Presence of SopE and mode of infection result in increased Salmonella-containing vacuole damage and cytosolic release during host cell infection by Salmonella enterica

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Abstract
Salmonella enterica serovar Typhimurium (STM) is an invasive, facultative intracellular pathogen that has evolved sophisticated molecular mechanisms to establish an intracellular niche within a specialised vesicular compartment, the Salmonella-containing vacuole (SCV). The loss of the SCV and release of STM into the cytosol of infected host cells was observed, and a bimodal intracellular lifestyle of STM in the SCV versus life in the cytosol is currently discussed. We set out to investigate the parameters affecting SCV integrity and cytosolic release. A fluorescent protein-based cytosolic reporter approach was established to quantify, time-resolved, and on a single cell level, the release of STM into the cytosol of host cells. We observed that the extent of SCV damage and cytosolic release is highly dependent on experimental conditions such as multiplicity of infection, type of host cell line, and STM strain background. Trigger invasion mediated by the Salmonella Pathogenicity Island 1-encoded type III secretion system (SPI1-T3SS) and its effector proteins promoted cytosolic release, whereas cytosolic bacteria were rarely observed if entry was mediated by zipper invasion. Presence of SPI1-T3SS effector SopE was identified as major factor for damage of the SCV in the early phase after STM invasion and sopE-expressing strains showed higher levels of cytosolic release.

Keywords
intracellular pathogen, invasion, pathogen-containing vacuole, type III secretion system

1 | INTRODUCTION

Bacterial pathogens have evolved distinct adaptations to their mammalian host, and a facultative intracellular style is a property of several clinically important pathogens. Two main form of intracellular lifestyle can be distinguished, that is, presence of the pathogen within the cytosol of the host cell or life within a membrane-bound, pathogen-containing vacuole (PCV). Shigella spp. and Listeria monocytogenes are paradigmatic representatives of the first group (Ray, Marteyn, Sansonetti, & Tang, 2009), whereas Chlamydia spp., Legionella pneumophila, Mycobacterium tuberculosis and Salmonella enterica are pathogens known to create and maintain specialised PCV (Diacovich & Gorvel, 2010). The cytosolic and the PCV-bound lifestyle each require specific adaptations to actively manipulate host cell functions for avoidance of host cell immune defences and to acquire nutrients (reviewed in Abu Kwaik & Bumann, 2015; Asrat, de Jesus, Hempstead, Ramabhadran, & Isberg, 2014; Baxt, Garza-Mayers, & Goldberg, 2013).
Salmonella enterica is an invasive, facultative intracellular pathogen that inhabits a PCV with a number of unique properties, and this compartment is referred to as Salmonella-containing vacuole or SCV (LaRock, Chaudhary, & Miller, 2015). S. enterica deploys the Salmonella Pathogenicity Island 1-encoded type III secretion system (SPI1-T3SS) to invade nonphagocytic cells (Galan & Curtiss, 1989; reviewed in Hume, Singh, Davidson, & Koronakis, 2017). Within mammalian host cells, function of the Salmonella Pathogenicity Island 2-encoded T3SS (SPI2-T3SS) is linked to formation and maintenance of the SCV (Beuzon et al., 2000). The S. enterica serovar Typhimurium (STM) is frequently used for infection models of Salmonella invasion of host cells and for the facultative intracellular lifestyle in mammalian host cells. The function of the SPI2-T3SS is of central importance of systemic pathogenesis of STM in a murine infection model (Shea, Hensel, Gleeson, & Holden, 1996), and mutant strains defective in SPI2-T3SS are highly attenuated in systemic virulence.

Within infected host cells, distinct subpopulations of STM can be observed (Birmingham, Smith, Bakowski, Yoshimori, & Brumell, 2006). Bacteria within the SCV are either actively replicating or dormant (Helaine & Holden, 2013). Rupture of the SCV and release into the host cell cytosol occurs in some host cells, resulting in hyper-replicating cytosolic STM (Knodler et al., 2010), targeting of bacteria by the host cell autophagy system (Birmingham et al., 2006), or induction of host cell pyroptosis (Fink & Cookson, 2006). The phenomenon of SCV rupture, cytosolic translocation of Salmonella, and bacterial replication in the host cell cytosol has recently attracted significant interest. A bimodal lifestyle of Salmonella in SCV and cytosol has been proposed (Malik-Kale, Winfree, & Steele-Mortimer, 2012).

Trigger invasion mediated by STM requires the concerted action of a subset of SPI1-T3SS effector proteins (Hume et al., 2017). Whereas two of the SPI1-T3SS effectors, that is, SipA and SipC directly nucleate actin, effector proteins SopE and highly related SopE2 act as GEF for host cell Rho GTPases Rac1 and Cdc42 (Bakshi et al., 2000; Hardt, Chen, Schuebel, Bustelo, & Galan, 1998; Stender et al., 2000; Wood, Rosqvist, Mullan, Edwards, & Galyov, 1996). Activation of Rac1 and Cdc42 by SopE and SopE2 initiate F-actin polymerisation, membrane ruffling, and bacterial uptake by macropinocytosis. Although sopE2 is present in most strains of STM, the distribution of sopE is restricted to a smaller number of strains and linked to lysogeny by the sopE phage (Mirot et al., 1999). Two STM isolates commonly used in infection biology differ in possession of sopE, with SL1344 harbouring the sopE phage, and NCTC 12023 (a.k.a. ATCC 14028) lacking sopE.

During the intracellular proliferation of STM, maintenance of SCV integrity depends on a subset of effector proteins of the SPI2-T3SS. Most important is SifA, and lack of this effector leads to defects in remodelling of the host cell endosomal system and inability to maintain intact SCV (Beuzon et al., 2000; Stein, Leung, Zwick, Garcia-del Portillo, & Finlay, 1996). SifA-deficient STM ultimately loose the SCV during intracellular replication, resulting in release into host cell cytosol. However, also cell invasion and activity of the SPI1-T3SS affect the integrity of the SCV in the early intracellular phase (Klein, Grenz, Slauch, & Knodler, 2017; Kreibich et al., 2015).

We set out to identify the factors that affect in the early intracellular phase SCV integrity or rupture and release of STM into the cytosol of infected mammalian cells. A reporter system for quantitative analyses of intracellular subpopulation was established using a promoter responsive to host cell cytosolic glucose-6-phosphate (G6P) to drive fluorescent protein expression. The G6P reporter was introduced by Schmutz et al. (Schmutz et al., 2013) and subsequently applied in several studies (Chong, Starr, Finn, & Steele-Mortimer, 2019; Cooper, Chong, Starr, Finn, & Steele-Mortimer, 2017; Lau et al., 2019; Spennenhirn et al., 2014). Here, we demonstrate that SCV rupture and cytosolic release of Salmonella in the early intracellular phase are functions of the experimental settings in cell culture models and linked to the presence of SPI1-T3SS effector protein SopE.

## RESULTS

### 2.1 A metabolic reporter for sensing cytosolic release of intracellular Salmonella

Integrity of the SCV was previously determined by scoring the presence of LAMP1-positive membranes enclosing intracellular STM or the presence or absence of Galectin 3 that accumulates at ruptured PCV. Both approaches are useful for analyses of the state of individual intracellular STM but limited for the analyses of larger populations. Thus, we deployed here a reporter system in STM that allows reliable distinction between the SCV-bound and cytosolic state of intracellular STM.

Glucose is a main substrate for mammalian cells, and analyses of STM metabolism during infection revealed that glucose is also central for STM nutrition. During uptake by cells, glucose is phosphorylated to G6P that is rapidly metabolised in bacterial cells, whereas higher levels of G6P are maintained in the cytosol of mammalian cells. STM is also able to take up G6P and expression of uhpT encoding the transporter for phosphorylated hexoses in controlled by the presence of G6P in the external medium and sensed by the UhpABC sensor system. In previous work on Shigella flexneri (Schmutz et al., 2013) and STM (Spennenhirn et al., 2014), the promoter of uhpT has been used to monitor the presence of these pathogens in the cytosol of host cells. During evaluation of initial reporter constructs, we noticed that expression of PsopE::DsRed was only detectable with delays, likely due to the long maturation time of the fluorescent protein. In contrast, sfGFP is a GFP derivative with very short maturation time of less than 14 min (Pedelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006). To enable sensitive detection of early events of cytosolic exposure, we generated a dual colour reporter plasmid with constitutive expression of DsRed and sfGFP under control of P_{uhpT}.

The response of STM strains harbouring the dual colour reporter plasmid to G6P was analysed in vitro (Figure 1a). Although induction of sfGFP was absent in Luria broth, addition of G6P at concentrations of 0.004% to 0.4% led to induction of the reporter. More than 95% of bacterial cells were super-folder green fluorescent protein sfGFP-positive after 120 min of culture with various concentrations of G6P.
In cultures supplemented with 0.004% or 0.04% G6P, the number of sfGFP-positive bacteria decreased continuously after 150 or 180 min of culture, respectively, indicating consumption of inductor G6P and dilution of sfGFP in dividing bacteria.

To test the dynamics of reporter response, the intensity of sfGFP expression was determined at various time points during growth in media with 0.4% G6P. The sfGFP-positive cells were detected after 60 min of culture. The fluorescence intensity per STM cell increased until 180 min of culture and decreased slightly at later time points of culture growth (Figure 1b).

We next tested the performance of the dual colour reporter for infection experiments (Figure 1c). STM harbouring the dual colour reporter was used to infect HeLa cells. For labelling of the lumen of SCV and SIF, pulse chase with fluid phase marker dextran-Alexa 647 was performed. Constitutive DsRed fluorescence allowed detection of all intracellular STM, and we observed infected cells that contained STM.
generally coincided with induction of cytosolic reporter Pmediated permeabilisation. The labelling by anti-O-antigen antibody but protection of vacuolar bacteria was analysed by digitonin-positive cells without dextran-Alexa 647 association increased over time and SCV integrity. Quantification indicate that the frequency of sfGFP-copy has to be considered for interpretation of fluid tracer colocalisation present. However, the limited spatial resolution of fluorescence microscopy has to be considered for interpretation of fluid tracer colocalisation and SCV integrity. Quantification indicate that the frequency of sfGFP-positive cells without dextran-Alexa 647 association increased over time p.i. (Figure 1e).

To further corroborate the dual colour reporter, we used previously described approaches to probe the presence of STM in host cell cytosol or in the SCV. Selective access of antibodies to cytosolic STM, but protection of vacuolar bacteria was analysed by digitonin-mediated permeabilisation. The labelling by anti-O-antigen antibody generally coincided with induction of cytosolic reporter P<sub>invC</sub>sfGFP, whereas noninduced bacteria were negative for antibody labelling (Figure S2).

Galectins are mammalian Lectin-like proteins that bind to glycostructures that become accessible after damage of compartments such as lysosomes (Paz et al., 2010; Thurston, Wandel, von Muhlinen, Foeglein, & Randow, 2012). Galectin-FP expressing cells were previously used to monitor PCV damage induced by intracellular pathogens. We transfected HeLa cells for expression of mCherry-Galectin 3 or mCherry-Galectin 8 and infected cells with STM reporter strains. Colocalisation of cytosolic-induced STM with either Galectin 3 and Galectin 8 was observed (Figure S3).

We also applied the cytosolic sensor to follow the fate of STM after phagocytic uptake by RAW264.7 macrophages. Flow cytometry did not detect increased number of cells positive for cytosolic bacte- ria, even if the ΔsiF strain was applied (data not shown). Time-lapse microscopy of RAW264.7 cells infected with STM harbouring the dual colour reporter revealed intracellular proliferation of STM WT (Movie 3) and occasionally the appearance of host cells with sfGFP-expressing cytosolic bacteria. RAW264.7 cells harbouring cytosolic STM rapidly showed cell rounding and completely lost adhesion to the substrate (Movie 4). These cells were lost in the subsequent preparation of samples for flow cytometry. We conclude that cytosolic presence of STM in RAW264.7 macrophages occurs transiently and rapidly induced pyroptotic cell death as previously described (Bergsbaken, Fink, & Cookson, 2009; Thurston et al., 2016). The response of the cytosolic reporter is sufficiently fast to record these events during microscopy analyses.

We next deployed an infection model using the human colonic epithelial cell line Caco2 BBBe1. If cultured as polarised monolayers, STM apical invasion requires cooperative function of the giant non-fimbrial adhesin SiiE and the SPI1-T3SS (Gerlach et al., 2008). After invasion of Caco2 BBBe1 cells, STM developed smaller microcolonies lacking sfGFP signals. In few cells, rapidly proliferating sfGFP-positive STM populations developed (Movie 5). In agreement with previous findings (Knodler et al., 2010), the corresponding host cell was expelled from the monolayer in later stages of infection, and intracellular bacteria were released from such host cells.

Taken together, our data show that the dual colour reporter can be used as faithful indicator of cytosolic release of STM from the SCV into the cytosol of host cells.

2.2 Trigger invasion promotes cytosolic release

STM invades nonphagocytic host cells by trigger invasion that lead to rapid local F-actin formation and membrane ruffle formation, resulting in a macropinocytosis-like uptake of the pathogen (reviewed in Hume et al., 2017). Other invasive bacteria deploy the zipper invasion mechanisms that is mediated by a high number of adhesion events of outer membrane proteins to host cell receptors. Invasin Inv is a well-characterised adhesin of Yersinia pseudotuberculosis, mediating zipper invasion (Isberg, 1985). We set out to evaluate the contribution of the mode of invasion to cytosolic release and compared STM entry by trigger versus zipper invasion. For this, a plasmid was used for expression of Y. pseudotuberculosis (Y.p.) Inv under control of SPI1 promoter P<sub>invF</sub>, allowing expression of Y.p. inv under similar conditions as the SPI1-T3SS. We observed that HeLa cells were efficiently invaded by STM expressing Y.p. inv (39.0% ± 0.7% of WT invasion, Figure S4a). After entry by SPI1-T3SS-mediated trigger invasion or Y. p. Inv-mediated zipper invasion, individual STM were located in LAMP1-positive SCV. Independent of the mode of entry, SPI2-T3SS-dependent formation of SIF was observed (Figure 2a).

Using strains harbouring the dual colour cytosolic reporter, we deployed flow cytometry to enumerate the populations of infected cells and of infected cells with cytosolic STM. We analysed ~100,000 HeLa cells recovered 8 hr p.i., and a representative data set is shown in Figures 2b and S4b. For WT NCTC, 7.6% and 0.77% of total host cells were infected with SCV-bound and cytosolic STM, respectively. For the ΔinvC strain, 0.28% of total host cells were infected with SCV-bound STM, and 0.03% of host cells harboured cytosolic-positive bacteria. It is not known how this low proportion of cells were invaded by a SPI1-T3SS-independent mechanism, and contribution of alternative invasion mechanisms such as Rck or PagN may be considered (Velge et al., 2012). Of the infected host cells, on average 8.4% ± 2.2% or 8.9% ± 0.3% harboured cytosolic WT or ΔinvC STM, respectively. If cells were infected with the ΔinvC strain expressing Y.p. inv, 8.8% of cells were infected with SCV-confined STM, but the population of cells with cytosolic STM was 0.02% of the total cells, and on average 0.5% ± 0.08% of the infected cells. The proportion of zipper-invaded cells with cytosolic STM was highly reduced compared with cells infected by trigger invasion.

Cytosolic release was analysed at various time points after trigger or zipper invasion (Figure 2c). For cells invaded by the SPI1-T3SS mechanism, an increased proportion of cytosolic WT STM was
detected at later stages after infection. For the low absolute number of host cells infected by invC or invC ssaV mutant strains, decrease in the percentage of cytosolic STM was recorded. If cells were invaded in Y.p. Inv-dependent manner, an increased population of host cells with cytosolic STM was observed 16 hr after infection, but this increase was absent if a SPI2-T3SS-deficient strain was used. We conclude that zipper invasion by STM allows formation of an SCV and leads to highly reduced SCV damage and cytosolic release. The intracellular population then deploys the SPI2-T3SS to manipulate host cells, replicates intracellularly, and eventually loses the SCV in the late stage of intracellular life. In agreement with a previous report by Birmingham et al. (2006), the results show that the sole intracellular presence of STM is not leading to release into the cytosol but rather the mode of cell entry. The SPI1-T3SS-mediated trigger invasion more frequently gives rise to cytosolic exposure.

2.3 | Extent of cytosolic release is a strain-specific phenomenon

Strains of STM differ in their equipment with SPI1-T3SS effector proteins, and commonly used WT strains such as NCTC 12023 (a.k.a. ATCC 14028) and SL1344 differ with respect to SopE. SopE is encoded by a prophage present in SL1344 and a low number of further isolates, whereas NCTC 12023 (NCTC) and the larger proportion of STM isolates are negative for the prophage and SopE (Mirold et al., 1999; Stender et al., 2000). The functional homologue SopE2 is conserved in STM isolates (Mirold et al., 2001). We investigated the frequency of cytosolic release of NCTC WT and are expressed as: n.s., not significant; ***, p < .001

![FIGURE 2](image)

Trigger invasion of host cells result in cytosolic release of Salmonella. HeLa cells were infected with STM NCTC WT or isogenic mutant strains, each harbouring cytosolic reporter p4889. If indicated, the strains additionally harboured a plasmid for the expression of Yersinia pseudotuberculosis invasin (Y.p. inv). (a) Infection of HeLa cells constitutively expressing LAMP1-GFP. Note the formation of SCV and SIF in representative infected cells. Scale bar, 10 μm. (b) Flow cytometry analyses of infected HeLa cells and HeLa cells harbouring cytosolic STM. The gates R2 and R3 as defined in Figure S1 are indicated. From a representative experiment with cells recovered 8 hr p.i., the total numbers of host cells harbouring DsRed-positive, sfGFP-negative STM (R2), and DsRed- and sfGFP-positive, that is, cytosolic STM (R3), are indicated. Values indicated above R3 indicate host cells with cytosolic STM as percentage of infected host cells. Means and standard deviations of three independent experiments are shown. Analyses for further strains are shown in Figure S4. (c) Time course of cytosolic release. HeLa cells were infected with various strains as indicated, fixed at 4, 8, or 16 hr p.i. and analysed by flow cytometry as for (b). Cytosolic release is expressed as percentage of infected host cells (sum of R2 + R3 gates) positive for sfGFP-induced STM (R3 gate). Means and standard deviations of the triplicate samples are shown and are representative for three independent experiments. Statistical analyses were performed by one-way ANOVA in comparison to NCTC WT and are expressed as: n.s., not significant; ***, p < .001.
of release from the SCV and replication in the cytosol of host cells (Beuzon et al., 2000; Brumell, Tang, Zaharik, & Finlay, 2002).

Infected cells were analysed for presence of cytosolic STM at various time points after infection (Figure 3). The proportion of HeLa cells harbouring cytosolic NCTC WT increased at a low rate over the time course. For WT SL1344, a rapid increase of cells harbouring cytosolic STM was observed at early time points after infection and remained at a high and continuously increasing level. At 4 hr p.i., 7.7% (±0.4%) and 18.1% (±2.1%) of NCTC- and SL1344-infected cells, respectively, were positive for cytosolic STM. Cytosolic release of NCTC ΔsifA started later than 6 hr p.i., and the proportion of cells with cytosolic STM showed a steep increase, reaching 65.5% (±0.3%) cells with cytosolic STM at 16 hr p.i. Representative micrographs of cells infected with various strains are shown in Figure S5.

To further address the role of host cell response to cytosolic STM, we investigated autophagic control of cytosolic STM. HeLa cells were transfected for expression of mTurquoise-LC3B as canonical marker of autophagosomal membranes and infected with STM strains harbouring

**Figure 3** Increased release into the cytosol of strain SL1344. HeLa cells were infected with STM strains NCTC WT (green) or ΔsifA (orange), or SL1344 WT (red), each harbouring cytosolic reporter p4889 at a multiplicity of infection (MOI) of 5. Cells were fixed at various time points p.i. and subjected to flow cytometry for quantification of infected HeLa cells with sfGFP-negative, SCV-bound bacteria (gate R2), and infected cells with sfGFP-positive (cytosolic) bacteria (gate R3). Shown are representative data for 2, 4, 8, and 16 hr p.i. (b) Quantification of infected cells harbouring cytosolic STM. Means and standard deviations from triplicates are shown.
We determined the growth kinetics of various strains used in this study. Under the conditions of preculture for induction in invasiveness, NCTC WT, SL1344 WT, and derived mutant strains showed very similar growth kinetics, entered the late log phase at 3.5 hr of subculture, and yielded similar cell densities (Figure S7). We thus exclude growth phase-dependent differences as cause for difference in cytosolic release.

Next, the frequency of cytosolic release in dependency of MOI ranging from 1 to 150 using NCTC and SL1344 WT strains was determined (Figure 4). For all MOI analysed, the percentage of host cells positive for cytosolic STM was markedly higher after infection by SL1344 than by NCTC. The percentage of cells with cytosolic STM increased continuously with increasing MOI. For infection by NCTC WT, the effect was less pronounced, and we determined 1.8% (±0.1%) and 6.3% (±0.5%) of cells with cytosolic STM at MOI 1 and 100, respectively. For SL1344 WT, 5.0% (±0.5%) and 16.9% (±2.5%) of cells at MOI 1 and 100, respectively, harboured cytosolic STM.

In conclusion, the experimental parameter MOI is a further factor that determines the rate of cytosolic release of STM.

2.5 | Effector protein SopE is an important driver of cytosolic release early after infection

Our experiments consistently showed that cytosolic release of SL1344 was higher than that of NCTC. Even infection by NCTC at high MOI did not lead to the frequency of cytosolic release observed for SL1344. To reveal the molecular basis of this phenotype, we focused on SopE, because this effector is present in SL1344 and absent in NCTC. Cytosolic release of the WT strains was compared with strains with different equipment with sopE (Figure 5a). Episomal expression of sopE in NCTC resulted in cytosolic release even higher than that of SL1344. Deletion of sopE in SL1344 reduced cytosolic release to the level observed for NCTC, and the sopE deletion could be fully restored by a plasmid for expression of sopE. We controlled the effect of SopE on the cytosolic replication of SL1344 (Figure 5b). Although the number of HeLa cells with cytosolic SL1344ΔsopE was lower than for SL1344-infected cells, we observed a similar increase of the bacterial load per cell. As shown in Figure 5b, the increase over time p.i. in DsRed levels of cells with SCV-bound or cytosolic STM is similar for both strains. These data confirm the role of SopE in mediating increased cytosolic release in the early phase of intracellular STM.

For further dissection of the role of SopE, we used a mutant allele of SopE defective in GEF activity due to G168 V exchange in the catalytic loop of the protein. The G168 V mutation of SopE was shown to lead to highly reduced formation of membrane ruffles during invasion and complete loss of catalytic activity (Schlumberger et al., 2003). We analysed membrane ruffle formation of NCTC, SL1344, and NCTC expressing sopE WT or sopEG168V. Induction of membrane ruffles during HeLa cell infection was very pronounced for SL1344 and NCTC [sopE] and subtle for NCTC. Ruffle induction by NCTC [sopEG168V] was morphologically similar to that of NCTC (Figure 6a).

Because STM translocates several effector proteins that manipulate the actin cytoskeleton and contribute to invasion, defects in SopE

2.4 | Cytosolic release of STM is affected by experimental conditions

Because the previous experiments indicate the contribution of the mode of entry and strain characteristics to cytosolic release, we next determined the effect of the experimental conditions. Protocols for infection of host cells vary for example in the culture conditions used to stimulate invasiveness of STM and in the multiplicity of infection (MOI) applied.

The degree of host cell invasion by Salmonella is a key factor for cytosolic release. HeLa cells were infected with STM NCTC WT (green) or SL1344 WT (red) harbouring cytosolic reporter p4889 at various MOIs and fixed 8 hr p.i. Subsequently, flow cytometry analyses were performed for the detection of infected HeLa cells and quantification of host cells with sfGFP-positive (cytosolic) STM. Means and standard deviations from triplicates are shown.
may be partially masked by functions of other effector proteins. As a reductionist system, we used a SL1344-derived mutant strain defective in five effector genes sipA, sopA, sopB, sopE, and sopE2. This strain, termed Δ5, is highly attenuated in invasion, and invasion can be partially restored by complementation with single effector proteins (Figure 6b). Invasion of Δ5[sopE] was about 200% of that of WT NCTC, whereas invasion of Δ5[sopEG168V] was 50%. Scoring of rates of infected HeLa cells by flow cytometry confirmed the efficient invasion mediated by SopE and the remaining activity of SopEG168V as invasion factor (Figure 6c). Surprisingly, scoring of infected cells with cytosolic STM revealed that a high proportion of cells infected with Δ5[sopEG168V] harboured cytosolic STM (17.0 ± 0.69%), whereas the frequency of cytosolic release for Δ5[sopE] much lower (5.4 ± 0.57%; Figure 6d). These data indicate that in non-invasive Δ5 background, SopE with GEF function is sufficient to mediate highly efficient invasion. Translocation of a GEF-deficient SopE allele only mediates low levels of invasion but leads to highly increased levels of SCV damage and cytosolic release.

In order to increase the temporal resolution of analyses of cytosolic release, we deployed a modified method, allowing the maturation of sfGFP synthesised early after exposure of STM to host cell cytosol. Infected host cells were treated with chloramphenicol + rifamycin at various time points after infection to stop bacterial transcription and translation, incubated overnight, then fixed and processed for flow cytometry (Figure 7). Increased cytosolic release was observed between 2 and 3 hr p.i., whereas only very low numbers of host cells with cytosolic STM were recorded at 1 hr p.i. (Figure 7a). In accordance with the data shown above, presence of SopE resulted in increased rates of cytosolic release (Figure 7b). In the SL1344 Δ5 background, presence of SopE resulted in slow increase of host cell with cytosolic STM over time, whereas presence of SopEG168V in this strain caused a steep increase of cytosolic STM already early after infection (Figure 7c).

3 DISCUSSION

We developed and applied a simple quantitative approach to investigate, on a single cell level, the integrity of the SCV and the occasional release of intracellular Salmonella into the cytosol of host cells. Our study shows that cytosolic release of Salmonella in the early intracellular phase is highly dependent on experimental conditions applied in cell culture infection models, on the mode of invasion, and on the presence of SPI1-T3SS effector protein SopE (summarised in Figure 8). These observations should be considered if a potential bimodal lifestyle of Salmonella in host cells is discussed. We showed that infection with high doses of Salmonella inoculum leads to a highly increased release into the cytosol during the early phase after

**FIGURE 5** SPI1-T3SS effector SopE contributes to cytosolic release of Salmonella. (a) HeLa cells were infected with STM strains expressing the reporter at a MOI of 5 and fixed at 4 hr p.i. Flow cytometry analyses were performed for quantification of infected HeLa cells with sfGFP-positive (cytosolic) bacteria. Means and standard deviations from triplicates are shown. Statistical analysis was performed as described for Figure 2c. Statistical indicators in green or red font indicate comparison to NCTC WT or SL1344 WT, respectively. (b) Cytosolic release and replication of NCTC WT (green), SL1344 WT (red), and SL1344 ΔsopE (orange). HeLa cells were infected with STM strains SL1344 WT or ΔsopE, each harbouring cytosolic reporter p4889 at a MOI of 5. Cells were fixed at various time points p.i. and subjected to flow cytometry for quantification of infected HeLa cells with sfGFP-positive (cytosolic) bacteria. The gate “cytosolic” indicates cells with high load of cytosolic bacteria. The increase in DsRed intensity per host cell over time p.i. indicates the increase in amounts of STM. Shown are representative data for 3, 4, 6, 8, 10, and 16 hr p.i. The percentages of host cells with sfGFP-positive STM are shown as means and standard deviations of three independent experiments.
invasion. Cytosolic release was almost absent after zipper invasion of Salmonella. We conclude that trigger invasion results in a more labile PCV. Further careful analyses of the intracellular lifestyle of Salmonella are required to clarify if the proposed bimodal lifestyle of Salmonella is an intrinsic pathogenic program of this pathogen or rather the result of experimental conditions applied in cell culture-based analyses.

Our conclusions are partially discordant with prior models for a role of SPI1-T3SS in mediating SCV damage in the absence of effector proteins (Knodler, Nair, & Steele-Mortimer, 2014). (Knodler et al., 2014) infected Caco2 BBe1 cells with a SPI1-T3SS-deficient strain, or a SPI1-T3SS-proficient, but effector-less strain at a MOI of 1,000 to achieve SPI1-T3SS-independent uptake. In analyses performed by immunolabelling after digitonin permeabilization and chloroquine resistance assays, increased amounts of cytosolic Salmonella were observed at early (1.5 hr p.i.) and late (7 hr p.i.) time points if the SPI1-T3SS was functional. A later study used a system for controlled SPI1-T3SS activity in intracellular Salmonella and observed a role of effector proteins SopB and SipA in cytosolic proliferation (Klein et al., 2017). Our data show that the SPI1-T3SS is involved in damage of the early SCV, and we propose a key role of SopE in the early phase of internalisation and intracellular presence. Further studies are required to test if presence of the SPI1-T3SS translocon is sufficient to induce SCV damage. However, the different degrees of cytosolic release of Salmonella NCTC and SL1344 strains and the role of SopE argue against a major role of SPI1-T3SS.

### 3.1 Stability of the early SCV

We showed that trigger invasion leads to formation of early SCV more prone to damage than compartments formed after zipper-mediated entry. The massive membrane reorganisation during Salmonella invasion is likely to a cause formation of macropinosomes and early SCV that are prone to membrane damage. The various events occurring in

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**FIGURE 6** SPI1-T3SS effector SopE has a major role in early cytosolic release of Salmonella. HeLa cells were infected with various STM strain isogenic to NCTC (green bars) or SL1344 (red bars), each harbouring cytosolic reporter p4889. If indicated, strains additionally harboured plasmids for expression of WT sopE or sopE<sub>G168V</sub> defective in the GEF catalytic centre. A mutant strain lacking SPI1-T3SS effector proteins SipA, SopA, SopB, SopE and SopE2 is indicated by Δ5. (a) HeLa cells expressing LifeAct-GFP were infected with various strains and imaged 30 min p.i. Representative invasion events indicate the degree of F-actin formation and extend of membrane ruffles induced by STM. (b) Host cell invasion was quantified by gentamicin protection assays at 1 hr p.i. Invasion is normalised as percentage of NCTC WT invasion. The percentage of infected cells was determined by flow cytometry by determination of DsRed-positive host cells (c), and of the population of infected HeLa cells harbouring cytosolic STM (d) was quantified 8 hr p.i. as described for Figure 2b. Means and standard deviations of the triplicate samples are shown and are representative for three independent experiment. Statistical analyses were performed as for Figure 2c
Salmonella-infected cells have been comprehensively described by Bir-
mingham et al. (2006). Detailed ultrastructural analyses showed that
invading Salmonella inhabits individual macropinosomes (Fredlund
et al., 2018) and works in our group showed that this individuality is
maintained in later stages of infection (Krieger et al., 2014). The
demand for membranes to build SCV with individual Salmonella
could exceed the supply in case to high rates of invasion, thus promoting
instability. A further factor leading to destabilisation of the early SCV
are translocon complexes with pore-forming activity if displaced from
the connecting T3SS needle (reviewed in Guignot & Tran Van Nhieu,
2016). Roles of T3SS in absence of effector proteins in damaging the
PCV of various intracellular pathogens has been reported by Du et al.
(2016). Again, increased invasion will levy a higher burden of pore-
forming activity on the SCV. Damaged SCV may be repaired by
recruitment of autophagosomal membranes (Kreibich et al., 2015), but
such repair mechanisms will be overrun by an increasing number of
damaged SCV (see Figure S6b).

### 3.2 SopE as driver of SCV instability

The difference in cytosolic release of WT strains NCTC and SL1344
should also be considered for interpretation of Salmonella-induced
phenotypes. The expression of the SPI1-T3SS is controlled by various
environmental factors (Ellermeier & Slauch, 2007) and additionally affected by stochastic expression (Ackermann et al., 2008). A lower frequency of SPI1-T3SS expressing cells was reported for NCTC compared with SL1344 (Clark et al., 2011). The presence of both effectors SopE and SopE2 is restricted to the minority of Salmonella isolates. SopE manipulates Cdc42 and Rac1, whereas SopE2 function is restricted to Rac1 (Friebel et al., 2001). This broader target range is a further reason for more severe cytoskeletal rearrangements that are likely to contribute to instability of macropinosomes and early SCV.

SopE translocation may be considered as hyper-virulence phenotype. SopE function may explain increased stimulation of inflammatory responses to the cytosolic presence of Salmonella (Keestra et al., 2013; Muller et al., 2009) and increased cell damage resulting from cytosolic hyper-replication (Knodler et al., 2010). We suggest to compare phenotypes of WT strains with and without sopE in order to dissect broader virulence phenotypes from SopE-specific effects on host cells and/or host organisms.

We made the unexpected observation that the GEF-deficient allele of SopE mediates highly reduced levels of invasion but increased frequency of cytosolic Salmonella. This observation indicates that SopE GEF function also contributes to stability of the SCV in the early phase of intracellular presence. The stability of this compartment may be affected by the amount of F-actin recruited to the sites of invasion and formation of early SCVs. If sufficient amounts of F-actin are missing, instability of the compartment is increased, and Salmonella is more frequently released into the cytosol.

Previous work by Vonaesch et al. (2014) demonstrated that translocation of SPI1-T3SS effector proteins continues after completion of invasion. A role of the ongoing translocation of SopE to the biogenesis of the SCV was proposed, and reduced intracellular proliferation was observed for mutant strains lacking SopE or SopE2. Investigating the subcellular localization of translocated SopE and SopEG168V, Vonaesch and coworkers found that SopE is retained at the early SCV, whereas retention of SopEG168V is highly reduced (Vonaesch et al., 2014). In light of our results, the simplest explanation is absence of a membrane compartment for interaction with SopEG168V. Indeed, a marker for an SCV membrane was not present in the prior analyses of SopEG168V.

The localization of functional SopE to membranes of early SCV can promote the SCV repair, and a role of autophagosomal membranes is sealing damaged SCV has been proposed (Kreibich et al., 2015). In our assays, SopEG168V was capable to mediate cell entry, but absence of GEF function led to highly increased rates of SCV damage and cytosolic release in the early phase of intracellular life.

A role of SPI1-T3SS effectors in the biogenesis of the early SCV has been reported by several groups. The contribution of these effectors to SCV stability has only been recognised recently. The balance between SCV-bound Salmonella and cytosolic Salmonella is directly affected by SPI1-T3SS activity and effector functions. Thus, care has to be taken in interpretation of population-based analysis such as intracellular proliferation, host cells responses, or immune responses of Salmonella-infected host organisms. These observations may be

![Diagram showing factors affecting the release of STM into the cytosol of infected host cells.](image)
skewed by the increased cytosolic fraction of *Salmonella*, rather than being direct effects of effector SopE.

Sensing of cytosolic bacteria is an important mechanism of innate immune defence. In phagocytic cells, cytosolic *Salmonella* induce cell death, and a sensing mechanisms for cytosolic lipopolysaccharide LPS-involving Caspases 1 and 11 leads to pyroptosis (Thurston et al., 2016).

3.3 | Cytosolic presence of *Salmonella*—detour or cul-de-sac?

The presence of *Salmonella* in the cytosol of host cells has been proposed as alternative intracellular lifestyle. We demonstrated that membrane damage and cytosolic presence of *Salmonella* is in part caused by the mode of entry but also highly dependent on experimental conditions and the effector repertoire of the strain investigated.

Would *Salmonella* be able to adopt a cytosolic lifestyle? A common trait of cytosolic pathogens is actin-based intracellular motility. The family of surface proteins has evolved that hijacks the host cell F-actin polymerising machinery in order to create F-actin polymerisation that propels the pathogen within the host cell cytosol (reviewed in Ray et al., 2009). This intracellular motility leads to intercellular spread and also protects the pathogen against host cell defence mechanisms such as autophagy (Huang & Brumell, 2014). Sensing of cytosolic bacteria is an important mechanism of innate immune defence. In phagocytic cells, cytosolic *Salmonella* induce cell death, and a sensing mechanisms for cytosolic lipopolysaccharide LPS-involving Caspases 1 and 11 leads to pyroptosis (Thurston et al., 2016).

**TABLE 1** Bacterial strains used in this study

| Designation | Relevant characteristic | Reference |
|-------------|-------------------------|-----------|
| NCTC 12023  | Wild type              | Lab collection |
| SL1344      | Wild type              | Lab collection |
| MvP503      | NCTC 12023 ΔsifA::FRT  | (Chakravortty, Hansen-Wester, & Hensel, 2002) |
| MvP509      | SL1344 ΔsifA::aph      | (Halici, Zenk, Jantsch, & Hensel, 2008) |
| MvP818      | NCTC 12023 ΔinvC::FRT  | (Gerlach et al., 2008) |
| MvP1412     | NCTC 12023 ΔsopE2::aph | (Zhang et al., 2018) |
| MvP1436     | NCTC 12023 ΔinvC::FRT ΔssaV::mTn5 | (Hölzer & Hensel, 2010) |
| MvP2473     | NCTC 12023 ΔsopE2::FRT, aph cassette in MvP1412 removed, FLP recombination | This study |
| MvP1459     | SL1344 ΔsopE2::aph, P22 transduction from MvP1412 | This study |
| M712        | SL1344 ΔsopA ΔsopB ΔsopE ΔsopE2 | Hardt lab, ETH Zürich |
| M1318       | SL1344 ΔsopE | Hardt lab, ETH Zürich |

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and growth conditions

*Salmonella enterica* serovar Typhimurium (STM) strains NCTC 12023 (identical to ATCC 14028) and SL1344 and isogenic mutant strains are summarized in Table 1. STM strains were routinely cultured in Luria-Bertani (LB) broth containing 50 μg/ml carbenicillin (Roth) and/or 12 μg/ml chloramphenicol (Sigma-Aldrich) if required for the selection of plasmids. Bacterial cultures were routinely grown in glass test tubes at 37 °C with aeration in a roller drum at ca. 60 rpm. For invasion of host cells, fresh LB medium was inoculated 1:31 with overnight cultures and incubated to 3.5 h with agitation in a roller drum.

To achieve zipper invasion by STM, the ΔinvC strain harboring p4880 for expression of *Yersinia pseudotuberculosis* Invasin under control of a SPI1 promoter was grown under the same conditions. To test induction of reporters by glucose-6-phosphate (G6P), LB medium was supplemented with various amounts of G6P ranging from 0.004 to 0.4 % (w/v).

4.2 | Construction of plasmids

Plasmids used in this work are listed in Table 2. Oligonucleotides for generation of recombinant DNA molecules were obtained from IDT.
are specified in Table S1. The promoter of uphT of STM was cloned as 251 bp fragment upstream of the translational start site of uphT. The fragment of fused by Gibson assembly (GA) to the RBS of DsRed in plasmid pMW211. GA was used to replace DsRed by sfGFP to achieve faster maturation kinetics, and to insert a cassette containing DsRed under control of the constitutive synthetic promoter EM7. This resulted in the dual fluorescence reporter p4889 harboring PEM7::DsRed for constitutive expression of DsRed and PuhpT::sfGFP, AmpR.

For exchange of the antibiotic resistance gene in pWSK29-derived plasmids, GA was performed using primers Vf-pWSK29-AB and Vr-pWSK29-AB to amplify the vector with bla gene, and 1f-pWSK-CAT and 1r-CAT-pWSK to amplify the CAT gene of pKD3 with overlaps to pWSK29.

### 4.3 | Cell lines and cultivation

Human epithelial cell line HeLa were maintained in DMEM containing 4.5 g x l$^{-1}$ glucose, 4 mM L-glutamine and sodium pyruvate (Biochrom) supplemented with 10 % FCS in an atmosphere of 5 % CO$_2$ and 90 % humidity at 37 °C. The murine macrophage cell line RAW264.7 (ATCC no. TIB-71) were cultured in DMEM containing 4.5 g x l$^{-1}$ glucose and 4 mM stable glutamine (Biochrom) supplemented with 6 % FCS. The human colonic polarized epithelial cell line Caco2Bbe1 (ATCC CRL-2102) is a derivate of Caco2 cells and was cultured in DMEM high glucose without pyruvate (PAA), containing Glutamax, 10 % FCS and 2.5 μg x ml$^{-1}$ human holo-transferrin (Sigma-Aldrich).

### 4.4 | Transfection

HeLa cells were cultured in 24-well plates with coverslips for one day. One μg of plasmid DNA (p5049, p5050) were solved in 50 μl DMEM without FCS and mixed with 2 μl FUGENE reagent (ratio of 1:2 for DNA to FUGENE). After incubation for 10 min at room temperature (RT), the transfection mix was added to the cells in DMEM with 10 %
4.5 | Infection of host cells

Host cell infections were performed as described previously (Rajasekhar, Liebl, Seitz, & Hensel, 2008). In short, HeLa cells were infected with 3.5 h subcultures of STM at a multiplicity of infection (MOI) ranging from 1 to 150. Infection was synchronized by centrifugation for 5 min at 500 x g, followed by incubation for 25 min at 37 °C in an atmosphere of 5 % CO2 before extracellular bacteria were removed by washing thrice with PBS. Subsequently, host cells were maintained in growth media containing 100 μg x ml⁻¹ gentamicin (AppliChem) for 1 h. Afterwards, cells were cultivated in growth media with a decreased gentamicin concentration of 10 μg x ml⁻¹ for the rest of the experiment.

4.6 | Pulse-chase with fluid phase markers

For tracing the endocytic pathway, fluid phase marker AlexaFluor 647-conjugated dextran (dextran-A647), molecular weight 10,000 (Molecular Probes) was used. HeLa cells were incubated with 100 μg x ml⁻¹ dextran-A647 overnight, washed, and incubated with dextran-free media. Cells were infected for 25 min, incubated 1 h with 100 μg x ml⁻¹ gentamicin and processed for imaging.

4.7 | Live cell imaging

HeLa cells cultured in 8 well-chamber slides were infected at a MOI of 5. The infection was performed as described above and 1 h after infection, the medium was replaced by Imaging Medium supplemented with 10 μg x ml⁻¹ gentamicin. At 1 h p.i., live cell imaging was carried out with a Zeiss microscope (63x objective) and an environmental incubation chamber at an atmosphere of 5 % CO2.

4.8 | Quantitation by flow cytometry analyses

HeLa cells were infected at a MOI of 1 to 150 for 25 min. At various time points from 1 to 16 h p.i., cells were detached with Biotase and fixed with 3 % PFA for subsequent flow cytometry analyses using the Attune Nxt Cytometer (Thermo Fischer). Experiments were performed in triplicates at least three times. Data were analyzed with Attune Nxt 2.5. Statistical analyses were performed using One Way ANOVA Bonferroni versus control with SigmaPlot 11 (Systat Software).

4.9 | Immunostaining

HeLa cells were infected with various STM strains as described above and at indicated time points p.i., cells were fixed with 3 % PFA for 10 min, washed with PBS and incubated in blocking solution (PBS containing 2 % goat serum, 2 % bovine serum albumin, 0.1 % saponin freshly added from 3 % stock solution in PBS). Permeabilized cells were stained with primary antibodies for 1 h, washed with PBS thrice, and staining was performed using secondary antibodies for 1 h. Antibodies and antiserum used in this study are listed in Table S2.

4.10 | Selective permeabilization of HeLa cells

A modified digitonin permeabilization method (Plutner, Davidson, Saraste, & Balch, 1992) was applied. The entire procedure was carried out on ice using ice-cold solutions. The infected cells were washed twice with KHM buffer (110 mM KAc, 20 mM HEPES, pH 7.2, 2 mM MgAc), and then incubated for 5 min in KHM buffer containing 10 μg x ml⁻¹ digitonin (Fluka). The detergent was removed and the cells were incubated for 20 min with KHM without digitonin to allow permeabilization. After a further washing step, cells were fixed with 3 % PFA in PBS and subsequently immuno-stained in blocking solution without saponin.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

J. R. and M. H. conceived the study, J. R. performed experimental work, J. R. and M. H. analysed the data, J. R. and M. H. wrote the manuscript.

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