Mechanism of SopE mediated recruitment of Rab5 on Salmonella containing phagosomes.
5.1. Introduction

Previous studies have shown that the intracellular survival in macrophages is an essential part of the pathogenesis of *Salmonella* and the mutants unable to survive in the macrophages were avirulent *in vivo* (Fields *et al.*, 1986). Irrespective of a few controversial findings it is an agreed view that the *Salmonella* survives in the macrophages by preventing its transport to the lysosomes (Holden, 2002). SpiC, *Salmonella* secretory protein encoded by the SPI-2, had been implicated in preventing the maturation of the *Salmonella* containing phagosome to phagolysosome. Thereby, SpiC was proposed to act as a general fusion inhibitor abrogating all vesicular fusion events in the cell (Uchiya *et al.*, 1999). However, later findings have questioned this observation since the modulation of trafficking of the SpiC knockout *Salmonella* could not be reproduced. Moreover it was also observed that the SpiC knockout *Salmonella* was unable to secrete the other SPI-2 encoded effectors as well and therefore questioning the validity of the inference of the former studies on SpiC modulated trafficking of *Salmonella* (Yu *et al.*, 2002). These results also indicate that there could be some other mechanism by which *Salmonella* inhibits their transport to the lysosomes.

Recent studies from our laboratory have shown that the *Salmonella* phagosome recruits Rab5 and there by prevents the maturation of the phagosome to phagolysosome and remains in a specialized compartment that interacts with the early endosome. However, the mechanism of recruitment of Rab5 by *Salmonella* containing phagosomes was not clear. In the previous chapter, we have identified that an effector protein from *Salmonella* encoded by the SPI-1 system, SopE, specifically binds with active GTP form of Rab5. Subsequently, SopE from the bacteria transported onto the surface of *Salmonella*-containing phagosomes and recruit Rab5 from the host cells. In the present study, we have delineated the mechanism of recruitment of GTP form of Rab5 on *Salmonella*-containing phagosomes and their physiological significance.
5.2. Materials and Methods

5.2.1. SopE-mediated nucleotide exchange of Rab5

The SopE-mediated nucleotide exchange of Rab5 was determined using an assay previously described (Hardt et al., 1998), with some modifications. First, the respective Rab 5 proteins were loaded with GDP and subsequently, the exchange of GDP to \([^{32}P]\) GTP was determined in the presence or absence of GST-SopE- (78-240). Briefly, different mutants of Rab5 (60 pmol of each protein) was incubated at room temperature for 30 min in loading buffer (20 mM Tris-HCl (pH 7.5), 5 μM GDP, 50 mM NaCl, 3 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA). Exchange reaction was carried out in 200 μl of exchange buffer (20 mM Tris-HCl (pH 7.5), 5 μM \([^{32}P]\) GTP, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin) containing 15 pmol of GDP-bound Rab proteins with/without an equimolar concentration of GST-SopE-(78-240) for 30 min at room temperature. Aliquots from the reaction were blotted onto a nitrocellulose membrane, and the membranes were extensively washed in ice-cold solution containing 20 mM Tris-HCl (pH 8), 100 mM NaCl, and 10 mM MgCl₂ to remove free radioactivity. The membranes were then transferred to scintillation vials, and counts were determined in the presence of scintillation fluid. A similar exchange reaction of Rab5 was carried out in the presence of different concentrations of SopE as indicated.

5.2.2. GTPase activation assay

The assay was similar to the one described earlier (Stenmark et al., 1994). To determine whether SopE acts as GTPase activating protein for Rab5, 5 nmoles of Rab5 WT protein immobilized on glutathione beads were incubated for 20min at 25°C in buffer A (20mM Tris-HCl pH 7.8, 100mM NaCl, 5mM MgCl₂, 1mM Na-Phosphate and 10mM 2-Mecaptoethanol). The beads were washed once with 1M guanidine hydrochloride and then twice with ice-cold buffer A to elute the bound nucleotide. The beads were then incubated in α-\([^{32}P]\)GTP and 5nmoles of SopE in 50 μl of buffer A for 10 min at 0°C. Then 50 μl of buffer A was added and the beads were incubated at 37°C for 1 hour to allow the
hydrolysis of bound GTP. The beads were washed thrice with buffer B (0.2% SDS, 2mM EDTA, 10mM GDP, 10mM GTP pH 7.5) heated at 70°C for 2 min. The supernatant was then analyzed by TLC in PEI-cellulose sheets in 0.6 M Na-phosphate buffer, pH 3.5. The sheets were dried and autoradiographed.

5.2.3. Removal of Rab5 from the cytosol

Immunodepletion of Rab5 from the cytosol was carried out using the procedure described previously (Mukherjee et al., 2000). Briefly, 100 µl of protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 10 µl of anti-Rab5 antibody in PBS overnight at 4°C. The antibody-protein A/G agarose complex was washed and centrifuged at 10,000 x g for 5 min at 4°C. Subsequently, 100 µl of J774E cytosol (600 µg) was added to the protein A/G agarose-antiRab5 complex and incubated for 2 h at 4°C to deplete the Rab5 from the cytosol. Subsequently, Rab5-depleted cytosol was separated from the agarose beads by centrifugation. Immunodepletion of Rab5 from the cytosol was confirmed by Western blot analysis using an anti-Rab5 antibody. Rab5-depleted cytosol was used for the in vitro fusion assay.

5.2.4. Preparation of biotinylated Salmonella

Salmonella grown in LB as described previously were biotinylated for use as a phagocytic probe for the phagosomes preparation using the standard method (Zurzolo et al., 1994). Briefly, bacteria were incubated with N-hydroxysuccinimidobiotin (0.5 mg/ml) in PBS-CM (10 mM PBS, pH 8 containing 0.1 mM CaCl₂ and 1 mM MgCl₂) for 1 h at 4°C. Then the cells were sequentially washed with PBS and 50 mM NH₄Cl to quench excess free biotin and resuspended in PBS. Biotinylation did not affect viability as shown by the ability to form similar number of colonies on LB agar plate before and after biotinylation. An aliquot of live biotinylated bacteria was killed by heat treatment followed by glutaraldehyde fixation. Both killed and live bacteria bound same amount of avidin-HRP, indicating a similar density of biotin in both the preparations. To determine
the biotinylated bacterial proteins in dead and live *Salmonella*, $1 \times 10^7$ bacteria were boiled in SDS sample buffer and aliquots were run on SDS-PAGE. In both the preparations, multiple and essentially identical proteins were biotinylated to similar extents as indicated by Western blotting with avidin-HRP.

5.2.5. Role of different mutants of Rab5 in fusion of early endosome with LSP *in vitro*

*In vitro* fusion of phagosomes containing the biotinylated *Salmonella* with early endosomes containing avidin-HRP were carried out using a procedure similar to that described previously (Mukherjee *et al.*, 2000). Briefly, phagosomes were purified from macrophages, and endogenous Rab proteins were stripped off from the phagosomes by GDI-GDP treatment. To determine the role of different mutant proteins of Rab5, fusion of Rab-stripped LSP with early endosomes was carried out in the presence of fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2, 1 mM dithiothreitol, 1.5 mM MgCl$_2$, 100 mM KCl, including an ATP-regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase, and 0.25 mg/ml avidin as scavenger) containing Rab5-immunodepleted gel-filtered cytosol supplemented with different mutants of Rab5 (10 µg of each GST-Rab5 preincubated in the presence of cytosol) as indicated. Fusion was carried out for 10 min at 37 °C, and the reaction was stopped by chilling on ice. The HRP-avidin-biotin bacterial complex was recovered by centrifugation (10,000 × g for 5 min) after solubilization of the membrane in solubilization buffer with 0.25 mg/ml avidin as scavenger. The enzymatic activity of avidin-HRP associated with the biotinylated bacteria was measured as fusion unit. The maximum fusion between endosomes and phagosomes (Control, LSP without GDI-GDP treatment) was observed at 0.5 mg/ml normal cytosol concentration, which was expressed as 1 unit of relative fusion. The HRP activity, corresponding to 1 unit, is mentioned in the legend to Fig. 11.
5.2.6. Preparation of purified phagosomes

Live or dead *Salmonella*-containing phagosomes were prepared using a method described previously (Alvarez-Dominguez et al., 1996, Sturgill-Koszycki et al., 1996). J774E clone macrophages (1 \times 10^8) were incubated with 1 \times 10^9 bacteria at 4 °C for 1 h in HBSA, and bacterial infection was synchronized by centrifugation at low speed. Then the cells were shifted to prewarmed medium and incubated for 5 min at 37 °C. The uptake was stopped by the addition of ice-cold medium. Cells were washed three times to remove unbound bacteria by centrifugation at low speed (300 \times g for 6 min). Subsequently, cells were resuspended in prewarmed medium and chased for different periods of time at 37 °C as indicated. Finally, cells were washed and resuspended (2 \times 10^8 cells/ml) in homogenization buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2) and homogenized in a ball bearing homogenizer (Pitt et al., 1992) at 4 °C. Homogenates were centrifuged at a low speed (400 \times g for 5 min) at 4 °C to remove nuclei and unbroken cells. The postnuclear supernatants (PNS) were quickly frozen in liquid nitrogen and stored at 70 °C. To obtain the phagosomal fraction, the PNS was quickly thawed and diluted with homogenization buffer (1:3) and centrifuged at 12,000 \times g for 1 min in a microcentrifuge at 4 °C as reported earlier (Pitt et al., 1992, Mayorga et al., 1991). Subsequently, phagosomes were further purified using the protocol as described previously (Sturgill-Koszycki et al., 1994). Briefly, the phagosomal fractions were resuspended in 100 µl of homogenization buffer containing protease inhibitors and loaded on 1 ml of 12% sucrose cushion. Samples were centrifuged at 1,700 rpm for 45 min at 4 °C, and the purified phagosomes were recovered from the bottom of the tube. The viability of bacteria in the phagosomes was determined by selective lysis of the phagosomal membrane with SB followed by cultivation of bacteria in LB-agar plate. Live *Salmonella* remained viable under these conditions. To determine the percentage of intact phagosomes, the phagosome preparations were treated with avidin at 4 °C to quench the biotin accessible in broken phagosomes. Subsequently, the phagosomes were washed and solubilized in SB in the presence of avidin-HRP. The difference in the HRP activity in avidin-untreated and avidin-treated phagosomes was used to determine the content of
intact phagosomes. The HRP activity associated with avidin-untreated solubilized phagosome preparation was taken as 100%. About 74% of DSP and 70% of LSP remained intact by this assay. The phagosomes purified in this way have already been shown to be free from contamination by plasma membrane, endosomes, Golgi and the endoplasmic reticulum (Hashim et al., 2000)

5.2.7. Analysis of phagosomal composition

To analyze the phagosomal composition at different time points, 40 μg of purified phagosomes from each time point were run on SDS-PAGE, transferred to nitrocellulose membranes, and incubated with appropriate dilution of respective monoclonal or polyclonal antibodies. Subsequently, membranes were incubated with secondary antibodies conjugated with peroxidase, and blots were visualized by using ECL (Amersham Pharmacia Biotech).

5.2.8. Electron microscopic observation of phagosome-lysosome fusion using MBSA-Gold as a lysosomal marker

J774E cells (1 × 10^6 cells) were incubated in the presence of mannosylated bovine serum albumin (MBSA) conjugated with 20-nm colloidal gold (100 μg/ml) for 30 min at 37 °C in prewarmed HBSA to allow uptake. Cell were washed three times with HBSA and chased for 80 min at 37 °C in the presence of 1 mg/ml mannan to label the lysosomes (Funato et al., 1997) After washing, cells were allowed to bind live or dead Salmonella (1 × 10^7 cells) at 4 °C for 1 h. Cells were resuspended in prewarmed medium, and uptake was carried out for 10 min at 37 °C. Cells were washed twice to remove the uninternalized bacteria and chased for 60 min at 37 °C. The cells were washed five times with cold PBS and fixed in 1% glutaraldehyde in 0.1M cacodylate buffer pH 7.2, washed, and postfixed with 1% osmium tetroxide in the same buffer. The cells were rinsed and dehydrated in ethanol and embedded in araldite (Mayorga et al., 1991). Thin sections were double stained with uranyl acetate and examined with an electron microscope.
5.3. Results

5.3.1. Prenylation-defective Rab5 mutant recruited by SopE is functionally active

To determine whether non-prenylated Rab5 (Rab5:ΔC4), recruited on the phagosomes is functionally active, we used an in vitro fusion assay. LSP (containing S. typhimurium WT) were incubated for 10 min with endosomes loaded with avidin- HRP at 37 °C in the presence of cytosol and an ATP-regenerating system. Results presented in Fig.11 show that LSP efficiently fuse with early endosomes in 10 min (Control). To establish the role of different forms of Rab5 in this fusion event, the endogenous Rab5 from the phagosomes were stripped off by Rab-GDI treatment, and fusion was carried out in Rab5- immunodepleted cytosol in the presence of indicated Rab5 mutant proteins. Data presented in Fig. 11 show that fusion of phagosomes with endosomes is inhibited in the Rab5- depleted condition Addition of Rab5: WT and Rab5: Q79L restored the fusion of the phagosomes with endosomes by more than 90%, whereas Rab5: S34N, which is locked in the GDP form did not stimulate the fusion (Fig.11). Interestingly, Rab5: ΔC4 stimulated the fusion of phagosomes with endosomes by more than 70% (Fig.11). Our finding that Rab5: ΔC4, which is identical...
with Rab5: WT excepting for the deletion of the C terminus cysteine motif that is essential for prenylation, promotes fusion suggests that non-prenylated Rab5 is functionally active. However, when Rab-striped LSP were pretreated with anti-SopE antibody, no fusion of LSP with endosomes could be detected even in the presence of Rab5 (Fig. 11). We also found that phagosomes containing the SopE knockout mutant Salmonella did not support fusion with the endosomes in Rab-depleted condition (data not shown), indicating that SopE-mediated recruitment of Rab5 by phagosomes is responsible for promoting fusion with early endosomes.

5.3.2. SopE acts as a GDP/GTP nucleotide exchange factor of Rab5

Our results (Fig. 12) demonstrated that Salmonella-containing phagosomes specifically bind Rab5 in its GTP form, the active form of the protein, which promotes

![Graphs showing nucleotide exchange of Rab5](image)

Fig. 12 A. Determination of SopE-mediated nucleotide exchange of Rab5. To determine the SopE-mediated nucleotide exchange of Rab5, GDP-loaded Rab5 and their mutants were incubated either in the presence (closed box) or in the absence (open box) of equimolar concentrations of GST-SopE-(78-240) in the presence of \[^{32}P\]GTP for 30 min. Aliquots from the reaction were blotted onto a nitrocellulose membrane to evaluate the binding of \[^{32}P\]GTP. Results are expressed as an average of GTP bound per pmol of Rab5 of three independent experiments ± S.D.

B. Concentration-dependent effect of SopE on nucleotide exchange of Rab5. A similar exchange reaction of Rab5 was carried out in the presence of indicated concentrations of GST-SopE as described in A. Aliquots from the reaction were blotted onto a nitrocellulose membrane to evaluate the binding of \[^{32}P\]GTP. Results are expressed as an average of GTP bound per pmol of Rab5 of three independent experiments ± S.D.
endosome-endosome fusion. Furthermore, SopE can induce the GDP to GTP exchange of Rho-GTPases (Hardt et al., 1998), which prompted us to investigate the role of SopE in the nucleotide exchange of Rab5. The results presented in Fig. 12A show that incubation of Rab5: WT and Rab5: Q79L in buffer alone significantly induces the nucleotide exchange of GDP to the GTP form over that obtained with Rab5: S34N, a mutant that is unable to exchange GDP to GTP. This is due to intrinsic activity of Rab GTPases. To determine the role of SopE, we measured the incorporation of $^{32}$P GTP molecules into GDP-loaded Rab5 in the presence of GST-SopE-(78-240). Our results demonstrated that Rab5: WT and Rab5: Q79L incorporated more than 2-fold of GTP in the presence of GST-SopE-(78-240) than did Rab5 alone, indicating that SopE enhances the nucleotide exchange of Rab5 (Fig. 12A). Furthermore, SopE was unable to induce the nucleotide exchange of Rab5: S34N, which is locked in the GDP form. Moreover, when Rab5 was incubated with increasing concentrations of SopE in the similar assay, the nucleotide exchange activity of Rab5 was proportional to the concentration of SopE present in the reaction (Fig. 12B).

5.3.3. SopE doesn’t influence the GTPase activity of Rab5

To check whether the SopE binding to Rab5 influences the inherent GTPase activity of the Rab5, an assay to measure the possible GAP (GTPase activating protein) activity of SopE was carried out. In short, 50 nmoles of Rab5: WT was first stripped off

![Fig 13. Determination of the effect of SopE on the GTPase activity of the Rab5. Rab 5 loaded with $^{32}$P GTP was incubated in the presence or absence of SopE. The protein bound nucleotides were resolved by TLC to separate GDP and GTP. The GTPase activity of the Rab5 was measured as a ratio of the protein bound GDP/GTP, as seen from the autoradiogram.](image)
5.3.5. Trafficking of SopE mutant *Salmonella* in macrophages

Since the SopE recruit Rab5 and promotes the interaction of *Salmonella* phagosome with the early endosome, we postulated that the phagosome containing the mutant bacteria would traffic differently in comparison to that containing the wild type bacteria. In order to demonstrate the role of SopE in intracellular trafficking of *Salmonella*, we have compared the intracellular route of WT and mutant *Salmonella* in macrophages by determining the presence or absence compartment specific markers at different stages of their maturation. The results presented in the Fig. 15 show that both phagosomes contain Rab5 in their early stages of maturation whereas at later stages only mutant *Salmonella*-containing phagosomes contain Rab7, which is a regulatory molecule for transporting the cargo towards late compartment. These results indicate that mutant *Salmonella* probably targeted to the late compartment while WT *Salmonella* inclined to stay more in some sort of early compartment. These results are further supported by the fact that WT *Salmonella*-containing phagosomes not only recruit Rab5 but also Rab5 interacting proteins like EEA1 and Rabaptin5. Similarly, Rab11 which is a Rab involved in...
in the recycling from early compartment, shown to be more in the WT *Salmonella*-containing phagosomes than mutant *Salmonella*-containing phagosomes.

5.3.6. SopE mutant *Salmonella* is trafficked to lysosomes

Studies discussed in the previous section that wild type *Salmonella* possibly resides in an early compartment and SopE mutant bacteria are probably targeted to the late compartment. To study whether the bacteria do reach the authentic lysosomes, the lysosomes were labeled with MBSA-gold and the co-localization of bacteria with gold was measured as indicator of the transport of bacteria to the lysosomes. Results shown in the Fig.16 indicate that the wild type bacteria do not co-localize with the
lysosomes and the mutant bacteria do. These results demonstrated that presence of SopE is essential to inhibit the transport of the bacteria to the lysosomes. Subsequently, mature phagosomal compartment containing either WT type or mutant bacteria were isolated and the nature compartment was determined. The results presented in the Fig.17 show that mutant *Salmonella* containing phagosomes contain more LAMP1 after 90 min than WT *Salmonella* containing phagosomes suggesting that SopE mutant bacteria probably target to the lysosomes. Simultaneously, we have also analysed the content of mature cathepsin D, a lysosomal enzymes in these phagosomes. These results also support the previous observations. However, our results also show that relatively higher amount of mature cathepsin D is also incorporated into Live *Salmonella dublin* WT containing phagosomes indicating some of the WT bacteria may be targeted to the lysosome like compartment. In contrast, live *Salmonella typhimurium* WT containing phagosome do not acquire any mature form of cathepsin D (Mukherjee *et al.*, 2000). These contrasting results obtained from two different WT *Salmonella* may be attributed the fact that *Salmonella dublin* which is not a mouse pathogen has a compromised survival in mouse.

5.4. Discussion

The experiments described in the previous section demonstrated that the SopE present on the phagosomal membrane specifically recruit Rab5. In the present chapter,
we have studied the mechanism of recruitment of Rab5 on phagosomes through a bacterial effector protein, SopE. Interestingly, results from data suggest that SopE only interact with GTP form of Rab5. So, the obvious question is that how inactive GDP form of Rab5 is converted to active GTP form. Is there any effector protein from the bacteria that is involved in the process? Current model of Rab GTPase functioning is that the Rab GTPases exists as active GTP bound form on the membrane or as inactive GDP bound form in the cytosol in complex with GDI. GDI presents the Rab protein on the membrane and then specific guanine nucleotide exchange factors (GEF) convert the inactive Rab to its active form by enhancing the exchange of GDP to GTP. Subsequently, at the later stages of the fusion, specific GTPase activating proteins (GAP) increase the inherent GTPase activity of the Rab protein to hydrolyse the bound GTP to GDP converting the Rab protein to its inactive form (Pfeffer, 2001). Thus, attempts were made to determine whether SopE has any role in the similar process. Our results show that the SopE acts as an exchange factor for Rab5 and convert inactive GDP bound Rab to active GTP bound confirmation. However, it has no effect on the inherent GTPase activity of Rab5.

These studies described in the present thesis have led us to propose a model for the mechanism of survival of Salmonella in macrophages (Fig. 18). SopE secreted by the

---

**Fig. 18. Model for SopE mediated modulation of the trafficking of the Salmonella containing phagosome**
*Salmonella* acts as an exchange factor for Rab5 converting it to active form in the cytosol and this activated Rab5 is then recruited by the SopE present on the phagosomal membrane. The recruited Rab5 then enhances the fusion of the *Salmonella* containing phagosome with the endosome and this prevents the trafficking of *Salmonella* to the lysosomes. The prediction of this model would be that if SopE is mutated then the trafficking of *Salmonella* in the macrophages would be different and this might lead to the killing of *Salmonella* by the macrophages. Further studies carried out partially validate this prediction.

To check for the changes in the modulation of trafficking of *Salmonella* in the macrophages, the interaction the phagosomes containing the wild type and SopE mutant *Salmonella* were assessed for the capability to interact with the early endosome, which according to our hypothesis is the major physiological event that prevents the maturation of the *Salmonella* phagosome to phagolysosome. Our results showed that SopE knock out *Salmonella* containing phagosomes do not efficiently fuse with early endosomes and are possibly transported to the lysosomes. Thus, the wild type *Salmonella* possibly will survive in some sort of early compartment which is indicated by the fact that wild type *Salmonella dublin* containing phagosome retains early compartment specific markers like Rab5, EEA1 and Rabaptin 5. Whereas mutant bacteria are targeted to a compartment, which is positive for Rab7 and contains more mature lysosomal enzymes and LAMP1. Electron microscopic studies further confirm the fact that SopE knock out bacteria is targeted to the lysosome like compartment similar to the dead bacteria.

Such a lysosomal trafficking of the bacteria would lead to the killing of the bacteria and so the mutant bacteria are expected not to survive in the macrophages or in the mice. Our experiments to test this prediction failed to show any discernible difference between the survival of the wild type and SopE mutant bacteria in the macrophages. At least three reasons could be attributed to the failure to see any difference in survival irrespective of the lysosomal trafficking of the mutant bacteria. First and foremost the *Salmonella* used for the study was *S.dublin* which is a veterinary pathogen and would have naturally adapted to survive in the bovine cells. So when the murine macrophage
cells were infected with the bovine pathogen the non-optimal pathogen-host interaction probably overshadowed the subtle differences in the survival between the wild type and the mutant *Salmonella* phagosomes.

The second reason owes to the presence of the recently identified SopE paralog SopE2 (Stender *et al.*, 2000). SopE2 is highly homologous to the SopE (69% sequence similarity) and was found to carry out similar exchange activities on cdc42, though there were subtle differences in the substrate specificity of the two proteins (Friebel *et al.*, 2001). SopE2 was found to be more conserved than the SopE (Stender *et al.*, 2000), which was found only in a few especially epidemic isolates (Ehrbar *et al.*, 2002). Studies on the interaction of Rab5 with SopE2 have to be carried out since it is the more conserved homologue and so would be expected to be more relevant to the studies on *Salmonella* survival in macrophages.

The third reason could be as pointed out by Falkow “We cannot deduce the contribution of a given bacterial gene to the infectious process simply by counting either the number of dead mice or the number of the bacteria in the organs” (Falkow, 2004). Host pathogen interaction is a complex process involving the regulation of many genes of both host and the pathogen. In case of *Salmonella* at least 4% of the genome is involved in virulence. It is possible that the system we are using to test the subtle contribution of the SopE to the survival in the macrophages is overridden by the effect of other virulence factors. In such a situation use of a knock-in would be better to study the contribution than a knock-out organism. Such a system improves the signal to noise ratio by removing the contribution of other genes to the virulence and thus making the effect of SopE visible. This would lead us to the question that if at all SopE had a small contribution to the survival of the bacteria, would that be enough to account for its fixation in the genome. The answer for this could be that the other contribution of SopE, in invasion of the epithelial cells is enough to fix the gene during evolution while the role of SopE in the modulation of trafficking might have been a side-effect.