SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling

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**Summary**

*Salmonella enterica* serovar Typhimurium (S. Typhimurium) replicates inside mammalian cells within membrane-bound compartments called *Salmonella*-containing vacuoles. Intracellular replication is dependent on the activities of several effector proteins translocated across the vacuolar membrane by the *Salmonella* pathogenicity island 2 (SPI-2)-type III secretion system (T3SS). This is accompanied by extensive rearrangements of the F-actin cytoskeleton meshwork around vacuoles. Numerous bacterial genes are required for intracellular survival, replication and virulence of this pathogen in mice. These include a multifunctional virulence system called the *Salmonella* pathogenicity island-2 (SPI-2) type III secretion system (T3SS; Waterman and Holden, 2003). The SPI-2 T3SS is induced intracellularly (Cirillo et al., 1998) and translocates several effectors into the vacuolar membrane and host cell cytosol (Waterman and Holden, 2003). These effectors are involved in several physiological activities, including the regulation of vacuolar membrane dynamics (Ruiz-Albert et al., 2002; Boucrot et al., 2005; Henry et al., 2006), inducing motility of infected cells (Worley et al., 2006), targeting SCVs to the Golgi apparatus in epithelial cells (Salcedo and Holden, 2003), and formation of an actin cytoskeleton meshwork around SCVs (Méresse et al., 2001; Unsworth et al., 2004).

At least two effectors, SseF and SseG, are encoded within SPI-2, but several others are encoded by genes located at different sites in the bacterial chromosome, and the full repertoire of effectors is unknown (Waterman and Holden, 2003; Kujat Choy et al., 2004; Geddes et al., 2005). The expression of SPI-2 genes and genes encoding effectors located outside the pathogenicity island requires the SPI-2-encoded two-component regulatory system SsrA–SsrB (Cirillo et al., 1998). In recent work we used a DNA microarray of *S. Typhimurium* to compare the levels of mRNAs in wild-type and *ssrA* mutant bacteria grown in conditions that result in strong expression of the SsrA–SsrB regulon. This led to the identification of an effector (SseL) with deubiquitinase activity (Rytkönén et al., 2007). Another gene whose RNA level was significantly lower in the *ssrA* mutant compared with the wild-type strain is *STM1698* (Rytkönén et al., 2007). *STM1698* was previously identified in a signature-tagged mutagenesis screen as a gene important in colonization of the chick intestine (Morgan et al., 2004). A subsequent study by Geddes et al. (2005) showed that the product of
STM1698 is translocated into host cells in a SPI-2 T3SS-dependent manner, and the gene was designated steC (Salmonella translocated effector C). In this study we have further characterized the product of steC. We show that SteC is a kinase that is required for SPI-2 T3SS-dependent actin meshwork formation in infected cells.

Results and discussion

SsrA-dependent intracellular expression of steC

A map of the chromosomal region encompassing steC is shown in Fig. 1A. To determine if intracellular expression of steC is regulated by SsrA–B, the steC open reading frame and 300 bp of DNA upstream from its start codon (see bar in Fig. 1A) was fused to a promoterless gfp gene. The fusion was ligated into a plasmid and introduced into wild-type or ssrA mutant strains, which were then used to infect HeLa cells. Infected cells were fixed at 2 h intervals following invasion, and examined by fluorescence and differential interference contrast (DIC) microscopy. Reporter activity was detected in intracellular but not extracellular wild-type bacteria, 8 h post invasion (Fig. 1B). No expression was detected in the ssrA mutant strain, confirming that steC is part of the SsrA–B regulon.

Secretion and translocation of SteC-2HA

To detect secreted and translocated SteC, a gene encoding a double haemagglutinin (2HA) epitope-tagged version of SteC (steC-2HA) was introduced into the chromosome in place of the steC allele in the wild-type strain and an isogenic strain carrying a mutation in ssaV, which encodes an essential component of the SPI-2 T3SS (Beuzón et al., 1999). Wild-type and ssaV mutant strains containing steC-2HA were grown in magnesium minimal MES medium (MgM-MES) at pH 5.0, which induces the expression of the SPI-2 T3SS and secretion of its effectors (Beuzón et al., 1999). Under these conditions, SPI-2 T3SS-secreted proteins accumulate on the plastic surface of the tube in which the bacteria are grown (Beuzón et al., 1999; Yu et al., 2004). Proteins were recovered from this location and from the bacterial cell pellet, separated by SDS-PAGE and analysed by immunoblotting (Fig. 2A). Rabbit anti-SseB antibody (Beuzón et al., 1999) was used as a positive control. SteC-2HA was detected extracellularly when expressed in the wild-type strain background, but was found only in the bacterial cell pellet when expressed in the ssaV mutant strain background. This shows that S. Typhimurium requires a functional SPI-2 T3SS to secrete SteC in vitro.

To examine the translocation of SteC-2HA in host cells, HeLa cells were infected with wild-type or ssaV mutant strains containing steC-2HA. At 8 h post invasion, cells were fixed, permeabilized with saponin to allow detection of translocated protein (Yu et al., 2004) and immunolabelled with anti-Salmonella and anti-HA antibodies. SteC-2HA was readily detectable in HeLa cells infected with wild-type bacteria, whereas cells infected with the ssaV mutant did not show any immunolabelling with the anti-HA antibody (Fig. 2B). This indicates that SteC requires a functional SPI-2 T3SS to be translocated into host cells. Cells infected with wild-type bacteria expressing SteC-2HA were also labelled for LAMP-1, a lysosomal membrane glycoprotein abundant on the SCV membrane and Salmonella-induced filaments (Sifs), which are tubules that extend from SCVs in infected epithelial cells (Garcia-del Portillo et al., 1993). There was extensive colocalization between SteC-2HA and SCV- and Sif-associated LAMP-1 (Fig. 2C). Therefore, SteC is a translocated SPI-2 effector that shows a similar localization pattern to that of several other SPI-2 effectors (Waterman and Holden, 2003).
Intracellular replication and virulence assays

The SPI-2 T3SS is required for replication of S. Typhimurium inside host cells (Waterman and Holden, 2003). Therefore, an steC knock-out mutant was constructed to investigate its intracellular growth, compared with that of the wild-type strain and an ssaV mutant. Replication assays were performed in epithelial (HeLa) cells and RAW macrophages. At 2 h and 16 h post uptake in each cell type, the growth of the steC mutant was indistinguishable from that of the wild-type strain, while the ssaV mutant displayed a strong replication defect in both cell types (data not shown). To determine the importance of SteC for virulence in the mouse model of systemic infection, the steC mutant strain was subjected to a virulence test involving mixed infections of mice. A competitive index (CI), which provides a value for the relative degree of virulence attenuation, was determined after recovering bacteria from spleens of infected animals, 48 h after intraperitoneal (i.p.) inoculation (Beuzón and Holden, 2001). The CI for the steC mutant strain versus the wild-type strain was 1.15% ± 0.08%, consistent with results of a previous study (Geddes et al., 2005). Furthermore, we failed to detect a virulence defect of the steC mutant when the mixed inoculum was administered by the oral route (data not shown).

SteC is required for SPI-2-dependent F-actin meshwork formation by intracellular bacteria

One characteristic of the SPI-2 T3SS, for which the corresponding effector(s) has not been identified, is the formation of an F-actin meshwork around SCVs (Méresse et al., 2001). Therefore, F-actin meshwork formation of the steC mutant was investigated. Swiss 3T3 fibroblasts, in which the SPI-2-dependent F-actin phenotype is particularly well defined (Méresse et al., 2001), were infected for 8 h with S. Typhimurium strains expressing 2HA-tagged SteC from the chromosome. Cells were fixed and immunolabelled to detect (B) the HA-epitope tag (red in merged image) and Salmonella (green in merged image) or (C) the HA-epitope tag (red in merged image), Salmonella (blue in merged image) and LAMP-1 (green in merged image). Boxed insets in (C) are magnifications of indicated regions showing colocalization between SteC-2HA and LAMP-1 on Sifs. Bars represent 5 μm.

Role of SteC in vacuole membrane integrity

Previous work from our laboratory has implicated SPI-2 T3SS-dependent F-actin reorganization in maintaining the integrity of the Salmonella vacuolar membrane (Méresse et al., 2001). We therefore investigated the integrity of intracellular vacuoles enclosing the steC mutant strain. RAW macrophages were infected for 12 h with GFP-expressing wild-type, steC or sifA mutant bacteria. The majority of sifA mutants lose their vacuolar membrane by 8 h post uptake (Beuzón et al., 2000) and therefore provide a positive control for vacuolar membrane loss. Cells were treated with digitonin to selectively permeabilize the host cell plasma membrane (Salcedo and Holden, 2003), and labelled with an anti-Salmonella antibody. Under these conditions, the majority (64.3% ± 4.1%) of wild-type bacteria failed to label with the anti-Salmonella antibody, confirming the presence of an intact
vacuolar membrane. However, only 28.6% ± 3.4% of the intracellular silA mutant strain failed to label with the antibody, indicating that the majority had lost vacuolar membrane integrity. Labelling of the steC mutant by this method was similar to that of the wild-type strain (60.2% ± 3.3%). The integrity of vacuoles containing steC mutant bacteria was also confirmed using LAMP-1 labelling as a marker for the Salmonella vacuolar membrane (data not shown). Therefore, loss of SteC does not result in noticeable destabilization of the SCV.

The evidence indicating a role for SPI-2-associated F-actin in stability of the vacuolar membrane came from experiments in which membranes enclosing wild-type but not ssaV mutant bacteria were destabilized when infected cells were treated with the actin-depolymerizing drugs cytochalasin D or latrunculin B (Méresse et al., 2001). However, prolonged exposure of host cells to these drugs results in depolymerization of the vast majority of cellular F-actin. In view of the results described above, it would appear that the effects of these drugs on SCV membranes are indirect and unrelated to SteC-directed actin remodelling.

SteC is a kinase

steC is predicted to encode a protein of 457 amino acids, originally annotated as a putative inner membrane protein (McClelland et al., 2001). Functional predictions using the InterProScan search engine (http://www.ebi.ac.uk/InterProScan/) identified a region in SteC with similarity to kinases. Visual alignment of this region (aa 232–280) with several eukaryotic and prokaryotic kinases revealed residues in SteC (indicated in bold, Fig. 4A) that are highly conserved in subdomains I, II and III of kinases (Hanks et al., 1988). The consensus Gly-X-Gly-X-X-Gly is found in subdomain I of many kinases and functions as a nucleotide positioning motif that has a critical role in ATP binding (Bossemeyer, 1994). Subdomain II contains an invariant Lys residue that anchors ATP and contributes to the correct orientation of the triphosphate by interacting with the α- and β-phosphates (Hanks and Hunter, 1995). Mutation of this residue invariably results in a loss of kinase activity (Bossemeyer, 1993; Iyer et al., 2005). The nearly invariant Glu residue in subdomain III helps stabilize interactions between the Lys residue and ATP (Bossemeyer and Hunter, 1995). The percentage identities of subdomains I-III of several kinases to SteC (Fig. 4A, far right brackets) indicate that this region of SteC has closest similarity to the human RAF proto-oncogene serine/threonine-protein kinase (Raf-1; Wellbrock et al., 2004). However, SteC lacks the conserved central core of the catalytic domain in subdomains VI through IX of eukaryotic kinases, including highly conserved residues in the catalytic loop, and the His-Arg-Asp, Asp-Phe-Gly and Ala-Pro-Glu motifs, found in subdomains VI, VII and VIII respectively, which are important for phosphotransfer activity and substrate recognition of the kinase (Hanks et al., 1988; Hanks and Hunter, 1995).
To determine if SteC is a kinase, SteC, SteCK256H (in which the putative ATP-anchoring Lys residue in subdomain II was replaced by His) and C-SteC (lacking the N-terminal 200 amino acids, which show no similarity to kinases) were expressed and highly enriched as 6-His fusion proteins (Fig. 4B). These were incubated separately with \([\gamma^32P]ATP\) and the general kinase substrate, myelin basic protein (MBP). Proteins were then separated by SDS-PAGE and analysed by autoradiography (Fig. 4B). SteC-6His and C-SteC-6His both phosphorylated MBP and also underwent autophosphorylation, a characteristic of many kinases (Hanks and Hunter, 1995; Morrison and Cutler, 1997; Chong et al., 2001). Mutation of Lys256 resulted in the complete loss of kinase activity. These results confirm that SteC is a kinase whose enzymatic activity is dependent on a conserved Lys residue in subdomain II, but not the N-terminal 200 amino acids of the protein. As for several other SPI-2 T3SS effectors, this region might be important for secretion, translocation and localization of SteC (Miao and Miller, 2000).

So far as we are aware, SteC is only the fourth T3SS effector to be identified displaying kinase activity. The others are OspG of *Shigella flexneri* (Kim et al., 2005), YopO/YpkA of the plasmid-encoded *Yersinia* T3SS (Galyov et al., 1993; Barz et al., 2000), and YspK, a recently identified effector of the chromosomally encoded Ysp T3SS of *Yersinia enterocolitica* (Matsumoto and Young, 2006). Of these kinases, OspG and YspK share...
90% identity within subdomains I–III (data not shown). Two additional effectors, NleH1-1SAKAI and NleH1-2SAKAI from Escherichia coli O157, are also predicted to be kinases on the basis of strong similarity to OspG (Tobe et al., 2006). However, SteC and YopO/YpkA are not members of this subclass of bacterial kinases, and SteC has greater similarity to eukaryotic kinases, especially Raf-1.

Intracellular F-actin meshwork formation by Salmonella is dependent on Lys\textsuperscript{256} of SteC

To determine the importance of SteC Lys\textsuperscript{256} in F-actin meshwork formation by Salmonella in infected cells, Swiss 3T3 cells were infected with an steC mutant containing plasmid-borne steC-2HA, or steCK256H-2HA. Cells were immunolabelled to detect Salmonella (green in merged image), F-actin was visualized by phalloidin-RRX staining (red in merged image). Results are the means ± SE of three independent experiments, in which a total of 300 infected cells were examined for each strain. Results for cells infected with wild-type or steC mutant strains are for comparison and are from Fig. 3B. As Lys\textsuperscript{256} is essential for kinase activity of SteC, SPI-2 T3SS-dependent F-actin meshwork formation almost certainly requires the kinase activity of SteC. To determine the localization of SteC-2HA in infected Swiss 3T3 cells, cells were fixed at 8 h post invasion and labelled to detect Salmonella, HA and F-actin. A substantial degree of colocalization between SteC-2HA and F-actin was observed (Fig. 5D). This indicates that in addition to its localization on or close to the SCV membrane (Fig. 2C), SteC-2HA also localizes to SPI-2-induced F-actin structures.

SteC is sufficient to cause ROCK-like F-actin reorganization

We next investigated whether SteC activity is sufficient to cause F-actin reorganization in host cells. Swiss 3T3 fibroblasts were transfected with vectors encoding c-myc-epitope-tagged SteC (SteC-myc), the kinase-inactive point mutant (SteCK256H-myc) or the kinase domain (C-SteC-myc). Cells were transfected for 20 h in medium containing serum, and then incubated for another 3 h in serum-free medium before fixation, immunolabelling and

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examination by confocal microscopy. Untransfected fibroblasts or cells expressing SteCK256H-myc contained very few organized actin filaments except in the cortical region (Fig. 6A). In contrast, expression of SteC-myc or C-SteC-myc resulted in the formation of thick actin cables connecting large clusters of highly condensed F-actin (Fig. 6A and B). In cells expressing SteC-myc, these structures appeared to be randomly distributed throughout the cytoplasm, but in cells expressing C-SteC, clusters and cables were mainly found at the cell periphery (Fig. 6A and B). Therefore, SteC is sufficient to induce substantial reorganization of the host cell actin cytoskeleton in the absence of other bacterial effectors and its N-terminal 200 amino acids appear to have a role in its regulation or localization.

Salmonella-containing vacuole-associated F-actin has different morphologies, depending at least in part on the host cell type. These include a meshwork of F-actin between SCVs of a bacterial microcolony, a cage-like structure enclosing several SCVs (Méresse et al., 2001), or a highly condensed cluster of F-actin, frequently positioned towards the centre of a bacterial microcolony (Miao et al., 2003), from which long cables or filaments sometimes extend (Figs 3A and 5A). This phenotype is interesting in relation to the similarity displayed by SteC to Raf-1. Raf-1 plays a central role in cell proliferation, differentiation and survival. Raf kinases also influence actin cytoskeleton dynamics by modulating signalling pathways involving the Rho effector ROCK (Pritchard et al., 2004; Ehrenreiter et al., 2005; Castellani et al., 2006). The F-actin clusters and cables that are formed in fibroblasts following expression of SteC are very similar in appearance to those produced upon expression of active ROCK (Fig. 6A lower panel; Amano et al., 1997). However, treatment of infected cells with the ROCK inhibitor Y-27632 did not affect SteC-dependent F-actin remodelling around Salmonella microcolonies (data not shown). We are therefore currently investigating the possibility that SteC targets other component(s) of a pathway involving ROCK and other signalling pathways. Previous work from our laboratory has shown that several proteins involved in actin assembly, including Cdc42, Rac, N-WASP, Scar/WAVE and Arp2/3 are not involved in SPI-2 T3SS-dependent F-actin meshwork formation (Unsworth et al., 2004).

As SteC does not appear to contribute significantly to virulence in the murine model of systemic infection, the broader physiological significance of the kinase activity of SteC and its effect on the actin cytoskeleton is currently unknown. However, homologues of steC are present in other S. enterica serovars, including S. Paratyphi, S. Typhi and S. Choleraesuis (Geddes et al., 2005), and it is possible that SteC has a role in colonization of the chick intestine (Morgan et al., 2004) and/or other hosts.

Fig. 6. SteC is sufficient to cause ROCK-like F-actin reorganization.
A. Representative confocal images of Swiss 3T3 cells transfected with vectors expressing myc-tagged SteC, C-SteC, SteCK256H or active ROCK (ROCK-K). Cells were immunolabelled with anti-Myc antibody (green in merged image) and F-actin was visualized by phalloidin-RRX staining (red in merged image). Bars represent 10 μm.
B. Quantification of transfected cells displaying F-actin reorganization (white bars) and also periphery F-actin reorganization (black bars). Results are the means ± SE of three independent experiments, in which a total of 300 transfected cells were examined for each transfection vector.
Experimental procedures

Bacterial strains and growth conditions

Bacteria (Table 1) were grown in Luria–Bertani (LB) medium supplemented with carbenicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or chloramphenicol (30 μg ml⁻¹), for strains resistant to these antibiotics (Ampr, Km and Cmr respectively). To induce SPI-2 gene expression and SPI-2-dependent secretion, bacteria were grown in MgM-MES, containing 170 mM 2-[N-morpholinol]ethane-sulfonic acid (MES) at pH 5.0, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 8 mM MgCl₂, 38 mM glycerol and 1% Casamino acids (Beuzón et al., 1999) with the corresponding antibiotics when appropriate. Bacteria were grown at 37°C overnight with aeration.

Primer and construction of mutant strain, epitope tagging, site-directed mutagenesis and expression vectors

The primers used in this study are listed in Table S1. Polymerase chain reactions (PCRs) were performed using either the Taq polymerase (Sigma) or the Expand Long-Template PCR system (Roche) protocols. The Quikchange II site-directed mutagenesis kit protocol (Stratagene) was used to construct the Lys to His point mutant. The chromosomal deletion of steC in S. Typhimurium 12023 was performed by using the one-step gene-disruption technique (Datsenko and Wanner, 2000). The influenza virus HA epitope DNA sequence was fused to the chromosomal copy of steC according to Uzzau et al. (2001). steC-2HA was transduced by bacteriophage P22 into the ssaV mutant strain following the method of Davis et al. (1980). Site-directed mutagenesis was performed according to manufacturer’s recommendations (Quikchange, Stratagene). Lys at residue 256 of SteC was changed to His using primers K256H-F and K256H-R. Primers for constructs are listed in Table S1.

Table 1. Bacterial strains used in this study.

| Strain | Description | Reference/source |
|--------|-------------|------------------|
| 12023  | Wild-type S. Typhimurium | NTCC (Colindale, UK) |
| 12023,psteC::gfp | steC::gfp under control of the steC promoter in 12023 (amp') | This study |
| P3F4   | ssaA::mTn5 (km') | Shea et al. (1996) |
| Rosetta | E. coli (cm') | Novagen |
| ΔssrA,psteC::gfp | steC::gfp under control of the steC promoter in P3F4 (km', amp') | This study |
| ΔsteC  | ΔsteC::km in 12023 (km') | This study |
| steC-2HA | steC-2HA in 12023 (km') | This study |
| ΔsteC,psteC | pWSK29 steC in ΔsteC (km', amp') | This study |
| ΔsteC,psteC-2HA | pWSK29 steC-2HA in ΔsteC (km', amp') | This study |
| ΔsteC,psteCK256H-2HA | pWSK29 steC-2HA in ΔsteC (km', amp') | This study |
| HH119  | ssaV::aphT (km) in 12023 | Deiwik et al. (1998) |
| ΔssaV,steC-2HA | steC-2HA in HH119 (km') | This study |
| TOP10  | E. coli | Invitrogen |

Table 2. Plasmids used in this study.

| Plasmid | Description | Reference/source |
|---------|-------------|------------------|
| pFPV25  | Promoter trap vector, used to fuse promoters to the green fluorescent protein gene, gfp | Valdivia and Falkow (1996) |
| pFPV25.1 | rpsM::gfpmut3a promoter fusion in pFPV25 | Valdivia and Falkow (1996) |
| psteC::gfp | steC promoter and open reading frame fused to promoterless gfp | This study |
| pSU315  | Template for HA-tagging of genes containing kanamycin cassette | Uzzau et al. (2001) |
| pWSK29  | Low copy plasmid | Wang and Kushner (1991) |
| psteC   | pWSK29 containing steC | This study |
| psteC-2HA | pWSK29 containing steC-2HA | This study |
| psteCK256H-2HA | pWSK29 containing steCK256H-2HA | This study |
| pRK5myc | Transfection vector containing multiple cloning site downstream of c-myc | Dr E Caron |
| pRK5steCmyc | steC tagged with N-terminal c-myc tag | This study |
| pRK5steCK256Hmyc | steC-2HA tagged with N-terminal c-myc tag | This study |
| pRK5steCK256Hmyc-C | C-SteC tagged with N-terminal c-myc tag | This study |
| Myc-ROCK-K | Active construct of ROCK tagged with N-terminal c-myc tag in pRK5myc | Dr E Caron |
| pET28b  | Expression vector containing an N-terminal 6-His tag | Olazabal et al. (2002) |
| pET28bsteC-6His | steC tagged with 6-His tag | Novagen |
| pET28bsteCK256H-6His | steC-2HA tagged with 6-His tag | This study |
| pET28bC-steC-6His | C-steC tagged with 6-His tag | This study |

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Antibodies and reagents

Anti-Salmonella goat polyclonal antibody CSA-1 (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used at a dilution of 1:200. Anti-HA mouse monoclonal antibody (HA.11; Covance) was used at a dilution of 1:200 (immunofluorescence microscopy) and 1:1000 (Western blot). Anti-HA rat monoclonal antibody (Roche) was used at a dilution of 1:200 for immunofluorescence. Anti-LAMP-1 mouse monoclonal antibody (HA43; Developmental Studies Hybridoma Bank), developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences), and was used at a dilution of 1:200. The rabbit polyclonal anti-LAMP-1 antibody 156 was kindly provided by Dr S. Méresse (Centre d'Immunologie de Marseille-Luminy, Marseille, France) and used at a dilution of 1:250. Rabbit polyclonal anti-SseB antibody (Beuzón et al., 1999) was used at a dilution of 1:1000. AMCA-, Cy2-, Cy5-, or Rhodamine red X (RRX)-conjugated donkey anti-goat, anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories) were used for immunofluorescence at a dilution of 1:400 for Cy5 antibody and 1:200 for the others. Anti-mouse (IgG) and anti-rabbit (IgG) horseradish peroxidase (Amersham Pharmacia Biociences) were used at a dilution of 1:10 000 for Western blot analysis.

Cell culture

HeLa (93021013) and RAW 264.7 (91062702) cells were obtained from the European Collection of Cell Cultures, Salisbury, UK. Swiss 3T3 murine fibroblast cells were kindly provided by Dr E. Caron (Imperial College London, UK). Cells were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FCS. Cells were grown at 37°C in 5% CO2.

Bacterial infection of cells and immunofluorescence microscopy and replication assays

HeLa and Swiss 3T3 cells were infected with exponential phase S. Typhimurium as described previously (Beuzón et al., 2000). Macrophages were infected with opsonized, stationary phase S. Typhimurium as described previously (Beuzón et al., 2000). To follow a synchronized population of bacteria, host cells were washed after 15 min (HeLa and Swiss 3T3 cells) or 25 min (macrophages) of exposure to S. Typhimurium and subsequently incubated in medium containing gentamicin to kill extracellular bacteria. For immunofluorescence, cells were fixed in paraformaldehyde, permeabilized, and incubated with antibodies as described (Beuzón et al., 2000). Labelled cells were analysed using a confocal laser scanning microscope (LSM510; Zeiss). For enumeration of intracellular bacteria, macrophages were washed three times with PBS, lysed with 0.1% Triton X-100 for 10 min and dilution series were plated onto LB agar.

Preparation of protein fractions from bacteria grown in vitro

Bacterial cell densities were determined by measurement of the OD600 after overnight growth. To ensure that protein from equal numbers of cells was analysed, in all experiments protein samples were adjusted to OD600 values such that a volume corresponding to 10 ml of a culture of OD600 0.6 was taken up in 100 µl of protein-denaturing buffer for gel electrophoresis. The secreted and total bacterial cell pellet were prepared as described before (Beuzón et al., 1999; Yu et al., 2004).

Protein purification and kinase assay

Plasmids for expression of 6-His fusion proteins (SteC-6His, SteCK256H-6His and C-SteC-6His) were constructed in the pET28b vector using the primers listed in Table S1 and introduced into E. coli Rosetta cells (Novagen). Protein expression was induced by using IPTG (0.1 mM) and cells were lysed using French press. Samples were centrifuged at 16 000 g for 45 min, and protein samples were samples were passed through a HisTrap HP column (GE Healthcare). Bound proteins were washed and eluted from the column using elution buffer (25 mM Hepes buffer pH 8.0, 500 mM KCl, 5% glycerol) with increasing concentration of imidazole (50–500 mM). Samples were dialysed in 40 mM Tris-HCl, pH 7.4, overnight and used directly for kinase assays. For assays, 10 µg of expressed protein (SteC-6His, SteCK256H-6His or C-SteC-6His) was added to a reagent mixture containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 100 mM NaCl, 1 mM Dithiothreitol, 20 µM ATP, 2 µCi [γ-32P]ATP (Amersham, 370 MBq ml-1, 3000 Ci mmol-1), 10 µg MBP (Sigma). The mixture was incubated for 30 min at 30°C and then subjected to SDS-PAGE followed by Coomassie blue staining and autoradiography.

Transfection of Swiss 3T3 cells

Transfection vectors for expression of c-myc-tagged SteC, SteCK256H and C-SteC were constructed in the pRK5myc vector using the primers listed in Table S1. Myc-tagged active ROCK (ROCK-K) was a gift from Dr E. Caron (Imperial College London, UK). Swiss 3T3 fibroblast cells were seeded onto glass coverslips (12 mm diameter) at a density of 5 × 104 cells ml-1, 24 h before transfection. The jetPEI (Autogen Bioclear) protocol for transfection was followed according to manufacturer’s recommendations: 3 µg of transfection vector DNA was added to 100 µl of sterile 150 mM NaCl. In a separate tube, 6 µl of jetPEI cations was added to 100 µl of sterile 150 mM NaCl. The jetPEI cations mixture was then added to the transfection vector mix and left for 30 min at room temperature. After incubation, the mixture was added to the cultured Swiss 3T3 cells, centrifuged at 180 g for 5 min, and then incubated at 37°C in 5% CO2. Cells were transfected for 20 h, after which serum-free medium was added for another 3 h before the cells were fixed in paraformaldehyde, permeabilized and labelled as described above.

Competitive Index assay

Female BALB/c mice (B and K Universal, Hull, UK) of 18–22 g were used for all infection studies and were challenged either by i.p. or by oral gavage (p.o.) with 0.2 ml of bacteria suspended in physiological saline solution. The bacterial inocula used were 1 × 109 (i.p.) or 1 × 108 (p.o.) cfu of each strain. At least five mice were inoculated per strain mixture for each experiment. Mice were sacrificed 48 h (i.p.) or 4 days (p.o.) after inoculation. Each CI value is the mean of three independent experiments.
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Supplementary material

The following supplementary material is available for this article online:

Table S1. Oligonucleotide primers used in this study.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1462-5822.2007.01010.x

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