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The *Salmonella*-containing Vacuole – Moving with the Times

Olivia Steele-Mortimer
Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840, USA

Summary

Salmonella pathogenesis is dependent on its ability to invade and replicate within host cells. Following invasion the bacteria remain within a modified phagosome known as the *Salmonella*-containing vacuole (SCV), within which they will survive and replicate. Invasion and SCV biogenesis are dependent on two Type III Secretion Systems, T3SS1 & T3SS2, which are used to translocate distinct cohorts of bacterial effector proteins into the host cell. Elucidating the roles of individual effector proteins in SCV biogenesis has proven difficult but several distinct themes are now emerging and it is apparent that SCV biogenesis is an extremely dynamic process involving; extensive membrane remodeling, interactions with the endolysosomal pathway, actin rearrangements and microtubule-based movement and tubule extension.

Introduction

The *Salmonella enterica* species is made up of a group of over 2,000 highly related serovars, which are 95–99% identical at the genetic level. Nevertheless, *S. enterica* serovars have different host specificities and cause a variety of different diseases in man and other animals. Typhoid fever, a severe systemic disease caused by the human restricted serovar Typhi, annually afflicts approximately 21 million people worldwide, almost all in the developing world. In contrast, non-typhoidal Salmonellosis, a usually self-limiting gastrointestinal infection, can be caused by a number of serovars and is endemic throughout the world. Serovar Typhimurium is one of the serovars most often associated with gastroenteritis in man but induces a typhoid-like systemic disease in susceptible mice. Almost all of what we know about the molecular basis of *Salmonella*-host cell interactions comes from studies using Typhimurium and cultured mammalian cells.

Salmonella virulence is dependent on the ability of the bacterium to invade non-phagocytic host cells and then to survive and replicate within a modified phagosome known as the *S*almonella-containing vacuole (SCV), although it should be noted that the factors determining systemic *vs* localized infection remain undetermined. Both invasion and intracellular survival/replication are mediated by chromosomally encoded virulence genes, which are clustered on several *Salmonella* pathogenicity islands. In particular two Type III secretion systems, T3SS1 and T3SS2, are used to translocate cohorts of bacterial effector proteins directly into host cells where they can manipulate host cell processes such as membrane trafficking or signal transduction. The two systems have different functions, T3SS1 is required for invasion of non-phagocytic cells whereas T3SS2 is implicated in intracellular survival and biogenesis of the

Corresponding author: Olivia Steele-Mortimer, Rocky Mountain Laboratories, 903 South 4th Street, Hamilton, MT 59840. Tel: 406-363 9292. Fax: 406-363 9380. omortimer@niaid.nih.gov.

SCV, and are expressed at different times. Recent studies have revealed a hitherto unexpected overlap in function of the two T3SS, particularly with respect to the role of T3SS1 in SCV biogenesis. Here we summarize and discuss what is known about SCV biogenesis and the roles of T3SS effectors in this process.

The canonical phagosome

Phagocytosis, or the internalization of particles by professional phagocytes such as macrophages and dendritic cells, is a critical host defense step during infection with bacteria. Depending on the cell type, a cascade of degradative anti-bacterial processes are initiated by the initial binding and engulfment of pathogens, including the production of superoxide and nitric oxide radicals. Concurrently the newly formed phagosome rapidly acidifies, due to the activity of the vacuolar ATPase (vATPase), a large macromolecular complex that is recruited to the phagosomal membrane. Fusion with lysosomes plays a critical role in this process and results in the delivery of lysosomal hydrolases, or endosomal proteases, that are active in the acidified (pH <5) lumen of the phagosome. Supplemented further by the activities of cationic proteins and antimicrobial peptides such as the defensins the mature phagosome is extremely bactericidal and in some cell types also functions as the source of antigenic peptides that are required for the development of an adaptive immune response against individual pathogens.

To survive inside the host cell intracellular bacterial pathogens have adopted a variety of mechanisms to evade intra-phagosomal killing and establish a replicative intracellular niche (for recent review [1]). Perhaps the most obvious approach to this problem is to lyse the phagosomal membrane and escape into the cytoplasm of the host cell, as exemplified by *Listeria* spp and *Shigella* spp. However, other bacteria, including *Salmonella*, have developed alternative ways to evade the arsenal of antimicrobial activities while still remaining within a membrane-bound vacuole or modified phagosome.

Because phagosome biogenesis is determined by its content, in addition to other factors including cell type (reviewed in [2]), there is no *de facto* default pathway; although latex bead-containing phagosomes have been used as a useful standard with which pathogen-containing phagosomes can be compared. Biogenesis of these phagosomes is a highly synchronous process typified by the sequential acquisition of specific cellular proteins delivered by fusion, either transient or complete, with different endosomal compartments ultimately resulting in the formation of a phagolysosome [3]. Thus early phagosomes (within 30 min following formation) transiently acquire a cohort of proteins typically restricted to early or sorting endosomes, including EEA1, Rab5 and Transferrin receptor. These proteins are then replaced by components of late endosomes and lysosomes, such as vATPase, lysosome-associated membrane proteins (Lamps; also known as LIMPs or lgps) and the lysosomal hydrolases such as the cathepsins. Although this is undoubtedly an oversimplified model, it is a useful benchmark with which pathogen-containing phagosomes can be compared.

The *Salmonella*-Containing Vacuole

Early studies of SCV biogenesis, which were carried out several years before proteomic analysis of LBP, found that while Lamps were enriched on SCV membranes, other endolysosomal marker proteins were not detected. In particular, neither the mannose 6-phosphate receptors nor lysosomal hydrolases could be detected at significant levels [4–6]. Based on these findings a model was developed based on the idea that *Salmonella* regulates SCV biogenesis by limiting interactions with the endocytic pathway and particularly by blocking SCV-lysosome fusion. This hypothesis rapidly gained acceptance and subsequent studies concentrated largely on identifying the bacterial factors involved in blocking lysosome fusion with the SCV. Recently, however, advances in live cell imaging technology together

with an improved understanding of eukaryotic membrane trafficking events are providing new insights into SCV biogenesis.

For the sake of simplification SCV biogenesis can be separated into 3 stages; early (<30 min p.i.), intermediate (30 min –approx 5 h p.i.) and the late (> 5h p.i.), each of which is associated with specific sets or subsets of T3SS effectors (Fig 1 and Table 1). By far the best-documented aspect of SCV biogenesis is the sequential delivery of endolysosomal membrane proteins that defines the conversion of early to intermediate SCVs. Thus the early SCV membrane is highly enriched in early endosome membrane markers, including EEA1, Rab5 and transferrin receptor, which are replaced within 20–40 min with late endosomal/lysosomal markers including Lamps and vATPase [7,8]. This change in membrane content is accompanied by a decrease in the luminal pH (pH_{scv}) to <4.5 [9,10] and redistribution of the SCV to a predominantly juxtanuclear position near the microtubule organizing center (MTOC) [11]. While this seems to hold true in most cell types studied, in phagocytic cells the mechanism of uptake, i.e. invasion *vs* phagocytosis, may affect SCV biogenesis especially with respect to acquisition of lysosomal proteins and acidification [9,12]. These data alone do not suggest any significant deviation from "canonical" phagosome biogenesis, particularly since factors such as particle size or surface charge can influence this process [13]. However, neither cathepsins nor the MPRs, which are responsible for trafficking cathepsins from the biosynthetic pathway to the endolysosomal pathway, were enriched in the SCV [4–6] suggesting a novel maturation pathway [4]. Conflicting with this model other studies have since suggested that SCVs can and do fuse with terminal lysosomes and maintain dynamic interactions with the endocytic pathway over many hours [14,15]. So how can these apparently mutually exclusive findings be resolved? One possibility is that *Salmonella* affects MPR trafficking/recycling rather than lysosome-SCV fusion. Indeed, at steady state MPR is primarily found in the TGN, since it is rapidly recycled following delivery of bound ligands (such as cathepsins) to the endolysosomal pathway. If recycling to the TGN is delayed the receptor is rapidly degraded and delivery of ligand to lysosomes is abrogated [16]. This would explain both low levels of MPR and cathepsins in the SCV. Alternatively, fusion with lysosomes, if not completely blocked may be delayed as indicated by several studies comparing delivery of lysosomal hydrolases, or content, to SCVs containing live wild type *Salmonella* with those containing non-replicative mutants or other similar particles [6,14,15]. A third possibility is that *Salmonella* have developed an alternative mechanism to reduce the bactericidal activity of the phagolysosomal environment, which could explain delayed maturation of cathepsins in SCVs [17,18].

Branching Out – *Salmonella* induced tubules radiate from the SCV into the host cell cytoplasm

The third stage of SCV biogenesis is characterized by bacterial replication and the centrifugal elongation of tubules from the surface of the SCV both of which are initiated within approximately 4–6 hr. Originally, named Sifs, for *S*almonella-induced filaments, these tubules have been best characterized in epithelial cells although they can form in other cell types [19]. Sifs have not yet been shown to form *in vivo* but in cultured epithelial cells they form a dramatic phenotype that distinguishes *Salmonella* from other intra vacuolar pathogens. Sifs were first discovered because they are highly enriched in Lamp, but they also contain vATPase, the late endosomal lipid lysobisphosphatidic acid, cathepsin D and Rab7 [20].

Centrifugal tubular extension of endolysosomal compartments is certainly not unique to *Salmonella*-infected cells. Similar tubular lysosomes or endosomes have been described in uninfected macrophages, monocytes, fibroblasts and dendritic cells [21–24]. Nevertheless, there is no doubt that *Salmonella* induces the formation of extensive Lamp enriched tubules and, although the physiological relevance of this phenomenon remains unclear, the phenotype has been useful in dissecting the roles of individual T3SS effector proteins.

Life in the balance – T3SS effector proteins in SCV/Sif biogenesis

While the debate over whether or not *Salmonella* block SCV-lysosome fusion continues there is no question that T3SS effectors are required to establish the intracellular niche. Nonetheless, the contributions of *individual* effectors to SCV biogenesis remain obscure, largely because of redundancy in function so that individual deletion mutants usually have little if any phenotype either *in vivo* or *in vitro*. Of the two T3SS in *Salmonella* it is T3SS2 that has been most conclusively shown to control intracellular events; it is also induced intracellularly and translocates effectors into the host cell across the SCV membrane. In contrast, the T3SS1 is induced extracellularly and translocates effectors across the plasma membrane in order to promote invasion of non-phagocytic cells. Following invasion the T3SS1 system is down regulated but can continue to translocate effectors across the SCV membrane for some time. Switching between T3SS1 and T3SS2 is not well understood, but it is now evident that there is overlap between the two systems and several T3SS1 effectors are implicated in SCV biogenesis and intracellular replication (Table 2).

T3SS1 effectors mediate early SCV biogenesis

At this time the four T3SS1 effectors that have been directly implicated in SCV biogenesis are; SipA/SspA, SopA, SopB/SigD and SopD. Following invasion SopA is translocated across SCV membrane into the cytosol where it can be ubiquitinated and rapidly degraded, and is associated with an increase in escape from the SCV, although the significance of this remains unclear [25].

In contrast, SopB and SipA can be detected in cells for many hours after infection [26,27]. SopB is a phosphoinositide (PI) phosphatase that contributes to efficient closing off of the phagocytic cup [28] by hydrolysis of $PI(4,5)P_2$ and has also been shown to be required for PI3P accumulation on the newly formed SCV [29]. The role of PI3P in SCV biogenesis remains unclear; it is required for EEA1, Vamp8 and Lamp1, but not rab5, recruitment [29–33]. It may well be critical for SCV biogenesis but this has not been conclusively demonstrated and furthermore the *in vivo* substrate, or substrates, of SopB phosphatase activity remain undefined. Nonetheless, since PIs are involved either directly or indirectly in all eukaryotic cell processes, it is likely that SopB activity in the SCV membrane has additional pleiotropic effects [34]. Another T3SS1 effector that is expressed following invasion is SopD [35], a protein that may act cooperatively with SopB at least during initial formation of the phagosome [36]. Lastly, SipA is an actin binding protein that has recently been shown to induce late endosome redistribution in infected cells and also to cooperate with the T3SS2 effector SifA to maintain SCV positioning at late time points [27].

T3SS2 effectors mediate intermediate and late SCV biogenesis

In comparison to the aforementioned T3SS1 effectors T3SS2 effectors seem to mediate generally later steps in SCV biogenesis, especially; movement of the SCV to the juxtanuclear region and the subsequent maintenance of this position, formation of an actin meshwork around the SCV and anterograde extension of Sifs along microtubules. The only T3SS2 effector that has been implicated in early SCV biogenesis is SpiC/SsaB, a protein that was shown to inhibit membrane fusion [37,38]. But other data suggests that SpiC is a component of the T3SS2 translocon rather than an effector [39] and this issue remains unresolved. Redistribution of SCVs from the cell periphery to the MTOC/juxtanuclear region is mediated at least in part by the small GTP-binding protein rab7 together with its effector RILP, which are required for recruitment of the microtubule-based motor dynein [40–42]. Once at the MTOC two T3SS2 effectors that have the ability to interact with one another, SseG and SseF, are required to maintain SCV positioning over longer periods of time [43–45]. SseF and SseG, together with SifA, have also been shown to be involved in redirecting exocytic cargo vesicles to the SCV

[46]. SifA is essential for Sif formation, since mutants lacking SifA do not form Sifs and eventually escape from the SCV. Although the molecular basis of SifA activity remains unclear at least one of its functions may be to down-regulate the recruitment the microtubule-based motor kinesin on the SCV via interactions with a host protein SKIP [47]. Certainly, at some point following dynein-dependent movement of the SCV to the MTOC, the emphasis switches to kinesin-dependent anterograde extension of Sif tubules away from the SCV/MTOC. The T3SS2 effector PipB2 enhances this process likely via direct interaction with kinesin light chain [48,49]. Sif formation involves at least one other T3SS2 effector, the deacylase SseJ, that seems to act as a counter balance to SifA since it down-regulates Sif tubule formation and, unlike the *sifA* deletion mutant, the double *sifA sseJ* deletion mutant does not escape from the SCV [50–52].

The third phenotype associated with T3SS2 effectors is the formation of an F-actin meshwork in the vicinity of the SCV, also known as vacuole-associated actin polymerizations (VAP), a process that is associated with SCV membrane integrity [53]. Three T3SS2 effectors, namely SteC, SseI and SspH2, have been implicated in this process although their roles remain obtuse. SteC has homology to the human kinase Raf-1 and its kinase activity is required for actin remodelling although not for SCV membrane integrity [54]. SseI and SspH2 can both interact with the actin cross-linking protein filamin yet neither of these effectors is essential for VAP formation, which instead requires another virulence factor SpvB that is encoded on the *Salmonella* virulence plasmid [55]. Interestingly, SspH2 co-localizes with VAP in infected cells and *in vitro* can interact with the actin binding protein profilin and decrease the rate of actin polymerization [55].

Conclusions

In spite of a spate of recent papers on the roles of T3SS2 effectors in SCV biogenesis we still know almost nothing about the molecular mechanisms involved. The roles of some effectors such as SseF and SseG are starting to unfold but at the same time two central paradigms are changing. Firstly, it is apparent that T3SS1 effectors are not only involved in invasion and at least some of them have potentially profound roles in SCV biogenesis. Secondly, live cell imaging studies are starting to reveal the dazzling dynamics of SCV and Sif biogenesis and have already shown that these organelles are much more accessible to the endolysosomal pathway than previously believed. So where is the future in SCV research? Almost all of what we know about the mechanisms involved in *Salmonella*-host cell interactions has come from studies using cultured mammalian cells, primarily non-polarized epithelial cells. Macrophages undoubtedly play an important role in *Salmonellosis* but it is likely that the outcome for the bacteria is determined by a number of factors such as activation state, mechanism of uptake or type of macrophage. For example, the intestinal macrophages encountered by Typhimurium in human gastroenteritis are likely to bear little resemblance to the circulating macrophages encountered in the mouse model of systemic Salmonellosis. Some macrophages may provide a replicative niche for *Salmonella*, whereas others do not. One well-documented difference between macrophages and epithelial cells is that *Salmonella* can induce rapid cell death in macrophages, via caspase 1, whereas in epithelial cells a prosurvival pathway is activated [34]. In addition to macrophages, *Salmonella* interact with a variety of highly differentiated cell types *in vivo* each of which may require specific adaptions by the bacteria. Although some studies have partially addressed these concerns by using polarized epithelial cells, cultured dendritic cells or activated macrophages, these one dimensional monocultures used in the lab still bear little resemblance to the highly 3 dimensional *in vivo* environment. It will be extremely interesting to see if SCV biogenesis *in vivo* is similar to that seen *in vitro*.

It is also important to consider the different disease outcomes when *Salmonella* serovars infect different hosts. For example, oral inoculation with serovar Typhimurium causes a localized

gastroenteritis or enterocolitis in higher primates, rabbits and young cattle but not in mice unless they are pretreated with streptomycin in which case a limited colonitis develops [56]. Since individual T3SS effector proteins may have roles only in certain cell types or host environments complete dissection of their roles, as well as resolution of the role of lysosome fusion, in SCV biogenesis may ultimately depend on the development of more sophisticated *in vitro* models or the analysis of appropriate *in vivo* infections.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest

•• of outstanding interest

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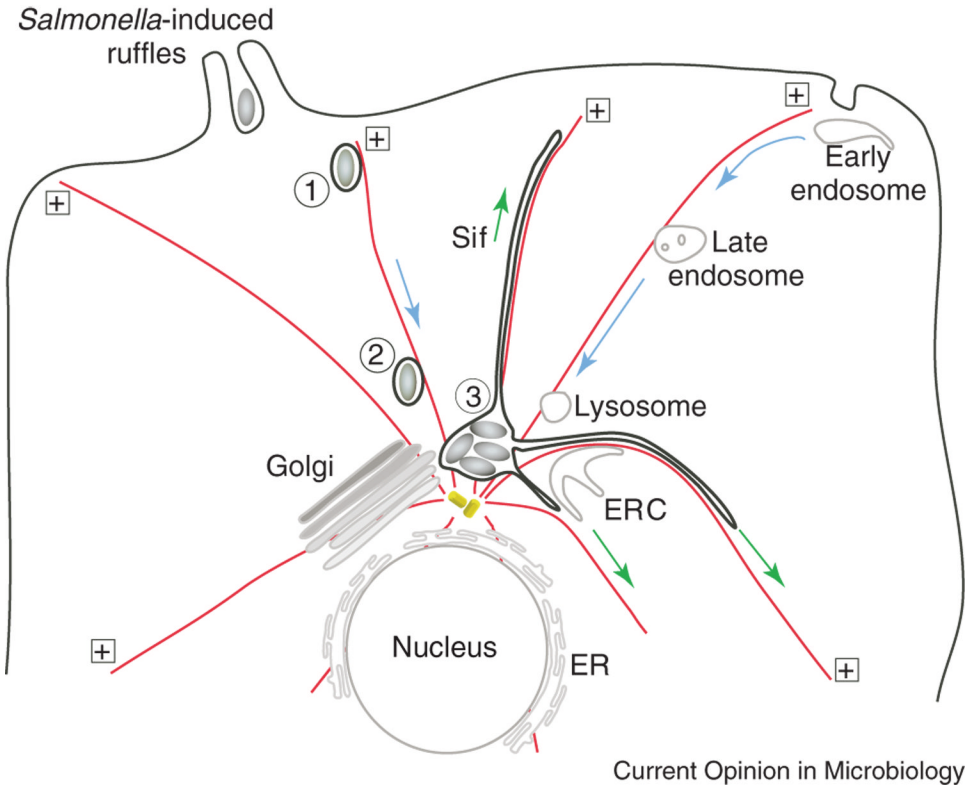

Figure 1.

Schematic representation of SCV biogenesis. *Salmonella* invade non phagocytic cells by inducing membrane ruffles on the plasma membrane. The bacteria are internalized into the early SCV (1), which is characterized by the presence of markers of the early endocytic pathway such as EEA1 and rab5. Within 15–60 min early endosome proteins are replaced by proteins normally found on late endosome or lysosomes, such as Lamps and vATPase, and SCVs are predominantly localized near the nucleus (2). Finally after 4–6 hr bacterial replication is initiated and Sif tubules extend radially along microtubules (3). In non-polarized epithelial cells microtubules emanate from the juxtanuclear MTOC forming a radial array of uniformly polarized microtubules whose plus ends are closest to the plasma membrane. In these cells the Golgi, lysosomes and endoplasmic recycling compartment (ERC) are concentrated near the MTOC. The localization of these compartments is mediated largely by interactions with microtubule-based motors such as dynein and kinesin, which mediate retrograde (blue arrows) and anterograde movement (green arrows) respectively. *Salmonella* utilize the same cellular system for translocation of SCVs towards the nucleus and subsequent extension of Sifs.

Table 1

Stages of SCV biogenesis

SCV stage	Characteristics	Host cell markers	T3SS effectors implicated	References
Early (<30 min)	Simple vacuole or spacious phagosome.	EEA1, rab5a, rab5b, rab5c, transferrin receptor	**T3SS1**: SipA, SopA, SopB, SpiC/SsaB T3SS2: SpiC	[7,27,38,57]
Intermediate (30 min – 5 h)	Vacuole primarily in juxtanuclear position	Lamps, vATPase, rab7, rab11a, rab11b	**T3SS1**: SopB, SipA **T3SS2**: SSeF, SseG, SpiC/SsaB, SteC, SseJ/SifC	[7,27,57]
Late (>5 h)	Initiation of intracellular replication and formation of tubules (Sifs) radiating throughout cells. Microtubule and actin accumulation around juxtanuclear SCV.	Lamps, vATPase, rab7, rab9	**T3SS2** PipB2, SifA, SopD2, SpiC/SsaB, SseF, SseG, SseJ/SifC, SteC,	[20,27,42,48,53,57]

Table 2

List of T3SS effector proteins that have been implicated in SCV biogenesis.

Effector	Intracellular localization		Enzymatic activity	Host target	Function with respect to SCV	References
	Translocated by *Salmonella*	Ectopically expressed				
SipA/SspA (T3SS1)	"Foci" in the vicinity of bacteria at early times (≤ 30 min p.i.), SCV at later times (≤ 8 h p.i.)	Cortical actin		Actin	SCV positioning	[27,58–60]
SopA (T3SS1)	Mitochondria	Mitochondria	E3 ubiquitin ligase	HsRMA1	SCV integrity	[25,61,62]
SopB/SigD (T3SS1)	SCV	Membranes of swollen vesicles, lamellipodia	Inositol polyphosphate phosphatase	Membrane phospholipids	Promotes membrane fission during invasion, PI(3)P production in and VAMP8 recruitment to ruffles, Lamp acquisition to SCV	[28,29,32,33,63–65]
SopD (T3SS1/T3SS2)	Cytosol	Cytosol and vesicles			Membrane fission and macropinosome formation during invasion	[35,36,66]
PipB (T3SS2)	SCV, Sifs					[67]
PipB2 (T3SS2)	SCV, Sifs	Vesicles at cell periphery		Kinesin-1	Anterograde movement of Sifs and late endosomes/ lysosomes	[19,48,49]
SifA (T3SS2)	SCV, Sifs	Lamp-enriched tubules and vesicles, plasma membrane, cytosol, nucleus		SKIP, Rab7	SCV positioning, membrane integrity and dynamics, Sif formation	[45,47,68–73]
SifB (T3SS2)	SCV, Sifs	Cytosol				[73]
SopD2 (T3SS2)	SCV, Sifs	Lamp-rich vesicles and tubules			Sif formation	[35,66]
SpiC/SsaB (T3SS2)	Conflicting data	Cytosol and nucleus		Hook 3, TassC	Inhibition of vesicle fusion with SCV, VAP formation, Sif formation	[37–39,74–77]
SseF (T3SS2)	SCV, Sifs	Microtubule bundles, Golgi network			SCV positioning and motility, Sif formation, microtubule bundling	[43–45,77–79]
SseG (T3SS2)	SCV, Sifs	Golgi network, perinuclear aggregates, microtubule bundles			SCV positioning, and motility, Sif formation, microtubule bundling	[11,43–45,77–79]
SseI/SrfH	VAP	Membrane ruffles and focal adhesions		Filamin, TRIP6	Actin rearrangements	[55,80,81]
SseJ/SifC (T3SS2)	SCV, Sifs	"Globular membranous compartments"	Deacylase		SCV membrane dynamics, negative regulation of Sifs	[50,51,73]
SspH2 (T3SS2)	VAP	Sites of actin polymerization, membrane ruffles and perinuclear region	Inhibits actin polymerization *in vitro*	Filamin, profilin		[55]
SteC (STM1698) (T3SS2)	SCV, Sifs, VAP	Cytosolic	Kinase	Actin cytoskeleton	Required for VAP	[54]

SKIP, SifA and kinesin-interacting protein; VAP, vacuole-associated actin polymerization.