The type III secretion system apparatus determines the intracellular niche of bacterial pathogens

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Upon entry into host cells, intracellular bacterial pathogens establish a variety of replicative niches. Although some remodel phagosomes, others rapidly escape into the cytosol of infected cells. Little is currently known regarding how professional intracytoplasmic pathogens, including *Shigella*, mediate phagosomal escape. *Shigella*, like many other Gram-negative bacterial pathogens, uses a type III secretion system to deliver multiple proteins, referred to as effectors, into host cells. Here, using an innovative reductionist-based approach, we demonstrate that the introduction of a functional *Shigella* type III secretion system, but none of its effectors, into a laboratory strain of *Escherichia coli* is sufficient to promote the efficient vacuole lysis and escape of the modified bacteria into the cytosol of epithelial cells. This establishes for the first time, to our knowledge, a direct physiologic role for the *Shigella* type III secretion apparatus (T3SA) in mediating phagosomal escape. Furthermore, although protein components of the T3SA share a moderate degree of structural and functional conservation across bacterial species, we show that vacuole lysis is not a common feature of T3SA, as an effectorless strain of *Yersinia* remains confined to phagosomes. Additionally, by exploiting the functional interchangeability of the translocator components of the T3SA of *Shigella*, *Salmonella*, and *Chromobacterium*, we demonstrate that a single protein component of the T3SA translocon—*Shigella* IpaC, *Salmonella* SipC, or *Chromobacterium* CipC—determines the fate of intracellular pathogens within both epithelial cells and macrophages. Thus, these findings have identified a likely paradigm by which the replicative niche of many intracellular bacterial pathogens is established.

type III secretion system | phagosomal escape | vacuole lysis | *Shigella* | *Salmonella*

Intracellular bacterial pathogens use a variety of elaborate means to survive within host cells. Postinvasion, some such as *Legionella*, *Salmonella*, and *Chlamydia* species modify bacteria-containing vacuoles to avoid death via phagosomal acidification or lysosomal fusion. Others, including *Shigella*, *Listeria*, *Rickettsia*, and *Burkholderia* species, rapidly escape from phagosomes into the cytosol of infected cells. Although escape from phagosomes by the classic intracytoplasmic Gram-positive bacterium *Listeria monocytogenes* is well understood (1), much less is known regarding how Gram-negative pathogens, including the model professional intracytoplasmic *Shigella* species, enter the cytosol.

During the course of an infection, many Gram-negative pathogens, including *Shigella*, *Salmonella*, enteropathogenic *Escherichia coli*, and *Yersinia* species, use type III secretion systems (T3SSs) as injection devices to deliver multiple virulence proteins, referred to as effectors, directly into the cytosol of infected cells (2). T3SSs are composed of ∼20 proteins and sense host cell contact via a tip complex at the distal end of a needle filament, which then acts as a scaffold for the formation of a translocon pore in the host cell membrane. Although components of their type III secretion apparatus (T3SA) are relatively well conserved, each pathogen delivers a unique repertoire of effectors into host cells, likely accounting for the establishment of a variety of replicative niches. For example, *Salmonella* and *Shigella* secreted effectors promote the uptake of these bacteria into nonphagocytic cells, whereas those from *Yersinia* inhibit phagocytosis by macrophages.

All four pathogenic *Shigella* species—*Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, and *Shigella dysenteriae*—deliver ∼30 effectors into host cells, the majority of which are encoded on a large virulence plasmid (VP) alongside the genes for all of the proteins needed to form a T3SA (3). These secreted proteins play major roles in *Shigella* pathogenesis, including host cell invasion and modulation of innate immune response. One effector, IpgD, promotes the efficiency of *Shigella* phagosomal escape, although it is not absolutely required for this process (4). Interestingly, IpaB and IpaC, components of the *Shigella* translocon, the portion of the T3SA that inserts into the host cell membrane, have been implicated to mediate phagosomal escape based on the behavior of recombinant proteins (5–7). The physiologic relevance of these findings has not yet been directly addressed, as strains that lack either of these two proteins are completely impaired in the delivery of *Shigella* effectors into host cells (8).

Here, using a reductionist approach, we directly tested a role for the *Shigella* translocon apparatus in phagosomal escape. Using an innovative reengineering approach, we introduced a functional effectorless *Shigella* T3SA into a nonpathogenic laboratory strain of DH10B *E. coli.* Remarkably, upon entry into host epithelial cells, these bacteria efficiently escape from phagosomes. This demonstrates for the first time, to our knowledge, in the context of an infection, a direct role for the *Shigella* T3SA in mediating vacuole lysis. Despite structural conservation across T3SS families, we further observed that, in the absence of any type III effectors, the Ysc T3SA mediates little to no *Yersinia*

Significance

Bacterial pathogens use a variety of strategies to evade host cell innate immune responses. For some, this includes the establishment of an intracellular replicative niche. Although many intracellular pathogens remodel phagosomes to support bacterial replication, others lyse their internalization vacuole to reside within the host cell cytosol. Little is currently known regarding how bacteria, particularly Gram-negative pathogens, mediate phagosomal escape. Using complementary reductionist and functional interchangeability experimental approaches, we demonstrate that the type III secretion system machinery itself directly modulates the extent to which bacteria escape from phagosomes. Given the high prevalence of type III secretion systems among intracellular bacterial pathogens, these studies have identified a potential means by which the intracellular niche of Gram-negative bacteria is defined.

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phagosomal escape, suggesting that not all injectisomes have equivalent functions. Lastly, by exploring the functional interchangeability of translocon components of the *Shigella*, *Salmonella*, and *Chromobacterium* T3SA, we demonstrate that one translocon protein controls the extent to which these intracellular pathogens escape into the cytosol of infected cells, thus demonstrating a major role for the T3SA in determining the site of the replicative niche of intracellular bacteria.

Results

mT3_*E. coli* Invade HeLa Cells at Levels Similar to WT *Shigella*. We recently reported the development of mT3 *E. coli*, laboratory strains of *E. coli* (i.e., DH10B) that express a functional *Shigella* T3SS (9). These strains carry a 31-kb region of the 220-kb *S. flexneri* VP, either on a plasmid (pmT3SS) or chromosomally integrated, plus a plasmid that encodes either the master *Shigella* T3SS transcriptional regulator, VirF, or its downstream target, VirB (10). All of the genes needed to form a functional T3SA, and four effectors are present within this fragment of transplanted *Shigella* DNA (Fig. 1*A*). Three of the effectors—IpaA, IpgB1, and IpgD—play roles in the invasion of *Shigella* into nonphagocytic epithelial cells (11), likely accounting for the ability of mT3 *E. coli* strains, like wild-type (WT) *Shigella*, to efficiently invade epithelial cells (9).

Here, we focused our efforts on further characterization of one of these strains, a variant of DH10B *E. coli* whereby the activity of T3SS is controlled by an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible allele of *virB*, a strain hereafter referred to as mT3_*E. coli* (Fig. 1*A*). As shown in Fig. S1, this strain as well as DH10B *E. coli* that carry the intact 220-kb *Shigella* VP (VP_*E. coli*) synthesize and secrete the three exported components of the *Shigella* T3SS—IpaD, the injectisome needle tip protein, as well as IpaB and IpaC—which form a translocon pore in the host membrane, at levels equivalent to WT *Shigella*.

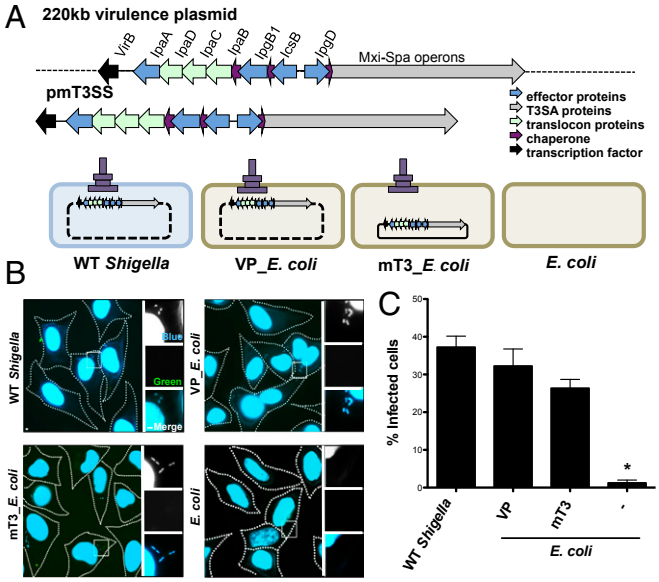

Fig. 1. mT3_*E. coli* efficiently invade epithelial cells. (*A*) Schematic of plasmids and strains. The dashed line represents the region of VP encoded outside of that present in mT3_*E. coli,* which encodes ∼21 *Shigella* effectors. (*B*) Representative images of HeLa cells infected with the designated strains that have been differentially labeled at 1 h p.i. using inside/outside microscopy assay to identify intra- (blue) versus extracellular (blue/green) bacteria. The HeLa cell perimeter is denoted by a dashed line. The enlarged images are from the boxed region in the larger panel. (*C*) Quantification of the percentage of cells that contain intracellular bacteria. For each infection, 100 HeLa cells were counted. *$P < 0.05$ for strains versus WT *Shigella* as determined by one-way ANOVA with Dunnett's post hoc analysis. Data are the mean ± SEM of four independent experiments.

We next assessed the functional activity of the *Shigella* T3SS in *E. coli* by comparing the ability of WT *Shigella*, VP_*E. coli*, and mT3_*E. coli* to invade epithelial cells, an early T3SS-dependent step. An inside/outside microscopy-based assay was used to differentiate between internalized and cell-associated bacteria to determine the percentage of HeLa cells that contain intracellular bacteria (Fig. 1*B*). At 1 h postinfection (p.i.), only strains that express a functional T3SS, mT3_*E. coli*, VP_*E. coli*, and WT *Shigella*, but not unmodified DH10B *E. coli*, were observed within epithelial cells (Fig. 1*C*). WT *Shigella*, VP_*E. coli*, and mT3_*E. coli* invaded epithelial cells at nearly equivalent levels (37% vs. 32% vs. 26%, respectively). Together, the results of the secretion and invasion assays demonstrate that the *Shigella* T3SS is expressed and exhibits similar levels of activity in the context of mT3_*E. coli*, VP_*E. coli*, and WT *Shigella*.

mT3_*E. coli* Efficiently Escape from Phagosomes Within Epithelial Cells. Using two complementary assays, we next investigated the intracellular fate of mT3_*E. coli*. Given our prior observation that these bacteria replicate poorly within host cells (9), we were interested in determining whether these bacteria, like WT *Shigella*, escape from phagosomes into the cytosol of epithelial cells. First, we investigated the sensitivity of intracellular WT *Shigella*, mT3_ *E. coli*, and VP_*E. coli* to chloroquine (CHQ), an antimicrobial agent that only reaches bactericidal levels when concentrated within phagosomes (12). When exposed to CHQ at 1 h p.i. for 1 h, almost all intracellular *Shigella* (90%) exhibited CHQ resistance (CHQ^R) (Fig. 2*A*), consistent with the observation that *Shigella* rapidly escape from phagosomes within epithelial cells (13). Similarly, 94% of VP_*E. coli* and 79% of mT3_*E. coli* exhibited CHQ^R, suggesting that these strains, like WT *Shigella*, also enter the cytosol of epithelial cells. In contrast, only 1% of DH10B *E. coli* that express *Yersinia* Invasin (Inv) (Inv_*E. coli*), a bacterial adhesin that promotes the uptake of *E. coli* into epithelial cells (14), exhibited CHQ^R.

To confirm the CHQ^R findings, we performed a previously established digitonin permeabilization fluorescent microscopy assay to deliver anti-LPS antibodies into the cytosol in order to directly visualize the location of intracellular bacteria within epithelial cells at 1 h p.i. (15, 16). As predicted, and consistent with our CHQ^R data, almost all WT *Shigella* (87%), as well as the majority of VP_*E. coli* (89%) and mT3_*E. coli* (69%), were detected by anti-LPS antibodies, indicating these bacteria were free in the cytosol or within phagosomes with compromised membranes (Fig. 2 *B* and *C*). However, only 4% of Inv_*E. coli* were labeled and thus the majority remained confined to intact phagosomes. The results from the CHQ^R and digitonin permeabilization assays demonstrate that all of the genetic information necessary to promote the uptake and entry of bacteria into the cytosol of host epithelial cells is present within the 31-kb fragment of *Shigella* DNA introduced into mT3_*E. coli*.

mT3Δeff_*E. coli* Are Unable to Attach to or Invade Epithelial Cells. The 31-kb region of *Shigella* DNA present in mT3_*E. coli* encodes proteins needed to form a functional T3SA and four effectors, three of which—IpgB1, IpgD, and IpaA—play roles in mediating host cell invasion and one—IpgD—that promotes phagosomal escape (4). Furthermore, studies with purified IpaB and IpaC have implicated these proteins in mediating vacuole lysis (5–7); however, given the absolute requirement of the IpaB–IpaC translocon for the delivery of effectors into host cells, a direct physiologic role for either protein in phagosomal escape has not yet been possible to assess.

To investigate whether effectors or the T3SA mediate phagosomal escape, we conducted a series of genetic modifications (Fig. S2 and *SI Materials and Methods*) to remove genes encoding the four effectors to generate mT3Δeff_*E. coli*, a variant of DH10B *E. coli* whereby the activity of an effectorless T3SS is controlled by an IPTG-inducible allele of VirB (Fig. 3*A*). As shown in Fig. S3*A*,

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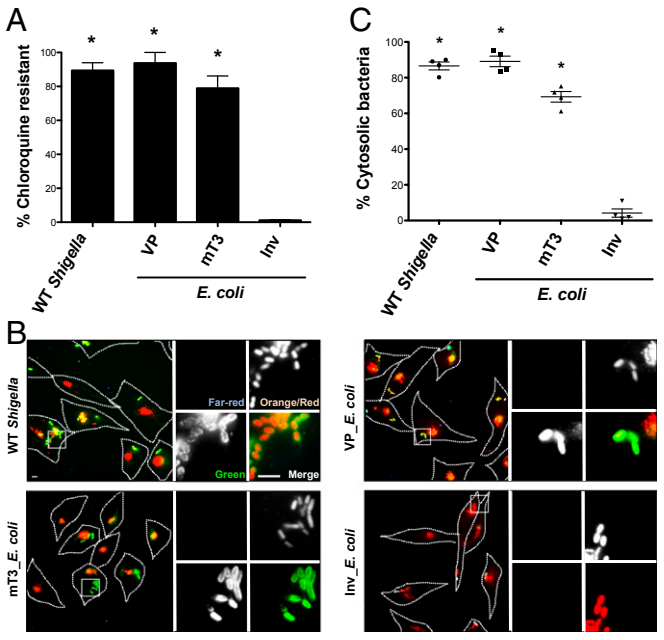

Fig. 2. mT3_*E. coli* efficiently escape from phagosomes in epithelial cells. HeLa cells were infected with the designated strains [multiplicity of infection (MOI) of 100 (WT *Shigella*, VP_*E. coli*, mT3_*E. coli*) or 10 (Inv_*E. coli*)] grown under conditions that induce expression of their T3SSs and assessed for phagosomal escape. (*A*) CHQ^R assay. HeLa cells were infected for 1 h and then treated with gentamicin (gent) ± CHQ for an additional 1 h before cell monolayers were lysed and bacteria enumerated. The percentage of CHQ^R bacteria was calculated as the ratio of (CHQ^R + $gent^R$)/($gent^R$) bacteria. Infections were conducted in duplicate for each experiment. (*B*) Representative images of infected cells visualized using the digitonin permeabilization assay. HeLa cells were infected with variants of the designated strains that constitutively express mCherry. Before fixation, cells were stained with Alexa Fluor 647 (far-red) goat-conjugated antibacterial antibodies to identify extracellular bacteria. Monolayers were then treated with digitonin to selectively permeabilize the plasma membrane and labeled with Alexa Fluor 555 (orange)-conjugated anti-GM130 (peripheral Golgi matrix protein) antibodies and antibacterial primary and Alexa Fluor 488 (green) secondary antibodies to identify permeabilized cells and label intracytoplasmic and extracellular bacteria (green). The enlarged images are from the boxed region in the larger panel. (*C*) Quantification of the percentage of cytoplasmic bacteria as defined by the ratio of intracytoplasmic to total intracellular (green/red)/(green/red + red) bacteria associated with digitonin-permeabilized (GM130-positive) cells. For each infection, 100 intracellular bacteria were counted; extracellular bacteria (far-red channel) were excluded from calculations. (*A* and *C*) $*P < 0.05$ for strains versus Inv_*E. coli* as determined by one-way ANOVA with Dunnett's post hoc analysis. The mean ± SEM of four independent experiments is shown.

mT3Δeff_*E. coli* secretes IpaB, IpaC, and IpaD at levels similar to WT *Shigella*. This strain expresses a fully functional translocon apparatus, as exogenously supplemented effectors are not constitutively secreted (a phenotype associated with *ΔipaB Shigella*) but rather are released from the bacteria only in the presence of the dye Congo red (CR), an in vitro activator of the *Shigella* T3SS (17) (Fig. S3*B*).

We next investigated whether this strain, like mT3_*E. coli*, can invade epithelial cells. Using the inside/outside microscopy assay, we observed very few mT3Δeff_*E. coli* bacteria within, or associated with, epithelial cells at 1 h p.i. (Fig. 3 *B* and *C*). Given our interest in assessing a role for the *Shigella* translocon apparatus in mediating phagosomal escape, we tested modifications that might promote the uptake of mT3Δeff_*E. coli* into host cells (Fig. 3 *B* and *C*). First, we investigated the fate of mT3Δeff_*E. coli* that express AfaI, an afimbrial adhesin from uropathogenic *E. coli* that promotes attachment to the extracellular surface of mammalian cells (18). This modification resulted in the adherence to, but not uptake of, mT3Δeff_*E. coli* into host cells. In contrast, mT3Δeff_Inv_*E. coli*, mT3Δeff_*E. coli* that express *Yersinia* Inv, entered HeLa cells at levels similar to Inv_*E. coli* (Fig. 3 *B* and *C*) and mT3_*E. coli* (Fig. 1*C*) (22% vs. 27% vs. 26%, respectively).

The *Shigella* T3SA Is Sufficient to Mediate Phagosomal Escape. Using the invasion-competent mT3Δeff_*E. coli* (mT3Δeff_Inv_*E. coli*), we next investigated the intracellular fate of bacteria expressing an effectorless *Shigella* T3SA using the CHQ^R and digitonin permeabilization assays. When infected cells were treated with CHQ at 1 h p.i. for 1 h, both VP_Inv_*E. coli* and mT3Δeff_Inv_*E. coli* exhibited high levels of CHQ^R, 93% and 99%, respectively, suggesting that both strains efficiently escape from their internalization vacuole into the cytosol of epithelial cells (Fig. 4*A*). Using the digitonin permeabilization assay, we similarly observed substantial numbers of intracytoplasmic mT3Δeff_Inv_*E. coli*, albeit at slightly lower levels than VP_Inv_*E. coli* (62% vs. 92%). In contrast, very few Inv_*E. coli* (4%) were detected within the cytosol of host cells, confirming that Inv alone in *E. coli* does not mediate phagosomal escape (14) (Fig. 4 *B* and *C*).

Inv mediates uptake of *Yersinia* into host cells via a "zipper"-type mechanism mediated by interactions with α5β1 integrins, a pathway that could potentially affect mT3Δeff_Inv_*E. coli* vacuolar lysis (19). To address this possibility, we developed a means to introduce mT3_*E. coli* into epithelial cells via a *Shigella*-like "trigger"-type mechanism (19). By sequentially deleting regions of DNA that encode three effectors present in mT3_*E. coli*, we generated mT3Δeff_IpaA_*E. coli*, a strain that encodes only a single effector (IpaA) (Fig. S4*A*). mT3Δeff_IpaA_*E. coli* invade epithelial cells about half as well as WT *Shigella* (Fig. S4*B*) (20% vs. 37%). The invasion defect is likely solely due to the absence of the two deleted effectors that are involved in invasion—IpgB1 and IpgD—as mT3Δeff_IpaA_*E. coli* and WT *Shigella* secrete similar levels of IpaB, IpaC, and IpaD (Fig. S4*C*). Intracellular mT3Δeff_IpaA_*E. coli*, like mT3Δeff_Inv_*E. coli*, exhibited high levels of CHQ^R (61%) (Fig. 4*A*). The slightly lower levels of CHQ^R observed for mT3Δeff_IpaA_*E. coli* versus mT3Δeff_Inv_*E. coli* might reflect differences due to the presence of IpaA or the nature of the phagosomes generated when uptake is mediated via zipper versus trigger mechanisms. Nevertheless, the complementary mT3Δeff_IpaA_*E. coli* and mT3Δeff_Inv_*E. coli* data demonstrate that the *Shigella* T3SA plays a major role in mediating phagosomal escape.

The *Yersinia* T3SA Does Not Mediate Phagosomal Escape. One model to account for the ability of the *Shigella* T3SA to mediate escape is through the formation of pores in the phagosomal membrane. If this is the case, then it seems likely that all intracellular bacteria that express a T3SS would escape from phagosomes into the cytosol, particularly in the absence of effectors that might prevent such lytic activity (i.e., *Yersinia* YopE) (20). To test this possibility, we investigated the behavior of YPIIIΔ6, an effectorless *Yersinia pseudotuberculosis* strain (21) that forms pores in host cell membranes (22). Although most *Yersinia* remain extracellular, some invade and establish a replicative niche within modified phagosomes in macrophages (23). When grown under conditions that activate its T3SS (Fig. S5*A*), we observed that, as previously reported, WT *Yersinia* rapidly induced the rounding and detachment of epithelial cells (24), precluding further analyses. However, YPIIIΔ6 did not and, like mT3Δeff_Inv_*E. coli*, efficiently invaded epithelial cells. In contrast, when exposed to CHQ at 1 h p.i. for 1 h, YPIIIΔ6 exhibited only 5% CHQ^R (Fig. S5*B*), indicating that effectorless *Yersinia* reside primarily within phagosomes, thus demonstrating that phagosomal escape is not a uniform property of all T3SA.

A Single Component of the Translocon Apparatus Mediates Phagosomal Escape Efficiency. *Salmonella* pathogenicity island 1 (SPI1) and *Shigella* T3SA belong to the Inv/Mxi-Spa family of T3SS (25), and accordingly, their proteins share a moderate degree of sequence similarity. Interestingly, although most *Salmonella enterica* serovar Typhimurium (*Salmonella*) reside within phagosomes, a significant

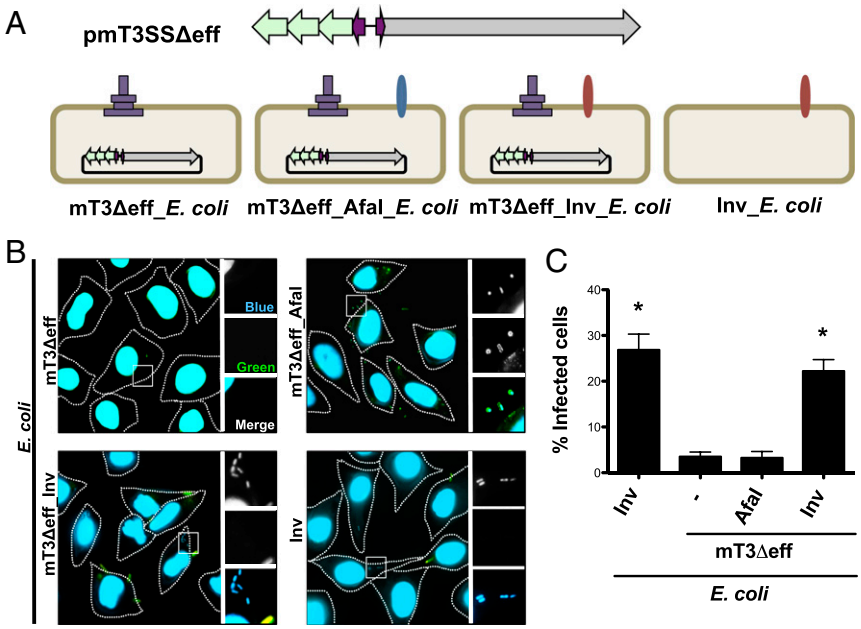

Fig. 3. Only mT3Δeff_*E. coli* that express *Yersinia* Inv enter epithelial cells. (*A*) Schematic representation of designated plasmids and strains. (*B*) Inside/outside microscopy assay as described in Fig. 1 of HeLa cells infected for 1 h with the designated strains at an MOI of 100 (mT3Δeff_*E. coli*) or 10 (mT3Δeff_AfaI_*E. coli*, mT3Δeff_Inv_*E. coli*, Inv_*E. coli*). (*C*) Quantification of the percentage of infected cells, defined as those that contain intracellular bacteria. For each infection, 100 HeLa cells were counted. $^*P < 0.05$ for strains versus mT3Δeff_*E. coli* as determined by one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as the mean ± SEM of four independent experiments.

number of *Salmonella* (~15–20%) escape from nascent vacuoles to establish an intracytoplasmic replicative niche in epithelial cells in a SPI1 T3SS-dependent manner (15, 26). There is evidence to suggest that this vacuole lysis is linked to the SPI1 T3SA itself, rather than seven of its translocated effectors (15). Given the observed differences in the ability of the phylogenetically related *Shigella* and *Salmonella* T3SA to mediate phagosomal escape, we hypothesized that the degree to which these pathogens enter the cytosol reflects the action of their T3SA, specifically one or more translocator proteins, the only three T3SA proteins that directly contact host cell membranes. To test this possibility, we investigated whether swapping the translocator proteins from *Salmonella* with their *Shigella* orthologs would promote *Salmonella* phagosomal escape, an approach that had previously suggested a role for *Shigella* IpaC in this process, at least when overexpressed in *Salmonella* (6).

We replaced *sipB*, *sipC*, and *sipD* from *Salmonella* with their respective *Shigella* orthologs (*ipaB*, *ipaC*, and *ipaD*) using allelic exchange to generate *sipB*::*ipaB*, *sipC*::*ipaC*, and *sipD*::*ipaD Salmonella* strains. Such chromosomal replacement preserves the genetic organization of the operon and stoichiometry of the translocator proteins. Each of these orthologs was expressed and recognized as a secreted substrate by the *Salmonella* SPI1 T3SS (Fig. S6*A*). Furthermore, the translocation of type III effectors (Fig. S6*B*) and invasion into epithelial cells (Fig. S6*C*) of *sipB*::*ipaB* and *sipC*::*ipaC Salmonella* indicated that IpaB and IpaC could functionally replace SipB and SipC, respectively. IpaD did not substitute for SipD (Fig. S6 *B* and *C*). We next investigated the intracellular fate of *sipB*::*ipaB* and *sipC*::*ipaC Salmonella* using the CHQ^R assay; the *sipD*::*ipaD* strain was not studied further given the lack of complementation. As shown in Fig. 5, replacement of SipC with IpaC, but not SipB with IpaB, significantly altered the fate of intracellular *Salmonella* in three different cell lines. Compared with WT *Salmonella*, at 1.5 h p.i., phagosomal escape by *sipC*::*ipaC Salmonella* was increased by 3.0-, 3.2-, and 11.6-fold within HeLa epithelial cells (Fig. 5*A*), HCT-8 intestinal epithelial cells (Fig. 5*B*), and J774A.1 murine macrophages (Fig. 5*C*), respectively. Therefore, the presence of IpaC, but not IpaB, leads to enhanced phagosome lysis by *Salmonella*.

T3SSs have been divided into eight distinct families based on phylogenetic analyses (25). The *Salmonella* SPI1 and *Shigella* Mxi-Spa systems are the best characterized members of the Inv/Mxi-Spa family, which also includes T3SSs from *Chromobacterium* (Cpi-1), *Yersinia* (Ysa), and *Burkholderia* (Bsa). Like *Salmonella* and *Shigella* spp., entry of *Chromobacterium violaceum* into nonphagocytic cells is dependent upon its Inv/Mxi-Spa T3SS (27), but its intracellular niche is unknown. By transmission electron microscopy (TEM), we identified that *Chromobacterium* is a cytosolic bacterium in epithelial cells, with 47% ($n = 30$ bacteria) and 97% ($n = 76$) free in the cytosol at 1 h and 3 h p.i., respectively. Furthermore, vacuole lysis is independent of the violacein pigment encoded by *vioA*; 53% ($n = 36$) and 96% ($n = 96$) of Δ*vioA* bacteria were in the cytosol at 1 h and 3 h p.i., respectively (Fig. S7). To test whether the ability of *Shigella* IpaC to mediate increased phagosomal escape might be a

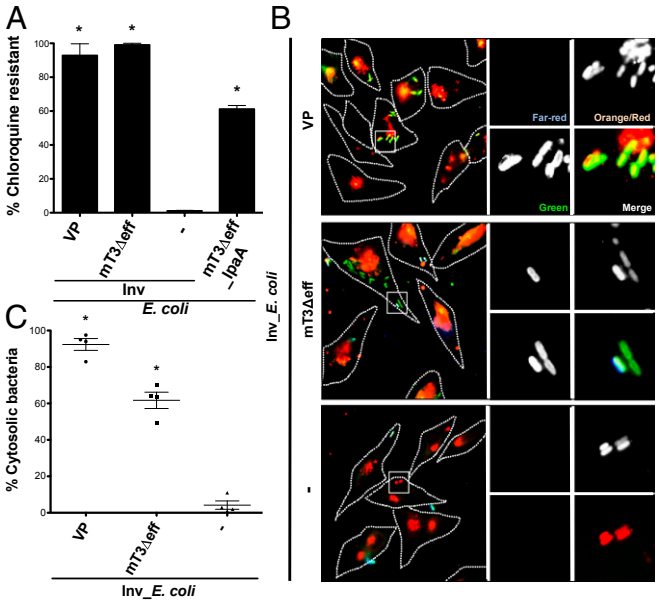

Fig. 4. The *Shigella* T3SA itself is sufficient to mediate phagosomal escape. HeLa cells were infected with the designated strains at an MOI of 100 (mT3Δeff_IpaA_*E. coli*) or 10 (VP_Inv_*E. coli*, mT3Δeff_Inv_*E. coli*, Inv_*E. coli*) and assessed for phagosomal escape. (*A*) CHQ^R and (*B* and *C*) digitonin permeabilization assays as described in Fig. 2. $^*P < 0.05$ for strains versus Inv_*E. coli* as determined by one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as the mean ± SEM of four independent experiments.

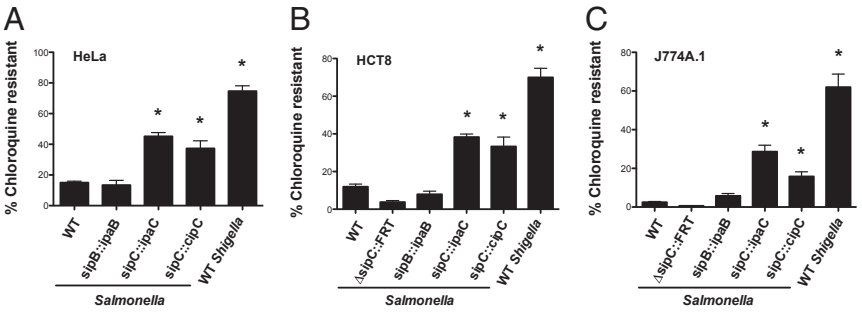

Fig. 5. The T3SA translocon plays a major role in determining the fate of intracellular bacterial pathogens. CHQ^R assays of (*A*) HeLa epithelial, (*B*) HCT-8 epithelial, or (*C*) J774A.1 macrophage-like cells infected with the designated strains grown under conditions that induce expression of the *Salmonella* SPI1 or *Shigella* Mxi-Spa T3SS. Cells were infected with WT *Shigella* (MOI of 20–100) or the *Salmonella* strains at an MOI of 10–50 and exposed to gent ± CHQ at 30 min p.i. for 1 h. $*P < 0.05$ for strains versus WT *Salmonella* as determined by one-way ANOVA with Dunnett's post hoc analysis. The mean ± SEM of at least three independent experiments is shown.

shared trait among Inv/Mxi-Spa translocator orthologs from cytosolic pathogens, we generated *sipC::cipC Salmonella*, a strain whereby *Salmonella* SipC is replaced with *Chromobacterium* CipC. CipC has greater amino acid sequence identity to SipC (41%) than does IpaC (30%), which likely explains its enhanced ability to substitute for *ΔsipC*::FRT *Salmonella* in both effector translocation (Fig. S6*B*) and invasion of epithelial cells (Fig. S6*C*). As observed for IpaC, the introduction of CipC into *Salmonella* resulted in a strain that escaped into the cytosol of both epithelial cells and macrophages at substantially elevated levels compared with WT *Salmonella*, with a 2.5-, 2.8-, and 6.4-fold increase in vacuole lysis in HeLa, HCT-8, and J774A.1 cells, respectively (Fig. 5). These observations demonstrate that the IpaC/SipC/CipC class of translocators plays a major role in determining the degree to which intracellular pathogens establish phagosomal versus cytoplasmic replicative niches within infected epithelial cells and macrophages.

Discussion

Despite its recognition as a model professional intracytoplasmic pathogen, our understanding of how *Shigella* rapidly escape from phagosomes into the host cell cytosol is currently limited. Here, to separate the established roles of the *Shigella* T3SS in effector delivery from phagosomal escape, we established a platform to study the behavior of the *Shigella* T3SA in the absence of all effectors. This technical feat was accomplished using recombineering and synthetic biology-based approaches to capture and transfer all of the genes needed to form a *Shigella* T3SA, but none of its effectors, into DH10B, a laboratory strain of *E. coli*. Our observation that these type 3 secretion-competent *E. coli* efficiently escape from phagosomes into the cytosol of infected cells demonstrates for the first time, to our knowledge, that the *Shigella* T3SA alone is sufficient to mediate phagosomal escape, a property we did not observe to be a conserved trait of the *Yersinia* T3SA.

To complement these reductionist-based studies, as well as to identify which component of the *Shigella* T3SA mediates escape, we examined the behavior of *Shigella* IpaB and IpaC, the two components of the T3SA that directly contact host cells and form a translocon pore in host cell membranes, when expressed at physiologic levels in place of their *Salmonella* orthologs. *Shigella* IpaB and IpaC each restored the ability of their corresponding *Salmonella* deletion strains to translocate effectors and invade nonphagocytic cells, but only expression of *Shigella* IpaC in *Salmonella* substantially altered the intracellular fate of this bacterium in epithelial cells and macrophages. This indicates that *Shigella* IpaC and *Salmonella* SipC confer different inherent vacuole lysis abilities to these two bacteria (6). Furthermore, enhanced phagosomal escape is not unique to *Shigella* IpaC, as complementation with the translocator protein CipC from another cytosolic pathogen, *Chromobacterium*, similarly enhanced vacuole lysis by *Salmonella*. Taken together, the results of our reductionist- and functional interchangeability-based experiments provide strong evidence that a single component of the T3SA translocon plays a major role in controlling the degree to which bacteria enter the cytosol of host cells.

The exact mechanism by which these proteins regulate phagosomal escape remains to be discovered. It is possible that translocon pore formation plays a role in this process, akin to the means by which *Listeria* pore-forming toxins mediate vacuole lysis. However, this seems unlikely, as the T3SSs of *Shigella*, *Salmonella*, and *Yersinia* are all well-known to form pores in host cell membranes (8, 24, 28), yet only *Shigella* efficiently lyses its phagosome. Our *Salmonella* complementation experiments indicate that it is not likely to be related to T3SS functionality, as there is no correlation between effector translocation and phagosomal escape; the *sipC::ipaC Salmonella* strain is much less efficient at translocating effectors and invading host cells than *sipC*::*cipC Salmonella*, yet both strains lyse their vacuole to a comparable extent. While it is possible that differences in the physical properties of the translocon pores formed by the bacteria result in altered stability of the phagosomal membrane, pore size does not seem to account for this, as the diameter of *Shigella* and *Salmonella* translocon pores is very similar (8, 24, 28).

Rather, we favor the possibility that interactions between IpaC, SipC, and CipC with host cell proteins play major roles in mediating phagosome escape. Besides being inserted into host cell membranes, each of these proteins is delivered directly into the cytosol of host cells. Interestingly, IpaC and SipC interact with both actin and intermediate filaments (29–31). It is possible that differences in the recruitment of cytoskeletal proteins to the internalization vacuole by IpaC and SipC play a role in determining phagosome stability. In this regard, it is intriguing that chemicals that disrupt the cytoskeletal cages that normally envelop *Chlamydia*-containing vacuoles, known as inclusions, result in the release of these pathogens into the host cell cytosol (32). Furthermore, SipC has been demonstrated to interact with Exo70, a component of the exocyst (33). Interactions with Exo70 promote localized membrane expansion at sites of *Salmonella* entry, perhaps resulting in differences in the lipid contents of *Salmonella* and *Shigella*-containing vacuoles, which could differentially affect the efficiency of phagosomal escape.

In summary, here we present complementary experimental approaches that together implicate a single component of the translocon, the outermost portion the T3SA, in mediating the efficiency of bacterial escape from phagosomes into the host cell cytosol. This work has broad scientific and technical implications, as it not only suggests a paradigm regarding how the intracellular niche of Gram-negative pathogens, many of which adopt an intracellular lifestyle as part of their colonization strategy, use their T3SA to mediate vacole lysis but also describes how an innovative "bottom–up" approach can be used to circumvent potential issues with effectors (and translocase) functional redundancy.

Materials and Methods

Bacterial Plasmids, Strains, and Cell Lines. Plasmids, strains, and oligonucleotides are summarized in Tables S1–S3. Cloning strategy and strain construction details are described in *SI Materials and Methods*. HeLa and HCT-8 epithelial cells and J774A.1 mouse macrophage-like cells were grown as recommended by American Type Culture Collection (ATCC).

Inside/Outside Microscopy Assay. As previously described (9), HeLa cells were infected with the designated bacteria grown under conditions that induce T3SSs. At 1 h p.i., infected cells were fixed and stained with rabbit anti-*E. coli*

or anti-*Shigella* antibodies followed by goat–anti-rabbit Alexa Fluor 488 (green) secondary antibody to label extracellular bacteria. The cells were subsequently permeabilized and treated with DAPI (4′,6-diamidino-2-phenylindole) to label intra- and extracellular bacteria as well as host cell nuclei. Images were captured digitally using a Nikon TE3000 microscope with Chroma Technology filters and a 60× objective. See *SI Materials and Methods* for additional details.

CHQ^R Assay. At 0.5–1 h p.i., infected cells were exposed to gentamicin (gent) (100 μg/mL) ± CHQ (200 μg/mL) for 1 h. Cell monolayers were then lysed and bacteria enumerated. See *SI Materials and Methods* for additional details.

Digitonin Permeablization Assay. HeLa cells were infected with bacterial strains that constitutively express mCherry. At 1 h p.i., the extracellular bacteria were labeled with Alexa Fluor 647 (far-red)-conjugated anti-*E. coli* or anti-*Shigella* antibodies, and the monolayers were then treated with digitonin (35 μg/mL) for 1 min to selectively permeabilize the plasma membrane. Cells were then labeled with Alexa Fluor 555 (orange)-conjugated anti-GM130 and Alexa Flour 488 (green)-labeled anti-*E. coli* or anti-*Shigella* antibodies to identify Golgi in permeabilized cells as well as label intracytoplasmic and extracellular bacteria. The percentage of cytoplasmic bacteria was determined by quantifying the ratio of intracytoplasmic to total intracellular (green/red)/(green/red + red) bacteria associated with digitonin-permeabilized cells. Far-red bacteria were not counted. See *SI Materials and Methods* for additional details.

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