Review

Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors

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Salmonella enterica serovars cause severe disease in humans, such as gastroenteritis and typhoid fever. The bacteria are able to invade and replicate within host cells, including epithelial cells and macrophages. Pathogenesis of *Salmonella* is facilitated by a type III secretion system (T3SS) encoded by genes of *Salmonella* pathogenicity island 2 (SPI-2). Intracellular replication occurs in a specialized membrane compartment, the *Salmonella*-containing vacuole (SCV), and depends on translocation of approximately 30 effector proteins via the SPI-2 T3SS into the host endomembrane system and cytoplasm. In this review we discuss the many different functions of these effectors, which range from maintaining the integrity of the SCV and its juxtanuclear location, to interference with the host cytoskeleton and immune signalling.

Introduction

Serovars of *Salmonella enterica* are Gram-negative facultative intracellular bacteria that infect a wide range of warm-blooded animals. In humans, infections by *Salmonella enterica* serovars Typhimurium and Typhi (referred to as *S.* Typhimurium and *S.* Typhi in this review) usually cause gastroenteritis and typhoid fever, respectively. These two pathogens alone are responsible for very large morbidity and mortality rates in humans worldwide (Crump *et al.*, 2004; Majowicz *et al.*, 2010).

Infection of certain mouse strains with *S.* Typhimurium produces a systemic disease with some similarities to human typhoid fever (Carter & Collins, 1974), and this has been used extensively over many years to study the pathogenesis of systemic *Salmonella* infections. Systemic infection depends on the ability of *S. enterica* to invade and grow within host cells such as epithelial cells and cells of the monocyte/granulocyte lineage, including macrophages (Fields *et al.*, 1986; Richter-Dahlfors *et al.*, 1997; Salcedo *et al.*, 2001). This in turn requires the activities of numerous virulence proteins encoded by up to 4 % of the *Salmonella* genome (Bowe *et al.*, 1998; Hensel *et al.*, 1995). These include many 'effector' proteins that are translocated into host cells by either one or both of two type III secretion systems (T3SSs), encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). T3SSs are multi-protein organelles that span the bacterial cell envelope and deliver effector proteins into host cells via a needle-like structure and an associated translocon pore that is formed in a target host membrane (Mueller *et al.*, 2008).

Genes of the SPI-1 T3SS are expressed in response to signals sensed by bacteria in the intestine of the infected host. This T3SS becomes active upon contact with

epithelial cells, translocating effectors across the host cell plasma membrane (Galán, 2001). Several of these effectors stimulate the assembly of actin filaments, whose extension causes localized membrane ruffling and bacterial invasion (Patel & Galán, 2005). In *S.* Typhimurium, SPI-1 T3SS effectors also trigger the activation of mitogen-activated protein kinase (MAPK) pathways, leading to the production of proinflammatory cytokines such as interleukin (IL)-8, stimulating the recruitment of polymorphonuclear leukocytes (PMNs) and inducing acute intestinal inflammation (Bruno *et al.*, 2009; Galyov *et al.*, 1997; Lee *et al.*, 2000b). This response is exacerbated by SPI-1-dependent induction of macrophage cell death (van der Velden *et al.*, 2000).

The SPI-2 T3SS is found in all subspecies of *S. enterica* (Hensel *et al.*, 1997; Ochman & Groisman, 1996) and is a major virulence factor of this group of pathogens. It was discovered as a result of a signature-tagged mutagenesis (STM) screen for genes that contribute to systemic growth of *S.* Typhimurium in the mouse (Hensel *et al.*, 1995). Numerous studies by many groups have established that it functions by translocating effectors across the membrane of the *Salmonella*-containing vacuole (SCV) in infected host cells such as epithelial cells and macrophages.

Following invasion or phagocytic uptake into host cells, the SCV gradually matures through interactions with the host endocytic pathway into a specialized compartment with some features of late endosomes, including an acidified lumen and the presence of lysosomal membrane glycoproteins such as LAMP1 in the SCV membrane. However, the SCV is not enriched in lysosomal hydrolases (Rathman *et al.*, 1997; Steele-Mortimer *et al.*, 1999; Thompson *et al.*, 2011), and is therefore considered a distinct compartment from a normal phagolysosome.

Delivery of SPI-2 T3SS effectors to the host cell cytosol is a precisely controlled process. It involves the ordered assembly of the secretion apparatus in the bacterial cell envelope, followed by the secretion of proteins that form the needle and translocon pore in the vacuolar membrane (Chakravortty *et al.*, 2005). Activation of genes encoding the secretion apparatus is mediated by two-component regulatory systems, including OmpR–EnvZ and SPI-2-encoded SsrA–B (Feng *et al.*, 2004; Garmendia *et al.*, 2003; Lee *et al.*, 2000a), in response to the acidic pH and poor nutritional status of the lumen of the SCV vacuole (Beuzón *et al.*, 1999; Cirillo *et al.*, 1998; Löber *et al.*, 2006). Following assembly of the translocon pore in the SCV membrane, the pH of the host cell cytosol is sensed by an unknown component of the T3SS, and a SPI-2-encoded regulatory complex in the bacterial cell (comprising the SpiC, SsaM and SsaL proteins) dissociates, derepressing the translocation of effectors into host cells (Yu *et al.*, 2010). Approximately 30 different effectors are delivered across the vacuolar membrane, but it is not known whether this occurs in an ordered hierarchy, as seems to be the case for *E. coli* (Mills *et al.*, 2008).

The full repertoire of SPI-2 T3SS effectors is not present in all *S. enterica* serovars. However, loss of function of the SPI-2 T3SS in different serovars invariably causes a strong virulence defect, and where tested, this is usually associated with an intracellular growth defect, regardless of host cell type. One recent study claims that the SPI-2 T3SS of *S.* Typhi is not involved in intracellular growth (Forest *et al.*, 2010), but most papers on the subject have reported intracellular growth defects of SPI-2 T3SS mutants (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Salcedo *et al.*, 2001), including mutants of *S.* Typhi (Khan *et al.*, 2003). Effectors of the SPI-2 T3SS carry out a large number of functions. These include maintaining the integrity of the SCV and its localization near the Golgi of host cells, as well as modulating the host cytoskeleton and interfering with immune signalling. Below we review our current knowledge of the functions of these effectors.

Effectors of the SPI-2 T3SS

Only two or three genes encoding effectors have been identified within SPI-2. The *spv* operon on the virulence plasmid also encodes at least two effectors, but the majority are scattered throughout the chromosome. Finding the full complement of effectors has been hampered by the lack of a universally conserved motif or other signature within their amino acid sequences. As a result, several different approaches have been used to identify SPI-2 T3SS effectors, with different degrees of success. The discovery of the first effector gene, *sspH1*, came from sequencing of a horizontally acquired region of chromosomal DNA (Miao *et al.*, 1999). The similarity between SspH1 and the *Shigella* IpaH family of T3SS effectors led to further investigation of this protein, and evidence for SPI-2 T3SS-dependent translocation was obtained using the adenylate cyclase (CyaA)

protein fusion strategy (Miao *et al.*, 1999; Sory & Cornelis, 1994). In the same study, *sspH1* was used as a probe to identify the related effector gene *sspH2* (Miao *et al.*, 1999). Using a conserved N-terminal amino acid sequence found in both SspH1 and SspH2, other SPI-2 T3SS effectors were identified, including SifA and SlrP, which were already known to be *S.* Typhimurium virulence proteins (Beuzón *et al.*, 2000; Brumell *et al.*, 2001; Miao & Miller, 2000). The N-terminal conserved sequence is required for secretion of these effectors, although the majority of SPI-2 T3SS effectors have since been shown to lack this region. Nevertheless, most SPI-2 T3SS effectors do require the two-component regulatory system SsrA/B for their expression, and genetic screens exploiting this dependency have identified several additional effectors (Coombes *et al.*, 2007; Rytkönen *et al.*, 2007; Worley *et al.*, 2000). Other methods include the use of CyaA transposon fusions to identify translocated proteins (Geddes *et al.*, 2005), and recent studies have taken advantage of the phenotype of an *ssaL* mutant strain (which is derepressed for effector secretion; Yu *et al.*, 2010) and conditions that induce SPI-2 gene expression, to identify known and new effectors by MS analysis of proteins in bacterial culture supernatants (Auweter *et al.*, 2011; Niemann *et al.*, 2011; Sherry *et al.*, 2011). Together, these and other approaches have identified 28 effectors of the SPI-2 T3SS (Table 1, Fig. 1). In most cases, translocation by the SPI-2 T3SS was confirmed through the use of CyaA fusion reporters (Sory & Cornelis, 1994) or by immunofluorescence imaging of epitope-tagged proteins. It is possible that other effectors exist, but in view of the different approaches that have been brought to bear on this question, there are unlikely to be many more.

Effectors that affect the vacuole membrane (SifA, PipB2, SseJ, SopD2)

Salmonella-induced filaments (Sifs) are lysosomal glycoprotein-containing membrane tubules induced by *Salmonella*. They are most easily apparent inside epithelial cells, where they extend centrifugally from SCVs along microtubules (Garcia-del Portillo *et al.*, 1993). The gene responsible for the formation of these structures was discovered using a genetic screen in 1996 and named *sifA* (*Salmonella*-induced filament gene A) (Stein *et al.*, 1996). SifA was originally implicated as a possible SPI-2 T3SS effector by virtue of its regulation by SsrA/B, functional link to SPI-2 (Beuzón *et al.*, 2000), and N-terminal amino acid sequence similarity to other SPI-2 effectors (Beuzón *et al.*, 2000; Miao & Miller, 2000). In addition to a complete lack of Sif formation, the *sifA* mutant has another very interesting phenotype: several hours after uptake by host cells, the vacuolar membrane surrounding these mutant bacteria is gradually lost, revealing that SifA has an essential function in maintaining the integrity of the SCV (Beuzón *et al.*, 2000). As a result, *sifA* mutant bacteria are released into the cytosol of host cells. In macrophages, cytosolic bacteria are rapidly killed (Beuzón *et al.*, 2002),

Table 1. Effectors of the SPI-2 T3SS

Effector	Function(s)	Biochemical activity	Host target
SCV localization			
SseF	SCV positioning at peri-Golgi region, microtubule bundling, Sif formation	?	?
SseG			
Vacuole membrane dynamics			
SifA	Sif formation, maintenance of vacuolar membrane, enhanced kinesin-dependent anterograde movement along microtubules	?	SKIP, RhoA
PipB2	Recruitment of kinesin-1 to the SCV, Sif extension	?	Kinesin-1
SopD2	Sif formation, vacuole integrity	?	?
SseJ	Esterification of cholesterol in infected cells, vacuole integrity	Acyltransferase	Phospholipids, cholesterol
Host immune signalling			
SpvC	Dephosphorylation of MAP kinases	Phosphothreonine lyase	MAPKs
SseL	Macrophage delayed cytotoxicity, downmodulation of NF-κB-dependent cytokine production, altered lipid metabolism in infected cells	Deubiquitinase	IκB, OSBP
SspH1	Inhibition of NF-κB-dependent gene transcription	E3 ubiquitin ligase	PKN1
Host cytoskeleton			
SteC	Formation of SCV-associated F-actin meshwork	Kinase	?
SspH2	Recruited to SCV-associated F-actin meshwork	E3 ubiquitin ligase	Profilin
SrfH/SseI	Recruited to SCV-associated F-actin meshwork, migration of infected phagocytes	?	TRIP6, IQGAP1
SpvB	Inhibition of actin polymerization, macrophage cytotoxicity, P-body disassembly in infected cells	Actin ribosyltransferase	?
Unknown function			
SlrP	?	E3 ubiquitin ligase	Thioredoxin, ERdj3
GtgE	Cleavage of Rab29 accumulated on SCV	Protease	?
PipB	?	?	?
SifB	?	?	?
SseK1	?	?	?
SseK2	?	?	?
SseK3	?	?	?
GogB	?	?	?
SteA	?	?	?
SteB	?	?	?
SteD	?	?	?
SteE	?	?	?
SpvD	?	?	?
GtgA	?	?	?
CigR	?	?	?

which explains the strong attenuation of virulence of a *sifA* mutant in mice. However SPI-2 T3SS null mutants, which also fail to translocate SifA, retain an intact vacuolar membrane, implying that other SPI-2 protein(s) are involved in modifying the SCV membrane (Beuzón *et al.*, 2000). At least three different SPI-2 effectors are now known to be involved in this activity (Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2010), and are discussed below.

Following its translocation, SifA is anchored in the SCV membrane and to Sifs through prenylation at a C-terminal hexapeptide motif (Boucrot *et al.*, 2003) by a host cell geranylgeranyltransferase (Reinicke *et al.*, 2005). A major

breakthrough in understanding the function of SifA was the discovery that it interacts with a host protein of 1020 aa called SKIP (SifA and kinesin-interacting protein) (Boucrot *et al.*, 2005). The N-terminal region of SifA binds to a C-terminal pleckstrin homology (PH) domain of SKIP (Diacovich *et al.*, 2009; Jackson *et al.*, 2008; Ohlson *et al.*, 2008). The 310 aa N-terminal region of SKIP was shown to interact with the tetratricopeptide repeat (TPR) domain of kinesin light chain (Diacovich *et al.*, 2009; Dumont *et al.*, 2010), a subunit of the kinesin motor that promotes anterograde transport on microtubules. Subsequent work showed that two WD motifs within the N-terminal region of SKIP are involved in
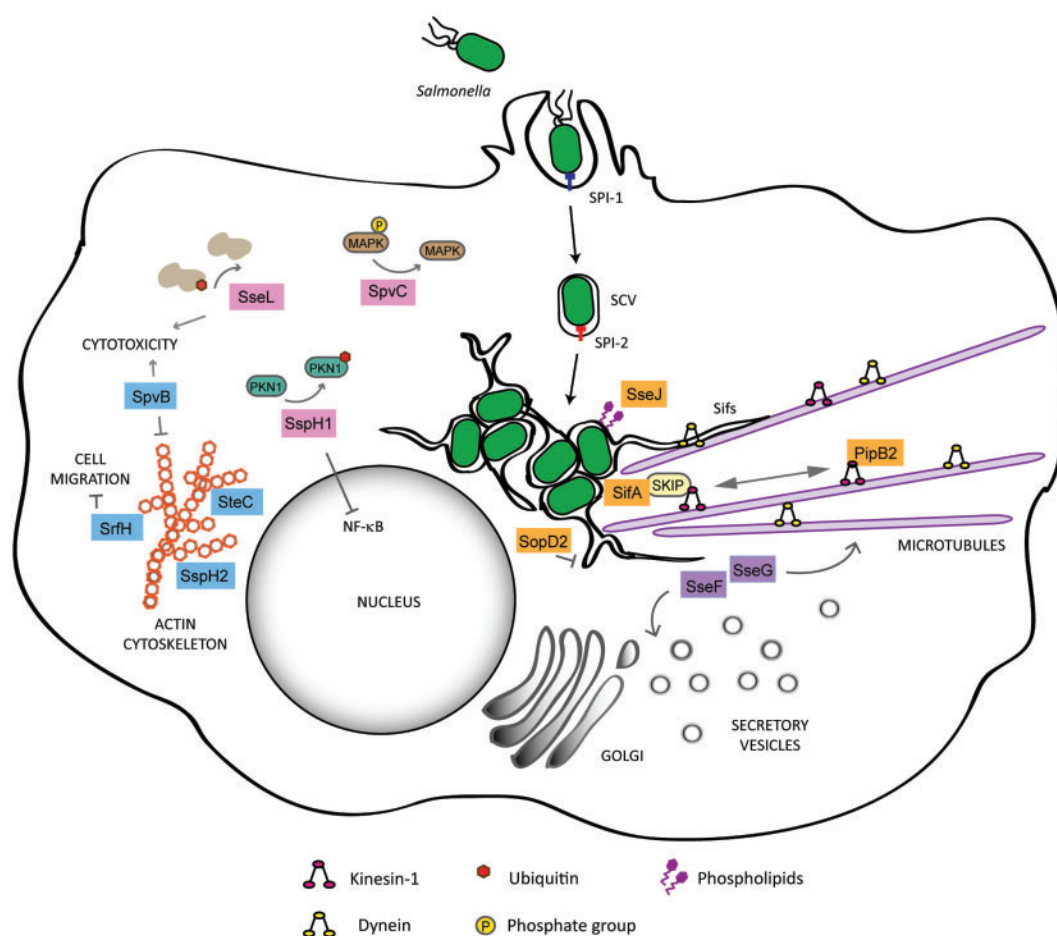
Fig. 1. Function of effectors of the SPI-2 T3SS. The SPI-2 T3SS functions mainly in maintenance of the SCV, through the action of the effectors SifA, SopD2, SseJ and PipB2 (orange boxes), and its localization near the Golgi of host cells, mediated by SseF and SseG (purple boxes). Other effectors are involved in modulation of host immune signalling (SpvC, SspH1 and SseL; pink boxes) or target the host cytoskeleton (SteC, SpvB, SspH2 and SrfH; blue boxes).

binding to kinesin-1 (Rosa-Ferreira & Munro, 2011). The SifA–SKIP interaction seems to promote fission of vesicles from the SCV and anterograde transport of kinesin-1-enriched vesicles (Dumont *et al.*, 2010). This and other work indicates that SKIP is a linker protein that binds and possibly activates kinesin-1. Recent work has shown that SKIP is also an effector of Arl8, a small GTPase present on lysosome membranes (Rosa-Ferreira & Munro, 2011). SKIP therefore provides a link between lysosomal membranes and kinesin-1, and is essential for peripheral distribution of lysosomes in the cell.

SifA also affects two GTPases: it binds GDP-bound RhoA (Ohlson *et al.*, 2008) and competes effectively with the endosomal GTPase Rab9 for binding to SKIP, thereby acting as a G-protein antagonist (Jackson *et al.*, 2008). SifA contains a potential cleavage site for caspase-3 between residues 127 and 141 (Srikanth *et al.*, 2010). This could divide the protein into separate functional domains following its translocation.

Increased recruitment of kinesin to the SCV membrane occurs in the absence of SifA or SKIP (Boucrot *et al.*, 2005). This is mediated by another SPI-2 T3SS effector, PipB2 (Henry *et al.*, 2006). PipB2 was discovered on the basis of sequence similarity to PipB. Both proteins contain several tandem pentapeptide repeats of unknown function in their C-terminal regions (Knodler *et al.*, 2003). PipB is an SPI-2 effector encoded by SPI-5, a pathogenicity island that contains another gene whose product (SopB) is translocated by the invasion-associated SPI-1 T3SS (Knodler *et al.*, 2002; Wood *et al.*, 1998). PipB also localizes to SCV membranes and Sifs (Knodler *et al.*, 2003), but its function is currently unknown. It does not appear to be involved in the development of systemic disease in mice (Knodler *et al.*, 2003), but does contribute to the inflammatory response in the bovine ileum (Wood *et al.*, 1998). On the other hand, PipB2 interacts with kinesin light chain (Henry *et al.*, 2006), which is consistent with the observations that this effector is found not only on the SCV membrane and Sifs, but also on vesicles at the periphery of infected cells

(Knodler *et al.*, 2003), and that overexpression of PipB2 results in the redistribution of late endosomal compartments to the cell periphery (Knodler & Steele-Mortimer, 2005). A functional link between PipB2 and SifA was established by the discovery that PipB2 is necessary and sufficient for recruitment of kinesin-1 to vacuoles containing *sifA* mutant bacteria (Henry *et al.*, 2006). Inhibition of kinesin activity prevented vacuolar membrane rupture around *sifA* mutant bacteria (Guignot *et al.*, 2004), but surprisingly the lack of PipB2 (and hence a failure to recruit kinesin to the SCV) did not rescue vacuolar membrane loss in a *pipB2*/*sifA* double mutant strain (Henry *et al.*, 2006). Hence the mechanism underlying vacuole membrane rupture is still unclear. Possibly some residual kinesin action on the SCV is responsible for the loss of the vacuolar membrane in the double mutant. Nevertheless, it is clear that PipB2 is involved in kinesin-driven SCV membrane dynamics and the radial extension of Sifs along microtubules (Knodler & Steele-Mortimer, 2005), and that it contributes to the systemic phase of growth of *S.* Typhimurium in the mouse (Knodler *et al.*, 2003).

SseJ is another effector that influences vacuolar membrane dynamics. It contains an N-terminal sequence conserved in several SPI-2 T3SS effectors, and its SPI-2 T3SS-dependent translocation into host cells was demonstrated using a CyaA fusion reporter (Miao & Miller, 2000). SseJ is absent from the genome of *S.* Typhi (Parkhill *et al.*, 2001), but it contributes to the virulence of *S.* Typhimurium in mice (Ruiz-Albert *et al.*, 2002). Intracellular vacuoles containing *sseJ* mutant bacteria appear to be morphologically normal, but when *sseJ* and *sifA* mutations were combined, the loss of vacuolar membrane around the double mutant strain was markedly reduced, indicating that loss of vacuolar membrane around *sifA* mutant bacteria requires the destabilizing action of SseJ (Ohlson *et al.*, 2005; Ruiz-Albert *et al.*, 2002; Schroeder *et al.*, 2010). Moreover, deletion of *sseJ* leads to an increased number of Sifs, suggesting that SseJ inhibits Sif formation (Birmingham *et al.*, 2005). The influence of SseJ on the vacuolar membrane and Sif formation indicates that SseJ attenuates or opposes the activity of SifA.

SseJ is a glycerophospholipid : cholesterol acyltransferase (GCAT) that esterifies cholesterol in infected cells (Lossi *et al.*, 2008; Nawabi *et al.*, 2008). Interestingly, this activity is strongly potentiated by a eukaryotic protein (Lossi *et al.*, 2008), which was subsequently identified as RhoA (Christen *et al.*, 2009). In *S.* Typhimurium-infected cells, a large proportion of total cellular cholesterol is recruited to the SCV (Catron *et al.*, 2002). The phospholipase A activity of SseJ (Lossi *et al.*, 2008) is likely to act on SCV membrane phospholipids, with the GCAT transferring acyl chains to cholesterol. Host cells infected with wild-type bacteria contain significantly more lipid droplets than cells infected with an *sseJ* mutant (Nawabi *et al.*, 2008). Since lipid droplets are enriched in esterified cholesterol, this suggests that the action of SseJ causes the removal of esterified cholesterol from SCVs to lipid droplets. This could then influence the anchoring of prenylated proteins

such as SifA and Rab proteins to the SCV membrane (Chen *et al.*, 2008), which in turn would have an effect on the recruitment of microtubule motors to SCVs. Furthermore, the rigidity of membranes is greatly affected by their cholesterol content (Petrache *et al.*, 2005). By depleting the SCV of cholesterol, SseJ would increase its rigidity, which could also help explain the role of SseJ in rupture of the vacuole in a *sifA* mutant. The effect of SseJ could also be mediated by its deacylation activity on membrane phospholipids, as the curvature of membranes reflects the composition of their phospholipid bilayer (McMahon & Gallop, 2005). Finally, changes at the level of host cell signalling pathways could be initiated by an effect on lipid rafts or by generation of lysophospholipid secondary messengers. The discovery that SifA binds GDP-RhoA, favouring GTP exchange, and that GTP-RhoA interacts with SseJ (Ohlson *et al.*, 2008), provides a potential biochemical link between these two effectors, and could point to the existence of a multi-protein signalling complex comprising SifA, SKIP, RhoA, SseJ and possibly other effectors such as PipB2 and SopD2, which could regulate the stability, expansion and tubulation of the vacuolar membrane.

SopD2 was identified through its conserved N-terminal secretion sequence (Brumell *et al.*, 2003). SopD2 localizes to the SCV membrane, Sifs, late endosomes and lysosomes. Deletion of *sopD2* interferes with Sif formation at an intermediate stage and has therefore been suggested to be necessary for fusion of late endosomes, leading to Sif formation (Jiang *et al.*, 2004). A *sopD2* mutant is attenuated for virulence in mice (Jiang *et al.*, 2004) and, as seen with an *sseJ* mutation, deletion of *sopD2* reduces the loss of membrane around *sifA* mutant bacteria, which implies that SopD2 has an important function in vacuolar membrane dynamics (Schroeder *et al.*, 2010). Interestingly, like *sseJ*, *sopD2* is a pseudogene in *S.* Typhi (Parkhill *et al.*, 2001), which suggests that *S.* Typhimurium and *S.* Typhi use different mechanisms to regulate membrane dynamics on their respective vacuoles.

The phenotypes and biochemical activities of SifA, PipB2, SseJ and SopD2 reveal that their apparently opposing actions actually work together to control vacuolar membrane dynamics. This maintains the integrity of the vacuolar membrane and presumably allows its controlled expansion, which must accompany bacterial cell division. It is also possible that by regulating vesicular fusion on the SCV these proteins ensure delivery of nutrients to the SCV lumen, thereby facilitating bacterial replication.

Effectors that affect SCV positioning (SseF, SseG)

In epithelial cells, maturation of SCVs is accompanied by their displacement towards the juxtanuclear region. This centripetal movement occurs along microtubules in the direction of the microtubule-organizing centre (MTOC), where Golgi stacks accumulate (Harrison *et al.*, 2004; Ramsden *et al.*, 2007). SCV movement is dependent on the

recruitment of dynein to the SCV via a Rab7–RILP interaction (Guignot *et al.*, 2004; Harrison *et al.*, 2004). Close apposition between SCVs and the Golgi/MTOC seems to be important for bacterial replication (Ramsden *et al.*, 2007; Salcedo & Holden, 2003), possibly by enabling interactions between SCVs and Golgi- or endosomal-associated vesicular traffic (Mota *et al.*, 2009; Salcedo & Holden, 2003). Bacterial cell division leads to the formation of microcolonies that are retained in the Golgi/MTOC region (Ramsden *et al.*, 2007) through the action of two SPI-2 T3SS effectors: SseF and SseG. Strains lacking either of these effectors undergo initial juxtanuclear migration and then scatter throughout the cell (Deiwick *et al.*, 2006; Ramsden *et al.*, 2007; Salcedo & Holden, 2003). Centrifugal scattering of *sifA* mutants also occurs, and this can be explained by the accumulation of kinesin on their vacuolar membranes prior to rupture (Boucrot *et al.*, 2005).

SseF and SseG are encoded by genes within SPI-2 (Hensel *et al.*, 1998) and were among the first proteins suggested to be effectors of the SPI-2 T3SS (Hensel *et al.*, 1998). The use of epitope-tagged versions of SseF and SseG confirmed their translocation into macrophages via the SPI-2 T3SS (Hansen-Wester *et al.*, 2002). *sseF* and *sseG* single mutant strains have similar levels of growth defect in macrophages and virulence attenuation in mice (Hensel *et al.*, 1998; Kuhle & Hensel, 2002), but an *sseF/sseG* double mutant is not more attenuated than either single mutant (Deiwick *et al.*, 2006). The similar phenotypes of single mutant strains and the lack of an additive effect on virulence attenuation in the double mutant strain support the hypothesis that SseF and SseG contribute to the same function. SseF and SseG are unusual effectors in that they are integral membrane proteins (Kuhle & Hensel, 2002) and interact (Deiwick *et al.*, 2006). They have also been shown to be involved in Sif formation (Kuhle & Hensel, 2002), and to co-localize with the microtubule network, leading to the creation of microtubule bundles (Kuhle *et al.*, 2004).

There are currently two models to explain how SseF and SseG could control the positioning of SCVs inside cells. The first is based on opposing activities of the microtubule motors kinesin and dynein. In this scenario, recruitment of kinesin (driving centrifugal movement) is modulated by SifA and PipB2, while that of dynein (driving centripetal movement) is controlled by SseF and SseG. The balance of these activities is normally in favour of SseF and SseG, which results in juxtanuclear positioning of SCVs. In support of this model, kinesin accumulates around vacuoles enclosing *sifA* mutant bacteria prior to their rupture (Boucrot *et al.*, 2005), and dynein recruitment to SCVs is reduced in *sseF* and *sseG* mutants (Abrahams *et al.*, 2006). Another possibility is a Golgi-tethering model, in which the complex formed by SseF and SseG, anchored in SCV membranes, interacts stably with Golgi-associated molecules to retain SCVs in that region of the cell. This model is based on live imaging observations (Ramsden *et al.*, 2007) and the fact that treatment of cells with Brefeldin A (which disassembles the Golgi complex) mimics the *sseF* and *sseG* positioning phenotype (Ramsden *et al.*, 2007). However, to date, no Golgi-associated protein has been shown to interact with SseF or SseG.

The positioning of *Salmonella* microcolonies at the MTOC/Golgi region could have an important physiological significance in enabling the acquisition of nutrients and membrane. Indeed a SPI-2-dependent redirection of post-Golgi traffic towards the SCV has been observed (Kuhle *et al.*, 2006), and the secretory carrier membrane protein (SCAMP)-3, which mainly localizes to the *trans*-Golgi network (TGN) in uninfected cells (Castle & Castle, 2005), is redistributed to the SPI-2 effector-induced tubular network emanating from SCVs (Mota *et al.*, 2009). This indicates that Sifs contain both endosomal and secretory pathway-derived molecules, and that SCVs interact with post-Golgi trafficking pathways (Mota *et al.*, 2009).

Effectors involved in ubiquitin modification (SspH1, SspH2, SlrP, SseL)

The post-translational reversible modification of eukaryotic proteins by covalent linkage of ubiquitin to lysine residues or to itself (to form poly-ubiquitin chains) regulates many important cellular processes (Fang & Weissman, 2004; Pickart & Eddins, 2004). Ubiquitination occurs via the sequential action of three enzymes: a ubiquitin-activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3). While there are only two E1 enzymes and E2 enzymes are relatively few, many E3 ligases exist which provide substrate specificity to the process. Ubiquitination can be reversed by the action of deubiquitinases. Ubiquitin is not found in proteobacteria, but several important pathogens, including *S. enterica*, have evolved both E3 ligase and deubiquitinating activity (Steele-Mortimer, 2011). These effectors are translocated via the SPI-1 and SPI-2 T3SSs and interfere with ubiquitin signalling in host cells.

Three *S. enterica* proteins have been discovered with E3 ligase activity: SspH1, SspH2 and SlrP. SspH1 and SspH2 share 69 % identity at the amino acid level (Miao *et al.*, 1999), and both contain leucine-rich repeats (LRRs), which are domains implicated in protein–protein binding (Kobe & Kajava, 2001). SlrP also contains LRRs, and was discovered through a signature-tagged mutagenesis screen for *S.* Typhimurium genes involved in virulence in mice and calves (Tsolis *et al.*, 1999). SlrP has been linked to the SPI-2 T3SS on the basis of similarity to SspH1 and SspH2, and is required for growth within murine but not bovine hosts (Tsolis *et al.*, 1999). The three proteins have been shown to be effectors of the SPI-2 T3SS through the use of CyaA fusion reporters (Miao *et al.*, 1999; Miao & Miller, 2000). However, SspH1 and SlrP can be translocated by both the SPI-2 and the SPI-1 T3SS. Similar LRRs are also present in some other T3SS effectors, including *Shigella* IpaH and *Yersinia* YopM (Tsolis *et al.*, 1999).

An important breakthrough with respect to these proteins was the discovery that IpaH and SspH1 have ubiquitin ligase activity (Rohde et al., 2007). IpaH and SspH1 have no primary amino acid sequence similarity to eukaryotic counterparts, and the crystal structures of IpaH (Singer et al., 2008; Zhu et al., 2008) and SspH2 (Quezada et al., 2009) reveal that they constitute a novel class of E3 ligase, containing a unique fold in the C-terminal region enclosing the catalytic site. Another unusual feature of these ligases is an autoinhibitory mechanism mediated by sequestering of the E3 ligase fold by the LRR domain. In the case of SspH2, the LRR motif also mediates localization of the protein to the apical side of the host cell plasma membrane (Quezada et al., 2009). Taking into account their different localization in the cell (nuclear for SspH1, cytoplasmic for SlrP, at the cell periphery for SspH2) and temporal regulation (SPI-1 or SPI-2 T3SS delivery), these effectors seem likely to affect host cell pathways in different ways. Nonetheless, each appears to have an impact on bacterial virulence. An slrP mutant is attenuated in mouse virulence (Tsolis et al., 1999) and an sspH1, sspH2 double mutant results in less mortality in calves (Miao et al., 1999).

Two lines of evidence indicate that SspH1 interferes with host cell inflammatory responses. Infection of epithelial cells with an sspH1 mutant strain results in increased secretion of the proinflammatory cytokine IL-8, compared with the wild-type strain (Haraga & Miller, 2003). Secondly, transfection-based expression of SspH1 in host cells causes an inhibition of NF-κB pathway-dependent gene expression (Haraga & Miller, 2003). A subsequent yeast two-hybrid screen identified the human serine/threonine protein kinase 1 (PKN1) as an interacting target for SspH1, and this interaction was confirmed by co-immunoprecipitation of endogenous PKN1 in infected HeLa cells (Haraga & Miller, 2006). The interaction is mediated by the LRR domain of SspH1 and is necessary for inhibition of NF-κB activation (Haraga & Miller, 2006). Interestingly, PKN1 has been reported to be involved in the TNFα-induced NF-κB signalling pathway (Gotoh et al., 2004). SspH1 has E3 ubiquitin ligase activity towards PKN1 (Rohde et al., 2007), but the relationship between ubiquitination of PKN1 and its possible effect on NF-κB activation remains to be established.

Unlike SspH1, the E3 ligases SspH2 and SlrP do not appear to influence activation of the NF-κB pathway (Haraga & Miller, 2003). SspH2 was instead found to localize to the host cell actin cytoskeleton and to function in actin polymerization (Miao et al., 2003). In vitro, SspH2 induces polymerization of K48-linked ubiquitin chains, which suggests that it can target proteins for proteasomal destruction (Levin et al., 2010).

SlrP has been reported to interact with the small redox protein thioredoxin (Bernal-Bayard & Ramos-Morales, 2009) and with ERdj3 (Bernal-Bayard et al., 2010), a chaperone of the Hsp40/DnaJ family. These two potential targets were identified by yeast two-hybrid screening and co-immunoprecipitation in host cells overexpressing SlrP. SlrP has been shown to ubiquitinate thioredoxin in vitro (Bernal-Bayard & Ramos-Morales, 2009). Thioredoxin is involved in functions such as cell proliferation, survival and apoptosis. In HeLa cells infected with S. Typhimurium, thioredoxin activity decreased, and this appeared to correlate with increased cell death (Bernal-Bayard & Ramos-Morales, 2009). However slrP mutants were not used to determine whether these phenotypes were dependent on SlrP or its E3 ligase activity. ERdj3 appears to be located in the endoplasmic reticulum (ER) (Bernal-Bayard et al., 2010). It has been suggested that SlrP, despite its largely cytoplasmic distribution, might also localize within the ER, where it could interact with the ERdj3. Although this interaction appears to be independent of the ubiquitination activity of SlrP, SlrP is able to compete for binding with ERdj3 substrates in vitro (Bernal-Bayard et al., 2010). However, neither ERdj3 nor thioredoxin has been shown to interact with, or be modified by, SlrP in host cells infected with bacteria. Therefore, these findings should be interpreted with caution, given the frequency with which yeast two-hybrid screens generate false positives and the fact that these conclusions were based on experiments involving overexpression of proteins following transfection and in vitro assays.

In addition to three ubiquitin E3 ligases, the SPI-2 T3SS translocates a protein with deubiquitinase activity. SseL was discovered as an SPI-2 T3SS effector on the basis of screens for genes regulated by SsrA/B (Coombes et al., 2007; Rytkönen et al., 2007). Purified SseL cleaved ubiquitin substrates in vitro with a clear preference for K63-linked chains over K48 linkages, which suggests that it is not involved in protecting proteins from proteasomal degradation (Rytkönen et al., 2007). Infection of epithelial cells and macrophages with an sseL mutant strain led to the accumulation of ubiquitin-modified proteins, indicating that SseL functions as a deubiquitinase in infected cells (Rytkönen et al., 2007). A strain lacking sseL did not display an intracellular replication defect in RAW 264.7 macrophages, but was defective for a SPI-2-dependent late-stage cytotoxic effect in macrophages and attenuated for virulence in the systemic phase of infection in mice (Rytkönen et al., 2007). How the deubiquitinase activity of SseL leads to cytotoxicity remains to be established. Although SseL did not appear to influence cytokine production or interfere with activation or degradation of IκBα upon infection of macrophages (Rytkönen et al., 2007), subsequent work by another group claimed that SseL counteracts IκBα ubiquitination and suppresses proinflammatory cytokine signalling (Le Negrate et al., 2008). Clearly, further work is required to resolve this controversy. A recent study involving stable isotope labelling of amino acids in cell culture (SILAC) and MS to screen for SPI-2 T3SS effectors and interacting host proteins identified oxysterol-binding protein (OSBP) as a possible interacting partner for SseL (Auweter et al., 2011). This could be related to the finding

that the deubiquitinase activity of SseL alters the lipid metabolism of infected cells, since lipid droplets accumulate in the gallbladders of infected mice in the absence of SseL (Arena *et al.*, 2011).

Effectors targeting the host cytoskeleton (SteC, SspH2, SrfH, SpvB)

Several SPI-1 effectors interact with host proteins to cause dramatic rearrangements of the actin cytoskeleton (Patel & Galán, 2005). The resulting membrane ruffling drives bacterial uptake. However, these changes are transient, and within a short period of time following bacterial uptake the cortical actin cytoskeleton resumes its normal morphology. Several hours later, *S.* Typhimurium induces a different form of actin reorganization. As the bacteria begin to replicate, a dense F-actin meshwork assembles in the vicinity of SCVs in several cell types, including epithelial cells, fibroblasts and macrophages (Méresse *et al.*, 2001). This meshwork depends on the SPI-2 T3SS (Méresse *et al.*, 2001) and SteC was subsequently identified as the effector mediating this phenomenon (Poh *et al.*, 2008), indicating that other SPI-2 T3SS effectors are not required for gross actin reorganization. SteC was first discovered in a CyaA-based transposon screen for translocated effectors (Geddes *et al.*, 2005). It is a kinase with weak similarity to human Raf-1 and other mammalian kinases (Poh *et al.*, 2008). Its kinase activity is necessary for actin meshwork formation, and overexpression of SteC in host cells causes massive alterations to the actin cytoskeleton (Poh *et al.*, 2008). However, an *steC* mutant strain does not have an obvious effect on virulence (Geddes *et al.*, 2005; Poh *et al.*, 2008) and its potential significance as an effector is currently unclear.

SspH2 and SrfH (also called SseI) (Miao & Miller, 2000; Worley *et al.*, 2000) are two SPI-2 T3SS effectors that also localize to the SCV-associated F-actin meshwork (Miao *et al.*, 2003). A yeast two-hybrid assay identified two actin-binding proteins (α-filamin and profilin-1) as interacting partners for SspH2, where the N-terminal region of SspH2 mediated binding to filamin and its C-terminal region interacted with profilin (Miao *et al.*, 2003). As SrfH shares a virtually identical N-terminal region with SspH2 (Miao & Miller, 2000), its interaction with filamin observed in the same study was not surprising (Miao *et al.*, 2003). However, while the binding between SspH2 and profilin was confirmed by glutathione *S*-transferase (GST) pull-down experiments, binding to filamin was not detected by this method (Miao *et al.*, 2003). Profilin enhances actin polymerization and filamin dimers act by cross-linking actin filaments in areas of active polymerization (Winder & Ayscough, 2005). SspH2 and SrfH did not appear to contribute to the formation of the actin meshwork, but purified SspH2 inhibited the rate of actin polymerization *in vitro* (Miao *et al.*, 2003), which suggests that it might counteract the effects of SteC.

SrfH has since been reported to influence host cell migration, although in contradictory ways. Yeast two-hybrid and overexpression assays in mammalian cells revealed an interaction between SrfH and thyroid receptor-interacting protein 6 (TRIP6) (Worley *et al.*, 2006). TRIP6 is an adaptor protein that localizes to focal adhesion sites and along actin stress fibres, serving as a platform for recruitment of molecules involved in actin polymerization and cell motility, amongst other functions. SrfH enhanced the ability of bacteria to induce motility of RAW 264.7 macrophages and facilitated bacterial spreading in the mouse model of systemic infection (Worley *et al.*, 2006). However, although TRIP6 was required for the effect of SrfH on cell motility, paradoxically, TRIP6-depleted cells carrying wild-type or *srfH* mutant bacteria migrated at levels similar to TRIP6-containing cells infected with wild-type bacteria (Worley *et al.*, 2006). This effect of SrfH on cell migration was not replicated in a more recent study in which SrfH was shown to interfere with the direction of movement of primary macrophages, and to inhibit the overall migration of both macrophages and dendritic cells (McLaughlin *et al.*, 2009). This inhibition was dependent on IQGAP1, a regulator of cell migration, which was shown to interact with SrfH both in a GST pull-down assay and when the effector was translocated from intracellular bacteria, which provides strong evidence that IQGAP1 is a physiological target of SrfH (McLaughlin *et al.*, 2009). However, the same study showed that, in RAW 264.7 macrophages, SrfH does not bind IQGAP1 and that its effect in this cell type is to mediate cell detachment (McLaughlin *et al.*, 2009), implying that the function of this effector is not the same in the two cell types. Moreover, SrfH has been shown to be required for long-term systemic infection in mice (Lawley *et al.*, 2006; McLaughlin *et al.*, 2009), and SrfH-dependent inhibition of dendritic cell migration *in vivo* correlates with a decrease in CD4+ T cells in the spleen of infected mice (McLaughlin *et al.*, 2009).

SpvB is a third effector that appears to interfere with the actin cytoskeleton. This protein is encoded by the *spv* operon in the *Salmonella* virulence plasmid (Krause *et al.*, 1992). While this operon (*spvRABCD*) has been known for many years to be important for *S.* Typhimurium virulence in the mouse (Gulig *et al.*, 1993; Gulig & Doyle, 1993; Matsui *et al.*, 2001; Rhen *et al.*, 1989; Roudier *et al.*, 1992), it has become clear only recently that at least two of its proteins are substrates for *Salmonella* T3SSs. SpvB has been studied in some detail and appears to have at least two functional domains: an N-terminal region that has similarity to a toxin from *Photorhabdus luminescens*, and a C-terminal region that acts on actin as an ADP-ribosylating toxin, inhibiting its polymerization (Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2001; Tezcan-Merdol *et al.*, 2005). SpvB causes cytotoxicity of macrophages, and although SPI-2 T3SS-dependent translocation of SpvB has not yet been demonstrated, this process has also been found to be dependent on the SPI-2 T3SS (Browne *et al.*, 2002, 2008). Interestingly, a *Salmonella spvB* mutant caused increased actin meshwork formation in HeLa cells (Miao *et al.*, 2003), suggesting that the target of SpvB might be the

F-actin meshwork formed as a result of the action of SteC. Furthermore, another link between SPI-2 and SpvB comes from work studying P-bodies, which are RNA/protein aggregates involved in post-transcriptional regulation. In *Salmonella*-infected human epithelial cells, the SPI-2 T3SS and SpvB were both found to be required for P-body disassembly (Eulalio *et al.*, 2011). However, infection did not affect P-body integrity in mouse macrophages, showing that its effects are cell type-specific.

Effector involved in immune signalling (SpvC)

SpvC is also encoded by the *spv* operon (Krause *et al.*, 1992). Its secretion from *Salmonella* requires either the SPI-1 or SPI-2 T3SS, depending on the conditions in which bacteria are grown (Mazurkiewicz *et al.*, 2008). However, SPI-2 T3SS-dependent translocation of SpvC into macrophages was only detected when its degradation was prevented by use of a proteasome inhibitor, which suggests that it is present in relatively small quantities. SpvC is a member of a family of effectors that have phosphothreonine lyase activity on MAPKs (Li *et al.*, 2007; Mazurkiewicz *et al.*, 2008). These enzymes use a process of irreversible phosphate removal, and are therefore potentially extremely powerful. This might provide an explanation for the apparently low amounts of SpvC in host cells. In the same study, SpvC was shown to be active on MAP kinases ERK and JNK, and overexpression of SpvC downregulated the release of TNF-α and IL-8 from infected macrophages and epithelial cells, respectively (Mazurkiewicz *et al.*, 2008). The anti-inflammatory effect of SpvC was confirmed in a recent study using a streptomycin-treated mouse model of enterocolitis (Haneda *et al.*, 2012). In the early stages of infection, SPI-1 T3SS-translocated SpvC led to reduced levels of cytokine mRNA and inflammation compared with an *spvC* mutant strain, and this correlated with systemic spread of the bacteria in mice.

It is very likely that additional SPI-2 effectors interfere with immune signalling. For example, it has been shown that infection of antigen-presenting cells by *S.* Typhimurium leads to poly-ubiquitination of major histocompatibility complex class II (MHC-II) molecules, resulting in removal of mature, peptide-loaded molecules from the cell surface. Although this process requires the SPI-2 T3SS, the specific effector(s) responsible have yet to be identified (Lapaque *et al.*, 2009).

Other effectors of the SPI-2 T3SS

Early studies involving *S.* Typhimurium and mouse macrophages implicated the SPI-2 T3SS in avoidance of SCV–lysosome fusion (Uchiya *et al.*, 1999), the respiratory burst (Vazquez-Torres *et al.*, 2000) and nitric oxide (Chakravortty *et al.*, 2002). The effectors mediating the latter two processes are currently unknown. The SPI-2-encoded protein SpiC was originally proposed to be responsible for SPI-2-mediated phagolysosome fusion avoidance (Uchiya *et al.*, 1999). Additional evidence pointed to SPI-2

T3SS-dependent translocation of SpiC into the cytosol of macrophages (Shotland *et al.*, 2003). Two host cell proteins, Hook3 (Shotland *et al.*, 2003) and NIPSNAP (NSP4) (Lee *et al.*, 2002), have been proposed to interact with SpiC. However, while Golgi-associated Hook3 has been implicated in vesicular trafficking (Xu *et al.*, 2008), NSP4 is a mitochondrial protein (Verhagen *et al.*, 2007). It is not clear how binding of SpiC to these proteins causes inhibition of phagolysosome fusion. It has also been established that SpiC is part of the three-protein regulatory complex that functions within the bacterial cell to mediate the switch from translocon protein secretion to effector translocation (Yu *et al.*, 2004, 2010). Moreover, attempts by our group and others to show that SpiC is itself a translocated protein have failed (Freeman *et al.*, 2002; Yu *et al.*, 2002), suggesting that SpiC is not an effector of the SPI-2 T3SS. The notion that the SPI-2 T3SS is involved in avoidance of or resistance to macrophage killing mechanisms is also difficult to reconcile with more recent work from our laboratory showing that an SPI-2 T3SS null mutant has an intracellular replication defect but does not undergo an increase in killing by macrophages (Helaine *et al.*, 2010). The link between the SPI-2 T3SS and avoidance of the respiratory burst has also been questioned recently (Aussel *et al.*, 2011).

Several other proteins have been implicated as being SPI-2 T3SS effectors, although their functions and physiological contributions to *Salmonella* virulence remain to be established. These include SifB, PipB, GogB, SseK1, SseK2, SseK3, GtgE, GtgA, SpvD, SteA, SteB, SteD, SteE and CigR.

Like SspH1 and some other effectors mentioned above, SifB was identified by its conserved, translocation-specific N-terminal sequence (Miao & Miller, 2000). It was named after its similarity to SifA, as they share 30 % identity over the length of the protein, and SifB was shown to be translocated into host cells via the SPI-2 T3SS (Miao & Miller, 2000). SifB localizes to the SCV and Sifs (Freeman *et al.*, 2003), although its potential function is unknown and a strain lacking *sifB* displayed the same level of virulence as a wild-type strain in mice (Ruiz-Albert *et al.*, 2002).

GogB is encoded by the gifsy-1 bacteriophage and localizes to the cytoplasm of infected cells in an SPI-2 T3SS-dependent manner (Coombes *et al.*, 2005). The protein has a partial LRR domain in its N-terminal region (Coombes *et al.*, 2005), and its C-terminal domain is similar to the T3SS effector EspK of enterohaemorrhagic *Escherichia coli* (EHEC) (Coombes *et al.*, 2005; Vlisidou *et al.*, 2006). A *gogB* mutant strain showed no major defect in colonization of the small intestine or spleen of infected mice (Figueroa-Bossi *et al.*, 2001).

SteA and SteB were, like SteC, identified in a screen based on transposon mutagenesis coupled with the CyaA fusion method (Geddes *et al.*, 2005). Both proteins were shown to be translocated by the SPI-1 and SPI-2 T3SSs, and SteA

localized to the TGN in transfected epithelial cells (Geddes *et al.*, 2005). In the same study, an *steA* mutant strain was found to be significantly attenuated in infection of mice spleens compared with a wild-type strain (Geddes *et al.*, 2005). However, no functional studies on SteA or SteB have been reported to date.

SseK1 and SseK2 were identified due to their similarity to the *E. coli* T3SS effector NleB, and their translocation via the SPI-2 T3SS was shown using the CyaA reporter system (Kujat Choy *et al.*, 2004). Recently, a third effector encoded on the *Salmonella* phage ST64B, named SseK3, was also shown to be translocated by the SPI-2 T3SS (Brown *et al.*, 2011). The three effectors show remarkable similarity at the amino acid level, but their functions are currently unknown. Interestingly, NleB contributes to inhibition of NF-κB activation in epithelial cells infected with EHEC (Newton *et al.*, 2010). Strains lacking SseK1 and SseK2 or all three SseK family members are strongly attenuated for virulence in mice (Brown *et al.*, 2011).

Another group of effectors was identified recently in a proteomic study of the secretome of an *ssaL* mutant strain, which is derepressed for effector secretion under conditions of SPI-2 activation (Niemann *et al.*, 2011; Yu *et al.*, 2010). Secreted proteins were analysed by MS, and six previously unidentified effectors of the SPI-2 T3SS were detected, namely CigR, GtgA, GtgE, SpvD, SteD and SteE (Niemann *et al.*, 2011). Translocation of all these proteins by the SPI-2 T3SS was confirmed by the CyaA method, and GtgE, SpvD and SteE were also found to be translocated by the SPI-1 T3SS (Niemann *et al.*, 2011). Moreover, mutant strains lacking *gtgE*, *spvD*, *steD* or *steE* were significantly attenuated for virulence in mice (Niemann *et al.*, 2011). In other work, GtgE was shown to be a protease that specifically cleaves Rab29 (Spanò *et al.*, 2011). In cells infected with *S.* Typhi, which does not contain *gtgE*, Rab29 is recruited to the SCV, and exogenous expression of GtgE in *S.* Typhi leads to its increased replication in macrophages.

SrfJ is another candidate SPI-2 T3SS effector. It is part of the SsrA/B regulon and has sequence similarity to a human glucosyl ceramidase (Worley *et al.*, 2000). A strain carrying a mutation in *srfJ* was mildly attenuated for virulence in mice (Ruiz-Albert *et al.*, 2002). However, translocation of this protein by the SPI-2 T3SS is yet to be established.

Conclusions

Nearly 30 effectors of the SPI-2 T3SS have been identified over the last decade. Thirteen of these carry out a broad range of functions, including the control of SCV membrane dynamics, the localization of SCVs within host cells, immune modulation, cytoskeletal modifications and affecting the motility of infected cells.

Some effectors of the SPI-2 T3SS appear to be extremely potent and have unique activities. For instance, the phosphothreonine lyase action of SpvC is irreversible, making it exceptionally efficient, despite being translocated in apparently small quantities (Mazurkiewicz *et al.*, 2008), and SifA is able to interact with SKIP, binding with even greater affinity than its cognate host cell binding partner Rab9 (Jackson *et al.*, 2008). It is remarkable that as a result of enormous selective pressure and convergent evolution, SPI-2 T3SS effectors have evolved to interfere both positively and negatively with the two major forms of post-translational modifications within eukaryotic cells: phosphorylation (SteC) and dephosphorylation (SpvC), and ubiquitination (SspH1, SspH2, SlrP) and deubiquitination (SseL).

However, despite the progress made to elucidate the biochemical functions of several SPI-2 T3SS effectors, we still know relatively little of their physiological consequences and how their coordinated activities contribute to SPI-2 T3SS-mediated intracellular replication (Helaine *et al.*, 2010). The continued study of these proteins is important from the perspectives of understanding the mechanisms of bacterial pathogenesis, in shedding light on host cell processes (Rosa-Ferreira & Munro, 2011) and, potentially, in the design of vaccines against *Salmonella* disease (Hindle *et al.*, 2002; Khan *et al.*, 2003; Lyon *et al.*, 2010) and as carriers of vaccines against other infectious organisms (Husseiny *et al.*, 2007; Panthel *et al.*, 2005; Xu *et al.*, 2010).

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