Rafts Can Trigger Contact-mediated Secretion of Bacterial Effectors via a Lipid-based Mechanism*

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Infection by the Gram-negative bacterial pathogen Shigella flexneri depends on its ability to invade host cells. Bacterial engulfment requires a functional type III secretion system (TTSS) allowing the translocation into host cells of bacterial effectors that activate cell-signaling cascades. We demonstrated previously that specialized lipid membrane domains enriched in cholesterol and sphingolipids (rafts) are involved during early steps of invasion, namely in binding and host cell entry. In this study, we addressed the issue of contact-mediated secretion by the TTSS. We show that contact-mediated and TTSS-induced hemolysis depend on the presence of cholesterol on the host cell surface. We found that purified detergent resistant membranes were able to activate TTSS. Finally, we found that artificial liposomes, devoid of proteins, were able to activate the TTSS but only when their composition mimicked that of lipid rafts. Altogether, these data indicate that specific lipid packing can trigger contact-mediated secretion by S. flexneri.

The Gram-negative bacterium Shigella flexneri is the etiological agent of human bacillary dysentery (1). Shigellosis is caused by invasion of epithelial cells of the colonic mucosa leading to an acute inflammatory reaction. Pathogenicity of Shigella is linked to a large virulence plasmid carrying the locus, considered as a pathogenicity island. The ipa and ipg genes encode effectors that mediate bacterial entry, as well as their chaperones. The mxi and spa genes encode the type III secretion system (TTSS)\(^1\) that is required to transport type III effectors from the bacterial cytoplasm into the host cell plasma membrane/cytosol (2). The IpaA-D and IpgP proteins trigger host signaling leading to actin cytoskeleton rearrangement and macropinocytic-like ruffle formation allowing the engulfment of the bacterium (3–5).

TTSSs, a wide spread feature of Gram-negative bacteria pathogenic for mammalian and plant hosts (5–9), constitutively assemble independently of contact with the host. Electron microscopy analysis of the Shigella TTSS indicated that it is composed of an internal cytoplasmic bulb, a transmembrane domain, and an external needle (2). The needle has a length reaching up to 500 Å with an external diameter of ~70 Å and an internal diameter of 20–30 Å (2, 10, 11) and is made up by a helical arrangement of its major component, i.e. the 10-kDa MxiH protein (10, 12, 13). TTSSs share features with the bacterium flagellum suggesting a common evolutionary origin (13, 14). Flagella are terminated by a flexible filament and a hook, the length of which is controlled by the fltK gene, i.e. FltK mutants show shorter hooks (15, 16). Interestingly, fltK is synthetically lethal to invJ in Salmonella and spa32 in Shigella, and spa32 mutants also show needle length defects (17–19). Recently, the Yersinia enterolitica yscP protein was shown to act as a molecular ruler with a strict linear relationship between the needle length and the number of amino acids in the central primary sequence of yscP (1.9 Å per amino acid) (20). Whether this mechanism applies also to FltK, InvJ, and Spa32 that behave as homologues remains to be demonstrated.

Although the TTSS of Shigella is preassembled, it does not secrete type III effectors (21) until it is activated by contact of the needle tip with the host cell surface (22, 23). Upon cell contact, IpaB and -C are thought to perforate the host cell membrane making a proteinaceous 25 Å in diameter pore as suggested by hemolysis assays using osmoprotectants of various sizes (2). Also, purified IpaB, IpaC, and IpaD are able to bind directly to liposomes (24, 25). Similarly, YopB and YopD, the Yersinia counterparts of IpaB and IpaC, possess hemolytic activity and channel-like activity in planar lipid bilayers (26–28). YopE and YopD can be replaced by the PopB and PopD of Pseudomonas (29). Interestingly, PopD was shown to form oligomeric complex and disrupts membranes at acidic pH (30). PopB and PopD together generate ring-like structures of 80 Å in outer diameter with a 40-Å-wide central hole (30).

We have recently shown that initial binding of S. flexneri and its subsequent cell entry critically depend on cholesterol and sphingolipid rich microdomains of the host cell membrane, so-called lipid rafts (31). A number of studies have now shown that rafts are preferential sites of entry for many bacteria (32) including Pseudomonas aeruginosa (33). Although raft cholesterol might be important for more than one aspect of invasion by these bacteria, it might be particularly important for perforation of the host cell membrane by the TTSS. This could in particular be the case for Pseudomonas because membrane lysis by PopB and PopD, in contrast to membrane binding, was found to be dependent on cholesterol, suggesting that rafts may play a key role in structural rearrangements leading to pore formation and membrane disruption (30). A similar mechanism could govern pore formation by IpaB and IpaC. Rafts would

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‡ The abbreviations used are: TTSS, type III secretion system; GFP, green fluorescent protein; GST, glutathione S-transferase; DRM, detergent-resistant membrane; RBC, red blood cell; MeCD, methyl-β-cyclo-dextrin; PNS, postnuclear supernatant; PBS, phosphate-buffered saline; SCRL, sphingolipid- and cholesterol-rich liposome.

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thus be important for the initial binding of *Shigella* (31) and for subsequent generation of the IpaB-IpaC pore. Here, we were interested in determining whether the intermediate step, contact-mediated secretion of IpaS, was also dependent on rafts.

**MATERIALS AND METHODS**

**Bacterial Strain, Eukaryotic Cells, and Reagents**

Bacteria used in this study were the invasive *S. flexneri* wild type M90T serotype 5 and an isogenic strain expressing the green fluorescent-protein (GFP-M90T) (34). Expression and purification of recombinant GST and GST-IpaB proteins were as described previously (31). For detergent-resistant membranes (DRMs) preparation we used HeLa cells. We also used the CD44 expression-deficient RPM-M cell line that is derived from a human recurrent cutaneous melanoma (35). The parental cells were stably transfected with the cDNA of the p85 CD44 standard form (35). The sphingolipid-based lipid deficient Chinese hamster ovary cDNA homolog of the yeast LCB1 gene (36, 37). Briefly, SPB-1 cells carry a temperature-sensitive mutation in the serine palmitoyltransferase enzyme that catalyzes the first step in the sphingolipid biosynthetic pathway, in which L-serine condenses with palmitoyl-CoA to produce 3-ketodihydrosphingosine. In SPB-1/LCB1 the serine palmitoyltransferase activity is restored with 0.5% Triton X-100 on ice for 30 min in TNE and then incubated for 30 min at 37 °C, then cells were centrifuged at 55,000 rpm (Sorvall RC M150GX) at 4 °C. Membranes from each fraction were pelleted by 30 min of centrifugation at 55,000 rpm (Sorvall RC M150GX) at 4 °C. These membranes were resuspended and incubated with bacteria. Proteins from each fraction were assayed (Bradford based protein assay, Bio-Rad).

**Liposome Preparation**

Multilamellar liposomes were prepared following a protocol described previously (42). Briefly, lipids were resuspended in CHCl₃, dried under N₂ gas, and resuspended in PBS at 85 °C. Lipids were vortexed for 30 s and incubated for 30 min, vortexed again and further incubated for 15 min at 85 °C, and finally allowed to cool to room temperature for at least 30 min. Liposomes used were: dioleoyl phosphatidylcholine:diopalmitoyl phosphatidylcholine, 1:1 (mole ratio); phosphatidylcholine:cholesterol, 7:1 (mole ratio). Liposomes were prepared with the corona containing phosphatidylcholine:sphingomyelin:ceresboids:cholesterol, 2:1:1:2 (mole ratio); and low SCRLs containing the same lipid in 56.7:5.5:33.3 mole ratio. That this protocol leads to multilamellar liposomes for all lipid compositions tested was controlled by electron microscopy (not shown).

**Activation of Secretion**

Secretion was analyzed according to an established assay using either cellular membranes or liposomes (43). Bacteria from overnight precultures diluted 1:100 and incubated at 37 °C under aerated condition in TSB until A₆₀₀ = 1 were used in the assays.

**Cellular Membranes**—Either step density fractions (yield analysis) or equal quantities (2 µg) of PNS and DRMs (enrichment analysis) were used in secretion assays. Bacteria (100 µl) washed in PBS were incubated with membranes at 37 °C for 45–60 min and further spun at 16,000 × g for 5 min. The secretion inducer Congo red (20 µM) was used as positive control (43). Proteins precipitated from supernatant using a MeOH/CHCl₃-MeOH procedure (44) and those from pellets were analyzed by immunoblotting.

**Liposomes**—100 µl of bacteria were pelleted and directly resuspended with 200 µg of liposomes, centrifuged 5 min at 1,500 × g, and incubated overnight at 37 °C. Samples were adjusted to 40% iodixanol (600 µl final) and layered with 25% (600 µl), 20% (400 µl), 10% (400 µl), and 5% iodixanol (200 µl), and PBS (200 µl). Samples were spun for 2 h at 55,000 rpm (Sorvall RC M150GX). Six fractions (400 µl each) were collected from the top and processed for immunoblotting analysis (proteins were precipitated as mentioned above), lipid extraction, or agarose plating.

For agar plate assay, 1 µl from an aliquot of each fraction, diluted 1:10, was plated on one agarose-TB-Congo red (0.01%) plate and incubated overnight at 37 °C. When experiments were performed with the M90T-GFP strain ampicillin (100 µg/ml) plates were used.

**Lipid Extraction and TLC Analysis**

For TLC analysis, liposomes or step density gradient fractions were adjusted to 800 µl PBS in a glass tube, mixed with 2 ml MeOH and 1 ml CHCl₃, and vortexed and incubated for at least 30 min. Then, 1 ml of CHCl₃ and 1 ml of acid salin (0.9% NaCl, 10 µm HCl) were added to the mix, vortexed, and incubated for 10 min before centrifugation for 5 min at 100 × g. The lipidic (lower) phase was transferred to a new glass tube and dried under N₂ flux. Migration was performed in a glass box saturated with a CHCl₃:MeOH:IPA 90:10:0.1 (v/v/v) mix and spotted with a capillary on a TLC plate (silica gel 60, Merck). Migration was performed in a glass box saturated with a CHCl₃:MeOH:NH₂OH 10:1:1.5 (v/v/v) mix. Dried plates were shortly (1 min) immersed in 8% H₃PO₄-CuAc (3g/100 ml), dried, and incubated at 160 °C. For cholesterol extraction efficiency determination, a quantitative TLC analysis was performed as described previously (45).

**RESULTS**

**Role of Cholesterol in Contact-mediated TTSS-induced Hemolysis**—The mechanism of contact-mediated secretion of *Shigella* effectors was studied using contact-mediated hemolysis, which was shown to be TTSS- and IpaB-, IpaC-, and IpaD-de-
pendent (2, 21, 46–48). Contact between wild-type S. flexneri (M90T strain) and RBCs was induced by centrifugation at 1,500 \( \times \) g, shown previously to be optimal (2). Centrifugation was indeed shown to deform the RBC membrane in a way that maximizes the interaction with the bacterial membranes (2).

We observed that after extraction of 40–60% of cellular cholesterol with MeCD (Fig. 1B), RBCs were far more resistant to contact-mediated hemolysis induced by S. flexneri (Fig. 1A). Indeed, this treatment was rather dramatic as hemolysis was reduced to about 10% for RBCs treated with 1.0 mM MeCD and infected at a multiplicity of infection of 20 (Fig. 1A). For the concentrations (0.5 mM and 1.0 mM) and treatment time (20 min at 37 °C) used, MeCD had no detectable hemolytic activity per se (data not shown). At concentration of MeCD higher than 1 mM, we noticed RBC hemolysis that can be explained by the recent study using atomic force microscopy showing that 1.25 mM MeCD leads to the formation of holes in model membranes (49).

**Detergent-resistant Membranes Elicit TTSS Activation**—The above results show a requirement for cholesterol in contact-mediated S. flexneri TTSS-dependent hemolysis suggesting a possible involvement of lipid rafts. Because rafts have the biochemical property of being insoluble in detergents at 4 °C (50), we next investigated whether purified DRMs could trigger TTSS-mediated secretion. As a positive control for secretion, we used Congo red, well documented as a potent inducer of secretion for TTSS (22, 23, 43, 51–53). Cells were solubilized in Triton X-100 at 4 °C and submitted to a step density floatation. Following a high speed centrifugation, sedimented material from each fraction was (A) submitted to protein quantification (n = 4, error bars represent the standard deviation) or (B) incubated with S. flexneri for 1 h at 37 °C prior to pelleting bacteria. Pellet and supernatant were then processed for immunoblotting. The same procedure was applied to MeCD-treated cells. CR, Congo red; TE, total extract; Ctrl, control (i.e. bacteria alone).
mitted to high speed centrifugation to recover the membranes, which were then resuspended. Each of these samples was submitted to protein quantification (Fig. 2A) and incubated with bacteria. The bacteria were then separated from the host cells membranes by low speed centrifugation, and both the pellet and the supernatant were analyzed by immunoblotting for the presence of IpaB used as a read out for secretion (Fig. 2B). The raft marker flotillin was used as control for DRM quality and was efficiently triggered secretion of IpaB, with the observation that cells that do not express CD44 can be infected by S. flexneri albeit with lower efficiency (54). Similar results were obtained with IpaC (data not shown).

Efficient DRM-triggered TTSS Activation Requires Sphingolipids—We have shown previously that S. flexneri entry was impaired into the deficient in sphingoid-based lipid biosynthesis SPB-1 cells (31). Therefore, we tested whether DRM4 contributed to TTSS activation. We used the CD44 deficient RPM-MC cell line and the cell line recomplemented by stable transfection with the p85 CD44 standard form (35). The absence of CD44 (Fig. 3B, top panel) only mildly affected the ability of DRMs to trigger IpaB secretion (Fig. 3B, middle and bottom panels) in agreement with the observation that cells that do not express CD44 can be infected by S. flexneri albeit with lower efficiency (54). Similar results were obtained with IpaC (data not shown).

To facilitate the comparison of the capacity of DRMs from different cell lines to activate secretion, we analyzed this capacity relative to that of a PNS using equal amounts of protein for each. As shown in Fig. 3A for HeLa cells, the ability to trigger secretion of IpaB was 2-fold higher for DRMs versus PNS. A similar observation (even more pronounced) was made for IpaC (Fig. 3A).

**DRM-triggered TTSS Activation Is Independent of CD44**—It has been shown previously that the hyaluronate receptor, CD 44, is able to mediate adhesion of Shigella to host cells (54), that this transmembrane protein partitions into rafts in epithelial cells (55), and that it is recruited, in conjunction with IpaB, to rafts upon infection (31). Therefore, we tested whether CD44 contributes to TTSS activation. We used the CD44 deficient RPM-MC cell line and the cell line recomplemented by stable transfection with the p85 CD44 standard form (35). The absence of CD44 (Fig. 3A) only mildly affected the ability of DRMs to trigger IpaB secretion (Fig. 3B, top panel) in agreement with the observation that cells that do not express CD44 can be infected by S. flexneri albeit with lower efficiency (54). Similar results were obtained with IpaC (data not shown).

**Raft-like Liposomes Activate TTSS**—The above-described experiments show that purified DRMs promote TTSS-mediated secretion and that this activity is diminished when cholesterol or sphingolipid levels are reduced, highlighting the importance of lipids in the process. Therefore, we decided to test whether artificial liposomes mimicking lipid rafts, or not, could trigger secretion. We tested four types of liposomes: dioleoyl phosphatidycholesterol liposomes mimicking lipid rafts, or not, could trigger secretion. We tested four types of liposomes: dioleoyl phosphatidylcholines with a palmitoyl group at the C2 position, dioleoyl phosphatidylcholines with a palmitoyl group at the C3 position, and dioleoyl phosphatidylcholines with a palmitoyl group at the C4 position. These liposomes were then incubated with bacteria, and the ability of DRMs to trigger secretion was assessed by immunoblotting for the presence of IpaB and IpaC in the supernatant and in the pellet. The results showed that the ability of DRMs to trigger secretion was abolished when the palmitoyl group was present at the C3 position, indicating that the presence of the palmitoyl group at the C2 position is required for DRM-mediated activation of TTSS.

**Raft-triggered Type III-mediated Secretion**
lipid: dipalmitoyl phosphatidylcholine and phosphatidylcholine: cholesterol liposomes in which no liquid-ordered phases form, SCRLs in which liquid-ordered domains do form (42) and a second type of SCRLs, low SCRLs, which have the same molar ratio of cholesterol as SCRLs but a 3-fold lower molar ratio of sphingolipids and thereby are less prone to the formation of liquid-ordered domains (42). Bacteria were incubated with liposomes and then centrifuged at low speed to promote contact as for the previously described contact mediated hemolysis assay. Samples were submitted to gradient density centrifugation to separate the bacteria from the liposomes (Fig. 4A). The efficiency of the separation was assessed by plating each fraction on agar plates. Irrespective of the type of liposomes used, bacteria were absent from the top fraction (Fig. 4B). This was confirmed by immunoblotting against GFP (GFP expressing Shigellas were used), which showed that GFP was found exclusively in fractions 3–6 (Fig. 4C). Interestingly, upon incubation of bacteria with SCRLs but not with other liposomes, a significant amount of IpaB was recovered in fraction 1 (Fig. 4C). Because soluble proteins remained in the bottom fractions of the gradient where the initial sample was loaded, the presence of IpaB in fractions 1 reflects the association with liposomes, which have low buoyancy. That SCRLs were indeed present mainly in fraction 1 and to a lesser extent in fraction 2, independent of their incubation with bacteria, is clearly illustrated in the experiment shown in Fig. 4D where the lipid content of each fraction from the density gradient was analyzed by one dimension thin layer chromatography. Interestingly, upon incubation with bacteria, some liposomes were trapped in fraction F4 (compare F4 in Fig. 4D left versus middle panel). Fig. 4E shows the IpaB secretion in the exact same experiment and also illustrates that in the absence of liposomes, IpaB does not float to the top of the density gradient. MxiH, a
Raft-triggered Type III-mediated Secretion

**DISCUSSION**

A variety of pathogenic bacteria for both animals and plants make use of a type III secretion system to inject bacterial proteins into the host cell to divert fundamental processes. An intriguing question that has remained unanswered is what signal triggers secretion from the preassembled TTSS. Because we have previously found that specialized cholesterol- and sphingolipid-rich domains from mammalian cells were required for efficient binding of *S. flexneri* and subsequent invasion, here we investigated whether lipid rafts might have an additional role in the invasion process, namely triggering secretion of effectors. We first showed that TTSS-dependent contact-mediated hemolysis by *Shigella* was sensitive to cholesterol extraction suggesting that the two effectors that mediate hemolysis, IpaB and IpaC, might be secreted in a cholesterol dependent manner or require cholesterol for membrane insertion.

A similar cholesterol requirement seems to apply to other TTSS harboring bacteria. In the contact-dependent hemolysis observed with *P. aeruginosa* cystic fibrosis isolates that depend on the TTSS for virulence, the PopB/PopD-secreted proteins indeed require cholesterol to form pores and disrupt membranes (30, 56, 57). Cholesterol-rich domains might also be required during infection by the enteropathogenic *Escherichia coli*, which involves the TTSS secreted proteins EspB, and EspD (58–61). Enteropathogenic *E. coli* TTSS also allows translocation into the host cell of Tir (translocated intimin receptor) that upon phosphorylation by a host kinase orchestrates cytoskeleton rearrangements (62). Enteropathogenic *E. coli* and mutants lacking a functional Tir protein are unable to trigger actin polymerization leading to full pedestal formation but are able to recruit raft-associated proteins at bacterial contact sites (63). This suggests that rafts act at an early step, possibly activating the secretion of the TTSS upon contact with the host surface.

In addition to cholesterol, we found that the presence of sphingolipids was important for DRMs to express their full secretory-activating capacity. In contrast, despite the fact that CD44 can mediate adhesion of *Shigella* to host cells (54) and partition within rafts upon infection (31), it did not interfere in

component of the needle, was used as a marker for the bacteria (10, 12).

The above experiments show that IpaB secretion is triggered by raft-like liposomes and that IpaB can associate with these liposomes, because it would otherwise not migrate to the top of the gradient. IpaB was not found in low buoyant fractions after incubation of bacteria with non-raft liposomes, because of either the lack of secretion or the subsequent inability of IpaB to associate with these membranes. To clarify this issue, we incubated bacteria with liposomes at 37 °C for 1 h and then spun down the bacteria by low speed centrifugation. Congo red was used as positive control. IpaB could be detected only in the supernatant containing SCRL, raft-like, liposomes (Fig. 5A) indicating that in Fig. 4, C and E, secretion with non-raft like liposomes did not occur. Then, we performed the complementary experiment, i.e. analyzed the capacity of purified IpaB to associate with the different type of liposomes. Liposomes (50 µg) were incubated with 100 ng of recombinant GST-IpaB for 1 h at 37 °C and then were separated from the soluble proteins by floatation on a density gradient. GST-IpaB was associated with the low density liposomes containing fractions only when using SCRLs, in marked contrast to what was observed with the phosphatidylcholine:cholesterol vesicles and in the absence of vesicles (Fig. 5B). This was not caused by the GST fusion because GST alone did not associate with SCRLs (Fig. 5C). Altogether, these experiments show that only raft-like liposomes are able to trigger IpaB secretion in these assays and moreover that IpaB, once secreted, preferentially associates with raft-like membranes.

**FIG. 5.** Raft-like liposomes induce IpaB secretion and promote its membrane association. A, liposomes and M90T-GFP bacteria were incubated at 37 °C for 1 h. After centrifugation, pelleted bacteria and the secreted material in the supernatant were analyzed by immunoblotting with anti-IpaB antibody. Congo red (20 µM) was used as positive control. Quantitation of three experiments is shown; error bars represent standard deviations. B, liposomes (50 µg) and purified recombinant GST-IpaB (100 ng) were incubated for 1 h at 37 °C, and the material was adjusted to 40% iodixanol in PBS and overlaid with a cushion of 30, 20, 10, 5, and 0% iodixanol. After centrifugation, fractions were collected from the top and protein was precipitated and analyzed by immunoblotting with the anti-IpaB antibodies. Note that only in presence of SCRLs can GST-IpaB be detected in the first fraction. GST-IpaB stays in the bottom fractions (F5 and F6) when submitted to step density floatation in the absence of liposomes. The absence of IpaB staining in the first fraction. GST-IpaB stays in the bottom fractions (F5 and F6) when submitted to step density floatation in the absence of liposomes. The absence of IpaB staining in F4 in the presence of SCRLs is caused by an experiment-to-experiment variation in the distribution of material between fractions 4–6. However, labeling was reproducibly evident in fractions 1–3 in the presence of SCRLs. C, the same experiment as in B was repeated when incubating GST with SCRLs and fractions were immunoblotted using anti-GST antibodies. GST remained in the bottom fractions indicating that GST itself does not stick to SCRLs.
secretion as measured in this study in agreement with the fact that it is not absolutely required for Shigella infection. Because no protein as yet been found to be essential for Shigella binding to the cell surface and secretion, we focused on the lipid components involved during initial steps of Shigella infection.

The insertion of bacterial effectors into liposomes has been investigated in several reports using reconstituted systems (25, 28, 30, 64). From these data, it appeared that oligomerization of lipid rafts can induce channel formation into the target lipid membrane. Especially, the subsequent vesicle lysis was shown to be cholesterol-dependent in the case of the PopB/PopD oligomers (30). The importance of the membrane lipid composition on the secretion process itself has never been addressed. The most remarkable and unexpected finding of this work is therefore the ability of protein-free artificial liposomes to trigger contact-mediated secretion by the TTSS. Interestingly, this secretion-activating capacity was restricted to liposomes having a lipid composition that allows the formation of liquid-ordered domains, mimicking lipid rafts. Therefore, specific lipid packing appears to be sufficient to trigger secretion. This, however, does not exclude the possibility that some host proteins further promote or regulate the secretion process. From a technical point of view, this in vitro system using artificial liposomes provides a useful assay for future studies on pore formation by IpaB and IpaC and the testing of host proteins that regulate this process. We also show that the step subsequent to IpaB secretion, namely membrane association, also preferentially occurs with raft-like domains, as do many pore-forming bacterial proteins (32).

Our data suggest that the TTSS of Shigella and possibly of other Gram-negative bacteria is sensitive to packing of lipids in the host cell membranes and shed new light on the mechanism involved in TTSS insertion in target cells. Combined with our previous work (31), this study shows that Shigella is critically dependent on cholesterol-rich raft-like membranes for three of the very early events in bacterial invasion: binding, activation of the TTSS, and perforation of the host plasma membrane to allow injection of effectors into the cytoplasm. Maybe this is not the last role of rafts in Shigella infection. Indeed, because the TTSS also plays a role during escape of Shigella from the phagosomal vacuole, and because effectors of other TTSS containing bacteria, such as Salmonella enterica, have been found associated with DRMs of phagosomal vacuoles (65), it will be of interest to investigate whether rafts promote escape of Shigella into the cytosol.

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