Role of *Salmonella* effector protein(s) in the modulation of host SNARE molecules during intracellular trafficking in macrophages

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CERTIFICATE

This is to certify that this thesis entitled **"Role of *Salmonella* effector protein(s) in the modulation of host SNARE molecules during intracellular trafficking in macrophages"** by **Richa Madan** towards partial fulfillment of the Ph.D. degree of the Jawaharlal Nehru University, embodies the work done by the candidate under my guidance at the National Institute of Immunology. This work is original and has not been submitted in part or in full for any other degree or diploma of any university.

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Richa.

Richa

Contents

List of abbreviations

µg	microgram
µl	microlitre
µM	micro Molar
µm	micrometer
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTPs	Deoxyribose nucleoside triphosphates
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
EEA-1	Early endosome associated autoantigen 1
EGTA	Ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic Reticulum
FCS	Fetal calf serum
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2ethane-sulphonic acid])
hr	Hour

HRP	Horse radish peroxidase
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kDa	kilo Dalton
LAMP	Lysosome associated membrane protein
LBPA	Lysobiphosphatic acid
LE	Late endosomes
LPS	Lipopolysaccharide
LTA	Lipotechoic acid
LB	Luria Bertani
M6PR	Mannose-6-phosphate receptor
MOI	Multiplicity of infection
mg	milligram
min	minutes
ml	millilitre
mM	milliMolar
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
NEM	N-ethyl maleimide
ng	nanogram
NHS	N-hydroxysuccinimide
nm	nanometre
NSF	NEM sensitive fusion protein
°C	Degree celsius
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PM	Plasma Membrane
RFP	Red fluorescent protein
RNA	Ribonucleic acids
RPMI	Roswell Park Memorial Institute

RT	Room Temperature (25°C)
RT-PCR	Reverse Transcription-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide electrophoresis
SNAP	soluble NSF attachment protein
SNAP-25	synaptosomal associated protein of 25kDa
SNARE	SNAP receptor
SopB	*Salmonella* outer protein B
SopE	*Salmonella* outer protein E
SipC	*Salmonella* invasion protein C
SPI	*Salmonella* pathogenicity island
T3SS	Type III secretion system
TCA	Tri-chloro acetic acid
TGN	trans-golgi network
Tris	Tris-hydroxy methyl- amino methyl
VAMP	Vesicle associated membrane protein
Vti1b	Vps 10 interacting protein 1b

Introduction

Typhoid fever which is caused by *Salmonella enterica* serovar *Typhi,* a food borne pathogen, is one of the major causes of human morbidity and mortality. *S. typhimurium,* which causes a similar systemic infection in mice, serves as an experimental model for studying *Salmonella* infections (Ohl and Miller, 2001). During the course of infection, *Salmonella* invades epithelial cells and activates the production of inflammatory cytokines, which attract the immune cells to the site of infection (Wallis and Galyov, 2000). The interaction between the pathogen and an activated macrophage is followed by overt responses from both bacteria and the host cell. The host response to infection is triggered by several cellular processes such as diverse as cytoskeletal rearrangements, alterations in membrane trafficking, activation of anti-microbial mechanisms, production of pro- and anti- inflammatory cytokines and chemokines, activation of apoptosis and production of molecules required for efficient antigen presentation to the adaptive immune system. The bacteria retort to the host mechanisms by secretion of molecules commonly called as effector proteins through a specialized system known as Type III secretion system. However, on encountering non-activated macrophages the pathogen is internalized and survives in a specialized membrane bound compartment inside the macrophage. This compartment has been termed as the *Salmonella* containing vacuole (SCV) or live *Salmonella* containing phagosome (LSP) (Cotter and DiRita, 2000; Hashim et al., 2000). *Salmonella* is able to survive and replicate in this intracellular niche in macrophages by inhibiting the maturation of the phagosome that contains it, into a phagolysosome (Fratti et al., 2002) which is carried out by modulating the expression of various intracellular transport molecules (Hashim et al., 2000).

It is well documented that the process of phagosome maturation involves sequential interactions of the phagosome with members of the endocytic pathway (Jahraus et al., 1998; Mayorga et al., 1991). Similar to the endocytic pathway, intracellular transport of the phagosome to its appropriate destination also requires a series of highly coordinated and specific vesicle fusion events. The process of vesicular fusion is regulated by small GTP binding proteins of the Rab family and various tethering factors in combination with soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins which confer specificity in these trafficking events (Pfeffer,

1999; Rothman and Sollner, 1997; Scott et al., 2003; Zerial and McBride, 2001). However, the exact mechanism by which intracellular pathogens like *Salmonella* modulate these transport molecules to survive within host cells is still not well characterized. Nonetheless, it is known that *Salmonella* secretes a number of bacterial effectors with a plethora of functions to enable efficient invasion and survival as an intracellular pathogen.

Previous studies from our laboratory have demonstrated that SopE, a *Salmonella* effector protein specifically binds and recruits Rab5 from host cells on the LSP. This promotes the fusion of LSP with early endosomal compartments thereby inhibiting the maturation of LSP to phagolysosomes (Mukherjee et al., 2000). Subsequent studies have shown that the recruitment of Rab5 on LSP helps the phagosome to acquire N-ethylmaleimide sensitive fusion protein (NSF), suggesting a role of SNAREs in the maturation of *Salmonella*-containing phagosomes. Consequently, we have observed that *Salmonella* recruits different SNARE molecules on the phagosomes as they mature. In the present study, we propose to understand the mechanisms by which *Salmonella* recruits these SNAREs on their phagosomes and the plausible role of different *Salmonella* effector proteins in this process.

Review of literature

Phagocytosis (Greek, *phagos*-eating; *cytes*-cells) is the process of uptake of a particle of large size (>0.5 μm) from its environment by a cell. The phagocytic machinery has been used in different organisms for varied purposes with unicellular organisms like amoeba deriving nutritional benefits while higher vertebrates and mammals exploit this machinery to fulfill additional functions such as clearance of apoptotic cells and aversion of infection (Cardelli, 2001; Underhill and Ozinsky, 2002). Phagocytosis in mammals is a specialized feature of so-called professional phagocytic cells, i.e. neutrophils, dendritic cells and macrophages but is not unique to these cells (Rabinovitch, 1995). Cells like the retinal epithelial cells which fall in the category of paraprofessional phagocytes have intermediate phagocytic ability unlike the professional phagocytes (Rabinovitch, 1995). In mammals, phagocytosis serves as a key host defense mechanism. When a non-self particle such as a bacterium enters the body, professional phagocytes are chemotactically attracted to the site of infection. Upon interaction of the bacterium with specific receptors on the phagocyte, actin polymerization is induced at the site of invasion followed by particle internalization via an actin-based mechanism. The phagocyte envelops the particle by a portion of its plasma membrane which finally pinches off to form a phagosome. After internalization actin is shed from the phagosome and the phagosome matures by a series of fusion events with members of the endocytic pathway, culminating in the formation of the mature phagolysosome (Conner and Schmid, 2003; Haas, 2007). The hostile environment of this compartment leads to the destruction of the internalized bacterium and the regurgitated bacterial peptides are then presented on the surface of macrophages to elicit the adaptive immune response.

2.1 Mechanism of phagocytosis

Two mechanisms have been considered for particle phagocytosis, namely 'Zipper' and 'Trigger'. The Zipper mechanism proposed by Griffin and Silverstein originated based on the studies of bacterial phagocytosis in erythrocytes (Griffin et al., 1975; Griffin and Silverstein, 1974). According to this mechanism, ingestion occurs as a result of continuous receptor-ligand interactions leading to formation of the phagoctyic cup. In contrast, the triggering process involves commencement of an all-or-none phagocytic response upon particle binding. This stimulates membrane ruffling at sites of

invagination, followed by formation of large endocytic structures, macropinosomes as membrane ruffles fold back against the cell surface (Racoosin and Swanson, 1989). This "non-zippering process" or the triggered phagocytosis has been exemplified by studies on *S. typhimurium* internalization into macrophages and epithelial cells (Takeuchi, 1967).

2.2 Receptor mediated phagocytosis

Due to restricted availability of predisposed phagocytic receptors and the propensity of pathogens to mutate, a variety of receptors are engaged in the phagocytic process by recognizing conserved motifs present specifically on pathogens, but absent on host cells. These conserved motifs called "pathogen-associated molecular patterns" (PAMPs) include mannans, formylated peptides and lipopolysaccharides of yeast, gram negative and positive bacteria and are recognized by the receptors commonly called as "pattern-recognition receptors" (PRRs) (Janeway, 1992). Both cellular and humoral immune components are involved in the recognition process. Cellular receptors that recognize these patterns include mannose receptor, integrins and scavenger receptors (Sastry and Ezekowitz, 1993; Stahl and Ezekowitz, 1998). C1q receptor and the transmembrane receptor, SPR210 are among the humoral recognition receptors (Epstein et al., 1996; Tenner et al., 1995). These receptors mainly mediate the specific recognition of cognate ligands, however the internalization is mediated via the Fc and the complement (C3b) receptors (Carroll, 1998; Ravetch, 1997). Similarly, a large number of receptors like class A scavenger receptors and class B scavenger receptor, CD36, the vitronectin receptor and CD14 (Devitt et al., 1998; Platt et al., 1996; Savill et al., 1992) assist the internalization of apoptotic cells by recognizing the phosphatidylserines, changes in the pattern of glycosylation of cell surface proteins and surface charge on the ailing cells (Platt et al., 1998).

2.2.1 Fc gamma receptor (FcγR) mediated phagocytosis

FcγR are members of the immunoreceptor class of receptor tyrosine kinases which recognize the Fc region of the immunoglobin. There are two categories of the FcγR: one that activates the effector function and one that inhibits it. The former class includes the FcγRI, FcγRIIA and FcγRIII and the latter class is represented by the

FcγRIIB (Ravetch, 1997). These receptors contain the tyrosine based activation motif (ITAM) within their cytoplasmic tails or in associated subunits (Ravetch, 1994). Clustering of the FcγR by IgG-opsonized particles induces phosphorylation of tyrosine within the ITAM motifs initiating a signaling cascade leading to recruitment of downstream effectors. The downstream effectors of the FcγR include the PI3-kinases required for phagosomal cup closure (Araki et al., 1996), members of the RhoGTPase family to regulate actin polymerization (Cox et al., 1997) and the protein kinase C (PKC) family of proteins (Zheleznyak and Brown, 1992).

2.2.2 Mannose receptor mediated phagocytosis

The mannose receptor (MR) on macrophages recognize branched mannose and fucose oligosaccharides as well as prototypic PAMPs with high affinity, making this phagocytic receptor of broad pathogen specificity (Stahl and Ezekowitz, 1998). The MR is a single chain receptor with a short cytoplasmic tail and an extracellular domain including 8 lectin-like carbohydrate-binding domains (Taylor et al., 1990). The cytoplasmic tail is crucial to both the endocytic and phagocytic functions of the receptor (Stahl and Ezekowitz, 1998). During mannose receptor mediated phagocytosis, proteins such as F-actin, talin, PKCα, MARCKS and Myosin I are recruited around the nascent phagosomes (Allen and Aderem, 1996b). The engagement of this receptor also leads to secretion of pro-inflammatory cytokines like IL-1β, IL-6, GM-CSF, TNFα, and IL-12 (Aderem and Underhill, 1999).

2.2.3 Scavenger receptor mediated phagocytosis

Scavenger receptors (SR) comprise a large family of structurally diverse transmembrane cell surface glycoproteins which mediate direct non-opsonic phagocytosis of pathogenic microbes. These receptors have the ability to recognize different microbial structures including PAMPs, LPS, LTA, bacterial CpG DNA and yeast zymosan (Areschoug and Gordon, 2008; Mukhopadhyay and Gordon, 2004). More recently, there have been reports suggesting that various bacterial surface proteins serve as major ligands for Class A SR (Areschoug et al., 2008; Jeannin et al., 2005; Peiser et al., 2006; Pluddemann et al., 2009) while the Class B SR recognizes LTA and diacylated

lipopeptide on bacteria (Hoebe et al., 2005). Some SRs also act as a co-receptor for TLRs, thus modulating the inflammatory response (Hoebe et al., 2005; Jeannin et al., 2005). As part of the phagocytic process, SRs mediate the activation of signaling cascades and the production of pro-inflammatory cytokines in response to specific receptor-ligand interaction.

2.2.4 Complement receptor mediated phagocytosis

Complement receptors (CR) opsonize bacteria by recognizing the complement proteins, C3b or C3bi. The receptor CR1 is involved in particle binding while CR3 and CR4 are involved in internalization of the particle (Aderem and Underhill, 1999). CR mediated phagocytosis is a relatively passive process forming only point like contact areas where the particles appear to sink into the cell (Kaplan, 1977) and a variety of cytoskeletal factors including F-actin, vinculin, α-actinin, paxillin and phosphotyrosine-containing proteins get recruited to the areas of contact (Allen and Aderem, 1996a). Unlike FcγR phagocytosis, CR mediated phagocytosis does not elicit a pro-inflammatory response (Aderem et al., 1985; Wright and Griffin, 1985). Moreover, CR mediated internalization requires intact microtubules and is accompanied by the accumulation of vesicles beneath the forming phagosome (Allen and Aderem, 1996b).

2.3 Macropinocytosis

Macropinocytosis, also known as triggered phagocytosis is not a receptor-ligand guided mechanism. It is however, an actin driven mechanism where in membrane ruffles fold back and fuse with the plasma membrane to generate large endocytic vesicles called macropinosomes (0.2-10 μm in diameter) (Weed and Parsons, 2001). Although macropinocytosis accompanies apparently disordered membrane ruffling, it is likely to be a highly controlled and regulated process, being driven by a cascade of signaling molecules, cytoskeletal proteins, actin and Rho-GTPases (Conner and Schmid, 2003). Macropinocytosis accomplishes diverse functions including a role in directed cell migration (Ridley, 2001), immune surveillance (Mellman and Steinman, 2001) and uptake of viruses like *Vaccinia* and some adenoviruses (Amstutz et al., 2008; Mercer and Helenius, 2008). It has also been shown that some bacteria like *S. typhimurium* and *L.*

pneumophila inject toxins into host cells triggering macropinocytosis to facilitate their own uptake into these macropinosomes, which are conducive for their survival and replication (Steele-Mortimer et al., 2000). Although the morphologies of phagosomes and macropinosomes vary, the molecules that regulate the dynamics of membrane and actin cytoskeleton have several shared features (Swanson, 2008).

2.4 Phagosome biogenesis and its maturation

The classical view of phagocytosis suggested the sole involvement of the plasma membrane pseudopods to internalize large particles (Cohn and Steinman, 1982; Jutras and Desjardins, 2005). However, subsequent studies have shown that the plasma membrane alone is insufficient to engulf large particles, thus, phagosome formation requires the contribution of other intracellular membranes. Several reports confirm the contribution of membranes by various intracellular compartments, including the endoplasmic reticulum (ER), recycling endosomes (VAMP-3 containing vesicles), late endosomes (VAMP-7 containing vesicles) and lysosomes, to nascent phagosomes (Bajno et al., 2000; Braun et al., 2004; Gagnon et al., 2002).

The biochemical composition of phagosomes is modified as it 'matures' into the phagolysosome via sequential interactions with compartments of the endocytic pathway (Desjardins et al., 1994). Shortly after their formation, phagosomes bind to microtubules (Goldstein et al., 1973; Hart et al., 1983; Pesanti and Axline, 1975) and engage in fusions with early endosomes followed by interactions with the late endosomes and lysosomes. During this process, there is continuous association and dissociation of proteins from different compartments. Finally, the phagosome develops lysosomal traits as it acidifies and accumulates different Rabs, lysosome associated membrane glycoproteins (LAMPs), Cathepsin D and other acid hydrolases, which efficiently degrade most of the invading microbes (Fig.1). The mechanism of fusion has been highly debated. The 'pre-existing compartment model' proposed that the endocytosed material is carried between a series of biochemically distinct pre-existing organelles and there occurs a complete mixing of the fusion partners, membranes and their luminal contents (Griffiths and Gruenberg, 1991). However, studies conducted by Desjardins on kinetics of acquisition and dissociation of molecules, transfer of contents and microscopical evidence demonstrating

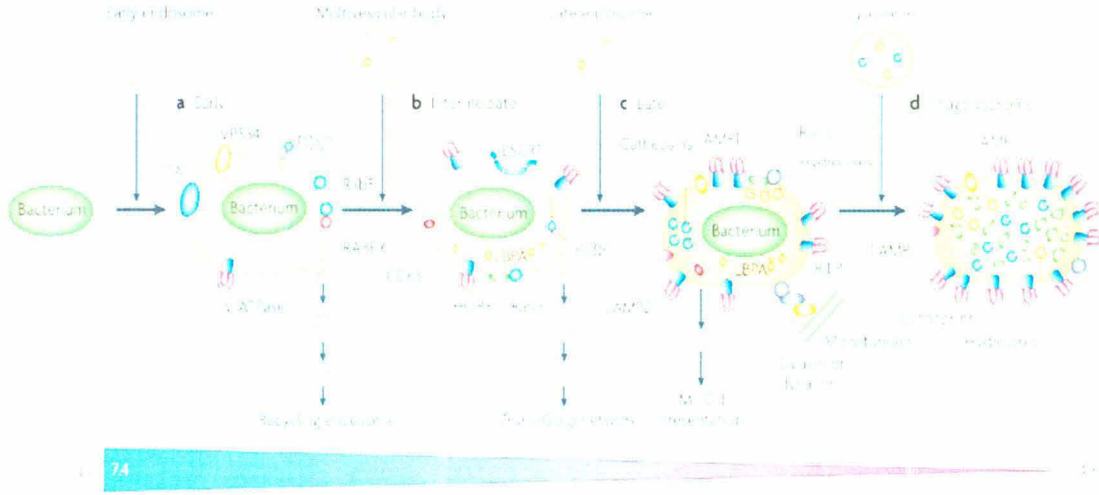


***Figure 1**: Phagosome maturation via sequential interactions with the endocytic pathway*
Adapted from Nature Reviews Microbiology; Ronald S. Flannagan, Gabriela Cosío, 2009.

engagement of phagosomes in multiple transient contacts led to another model, 'the kiss and run' hypothesis. This model proposes that lipid bilayers of the fusing organelles intermingle transiently and after a short mixing of contents ('kiss'), the fusion vesicle retracts ('run') to re-fuse with the target membrane (Desjardins, 1995). Hence, fusion events involve multiple transient interactions to exchange luminal and membrane material while the organelles still maintain their identities.

2.5 Regulation of phagocytosis

The processes of phagocytosis and macropinocytosis are driven by a combination of localized cytoskeletal rearrangements. These include actin polymerization, depolymerization and contraction of actin filament networks. The microtubules and other motor proteins contribute to the interactions of phagosomes with the endocytic pathway by driving the intravesicular fusion events. The cytoskeletal rearrangements are controlled by accessory molecules, which include the small GTPases and their regulators, the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). As phagocytosis proceeds, phagosomes mature, undergoing a series of membrane fusion events. For the proper flow of cargo within the cell, it is essential to maintain the

specificity of membrane fusion. This is regulated by small GTP binding proteins of the Rab family (Rothman and Sollner, 1997; Zerial and McBride, 2001), proteins of the Sec1p family and various tethering factors in combination with soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins (Pfeffer, 1999).

2.5.1 Cytoskeletal proteins: actin and microtubules

The cytoskeletal proteins, actin, microtubules and their regulatory molecules have been implicated to play a role in phagocytosis as well as macropinocytosis. It is well established that the actin cytoskeleton is important for the initial steps of phagocytosis (Greenberg et al., 1988). Actin polymerization in phagocytosis is in turn regulated by accessory molecules, Arp2/3, formins and other proteins including WASP108, WAVE2, amphiphysin and coronin in concert with several myosins (Swanson, 2008). However, evidence suggests that later transport events require microtubules as they facilitate interactions between phagosomes and organelles of the endocytic pathway (Goldstein et al., 1973; Hart et al., 1987; Hart et al., 1983; Pesanti and Axline, 1975). It is now well established that the cytoplasmic dynein and kinesin motors can interact with membrane organelles indicating a role of these molecules in driving the transport events (Hollenbeck, 1989; Lacey and Haimo, 1992; Morin et al., 1993; Neighbors et al., 1988).

2.5.2 Lipid rafts

Phagosome maturation is also accompanied by the acquisition of sets of proteins and lipids that contribute to the segregation of certain phagosome constituents in membrane microdomains. Lipid microdomains on phagosomes have been proposed to serve as platforms for the assembly and nucleation of actin (Defacque et al., 2002) and for the assembly of an active NADPH oxidase complex (Shao et al., 2003; Vilhardt and van Deurs, 2004) which is a crucial enzyme for the microbicidal function of phagosomes and innate immune defense against infections.

2.5.3 Rab GTPases and their effectors

Rab proteins form the largest subfamily of Ras superfamily of small GTP binding proteins. As many as 70 members of this family have been identified in mammals. These

proteins are present on specific vesicle compartments and regulate the transport of cargo molecules within the cells (Deneka et al., 2003; Zerial and McBride, 2001). The regulation of intracellular transport is attributed to their ability to function as molecular switches, oscillating between the GTP-bound 'active' and the GDP-bound 'inactive' form. This cycling between the two forms requires nucleotide exchange and hydrolysis which is regulated by the GEFs and GAPs (Seabra and Wasmeier, 2004).

***Figure 2**: Intracellular localization of various Rab proteins*
Adapted from http://www.umassmed.edu/igp/faculty/lambright

At steady state, Rabs are localized on specific subcellular compartments in eukaryotic cells (Fig.2) (Pfeffer, 2001; Somsel Rodman and Wandinger-Ness, 2000). However, multiple Rabs have been reported to be present on a single intracellular compartment occupying distinct "microdomains" (Sonnichsen et al., 2000). For instance, Rab5 associates predominantly with the sorting endosomes, Rab4 and Rab11 locate preferentially to the recycling endosomes, while Rab7 is localized to late endosomes and to the lysosomes (Bucci et al., 2000; Somsel Rodman and Wandinger-Ness, 2000) (Fig. 2). Targeting of Rabs to distinct compartments is mediated through the post-translational modifications of these proteins by the addition of one or two prenyl groups at the C-

terminal cysteine residue(s) (Gurkan et al., 2005). On the other hand, targeting to specific microdomains occurs via interactions with their effectors and lipids present on the membrane (Pfeffer, 2003; Pfeffer and Aivazian, 2004).

Rab GTPases regulate intracellular trafficking based on their subcellular localization and by interactions with effectors and lipids present in the compartment. Rabs, in their active form, bind to soluble molecules that act as 'effectors' and transduce the signal of the Rab GTPase to drive the transport mechanism. Many Rab effectors have been identified including Rabaptin-5, Rabex-5, Rabenosyn-5 and EEA-1 which act as effectors of the early endosomal Rab5, playing important roles in recycling and endosome fusion (Horiuchi et al., 1997; Mills et al., 1998; Nielsen et al., 2000; Simonsen et al., 1998; Stenmark et al., 1995). Specialized structural features of Rab effectors mediate cellular trafficking events. For instance, in the Rab7 effector, RILP coiled coil domains have been found to recruit functional dynein-dynactin motor complexes to late endosomes thereby inhibiting their transport towards the cell periphery (Jordens et al., 2001). Similarly, TIP47 is a cytosolic protein which can bind both Rab9 and M6PR to regulate LE to Golgi transport (Carroll et al., 2001; Diaz and Pfeffer, 1998). Table 1 represents various Rabs and their effectors involved in different transport events.

Rab proteins control many aspects of membrane traffic including vesicle formation, vesicle motility along the actin/microtubule cytoskeleton, tethering, transport and fusion (Gurkan et al., 2005; Somsel Rodman and Wandinger-Ness, 2000; Zerial and McBride, 2001). There are also functional connections between Rab proteins and motors of the actin cytoskeleton (Pruyne et al., 1998; Schott et al., 1999). These GTPases also determine the distribution of cellular compartments by regulating the movement of vesicles and organelles along cytoskeletal filaments. Rab5 regulates both the attachment of early endosomes and their motility along microtubules (Nielsen et al., 1999). A role for Rab6 in microtubule-dependent transport has been suggested from the discovery that this GTPase interacts with a kinesin-like protein, Rabkinesin-6 (Echard et al., 1998), which is important for cytokinesis. Rab proteins coordinate the membrane tethering and docking via their effectors. To drive vesicle fusion, Rab effectors interact with specific SNARE molecules within the Rab domain to selectively enrich the *cis*-SNARE

complexes at sites of their function, a prerequisite for cognate SNAREs to pair in *trans* upon tethering (Zerial and McBride, 2001).

Rab	Rab function	Direct effector	Effector function	Rab specificity	Effector partners	Partner features
Rab1	• ER–Golgi transport	p115	• Tethering • Recruits/clusters SNAREs into budded vesicles	Rab1–GTP	Giantin GM130	• Tethering of COPI coated vesicles to Golgi
		PRA1	• Rab receptor (proposed)	Rab1 Rab3 Rab4b Rab8a Rab8c	VAMP2	• v-SNARE involved in bilayer fusion
Rab3	• Rab3a: synaptic vesicle and chromaffin granule secretion • Rab3b, c, d: regulated secretion	Rabphilin-3	• Potentiates fusion	Rab3–GTP	α-actinin Rabaptin-5	• Crosslinks actin filaments into bundles • Stimulated by Rabphilin-3 interactions • Also binds Rabaptin-5, an effector of Rab5 and Rab4
		RIM1 RIM2	• Membrane fusion	Rab3–GTP	RIM–BP1	• Contains fibronectin type III repeats and SH3 domains
		Calmodulin	• Confers calcium sensitivity to protein interactions	Rab3	Many	• Multiple functions
Rab4	• Localized to early recycling endosomes • Role in sorting/recycling in early endosomes	Rabaptin-5 Rabaptin-5β Rabaptin-4	• Activates Rab5 through complex with Rabex-5 • Implicated in protein sorting and recycling	Rab4–GTP Rab5–GTP	Rabex-5	• Nucleotide exchange factor
Rab5	• Ligand sequestration at plasma membrane • CCV–EE and EE–EE fusion • Endosome motility	Rabaptin-5 Rabaptin-5β	• Stabilizes Rabex-5 recruitment	Rab5–GTP Rab4–GTP	Rabex-5 Rabphilin-3	• Nucleotide exchange factor
		EEA1	• Tethering; core fusion component	Rab5–GTP	Syntaxin13 Syntaxin6	• t-SNAREs essential for bilayer fusion
		p150	• Class III PI(3)K regulatory subunit	Rab5–GTP	hVps34	• Class III PI(3)K catalytic subunit
		p110β	• Class I PI(3)K catalytic subunit	Rab5–GTP	p85-α	• Class I PI(3)K regulatory subunit
		Rabenosyn-5	• Required for CCV–EE and EE–EE fusion	Rab5–GTP Rab4–GTP	hVps45	• Regulates SNARE complex formation or disassembly
Rab6	• Retrograde Golgi–ER and intra-Golgi transport	Rabkinesin-6	• Vesicle motility • Cytokinesis	Rab6–GTP	Microtubules	
Rab8	• TGN–plasma membrane traffic (basolateral in epithelial cells)	Rab8IP	• Stress-activated protein kinase	Rab8–GTP		
Rab9	• Late endosome to Golgi	p40	• Stimulates fusion	Rab9–GTP		
Rab11	• Recycling through perinuclear recycling endosomes • Plasma membrane–Golgi traffic	Rab11BP	• Unclear	Rab11–GTP	mSec13	• Coat component of COPII vesicles
Rab13	• Involved in the formation of the tight junction	δ PDE	• Extracts Rab13 from membrane	Rab13		
Rab33b	• Intra-Golgi transport	Rab33b-BP	• Probably regulates motility of Rab33 vesicles	Rab33b–GTP		

Table 1 *Rab proteins and their effector molecules. Abbreviations used: CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; PDE, phosphodiesterase; PI(3)K, phosphoinositol-3-kinase; SH3, Src homology region 3 domain; TGN, trans-Golgi network; Vamp, vesicle-associated membrane protein.*
Adapted from Nature reviews Molecular Cell biology; Zerial and McBride Feb, 2001.

2.5.4 SNAREs

SNAREs comprise a large family of coiled coil proteins (Jahn and Sudhof, 1999) which play a central role in intracellular membrane trafficking by conferring specificity to vesicular fusion events in conjunction with Rab GTPases. Around 36 members of this super family of proteins are known in mammals. Most SNAREs have a membrane-spanning region, an N-terminal domain and a membrane proximal SNARE motif domain

which contains conserved heptad repeat sequences and is critical for SNARE complex formation (Fasshauer et al., 1998). SNARE molecules have been divided into two groups, vesicular (v)-SNAREs on donor membranes and target (t)-SNAREs on target membranes (Sollner et al., 1993) based on their functionality; and as R-SNAREs (arginine containing SNAREs) and Q-SNAREs (glutamine-containing SNAREs) depending on the conserved residue in the SNARE motif. The Q-SNAREs have been further sub classified into Qa, Qb and Qc SNAREs on the basis of their N-terminus domain (Bock et al., 2001; Fasshauer et al., 1998).

Specific members of the SNARE families are localized to distinct subcellular compartments (Advani et al., 1998) to function in specific intracellular fusion steps (Fig.3). Examples include syntaxin 1, syntaxin 2, syntaxin 4, SNAP-23 and SNAP-25 at the plasma membrane, as well as VAMP/synaptobrevin on synaptic and neurosecretory vesicles, and syntaxin 5 and VAMP 4 in the Golgi apparatus (Hong, 2005). Syntaxin 13 is shown to be present on the early endosomes and mediates the trafficking from early endosomes to recycling endosomes (Prekeris et al., 1998) whereas syntaxin 8 is predominantly located on early endosomes and regulates trafficking between early compartments (Prekeris et al., 1999). Syntaxin 7 is shown to play a major role in regulating trafficking from endosomes to lysosomes (Ward et al., 2000).

After completing most fusion reactions, some SNAREs are need for the next fusion event and hence, are required to be returned to their donor compartments. Consequently, SNAREs reside not only on the organelle for which they mediate fusion, but they also reside in the membranes of the organelles that are part of their recycling pathway. This has been exemplified by studies on SNAREs that are involved in trafficking between the ER and the Golgi. These SNAREs are found in ER, Golgi and in the intermediate trafficking vesicles (Cao and Barlowe, 2000; Hay et al., 1998). Specific localization of SNAREs is therefore dependent on the steady state between SNARE biosynthesis, fusion and recycling.

Vesicular fusion events require one member each of the Qa-, Qb-, Qc- and R-SNAREs to form functional hetero-oligomeric complex held together by parallel four-helix bundles. This brings the membranes close together and leads to changes in free energy needed to drive membrane fusion, as detailed in the following section. For various

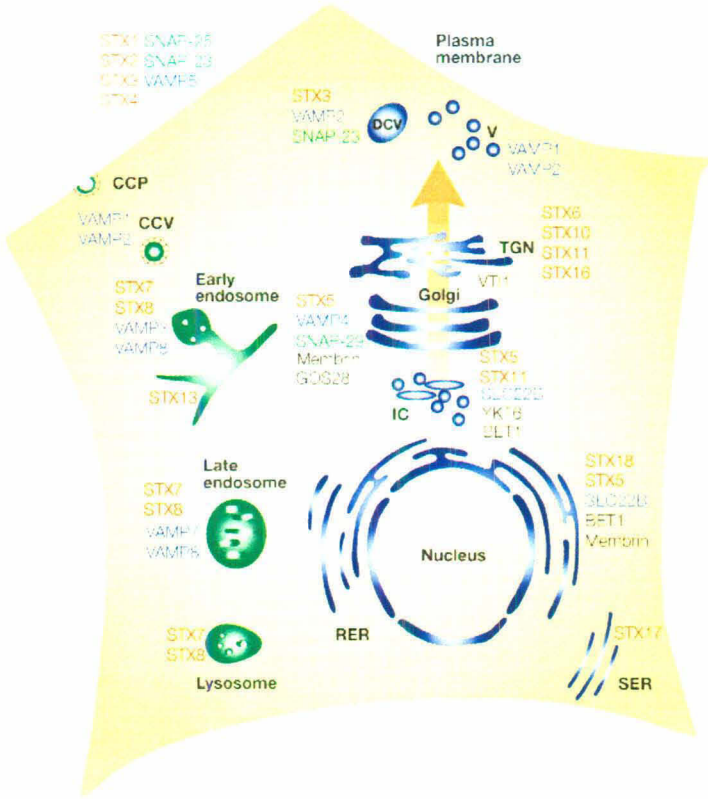


***Figure 3**: Intracellular localization of different SNARE molecules*
Adapted from Nature Reviews; Chen and Scheller, Feb, 2001.

fusion reactions, it has been demonstrated that only matching R/Q-SNARE combinations can accomplish the fusion event, suggesting that the specificity is derived from the pairing process (McNew et al., 2000). Furthermore, the pairing process is made exclusive by SNAREs exhibiting multiple configurational, conformational, and oliogomeric states that govern interactions only with their matching SNARE partners, auxiliary proteins, or with other SNARE domains. However, some SNAREs display flexibility in their choice of partners thus exhibiting promiscuity. One such molecule is syntaxin 6 which has been shown to form several fusion complexes with different SNAREs and is involved in several fusion events including post Golgi fusion and early and late endosomal fusion (Wendler and Tooze, 2001). This indicates that SNAREs are probably not the sole determinants of vesicle targeting specificity, but this view needs further research.

2.5.5 Regulation of vesicular fusion by Rabs and SNAREs

Membrane trafficking events are tightly regulated by a complex interplay of several proteins. The first level of specificity is conferred by Rabs which mediate tethering of an incoming vesicle to the correct target organelle. The specific topological pairing of cognate SNAREs ensures precision in the subsequent fusion event. According to the model of membrane fusion, a cascade of protein-protein interactions is required to ensure that appropriate SNARE partners are made available to each other (Fig.4). Firstly, *cis*-SNARE complexes, the products of previous fusion reactions that consequently contain both v- and t-SNAREs within the same membrane, must be disassembled. This task is performed by the soluble co-factor SNAP (soluble NSF attachment protein), which specifically binds NSF (Nichols and Pelham, 1998). Binding of these proteins to *cis*-SNARE complexes is followed by NSF-dependent ATP hydrolysis that uncoils the core complex so that SNAREs are released from each other (Weber et al., 1998).

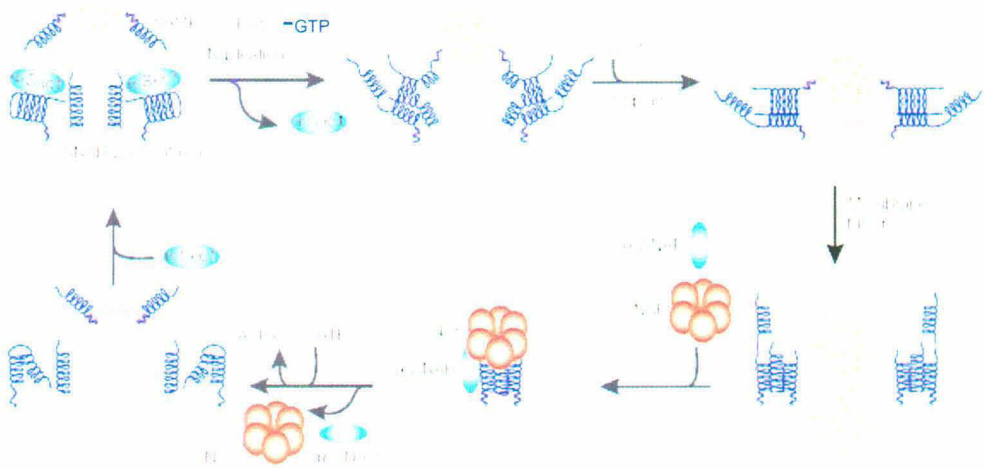


***Figure 4**: Events in vesicular fusion*
Adapted from Nature Reviews; Chen and Scheller, Feb, 2001.

This is accompanied by a conformational change in the t-SNARE that prevents it from rebinding to its partner v-SNARE. The unpaired t-SNARE is further stabilized by a member of the Sec1p family of proteins (Chen and Scheller, 2001). Incoming vesicles initially interact with the target membrane via a specific tether molecule, which is unique to each transport event, in conjunction with the Rab proteins. Subsequently Sec1p is

released by an activated Rab, coaxing the t-SNARE into an open conformation, which eventually engages in *trans*-interactions with a specific cognate v-SNARE via SNAREpins (Weber et al., 1998). These interactions lead to closely apposed membranes resulting in membrane fusion.

2.5.6 Signaling molecules

During the process of phagocystosis, a cascade of signaling events are initiated and literature on this subject is quite vast. Some of the signaling molecules which regulate transport molecules like the Rab GTPases and cytoskeletal proteins such as actin are discussed. Phosphatidyl inositol-3 (PI-3) kinases regulate a variety of intracellular trafficking events that include cargo selection, vesicle formation, vesicle movement and membrane fusion (Lindmo and Stenmark, 2006). It has been reported that dissociation of Rab5 from membranes requires products of PI3K (Vieira et al., 2003). VPS34 has been implicated a role in PI(3)P synthesis, essential for phagolysosome formation (Vieira et al., 2001). PI3 kinase and its effectors are also required for the phagosomal cup closure (Araki et al., 1996). The other signaling molecules such as the p38 MAPK, upon induction, reduces recruitment of EEA1 to the phagosomal membrane (Fratti et al., 2003a) thus, affecting phagosome maturation. Others have also reported the role of phospholipase D in phagosome formation (Corrotte et al., 2006) and tyrosine kinase signaling in controlling phagosome maturation (Fang et al., 2007). PIPKI α regulates the recruitment of actin modulating proteins by controlling changes in PIP2 levels (Coppolino et al., 2002). Thus, signaling molecules act at different sites to regulate the phagocytic machinery.

2.6 Modulation of phagosome maturation by intracellular pathogens

During its course of maturation, phagosomes acquire a full arsenal of antimicrobial features, including the acidification of the phagosome, production of reactive oxygen and nitrogen species, production of antimicrobial proteins and peptides and an assortment of endopeptidases, exopeptidases and hydrolases to degrade the invading microbes. Despite the presence of numerous host antimicrobial factors, certain

organisms can survive efficiently as intracellular pathogens. Some bacterial species interfere with engulfment while others survive by impairing the phagosomal machinery. These pathogens have evolved a myriad of strategies to protect them from the hostile environment within the cells which can be broadly categorized into four types:

A) Arrest of phagosome maturation into the phagolysosome is the survival strategy adopted by *Mycobacterium* and *Salmonella.*

B) Reprogramming the phagosome maturation pathway is characteristic of *Legionella, Chlamydia* and *Brucella.*

C) Escape from the phagocytic vacuole to survive within the cytoplasm as exemplified by *Listeria* and *Shigella.*

D) Surviving in the hostile phagolysosomal compartment typified by *Coxiella* and *Leishmania.*

The details of survival mechanism of one representative organism from each group i.e., *Mycobacterium, Legionella, Listeria and Coxiella* are represented in the following sections (Fig.5).

2.6.1 Arrest of phagosome maturation by *Mycobacterium*

M. tuberculosis, another facultative intracellular pathogen survives and replicates within macrophages by arresting phagosomal maturation to the phagolysosome (Hart et al., 1987; Pethe et al., 2004). The phagosomes containing the *Mycobacteria* are arrested at an early stage and retain early endosomal molecules such as Rab5a; however, the recruitment of Rab5a effectors like EEA-1 and hVPS34 gets impaired (Fratti et al., 2001; Fratti et al., 2003b). This prevents the accumulation of the signaling molecule PI-3-P that is required for phagosome maturation. Another proposed model invokes the role of mycobacterial lipid glycosylated phosphatidyl inositol in inhibiting Ca^{2+}/Calmodulin dependent production of PI-3-P by hVPS34 (Vergne et al., 2003). *M. tuberculosis* also produces the phophatase SapM, which specifically hydrolyses PI-3-P (Vergne et al., 2005). The combined effects of all these effectively depletes PI(3)P from the early phagosomes preventing the transition to late and phagolysosomal stages. Recently, it has also been observed that *M. tuberculosis* phagosomes accumulate Rab22a on their membranes. This GTPase has been shown to be critical for regulation of Rab7 conversion

and subsequently, phagosome maturation (Roberts et al., 2006). Thus, the phagosomal maturation is blocked at the step between Rab5 and Rab7 mediated trafficking (Via et al., 1997). It has been observed that *Mycobacteria* containing phagosomes retain a protein called TACO (homologue of Coronin) on their surface and this protein might behave like a rigid coat to prevent interaction with the other vesicular compartments (Ferrari et al., 1999). *Mycobacteria* have also been shown to prevent the acquisition of actin (Anes et al., 2003) and Hrs, a signal needed for late endosomal targeting (Vieira et al., 2004) on the phagosomal surface and this has been correlated with the impaired maturation of the phagosome. Moreover, the pathogen secretes ZmpA, a predicted zinc metalloprotease that inhibits IL-1β processing by host cells, as a mechanism to counteract the inflammatory response (Master et al., 2008). Apart from arresting phagosome maturation, reports suggest the ability of the bacterium to escape from the phagosomes via the expression of a novel bacterial system-ESX (van der Wel et al., 2007).

2.6.2 Reprogramming the phagosome maturation pathway by *Legionella*

L. pnuemophilia is a facultative intracellular pathogen which can survive and replicate within macrophages (Bruggemann et al., 2006). Internalized *Legionella* rapidly modulates the maturation of *Legionella*-containing vacuoles (LCVs) by avoiding interaction with the default endolysosomal pathway (Clemens et al., 2000; Joshi et al., 2001). The pathogen encodes a specialized protein secretion system, T4SS, the products of which are essential for its survival (Robinson and Roy, 2006). T4SS effectors like DrrA, LidA, LepB and RalF help to recruit active Rab1 and ARF1 to the LCV. Recruitment of these GTPases on the phagosomal membrane induces fusion of LCV with ER-derived vesicles (Ingmundson et al., 2007; Murata et al., 2006; Nagai et al., 2002). Additional virulence factors such as AnkX disrupt the normal microtubule-dependent organeller transport of host cell (Pan et al., 2008). Ultimately, *L. pnuemophilia* replicates intracellularly within large, acidic vacuoles having some lysosomal properties (Sturgill-Koszycki and Swanson, 2000). The delayed entry into the acidified compartment probably allows the pathogen to develop resistance to the vacuolar environment. Some other T4SS effectors also have been implicated in *Legionella* pathogenesis, which have motifs commonly identified by eukaryotic proteins (Albert-Weissenberger et al., 2007),

suggesting the potential of *Legionella* to manipulate additional host processes for its intracellular survival.

2.6.3 Escape of *Listeria* from the phagosomes

L. monocytogenes, a facultative intracellular pathogen, survives intracellularly by modifying the phagosomal membrane to escape into the cytoplasm. At very early stages post infection, the pathogen secretes cholesterol-dependent cytolysin, listeriolysin O (LLO) (Beauregard et al., 1997). Secretion of LLO, along with recruitment of Rab5 on the phagosomal membrane inhibits the maturation of phagosomes (Henry et al., 2006), owing to a loss of luminal H^+ and Ca^{2+} required for fusion events. LLO, together with phopholipase C enzymes expressed by *Listeria* causes the breakdown of the phagosomal membrane. Upon lysis of the phagosomal membrane, the pathogen escapes into the cytoplasm where the bacterial replication occurs. (Shaughnessy et al., 2006; Tilney and Portnoy, 1989). Once inside the cytosol, the bacteria move around by seizuring the host's cytoskeletal machinery. The bacterial surface protein ActA, activates host Arp2/3 complex, G-actin and VASP family members to recruit actin (Lambrechts et al., 2008). *Listeria* has perfected the art of surviving in the host cell cytosol and can survive even in the hostile environment of the macrophage cytosol, where it has to resist the microbicidal proteins and peptides.

2.6.4 Survival of *Coxiella* in the hostile environment

C. burnetti is an obligate intracellular pathogen with a biphasic developmental cycle, consisting of an infectious (phase1 *Coxiella*) and a replicative (phase 2 *Coxiella*) (Voth and Heinzen, 2007) phase. After formation of the *Coxiella* phagosome, it interacts with the default endocytic machinery (Heinzen et al., 1996). As the phagosome matures, it acquires lysosomal markers such as LAMP-1, LAMP-2, LAMP-3, vacuolar ATPase and Rab7 (Beron et al., 2002; Ghigo et al., 2002; Heinzen et al., 1996). However, the compartment is not a proper lysosome but is a bacterium modified phagolysosome having properties of autophagosomes. *Coxiella* specifically recruits the autophagic protein LC3 to the phagosomal membrane which increases interactions of the autophagic and phagocytic pathways, altering phagosome maturation by delaying the lysosomal fusion

event (Gutierrez et al., 2005; Romano et al., 2007). The delay in the fusion allows the transition into the replicative form of the pathogen. The replicative *C. burnetti* resides in a large spacious compartment termed as RCV that contains several lysosomal proteins. It survives within this hostile environment by adapting as an acidophile for certain metabolic activities (Hackstadt and Williams, 1981) and utilizing virulence factors encoded by its secretion system (T4SS) to nullify the effects of various antimicrobial agents which it encounters within the RCV.

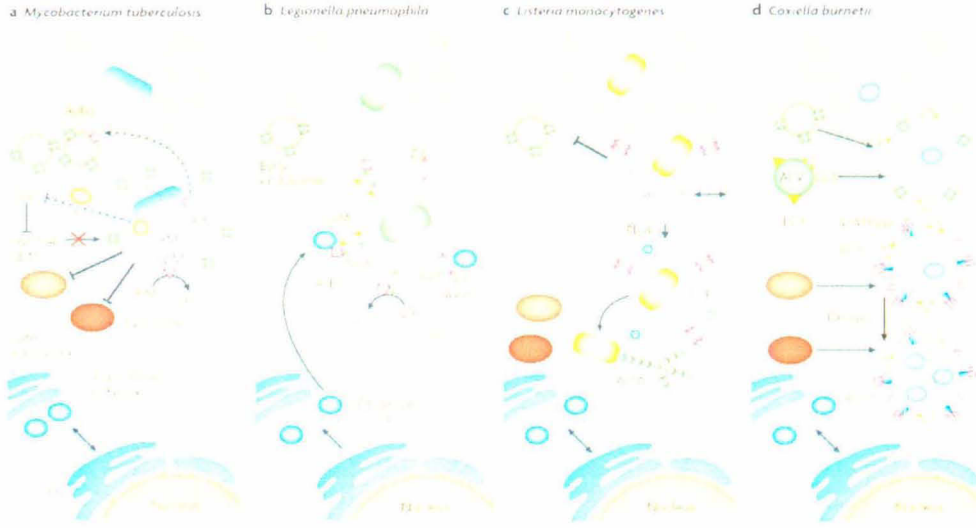


***Figure 5**: Survival strategies of different intracellular pathogens*
Adapted from Nature Reviews Microbiology; Ronald S. Flannagan, Gabriela Cosío, 2009.

2.7 *Salmonella* pathogenesis

Salmonella is a gram negative intracellular facultative food borne pathogens capable of infecting a wide range of animals. *Salmonella enterica* serovar *typhi* and *paratyphi* cause human typhoid fever while *S. typhimurium* causes gastroenteritis in humans and typhoid like fever in mice (Miller and Pegues, 2000) (Fig.6). *Salmonella* enter the host body via contaminated food and water. The bacterium has an adaptive acid-tolerance response and can survive in the acidic milieu of the stomach (Garcia-del Portillo et al., 1993a). During the course of infection, *Salmonella* can invade the non-phagocytic enterocytes of the intestinal epithelium by bacteria-mediated endocytosis,

***Figure 6**: Pathogenesis of Salmonella*
Adapted from Nature Reviews Microbiology; Haraga, A, Ohlson, M.B., 2008.

involving membrane ruffling and uptake of the bacterium in large vesicles (Francis et al., 1992). It has been observed that *Salmonella* preferentially adhere to the Microfold (M)cells of the intestinal epithelium which further transport them to lymphoid cells in the underlying Peyer's patches (Jones et al., 1994; Kohbata et al., 1986) . After infringing the epithelial barrier, *Salmonella* can enter intestinal macrophages by inducing macropinocytosis, activating various virulence mechanisms for its survival in the microbicidal environment, intracellular replication and subsequent dissemination within the host macrophages (Haraga et al., 2008).

2.7.1 *Salmonella* virulence mechanism

The pathogenicity of the organism is attributed to the presence of virulence gene clusters in localized regions of the chromosome termed as pathogenicity islands (Groisman and Ochman, 1996). These pathogenicity islands encode specialized devices for the delivery of virulence factors within host cells. *Salmonella* encode two distinct virulence Type III secretion systems (T3SS) within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). The T3SS mediates the transfer of bacterial virulence proteins, known as effectors from the bacterial cell into the host cell cytoplasm (Hansen-Wester and Hensel, 2001). T3SS is a complex needle like injectisome which spans the bacterial membrane and delivers the effectors into the translocon pore within the host cellular

membrane. This process is aided by an ATPase located at the base of the needle complex and *Salmonella* effectors, which are part of the translocon pore (Akeda and Galan, 2005; Kubori et al., 1998). SPI-1 and SPI-2 are known to function at different times during infection (Hansen-Wester and Hensel, 2001). SPI-1 encoded T3SS is active on contact with the host cells and delivers bacterial effectors across the plasma membrane, while SPI-2 system is expressed within the phagosome and translocates effectors across the vacoular membrane. Thus, SPI-1 has been shown to be essential for the invasion process (Galyov et al., 1997; Watson et al., 1995), while SPI-2 plays an important role in bacterial survival and the establishment of disease (Cirillo et al., 1998; Hensel et al., 1997). However, recent studies demonstrated the expression of SPI-2 even in early stages of *S. typhimurium* infection in mice (Brown et al., 2005). Several other reports also suggest that some of the SPI-1 effectors are expressed and persist within host cells long after infection, thought to be contributing to functions previously attributed exclusively to SPI-2 effectors (Brawn et al., 2007; Drecktrah et al., 2005; Giacomodonato et al., 2007; Hernandez et al., 2004; Lawley et al., 2006; Steele-Mortimer et al., 2002). Thus, the line demarcating the functional difference between the two T3SS is slowly being diminished.

The intracellular niche where the *Salmonella* resides has an acidic environment making it difficult for pathogen survival. However, to promote its intracellular survival, *Salmonella* adapts to this hostile environment by bringing about changes in the bacterial envelope components by surface modelling (Alpuche Aranda et al., 1992). This is achieved by the activation of different regulatory systems as the PhoP/PhoQ system (Miller et al., 1989). The PhoQ sensor promotes resistance to antimicrobial domains and also responds to pH fluctuations by incorporating structural changes for efficient survival (Bader et al., 2003; Miller et al., 1989; Prost et al., 2007).

2.7.2 Regulation of intracellular trafficking by effector proteins

Once inside the host cells, these effectors can alter several host cellular functions, such as cytoskeleton, membrane trafficking, signal transduction and cytokine gene expression to promote bacterial survival as an intracellular pathogen. Many of these effectors have been known to exert their function by mimicking activities of host cellular

proteins (Stebbins and Galan, 2001). The functions and host cell targets of some of the effectors have been summarized in Table 2.

Effector	Cellular function	Host-cell target
SPI1 T3SS		
AvrA	Inhibits nuclear factor (NF)-κB signalling and interleukin (IL)-8 production; also prevents ubiquitination of β-catenin	Unknown
SopA or SopA*	Decreases the critical concentration of G-actin and increases the stability of F-actin; also induces PMN transepithelial migration and disrupts tight junctions	F-actin; Tgiantin
SopB or SspB*	Binds and activates caspase-1 and induces autophagy in macrophages	Caspase-1; cholesterol
SopC or SspC*	Nucleates and bundles actin	F-actin; cytokeratin 8 and cytokeratin 18
SopA	Stimulates PMN transmigration via HECT-like E3 ubiquitin ligase activity	Unknown
SopB or SigD	Activates Cdc42, Rho G, Akt/PKB and Ilk; modulates actin through its inositol phosphatase activity and disrupts tight junctions	Unknown
SopD	Stimulates fluid accumulation in bovine ligated ileal loops and contributes to diarrhoea in calves and systemic disease in mice	Unknown
SopE	Activates Cdc42, Rac1 and RhoG by its GEF activity and disrupts tight junctions	Cdc42, Rac1 and RhoG
SopE2	Activates Cdc42, Rac1 and RhoG by its GEF activity and disrupts tight junctions	Cdc42 and Rac1
SptP	Inhibits Cdc42 and Rac1 by its GAP activity and MAPK signalling and IL-8 secretion; through its tyrosine phosphatase activity	Rac1
SPI2 T3SS		
CigB	Unknown	Unknown
EipB	Unknown	Unknown
EipBZ	Contributes to Sif formation	Kinesin-1
SifA	Induces Sif formation; maintains integrity of the SCV and downregulates kinesin recruitment to the SCV	SKIP and RhoF
SifB	Unknown	Unknown
SopD2	Contributes to Sif formation	Unknown
SpiC*	Interferes with endosomal trafficking	Hook3
SpvB*	Actin-specific ADP-ribosyltransferase and downregulates Sif formation	Actin
SseI	Contributes to Sif formation and microtubule bundling	Unknown
SseG	Contributes to Sif formation and microtubule bundling	Unknown
SseF or SrfH	Contributes to host cell localization	Filamin and ERIEs
SseJ	Maintains integrity of the SCV and has deacylase activity	Unknown
SseK1	Unknown	Unknown
SseK2	Unknown	Unknown
SseL	Deubiquitinase	Ubiquitin
SspH2	Inhibits the rate of actin polymerization and contributes to virulence in calves	Filamin and profilin
SteA	Unknown	Unknown
SteB	Unknown	Unknown
SteC	Unknown	Unknown
SPI1 and SPI2 T3SS		
SlrP	Contributes to virulence in calves	Unknown
SspH1	Inhibits NF-κB signalling and IL-8 secretion; contributes to virulence in calves and has E3 ubiquitin ligase activity	PKN1

*Also a component of the secretion apparatus. ‡Has not been definitively shown to be an SPI2 T3SS effector. GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; HECT, homologous to E6-AP carboxyl terminus; MAPK, mitogen-activated protein kinase; PMN, polymorphonuclear leukocyte; SCV, *Salmonella*-containing vacuole; Sif, *Salmonella*-induced filament; SPI, *Salmonella* pathogenicity island.

***Table 2**: Functions of different Salmonella effectors*
Adapted from Nature Reviews Microbiology; Haraga, A, Ohlson, M.B., 2008.

2.7.2.1 *Salmonella* Pathogenicity island-1

SPI-1 T3SS effectors are translocated across the host plasma membrane and are majorly involved in the bacterial invasion process. Some of the effectors SopE, SopE2 and SopB are known to activate the host Rho GTPases cdc42, Rac1 and RhoG, which induces actin cytoskeletal rearrangements, promoting bacterial uptake (Bakshi et al., 2000; Friebel et al., 2001; Hardt et al., 1998; Patel and Galan, 2006; Stender et al., 2000; Zhou et al., 2001). Similarly, SipA and SipC also promote bacterial internalization but they do so by modulating actin dynamics (Hayward and Koronakis, 1999; Scherer et al., 2000; Zhou et al., 1999). All these effectors act in concert with each other to induce formation of membrane ruffles, thus, encouraging bacterial uptake. The stimulation of cdc42 by SopE, SopE2 and SopB also triggers several signaling cascades, including p38, Erk and Jnk pathway, resulting in the activation of various transcription factors like AP-1 and NF-κB (Chen et al., 1996a; Hobbie et al., 1997; Patel and Galan, 2006) which direct the production of pro-inflammatory cytokines and the manifestation of disease symptoms. Another effector, SptP also acts on the host GTPases, cdc42 and Rac1; however, it functions as a GAP acting antagonistically to SopE, thus restoring actin cytoskeleton to maintain cellular homeostasis (Fu and Galan, 1999). SopD encoded by SPI-1 T3SS has been reported to be expressed under SPI-2 T3SS conditions as well. It has been shown to persist within cells even at later stages after infection, however, the role is still not clear (Brumell et al., 2003; Jiang et al., 2004). Some of the SPI-1 effectors are also part of the translocation assembly and help to translocate other effectors into the host cytoplasm.

2.7.2.2 *Salmonella* Pathogenicity island-2

The expression and assembly of SPI-2 encoded T3SS is induced as a result of sensing the phagosomal environment (Cirillo et al., 1998; Lee et al., 2000). SPI-2 T3SS effectors are delivered into the phagosome and their interference with the host cellular processes determine the intracellular fate and the ability of the pathogen to cause systemic infection. Although the function of this T3SS in pathogenesis is poorly understood, it has been shown to be essential for virulence in a mouse infection model (Cirillo et al., 1998; Shea et al., 1996). It has been well established that SPI-2 induces the formation of long filamentous membrane structures commonly known as *Salmonella-*

induced filaments (Sifs) (Garcia-del Portillo et al., 1993b; Knodler et al., 2003), which function to increase the size of the phagosome to accommodate bacterial replication during systemic infection. The formation of Sifs is dependent on the functions of SifA, SseF, SseG, SopD2 and PipB2. While, the centrifugal extension of Sifs is promoted by the activity of PipB2, SifA interacts with host molecule SKIP to displace kinesin from the vacoular membrane and help in tubular extensions (Jiang et al., 2004; Knodler et al., 2005; Stein et al., 1996). While these molecules manipulate the host microtubules involved in Sif formation, there are few SPI-2 T3SS effectors which are involved in actin rearrangements. SspH2 and SseI are two such effectors which interact with filamin, an F-actin crosslinking protein to inhibit actin polymerization and thus, reduce the *Salmonella*-containing phagosome associated actin (Miao et al., 2003). Another effector, SpvB has also been reported in actin inhibitory activity by ADP-ribosylating actin and promoting its depolymerization, which seems to be important for the intracellular lifestyle of *Salmonella* (Lesnick et al., 2001; Miao et al., 2003). The pathogen survives and replicates in the intracellular niche and the bacterial effectors SseF and SseG have been implicated to play a role in this process. SseG is targeted to the TGN and helps to maintain *Salmonella* microcolonies in juxtanuclear, Golgi associated position, further aiding the intracellular replication of the pathogen (Deiwick et al., 2006; Salcedo and Holden, 2003). *Salmonella* survives within the host cells by altering the intracellular trafficking. The product of *spiC*, a gene located within SPI-2, was reported to be an inhibitor of a variety of cellular trafficking events, including phagosome-lysosome fusion, phagosome-endosome fusion, normal vesicular trafficking in the degradative pathway and endocytosis and recycling of transferrin (Uchiya et al., 1999). The multitude of alterations caused by SpiC suggests that it is an important player in *S. typhimurium* trafficking within macrophages. However, the status of SpiC as an effector is being debated as it forms an important part of the translocon machinery and promotes the translocation of many other SPI-2 effectors (Freeman et al., 2002; Yu et al., 2002). The SPI-2 T3SS also has an important role in preventing trafficking of the macrophage NADPH oxidase to the *Salmonella*-containing phagosome, avoiding exposure of the pathogen to the damaging effects of the respiratory burst (Vazquez-Torres et al., 2001). Fig.7 summarizes the changes in host cells induced via the *Salmonella* effector proteins.

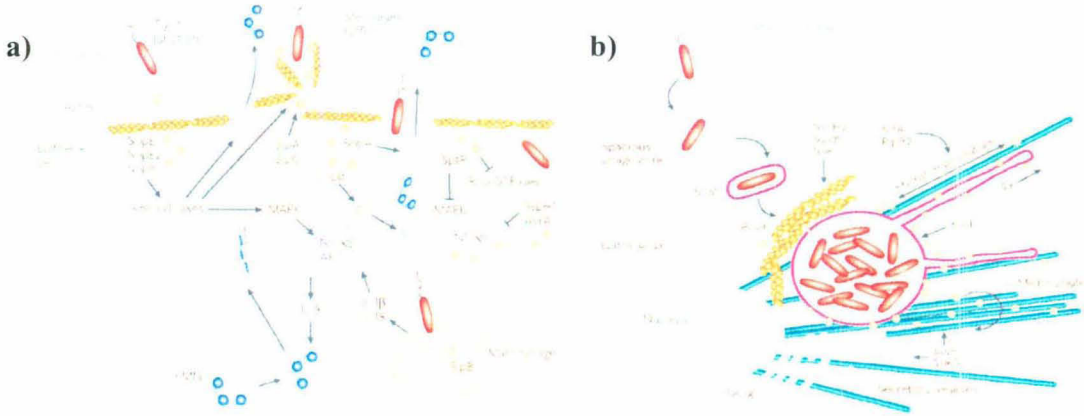


***Figure 7**: SPI- a)-1 and b)-2 T3SS induced changes in host cells*
Adapted from Nature Reviews Microbiology; Haraga, A, Ohlson, M.B., 2008.

2.7.3 Survival of *Salmonella* within host macrophages

S. typhimurium invades and survives within host epithelial cells and macrophages in a specialized compartment termed as the *Salmonella* containing vacuole (SCV) or Live *Salmonella*-containing phagosome (LSP). There have been reported differences in the trafficking pattern and interaction of *Salmonella* containing phagosomes with the host cellular machinery depending on the cell type as well as mode of entry of the bacteria in the host cells (Brawn et al., 2007; Dukes et al., 2006; Giacomodonato et al., 2007; Lawley et al., 2006; Ly and Casanova, 2007; Steele-Mortimer, 2008). However, due to heterogeneous intracellular behavior of the pathogen in terms of bacterial uptake and association of the SCV with members of the endocytic pathway within host macrophages (Holden, 2002), studies have been limited.

It has been documented that the pathogen survives within host macrophages by halting the phagosome maturation into a phagolysosome. To achieve this, live *Salmonella* modulate the expression of various Rabs (e.g. Rab5, Rab7, Rab9 and Rab18) on the phagosomes and reside in a specialized compartment that is devoid of actin, lysosomal enzymes and transferrin receptors but that retains Rab5 and Rab18. They also selectively deplete Rab7 from the phagosomal membrane and therefore, inhibit their transport to the late endocytic compartment (Hashim et al., 2000); However the association of mannose-

6-phosphate receptor (M6PR), lysobiphosphatic acid (LBPA) and the lysosomal hydrolase Cathepsin D with the LSP is still debated (Brumell et al., 2001b; Cuellar-Mata et al., 2002; Garcia-del Portillo and Finlay, 1995; Garvis et al., 2001; Hashim et al., 2000). Previous studies from our laboratory have shown that SopE, an effector protein from *Salmonella* specifically binds to host Rab5 (Mukherjee et al., 2000) and further, LSP specifically recruits Rab5 and NSF on the phagosomal membrane to promote efficient fusion with early endosomes (Mukherjee et al., 2001). Thus, different *Salmonella* effectors interact with and modulate the host transport molecules to their benefit, promoting their intracellular survival within macrophages. Though intracellular trafficking is regulated by Rabs, SNAREs and their interacting proteins, most of the studies regarding maturation of *Salmonella*-containing phagosomes have been restricted to the modulation/recruitment of Rab proteins on phagosomes by bacterial effectors. In the present study, we have tried to delineate the role of SNARE proteins on the maturation of *Salmonella*-containing phagosomes in macrophages and how this process is regulated by *Salmonella* effector proteins.

Objectives

Microbes are phagocytosed inside macrophages and degraded in the acidic environment of the lysosomes. However, several pathogens manipulate host cellular processes to their advantage and evade transport to the degradative compartment and survive as intracellular pathogens. It is now well established that *Salmonella* enters macrophages by triggering its own uptake via cytoskeletal rearrangements and subsequently, establishes an intracellular niche by inhibiting its transport to lysosomes. To this effect, *Salmonella* secretes several effectors into the host cytoplasm by a specialized secretion system. A complex interplay between a number of host and pathogen encoded factors is envisaged as part of *Salmonella* survival mechanism.

Recent findings from our laboratory have shown that a *Salmonella* effector, SopE recruits the host transport molecule, Rab5 on the *Salmonella*-containing phagosomes and subverts targeting to the lysosomes (Mukherjee et al., 2001). Moreover, temporal acquisition of another family of transport molecules, SNAREs on *Salmonella*-containing phagosomes speculated the involvement of different effectors in this process. However, the mechanism of recruitment of SNAREs by *Salmonella* on its phagosomes needs to be elucidated. Accordingly, studies were initiated in the present thesis to achieve the following objectives:

1. Identification and characterization of *Salmonella* effector molecules which are involved in the recruitment of SNARE(s) on phagosomes.
2. Determination of the role of the identified effector molecule(s) in *Salmonella* trafficking in macrophages.

Chapter 1

Identification of Salmonella effector molecules interacting with host SNAREs

4.1 Introduction

Salmonella is a gram negative, facultative intracellular pathogen that survives in the splenic and liver macrophages of a susceptible host. It causes infection by invading intestinal epithelial cells and host macrophages where the bacterium resides. The invasion and infection processes of *Salmonella* are attributed to the products of virulence genes which are clustered in localized regions at centisome 63 of the bacterial chromosome (Mills et al., 1995), commonly called as *Salmonella* pathogenicity islands, SPI-1 and SPI-2. Genes in these regions encode a specialized system for the delivery of virulence proteins into host cells termed as type III secretion system (Aderem and Underhill, 1999; Galyov *et al.*, 1997; Watson *et al.*, 1995).

Various *Salmonella* effector proteins manipulate different host molecules to aid in its survival as an intracellular pathogen. Conventionally, SPI-1 effectors (T3SS1) are known to regulate the invasion within epithelial cells, whereas SPI-2 effectors (T3SS2) aid in survival within host macrophages. For instance, T3SS1 effector SptP acts as a RhoGAP for Rac1 and Cdc42 and helps to recover the host actin cytoskeleton post infection (Fu and Galan, 1999). On the other hand SopB and SigD, both T3SS2 molecules induce membrane ruffling and modulate vacuolar traffic with their inositol phosphatase activity (Hernandez et al., 2004; Knodler et al., 2005). Similarly, the effectors SipA and SipC induce membrane ruffling by depolymerization of actin filaments and actin bundling (Hayward and Koronakis, 1999; Zhou et al., 1999). SipB is reported to cause autophagy induced cell death of host cells (Hayward et al., 2000; Hernandez et al., 2003; Hersh et al., 1999). Another T3SS2 effector, SpiC helps in intracellular survival by inhibition of phagosome-lysosome fusion (Lee et al., 2002; Shotland et al., 2003), while SifA and SSeG help in replication within macrophages (Beuzon et al., 2000; Boucrot et al., 2003; Brumell et al., 2001a; Deiwick et al., 2006). However, more recent reports have indicated that *Salmonella* survival within macrophages also involves several T3SS1 effectors, thus, diminishing the functionality difference between the T3SS1 and T3SS2 (Brawn et al., 2007; Dukes et al., 2006; Giacomodonato et al., 2007; Lawley et al., 2006).

Previously T3SS1, SopE and SopE2 were known GEFs for Rac1 and Cdc42 (Friebel et al., 2001; Hardt et al., 1998) and were shown to aid infection by inducing membrane ruffling. In addition, studies from our laboratory have shown that SopE also acts as a GEF for host Rab5 and recruits it in GTP-bound form on the phagosomal membrane thereby inhibiting its transport to the lysosomes (Mukherjee et al., 2001). Thus, a number of *Salmonella* effector proteins interact with/modulate host proteins to inhibit the phagosomal maturation process and enable the bacterium to survive within host macrophages.

Like Rab GTPases, SNARE proteins play a key role in intracellular trafficking by driving intravesicular membrane fusion events. Several groups have recently reported the recruitment of certain SNARE molecules on the phagosomal membrane of different intracellular pathogens, including *Salmonella, Mycobacterium* and *Chlamydia* (Delevoye et al., 2008; Fratti et al., 2002; Fratti et al., 2003b; Smith et al., 2005). Contemporary studies from our laboratory suggest a temporal specificity in the acquisition of some of the SNARE molecules; syntaxin 6, syntaxin 7 and syntaxin 8 by live *Salmonella*-containing phagosomes during the phagosomal maturation process. Between the three, relatively higher amount of syntaxin 6 was present on phagosomes at 90 min of maturation (unpublished data).

However, it still needs to be found, how SNARE molecules are acquired by these *Salmonella*-containing phagosomes. Thus, in the present section, attempts have been made to identify the mechanism of recruitment of syntaxin 6 on *Salmonella*-containing phagosomes that presumably involves effector molecules from the bacteria.

4.2 Materials

4.2.1 Reagents and chemicals

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were obtained from Griener Bio-one (Wemmel, Belgium) and Biological Industries, Israel. TRIzol reagent and Platinum HiFidelity *Taq* polymerase were procured from Invitrogen (Carlsbad, CA). pGEM-T easy cloning vector and restriction enzymes were purchased from Promega Life Science (Madison, WI). Gel extraction kit and Ni-NTA agarose were supplied by Qiagen (Valencia, CA). SDS–PAGE

markers, RPN 756 and RPN 800, glutathione sepharose, as well as ECL reagents and photographic HyperfilmMP were procured from Amersham Biosciences (Amersham, UK). Agarose gel markers were obtained from MBI Fermentas (Canada). Luria-Bertani (LB) broth and LB-Agar were supplied by Difco Laboratories (New Jersey, USA). Bradford reagent was procured from Bio-Rad Laboratories (Hercules, CA). Bicinchoninic acid (BCA) reagents and *N*-Hydroxysuccinimidobiotin (NHS-biotin) were obtained from Pierce Biochemicals (Rockford, IL). IFA and CFA were obtained from Difco (Detroit, MI, USA). All other reagents used were of analytical grade.

4.2.2 Antibodies, vectors and recombinant proteins

SipC plasmid was received as a kind gift from Dr. Bobby J. Cherayil of Massachusetts General Hospital, Charlestown, MA. Antibodies against *Salmonella* effectors (anti-SopE, anti-SopB, and anti-SipC) were kindly provided by Dr. E. E. Galyov from the Institute for Animal Health, Berkshire, UK. Anti-syntaxin 6 antibody was purchased from Synaptic Systems, Germany. 12 nm colloidal gold conjugated goat anti-mouse IgG and HRP labeled secondary antibodies were purchased from Jackson Immuno Research Laboratory, West Grove, PA. Expression vectors, pET-28a and pGEX-4T2 were purchased from Novagen (San Diego, CA) and Amersham Biosciences (Amersham, UK), respectively.

4.2.3 Bacterial strains

The virulent wild type (WT) *Salmonella typhimurium* strain, SL1344 was obtained from Dr. Ayub Qadri of the National Institute of Immunology, New Delhi, India.

4.2.4 Cells

J774E, a mannose receptor positive murine macrophage cell line was kindly provided by Dr. Philip Stahl of Washington University (St. Louis, USA).

4.3 Methods

4.3.1 Culture of bacterial strains

The virulent WT *S. typhimurium* strain SL1344 and all *E. coli* strains were grown in LB broth containing appropriate antibiotics at 37°C with constant shaking (250 rpm).

4.3.2 Culture of cell lines

J774E cells were cultured in RPMI-1640 containing 10% FCS and 50 μg/ml gentamycin at 37°C in a humidified incubator with 5% CO_2. The average doubling time of the cells is 24 hrs and the cells were sub cultured every 48 hrs by seeding 10 million cells in 12 ml media in tissue culture flasks with an area of 75 cm^2 (T-75).

4.3.3 Cloning of syntaxin 6, 7 and 8 from J774E murine macrophage cell line

In order to clone syntaxin 6, 7 and 8 from J774E macrophages, RNA was prepared from the cell line by a standard procedure using TRIzol reagent. Briefly, J774E macrophages were scraped using a cell scraper from a T-75 flask. Cells were washed thrice with PBS at 1,000 rpm for 6 min and lysed in 1 ml of TRIzol reagent by gentle pipetting. To separate RNA from the protein complexes, 200 μl of chloroform was added to the tube, mixed vigorously and centrifuged at 12,000 rpm for 15 min at 4°C. A clear aqueous phase containing RNA obtained as the upper layer was carefully removed and mixed with 500 μl of isopropanol. Subsequently, RNA was precipitated by centrifugation at 12,000 rpm for 10 min. The RNA pellet obtained was washed with 500 μl of 70% ethanol, air dried and re-suspended in 50 μl RNase free water.

cDNA was prepared from RNA using Reverse Transcription kit from Invitrogen according to the manufacturer's instructions. Briefly, 2 μg of total RNA was mixed with 1 μl of poly-$(dT)_{20}$ primer, 2 μl of 10 mM dNTPs mix and incubated at 65°C for 5 min to melt any secondary structures in the RNA. Subsequently, 4 μl of 5X first strand synthesis buffer, 1 μl of 100 mM DTT, 1 μl of 'RNase-out' inhibitor and 1 μl of Reverse Transcriptase (RTase) enzyme (15 U/μl) were added and the mixture was incubated at 50°C for 1 hr to facilitate synthesis of the first strand cDNA. Following this, RTase enzyme was heat inactivated at 85°C for 5 min and the reaction was stopped by

incubation at 4°C for 5 min. RNaseH (1 µl of 1 U/µl stock) was added to the reaction mixture at 37°C for 20 min to cleave any RNA associated with the cDNA hybrid.

Finally, using specific forward and reverse primers as detailed in Table 3, full length syntaxin 6, syntaxin 7 and syntaxin 8 were amplified from cDNA by PCR cycling in a Perkin Elmer Lifesciences thermocycler for 30 cycles (denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and extension at 68°C for 1 min) using Hifidelity *Taq* according to the manufacturer's protocol. The amplified PCR products were analyzed on a 0.8% agarose gel. The PCR products (~800 bp) were digested with *Bam*HI/*Eco*RI for 2 hrs at 37°C and ligated into linearized pGEX-4T2 (~5 kb). The positive clones obtained after ligation were confirmed by restriction digestion of the plasmid with *Bam*HI/*Eco*RI and checked for the release of an appropriate size insert. The clones were sequenced using gene specific end to end primers and the sequences were analyzed using BLAST program.

TABLE 3

Primer Name	Sequence 5'-3'	Enzyme site
Syntaxin 6 Forward	GTGGATCCATGTCCATGGAGGACCCCTTC	*Bam*HI
Syntaxin 6 Reverse	GTGAATTCTCACAGCACTAGGAAGAGGAT	*Eco*RI
Syntaxin 7 Forward	GTGGATCCATGTCTTACACTCCGGGGATT	*Bam*HI
Syntaxin 7 Reverse	GTGAATTCTCAGCCTTTCAGTCCCCATAC	*Eco*RI
Syntaxin 8 Forward	GTGGATCCATGGCCCCGGACCCCTGG	*Bam*HI
Syntaxin 8 Reverse	GTGAATTCTCAGTTGGTTGGCCACACTGC	*Eco*RI

4.3.4 Expression and purification of syntaxins as GST-tagged fusion protein

The full length syntaxin 6, 7 and 8 were cloned into the *Bam*HI/*Eco*RI sites of pGEX-4T2 vector and transformed into *E. coli* BL21 cells (Stratagene, USA) for expression as GST fusion proteins. *E. coli* BL21 cells containing the appropriate plasmid were grown in LB to an $O.D._{600}$ of 0.5 and induced with 0.5 mM IPTG for 3 hrs at 37°C to allow expression of the recombinant syntaxin fusion protein. Cells were harvested by centrifugation at 6,000 rpm for 20 min at 4°C, washed and re-suspended in PBS containing lysozyme (1 mg/ml) for 30 min on ice to lyse the cells. Subsequently, cell lysates were treated with DTT (1 mg/ml) and unbroken cells were lysed by sonication (10

sec pulses for 2 min). Lysates were clarified by centrifugation at 10,000 rpm for 15 min at 4°C and the resulting supernatants containing the recombinant proteins were incubated with glutathione sepharose for 1 hr at 4°C to facilitate binding of the GST-tagged syntaxin proteins to the beads. Following extensive washes with PBS, recombinant GST-tagged syntaxin proteins were eluted by 30 mM glutathione from the beads according to manufacturer's recommendations.

The purified proteins were dialyzed against PBS and analyzed by SDS-PAGE. Protein content in the preparations was determined using Bradford protein detection assay. The protein samples were re-suspended in SDS sample buffer (0.0625 M Tris, pH-6.8, 2% SDS w/v, 10% glycerol v/v, 5% β-mercaptoethanol v/v, and 0.001% bromophenol blue w/v), boiled for 10 min at 100°C, resolved on a 12% polyacrylamide gel and visualized by Coomassie staining.

4.3.5 Preparation of *Salmonella* secreted proteins

A single colony of *Salmonella* was inoculated into 5 ml of LB and grown overnight at 37°C with constant shaking (300 rpm). Subsequently, this seed culture was inoculated into 2 L of fresh LB containing 300 mM NaCl and grown for an additional 16 hrs at 37°C with constant shaking. The high salt concentration in the medium was used to induce the secretion of *Salmonella* effector proteins into the medium (Chen et al., 1996b). Subsequently, the spent medium containing *Salmonella* secreted proteins was separated from the bacterial cells by centrifugation and concentrated through a 3 kDa cut off filtration membrane (Amicon) at 3,000 rpm, 4°C. After concentration of the spent medium, protein content was estimated by BCA and the concentrated proteins were snap frozen in liquid nitrogen and stored at -80°C.

4.3.6 Biotinylation of *Salmonella* secretory proteins

The concentrated secretory proteins of *Salmonella* were biotinylated using NHS-biotin by a standard procedure (Gruenberg *et al.*, 1989). Briefly, 20 mg of secretory proteins were dissolved in 9.5 ml of 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.3 and the proteins were biotinylated by drop wise addition of NHS-biotin (11.5 mg dissolved in 0.5 ml DMSO). The mixture was incubated for 2 hrs at RT with gentle stirring. Thereafter,

unreacted active groups were quenched by incubating the mixture for an additional 30 min in the presence of 1 ml of 0.2 M glycine. Finally, biotinylated secretory proteins were separated from other residual reactants by dialysis against PBS and concentrated using Centriprep YM 10 (Millipore, USA). The proteins were stored in small aliquots at -80°C. Biotinylation of secretory proteins was confirmed by Western blotting using avidin-HRP as the probe.

4.3.7 Identification of effector molecules from *Salmonella* recognized by host syntaxins

To identify the effector molecules from *Salmonella* interacting with host syntaxins, GST-syntaxins were incubated in the presence of biotinylated secretory proteins of *Salmonella.* Briefly, respective GST-syntaxin (100 µg) or GST (50 µg) was immobilized on glutathione sepharose beads (100 µl) by incubating them at 4°C for 1 hr in PBS and the unbound protein was removed by washing with PBS. Subsequently, immobilized syntaxin was incubated with biotinylated secretory proteins (5 mg) in 500 µl of PBS for 2 hrs at RT. Beads were washed with PBS to remove non-specifically bound proteins. In order to determine the binding of biotinylated secretory protein(s) with syntaxin-immobilized beads, the beads were boiled in SDS sample buffer and proteins were separated on a 12% SDS-gel and transferred onto nitrocellulose membrane. Finally, Western blot analysis was carried out with avidin-HRP to detect the presence of biotinylated-*Salmonella* protein(s) bound with syntaxin molecules. Once identified, a similar experiment was performed with non-biotinylated secretory proteins and the ones interacting with the GST-syntaxins were identified by Western blot analysis using an array of specific antibodies against different *Salmonella* effector proteins.

4.3.8 Western blotting

Nitrocellulose membrane (0.45 µm; Millipore, USA) and the polyacrylamide gel containing the resolved proteins were soaked in Tris-glycine buffer (25 mM Tris, 200 mM glycine containing 20% methanol) for 15 min and the proteins were electrophoretically transferred to the nitrocellulose membrane at a constant current of 120 mA for 12 hrs at 4°C using a wet transfer cell (Bio-Rad, CA, USA). The efficiency of

protein transfer was verified by staining the membrane with Ponceau. The membrane containing the transferred proteins was blocked with 5% BSA in PBST (PBS containing 0.1% Tween-20) at room temperature for 1 hr. Thereafter, it was washed three times with PBST and incubated with primary antibody for 1 hr at RT. Non-specifically bound antibody was removed by washing the membrane thrice with PBST after which it was incubated with HRP conjugated secondary antibody for 1 hr at RT. Following washes, the blot was developed with ECL reagents according to the manufacturer's protocol and exposed to photographic film to capture the signals.

4.3.9 Sub-cloning of SipC in pET28a expression vector

Full length SipC gene was received as a kind gift from Dr. Bobby J. Cherayil in pBH vector. It was sub-cloned into the pET28a vector for expression as a His_6-tagged fusion protein. SipC gene digested with *Bam*HI/*Eco*RI was ligated into the same sites of the linearized pET28a vector. The clones obtained were screened for SipC insertion by restriction enzyme digestion to obtain an insert of appropriate size.

4.3.10 Expression and purification of recombinant SipC as His_6-tagged fusion protein

Full length SipC was cloned into the pET28a vector for expression as a His_6-tagged fusion protein. Competent *E. coli* BL21 cells transformed with pET28a-SipC construct were grown in LB to an $O.D._{600}$ of 0.5 and induced with 0.5 mM IPTG for 4 hrs at 37°C to allow expression of the recombinant SipC fusion protein. Cells were harvested by centrifugation at 6,000 rpm for 20 min at 4°C and the protein was purified under denaturing conditions using Ni-NTA agarose as per manufacturer's instructions. Briefly, the cell pellet was re-suspended in lysis buffer (6M GuHCl, 20 mM sodium phosphate, pH 7.8 and 500 mM NaCl) and incubated for 10 min to lyse the cells. Unbroken cells were lysed by sonication (3 pulses of 5 secs each). Subsequently, lysates were clarified by centrifugation at 10,000 rpm for 20 min at 4°C and the resulting supernatants were incubated with equilibrated Ni-NTA agarose in equilibration buffer (8 M urea, 20 mM sodium phosphate, pH 7.8 and 500 mM NaCl) for 30 min at RT to facilitate binding of the recombinant protein to the beads. Following extensive washes with wash buffer (8 M

urea, 20 mM sodium phosphate, pH 6.0 and 500 mM NaCl), recombinant His_6-SipC was eluted from the beads in elution buffer (8 M urea, 20 mM sodium phosphate, pH 4.0 and 500 mM NaCl). The eluate was diluted 1:10 in dialysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl and 2 M urea) and the purified protein was step dialyzed against this buffer with reducing amounts of urea from 2 M to 0 M to renature the protein.

Protein content in the preparations was determined using Bradford protein detection assay and the purified proteins were analyzed by SDS-PAGE.

4.3.11 Generation of polyclonal sera against SipC

In order to raise polyclonal sera against SipC, mice were immunized according to the standard protocol (Overkamp et al., 1988). Briefly, adult BALB/c mice (4-6 weeks) were immunized subcutaneously with approximately 10 μg of the purified His_6-SipC emulsified with CFA. Subsequently, mice were injected subcutaneously with the same amount of antigen emulsified in IFA thrice at three week intervals. Blood samples were collected five days after the last booster and polyclonal sera was separated by a standard method. The blood samples were incubated at 37°C for 2 hrs and subsequently, the sera was collected after centrifugation at 1,500 rpm for 10 min. Antibody specificity was determined by ELISA and Western blot analysis using purified SipC (2 μg) protein and secreted proteins of *Salmonella* (200 μg).

4.3.12 Relative interaction of different syntaxins with SipC

4.3.12.1 ELISA

The relative interaction of different syntaxin proteins with SipC was determined by a modified ELISA. The recombinant syntaxin 6-GST, syntaxin 7-GST and syntaxin 8-GST (500 ng/well) were coated in 100 μl in an ELISA plate overnight at 4°C in coating buffer (0.1 N sodium carbonate buffer, pH 9.5). Subsequently, wells were washed thrice with PBST (PBS containing 0.2% Tween-20) and incubated for 2 hrs at 37°C in blocking buffer (PBS containing 1% BSA and 3% milk protein). Wells were washed four times with PBST and incubated with or without SipC (250 ng/well in Tris-Cl, pH 8.0) for 1 hr at 37°C to allow binding. To determine the binding of syntaxins with SipC, wells were incubated with SipC specific polyclonal antibody (1:5,000 dilution) in PBS for 1 hr at

37°C. Excess antibody molecules were removed by washing the wells four times with PBST. Subsequently, HRP labeled anti-mouse IgG secondary antibody (1:10,000 dilution) was added to the wells for 1 hr at 37°C, washed four times with PBST, followed by three washes with PBS. Finally, the HRP activity present in each well was measured by a standard procedure (Gruenberg *et al.*, 1989). In the same assay, wells were also coated with equimolar concentration of free GST (250 ng/well) as a negative control. After subtracting the background readings obtained with free GST, the HRP activity associated with the syntaxin-SipC complexes was expressed as the relative binding of SipC with the recombinant syntaxins.

4.3.12.2 Western analysis

To confirm the direct interaction of different syntaxin proteins with SipC, 100 μg of syntaxin 6-GST, syntaxin 7-GST and syntaxin 8-GST was immobilized on glutathione sepharose beads by incubating the beads with the respective protein for 1 hr at 4°C in the presence of protease inhibitors. The unbound protein was removed by giving three washes with PBS. Syntaxin bound sepharose beads were blocked with 2% BSA in PBS for 1 hr at 4°C. After subsequent washes, the beads were incubated with or without SipC (2 μg) for 2 hrs at 4°C. Finally, the beads were given three washes with PBST, followed by three washes with PBS. The beads were boiled in SDS buffer and the syntaxin-bound protein(s) were resolved on a 12% SDS gel. The proteins were then transferred onto a nitrocellulose membrane and probed with anti-SipC antibody. In the same experiment, free GST was also immobilized on the beads as a negative control. The relative binding property of SipC with various syntaxins was compared.

4.3.12.3 Immuno-precipitation

To confirm the SipC-syntaxin 6 interaction, immuno-precipitaion was done. Briefly, anti-SipC polyclonal serum (10 μl) was immobilized on 20 μl bed volume of Protein G beads at 4°C for 2 hrs. The unbound antibody was washed thrice (1,000 rpm, 1 min) with lysis buffer (PBS containing 0.1% Tx100). Subsequently, SipC was immobilized on the beads by incubating 2 mg of *Salmonella* secretory proteins at 4°C for 12 hrs followed by extensive washes to remove unbound proteins. The beads were then

incubated with 4 mg of macrophage lysate for 8 hrs at 4°C in the presence of protease inhibitors. The beads were washed thrice with lysis buffer, followed by three washes with PBS. Finally, the beads were boiled in 1X SDS non reducing sample buffer (such that the antibody does not reduce into its heavy and light chains of 50 kDa and 25 kDa, respectively), separated on a 12% SDS gel, transferred onto a nitrocellulose membrane and probed with anti-syntaxin 6 antibody to check for the presence of bound syntaxin 6 to SipC. Similar experiment was carried out with beads without SipC polyclonal sera to determine the nonspecific binding.

4.3.13 Preparation of purified *Salmonella*-containing phagosomes

Phagosomes containing WT *S. typhimurium*, were prepared using a procedure described previously (Mukherjee et al., 2000). Briefly, *Salmonella* (2×10^9) were internalized into J774E cells (1×10^8) for 5 min at 37°C. Finally, cells were washed with plain media three times (1,000 rpm for 6 min at 37°C) to remove uninternalized bacteria. The late phagosomes (60 min and 120 min) were prepared by incubating the infected cells for indicated periods of time in RPMI at 37°C. At respective periods of time (5 min, 60 min and 120 min), *Salmonella* infected cells were diluted with three volumes of homogenization buffer (HB: 250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2) and homogenized in a ball bearing homogenizer at 4°C. Homogenates were centrifuged at a low speed (2,000 rpm for 10 min) at 4°C to remove nuclei and unbroken cells (Mayorga et al., 1991; Pitt et al., 1992). Subsequently, phagosomes were purified using the protocol as described previously (Sturgill-Koszycki et al., 1994). Briefly, enriched phagosomal fractions were re-suspended in 100 µl of HB containing protease inhibitors and layered onto a 1 ml continuous 12% sucrose gradient. Samples were centrifuged at 1,700 g for 45 min at 4°C and the purified phagosomes were recovered from the bottom of the tube. Biochemical characterization of these phagosomes was carried out by the standard techniques established in the lab previously (Mukherjee et al., 2000).

4.3.14 Immuno-labeling of SipC on *Salmonella*-containing phagosomes

SipC present on the purified *Salmonella*-containing phagosomes was detected by immunogold labeling using a negative staining technique as described previously (Mukherjee et al., 2000). Briefly, phagosomes were purified and washed five times with ice-cold HB and sedimented by centrifugation. First, the purified phagosomes were adsorbed on to carbon-coated nickel grids supported by a film of glow-discharged formvar, and the specimens were quickly rinsed twice with HB and incubated for 30 min in blocking buffer (HB containing 3% skimmed milk and 0.1% gelatin). The samples were then incubated for 2 hrs with anti-SipC antibody (monoclonal) diluted 1:20 in blocking buffer. Subsequently, the specimens were rinsed three times (5 min each) with blocking buffer and incubated for 1 hr with goat anti-mouse conjugated with 12 nm colloidal gold at a 1:20 dilution. After two washes, the specimens were fixed in 1% glutaraldehyde in HB for 10 min. Finally, samples were sequentially washed with HB and distilled water, stained with 0.5% aqueous uranyl acetate for 1 min, blotted onto filter paper and air-dried. In the same experiment, anti-SopE antibody at a dilution of 1:40 is used as a positive control. The samples were examined in a transmission electron microscope (JEOL 1200 EX 11).

4.4 Results

4.4.1 Cloning, expression and purification of syntaxin 6 from J774E murine macrophages

To clone syntaxin 6 from the murine macrophage cell line, gene specific end to end primers were designed as mentioned in the methods to amplify the appropriate fragment of 767 bp from J774E cDNA by RT-PCR and analyzed on a 0.8% agarose gel (Fig.8a). The PCR product was digested by *Bam*HI/*Eco*RI and cloned into pGEX-4T2 vector to be expressed as a GST-tagged fusion protein. The positive clones were confirmed by release of an insert of appropriate size upon restriction digestion. The final clones were sequenced using gene specific forward and reverse primers. The obtained sequence was *in-silico* translated into the amino acid sequence and was analyzed by BLAST to determine its homology with the known sequences from the database. The

sequence was found to be completely identical to the reported mouse sequence of syntaxin 6 (Fig.8c).

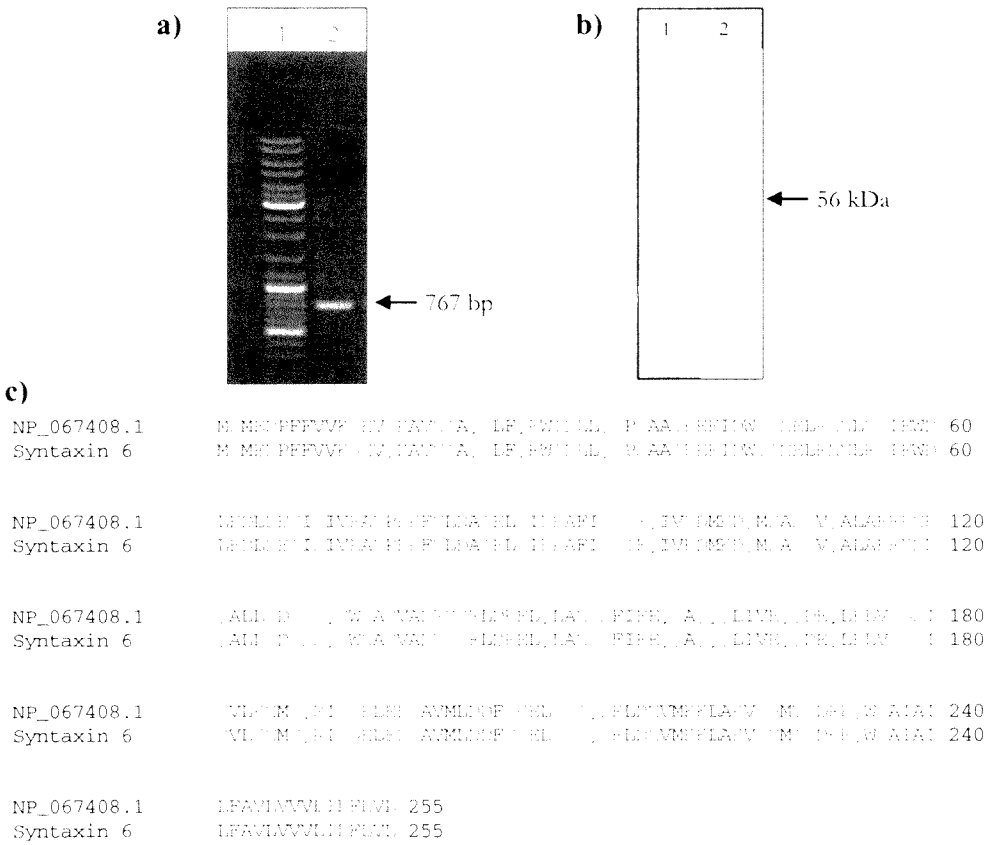


Figure 8: Cloning and expression of syntaxin 6 from J774E macrophages

a) PCR amplification of syntaxin 6 *Lane1: 1 kb DNA Ladder; Lane2: PCR amplified fragment of syntaxin 6*

b) Purification of syntaxin 6-GST *Lane1: RPN756 marker; Lane2: Purified syntaxin 6-GST*

c) CLUSTALW *alignment of sequence of cloned syntaxin 6 with reported sequence of mouse syntaxin 6 (Accession no. NP_067408.1)*

To prepare syntaxin 6 as GST fusion protein, *E. coli* BL21 cells transformed with pGEX-4T2-syntaxin 6 construct were grown and incubated in the presence of IPTG to induce the expression of the fusion protein. Subsequently, syntaxin 6-GST fusion protein was purified to homogeneity by affinity chromatography using glutathione sepharose beads; proteins were eluted and dialyzed against PBS. The SDS-PAGE analysis presented in Fig.8b concurred with purified syntaxin 6-GST being a 56 kDa protein.

4.4.2 Cloning, expression and purification of syntaxin 7 from J774E murine macrophages

Similarly, gene specific end to end primers of syntaxin 7 were designed and used to amplify a 785 bp fragment from cDNA prepared from J774E by RT-PCR. The PCR product was analyzed on a 0.8% agarose gel (Fig.9a). The PCR product was digested by *Bam*HI/*Eco*RI restriction enzymes and cloned into pGEX-4T2 vector to be expressed as a GST tagged fusion protein. The positive clones containing appropriate insert were sequenced using gene specific forward and reverse primers. The obtained sequence was translated *in-silico* into amino acid sequence and the sequence was analyzed by BLAST. It was found to be identical to the reported sequence of mouse syntaxin 7 (Fig.9c).

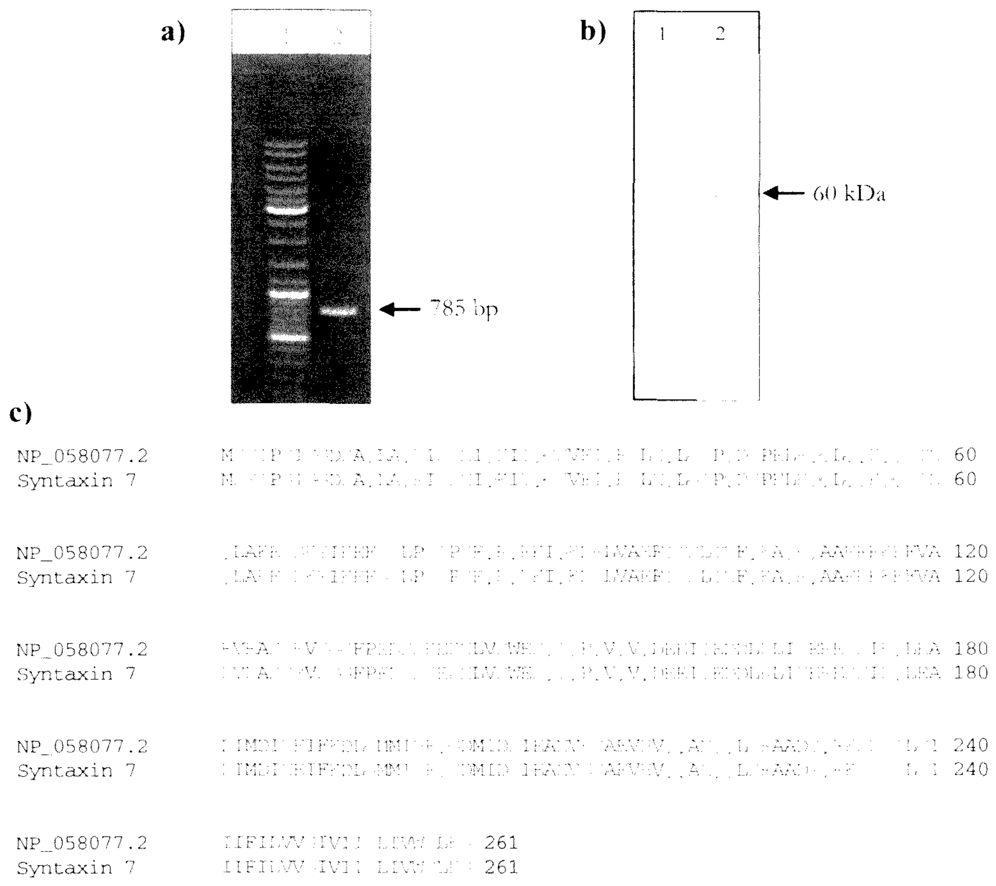


Figure 9: Cloning and expression of syntaxin 7 from J774E macrophages

a) PCR amplification of syntaxin *7 Lane1: 1 kb DNA Ladder; Lane2: PCR amplified fragment of syntaxin 7*

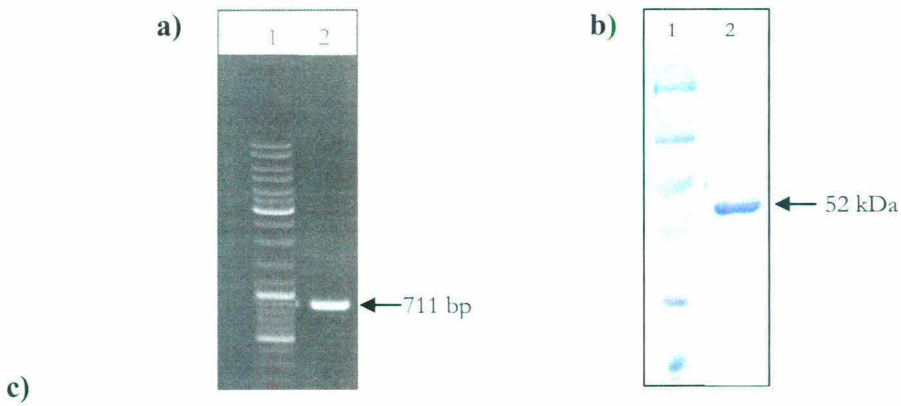
b) Purification of GST-syntaxin *7 Lane1: RPN756 marker; Lane2: Purified syntaxin 7-GST*

c) CLUSTALW *alignment of sequence of cloned syntaxin 7 with reported sequence of mouse syntaxin 7 (Accession no. NP_058077.2)*

To prepare syntaxin 7 as GST fusion protein, *E. coli* BL21 cells transformed with pGEX-4T2-syntaxin 7 construct were grown and incubated in the presence of IPTG to induce the expression of fusion protein. Subsequently, syntaxin 7-GST fusion protein was purified to homogeneity by affinity chromatography using glutathione sepharose beads; proteins were eluted and dialyzed against PBS. The SDS-PAGE analysis presented in the Fig.9b was in concordance with purified syntaxin 7-GST being a 60 kDa protein.

4.4.3 Cloning, expression and purification of syntaxin 8 from J774E murine macrophages

To clone syntaxin 8 from the murine macrophage cell line, gene specific end to end primers were designed to amplify the appropriate syntaxin 8 fragment using cDNA prepared from J774E cells by RT-PCR. The amplified fragment was found to be 711 bp when analyzed on a 0.8% agarose gel (Fig.10a).



c)

```
NP_061238.1     MAPDPWFSTYDSTCQIAQEIAEKIQERNQCERRGEKTPKLTLTIRTLLKNLKVKIDLLKD 60
Syntaxin 8      MAPDPWFSTYDSTCQIAQEIAEKIQERNQCERRGEKTPKLTLTIRTLLKNLKVKIDLLKD 60

NP_061238.1     LLLRAVSTRQITQLEGDRRQNLLDDLVTRERLLLASFKNEGAEPDLIRSSLMSEEAKRGT 120
Syntaxin 8      LLLRAVSTRQITQLEGDRRQNLLDDLVTRERLLLASFKNEGAEPDLIRSSLMSEEAKRGT 120

NP_061238.1     PNPWLCEEPEETRGLGFDEIRQQQQKIIQEQDAGLDALSSIISRQKQMGQEIGNELDEQN 180
Syntaxin 8      PNPWLCEEPEETRGLGFDEIRQQQQKIIQEQDAGLDALSSIISRQKQMGQEIGNELDEQN 180

NP_061238.1     EIIDDLANLVENTDEKLRTEARRVTLVDRKSTSCGMIMVILLLLVAIVVVAVWPTN 236
Syntaxin 8      EIIDDLANLVENTDEKLRTEARRVTLVDRKSTSCGMIMVILLLLVAIVVVAVWPTN 236
```

Figure 10: Cloning and expression of Syntaxin 8 from J774E macrophages

a) PCR amplification of syntaxin 8 *Lane1: 1 kb DNA Ladder; Lane2: PCR amplified fragment of syntaxin 8*

b) Purification of syntaxin 8-GST *Lane1: RPN756 marker; Lane2: Purified syntaxin 8-GST*

c) CLUSTALW *alignment cloned syntaxin 8 mouse syntaxin 8 (Accession no. NP_061238.1) sequences*

The PCR product was digested with *Bam*HI/*Eco*RI restriction enzymes and cloned into pGEX-4T2 vector to be expressed as a GST tagged fusion protein. The positive clones were selected and sequenced using gene specific forward and reverse primers. The obtained sequence was translated *in-silico* into the amino acid sequence. BLAST analysis of the obtained sequence was found to be completely identical to the reported mouse sequence of syntaxin 8 (Fig.10c).

Subsequently, *E. coli* BL21 cells transformed with pGEX-4T2-syntaxin 8 construct were grown in the presence of IPTG to induce the expression of fusion protein. The induced syntaxin 8-GST fusion protein was purified to homogeneity by affinity chromatography using glutathione sepharose beads. Proteins were eluted from the beads using a standard procedure. The SDS-PAGE analysis presented in the Fig.10b is in accordance with purified syntaxin 8-GST being a 52 kDa protein.

4.4.4 Identification of effector molecule(s) from *Salmonella* interacting with host syntaxins

To identify the possible effector molecule(s) from *Salmonella* involved in interacting with syntaxin 6, a GST pull down assay was performed. Syntaxin 6-GST was immobilized on beads and incubated in the presence of biotinylated secretory proteins of *Salmonella*. Finally, biotinylated secretory proteins bound with immobilized syntaxin 6 were detected by Western blot using avidin-HRP. The results presented in Fig.11a show that syntaxin 6-GST specifically interacts with a ~42 kDa effector protein from *Salmonella*. GST alone was unable to pull down any bacterial effector protein.

In order to identify the ~42 kDa effector protein, a similar experiment was carried out using non-biotinylated *Salmonella* effector proteins. Effector proteins bound with syntaxin 6 were probed with specific antibodies against different *Salmonella* effector molecules e.g., SopE, SipC and SopB. Our results showed that the 42 kDa protein is specifically recognized by anti-SipC antibody but not by anti-SopE or anti-SopB antibodies (Fig.11b). A similar experiment was also carried out using immobilized syntaxin 7-GST and syntaxin 8-GST to identify the possible *Salmonella* effector protein(s) interacting with these SNAREs. Interestingly, we found that SipC also interacts with host syntaxin 7 and syntaxin 8 (Fig.11c).

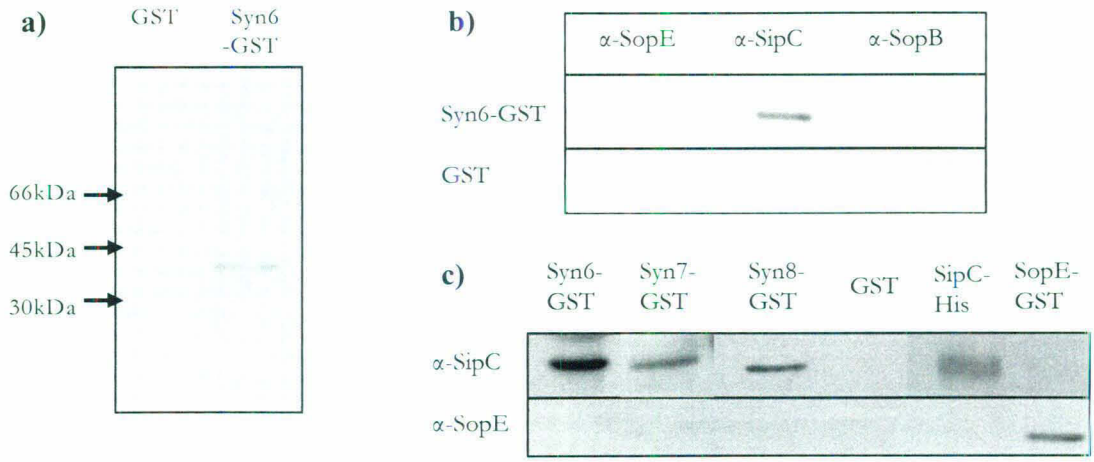


Figure 11: Identification of Salmonella effector protein(s) interacting with host syntaxins

a) *Detection of effectors interacting with syntaxin 6 by Western blot analysis using avidin HRP (1:10,000).*

b) *Identification of the ~42 kDa interacting protein as Salmonella Invasion Protein C (SipC) using α-SipC (1:60),α-SopE (1:100) and α-SopB (1:100) antibodies.*

c) *Western analysis of pull down assay with syntaxin 6-GST, syntaxin 7-GST and syntaxin 8-GST to identify interacting molecules from Salmonella.*

4.4.5 Expression and purification of SipC fusion protein

In order to further characterize the SipC-syntaxin interaction and understand its importance, SipC was cloned and expressed as a recombinant protein. Full-length SipC gene (1.2 kb) was sub-cloned into the linearized pET28a (~5 kb) vector for expression as a His_6–tagged fusion protein (Fig.12a).

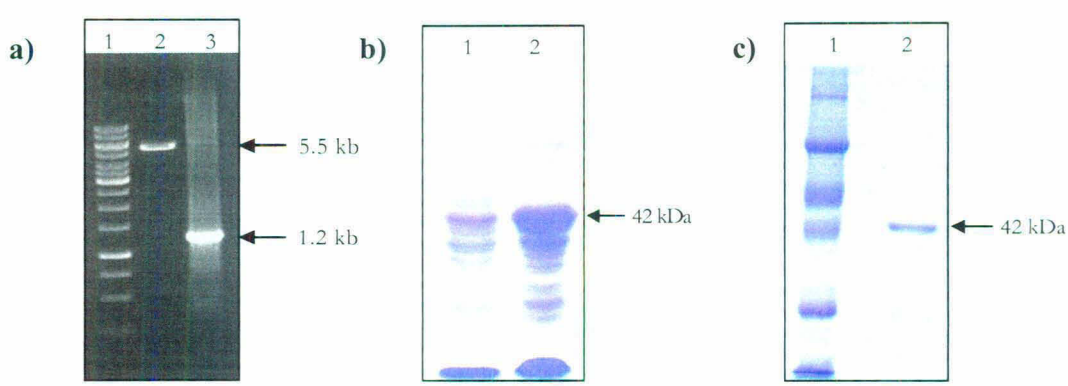


Figure 12: a) Cloning of SipC in pET28a *Lane1:1 kb DNA ladder; Lane2: pET28a linearized with BamHI/EcoRI, Lane3: SipC digested with BamHI/EcoRI*

b) Expression of SipC *as a recombinant His tagged protein Lane1: Uninduced sample; Lane 2: Induced SipC*

c) Purification of SipC *Lane1: RPN 756; Lane 2: Purified His_6-SipC*

Finally, the His_6-SipC fusion protein was purified to homogeneity by affinity chromatography and analyzed on a 12% SDS gel. Our results showed that the purified protein has a molecular weight of ~42 kDa, which is the expected size for SipC (Fig.12c).

4.5.6 Specificity of polyclonal sera generated against recombinant SipC

Purified His_6-SipC protein was used to generate polyclonal anti-serum against the protein in mice. The reactivity of the sera generated in different mice was checked by ELISA. The results in Fig.13a represent the detection of specific anti-SipC antibodies, post immunization. Their specificity was further confirmed by Western blot analysis using an enriched preparation of *Salmonella* effector proteins as well as purified His_6-SipC. Another affinity purified *Salmonella* effector protein SopE-GST was used as a control. Indeed, antibodies in the polyclonal sera could recognize the 42 kDa SipC protein both in the enriched preparation and in the purified His_6-SipC form. No crossreactivity to SopE-GST was observed (Fig.13b). Hence, the serum was used in subsequent experiments.

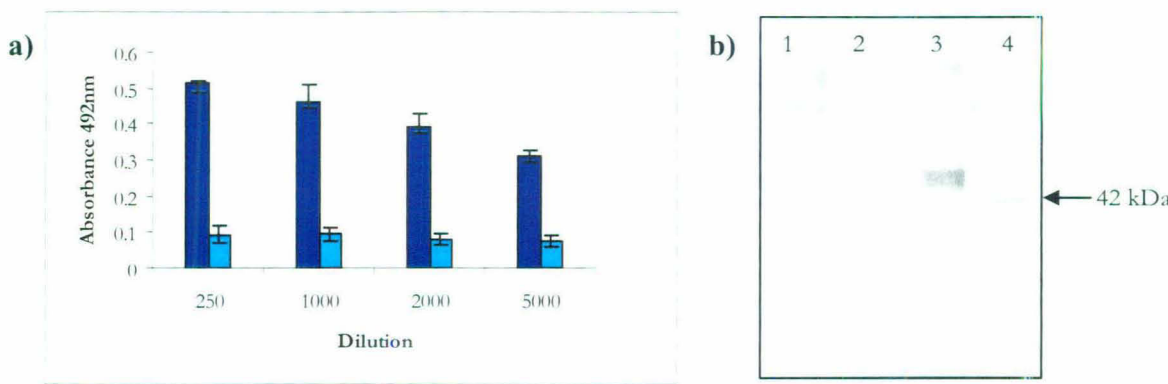


Figure 13: Generation of specific antibody against SipC

a) *ELISA to check the specificity of the polyclonal sera (dark blue bars) raised against recombinant SipC. Light blue bars represent pre-immune sera.*

b) *Western blot to check the specificity of the polyclonal sera using α-SipC antibody (1:500). Lane1: RPN 800; Lane 2: Purified SopE-GST (2μg); Lane 3: Purified His_6-SipC (2μg); Lane 4: Salmonella secretory proteins (300μg)*

4.4.7 Relative interaction of different Syntaxins with SipC

As established by the GST pull down, SipC was identified to be interacting with host SNARE molecules, syntaxin 6, 7 and 8. Subsequently, attempts were made to determine the relative binding of SipC with different syntaxins using direct protein-protein interactions. GST-syntaxins were immobilized on glutathione sepharose beads and incubated with purified His_6-SipC. Unbound SipC was washed away and the protein complexes were subjected to Western blot analysis using anti-SipC antibody. GST bound beads were used as controls. The results presented in the Fig.14a show that syntaxin 6 binds to SipC with higher affinity. However, relatively less binding of SipC was also observed with syntaxin 7 and syntaxin 8. No interaction was detected with free GST as well as no signal was observed when the beads were incubated without SipC ruling out any possible cross reactivity of the antibodies in the polyclonal sera with the purified GST-syntaxins or glutathione beads.

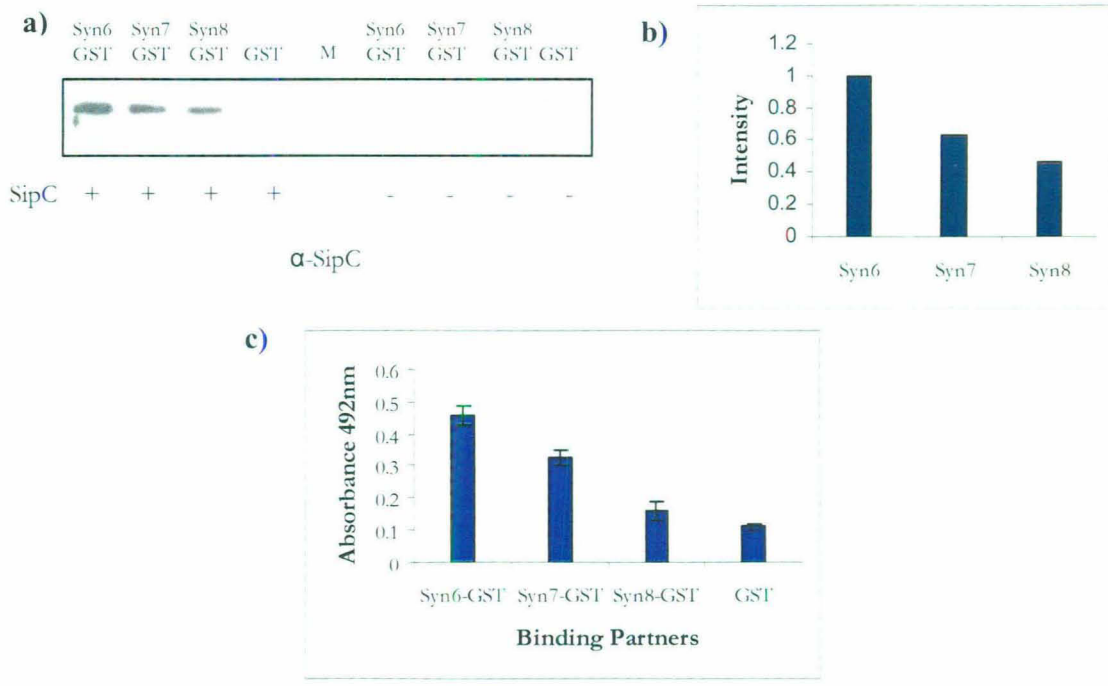


Figure 14: Relative binding of SipC with different syntaxins

a) *Western blot showing the relative interaction of various syntaxins with SipC in a direct protein interaction.*

b) *Quantification of the Western blot.*

c) *Determination of relative binding of SipC with various syntaxins by ELISA using α-SipC (1:5,000) antibody.*

These results suggest that SipC binding with various syntaxins is specific. Further quantification of the Western blot revealed that syntaxin 6 binds 1.5 folds and 2.5 folds more SipC than syntaxin 7 and syntaxin 8, respectively (Fig.14b). These results were further confirmed by using a modified ELISA. The purified GST-syntaxins or free GST were coated in equimolar amounts in an ELISA plate, incubated with equal amounts of His_6-SipC and the complexes formed were probed with anti-SipC antibody followed by secondary antibody labeled with HRP. The HRP activity associated with the complex determined the relative amount of SipC bound with syntaxins. Similar to previous results, we found that syntaxin 6 binds relatively higher amount of SipC in comparison to syntaxin 7. However, binding of syntaxin 8 with SipC was negligible and almost equal to binding with GST (Fig.14c).

4.4.8 Binding of SipC with syntaxin 6 from macrophages

Results presented above have demonstrated quite convincingly that at least *in vitro*, SipC specifically binds with syntaxin 6 with higher affinity. Therefore, we concentrated on the SipC-syntaxin 6 interaction and tried to find out its implication in the survival of *Salmonella* in macrophages. To test, whether the specific interaction could also be detected *in vivo* in macrophages, we carried out an immuno-precipitation using macrophage cell lysate. Briefly, anti-SipC antibody coated Protein G agarose beads were incubated with *Salmonella* secretory proteins to immobilize SipC on beads. The immobilized SipC was incubated with macrophage lysate and finally binding of syntaxin 6 with SipC was determined by Western blot analysis using anti-syntaxin 6 antibody. Antibody coated beads without immobilized SipC was used as control. The appearance of a ~30 kDa band corresponding to syntaxin 6 in the Western blot (Fig.15) confirms that SipC could specifically pull out syntaxin 6 from the pool of host proteins.

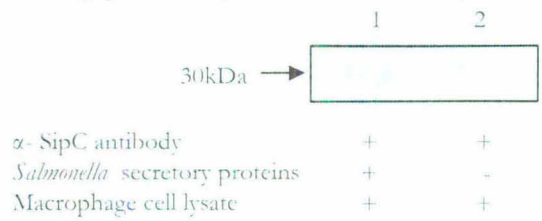


Figure 15: Co-immunoprecipitation to confirm SipC-syntaxin 6 interaction: *Western blot probed with α-syntaxin 6 (1:2,500) Lane1: Immunoprecipitation using Salmonella effector proteins and macrophage cell lysate; Lane 2: Control immunoprecipitation.*

The interaction is specific as absence of SipC from the beads was unable to capture syntaxin 6 from macrophage lysate. These results suggest that a similar SipC-syntaxin 6 interaction is possibly modulating the trafficking of *Salmonella* inside macrophages.

4.4.9 Localization of SipC on *Salmonella*-containing phagosomes

It is well established that *Salmonella* effector protein, SipC is secreted out of the bacteria through T3SS. However, in order to interact with host syntaxins, SipC must cross the phagosomal membrane and enter host cytosol or at least be present on *Salmonella*-containing phagosomal membrane. Therefore, to determine the localization of SipC within host cells, immuno-localization was done. Briefly, live *Salmonella*-containing phagosomes were purified and probed with anti-SipC antibody followed by a secondary antibody conjugated with colloidal gold particles. The experiment reveals that the effector protein of the pathogen was localized on the membranes of the phagosomes (Fig.16a). We used anti-SopE antibody as a positive control in the same experiment (Fig.16b) since SopE which is also present on phagosomal membrane has been reported to interact with host Rab5 (Mukherjee et al., 2001).

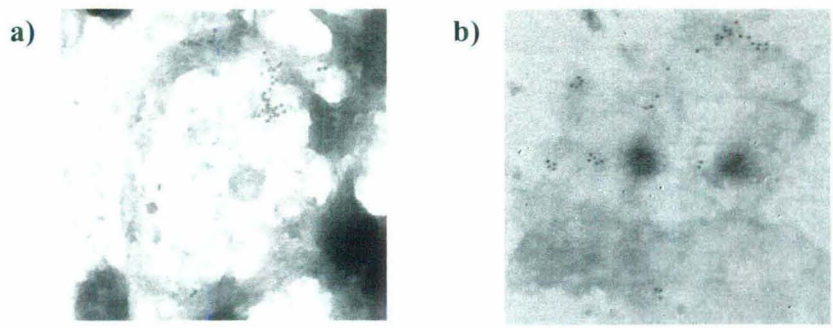


Figure 16: Immunolabelling of phagosomes

Purified phagosomes probed with ***a)*** *α-SipC (1:20), and* ***b)*** *α-SopE (1:40) antibodies followed by 12 nm gold labeled secondary antibody (1:40). The proteins were visualized as small spherical black dots.*

4.5 Discussion

Phagosomes, during maturation undergo a series of intravesicular fusion events and acquire/modulate different host molecules which aid in the survival of *Salmonella* as an intracellular pathogen. *Salmonella* has evolved a complex protein secretion system

termed TT3SS to deliver bacterial effector proteins into host cells, which serve to modulate host cellular functions (Zhou et al., 1999; Galan and Collmer, 1999) and support pathogen survival. As part of its evasion mechanism, the pathogen modulates host cellular functions by targeting Rab GTPases, SNARE molecules and signaling pathways, of which Rab GTPases and SNARE proteins are the key regulators of intravesicular fusion events.

Earlier studies from the lab suggest that *Salmonella* containing phagosomes recruit Rab5 and promote fusion with early endosomes, thereby preventing their transport to the lysosomes (Hashim et al., 2000; Mukherjee et al., 2000). Subsequently, SopE, a T3SS1 *Salmonella* effector protein was identified in the lab as the mediator of this process (Mukherjee et al., 2001). In addition, *Salmonella*-containing phagosomes have also been demonstrated to recruit NSF (N-ethylmaleimide sensitive fusion factor) on their phagosomes. NSF involves SNAP receptors (SNAREs) in driving the vesicular fusion events (Nichols and Pelham, 1998). Thus, recruitment of NSF on the phagosomes indicated that SNARE proteins might also play a role in the trafficking of *Salmonella* in macrophages.

Subsequently, we and others have shown that NSF mediated SNARE function is also necessary for phagosome maturation (Mukherjee et al., 2000; Nichols and Pelham, 1998). Moreover, recent studies from our lab have shown that *Salmonella*-containing phagosomes also recruits higher amount of syntaxin 6, syntaxin 7 and syntaxin 8 than dead *Salmonella*-containing phagosomes suggesting that live *Salmonella* driven processes might be responsible for enhanced recruitment of these syntaxins on phagosomes. Even though few, but there are some reports regarding the recruitment of host SNARE molecules on bacteria-containing phagosomes via the bacterial effector proteins. Zhou *et al* showed that *Salmonella* effector protein, SopB recruits host SNARE, VAMP-8 (Dai et al., 2007). Similarly, IncA, a *Chlamydial* inclusion protein interacts with and recruits many host SNAREs to the *Chlamydia* inclusion (Delevoye et al., 2008).

Among the three Syntaxins, syntaxin 6 was found to be recruited on *Salmonella*-containing phagosomes with higher affinity. With this background knowledge, studies were initiated to decipher the mechanism of recruitment of syntaxin 6 on phagosomes. Based on the experimental evidence as provided by pull down assays, direct protein

interactions and immuno-precipitation, we could identify that SPI-1 effector, *Salmonella* Invasion Protein C (SipC), interacts with host syntaxin 6 with higher affinity than other syntaxins suggesting a plausible mechanism of recruitment of syntaxin 6 on phagosomes (Fig.11,14). With previous understanding regarding involvement of bacterial effectors in recruitment of host SNAREs on the phagosomes and the current finding, it is tempting to speculate that SipC might be involved in the recruitment of syntaxin 6 on the *Salmonella*-containing phagosomes. Presence of SipC on the *Salmonella*-containing phagosomal membrane and immuno-precipitation of syntaxin 6 from the macrophage lysate by immobilized SipC (Fig.15,16) support our hypothesis of similar events happening *in vivo* which we have tried to address in the following studies.

Chapter 2

Generation of sipC knockout Salmonella

5.1 Introduction

It is well established that various *Salmonella* effector molecules modulate host proteins to their benefit to survive in the intracellular environment in macrophages as well as epithelial cells. Protein-protein interaction studies reported in the previous chapter have demonstrated that T3SS1 effector of *Salmonella*, SipC specifically binds with host syntaxin 6 with high affinity. Nevertheless, the significance of SipC interaction with host syntaxin 6 in the maturation of *Salmonella*-containing phagosomes in macrophages needs to be elucidated. The best way to determine the function of a bacterial effector molecule in host cells is to delete or silence the corresponding gene in the bacteria and study the behavior of the mutant bacteria in the host cells. Therefore, efforts were made to delete *sipC* from *Salmonella*.

Bacterial gene knock out can be generated by a number of allele replacement methods based on homologous recombination (Datsenko and Wanner, 2000; Hamilton et al., 1989; Russell et al., 1989; Skorupski and Taylor, 1996). Usually, a mutated construct containing a portion of the upstream and downstream flanking regions of the target gene with a selection marker is used to recombine with the bacterial genome and the knockout generated is selected appropriately. Here, we have used suicide vector based allelic exchange method by homologous recombination to generate *sipC* knockout *Salmonella* (*sipC* knockout). (Skorupski and Taylor, 1996). Suicide vectors typically contain an *ori* that can replicate only under specific conditions. Moreover, the vector has a positive selection marker, usually an antibiotic resistance gene. These two properties allow direct selection of the conjugants obtained after homologous recombination. In the first recombination event, the upstream flanking region of the gene of interest recombines with the complementary upstream region in the genome. In the subsequent recombination event, the other flanking region recombines with the complementary downstream region of the genome. The region containing the gene-suicide vector is excised from the genome upon further selection. The suicide vector used in this study, pRE112 has a *sacB* gene of *Bacillus sp.* (Gay et al., 1983). SacB is a levan sucrase which polymerizes levan, a product of sucrose catabolism and accumulates it in the cell periplasm, which is toxic for the cells (Donnenberg and Kaper, 1991; Gay et al., 1983). Hence, conjugants which get selected on sucrose containing media are those that have successfully lost the suicide

vector containing the gene to be knocked out. In the current chapter, we discuss the deletion of *sipC* gene from *Salmonella* genome and characterization of the strain obtained. Fig.17 depicts a schematic representation of the process of generating a bacterial gene knockout, in the current situation, *sipC* deletion from *Salmonella*.

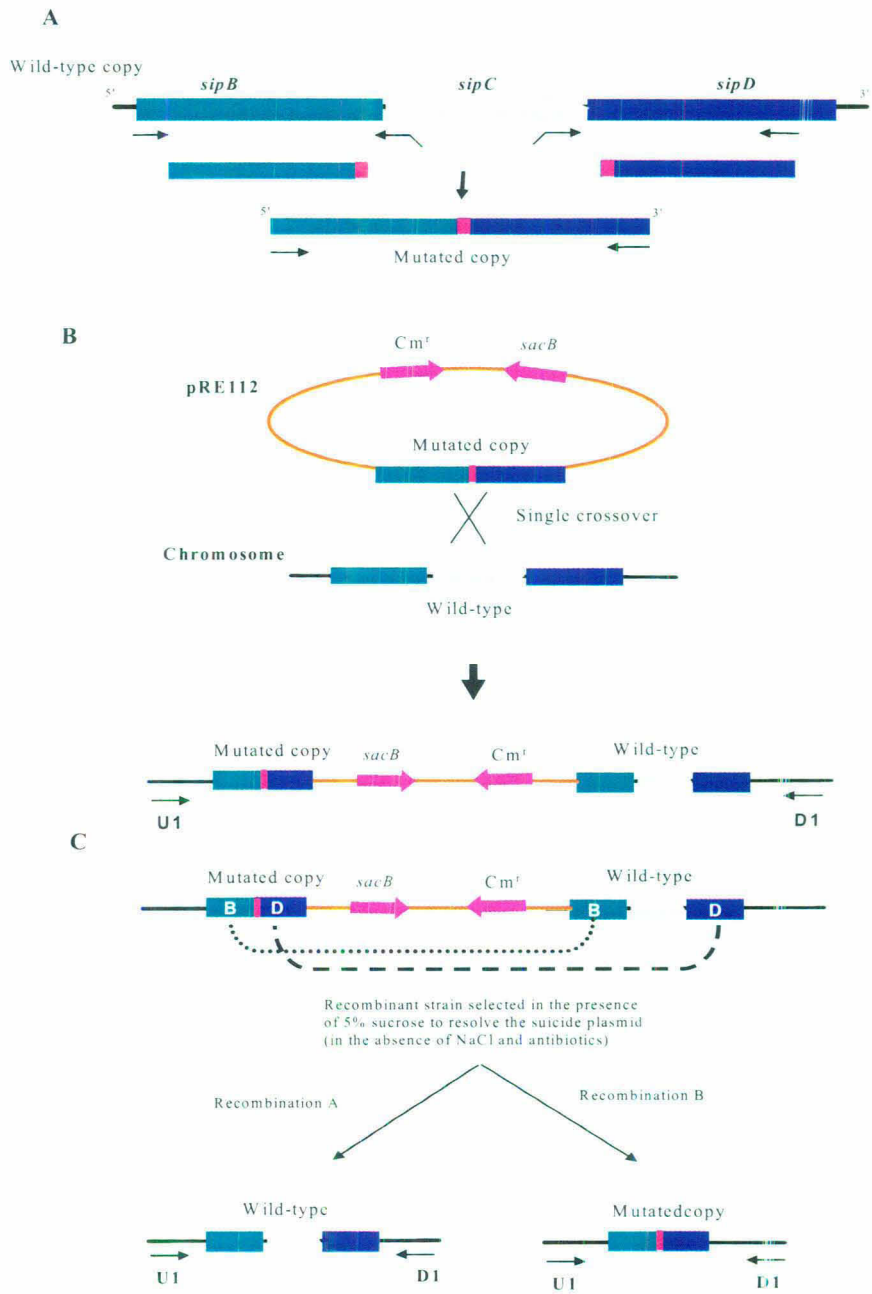


Figure 17: Generation of bacterial gene knockout by suicide vector based allelic exchange.

A. Generation of mutated copy. B. First recombination to generate intermediate strand.

C. Second recombination event by negative selection to generate knockout.

5.2 Materials

5.2.1 Reagents and chemicals

The labeled probes Phalloidin, Hoechst and the mounting reagent Prolong Gold antifade was procured from Molecular probes, Invitrogen. Mouse anti-actin antibody was purchased from Calbiochem (La Jolla, CA). All the secondary antibodies labeled with HRP were purchased from Jackson Immunoresearch. All other reagents used were of analytical grade and have been described previously.

5.2.2 Vectors

Suicide vector pRE112 was kindly provided by Dr. Olivia S. Mortimer of National Institutes of Health, Washington. Plasmid blue script (pBSK+) was purchased from Stratagene, La Jolla, CA.

5.2.3 Cells

Human epithelial carcinoma cell line, HeLa was obtained from American Type Culture collection (ATCC), Manassas, VA. The cell line was cultured in DMEM containing 10% FCS and 50 µg/ml gentamycin at 37°C in a humidified incubator with 5% CO_2. J774E murine macrophage cell was maintained as described previously.

5.2.4 Bacterial strains

E. coli strains SM10λpir and SY327λpir, required for the generation of bacterial gene knockout were kindly provided by Dr. Olivia S. Mortimer of National Institutes of Health, Washington.

5.3 Methods

5.3.1 Preparation of constructs for deleting *sipC* from *Salmonella* genome

In order to delete *sipC* from the *Salmonella* genome, regions 1 kb upstream and downstream of *sipC* containing some region of the *sipC* were PCR amplified and sequentially cloned into the suicide vector pRE112. For this targeted replacement, two sets of primers were designed. The first set of primers (SipB-C forward and reverse) was

used to amplify the region 1kb upstream of *sipC* (i.e. *sipB*) along with ~150 bp of 5′ end of *sipC*. Similarly, the second set of primers (SipC-D forward and reverse) was designed to amplify the region 1 kb downstream of *sipC* (i.e. *sipD*) containing ~150 bp of the 3′ end of *sipC*. The primer details are as mentioned in Table 4.

TABLE 4

Primer Name	Sequence 5′-3′	Enzyme site
SipB-C Forward	GTAAGCTTACGCCTTGCAGGAAGGGCG	*Hind*III
SipB-C Reverse	GTGATATCGGTCACTGACTTTACTGCTGC	*Eco*RV
SipC-D Forward	GTCCCGGGGTGAAAGTTCACGTAAATCGACC	*Sma*I
SipC-D Reverse	GTTCTAGATGCCAGGCTTGATATTTGGCG	*Xba*I
SipC Forward	GTGAATTCATGTTAATTAGTAATGTGGGAATAAATCCC	*Bam*HI
SipC Reverse	GTGGATCCTTAAGCGCGAATATTGCCTGCGATAGC	*Eco*RI
U1	GGCAACGAAAGCGGGCGACC	-
D1	CGGTTTCCAGGCTGCTACTTATATCG	-

Appropriate fragments were PCR amplified from SL1344 genomic DNA (PCR cycling conditions of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 68°C for 60 sec were used for 30 cycles). The upstream and downstream fragments, *sipB-C* and *sipC-D* respectively, were sequentially cloned into the *Hind*III/*Eco*RV and *Sma*I/*Xba*I sites of a cloning vector pBSK+ to generate a pBSK+Δ*sipC* construct.

The insert Δ*sipC* (~2kb) was subcloned from the cloning vector, pBSK+ into the *Xba*I digested and *Hind*III partially digested suicide vector, pRE112 to obtain pRE112Δ*sipC*. Since the suicide vector can propagate only in specific strains, hence this plasmid was transformed into competent *E. coli* SY327λ cells. Confirmation of the successful clone was obtained by subjecting the resultant clones to *Hind*III/*Xba*I digestion. The plasmid, pRE112 Δ*sipC* was transformed into the *E. coli* donor strain SM10λpir for subsequent conjugation with the *Salmonella* strain. The schematic representation of the cloning strategy used to generate these constructs for targeted deletion of *sipC* is depicted in Fig.18.

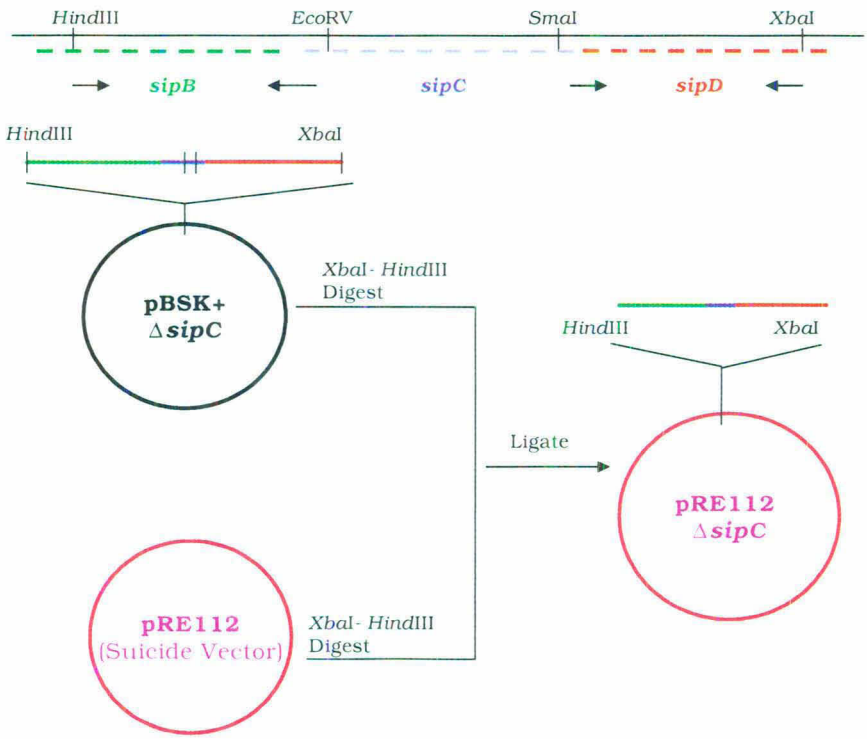


Figure 18: *Cloning strategy adopted to generate constructs for knocking out sipC*

5.3.2 Conjugation

Salmonella SL1344 strain and the *E. coli* donor strain SM10λpir containing pRE112 Δ*sipC* were grown in appropriate antibiotic containing media for 8 hrs at 37°C. Cells from both the cultures were mixed and propagated on LB agar plates without any antibiotic. Similarly, *Salmonella* SL1344 and *E. coli* SM10λpir containing pRE112 Δ*sipC* were also grown on the same media and used as controls. After incubation at 37°C for 24 hrs, bacterial cells were diluted in PBS and the conjugants were selected on antibiotic (streptomycin and chloramphenicol) containing media at 37°C. The *E. coli* donor strain is resistant to kanamycin. At the same time, the plasmid pRE112 contains the gene for chloramphenicol resistance while SL1344 is resistant to streptomycin. Subsequently, the conjugants obtained were further selected on media containing both streptomycin and choramphenicol while they were simultaneously screened by replica plating for the loss of kanamycin resistance which indicated elimination of the donor strain.

Single colonies of the conjugants obtained after the first recombination event, pRE112 Δ*sipC* -SL1344 were grown in LB for 4 hrs at 37°C and serial dilutions made in PBS (10^{-1} and 10^{-3}) were grown overnight at 30°C and selected on nutritional media lacking sodium chloride but containing 5% sucrose and streptomycin as the selection marker. Cells were grown in the presence of sucrose as toxicity mediated by sucrose metabolites leads to the second recombination event resulting in the excision of the suicide vector containing the target gene from the genome. Moreover, sucrose sensitivity is highly dependent on the incubation temperature and sodium chloride concentration (Blomfield et al., 1991). The colonies obtained after this round of negative selection were streaked on LB containing either streptomycin or chloramphenicol to confirm the excision of suicide vector containing *sipC* from the *Salmonella* genome.

5.3.3 Confirmation of deletion of *sipC* from *Salmonella*

The positive clones were screened for the successful deletion of *sipC* by PCR using genomic DNA as the template. The first set of PCR was carried out using *sipC* gene specific end to end primers (SipC forward and reverse). Another set of PCR was done using *sipB* forward and *sipD* reverse primers (U1 and D1). The PCR product was sequenced using *sipC* gene specific forward and reverse primers. Primer details are given in Table 4.

To further confirm the deletion of *sipC*, the bacteria were grown in LB for 16 hrs at 37°C and the secreted proteins in the culture supernatant were TCA precipitated. This involved addition of TCA to a final concentration of 10% and incubation at 4°C overnight followed by centrifugation at 10,000 rpm for 30 min at 4°C. The pellet thus obtained was washed thrice with PBS and re-suspended in SDS sample buffer. The proteins were separated on a 12% SDS gel and transferred onto nitrocellulose membrane. The membrane was probed with anti-SipC and anti-SopE (positive control) antibodies to determine the presence of these proteins in the pool of secreted proteins of *Salmonella* in the generated knock out strain.

5.3.4 Characterization of *sipC* knockout *Salmonella* strain

To characterize the *sipC* knockout *Salmonella*, actin bundling properties of the WT and mutant *Salmonella* were compared by staining F-actin with fluorescent labeled phalloidin. Briefly, 50,000 J774E macrophages or HeLa cells were plated overnight on a coverslip under normal growth conditions. The cells were infected with late log phase ($O.D._{600}$ ~0.8-0.9) GFP: WT or GFP: *sipC* knockout *Salmonella* at an MOI of 10 for 5 min at 37°C. The uninternalized bacteria were removed by washing thrice with PBS. The cells were fixed with 4% para formaldehyde for 20 min at RT. The fixed cells were stained with Ax-546 phallodin (1:2000 dilution of 6.6 µM stock solution) and Hoechst, a nuclear stain (1:1000 dilution of 10 mg/ml) in blocking buffer (PBS containing 2% BSA, and 0.1% saponin, a permeabilizing agent) for 45 min at RT. The non-specifically bound probe was removed by washing with PBS. The coverslips were mounted in Prolong gold antifade and observed under a LSM 510 Meta confocal scanning microscope.

The levels of actin were also checked on purified phagosomes at different stages of maturation. Phagosomes containing WT or *sipC* knockout bacteria were isolated at different times of maturation (5 min, 60 min and 120 min). The phagosomal protein content was estimated by BCA protein assay. 40 µg of the purified phagosomal proteins were separated on a 12% SDS gel, transferred to nitrocellulose membranes and incubated with anti-actin antibody, followed by HRP conjugated anti-mouse secondary antibody. The signal was detected by ECL and the levels of actin present on WT and *sipC* knockout *Salmonella*-containing phagosomes at different stages of maturation were compared. The membrane was also probed for a *Salmonella* structural protein, flagellin as a loading control for the experiment.

5.4 Results

5.4.1 Generation of constructs

The upstream and downstream flanking regions along with small portions of *sipC* were PCR amplified using specific primers to generate amplicons of the sizes 1107 bp and 1058 bp, respectively (Fig.19a). *sipB-C* was cloned into the *Hind*III/*Eco*RV sites of the cloning vector, pBSK+ to generate pBSK+*sipB-C* (Fig.19b). Subsequently, *sipC-D*

was cloned into the *Sma*I/*Xba*I sites of pBSK+*sipB-C* to generate pBSK+Δ*sipC* containing the upstream and downstream flanking regions of *sipC* (Fig.19c).

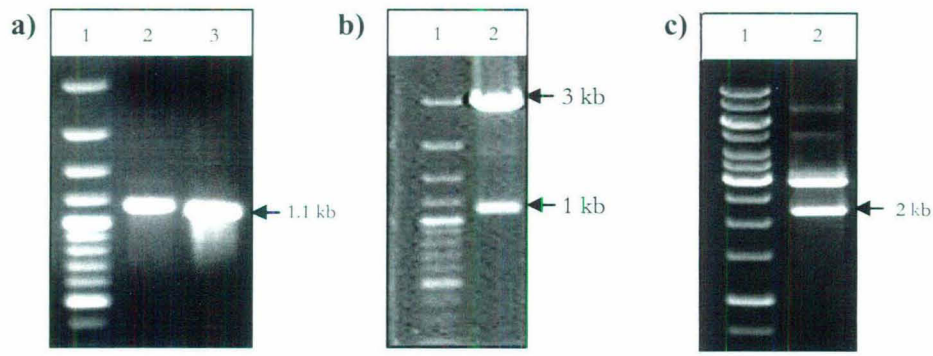


Figure 19: a) PCR amplification of flanking regions *Lane1: 100 bp DNA Ladder; Lane2-3: PCR amplified sipB-C and sipC-D, respectively using specific primers.*

Generation of pBSK+ΔsipC by sequential cloning

b) *Lane1:100 bp Ladder; Lane2: pBSK+ sipB-C EcoRV/ Hind III digest.*

c) *Lane1:100 bp Ladder; Lane 2: pBSK+ΔsipC Hind III /XbaI digest.*

5.4.2 Sub-cloning of Δ*sipC* into the suicide vector pRE112

For the process of allelic exchange, the clone generated above containing the flanking regions of *sipC*, Δ*sipC* was sub-cloned into the suicide vector, pRE112. The Δ*sipC* insert (~2 kb) was obtained from the cloning vector, pBSK+ by *Hind*III/*Xba*I digestion and cloned into the corresponding sites of the suicide vector, pRE112 to generate pRE112Δ*sipC* which was propagated in *E. coli* SY327λ strain. (Fig.20a,b)

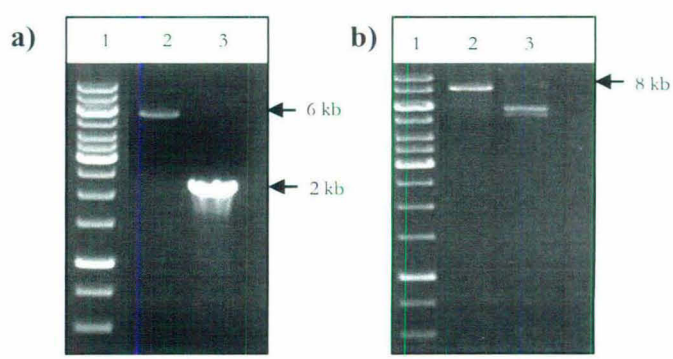


Figure 20: Sub-cloning of ΔsipC into pRE112

a) *Lane1:1 kb Ladder; Lane2: pRE112 HindIII(partial)/XbaI digest; Lane 3: ΔsipC HindIII/ XbaI digest*

b) *Lane1:1 kb Ladder; Lane 2: pRE112 XbaI digest; Lane 3: pRE112 ΔsipC XbaI digest*

5.4.3 Conjugation

pRE112Δ*sipC* was propagated in *E. coli* SY327λ strain which has the necessary machinery for propagation of the vector and a high transformation efficiency. However, this strain lacks the conjugation ability due to the absence of transfer genes for broad range hosts. Hence, for conjugation with *Salmonella* strain SL1344, pRE112Δ*sipC* was transformed into the *E. coli* donor strain SM10λpir. After the first recombination event between SL1344 WT *Salmonella* and pRE112Δ*sipC* SM10λpir *E. coli*, 97 conjugants were obtained on selection media containing streptomycin and chloramphenicol. SL1344 WT *Salmonella* and pRE112Δ*sipC* SM10λpir *E. coli* alone were used as negative controls for the conjugation. As expected, the negative controls did not grow on the selection media. The conjugants obtained were further screened individually on both kanamycin as well as on streptomycin-chloramphenicol containing media. It was observed that only 10 conjugants could specifically grow on streptomycin and chloramphenicol selection media and not on kanamycin containing media suggesting that only these conjugants represent SL1344 containing pRE112Δ*sipC* that had lost the *E. coli* donor strain SM10λpir which has kanamycin as the selectable marker. Of the 10 conjugants obtained, four were randomly selected and diluted in PBS. Finally, these clones were propagated on negative selection media (LB without sodium chloride containing sucrose and streptomycin) to select for the excision of pRE112-*sipC*. Of the many colonies obtained, 44 large colonies were streaked individually on streptomycin and on chloramphenicol containing media. The deletion mutants which had lost pRE112-*sipC* did not grow on chloramphenicol containing media. 11 such positive clones were obtained after the second recombination event and these were screened for the deletion of *sipC* gene from *Salmonella* as described in the subsequent sections.

5.4.4 Confirmation of deletion of *sipC* from *Salmonella* by PCR and sequencing

The positive clones were screened by PCR using genomic DNA as the template and *sipC* gene specific primers (SipC-F and SipC-R). Of the positive clones obtained, few clones amplified a 1.2 kb region corresponding to full length *sipC*, suggesting that

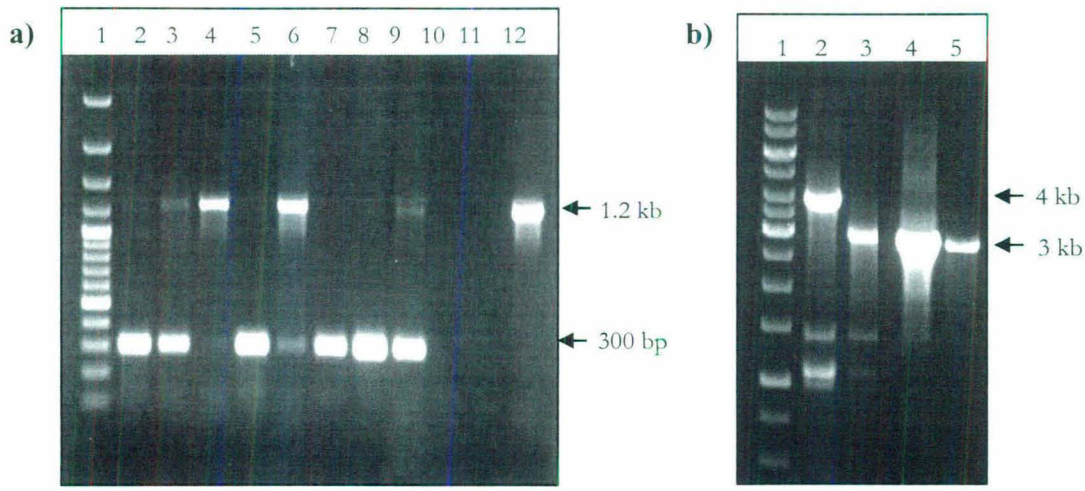


Figure 21: Confirmation of sipC knockout by PCR

a) *Lane1:100 bp Ladder; Lane 2-11,12: PCR amplification from genomic DNA of the conjugants and Wild type Salmonella, respectively using SipC gene specific primers.*

b) *Lane1:1kb Ladder; Lane2-4,5: PCR amplification from knockout clones and Wild type, respectively using SipB and SipD primers*

the gene had not been knocked out. However, three clones (lanes 5, 7, 8) yielded an amplicon of ~300 bp indicating the successful deletion of *sipC* gene (Fig.21a). Another round of confirmatory PCR for these clones using forward primer of *sipB* (U1) and reverse primer of *sipD* (D1) was carried out. As seen in Fig.21b, the deletion mutants amplified a fragment of ~3.1 kb as opposed to 4.2 kb in the wild type confirming the loss of a region of around 900 bp in between *sipB* and *sipD*. PCR products thus obtained were sequenced using *sipC* gene specific primers (SipC-F and SipC-R). The sequencing results confirmed the loss of a major portion of *sipC* (~900 bp) from the *Salmonella* genome (Fig.22).

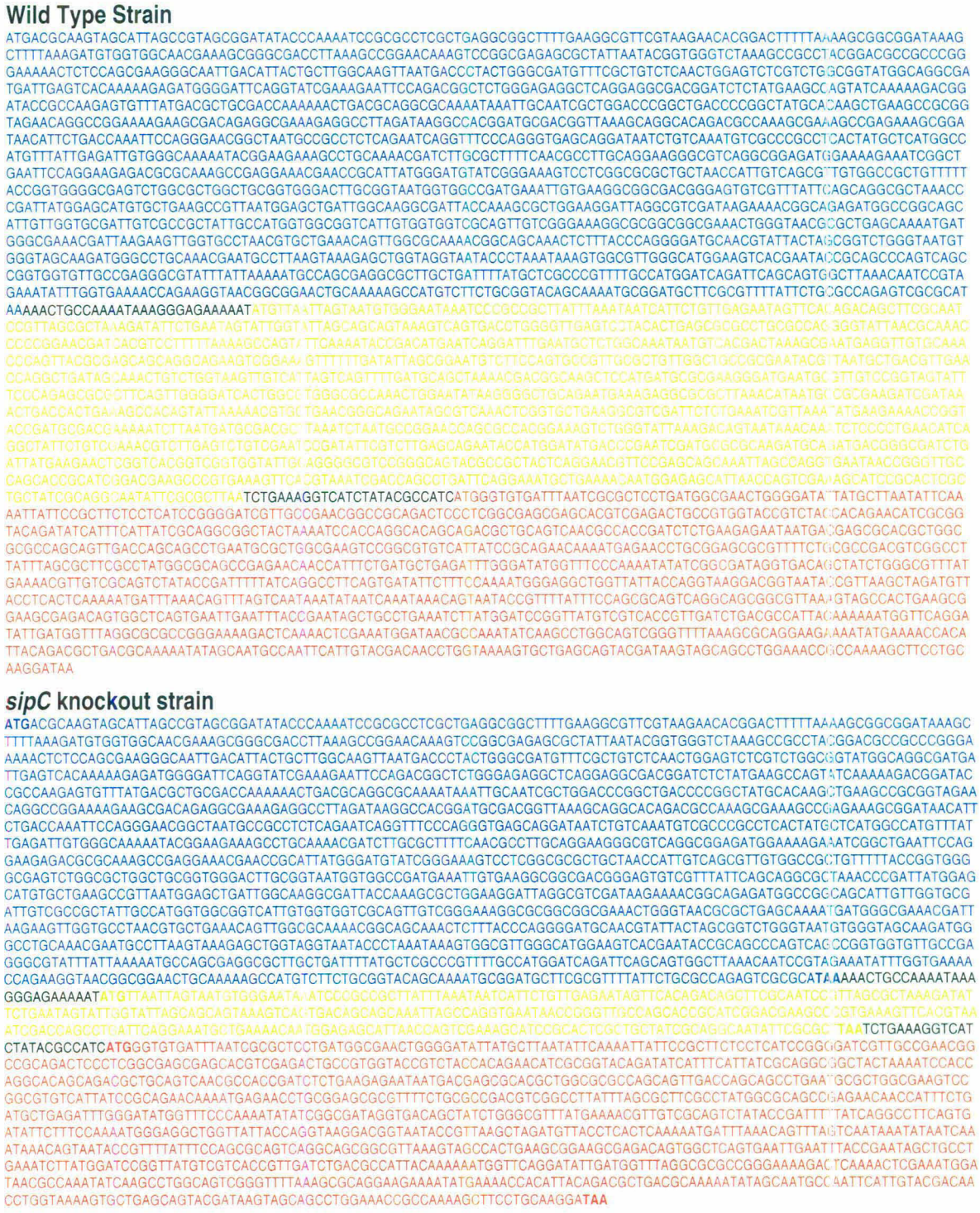


Figure 22: Sequencing results of Wild type and sipC knockout clones

Upper panel shows the sequencing result of the Wild type clone. Lower panel shows the sequencing result of the sipC knockout clone. Regions in blue, green and red correspond to sipB, sipC and sipD respectively.

5.4.5 Confirmation of knocking out of *SipC* from *Salmonella* by Western blotting

SipC is secreted outside the bacterial cell by the Type III secretion system along with other secretory proteins of *Salmonella*. Thus, the presence of SipC in the secretory proteins was determined using WT and *sipC* knockout *Salmonella* strains by Western blotting. Analysis of the secreted proteins demonstrated that *sipC* knockout *Salmonella* strain was unable to secrete SipC, which was otherwise successfully secreted by the WT strain (Fig.23, upper panel). However, both the strains could efficiently secrete out another *Salmonella* effector, SopE (Fig.23, lower panel), illustrating that deletion of *sipC* did not alter the Type III secretion machinery. These results reconfirmed the successful deletion of *sipC* from the *Salmonella* genome.

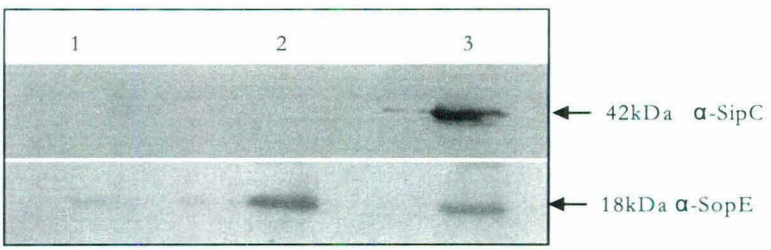


Figure 23: Protein secretion by sipC knockout Salmonella

Lane 1:RPN800; Lane2,3: Secreted proteins by sipC knockout and Wild type Salmonella, respectively using anti-SipC(1:500) and anti-SopE (1:100) antibodies.

5.4.6 Characterization of *sipC* knockout *Salmonella* strain

Further studies were carried out to confirm the *sipC* knockout strain using previous knowledge regarding the role of SipC mediated polymerization of actin in epithelial cells. Accordingly, J774E macrophages were infected with WT or *sipC* knockout *Salmonella* followed by phalloidin staining to label F-actin and look at the actin bundling ability of both the bacterial strains. It was observed that infection of macrophages with WT *Salmonella* leads to the bundling of actin at the site of infection. In contrast, no such bundling of actin at the site of infection was observed when infection was carried out with *sipC* knockout *Salmonella* and actin was found to be distributed evenly throughout the cell boundary as observed in uninfected control cells (Fig.24a). This indicated that

SipC is involved in actin bundling and that *sipC* knockout *Salmonella* lose this property. These observations were not just restricted to macrophages. Our results in Fig.24b confirm the loss of actin bundling by *sipC* knockout *Salmonella* in epithelial cells as well. These results are in accordance with previous reports where *sipC* knockout *Salmonella* failed to induce bundling of actin at the site of infection in the epithelial cells (Hayward and Koronakis, 1999). Thus, our results in macrophages and epithelial cells further confirmed the generation of *sipC* knockout *Salmonella* in the present investigation.

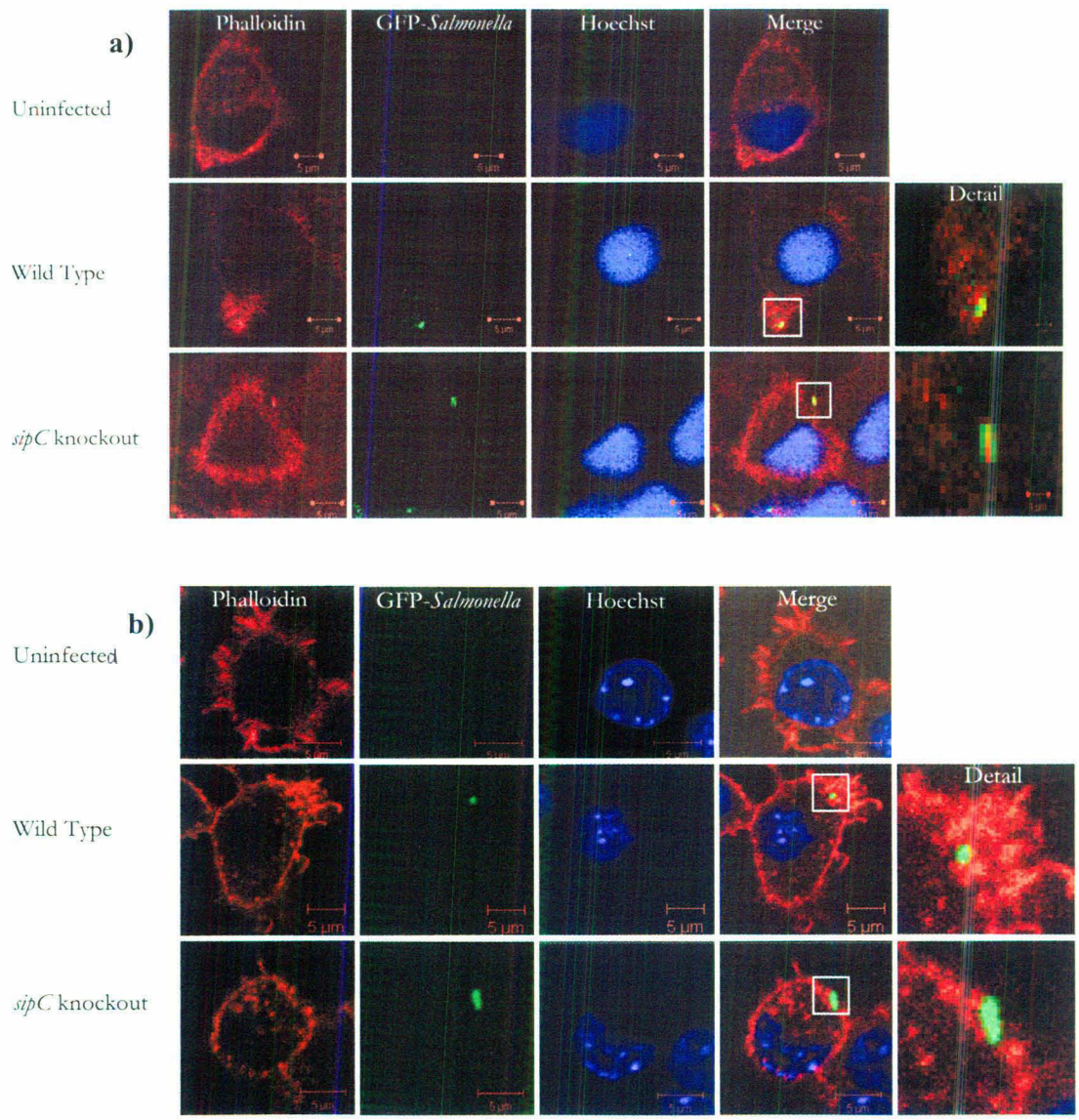


Figure 24: Actin bundling as visualized by phalloidin staining at sites of bacterial infection in a) *J774E macrophages and* ***b)*** *Hela cells. Last panel shows the enlarged region.*

To understand the role of SipC in the recruitment of actin during the maturation of *Salmonella*-containing phagosomes in macrophages, Western blot analysis was carried out with anti-actin antibody using purified phagosomes containing WT or *sipC* knockout *Salmonella*. The Western blot and its quantification presented in Fig.25 shows that the levels of actin drop by nearly 50% as the WT *Salmonella*-containing phagosomes mature towards a late compartment. In contrast, nearly 80% lesser amounts of actin were found on *sipC* knockout *Salmonella*-containing phagosomes even at early time of maturation which further dropped as the phagosomes mature. Presence of equal levels of flagellin, a *Salmonella* structural protein, on all the phagosomes demonstrated the equal loading and was used as an internal loading control. Furthermore, flagellin levels were used to normalize the levels of actin for quantification. Both immunofluorescence and Western blots results are in concordance with each other and implicate a role of SipC in the bacterial invasion process and probably phagosome maturation.

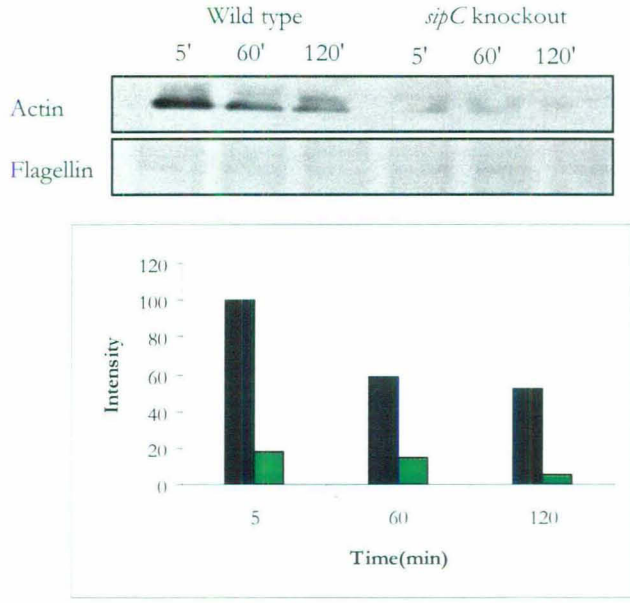


Figure 25: Actin recruitment on phagosomes:

Levels of actin in phagosomes containing WT or sipC knockout Salmonella at different maturation stages. Western blot probed by α-actin (1:5000) and α-flagellin (1:1000) antibodies. Graph shows the quantification of the Western blot after normalizing with flagellin values. Black bars represent ***wildtype;*** *Green bars represent* ***sipC knockout***

5.5 Discussion

Previously, we had observed that host SNARE molecule, syntaxin 6 is specifically recruited on live *Salmonella*-containing phagosomes as they mature inside macrophages indicating the involvement of some bacterial effector for binding with host syntaxins. Consequently, our initial studies reported in the previous chapter have established that *Salmonella* T3SS1 effector, SipC interacts with this SNARE. These findings led us to speculate a role of SipC in the recruitment of syntaxin 6 on live *Salmonella*-containing phagosomes. To verify this experimentally, a *Salmonella* strain with the *sipC* gene knocked out was successfully generated by homologous recombination. PCR amplification of the ORF (Fig.21) along with DNA sequencing of the region (Fig.22) has shown the successful deletion of *sipC* in the knockout strain. This has been further validated by the observation that the knockout strain is unable to synthesize and secrete SipC from the cells as demonstrated by Western blot of the secreted proteins (Fig.23). We also observed that the mutant strain obtained can secrete other T3SS effectors efficiently suggesting the specific deletion of *sipC* from the genome as well as indicating that the TTSS has not been affected by this deletion.

Previous studies have shown that SipC is involved in actin bundling and membrane ruffling at sites of bacterial infection (Hayward and Koronakis, 1999). It has also been reported in epithelial cells that SipC acts in concert with another T3SS1 effector, SipA and downstream cellular effectors of Rho GTPases to initiate and localize this actin rearrangement associated with membrane ruffling (Collazo and Galan, 1997). In the same study, authors have reported that *sipC* knockout *Salmonella* failed to carry out the actin rearrangement and assembly. Further studies have shown that F-actin and microtubules organize in a meshwork outside the *Salmonella* phagosomes (Galan, 2001; Guignot et al., 2004) and this organization is coordinated by some of the T3SS1 and T3SS2 effectors. In concordance with the results of Hayward *et al*, we also observed that *sipC* knockout *Salmonella* strain generated in the present investigation loses its property of actin bundling at sites of bacterial infection not only in macrophages but also in epithelial cells. Thus, our results indicate that SipC possibly plays a similar role as observed in epithelial cells when it is phagocytosed within host macrophages. Moreover, we have found that the levels of actin are considerably lower in *sipC* knockout

Salmonella-containing phagosomes. Hence, both *in vitro* and *ex vivo* data unequivocally prove that *sipC* gene is specifically deleted from *Salmonella* genome in the *sipC* knockout strain generated in the present investigation. Subsequent studies are carried out using this mutant strain to determine the role of SipC in the modulation of intracellular trafficking of *Salmonella* in macrophages.

Chapter 3

Role of SipC in the maturation of Salmonella-containing phagosomes in macrophages

6.1 Introduction

Salmonella, after entry into macrophages reside in a specialized compartment known as live *Salmonella* containing phagosome (Hashim et al., 2000). As these phagosomes mature, they undergo a series of sequential vesicular fusion events with various compartments of the endocytic and the secretory pathway, resulting in continuous association and dissociation of different transport molecules on the maturing phagosome. The process of intravesicular fusion is highly specific and regulated by members of the Rab GTPase and SNARE family of proteins. Several pathogens target host Rabs and SNAREs and modulate these molecules for their own benefit to enable efficient intracellular survival. This modulation of different transport molecules on the maturing phagosomes is carried out by several effector proteins which are secreted by the pathogen into the host cytoplasm. It has been previously reported that live *Salmonella,* as part of its survival mechanism, modulate the expression of various Rabs (e.g. Rab5, Rab7, Rab9, and Rab18) on the phagosomes within host cells (Hashim et al., 2000); and *Salmonella* effector, SopE is involved in the recruitment of Rab5 on the phagosomal membrane (Mukherjee et al., 2001).

In the previous chapters, we could establish that SipC, a T3SS1 *Salmonella* effector protein specifically interacts with host syntaxin 6 implicating a plausible role of SipC in the modulating this transport molecule and hence, contributing to phagosome maturation. Syntaxin 6 is an important SNARE regulating intracellular trafficking at the TGN (Watson and Pessin, 2000). It can interact with several endocytic SNAREs to become part of different fusion/SNARE complexes and thus, aids in driving many vesicular fusion events (Wendler and Tooze, 2001). To understand the role of SipC in the process of phagosome maturation, we knocked out the gene from the *Salmonella* genome. In the current section, we have tried to decipher the physiological significance of SipC in the maturation of *Salmonella*-containing phagosomes by comparing the trafficking of WT and *sipC* knockout *Salmonella* in macrophages.

6.2 Materials

6.2.1 Antibodies and vectors

Salmonella expression vectors, pFPV25.1 and pIZ1590 for constitutive expression of GFP and RFP were kindly provided by Dr. Raphael Valdivia (Duke Centre for microbial pathogenesis, Durham, NC) and Dr. Fransisco Ramos-Morales (Universidad de Sevilla, Spain). LAMP-1 GFP was a kind gift from Dr. Alberto Luini of Consorzio Mario, Negrusid, Italy. pBAD24 vector for over expression in *Salmonella* was kindly provided by Dr. A. Surolia of National Institute of Immunology.

Antibodies against mammalian Rab7 and LBPA were a kind gift from Dr. J. Gruenberg (EMBL, Heidelberg, Germany). Antibodies against mammalian Rab5 and EEA-1 were provided by Dr. A. Wandinger-Ness (University of New Mexico, Albuquerque, NM) and Dr. Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden). Commercial antibodies against various markers, GM130, Vti1b, LAMP-1 and syntaxin 6 were purchased from BD Biosciences (Bedford, MA). Antibodies against Cathepsin D and Rab6 were obtained from Neuromics and Santa Cruz, respectively. Polyclonal sera against the *Salmonella* protein, flagellin was purchased from Difco. All the secondary antibodies labeled with HRP were purchased from Jackson Immunoresearch. All the labeled probes including Texas red labeled Dextran, LysoTracker Red, Hoechst, fluorescent tagged secondary antibodies along with the mounting reagent Prolong Gold antifade were procured from Molecular probes, Invitrogen.

6.2.2 Cells

RAW 264.7, a murine macrophage cell line was obtained from American Type Culture collection (ATCC), Manassas, VA. The cell line was cultured in RPMI-1640 containing 10% FCS and 50 μg/ml gentamycin at 37°C in a humidified incubator with 5% CO_2. The average doubling time of the cells is 20 hrs and the cells were sub cultured every 48 hrs. J774E murine macrophage cell was maintained as described previously.

6.3 Methods

To understand the role of SipC in phagosome maturation, we compared the trafficking of WT or *sipC* knockout *Salmonella* in macrophages by *in vitro* and *ex vivo* approaches. The trafficking pattern of the bacteria inside host cells was analyzed by the acquisition of different transport related molecules on the phagosomes by direct and indirect immunofluorescence as well as by Western blot analysis of purified phagosomes at different stages of their maturation in macrophages.

6.3.1 Over expression of GFP and RFP in *Salmonella* strains

Salmonella constitutively expressing GFP or RFP were prepared for efficient visualization in the immunofluorescence experiments. To achieve this, WT as well as *sipC* knockout *Salmonella* were electroporated with plasmids, pFPV25.1 or pIZ1590 for GFP and RFP expression, respectively. Briefly, *Salmonellae* were propagated in 10 ml of LB till they reached log phase ($O.D._{600}$ of 0.5-0.6). The log phase cells were washed thrice with chilled water at 4°C (6,000 rpm for 6 min) to remove the salts contained in the medium. The bacterial cells thus obtained were re-suspended in 50 µl of chilled water and used for a single transformation. The electrocompetent *Salmonellae* were transformed with 1 µg of DNA using manufacturer's preset protocols for bacterial cells in a 2 mm gap cuvette in Biorad gene Pulser (Voltage- 2.5 kV, Capacitance-25 F, Resistance-200 Ω). After electroporation, the bacterial cells were allowed to recover for 1 hr at 37°C in 1 ml LB and subsequently the positive clones were selected on appropriate antibiotic containing media. The transformed bacteria were checked for GFP or RFP expression under a fluorescence microscope.

6.3.2 Trafficking of WT and *sipC* knockout *Salmonella* inside macrophages

6.3.2.1 Direct immunofluorescence

Briefly, 50,000 J774E macrophages were plated on a cover slip overnight under normal growth conditions. The cells were infected with late log phase ($O.D._{600}$ of 0.8-0.9) GFP: WT or GFP: *sipC* knockout bacteria at a MOI of 10 for 5 min at 37°C in plain RPMI containing Texas Red labeled dextran (MW 70,000). Following infection, uninternalized bacteria and dextran were removed by washing thrice with PBS.

Subsequently, both bacteria and dextran were allowed to traffic within the cells for indicated periods of time at 37°C (5 min, 30 min, 90 min and 120 min). The transport was stopped by fixing the cells at specific time points in 4% para formaldehyde for 20 min at RT. The fixed cells on the cover slips were mounted in Prolong gold antifade and observed under a LSM 510 Meta confocal scanning microscope. To follow the intracellular trafficking of WT or *sipC* knockout *Salmonella* towards the lysosomes, respective bacteria were internalized into macrophages for 5 min, washed and chased for additional 2 hrs at 37°C in the presence of LysoTracker Red (5 μM) in RPMI. To characterize the trafficking pattern of the bacteria within host cells, approximately 100 bacteria were scored for co-localization at each time point with the labeled probes mentioned above.

6.3.2.2 Indirect Immunofluorescence

As mentioned above, 50,000 J774E macrophages were infected with GFP: WT or GFP: *sipC* knockout bacteria at a MOI of 10 for 5 min at 37°C. After internalization, respective bacteria were chased inside cells for varying periods of time (5 min, 30 min, 90 min and 120 min). Subsequently, infected cells were washed and fixed with 4% para formaldehyde for 20 min at RT. The fixed cells were blocked for 1 hr in blocking buffer (PBS containing 2% BSA and 0.1% saponin, a permeabilization agent) at RT. These cells were then incubated with appropriately diluted primary antibody against different proteins like Rab5, LBPA, syntaxin 6, GM130 and LAMP-1 in blocking buffer for 1 hr at RT. The non-specifically bound antibody was removed by washing thrice with PBS. Following the binding of primary antibody, the cells were probed with specific Alexa conjugated secondary antibody (1:1,000) and counter stained with a nuclear dye, Hoechst (1:1,000). Fixed cells on the cover slips were mounted in Prolong gold antifade and observed under a LSM 510 Meta confocal scanning microscope. Percentage co-localization of bacteria with different transport molecules/organelles was calculated by analyzing approximately 100 bacteria under each condition.

6.3.3 Detection of transport related proteins on WT and *sipC* knockout *Salmonella*-containing phagosomes at different stages of maturation

Phagosomes containing WT or *sipC* knockout *Salmonella* were isolated and purified at different stages of maturation (5 min, 60 min and 120 min) as described previously. To detect the presence of host proteins on purified phagosomes, 40 µg of the purified phagosomal proteins were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was probed with specific antibodies against various transport molecules including Rab5, Cathepsin D, syntaxin 6, Rab6, EEA-1, Vti1b, LAMP-1 and a *Salmonella* structural protein, flagellin, which was used as the loading control. This was followed by addition of corresponding HRP conjugated secondary antibodies and the signal obtained after ECL detection was quantified using ImageJ software. The values obtained were normalized against the corresponding values for control and the recruitment of different transport proteins on the *Salmonella*-containing phagosomes was analyzed.

6.3.4 Over expression of LAMP-1 GFP in macrophages

LAMP-1 GFP was transiently over expressed in RAW 264.7 macrophage cells by electroporation. The cells were grown to 80% confluency and harvested. $10x10^6$ cells were washed with plain RPMI (100 g for 10 min at RT) and re-suspended in 400 µl of RPMI. 20 µg of purified LAMP-1 GFP plasmid was added to this cell suspension in a 4 mm gap cuvette. After gentle mixing of the contents, the DNA was transfected into the cells at 300 V, 975 µFD by Biorad Gene Pulser. After electroporation, the cells were allowed to recover at RT for 5 min. Following this, the goblet of dead cells was removed and the tranfectants were re-suspended in RPMI 1640 containing 10% FCS and plated on cover slips. The media was replaced after 6 hrs and the cells were checked for over expression after 20 hrs under a fluorescence microscope.

6.3.5 LAMP-1 transport from Golgi derived vesicles

Intracellular transport of molecules can be synchronized in Golgi by a temperature stress at 15°C (Trucco et al., 2004). LAMP-1 was synchronized in the Golgi and its transport from the Golgi was studied as described below. LAMP-1 was over

expressed in the RAW264.7 cells and incubated for 20 hrs at 37°C. LAMP-1 GFP over expressing cells were incubated at 15°C for 20 min to synchronize LAMP-1 at Golgi, followed by a chase at 37°C for another 30 min to allow vesicle budding from the Golgi. To confirm the transport block, cells were fixed at different time points and stained with Golgi specific GM130 antibody, followed by Alexa-546 labeled anti-mouse secondary antibody to be visualized by indirect immunofluorescence as described previously. The samples were analyzed for co-localization of LAMP-1 with GM130 to confirm the synchronization of LAMP-1 at Golgi.

6.3.6 Determination of the recruitment of LAMP-1 on *Salmonella*-containing phagosomes

To monitor the acquisition of LAMP-1 by WT or *sipC* knockout *Salmonella*-containing phagosomes from Golgi derived vesicles, LAMP-1 GFP was over expressed in macrophages. 18 hrs after transfection, these cells were infected with RFP: WT or RFP: *sipC* knockout *Salmonella* for 5 min at 37°C. After infection, uninternalized bacteria were removed by washing with plain RPMI and the infected cells were incubated at 37°C for 2 hrs to allow bacterial transport towards the appropriate late compartments inside the cells. Subsequently, cells were shifted to 15°C for 20 min to synchronize LAMP-1 in Golgi. Finally, the cells were incubated at 37°C upto 40 min to allow budding of LAMP-1 containing vesicles from Golgi. At different times after vesicle budding, the cells were fixed in 4% para formaldehyde and mounted as described previously. Cells were observed under a LSM 510 Meta confocal scanning microscope. Percentage co-localization of *Salmonella*-containing phagosomes with Golgi derived vesicles was calculated by analyzing nearly 100 cells, which were both transfected as well infected, under each condition.

6.3.7 Complementation of *sipC* in *sipC* knockout *Salmonella* strain

To unequivocally prove the role of SipC, *sipC* was complemented in the *sipC* knockout *Salmonella* strain to assess the gain of function by this protein. For complementation, *sipC* was cloned into an arabinose inducible *Salmonella* expression vector, pBAD24. *sipC* was PCR amplified using gene specific forward and reverse

primers (Table 5) from *Salmonella* genomic DNA and this product was cloned into the *Hind*III/*Eco*RI sites of the vector, pBAD24. The positive clones were checked for insert release of 1.2 kb by *Hind*III/*Eco*RI digestion. Purified DNA of the positive clone was transformed in the *sipC* knockout *Salmonella* cells by electroporation as described in section 6.3.1 to generate *sipC* knock-in *Salmonella.*

TABLE 5

Primer Name	Sequence 5'-3'	Enzyme site
SipC Forward	GTGAATTCATGTTAATTAGTAATGTGGGAATAAATCCC	*Bam*HI
SipC Reverse	GTAAGCTTTTAAGCGCGAATATTGCCTGCGATAGC	*Hind*III

The secretion of SipC was induced in the *sipC* knock-in *Salmonella* strain under different conditions, with varying arabinose concentrations and time periods. After the culture was induced and grown to late log phase (OD_{600} of 0.8-0.9), the secreted proteins were TCA precipitated as described previously. The secretion of SipC by *sipC* knock-in *Salmonella* was checked in 200 μg of the TCA precipitated secretory protein preparation on a Western blot probed with anti-SipC antibody. The secreted proteins from WT and *sipC* knockout *Salmonella* were used as controls in the experiment.

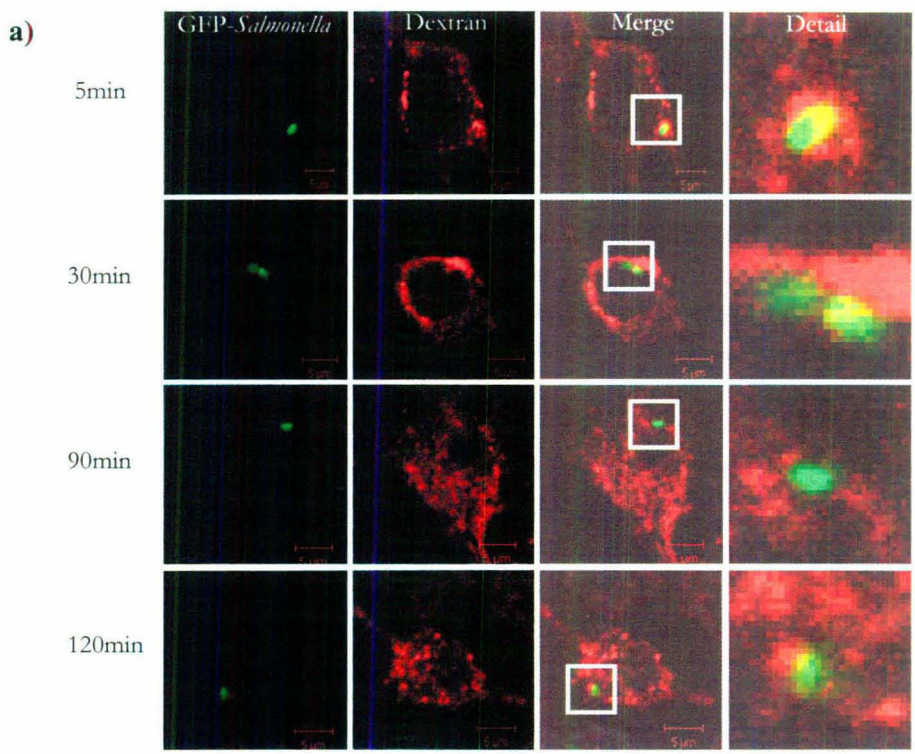
6.4 Results

6.4.1 Comparative trafficking of WT and *sipC* knockout *Salmonella* in the endocytic route

It is well established that as the phagosome matures, it interacts with various endocytic compartments, leading to a continuous association/dissociation of molecules on the phagocytic compartment. Thus, attempts were made to determine the interactions of WT and *sipC* knockout *Salmonella*-containing phagosomes with different endocytic compartments. To achieve this, GFP over expressing bacteria were chased within cells labeled with markers for specific compartments. Interaction

of the phagosomes with different compartments was characterized by calculating percentage co-localization.

Initially, the interaction of WT or *sipC* knockout *Salmonella*-containing phagosomes with the endocytic cargo was studied using a fluid phase probe, dextran labeled with Texas red. The results presented in Fig.26 depict that nearly 40% of both the WT and *sipC* knockout *Salmonella*-containing phagosomes co-localize with the endocytic cargo at early stages of entry inside the cell (5 min). However, as the phagosome matures with time (120 min), the percentage of bacteria co-localizing with dextran drops to a mere 20%.



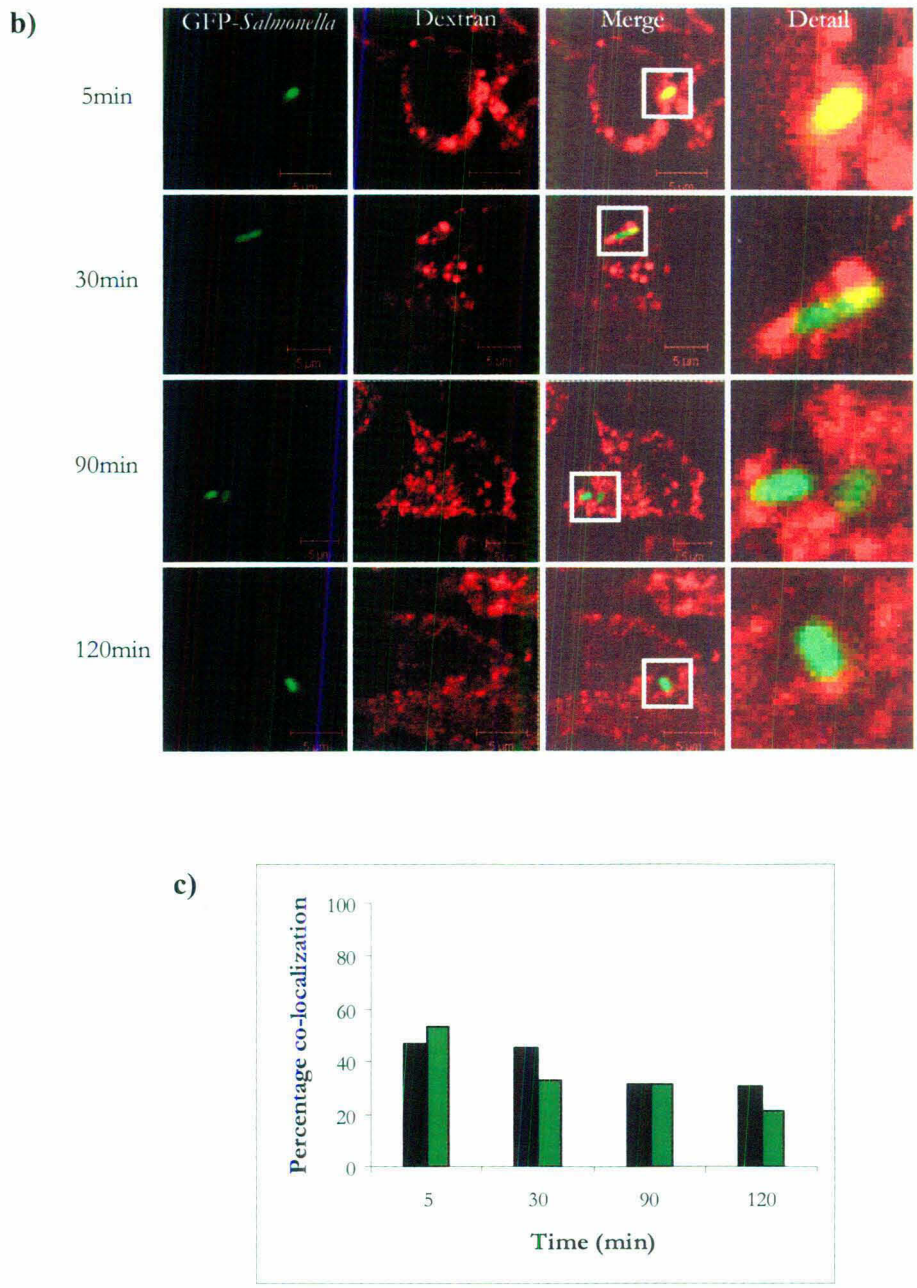


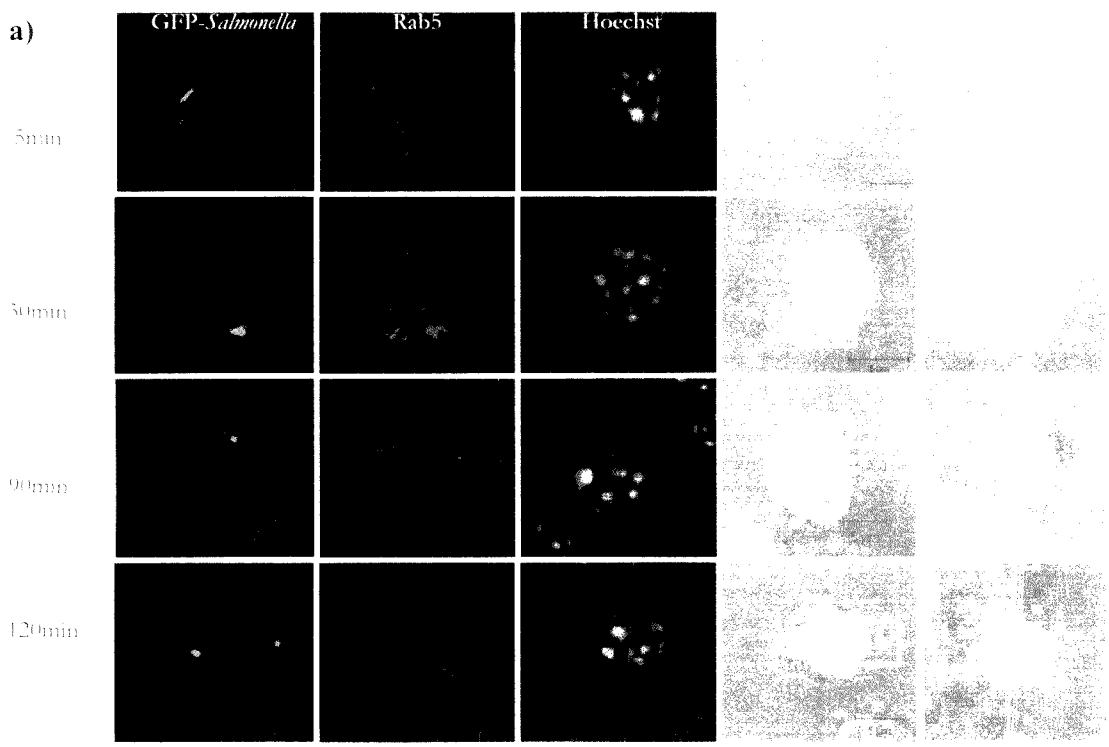
Figure 26: Interaction of WT or sipC knockout Salmonella-containing phagosomes with endocytic cargo.

a) *Wild Type Salmonella;* ***b)*** *sipC knockout Salmonella; Last panel shows the enlarged region.*

c) *Graph shows the percentage of bacteria co-localizing with dextran, n=100.*

Black bars represent ***wildtype****; Green bars represent* ***sipC knockout****.*

Following this, studies were carried out to characterize the interactions between *Salmonella*-containing phagosomes and different intracellular compartments. Rab5 is known to be associated with early endosomal compartments. Initial experiments were performed to determine the recruitment of Rab5 on WT or *sipC* knockout *Salmonella*-containing phagosomes. The results presented in Fig.27 illustrate that both WT (Fig.27a) and *sipC* knockout (Fig.27b) *Salmonella*-containing phagosomes recruit Rab5 within 5 min of bacterial internalization and retain this molecule even as the phagosome matured to 120 min.



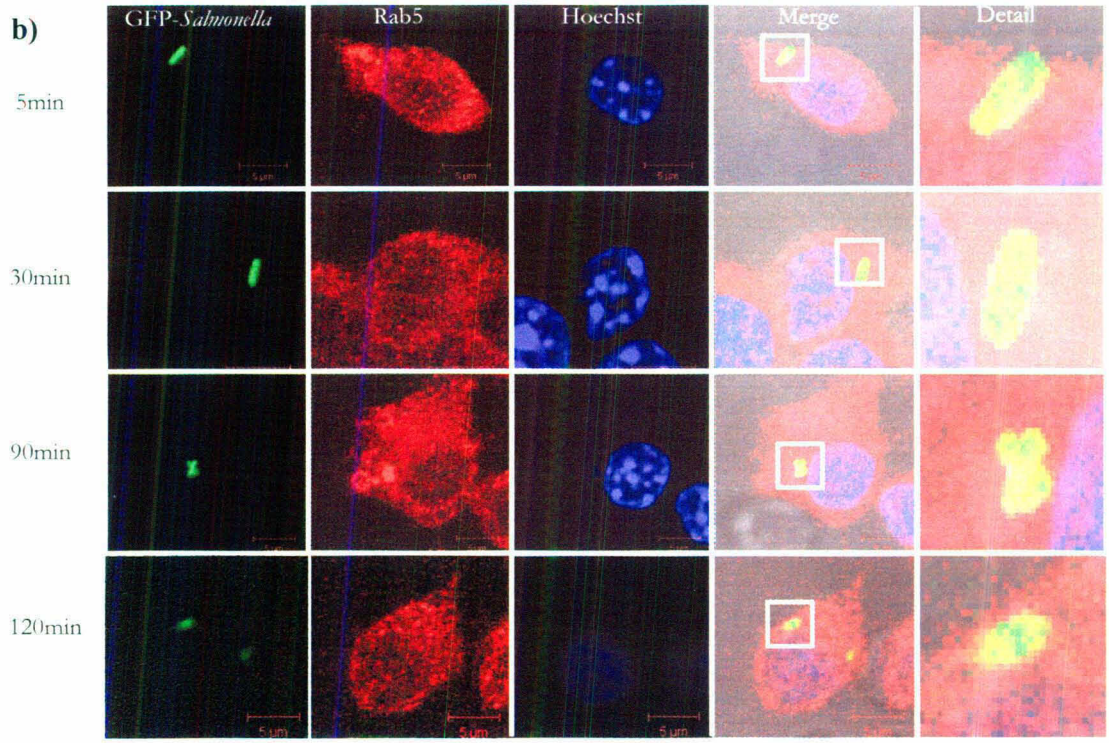


Figure 27: Recruitment of early endosomal marker, Rab5 on WT or sipC knockout Salmonella-containing phagosomes.

a) *Wildtype Salmonella;* ***b)*** *sipC knockout Salmonella; Last panel shows the enlarged region.*

These results along with previous observations (Hashim et al., 2000) indicated that though S*almonella* resides in a specialized compartment at later stages of their maturation in macrophages but it still retains some characteristics of the early compartment. During endocytosis as well as phagocytosis, cargo is transported from the early compartment to the lysosomes via a late endocytic compartment. Thus, we analyzed the association of a late endocytic marker, Lysobiphosphatic acid (LBPA), with WT or *sipC* knockout *Salmonella*-containing phagosomes at later stages (90 and 120 min after internalization) of their maturation in macrophages. The immunofluorescence data demonstrates that around 70%-80% of WT *Salmonella* co-localize with LBPA positive compartments at these time points (Fig.28a,c). No deviation in the recruitment of LBPA was observed with *sipC* knockout *Salmonella*-containing phagosomes (Fig.28b,c). These results indicate that both WT and *sipC* knockout *Salmonella*-containing phagosomes undergo fusion with and acquire molecules from the late endocytic compartments on their respective phagosomes.

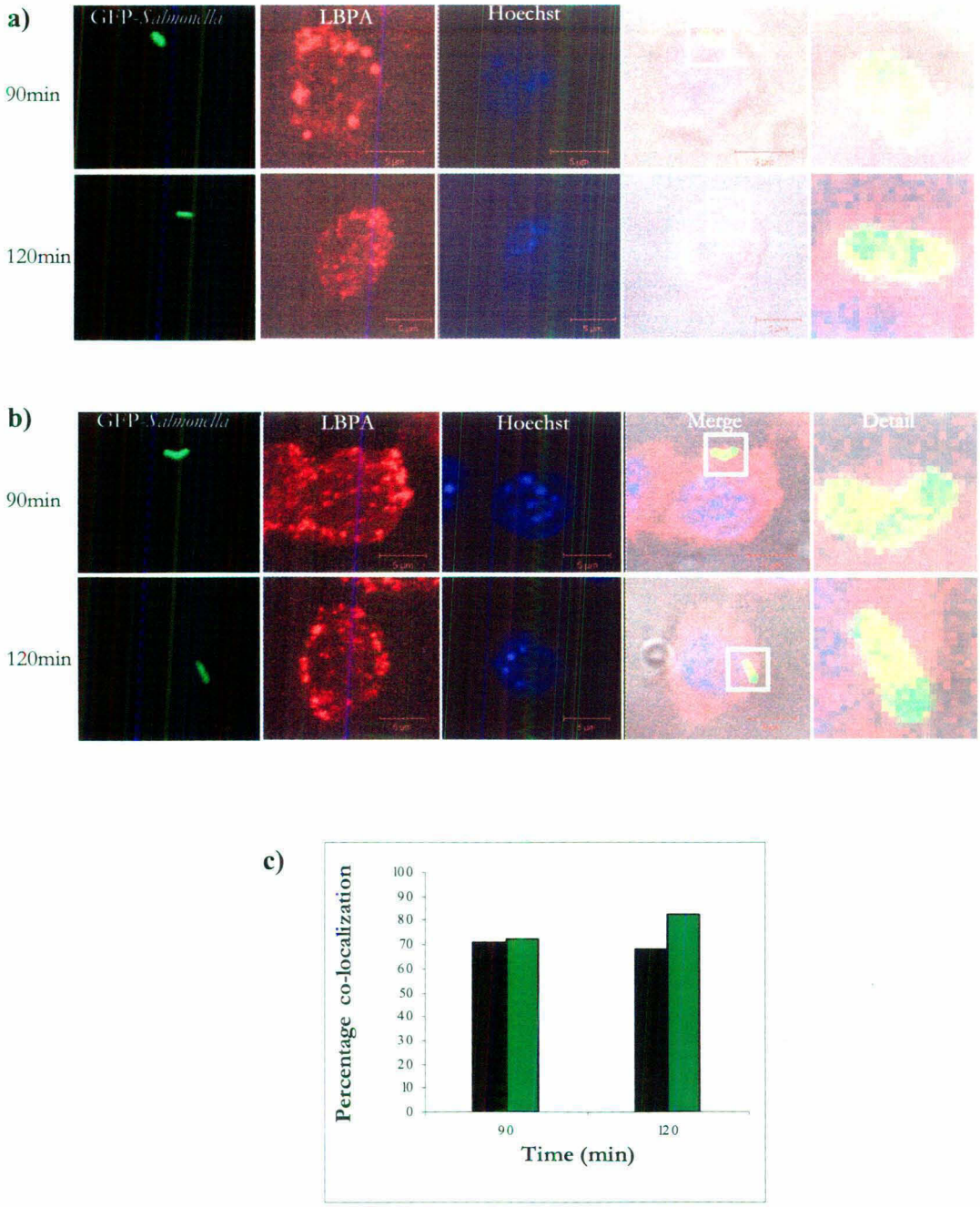


Figure 28: Acquisition of LBPA on WT or sipC knockout Salmonella-containing phagosomes.

a) *Wild Type Salmonella;* ***b)*** *sipC knockout Salmonella; Last panel shows the enlarged region.*

c) *Graph shows the percentage of bacteria co-localizing with LBPA, n=100.*

Black bars represent ***wildtype;*** *Green bars represent* ***sipC knockout****.*

The endocytic pathway finally culminates into an acidic compartment, the lysosome, where the internalized cargo is degraded. It is well established that WT *Salmonella* avert targeting to the lysosomes by avoiding phagosome-lysosome fusion. Accordingly, studies were carried out to determine whether deletion of *sipC* from *Salmonella* facilitated its targeting to the lysosomes. To examine this, *sipC* knockout *Salmonella* were chased inside macrophages for 120 min and the intracellular lysosomes were stained with LysoTracker Red. No apparent co-localization of *sipC* knockout bacteria with LysoTracker Red was observed indicating that like WT *Salmonella*, the mutant bacteria also prevented its transport to the lysosomes to survive within host cells (Fig.29a,b).

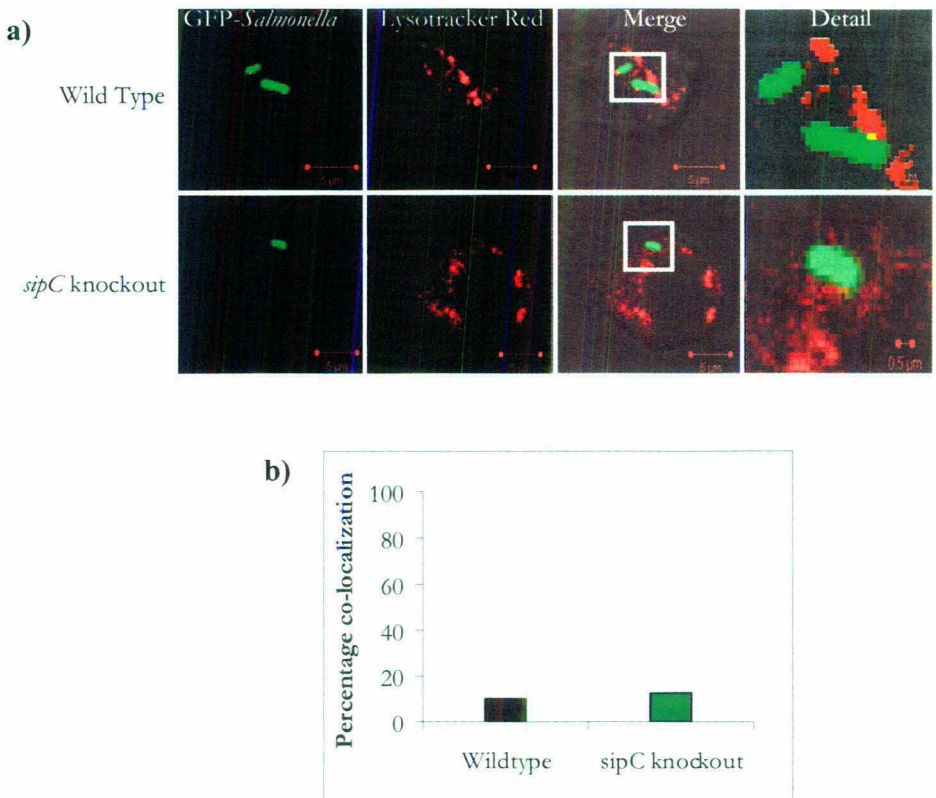


Figure 29: Transport of WT or sipC knockout Salmonella to the lysosomes.

a) Immunofluorescence showing co-localization of wildtype and sipC knockout Salmonella with lysosomes. Last panel shows the enlarged region. b) Graph shows the percentage of bacteria co-localizing with LysoTracker red, n=100. Black bars represent ***wildtype;*** *Green bars represent* ***sipC knockout****.*

Taken together, our results demonstrated that both WT and *sipC* knockout *Salmonella* retained the early endosomal marker, Rab5 on their phagosomes, followed by acquisition of LBPA from the late endocytic compartments. However, both WT as well as *sipC* knockout *Salmonella* prevented their transport to the lysosomes. Thus, suggesting that SipC plays no role in altering the endocytic pathway in macrophages. In order to validate these observations, WT or *sipC* knockout *Salmonella*-containing phagosomes were purified at different stages of their maturation and these were analyzed for the presence of host compartment specific molecules by Western blotting. The results show that Rab5 is recruited by both WT and *sipC* knockout *Salmonella* on their phagosomes at early stages and retained even as the phagosome matures (Fig.30, upper panel) which is in accordance with the immunofluorescence data presented above. To determine targeting to lysosomes, the presence of mature Cathepsin D on the phagosomes was analyzed. Cathepsin D is an acid hydrolase which after synthesis is transported through various endocytic compartments where it gets differentially cleaved depending on the acidity of the compartment. Finally, the mature protein is found in the lysosomes thus, serving as a lysosomal marker. The Western blot (Fig.30, middle panel) results reveal that phagosomes containing WT or *sipC* knockout *Salmonella*, even at later stages of their maturation do not acquire the terminally cleaved form of Cathepsin D (~17 kDa) suggesting the inhibition of transport of these bacteria to the lysosomal compartment.

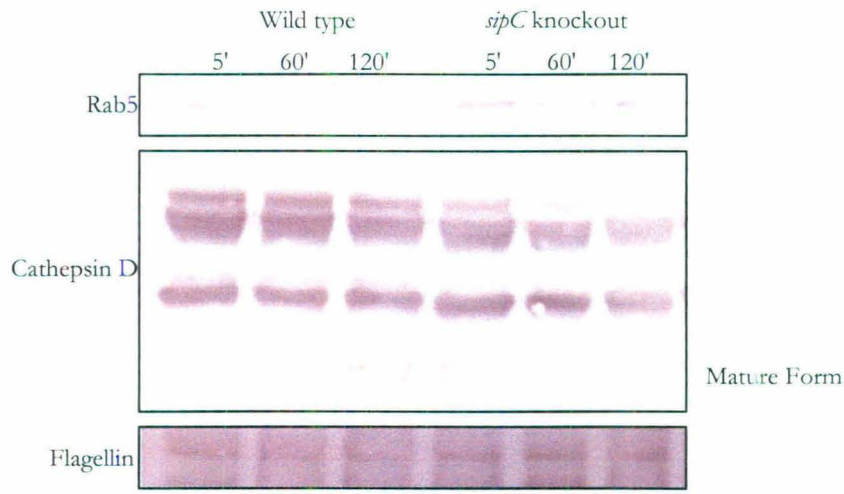


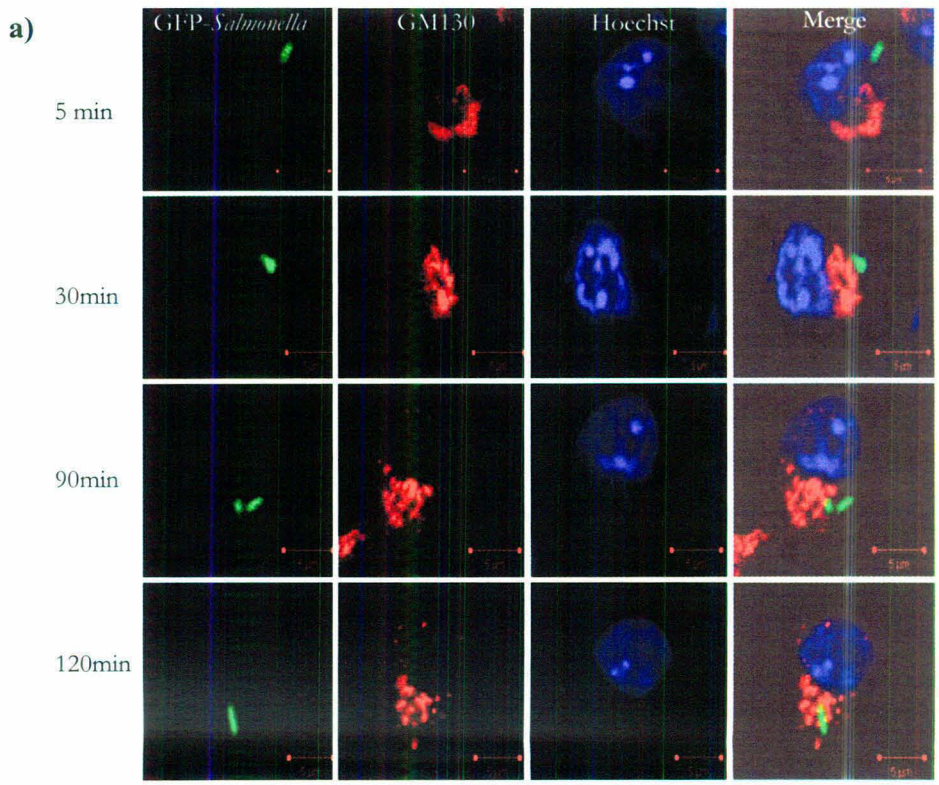
Figure 30: Recruitment of different endocytic molecules on WT or sipC knockout Salmonella-containing phagosomes at different stages of maturation.

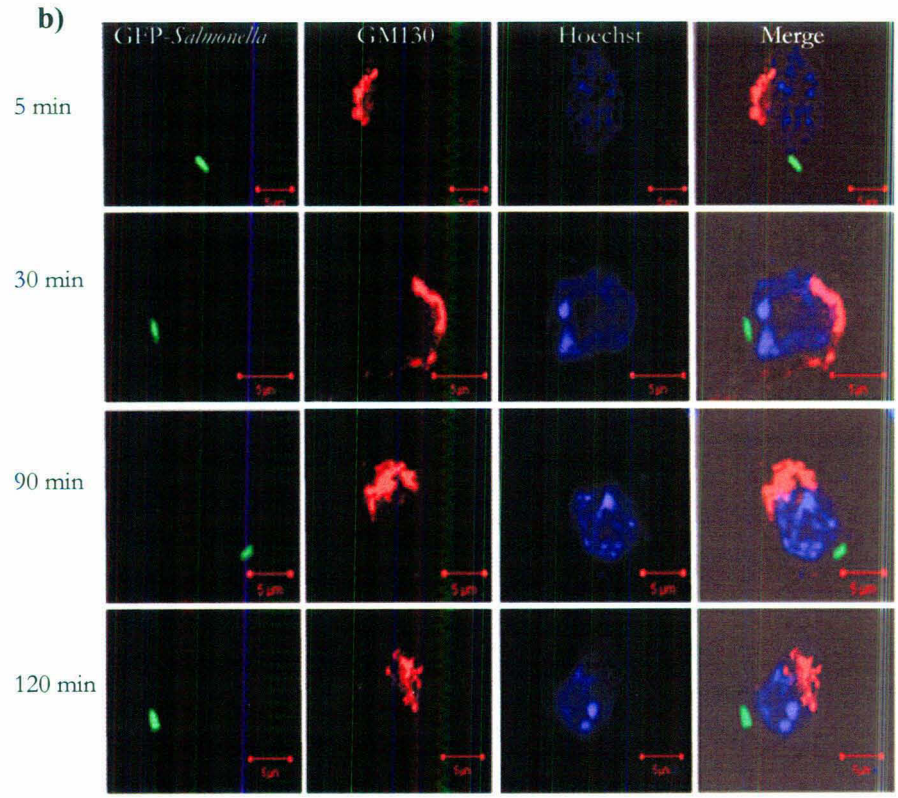
Western blot probed with α-Rab5 (1:5,000), α-Cathepsin D (1:500) and α-flagellin (1:1,000) antibodies.

A *Salmonella* structural protein, flagellin was used as the loading control for the Western blots. Similar levels of this molecule rule out experimental error due to unequal loading (Fig.30, lower panel).

6.4.2 Comparative trafficking of WT and *sipC* knockout *Salmonella* towards Golgi

The results presented in the preceding section suggested that *Salmonella*-containing phagosomes initially followed the endocytic pathway, but ultimately segregated from the endocytic route and homed into a specialized compartment. Previous studies have shown that the pathogen resides in the vicinity of the Golgi, which is conducive for bacterial replication (Deiwick et al., 2006). Thus, *ex vivo* studies were carried out to follow the movement of WT and mutant bacteria towards the Golgi which was labeled with GM130. In contrast to WT *Salmonella* (Fig.31a), the *sipC* knockout *Salmonella* (Fig.31b) failed to reach the juxtanuclear Golgi location. This altered trafficking was confirmed further by quantification. Bacteria residing within a distance of 1 μm from the Golgi were considered to be in the vicinity of the organelle.





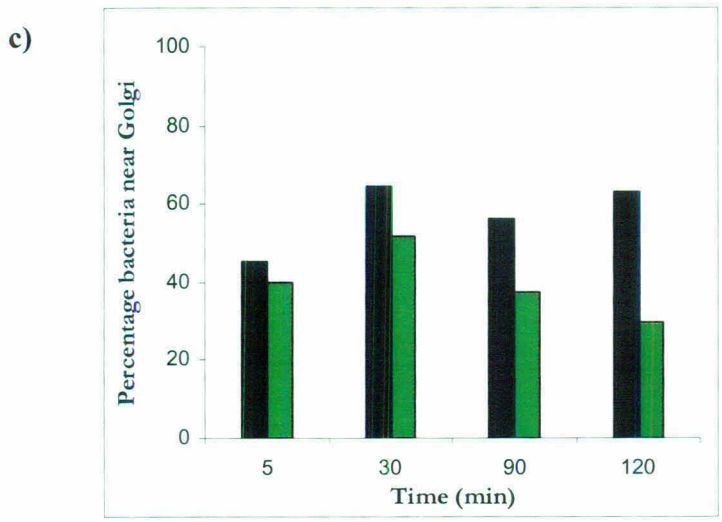


Figure 31: Trafficking on WT or sipC knockout Salmonella towards Golgi.

a) *Wild Type Salmonella;* ***b)*** *sipC knockout Salmonella; Last panel shows the enlarged region.*

c) *Graph shows the percentage of bacteria in vicinity of Golgi, n=100.*

Black bars represent ***wildtype;*** *Green bars represent sipC knockout*

We observed that nearly 65% of the bacteria trafficked towards the Golgi within 30 min of internalization and resided there even at later time points (120 min). On the other hand, only 30% of *sipC* knockout *Salmonella* moved towards the Golgi even after 120 min of internalization inside cells (Fig.31c). These results clearly suggest that *sipC* knockout *Salmonella* do not move towards the Golgi, indicating a plausible role of this protein in targeting the bacteria towards this region.

6.4.3 Understanding the role of SipC in the maturation of *Salmonella*-containing phagosomes

The results presented in the previous section clearly demonstrated that SipC is involved in the targeting of *Salmonella* near the Golgi compartment. In the following section, we have tried to understand the mechanism of *Salmonella* trafficking towards Golgi. This has been achieved by comparing the levels of different TGN associated Rabs, SNAREs and other interacting molecules on the phagosomes containing WT or *sipC* knockout *Salmonella*.

The Western blot analysis of purified phagosomes containing WT or *sipC* knockout *Salmonella* shows that WT *Salmonella* recruit syntaxin 6 on their phagosomes as they mature from 5 min to 60 min. However, the levels of syntaxin 6 dropped as the phagosomes matured further (Fig.32a). Interestingly, a completely different profile was observed for *sipC* knockout *Salmonella*-containing phagosomes, which had considerably lower amounts of syntaxin 6 at 60 min of phagosome maturation. However by 120 min, these bacteria could recruit higher amounts of syntaxin 6 on their phagosomes (Fig.32a). Quantification of the blots suggested that WT *Salmonella* had two-fold more syntaxin 6 than *sipC* knockout *Salmonella*-containing phagosomes at 60 min of maturation. However, as the WT *Salmonella*-containing phagosomes matured further, they lost up to 40% of the recruited syntaxin 6, whereas phagosomes containing *sipC* knockout *Salmonella* could recruit around 30% more syntaxin 6 in a temporal manner (Fig.32b).

Similarly, the differences in the acquisition of Rab6, another TGN related molecule, was also observed. WT *Salmonella*-containing phagosomes could recruit Rab6 at the early onset of phagosome maturation and similar levels were retained on the mature phagosomes (Fig.32a). Though *sipC* knockout *Salmonella* could recruit similar amounts

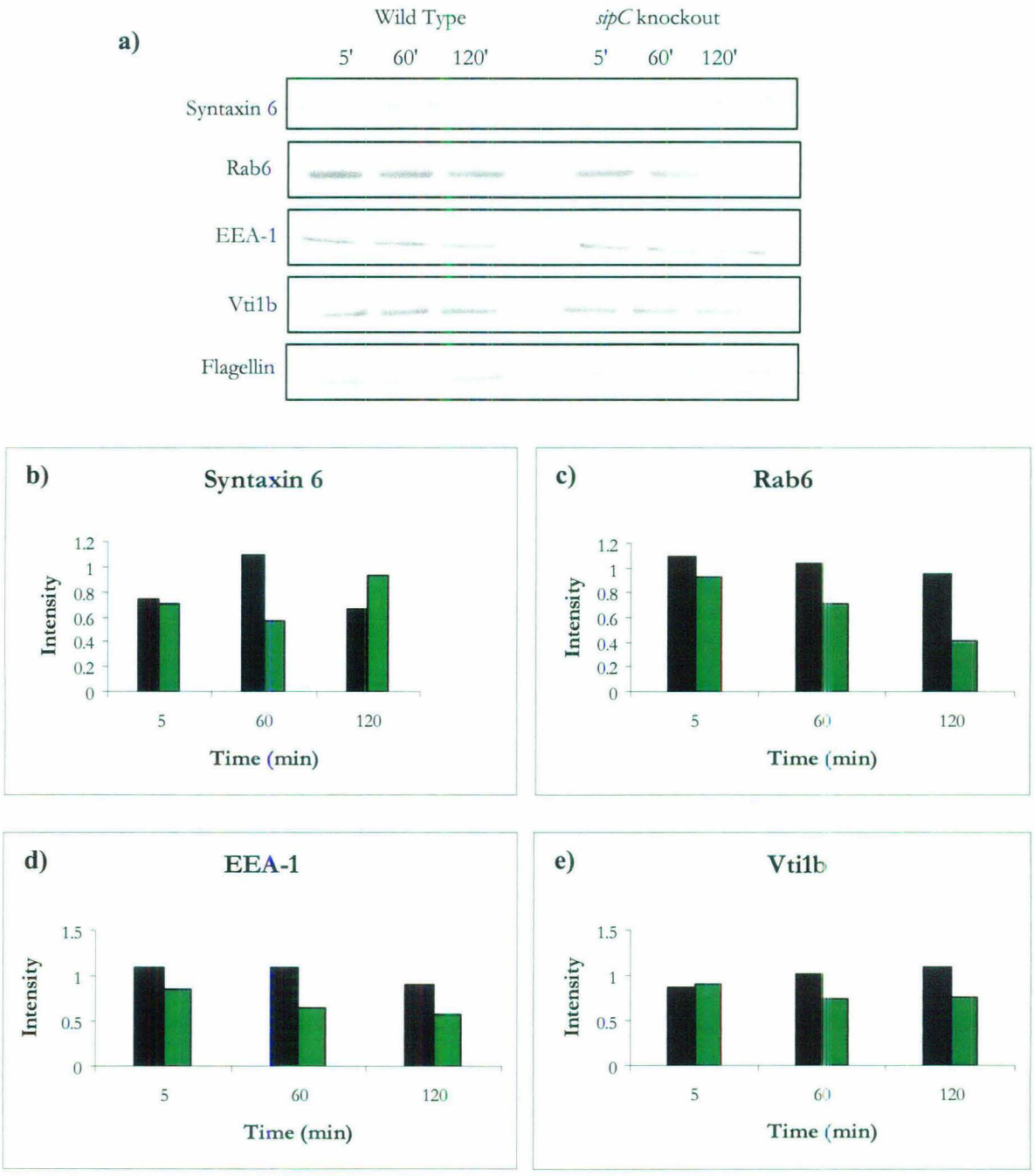


Figure 32: Acquisition of TGN associated transport molecules on WT or sipC knockout Salmonella-containing phagosomes.

a) *Western blot probed with α-syntaxin 6(1:2,500), α-Rab6 (1:500), α-EEA-1(1:500), α-Vti1b (1:1000) and α-flagellin (1:1,000) antibodies; Graphs show the quantification of the Western blots after normalizing with flagellin values.* ***b)*** *Syntaxin 6;* ***c)*** *Rab6;* ***d)*** *EEA-1;* ***e)*** *Vti1b.*

Black bars represent ***wildtype;*** *Green bars represent* ***sipC knockout***

of Rab6 at early stages of phagosome maturation as WT bacteria, they were unable to retain these levels as the phagosomes matured in time (120 min). As the *sipC* knockout *Salmonella*-containing phagosomes matured to 60 min, there was a 40% drop in the levels of Rab6. Further maturation of the phagosomes led to as significant as a 70% reduction in the levels of Rab6 (Fig.32c).

In addition, our results showed that acquisition of both EEA-1 and Vti1b was affected by deletion of *sipC* from *Salmonella* (Fig.32a). There was no significant change in the recruitment of EEA-1 and Vti1b on WT *Salmonella*-containing phagosomes during their maturation. However, a significant time dependent drop (approximately 50%) in the levels of EEA-1 (Fig.32d) and a marginal reduction (nearly 30%) in the levels of Vti1b (Fig.32e) was observed in *sipC* knockout *Salmonella*-containing phagosomes. As before, flagellin was used as loading control (Fig.32a). Taken together, these results indicate that the function of SipC is required for recruiting and retaining these host transport molecules on *Salmonella*-containing phagosomes.

Previous studies from our lab have shown that as the *Salmonella*-containing phagosomes mature, they acquire LAMP-1, a lysosomal membrane protein, without being targeted to the lysosomes (Hashim et al., 2000). It has been established by earlier studies that LAMP-1, though predominantly present on the late endosomes/lysosomes, is ubiquitously distributed in various endocytic compartments. This is because, LAMP-1 after synthesis in the ER and maturation in the TGN, trafficks to the late endosomal/lysosomal compartment via the early endosome (Cook et al., 2004). Thus, it was tempting to speculate that *Salmonella*-containing phagosomes might be recruiting LAMP-1 from the Golgi while they are in close vicinity of this compartment. Thus, we proposed to compare the association of LAMP-1 with WT and *sipC* knockout *Salmonella*-containing phagosomes.

Western blot analysis of purified phagosomes at different stages of maturation shows that WT *Salmonella* recruit LAMP-1 both at early and late stages of their maturation (Fig.33a). In the same experiment, it was also observed that *sipC* knockout *Salmonella* initially obtained LAMP-1 on their phagosomes, but failed to retain this molecule on their phagosomes as efficiently as WT *Salmonella*, with nearly a 50% drop

in the levels at 120 min of maturation (Fig.33b). As earlier, flagellin was used as an internal loading control.

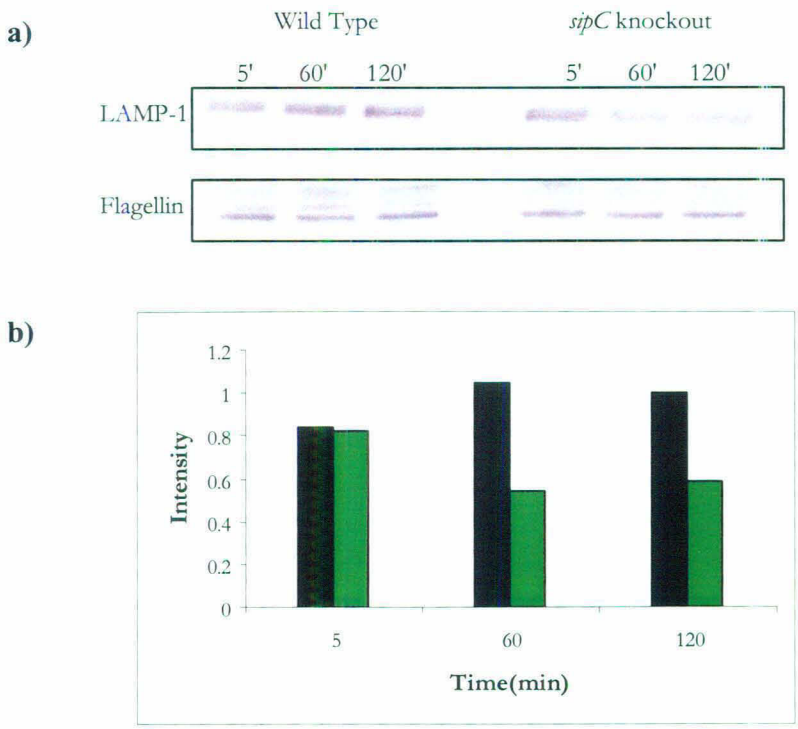
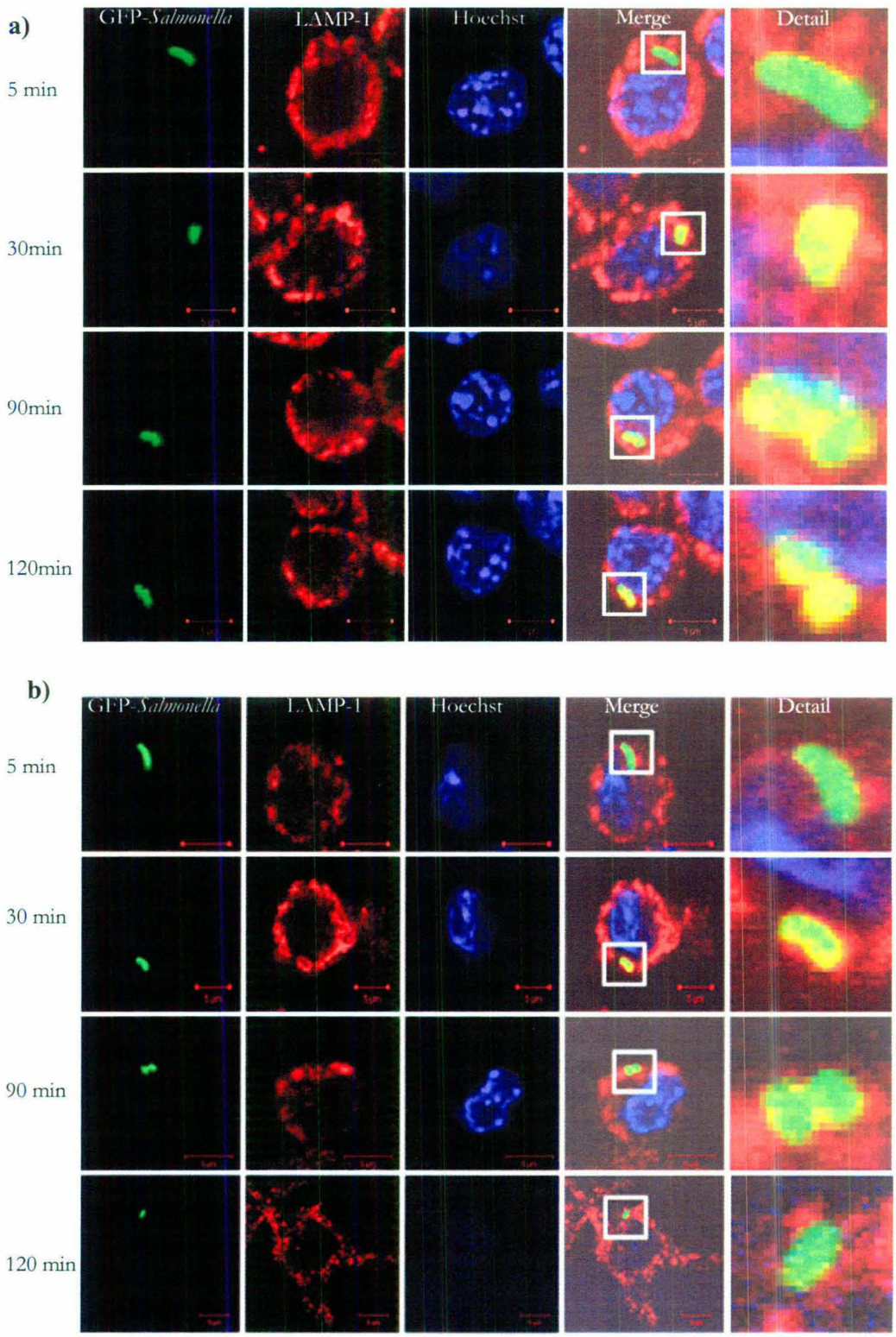


Figure 33: Recruitment of LAMP-1 on WT or sipC knockout Salmonella-containing phagosomes.

a) *Western blot probed with α-LAMP-1(1:2,000) and α-flagellin (1:1,000) antibodies;*

b) *Graph shows the quantification of the Western blot after normalizing with flagellin values. Black bars represent* ***wildtype;*** *Green bars represent* ***sipC knockout***

These findings were further confirmed by immunofluorescence studies which illustrated that within 30 min after the pathogen had trafficked inside the cell, nearly 80% of the phagosomes containing WT or *sipC* knockout *Salmonella* could recruit LAMP-1 (Fig.34a,b). However, only 40% of *sipC* knockout bacteria could retain this molecule on the mature phagosomes (Fig.34c) as opposed to nearly 70% of WT bacteria retaining LAMP-1 on their phagosomes temporally. This data highlights the inability of *sipC* knockout *Salmonella* to retain LAMP-1 on its phagosomes at later stages of maturation.


a)
GFP-Salmonella
LAMP-1
Hoechst
Merge
Detail
5 min
30min
90min
120min
b)
GFP-Salmonella
LAMP-1
Hoechst
Merge
Detail
5 min
30 min
90 min
120 min

c)

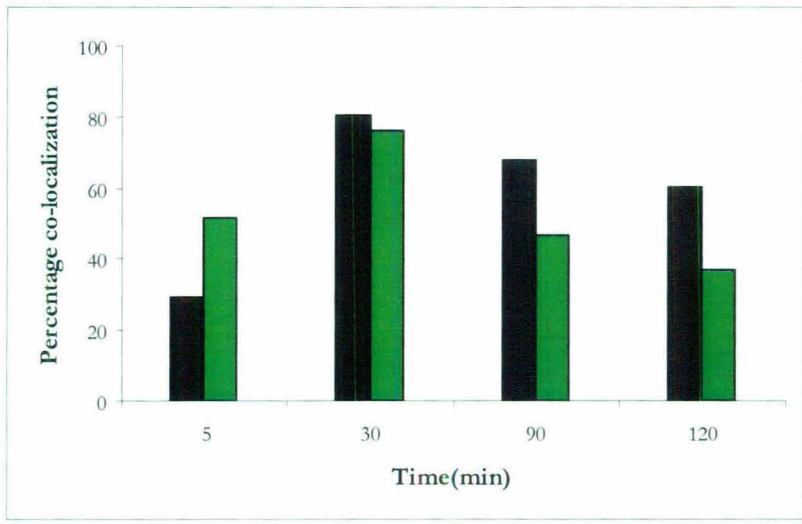


Figure 34: Recruitment of LAMP-1 on WT or sipC knockout Salmonella-containing phagosomes.
a) *WT Salmonella;* ***b)*** *sipC knockout Salmonella. Last panel shows enlarged region.*
c) *Graph shows the percentage co-localization of bacteria with LAMP-1, n=100.*
Black bars represent ***wildtype;*** *Green bars represent sipC knockout*

These results supported our previous proposition that *Salmonella*-containing early phagosomes possibly recruit LAMP-1 through fusion with some LAMP-1 containing vesicles present either in the early or late endocytic compartments. Significant reduction in the level of LAMP-1 on *sipC* knockout *Salmonella*-containing mature phagosomes indicated that *sipC* knockout *Salmonella* was unable to recruit LAMP-1 possibly from Golgi derived vesicles as they are not targeted to a near Golgi location. This is reinforced by the fact that *sipC* knockout *Salmonella*-containing phagosomes are unable to efficiently recruit Rab6 and syntaxin 6, the molecules required for fusion with Golgi derived vesicles.

6.4.4 Role of SipC in the recruitment of LAMP-1 from Golgi

To understand the mechanism of LAMP-1 recruitment on *Salmonella*-containing phagosomes, LAMP-1 was synchronized in Golgi, followed by analyzing the fusion of Golgi derived vesicles containing LAMP-1 with WT or *sipC* knockout *Salmonella*-containing phagosomes.

To follow the transport of LAMP-1 containing vesicles from Golgi, conditions were standardized for synchronization of this molecule in Golgi. LAMP-1 GFP was transiently over expressed in RAW 264.7 macrophages by electroporation and 20 hrs post transfection, fluorescent punctuate structures could be seen throughout the cell (Fig.35a), a profile similar to that as observed previously by indirect immunofluorescence using specific antibody against LAMP-1. Subsequently, attempts were made to synchronize the transport of this molecule in Golgi by a temperature shock. For the same, 20 hrs after transfection, over expressing cells were shifted to 15°C for 20 min and we could achieve partial co-localization of LAMP-1 with Golgi labeled by GM130 (Fig.35b). Complete co-localization was observed after shifting the cells back to 37°C for 20 min (Fig.35c). Budding of LAMP-1 containing vesicles from the Golgi was detected when the cells were incubated at 37°C for another 10 min (Fig.35d).

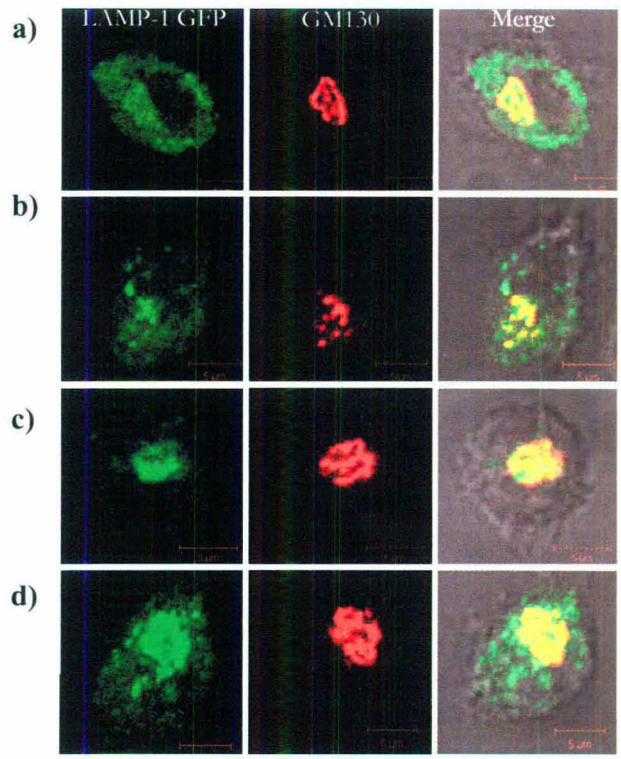
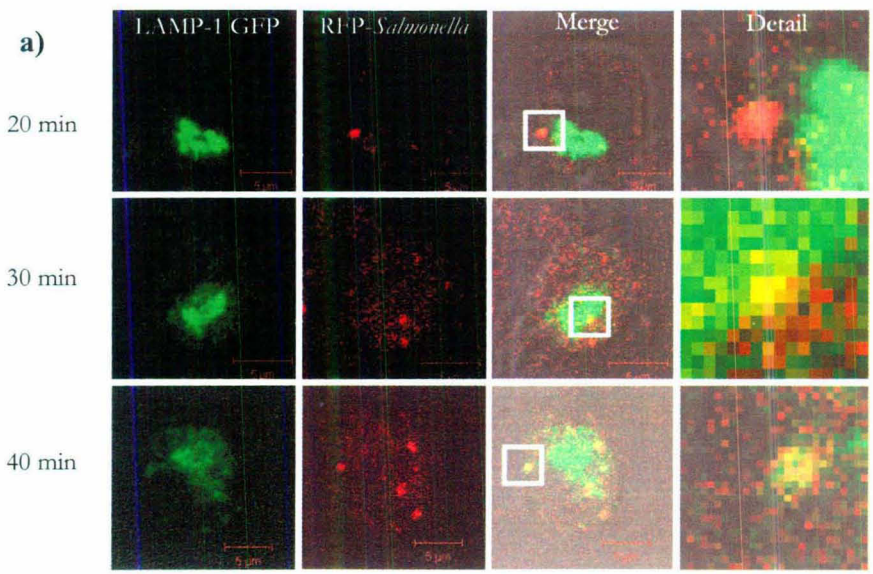


Figure 35: Synchronization of LAMP-1 transport in Golgi. *Immuno-stained with the Golgi marker GM130, LAMP-1 GFP over expressing cells were synchronized by exposure to different temperatures for varying times intervals in the folliowing sequence -* ***a)*** *37°C, 20 hrs;* ***b)*** *15°C, 20 min;* ***c)*** *37°C, 20 min;* ***d)*** *Snpashot 10 min post synchronization..*

Subsequently, cells with synchronized LAMP-1 in the Golgi compartment were used to study the recruitment of LAMP-1 from the Golgi derived vesicles by *Salmonella*-containing phagosomes.

After synchronizing the transport, interaction between Golgi derived vesicles containing LAMP-1 and *Salmonella*-containing phagosomes was studied. To achieve this, RAW 264.7 macrophages were transfected with plasmid LAMP-1 GFP and incubated at 37°C for 18 hrs for the expression of LAMP-1 GFP fusion protein (green). Subsequently, cells were infected with *S. typhimurium* WT or *sipC* knockout expressing RFP (red) and the bacteria were allowed to chase inside cells for 2 hrs at 37°C. The infected cells were then given a temperature shock to synchronize LAMP-1 in Golgi. Finally, cells were incubated for indicated periods of time at 37°C to allow the budding of LAMP-1 containing Golgi derived vesicles. At different intervals, cells were fixed and co-localization of LAMP-1 GFP containing compartments with RFP expressing WT or *sipC* knockout *Salmonella* was determined. Our results illustrate that initially (20 mins of incubation at 37°C) LAMP-1 is predominantly restricted in the Golgi and both WT and *sipC* knockout *Salmonella* are segregated from this molecule. Interestingly, LAMP-1 was found to be associated with WT *Salmonella* nearly after 40 min of incubation at 37°C (Fig.36a) suggesting the fusion of LAMP-1 containing Golgi derived vesicles with WT *Salmonella*-containing phagosomes.



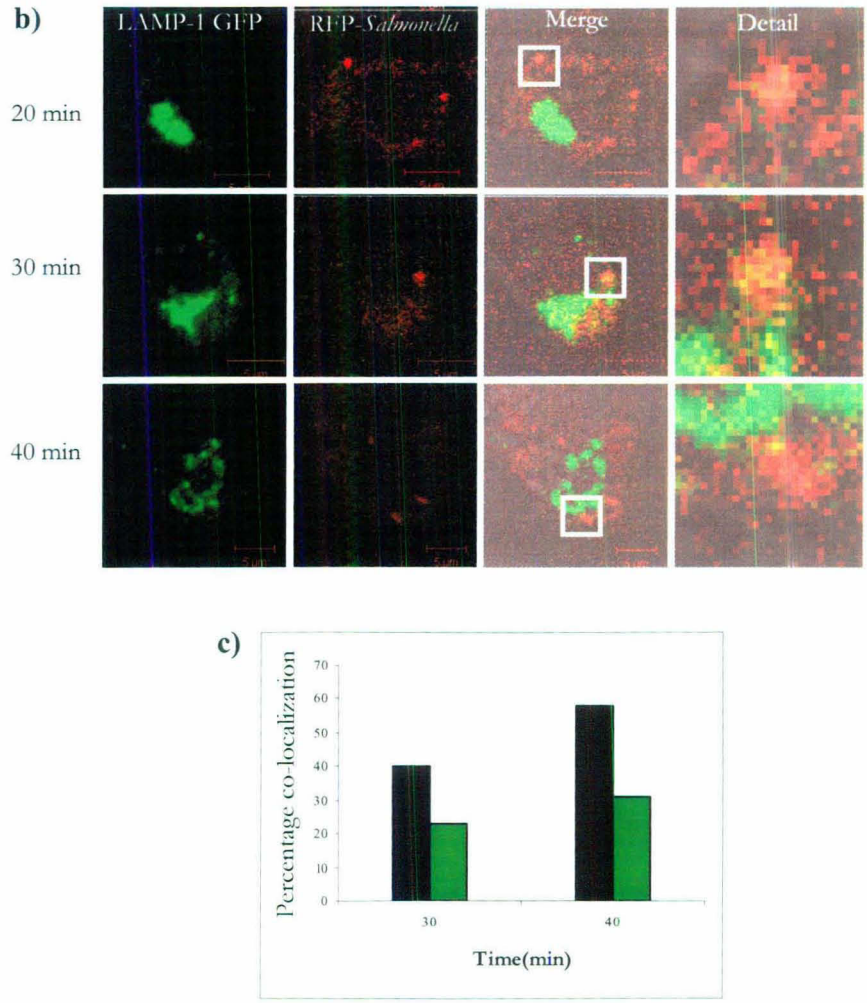


Figure 36: Interaction of Golgi derived vesicles containing LAMP-1 with WT or sipC knockout Salmonella-containing phagosomes.

a) *Wild Type Salmonella;* ***b)*** *sipC knockout Salmonella. Last panel shows enlarged image.*

c) *Graph showing percentage co-localization of Salmonella-containing phagosomes with Golgi derived vesicles containing LAMP-1. Black bars represent* ***wildtype;*** *Green bars represent sipC knockout*

contrast, no co-localization of LAMP-1 with *sipC* knockout *Salmonella*-containing phagosomes was observed after 40 min of incubation at 37°C (Fig. 36b). Further quantification of at least 100 bacteria under each condition revealed that nearly 60% WT *Salmonella* could co-localize with LAMP-1 containing Golgi derived vesicles as opposed to only 30% *sipC* knockout *Salmonella* co-localizing with these vesicles (Fig.36c), illustrating that *Salmonella*-containing phagosomes recruit LAMP-1 from Golgi through a SipC mediated process.

6.4.5 Complementation of *sipC* in the *sipC* knockout *Salmonella*

In order to validate the observed role of SipC on the maturation of *Salmonella*-containing phagosomes in macrophages, *sipC* was complemented in *sipC* knockout *Salmonella* strain. Subsequently, the content of purified phagosomes containing *sipC* knock-in *Salmonella* was analyzed to determine the gain of function.

To generate *sipC* knock-in *Salmonella*, *sipC* was cloned into pBAD24, an arabinose inducible *Salmonella* expression vector. Full length *sipC* (1.2 kb) was cloned into *Eco*RI/*Hind*III sites of pBAD24 (4.5 kb) (Fig.37a). The gel profile in Fig.37b shows the confirmation of the positive clone by release of a 1.2 kb insert upon restriction digestion. pBAD24-*sipC*, thus obtained, was over expressed in the *sipC* knockout strain by electroporation as elaborated in the methods section to generate *sipC* knock-in *Salmonella*. To attain similar levels of SipC secretion in *sipC* knock-in strain like WT *Salmonella*, different conditions of induction, including varying arabinose concentrations and induction times were tried. The Western blot presented in Fig.37c shows that the expression

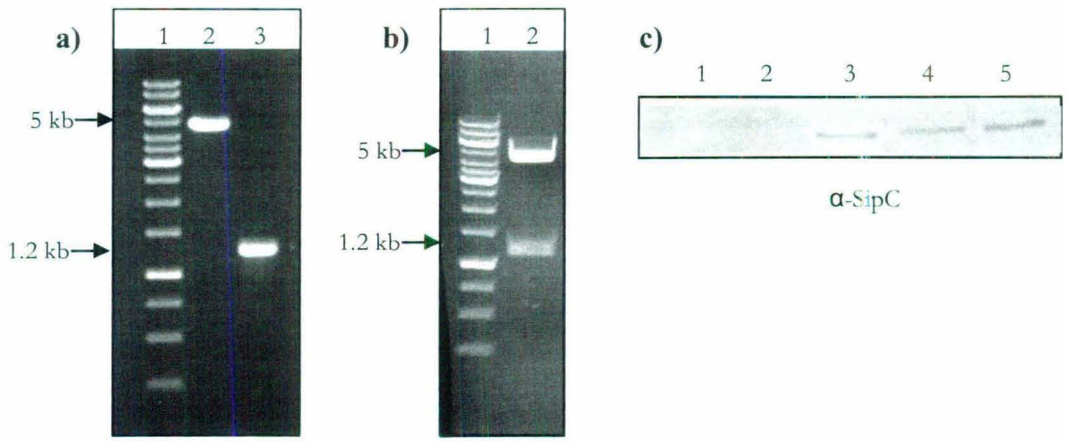


Figure 37: Cloning and expression of SipC in arabinose inducible Salmonella expression vector, pBAD24 to generate sipC knock-in Salmonella.

a) *Lane 1: 1 kb DNA ladder; Lane2: pBAD24 EcoRI/HindIII digested; Lane 3: PCR amplified sipC*

b) *Lane 1: 1 kb DNA ladder; Lane2: pBAD24-sipC EcoRI/HindIII digested*

c) *Western blot probed with α-SipC (1:500) antibody demonstrating induction and expression of SipC in sipC knock-in Salmonella. Lane 1-RPN 800; Lane 2-secretory proteins of sipC knockout; Lane 3-secretory proteins of Wild Type t; Lane 4- secretory proteins of sipC knock-in (0.0005% Arabinose, 2hrs); Lane5-secretory proteins of sipC knock-in (0.001% Arabinose, 30 min).*

of SipC (~42 kDa) was induced by arabinose addition and the levels were comparable to WT *Salmonella* when the culture was induced with 0.0005% arabinose for 2 hrs while the bacteria reached late log phase (O.D.$_{600}$ of 0.8-0.9).

6.4.6 Restoration of function in *sipC* knock-in *Salmonella*

In order to confirm the role of SipC, phagosomes were prepared using *sipC* knock-in or *sipC* knockout *Salmonella* at different stages of their maturation in macrophages and the recruitment of some vital host molecules like Rab6 and LAMP-1 was compared. The Western blots presented in Fig.38 show that both Rab6 and LAMP-1 are recruited and retained by *sipC* knock-in *Salmonella*-containing phagosomes in a

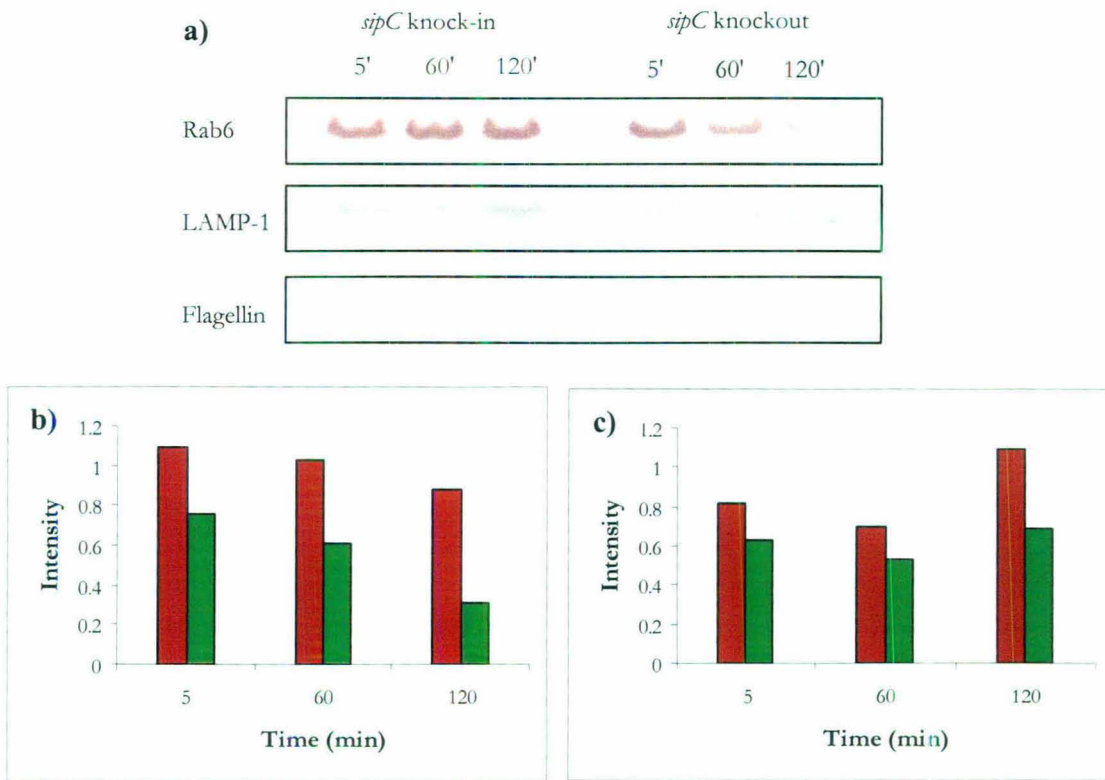


Figure 38: Recruitment of transport molecules on sipC knockout or sipC knock-in Salmonella-containing phagosomes. a) *Western blots probed with α-LAMP-1(1:1,000), α-Rab6 (1:500) and α-flagellin (1:1,000) antibodies. Graphs showing the quantification of the Western blots after normalizing with flagellin values* ***b)*** *Rab6* ***c)*** *LAMP-1. Brown bars represent* ***sipC knock-in****; Green bars represent* ***sipC knockout***

temporal fashion almost similar to that observed with WT *Salmonella*-containing phagosomes. *sipC* knockout *Salmonella*-containing phagosomes were used as a control to highlight this gain of function by *sipC* complementation in mutant bacteria. These results further reinforce the observations presented in the preceding sections that SipC is involved in the recruitment of different transport molecules on the maturing phagosomes.

6.5 Discussion

Intracellular trafficking of phagosomes depends on vesicular membrane composition as well as intravesicular content (Desjardins et al., 1994; Garcia-del Portillo and Finlay, 1995) and involves dynamic modulations of the phagosomal membrane brought about by fusion with other endocytic vesicles and recruitment of various transport proteins. Membrane fusion events are highly co-ordinated and are regulated by a complex interplay of Rab GTPases and SNARE proteins (Pfeffer, 1999; Rothman and Sollner, 1997; Schimmoller et al., 1998; Zerial and McBride, 2001).

Intracellular pathogens during the course of evolution have learnt to modulate the recruitment of these proteins on phagosomes for their survival by avoiding or inducing specific interactions of phagosomes with other intracellular compartments (Uchiya et al., 1999; Via et al., 1997). The ability to modulate host cellular machinery is attributed to the evolution of a complex protein secretion system termed TTSS which deliver bacterial effector proteins into host cells (Galan, 2001; Zhou et al., 1999). Previously, we have reported that live *Salmonella*-containing phagosomes modulate the expression of different Rabs for their benefit to persist in a low acidity compartment lacking active lysosomal enzymes (Hashim et al., 2000). Among the different Rabs, we have observed that Rab5 is recruited on the phagosomal membrane by participation of *Salmonella* effector molecule, SopE (Mukherjee et al., 2001) in this process.

We have also observed a temporal acquisition of different SNAREs on the maturing phagosomes. Particularly, the levels of syntaxin 6, syntaxin 7 and syntaxin 8 were found to be comparatively more on maturing *Salmonella*-containing phagosomes (unpublished data). The employment of various syntaxins on the phagosomal membrane by *Salmonella* could possibly be controlled by some of its effectors. In the previous chapters, we could identify SipC, a SPI-1 T3SS effector, specifically interacting with host

syntaxin 6. The presence of SipC on the phagosomal membrane implicated its possible function in the recruitment of syntaxin 6 during phagosome maturation. To address the functionality of SipC in this process, we have generated a mutant *Salmonella* strain with the *sipC* gene knocked out. In the present chapter, we have tried to decipher the role of SipC in phagosome maturation by studying the behavioral differences of the or *sipC* knockout *Salmonella* in terms of intracellular trafficking and attainment of different transport molecules on its phagosomes.

The uptake of endocytic cargo inside cells takes place via receptor mediated endocytosis as well as fluid phase endocytosis while *S. typhimurium* enters macrophages through macropinocytosis (Conner and Schmid, 2003). Following the intracellular trafficking of the bacteria and the endocytic cargo, we observed that immediately after internalization into the host cell, the pathogen may follow the route commonly used by the endocytic cargo or traverse through an altogether different pathway. However, it is clear that the path of maturing phagosomes and the endocytic cargo segregate early on, suggesting that there aren't a significant number of phagosomes fusing with the incoming traffic (Fig.26). This pattern was observed for both WT and or *sipC* knockout *Salmonella.*

After phagocytic uptake, the bacterium resides in a specialized compartment which interacts with different endocytic compartments as it trafficks within the cell. We compared the ability of WT or *sipC* knockout *Salmonella*-containing phagosomes to interact with early and late endocytic compartments which has been extrapolated by analyzing their ability to recruit specific endocytic markers. Phagosomes during maturation first interact with early compartments of the endocytic pathway, the early endosomes, and Rab5 is a universally known marker for this compartment (Somsel Rodman and Wandinger-Ness, 2000). Our results suggest that both WT and *sipC* knockout *Salmonella* recruit Rab5 on their phagosomes at early onset and retain this marker significantly even after 120 min of internalization inside cells (Fig.27, 30). After interaction with early compartments, the phagosome propels further and interacts with other members of the endocytic pathway. We observed that both WT and *sipC* knockout *Salmonella* travel to the late endocytic compartments and obtain LBPA from them at later time points (Fig.28).

Particles phagocytosed inside macrophages are ultimately transported to the lysosome and degraded by the acid hydrolases present in this compartment. It is well established that *Salmonella* bypass this mechanism of transport to the lysosomes and survive within the host cells. To determine whether the mutant bacteria also bypass lysosomal targeting, we measured their co-localization with labeled lysosomes and assessed the levels of the mature form of Cathepsin D on purified phagosomes. Cathepsin D is an acid hydrolase synthesized in the TGN as a molecule of 51 kDa which then cleaves into a 48 kDa molecule as it is transported to the early endosomes. The molecule matures further by proteolytic cleavage into a 31 kDa molecule and ultimately a 17 kDa mature form chiefly present in the lysosomes (Gieselmann et al., 1983). The findings from these experiments show that like WT *Salmonella*, the mutant strain also evades transport to the lysosomes (Fig.29), confirmed by the fact that both WT and *sipC* knockout *Salmonella* do not co-localize with LysoTracker Red and the phagosomes even after maturation do not acquire the terminally cleaved form of Cathepsin D (Fig.30), which is otherwise predominant on late phagosomes (60 min and 120 min) containing an inert probe or a dead bacterium destined to be degraded in the lysosomes (Hashim et al., 2000). From the above mentioned observations, it is evident that both *sipC* knockout and WT *Salmonella* travel to the late compartments but escape transport to the lysosomes.

After examining interactions of *sipC* knockout and WT *Salmonella* with the endocytic pathway, we tracked the movement of bacteria towards Golgi. It has been well established that WT *Salmonella*-containing phagosomes finally reside in a juxtanuclear, Golgi associated localization which serves as the intracellular replicative niche for the pathogen. Targeting to this location is attributed to the *Salmonella* effectors, SseG, SseF and SifA (Abrahams et al., 2006; Salcedo and Holden, 2003). Interestingly, we found that the *sipC* knockout *Salmonella* fails to be targeted towards the Golgi (Fig.31). To understand the possible mechanism of altered trafficking of *sipC* knockout *Salmonella*, we analyzed the content of some of the TGN related Rabs and SNAREs on the phagosomes. From the *in vitro* characterization of WT and *sipC* knockout *Salmonella*-containing phagosomes, we observed a differential pattern in the employment of various TGN related markers, particularly a defect in the recruitment of syntaxin 6 and Rab6.

Syntaxin 6 is a Qc SNARE which has been implicated to function in several trafficking pathways. This molecule primarily localizes to the TGN, regulating intracellular trafficking from TGN to the endocytic pathway and *vice versa*. However, this molecule is also found on endosomes and is known to associate *in vivo* with a variety of SNAREs including VAMP 7, VAMP 8, VAMP 2 and syntaxin 7. Thus, it is involved in diverse cellular processes like homotypic fusion of immature secretory granules as well as in regulating early and late fusion events by changing its SNARE binding specificities (Bock et al., 1997; Steegmaier et al., 1999; Wade et al., 2001). We analyzed the levels of syntaxin 6 on the maturing phagosomes and have shown that WT *Salmonella*-containing phagosomes acquire syntaxin 6 as the phagosomes mature to 60 min which is consistent with previous lab observations. However, the levels dropped as the phagosomes matured further. This dissociation of syntaxin 6 from the phagosomal membrane could be due to the interaction of the maturing phagosome with other intracellular compartments. Interestingly, *sipC* knockout *Salmonella* do not recruit syntaxin 6 efficiently on their phagosomes within 60 min of maturation (Fig.32a,b). This correlates well with our initial proposition that SipC interacts with and aids in the recruitment of syntaxin 6 on the phagosomes. However, as the phagosome matured with time, even the mutant bacteria could recruit some more molecules of syntaxin 6. This was surprising because we have found that SipC is involved in the recruitment of syntaxin 6 on the phagosomal membrane. Thus, it remains unclear as to how mature phagosomes (120 min) recruit syntaxin 6 in the absence of SipC. A possible reason for this observation could be that *Salmonella* secretes some other effector protein in higher amounts to compensate for/mimic the role of SipC in phagosome maturation. However, the prospect of involvement of other effector proteins in this process still needs to be explored.

Rab6 is a TGN associated protein involved in regulating transport events at the TGN (Martinez et al., 1994; Opdam et al., 2000). It has also been reported that Rab6 interacts with a molecular motor protein, Rabkinesin-6 to mediate microtubule dependent transport at the Golgi network (Echard et al., 1998). On analyzing the levels of Rab6, we found that WT *Salmonella*-containing phagosomes could recruit Rab6 at the early onset of phagosome formation probably by dynamic interactions with members of the

endocytic pathway and retain this protein even as the phagosome matures. We know that the maturing phagosome is targeted towards the Golgi, thus Rab6 present on the mature phagosomes must have been obtained via interactions with the secretory pathway. Remarkably, *sipC* knockout *Salmonella*-containing phagosomes showed similar amounts of Rab6 as compared to WT phagosomes at early time points, but the levels dropped significantly in a time dependent manner (Fig.32a,c). This can be explained by the fact that *sipC* knockout *Salmonella*-containing phagosomes interact efficiently with members of the endocytic pathway to attain Rab6 on early phagosomes. However, *sipC* knockout *Salmonella*-containing phagosomes fail to move near Golgi even at later stages, and thus are unable to recruit Rab6 from the vesicles originating from TGN. Thus, the inability of *sipC* knockout *Salmonella* to traffic towards Golgi and the altered recruitment of syntaxin 6 and Rab6 on the maturing phagosomes led us to speculate that this could lead to a defect in acquiring host transport molecules from the TGN.

Since the recruitment of the SNARE molecule, syntaxin 6 was altered on *sipC* knockout *Salmonella*-containing phagosomes, we assessed the status of syntaxin 6 interacting molecules such as EEA-1 and Vti1b, a syntaxin 6 associated fusion complex partner on the maturing phagosomes. EEA-1 is a Rab5 effector molecule, which has been shown to be interacting with syntaxin 6 and co-localizes with syntaxin 6 present on the endosomal structures. This molecule has a common binding motif for both Rab5 and syntaxin 6 which possibly mediates the recruitment of syntaxin 6 on the maturing phagosome by dissociation of Rab5. The EEA-1-syntaxin 6 interaction could mediate tethering of a post Golgi vesicle to endosomes (Simonsen et al., 1999). Our findings indicate that EEA-1 is recruited on to the phagosomal membrane at early stages of maturation and is retained on the compartment with a marginal drop in the levels at later stages. The association of EEA-1 with the phagosomes can be attributed to the presence of Rab5 on the mature phagosomes which helps to recruit this effector. Interestingly, or *sipC* knockout *Salmonella*-containing phagosomes lose up to 50% EEA-1 in a temporal manner (Fig.32a,d). This dissociation of EEA-1 from the mature phagosomes could be a possible reason for lesser amounts of syntaxin 6 on the *sipC* knockout *Salmonella*-containing phagosomes.

Vti1b, a Qb SNARE takes part in the formation of a fusion complex with syntaxin 6. This complex has been reported to be involved in the exocytic transport of TNFα containing post Golgi vesicles (Murray et al., 2005). Our results suggested that the levels of Vti1b are similar initially but decrease on the maturing phagosomes containing *sipC* knockout *Salmonella* (Fig.32a,e). Vti1b is also known to be part of another endosomal fusion complex involving syntaxin 7, syntaxin 8 and VAMP-8, regulating the endosomal transport (Wade et al., 2001). Thus, significant levels of Vti1b on the early phagosomes could be attributed to its role in this fusion complex driving the intracellular trafficking event. However, the dissociation of Vti1b from the mature *sipC* knockout *Salmonella*-containing phagosomes could be due to the reduced availability of the other fusion partner, syntaxin 6, resulting in inefficient recruitment of this molecule on the mature phagosomes. Thus, we find an impaired recruitment of syntaxin 6, Rab6, EEA-1 and Vti1b in the absence of SipC, implicating some defect in the fusion machinery regulating the transport at TGN.

LAMP-1 is a known marker for late endosomes and lysosomes. However, it is well demonstrated that LAMP-1, after initial synthesis in the ER, moves towards early endosomal compartments and the plasma membrane via TGN using the secretary pathway and is finally trafficked to the lysosomes (Cook et al., 2004). It has also been reported that the presence of different LAMP molecules on *Salmonella*-containing phagosomes helps to stabilize the phagosomal compartment (Chakraborty et al., 1994; Roark and Haldar, 2008). Our findings of reduced interactions of *sipC* knockout *Salmonella*-containing phagosomes with TGN associated trafficking molecules like Rab6, syntaxin 6 and Vti1b suggest that there might be a defect in acquisition of transport molecules like LAMP-1 by these phagosomes from the TGN. Thus, we analyzed the status of LAMP-1 recruitment by the mutant *Salmonella* on its phagosomes. We observe that both WT and *sipC* knockout *Salmonella* could recruit LAMP-1 on their phagosomes initially, obtained by interactions with the early endosomes. In accordance with previous lab reports, we also observed that WT *Salmonella*-containing phagosomes could retain LAMP-1 on the maturing phagosomes. At the same time, *sipC* knockout *Salmonella*-containing mature phagosomes could not attain substantial levels of LAMP-1 (Fig.33,34), possibly due to crippled interactions with members of the secretory pathway. These

results validate our hypothesis of impaired fusion of LAMP-1 containing vesicles from TGN with the phagosomes in the absence of SipC.

In order to delineate the role of SipC in the recruitment of LAMP-1 on *Salmonella*-containing phagosomes by fusion with the LAMP-1 containing vesicles derived from TGN, initially we over expressed and synchronized LAMP-1 in Golgi. Subsequently, we allowed the LAMP-1 containing vesicles to bud from the Golgi and finally looked for this molecule on *Salmonella*-containing phagosomes. Our results show that WT *Salmonella*-containing phagosomes co-localize with LAMP-1, 20 min post vesicle budding from Golgi, indicating the fusion of *Salmonella*-containing phagosomes with LAMP-1 containing vesicles. In contrast, *sipC* knockout *Salmonella* failed to fuse with LAMP-1 containing vesicles from Golgi (Fig.36). The role of SipC in this process is further confirmed by complementing *sipC* in the *sipC* knockout strain and our results have shown that *sipC* knock-in strain behaves similar to the WT strain and efficiently recruits transport molecules like Rab6 and LAMP-1 (Fig.38).

The interactions of the WT and *sipC* knockout *Salmonella*-containing phagosomes with different intracellular compartments were examined in this chapter. Taken together, these results unequivocally prove that *Salmonella*-containing phagosomes acquire LAMP-1 by fusion with LAMP-1 containing vesicles derived from the Golgi through SipC mediated recruitment of syntaxin 6 and Rab6.

Summary

The intracellular pathogen *Salmonella,* during its course of infection, invades the intestinal epithelial cells and macrophages of the spleen and liver, where the bacterium resides and replicates (Richter-Dahlfors et al., 1997). Survival within macrophages is an essential part of *Salmonella* pathogenesis since mutants lacking this ability have been shown to be avirulent (Fields et al., 1986). Studies over the years have led to the conclusion that *Salmonella* survives in an intracellular niche inside macrophages by inhibiting its transport to the lysosomes (Buchmeier and Heffron, 1991). Though, several groups have tried to elucidate the mechanism of inhibition of transport, it has still not been fully comprehended. However, a general consensus has developed on the role of certain effector molecules secreted by *Salmonella* that modulate the host cellular processes, accomplishing pathogen survival in macrophages.

Previous studies from our lab have identified one such *Salmonella* effector protein, SopE. It has been shown to recruit one of the regulators of host intracellular transport, Rab5, onto the phagosomal membrane and promote fusion of the phagosomes with early endosomes, subverting the compartment from being targeted to lysosomes (Hashim et al., 2000; Mukherjee et al., 2000). Recent data from the lab has shown a temporal acquisition of host SNARE molecules during the maturation of *Salmonella*-containing phagosomes. Based on the premise that SNARE recruitment must have been brought about by some bacterial effectors, the present study aimed at identifying them and determining their role in bacterial survival inside macrophages.

Initially, three different molecules from a subclass of host SNARE proteins, namely, syntaxin 6, syntaxin 7 and syntaxin 8, were cloned and expressed as GST-tagged fusion proteins. Subsequently, these fusion proteins were used as baits to pull down effector molecules from *Salmonella* and characterize them. Our results demonstrate that SipC, a SPI-1 effector protein of *Salmonella,* specifically interacts with host syntaxin 6. This interaction was confirmed *in vitro* by Western blotting using recombinant proteins as well as *in vivo* by immunoprecipitation where *Salmonella* SipC could specifically pull out syntaxin 6 from macrophage lysate. Thereafter, the presence of SipC on the phagosomes implicated its role in regulating the host molecules and thus, contributing to the phagosomal maturation process. To decipher the physiological role(s) of SipC, we deleted this molecule from the *Salmonella* genome.

Interactions of *Salmonella*-containing phagosomes with various intracellular compartments were then compared between WT as well as *sipC* knockout bacteria. However, no significant differences were found; the mutant bacteria were still able to interact efficiently with members of the host endocytic pathway. This has been illustrated by the observations that *sipC* knockout *Salmonella* obtained early endosomal markers such as Rab5, followed by subsequent dissociation of these molecules and acquisition of LBPA from the late endosomes in a similar fashion as WT *Salmonella*. This clearly indicates that SipC does not modulate the endocytic pathway to the bacteria's advantage. On the other hand, we were able to confirm that the loss of SipC did not lead to targeting of the bacteria to the lysosomes and hence, the mutants could survive efficiently within host macrophages.

Syntaxin 6 is a TGN associated SNARE molecule, involved in regulating transport at this compartment. Considering that SipC interacts specifically with host syntaxin 6, we postulated that SipC might be involved in regulating interactions of the phagosomes with intracellular compartments of the secretory pathway. Interestingly, we observed that deletion of *sipC* leads to altered intracellular trafficking and the bacterium is not targeted to a juxtanuclear Golgi localization which, under normal circumstances, serves as its replicative niche (Salcedo and Holden, 2003). To explore this further, we compared the recruitment of different TGN related transport molecules on the maturing phagosomes and observed a differential association of many of these molecules with WT and *sipC* knockout *Salmonella*-containing phagosomes by Western blotting. The mutant *Salmonella* are unable to recruit syntaxin 6 and Rab6 on to the mature phagosomes. Moreover, the recruitment of Vti1b, a syntaxin 6 fusion complex partner as well as EEA-1, a syntaxin 6 interacting molecule, was also hampered. Taken together these results confirmed the previous observation that there was no defect in interactions of *Salmonella*-containing phagosomes with members of the host endocytic machinery. On the other hand, it was the TGN associated transport molecules whose acquisition was crippled only on mature phagosomes, implicating the inability of *sipC* knockout *Salmonella*-containing phagosomes to interact efficiently with members of the secretory pathway.

Finally, we were able to restore the recruitment of host transport molecules with a *sipC* knock-in *Salmonella*. By regaining the function with a 'knock-in' phenotype, we have established beyond doubt that this bacterial effector protein is responsible for recruitment

of molecules from TGN. In other words, SipC mediated processes might be essential to import molecules on to the phagosomes from the secretory pathway. These observations led us to speculate that the transport of host molecules via this pathway might be hampered. So, efforts were made to monitor the transport of some host molecules from this compartment.

It has been shown that LAMP-1, after being synthesized in the ER, moves towards the early endosomal compartment and plasma membrane via TGN before being trafficked to the lysosomes (Cook et al., 2004). It has also been suggested that LAMP proteins are involved in the stabilization of the phagosomal compartment (Chakraborty et al., 1994; Roark and Haldar, 2008) and maturing phagosomes acquire LAMP-1 (Hashim et al., 2000), possibly by interaction with the secretory pathway. Hence, we chose this molecule as a marker for intracellular transport via TGN. Initial experiments examined the recruitment of LAMP-1 on maturing phagosomes containing either the WT or *sipC* knockout *Salmonella*. The results obtained show that the mutant bacteria could acquire LAMP-1 efficiently on early phagosomes. However, it failed to recruit this molecule on the mature phagosomes. This reaffirmed that SipC modulated the components of secretory pathway to help establish the bacterial intracellular niche. We validated this proposition by monitoring intravesicular fusion between LAMP-1 containing Golgi derived vesicles and *Salmonella*-containing phagosomes by microscopy. It turned out that whereas the two compartments could efficiently fuse in case of the WT bacteria, phagosomes containing the *sipC* knockout *Salmonella* were unable to fuse and acquire LAMP-1.

In conclusion, our results have shown that SipC is required for the trafficking of *Salmonella* near the Golgi in macrophages. Previously, SipC has been implicated in modulation of host actin cytoskeleton in concert with other T3SS1 effectors (Hayward and Koronakis, 1999). It has also been established that the final niche where *Salmonella* replicates is near Golgi and this has been attributed to the *Salmonella* effectors SseF, SseG and SifA. These proteins mediate the precise positioning of the phagosomes by differentially modulating the recruitment of microtubule motor proteins (Abrahams et al., 2006; Salcedo and Holden, 2003). However, in this work, we have identified another *Salmonella* effector, SipC, that is involved in the targeting of *Salmonella*-containing phagosomes towards Golgi, which might be a consequence of a similar modulation of host

cytoskeleton. Finally, we have addressed the significance of *Salmonella* homing near Golgi and shown for the first time that SipC mediated processes recruit syntaxin 6 and Rab6 to obtain LAMP-1 on *Salmonella*-containing phagosomes which might stabilize this specialized intracellular compartment in macrophages (Fig.39).

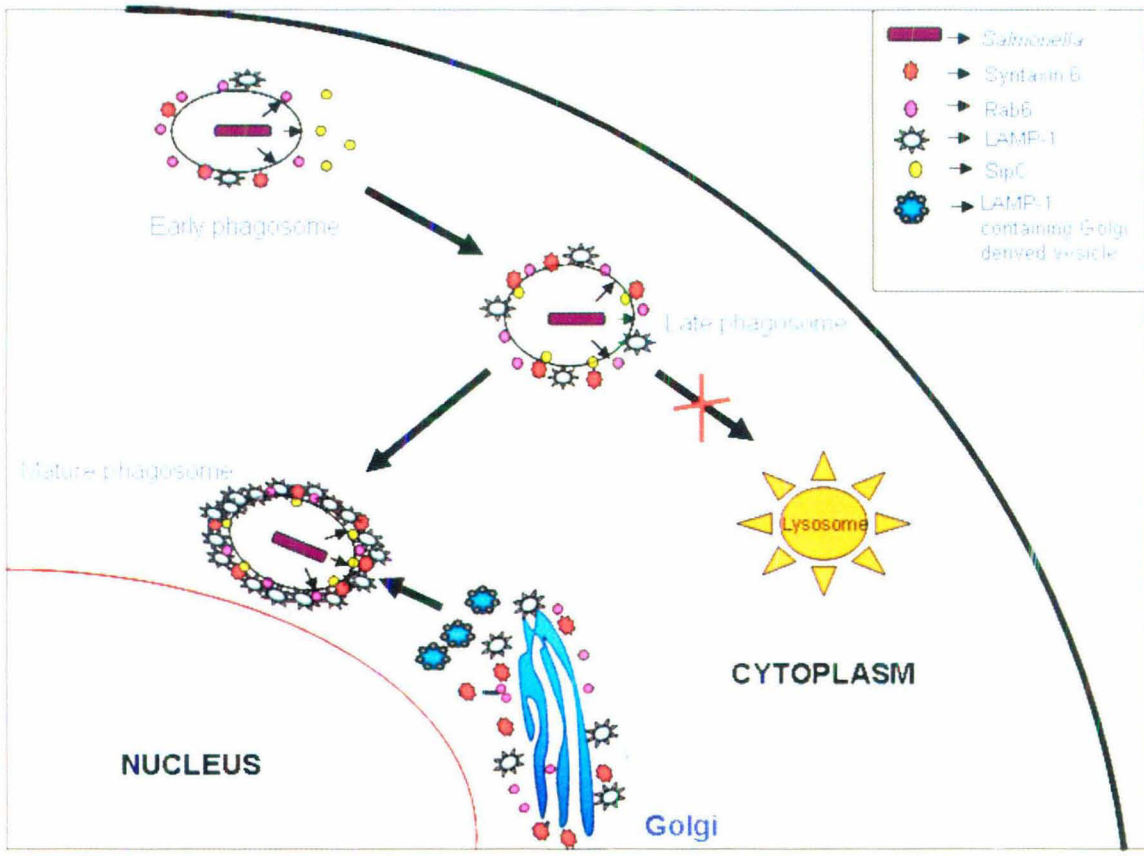


Figure 39: Schematic of SipC mediated subversion of host transport machinery for efficient survival of Salmonella within macrophages.

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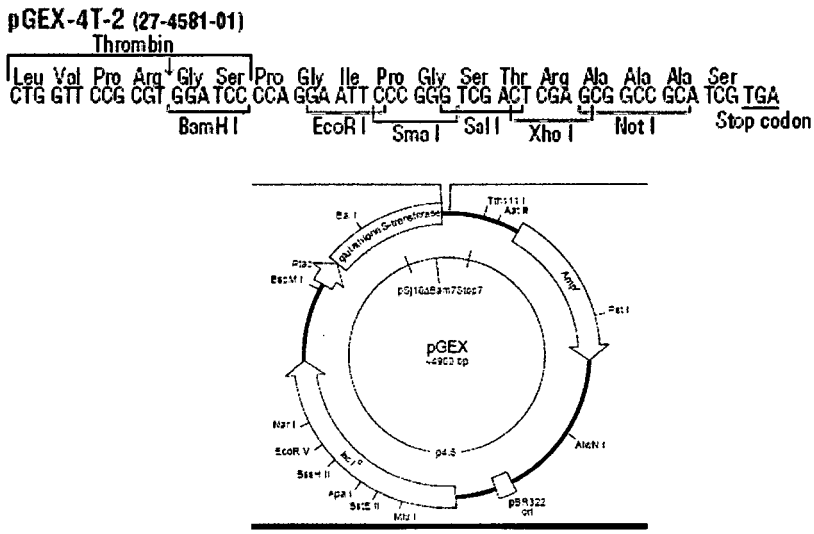
Appendices

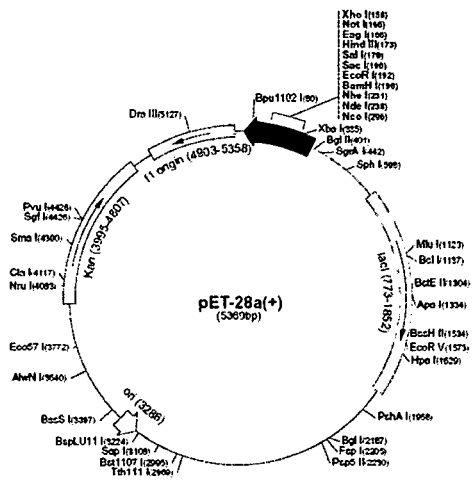
Appendix A

Vector maps of plasmids used in the study

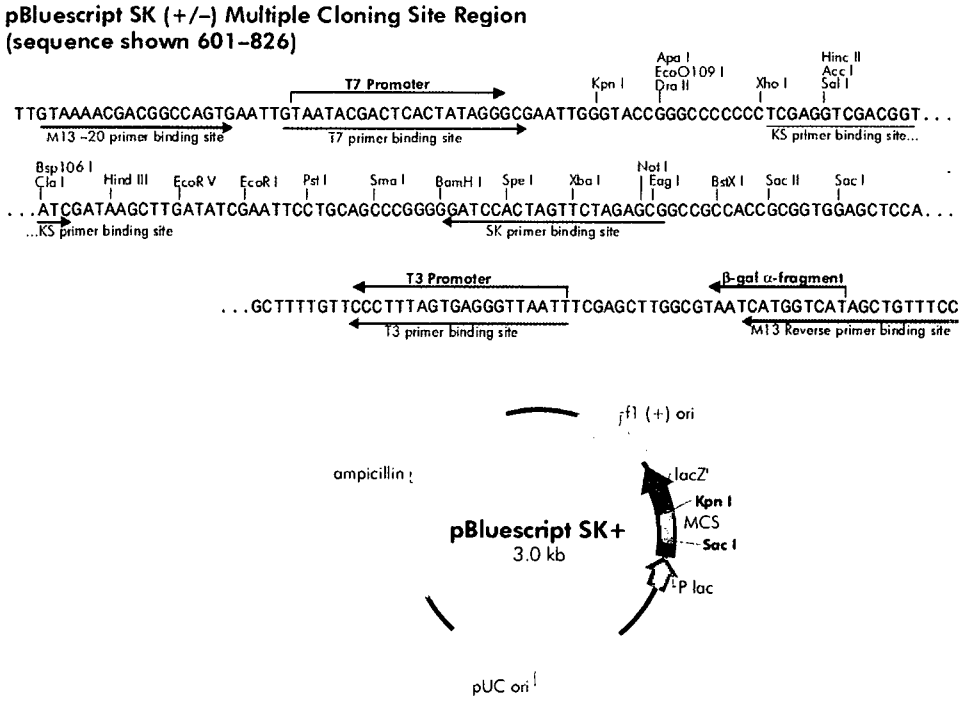
A. GST-tagged expression vector, pGEX-4T2



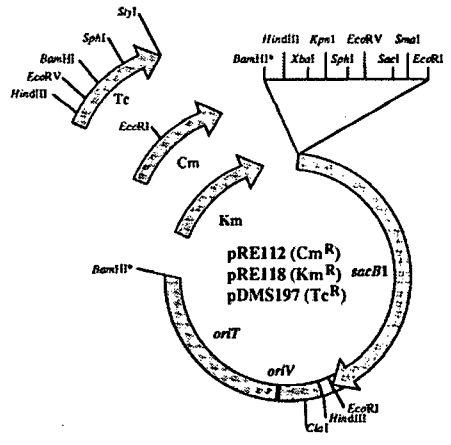
B. His_6-tagged expression vector, pET-28a



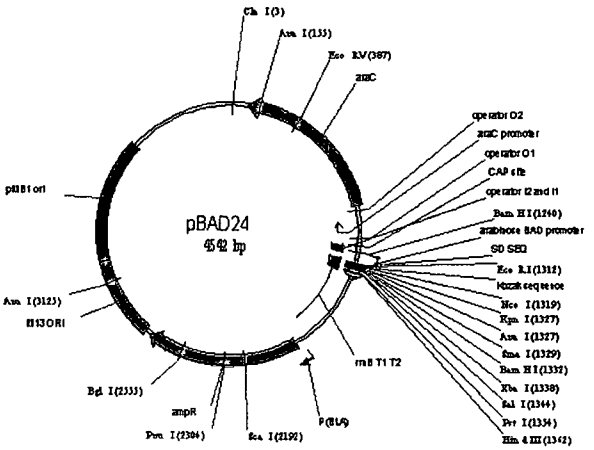
C. Plasmid blue script (pBSK+)



D. Suicide vector, pRE112



E. *Salmonella* expression vector, pBAD24



Appendix B

Antibiotic selection of different bacterial strains, vectors and plasmids used in the study

Bacterial strain/Construct	Antibiotic (concentration)
SL1344 *Salmonella*	Streptomycin (100 µg/ml)
sipC knockout *Salmonella*	Streptomycin (100 µg/ml)
sipC knock-in *Salmonella*	Streptomycin (100 µg/ml) and Amplicillin (100 µg/ml)
pGEX-4T2 clones	Amplicillin (100 µg/ml)
pET28a-SipC	Kanamycin (50 µg/ml)
pBSK+	Amplicillin (100 µg/ml)
pRE112	Chloramphenicol (30 µg/ml)
SM10λpir *E. coli*	Kanamycin (50 µg/ml)
pFPV25.1	Amplicillin (100 µg/ml)
pIZ1590	Amplicillin (100 µg/ml)
LAMP-1GFP	Kanamycin (50 µg/ml)
pBAD24	Amplicillin (100 µg/ml)

Appendix C

Antibodies used in various experiments

Figure	Antibody	Dilution	Company	Secondary antibody
11	Avidin	1:25,000	Sigma	-
11	SipC	1:60	Gifted	Mouse
11	SopE	1:100	Gifted	Mouse
11	SopB	1:100	Gifted	Mouse
14a	SipC	1:500	Lab generated	Mouse
14c	SipC	1:5000	Lab generated	Mouse
15	Syntaxin 6	1:500	Synaptic Systems	Rabbit
16a	SipC	1:20	Gifted	Mouse
16b	SopE	1:20	Gifted	Mouse
25	Actin	1:5000	Oncogene	Mouse
27	Rab5	1:50	BD Biosciences	Mouse
30	Rab5	1:5000	Gifted	Mouse
28	LBPA	1:50	Gifted	Mouse
30	Cathepsin D	1:500	Neuromics	Goat
30	Flagellin	1:500	DIFCO	Rabbit
31	GM130	1:200	BD Biosciences	Mouse
32	Syntaxin 6	1:2000	BD Biosciences	Mouse
32	Rab6	1:250	Santa Cruz	Rabbit
32	Vti1b	1:1000	BD Biosciences	Mouse
32	EEA-1	1:250	Gifted	Rabbit
33	LAMP-1	1:2000	BD Biosciences	Mouse
34	LAMP-1	1:200	BD Biosciences	Mouse

All secondary antibodies were used at 1:10,000 unless specified separately in text.