of T3SS (Mueller et al.,
2005). It has been shown that LcrV is required for translocation
and promotes insertion of the two hydrophilic translocators
into the host cell membrane (Pettersson et al., 1999; Broz et al.,
2007). According to the injection model, the needle complex
extends to contact the YopB/YopD induced pore and form a
conduit for direct transport of the effectors from the cytosol of
the bacterium into the host cell. Interestingly, studies from P. aeruginosa and Shigella indicate an active role of the tip proteins,
PcrV and IpaD respectively, in sensing host cell contact (Lee et al.,
2010; Schiavolin et al., 2013). In addition, it was recently proposed in P. aeruginosa that a complex of PcrV and the
hydrophobic translocator PopD is involved in sensing host cell
contact (Armentrout and Rietsch, 2016).

In human pathogenic Yersinia spp., LcrV has an additional
regulatory role since ΔlcrV mutants are down-regulated for yop
expression (Bergman et al., 1991; Skrzypek and Straley, 1995).
To study the role of LcrV in translocation and virulence, it is
therefore crucial to construct mutants with retained regulatory
competence. Indeed, the two previously characterized lcrV
mutants (LcrV+1 and LcrV−1) with completely altered amino
acid sequences within the first 15 amino acids of the protein
(Figure S1) maintained full control of both expression and
secretion of translocators, including LcrV itself and effectors
(Broms et al., 2007). In addition, these mutants induced YopE
mediated cytotoxicity on infected HeLa cells, indicating that
they retained an ability to translocate into infected cells (Broms
et al., 2007). When revisiting these mutants we observed that
they were devoid of hemolytic activity and did not insert
YopB or YopD into erythrocyte membrane, not even in a
ΔyopK mutant background. We could however, confirm that
both the LcrV+1 and the LcrV−1 strain translocated YopE
and YopH at levels indistinguishable from the parental strain
and we also verified that translocation was still dependent on
YopB and YopD. Importantly, we also showed that translocation
by the LcrV+/−1 strains was up-regulated in the absence of
YopK. Collectively, these results show that translocation by
the LcrV+/−1 strains is regulated in the same way as in the
isogenic wild type strain, despite the lack of pore formation
in erythrocytes. Remarkably, when we analyzed YopB/YopD
membrane localization in nucleated cells we found that the
LcrV+/−1 strains inserted similar levels of YopD in the HeLa
cell membranes as the parental strain. Analyzing nucleated cells
is far more challenging compared to erythrocytes and YopB
levels proved to be below the detection limit in the Western blot
analysis. Although we could not verify YopB insertion in HeLa
cell membranes, we find it likely that the LcrV+/−1 strains also
insert YopB along with YopD into the cell membrane. These
results are important as they show that studies of pore formation
in erythrocytes does not necessarily correlate to T3SS activity.
This observation is reinforced by studies on YopD mutants that
had a reduced hemolytic activity but maintained efficient and
functional effector translocation (Costa et al., 2010). Numerous
studies have used erythrocytes as the sole model system to
study pore formation and its role in the T3SS mechanism of
effector translocation across the host cell membrane. Those
studies may need to be revisited to verify the findings in nucleated
cells.

Y. pseudotuberculosis is an extracellular pathogen that
replicates in the lymphatic tissues of the infected animal (Simonet et al., 1990). Therefore, the anti-phagocytic action mediated
by the plasmid encoded T3SS, in Yersinia, is tremendously
important (Rosqvist et al., 1988). Uptake of bacteria is triggered
immediately after binding of the bacteria to the phagocytic cell.
Hence, to block uptake, Yersinia must rapidly translocate the
effectors upon cell contact. Critically, Andersson et al. have
shown that YopH is rapidly translocated, reaching its eukaryotic
targets within minutes after cell contact (Andersson et al., 1996;
Persson et al., 1999). Here, we first established that the LcrV+1
and LcrV−1 strains translocated YopE and YopH as efficiently
as the wild type strain when measured after 1–2 h of infection.
However, this does not exclude the possibility that these lcrV
mutations may have an effect on the early translocation after
cell contact. Importantly, prolonged measurements of effector
translocation may not always be a true reflection of the T3SS
functional status. This is exemplified by the study of Thorslund
and co-workers, where it was demonstrated that the ability to
induce cytotoxicity did not correlate with the ability to block
phagocytosis (Thorslund et al., 2011). Consistent with this, when
we analyzed the LcrV+1 and LcrV−1 strains for phagocytosis
inhibition, we found that they were significantly impaired in their
ability to block phagocytosis. Together, these data demonstrate
unequivocally that in vitro translocation levels after prolonged
infection do not necessarily predict the outcome of an in vivo
infection.

The YopE and YopH effectors mediate anti-phagocytic
actions through different mechanisms. To block phagocytosis
by macrophages, Yersinia relies heavily on YopH (Fahlgren et al.,
2009), and further it has been shown that YopH is the
effector that has the greatest impact on in vivo virulence
(Logsdon and Mecsas, 2003). YopH is translocated immediately
after cell contact and is actively targeted to the focal adhesion
sites by the T3SS (Thorslund et al., 2011). In line with our
results on phagocytosis inhibition, the early targeting of YopH
to FAK was impaired in the LcrV+/−1 mutants, which suggests
that the inability of the lcrV mutants to block phagocytosis
is due to a defect in the intracellular targeting of translocated
YopH. Thus, despite there being no disparity in the levels
of translocated YopH accumulated after prolonged infection,
by wild type and mutant strains, the early targeting of YopH
was dramatically affected in our mutants. As evidenced from
this work, future studies must recognize the importance of
analyzing translocation at the early time points of infection.
Using our in vivo infection model, LcrV+1 and LcrV−1 strains
were almost fully attenuated for virulence and equivalent to
the non-translocating ΔlcrV mutant. Therefore, it seems that
the consequence of inefficient targeting of YopH is as severe
as not being able to translocate at all. In agreement with this,
Persson and co-workers have previously shown that a YopH
variant, which failed to associate YopH with the focal adhesion
sites was strongly reduced in phagocytosis inhibition and was
also attenuated for virulence in vivo (Persson et al., 1999). Interestingly, our results show that these lcrV mutations result in a comparable phenotype.

Based on the results presented here, we propose that LcrV, in addition to promoting membrane insertion of YopB and YopD, also has a role in the early targeting of YopH in the eukaryotic cell. The LcrV+/−1 mutations are positioned within a postulated unstructured region that is somehow recognized for secretion. This region is not part of the solved LcrV crystal structure (Derewenda et al., 2004; Chaudhury et al., 2013), and this makes it difficult to predict what impact the mutations could have on the overall structure/function of LcrV. However, it is feasible that it could have some impact on the LcrV/YopD interaction, which is known to be mediated primarily by the N-terminal domain immediately downstream of the LcrV+/−1 mutations (Broz et al., 2007; Armentrout and Rietsch, 2016). This could in turn have an impact on the cell contact-mediated induction and effector translocation in line with what was recently proposed in Pseudomonas (Armentrout and Rietsch, 2016). Considering the proposed model where YopK directs YopH intracellularly by bridging RACK1 to the translocon, through YopD (Thorlund et al., 2011; Dewoody et al., 2013), it is possible that LcrV via its interaction with YopD impacts on the YopH targeting efficiency.

It is very intriguing that the LcrV+/−1 mutants, which initially appeared to possess very subtle phenotypes using established assays to study T3SS function, in fact turned out to have a major impact on phagocytosis inhibition and in vivo virulence. This highlights that for an extracellular pathogen like Yersinia, it is the early events directly after cell contact that are crucial for the outcome of the infection. This is very important since most studies of T3SS secretion and effector translocation are routinely performed after a few hours of host cell infection. One obvious reason for this is that protein levels are only readily detectable at these time points. Yet, our findings clearly signal that for future research on the molecular mechanism of T3SSs it is critical to assess the early events after bacteria host cell contact. We also conclude that using erythrocytes to study pore formation in relation to T3SS mechanism and function is not recommended if future research in the field is to yield meaningful data.

### MATERIALS AND METHODS

#### Growth Conditions

The bacterial strains and plasmids used in this study are presented in Table 1. Bacteria were routinely grown at 26°C in Luria Bertani (LB) agar or LB broth supplemented with 1 mM CaCl₂ and 75 mM NaCl. For induction of the T3SS, bacteria were grown at 37°C in secretion permissive medium (5 mM EGTA and 20 mM MgCl₂) (LB–Ca²⁺) unless otherwise stated. Where appropriate the following antibiotics were added to the final concentrations; kanamycin 30 µg/ml, chloramphenicol 25 µg/ml.

HeLa cells were cultivated in Minimum essential medium (MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 2 mM glutamine, 0.035% sodium bicarbonate and 100 IU penicillin. J774.1 cells were cultivated in Dulbecco's modified eagle medium (DMEM glutaMAX) (Gibco) supplemented with 10% FBS and 100 IU penicillin. The cells were kept at 37°C with 5% CO₂. For infection assays cells were seeded in plates 1 day before experiment and 0.5 hour before infection the cells were washed and incubated in non-supplemented MEM or DMEM.

#### Construction of Mutants

*E. coli* S17-1 λpir was used to conjugate the suicide plasmids pJE368 and pJE369 into YPIII/pIB155 (ΔyopK) to generate ΔyopK/LcrV+1 and ΔyopK/LcrV−1 mutants. ΔyopB and ΔyopD double mutants were constructed by conjugating the suicide plasmids pMF024 and pMF463 into YPIII/pIB10201 (LcrV+1) and YPIII/pIB10202 (LcrV−1). The YopH-Bla fusion was constructed by overlap PCR using the primers listed in Table S1, yielding a DNA fragment corresponding to codons 6–468 of YopH fused to beta-lactamase codons 24–286 (Akopyan et al., 2011). The PCR fragment was cloned into the pcr2.1 TOPO vector (Invitrogen) for amplification, and then sub-cloned into the pNQ705 suicide vector (Milton et al., 1992). S17-1 λpir was used as the donor strain in conjugation with *Yersinia pseudotuberculosis* wild type (YPIII pIB102), LcrV+1 (YPIII pIB10201) and LcrV−1 (YPIII pIB10202) strains and single recombination of the pNQ-YopHBLA inactivated the wild type copy of YopH.

#### Lytic Activity and Membrane Isolation from Erythrocytes

Sheep erythrocytes were washed and resuspended in LB–Ca²⁺ Supplemented with 75 mM NaCl and Complete Mini protease inhibitor cocktail (PIC) (Roche). A total of 5 × 10⁹ induced *Yersinia* bacteria were harvested and resuspended in 5 ml erythrocytes suspension (10¹¹ cells) and centrifuged to create contact between bacteria and cells before incubation at 37°C. Samples were repeatedly resuspended and centrifuged during infection to increase contact between bacteria and cells. After 2 h 100 µL samples were taken and mixed 1:1 with PBS and centrifuged at 3500 g, 5 min, after which 100 µL of the supernatant was transferred to a flat bottom plate and the absorbance at 570 nm was measured to determine the hemolysis.

To analyze total levels of YopB and YopD during infection, 500 µl samples were collected, mixed 1:1 with MQ H₂O and vortexed to lyse the cells. The samples were treated with DNAseI (Thermo Fisher Scientific) according to the manufactures instructions. The samples were centrifuged at 12,000 g, 5 min and the supernatants were collected and subjected to SDS-PAGE and Western blot using an antisera which recognizes both YopB and YopD (ASTI).

To isolate the erythrocytes membranes, 35 ml ice-cold lysis buffer (5 mM Tris-HCl, PIC, pH 7.5) was added to the remaining reactions followed by shaking and incubation on ice 10 min, then 4.5 ml 10X TBS (200 mM Tris-HCl, 1.5 M NaCl, pH 7.5) was added to restore the pH and salt concentration and the samples were centrifuged at 3500 g, 20 min. The supernatants were transferred to thick-wall centrifugation tubes
TABLE 1 | Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
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<tr>
<td>S17-1pir</td>
<td>recA, thi, pro, hasdR&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, &lt;RP4:2-Tc::Mu::Ku::Tn7::Tp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YPIII&lt;sub&gt;C&lt;/sub&gt;</td>
<td>Plasmid cured strain</td>
<td>Bölin et al., 1982</td>
</tr>
<tr>
<td>YPIII pIB102</td>
<td>yadA; Km&lt;sup&gt;r&lt;/sup&gt; (wild type)</td>
<td>Bölin and Wolf-Watz, 1984</td>
</tr>
<tr>
<td>YPIII pIB155</td>
<td>plB102: lcrV full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Pettersson et al., 1999</td>
</tr>
<tr>
<td>YPIII pIB621</td>
<td>plB102: yopD full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Olsson et al., 2004</td>
</tr>
<tr>
<td>YPIII pIB615</td>
<td>plB102: yopB full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Bröms et al., 2003</td>
</tr>
<tr>
<td>YPIII pIB30</td>
<td>plB102: yopH full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>YPIII pIB526</td>
<td>plB102: yopE full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Ali et al., 2008</td>
</tr>
<tr>
<td>YPIII pIB15519</td>
<td>plB155: lcrV full-length in frame deletion; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Holmstrom et al., 2001</td>
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<td>YPIII pIB10201</td>
<td>plB102: lcrV&lt;sup&gt;+&lt;/sup&gt;1 frameshift mutation in codons 4–13; Km&lt;sup&gt;r&lt;/sup&gt; (V&lt;sup&gt;+&lt;/sup&gt;1)</td>
<td>Bröms et al., 2007</td>
</tr>
<tr>
<td>YPIII pIB10202</td>
<td>plB102: lcrV&lt;sup&gt;−&lt;/sup&gt;1 frameshift mutation in codons 2–15; Km&lt;sup&gt;r&lt;/sup&gt; (V&lt;sup&gt;−&lt;/sup&gt;1)</td>
<td>Bröms et al., 2007</td>
</tr>
<tr>
<td>YPIII pIB1550201</td>
<td>plB155: lcrV&lt;sup&gt;+&lt;/sup&gt;1; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>YPIII pIB1550202</td>
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<tr>
<td>YPIII pIB6210201</td>
<td>plB621: lcrV&lt;sup&gt;+&lt;/sup&gt;1; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>YPIII pIB6210202</td>
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<td>This study</td>
</tr>
<tr>
<td>YPIII pIB6150201</td>
<td>plB615: lcrV&lt;sup&gt;+&lt;/sup&gt;1; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>YPIII pIB6150202</td>
<td>plB615: lcrV&lt;sup&gt;−&lt;/sup&gt;1; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>YPIII pIB102YopH-Bla</td>
<td>Wild type, expressing YopH&lt;sub&gt;1&lt;/sub&gt;-Bla</td>
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<tr>
<td>YPIII pIB10201YopH-Bla</td>
<td>LcrV&lt;sup&gt;+&lt;/sup&gt;1, expressing YopH&lt;sub&gt;1&lt;/sub&gt;-Bla</td>
<td>This study</td>
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<tr>
<td>YPIII pIB10202YopH-Bla</td>
<td>LcrV&lt;sup&gt;−&lt;/sup&gt;1, expressing YopH&lt;sub&gt;1&lt;/sub&gt;-Bla</td>
<td>This study</td>
</tr>
<tr>
<td>YPIII pIB102 pYopH&lt;sub&gt;C403A&lt;/sub&gt;</td>
<td>Wild type, expressing catalytically inactive YopH</td>
<td>This study</td>
</tr>
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<td><strong>Plasmid</strong></td>
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<td>pJEB368</td>
<td>pDM4 lcrV&lt;sup&gt;+&lt;/sup&gt;1 frameshift encompassing codon 4–13</td>
<td>Bröms et al., 2007</td>
</tr>
<tr>
<td>pJEB369</td>
<td>pDM4 lcrV&lt;sup&gt;−&lt;/sup&gt;1 frameshift encompassing codon 2–15</td>
<td>Bröms et al., 2007</td>
</tr>
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<td>pMF024</td>
<td>pDM4 yopD full-length in frame deletion</td>
<td>Francis and Wolf-Watz, 1998</td>
</tr>
<tr>
<td>pMF463</td>
<td>pDM4 yopB full-length in frame deletion</td>
<td>Bröms et al., 2003</td>
</tr>
<tr>
<td>pDM4-ΔyopH</td>
<td>pDM4 yopH full-length in frame deletion</td>
<td>Westermark et al., 2014</td>
</tr>
<tr>
<td>pNQ-YopH&lt;sub&gt;C403A&lt;/sub&gt;-Bla</td>
<td>pNQ705 with YopH&lt;sub&gt;1&lt;/sub&gt;...&lt;sub&gt;468&lt;/sub&gt;-Bla&lt;sub&gt;24&lt;/sub&gt;...&lt;sub&gt;286&lt;/sub&gt; fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pYopH&lt;sub&gt;C403A&lt;/sub&gt;</td>
<td>Point mutation in the catalytic site of YopH, cloned under its own promoter</td>
<td>Persson et al., 1997</td>
</tr>
</tbody>
</table>

(Beckman) and spun at 100,000 g for 2 h at 4°C. The pellet was resuspended in 50% sucrose in TBS (20 mM Tris-HCl, 150 mM NaCl, PIC, pH 7.5) and sonicated. A step-wise gradient was established in ultra-clear centrifugation tubes by adding the resuspended sample on top of 2 ml 65% sucrose-TBS and overlay with 15 ml 44% sucrose-TBS followed by 5 ml 25% sucrose-TBS. Gradients were centrifuged at 15,000 g for 16 h at 4°C using a Sw-28 rotor (Beckman). The membranes were isolated from the 25/44% interface and washed with TBS before resuspension in 150 µl TBS. The total protein concentration was estimated in a NanoDrop spectrophotometer at A<sub>280</sub> and 20 µg was loaded on a 12% SDS-PAGE and analyzed by Western blot using YopD polyclonal antisera and YopB monoclonal antibody.

**Intracellular Localization of YopE and YopH**

A total of 10<sup>5</sup> HeLa cells were infected with induced *Yersinia* strains at MOI ranging from 2.5:1 to 40:1 and the level of cytotoxicity was observed in an inverted light microscope, repeatedly during up to 4 h post infection. For immunostaining a MOI of 25:1 was used. The cells were washed and fixed in 2% paraformaldehyde after 1.5 h infection. The cell membranes were stained with TexasRed conjugated wheat germ agglutinin (Molecular Probes, Invitrogen) before they were permeabilised in TSB (0.5% Triton X-100 in a buffer consisting of 4% PEG 6000, 1 mM EGTA and 100 mM Pipes pH 6.8). Intracellular YopE was detected using an affinity purified polyclonal YopE antibody followed by an Alexa488-conjugated secondary antibody (Molecular probes, Invitrogen). The nucleus
and bacteria were stained with DAPI. Images were taken with laser scanning confocal microscopy (Leica).

YopH translocation was analyzed using a beta-lactamase reporter system (Charpentier and Oswald, 2004; Marketon et al., 2005) where full length YopH was fused to beta-lactamase. HeLa cells were seeded in 35 mm glass bottom dishes (MatTek) 1 day before the experiment. Prior to infection the cells were labeled with the FRET substrate CCF4-AM (Invitrogen) according to the manufactures instructions. Induced Yersinia strains were added to the cells at a MOI of 50:1 and incubated 40 min at room temperature. Images were taken with a live cell microscope (Nikon Eclipse Ti-E), equipped with a true color camera, using a long pass filter to detect the two wave length of the FRET substrate.

**Proteinase K Protection Assay**

The experiment was performed essentially as described before (Nordfeldt and Wolf-Watz, 2001). In short, 5 × 10⁶ HeLa cells were infected with induced Yersinia strains at a MOI of 50:1 in the presence of Cytochalasin D (0.5 µg/ml). After infection the cells were washed in PBS and treated with 0.5 mg/ml Proteinase K (Roche Diagnostics, Gmbh) for 1 min. The cells were aspirated and incubated 20 min at room temperature, after which, 4 mM PMSF was added to inactivate remaining Proteinase K. The cells were lysed with 0.4% Digitonin in a total volume of 1 ml. Cell debris and bacteria were pelleted by centrifugation at 15,000 g, 10 min, 4°C. The supernatant was separated in a 12% SDS-PAGE and analyzed by Western blot using affinity purified antisera against YopE and YopH or monoclonal Tubulin antibody (Sigma Aldrich). The protein levels were quantified by use of the LAS4000 image reader and Multi Gauge-Image software (Fujifilm). The levels of YopE and YopH were normalized against Tubulin levels. The results were analyzed using the paired Student's t-test with the significance set at p ≤ 0.05*, p ≤ 0.01** and p ≤ 0.001***. NS, No significant difference.

**Isolation of Plasma Membrane from HeLa Cells**

A total of 10⁶ HeLa cells were infected with Yersinia strains at a MOI of 50:1 in 15 cm dishes. After 1 h at 37°C the cells were put on ice for 5 min and washed twice with ice cold PBS. The cells were scraped off in 5 ml ice-cold PBS and suspension from the duplicate dishes were pooled in a centrifugation tube. The cells were pelleted at 290 g, 5 min, resuspended in 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, and incubated on ice for 8 min after which the cells were pelleted again at 290 g, 5 min. The cells were resuspended in 0.3 ml 250 mM sucrose, 3 mM Imidazole and Complete Mini protease inhibitor cocktail (Roche) (total volume ~0.4 ml) and lysed by passing 40 times through a 23 G needle. The lysate was centrifuged at 900 g, 5 min and the supernatant was collected. The pellet was washed with 1 ml sucrose buffer and spun again at 900 g and the supernatant was pooled to the previous one and the samples were spun at 14000 g, 5 min. The resulting supernatant was centrifuged at 125000 g for 1 h using a Ti70.1 rotor (Beckman). The pellet containing the cell membranes was resuspended in 100 µL 2X SDS loading buffer. Equal amounts of protein were loaded on a 12% SDS-gel and analyzed with Western blot using YopD polyclonal antisera. The levels of YopD were quantified using the Multi Gauge-Image software (Fujifilm) and normalized to the unspecific band. The results were analyzed using the paired Student’s t-test with the significance set at p ≤ 0.05*, p ≤ 0.01** and p ≤ 0.001***. NS, No significant difference.

**Phagocytosis Inhibition Assay**

A total of 10⁵ J774a.1 cells were infected with Yersinia strains at a MOI of 20:1. After 30 min infection unattached bacteria were washed away with PBS and the cells were fixed in 0.4% paraformaldehyde. Extracellular bacteria were stained using Yersinia antisera followed by Alexa568-conjugated antibody (Molecular Probes, Invitrogen). The cells were permeabilised with 0.5% Triton X-100 and both extra- and intracellular bacteria were stained with Yersinia antisera followed by Alexa488-conjugated antibody (Molecular Probes, Invitrogen). Samples were viewed in a fluorescence microscope and the total amount of cell associated bacteria and extracellular bacteria were counted manually. The results were analyzed using the Wilcoxon signed-rank test. Each experiment was analyzed separately and significance was set at p ≤ 0.05*, p ≤ 0.01** and p ≤ 0.001***.

**Immunoprecipitation**

A total of 4 × 10⁶ HeLa cells were infected with Yersinia strains at a MOI of 100:1 in a small volume to create instant contact between bacteria and cells. The infection was terminated at different time points by washing two times with ice-cold PBS + 0.1 mM Na₃VO₄. The cells were lysed in ice-cold precipitation buffer [50 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Nonidet NP-40, 0.25% sodium deoxycholate, 1 mM Na₃VO₄ and Complete Mini protease inhibitor cocktail (Roche Diagnostics, Gmbh)] at 4°C for 20 min after which the cells were scraped off and centrifuged at 16000 g for 10 min. The lysate was pre-incubated with mouse IgG coated protein G Sepharose beads (4 Fast Flow, Amersham Biosciences, Sweden) at 4°C for 1 h. The beads were spun down and the pre cleared lysate was incubated with anti-FAK (clone 2A7, Upstate Biotechnology, Lake Placid, NY) coated proteinG sepharose beads at 4°C for 3 h. The beads were washed two times in precipitation buffer and the bound material was eluted in a small volume of 2X SDS loading buffer at 95°C for 5 min. Samples were subjected to SDS-PAGE and the amount of phosphorylated FAK was analyzed by Western Blot using a phosphotyrosine antibody (Clone 4G10) (Millipore). Images were acquired in a LAS4000 image reader and the signal intensity was quantified using Multi Gauge-Image software (Fujifilm). The results were analyzed using the paired Student’s t-test with the significance set at p ≤ 0.05*, p ≤ 0.01** and p ≤ 0.001***. NS, No significant difference.

**Mouse Infection Model**

Bacteria were grown overnight in unsupplemented LB at room temperature and harvested and diluted in PBS to final concentrations ranging from 10⁴ to 10⁶ bacteria/ml for the wild type and LcrV frameshift mutants. The attenuated ΔlcrV mutant was used as a negative control at concentrations 10⁶–10⁷ bacteria/ml. Five C57BL/6 mice were infected intraperitoneally
with 100 µl for each strain and dilution. Bacteria were plated on LA plates in parallel to determine the exact infection dose. The experiments were conducted in accordance with the ethical permission and all mice were monitored for symptoms twice daily and mice showing severe symptoms were sacrificed. Based on our previous experience mice with severe symptoms normally succumb to the infection within 24 h. The animal experiment was approved by the local animal ethics committee at Umeå University (Dnr A144-12).

**AUTHOR CONTRIBUTIONS**

SE, JB, TE, MF, MSF and ÅF conceived and designed the experiments. SE, JB, and ÅF performed the experiments. SE and JB analyzed the data. SE, MF, MSF and ÅF wrote the manuscript.

**REFERENCES**


FUNDING

This work was supported by grants from the Swedish Research Council, 2011-3439, and the Swedish Foundation for Strategic Research, SB12-0022.

**ACKNOWLEDGMENTS**

This study is dedicated to Roland Rosqvist, our dear friend and colleague. We acknowledge Ingergerd Söderström for help with the statistical calculations.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2016.00175/full#supplementary-material

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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