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The roles of SsrA–SsrB and OmpR–EnvZ in the regulation of genes encoding the *Salmonella typhimurium* SPI-2 type III secretion system

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The type III secretion system (TTSS) encoded by *Salmonella typhimurium* pathogenicity island 2 (SPI-2) is expressed after bacterial entry into host cells. The SPI-2 TTSS secretes the translocon components SseBCD, which translocate across the vacuolar membrane a number of effector proteins whose action is required for intracellular bacterial replication. Several of these effectors, including SifA and SifB, are encoded outside SPI-2. The two-component regulatory system SsrA–SsrB, encoded within SPI-2, controls the expression of components of the SPI-2 TTSS apparatus as well as its translocated effectors. The expression of SsrA–B is in turn regulated by the OmpR–EnvZ two-component system, by direct binding of OmpR to the *ssrAB* promoter. Several environmental signals have been shown to induce *in vitro* expression of genes regulated by the SsrA–B or OmpR–EnvZ systems. In this work, immunoblotting and flow cytometry were used to analyse the roles of SsrA–B and OmpR–EnvZ in coupling different environmental signals to changes in expression of a SPI-2 TTSS translocon component (SseB) and two effector genes (*sifA* and *sifB*). Using single and double mutant strains the relative contribution of each regulatory system to the response generated by low osmolarity, acidic pH or the absence of Ca^{2+} was determined. SsrA–B was found to be essential for the induction of SPI-2 gene expression in response to each of these individual signals. OmpR–EnvZ was found to play a minor role in sensing these signals and to require a functional SsrA–B system to mediate their effect on SPI-2 TTSS gene expression.

INTRODUCTION

Type III secretion systems (TTSSs) mediate translocation of effector proteins from the bacterial cytoplasm of Gram-negative pathogens into the eukaryotic host cell. Once delivered into the host cell, effectors alter host cell functions to the advantage of the pathogen. TTSSs comprise a needle-like secreton for exporting proteins across the bacterial cell envelope, a translocon for transferring effector proteins into the host cell, several regulators that control gene transcription and protein secretion, chaperones that bind to and are needed for secretion of translocator and effector proteins, and the effector proteins themselves (Cornelis & Van Gijsegem, 2000; Hueck, 1998).

Serovars of *Salmonella enterica* encode two distinct virulence-associated TTSSs located within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), which are involved in different aspects of *S. enterica* pathogenicity. The SPI-1 TTSS of *S. enterica* serovar Typhimurium (*S. typhimurium*) translocates at least eight effector proteins that control several processes, including host cell invasion, an apoptotic-like effect in macrophages, and trans-epithelial migration of neutrophils (Hersh *et al.*, 1999; Zhou & Galán, 2001). The SPI-1 TTSS is expressed optimally in growth conditions that reflect those in the lumen of the small intestine, including low oxygen, high osmolarity and slight alkalinity (pH 8) (Bajaj *et al.*, 1996). The *S. typhimurium* SPI-2 TTSS is required for systemic infection of mice, and intracellular replication in both macrophages and epithelial cells (Cirillo *et al.*, 1998; Hensel *et al.*, 1995, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI-2 TTSS gene expression is induced inside the host cell and requires the two-component regulatory system SsrA–B, also encoded within SPI-2 (Cirillo *et al.*, 1998). SsrA–B controls the expression of genes encoding the components of the SPI-2 TTSS, as well as genes encoding SPI-2 effectors located both in SPI-2 and elsewhere in the chromosome (Beuzón *et al.*, 2000; Brumell *et al.*, 2003; Cirillo *et al.*, 1998;

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Abbreviations: FCS, fetal calf serum; GFP, green fluorescent protein; SCV, *Salmonella*-containing vacuole; SPI-2, *Salmonella typhimurium* pathogenicity island 2; TRSC, Texas red sulfonyl chloride; TTSS, type III secretion system.

Knodler *et al.*, 2002; Miao & Miller, 2000; Worley *et al.*, 2000). Two effector proteins, SlrP and SspH1, which have been shown to be translocated via both SPI-1 and SPI-2 TTSS, are expressed constitutively, in a SsrA–B-independent manner (Miao & Miller, 2000).

The expression of SsrA–B is regulated by the OmpR–EnvZ two-component system. OmpR binds directly to the *ssrAB* promoter (Lee *et al.*, 2000). In *Escherichia coli*, OmpR–EnvZ has been shown to be responsible for both activation and repression of gene expression, in response to changes in osmolarity and pH (Heyde & Portalier, 1987). The OmpR–EnvZ system is required for *Salmonella* replication and survival within macrophages (Lee *et al.*, 2000) and is necessary for full virulence in mice (Chatfield *et al.*, 1991; Dorman *et al.*, 1989).

Several studies have analysed SPI-2 TTSS gene expression in different conditions, thought to reflect the environment within the *Salmonella*-containing vacuole (SCV). Bacteria grown in different minimal media express SPI-2 TTSS genes when reaching stationary phase (Beuzón *et al.*, 1999; Deiwick & Hensel, 1999; Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*, 2002). In addition, low osmolarity in the growth medium has been shown to play a role in the induction of SPI-2 TTSS gene expression (Lee *et al.*, 2000). Low concentrations of Mg^{2+}, Ca^{2+} or PO_4^{3-} in growth media have also been reported to stimulate SPI-2 TTSS gene expression (Deiwick *et al.*, 1999), although more recent studies have failed to reproduce the effect of low concentrations of Mg^{2+} (Lee *et al.*, 2000; Miao *et al.*, 2002). It has been shown that the SCV undergoes acidification to a pH between 4·0 and 5·0 (Rathman *et al.*, 1996). Using transcriptional fusions of several SPI-2 genes to a gene encoding the green fluorescent protein (GFP), Cirillo *et al.* (1998) reported that inhibition of SCV acidification abolished SPI-2 TTSS gene expression inside the host cell. Using fusions to different reporters Lee *et al.* (2000) and Miao *et al.* (2002) found that acidic pH induced SPI-2 TTSS gene expression *in vitro* but Deiwick *et al.* (1999) found no significant differences in SPI-2 gene expression in response to pH changes.

In this study, we have determined the effect of [Ca^{2+}], osmolarity and pH on SPI-2 gene expression and the relative influence exerted by the SsrA–B and OmpR–EnvZ two-component regulatory systems in this process. Our results show that the effects of these signals on SPI-2 TTSS gene expression are completely dependent on SsrA–B but only partially dependent on OmpR–EnvZ. Futhermore, the effect of OmpR–EnvZ on SPI-2 TTSS gene expression requires a functional SsrA–B system, which indicates that the effect of OmpR–EnvZ on SPI-2 gene expression is mediated through *ssrAB*.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strain HJG1 was constructed by P22-mediated transduction of an *ompR1009*::Tn*10* mutation from strain HH182 to strain P3F4. Bacteria were grown in Luria–Bertani (LB) medium or in MES-buffered magnesium minimal medium (MgM) containing 170 mM MES at the corresponding pH (7·5 or 4·5), 5 mM KCl, 7·5 mM $(NH_4)_2SO_4$, 0·5 mM K_2SO_4, 1 mM KH_2PO_4, 8 μM $MgCl_2$, 38 mM glycerol and 0·1 % Casamino acids (Hmiel *et al.*, 1986). Antibiotics were added at the following concentrations, as appropriate: ampicillin, 50 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 25 μg ml^{-1}. Bacteria were grown at 37 °C overnight with aeration.

Table 1. Strains used in this study

Name	Description	Source or reference
12023	Wild-type	NTCC
P3F4	*ssrA*::mTn*5*	Hensel *et al.* (1995)
HH102	Δ*sseB*::*aphT*	Hensel *et al.* (1998)
HH182	*ompR1009*::Tn*10*	Beuzón *et al.* (2000)
HJG1	*ssrA*::mTn*5*, *ompR1009*::Tn*10*	This study

Plasmids. Plasmid pFVP25.1, carrying *gfpmut3A* under the control of a constitutive promoter, was introduced into bacterial strains for fluorescence visualization where indicated (Valdivia & Falkow, 1997). pID835 is a derivative of pFVP25, a vector carrying a promoterless *gfpmut3A* (Valdivia *et al.*, 1996). pID835 contains a transcriptional fusion of the promoter region of the *sifA* region to the *gfp* gene. A fragment including 356 bp of the *sifA* promoter region and the sequence encoding the 5′ −27 bp of *sifA* was amplified by PCR from 12023 genomic DNA using the primers SIFA-Prf1 (5′-CCGGAATTCTAATTGCGCAACGCTAAC-3′) and SIFA-Prr1 (5′-CGCGGATCCAACCATTCCCTATAGTAA-3′). The 374 bp PCR product, containing terminal *Eco*RI and *Bam*HI sites, was digested and ligated into pFVP25, generating pID835. pID836 is a derivative of pFVP25, containing a transcriptional fusion of the promoter region of *sifB* to the *gfp* gene. A 1·5 kb fragment containing the *sifB* promoter region and the sequence encoding the 5′ −27 bp of the *sifB* gene was amplified by PCR from 12023 genomic DNA using the primers SifB-2 (5′-GGATCCTTTGAGCCTCCTCGCAGG-3′) and SifB-5 (5′-CATATGACTCTGGTGATGAGCCTC-3′), and ligated into pCR2.1 topo vector (Novagen). The 1·5 kb fragment was excised from this intermediate plasmid by *Eco*RI digestion and then digested with *Dra*I, generating an *Eco*RI–*Dra*I 562 bp product, which was ligated into *Eco*RI–*Dra*I-digested pFPV25, generating pID836.

Preparation of protein samples. Bacterial cell densities were determined by measurement of the OD_{600}. To ensure that protein from equal numbers of cells was analysed, in all experiments protein samples were adjusted to OD_{600} values such that each fraction from a 10 ml culture of OD_{600} 0·6 was taken up in 100 μl protein-denaturing buffer for gel electrophoresis.

PAGE and Western analysis of proteins. Protein samples were dissolved in the appropriate volume of protein-denaturing buffer containing 62·5 mM Tris/HCl pH 6·8, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol and 0·02 % bromophenol blue and held at 100 °C for 5 min. Proteins were immediately separated on a 12 % SDS-polyacrylamide gel (Laemmli, 1970). Proteins were transferred from gels to Immobilon-P membranes (Millipore) using a semi-dry blotting apparatus (Bio-Rad) with the buffer described by Kyhse-Andersen (1984). Westerns were developed using the ECL detection system under the conditions recommended by the manufacturer (Amersham Life Science). Rabbit anti-SseB (Beuzón *et al.*, 1999) or anti-RecA (a gift from Kenji Adzuma, The Rockefeller University, New York, USA) polyclonal antibodies, or mouse monoclonal anti-GFP (Clontech) were used as primary antibodies.

Donkey anti-rabbit or anti-mouse horseradish-peroxidase-conjugated antibodies (Amersham Life Science) were used as secondary antibodies. Affinity purification of anti-SseB antibody was performed using the method described by Ruiz-Albert *et al.* (2003).

Preparation of bacteria for flow cytometric analysis. *S. typhimurium* 12023 wild-type and mutant strains carrying pID835 or pID836 plasmids were centrifuged at 4000 ***g***, and pellets were resuspended in PBS. In each experiment, strain 12023 and strain 12023 carrying pFVP25.1 were used as negative and positive controls for fluorescence, respectively. For each sample, 10^5 cells were analysed on a FACS Calibur cytometer (Becton Dickinson). GFP was detected at 525 nm in the FL1 channel. Data were analysed with CellQuest software. Flow cytometric data were analysed as follows. The geometric mean of the fluorescence of each strain in three independent experiments was calculated. The fold increase in fluorescence of the *ssrA*, *ompR*, or *ssrA ompR* mutant strains versus that of the wild-type was calculated by dividing the geometric mean fluorescence of the wild-type strain by the geometric mean fluorescence of the mutants.

Antibodies and reagents. Anti-*Salmonella* goat polyclonal antibody CSA-1 was purchased from Kirkegaard and Perry Laboratories and was used at a dilution of 1 : 400. Texas red sulfonyl chloride (TRSC)-conjugated donkey anti-goat antibody was purchased from Jackson Immunoresearch Laboratories and used at a dilution of 1 : 400.

Cell culture. RAW 264.7 cells were obtained from ECACC (ECACC 91062702). HeLa cells (clone HtTA1) were kindly provided by Dr H. Bujard (Heidelberg, Germany). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 2 mM glutamine at 37 °C in 5 % CO_2.

Bacterial infection of HeLa cells. HeLa cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5×10^4 cells per well, 24 h before infection. Bacteria were incubated for 16 h at 37 °C with aeration, diluted 1 : 33 in fresh LB broth and incubated in the same conditions for 3·5 h. Cultures were diluted in Earle's buffered salt solution (EBSS) pH 7·4 and added to the HeLa cells at a m.o.i. of 100 : 1. The infection was allowed to proceed for 15 min at 37 °C in 5 % CO_2. The monolayers were washed once with DMEM containing FCS and 100 μg gentamicin ml^{-1} and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 16 μg ml^{-1}.

Immunofluorescence. For immunofluorescence, cell monolayers were fixed in 3·7 % paraformaldehyde in phosphate-buffered saline (PBS) pH 7·4, for 15 min at room temperature and washed three times in PBS. Antibodies were diluted in 10 % horse serum, 1 % bovine serum albumin, 0·1 % saponin in PBS. Coverslips were washed twice in PBS containing 0·1 % saponin, incubated for 30 min with primary antibodies, washed twice with 0·1 % saponin in PBS and incubated for 30 min with secondary antibodies. Coverslips were washed twice in 0·1 % saponin in PBS, once in PBS and once in H_2O, and mounted on Mowiol. Samples were analysed using an Olympus BX50 fluorescence microscope or a Zeiss LSM510 confocal laser scanning microscope. For staining with anti-SseB antibody, cells were permeabilized for 10 min by incubation with 0·1 % Triton X-100 in PBS, prior to incubation with the antibody.

Bacterial infection of macrophages for flow cytometric analysis. Macrophages were seeded at a density of 4×10^5 cells per well in 24-well tissue culture plates, 24 h before use. Bacteria were cultured at 37 °C with shaking until they reached an OD_{600} of 2·0. The cultures were diluted to an OD_{600} of 1·0 and opsonized in DMEM containing FCS and 10 % normal mouse serum for 20 min. Bacteria were added to the monolayers at a m.o.i. of 100 : 1, centrifuged at 170 ***g*** for 5 min at room temperature and incubated for 25 min at 37 °C in 5 % CO_2. Macrophages were washed once with DMEM containing FCS and 100 μg ml^{-1} gentamicin and incubated in this medium for 1 h. The medium was replaced with DMEM containing FCS and 16 μg ml^{-1} gentamicin for the rest of the experiment. Bafilomycin A1 was added to cell monolayers 15 min prior to the addition of the bacteria, to a final concentration of 100 nM, where indicated. At 2 h after bacterial uptake, cells were lysed with 0·1 % Triton X-100 for 10 min, and used for flow cytometry.

Flow cytometric analysis of infected macrophages. Infected macrophage lysates were resuspended in a mixture of 250 μl 0·1 % Triton X-100 and 250 μl PBS and kept on ice for immediate analysis. For each sample, 10^4 bacterial-sized particles were analysed on a FACS Calibur cytometer (Becton Dickinson). GFP was detected at 525 nm in the FL1 channel. Data were analysed with CellQuest software.

RESULTS

Effect of SsrA–B and OmpR–EnvZ on SPI-2 TTSS gene expression in infected cells

To analyse the expression of SPI-2 TTSS effector genes, transcriptional fusions of the *sifA* and *sifB* promoters to a gene encoding GFP were generated in the promoter trap vector pFPV25 (Valdivia & Falkow, 1997; Valdivia *et al.*, 1996). SifA is translocated into the host cell by the SPI-2 TTSS (Brumell *et al.*, 2001), and is necessary for the maintenance of the SCV membrane (Beuzón *et al.*, 2000) and for Sif (*Salmonella*-induced filament) formation (Stein *et al.*, 1996). SifB is also translocated by the SPI-2 TTSS and is expressed *in vitro* in an SsrA–B-dependent manner (Miao & Miller, 2000). Translocated SifB is localized on the SCV membrane and Sifs (Freeman *et al.*, 2003); however, its function is unknown (Ruiz-Albert *et al.*, 2002). The expression patterns of the P_{sifA} : : *gfp* and P_{sifB} : : *gfp* transcriptional fusions were analysed in HeLa cells infected with wild-type *S. typhimurium* harbouring the respective plasmids. Expression of P_{sifA} : : *gfp* was only detected intracellularly, and was completely dependent on SsrA–B and partially dependent on OmpR–EnvZ, a pattern similar to that reported for a SifA : : GFP translational fusion (Beuzón *et al.*, 2000). GFP could be detected as early as 2 h after invasion, and reached a peak 6 h after invasion (data not shown). Similarly, P_{sifB} : : *gfp* expression was only detected intracellularly, and was completely dependent on SsrA–B (Fig. 1a). The intensity of fluorescence in bacteria carrying the P_{sifB} : : *gfp* fusion was clearly higher than that in bacteria carrying P_{sifA} : : *gfp* (data not shown). Approximately 50 % of *ompR* mutant bacteria carrying P_{sifB} : : *gfp* displayed GFP expression inside the host cell, and the overall intensity of the fluorescence was noticeably lower than that displayed by wild-type bacteria carrying the same plasmid (data not shown). These results are similar to those reported for a SifA : : GFP translational fusion (Beuzón *et al.*, 2000) and for a P_{ssaH} : : *gfp* transcriptional fusion (Cirillo *et al.*, 1998).

As an independent method to monitor the levels of another SPI-2-secreted protein, we used an anti-SseB antibody. SseB is a translocon component of the SPI-2

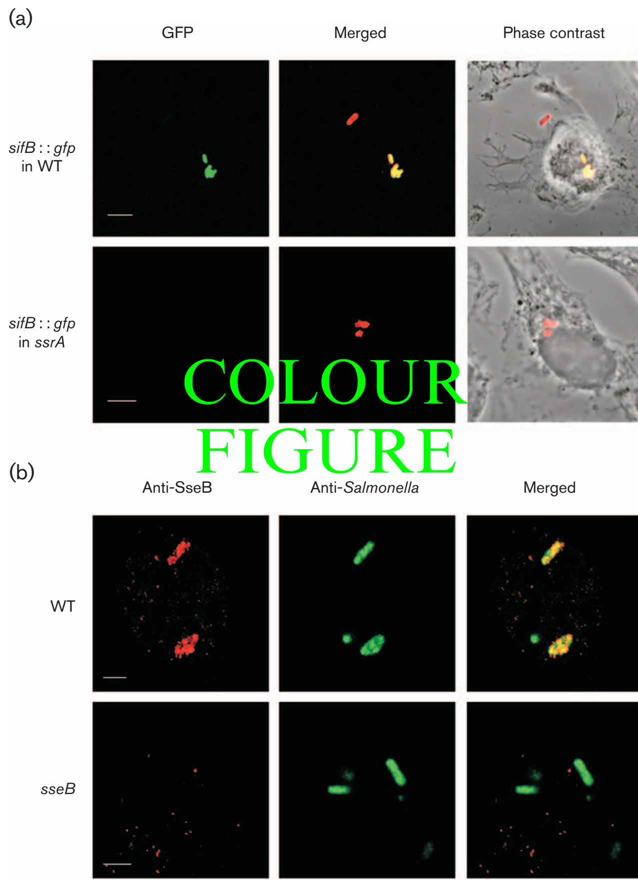

Fig. 1. (a) P_{sifB}::*gfp* expression in *S. typhimurium* wild-type (WT; 12023) and *ssrA* mutant strains. Confocal immunofluorescence analysis in HeLa cells 6 h after invasion with different strains carrying a plasmid-borne P_{sifB}::*gfp* transcriptional fusion. *S. typhimurium* was detected with goat anti-*Salmonella* and TRSC-conjugated donkey anti-goat antibodies (red). The right upper panel shows one extracellular bacterium not expressing GFP, and several GFP-positive intracellular bacteria. The lower panel shows lack of intracellular P_{sifB}::*gfp* expression in an *ssrA* mutant strain. Scale bars, 5 μm. (b) SseB was detected intracellularly in HeLa cells 6 h after invasion with wild-type but not with an *sseB* mutant strain. Bacteria were detected with goat anti-*Salmonella* and FITC-conjugated donkey anti-goat antibodies (green). SseB was detected with rabbit anti-SseB polyclonal affinity purified-antibody and TRSC-conjugated donkey anti-rabbit antibodies (red). Scale bars, 1 μm.

TTSS (Nikolaus *et al.*, 2001), which is secreted *in vitro* in response to acidic pH (Beuzón *et al.*, 1999). SseB is encoded within the SPI-2 *sseABCDEFG* operon, and displays the same expression profile as other components of the SPI-2 TTSS (Deiwick *et al.*, 1999). Affinity-purified anti-SseB antibody was used to detect SseB by immunofluorescence microscopy in wild-type *S. typhimurium* after infection of host cells. SseB was detected in some wild-type bacteria 2 h after invasion, and was detected maximally at 6 h after invasion (Fig. 1b and data not shown). At this time point, SseB was detected in 50±2·1 % of wild-type, 0 % of *ssrA* mutant, and 19·5±3·9 % of *ompR* mutant bacteria (mean±SEM, $n=100$) in four independent experiments. These numbers are similar to those reported for SifA::GFP (Beuzón *et al.*, 2000) and *ssaH*::*gfp* expression (Cirillo *et al.*, 1998).

Regulation of *sifA* and *sifB* expression in vitro

Mg minimal (MgM) salts medium (see Methods) has been used to stimulate SPI-2 TTSS gene expression and to attempt to identify the signals that trigger expression of the SPI-2 TTSS inside the SCV (Deiwick & Hensel, 1999; Deiwick *et al.*, 1999; Miao *et al.*, 2002; Beuzón *et al.*, 1999). Growth of bacteria in this medium results in a strong induction of SPI-2 TTSS expression. Therefore, this medium was used in this study as a basis to further investigate the signals stimulating SPI-2 gene expression. The pH of bacterial cultures after overnight growth in MgM medium buffered to pH 7·5 before inoculation remained unchanged.

Wild-type *S. typhimurium* carrying either P_{sifA}::*gfp* or P_{sifB}::*gfp* transcriptional fusions were grown overnight in MgM medium. Expression from each plasmid was detected by flow cytometry (Fig. 2a, b) and immunoblotting using an anti-GFP antibody (Fig. 3a, lower panel). As observed in infected cells (Fig. 1a), we found that expression driven by the *sifB* promoter was higher than that driven by P_{sifA} (Fig. 2a, b, upper panels).

P_{sifA}::*gfp* and P_{sifB}::*gfp* expression was then analysed in *ssrA* or *ompR* single mutant strains, and *ssrA ompR* double mutant strains after growth in the same culture conditions. The expression of P_{sifA}::*gfp* was reduced to levels undetectable by flow cytometry in any of these three mutant strains (Fig. 2a). P_{sifB} expression levels were reduced but were reproducibly detectable in *ssrA*, *ompR* and *ssrA ompR* mutant strains (Fig. 2b). Expression of P_{sifB}::*gfp* was reduced more than tenfold in the *ssrA* mutant strain and twofold in the *ompR* mutant. The expression in the *ssrA ompR* double mutant strain was equivalent to that in the *ssrA* single mutant (Fig. 2b), indicating that the effect of OmpR is dependent on a functional SsrA–B system, in agreement with previous work (Lee *et al.*, 2000), and that the signals that OmpR–EnvZ responds to are routed through SsrA–B. These results indicate that the SsrA–B and OmpR–EnvZ-dependent expression displayed in infected cells by the SPI-2 TTSS genes (Fig. 1) can be replicated *in vitro* by using MgM as bacterial growth medium.

Contribution of different environmental signals to SPI-2 TTSS gene expression *in vitro*

We next investigated the contribution of $[Mg^{2+}]$ to SPI-2 TTSS gene expression by growing bacterial strains in MgM medium containing either 8 μM or 200 μM $MgCl_2$. Flow cytometry was carried out using wild-type bacteria harbouring either P_{sifA}::*gfp* or P_{sifB}::*gfp* fusions, after

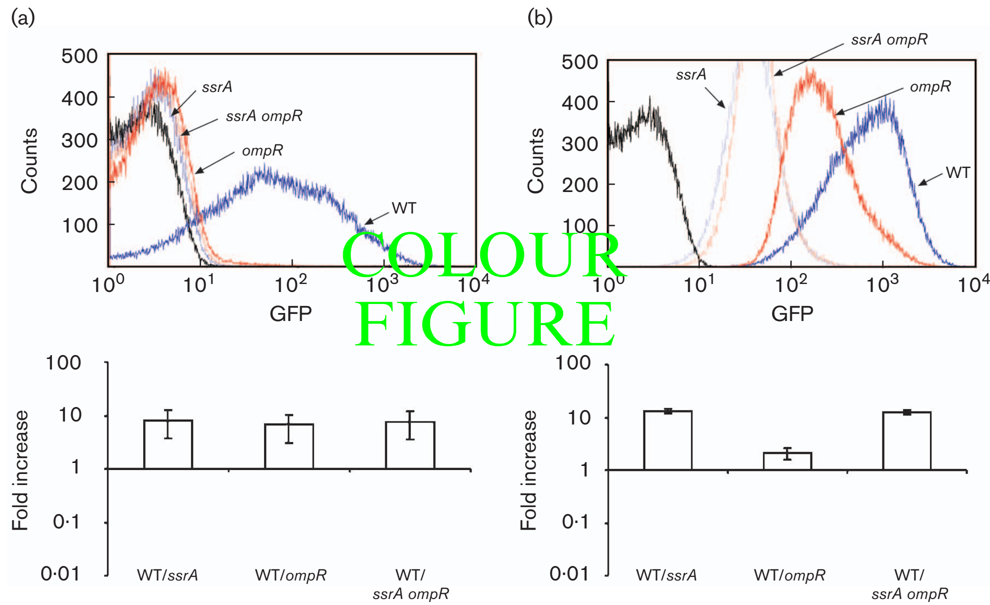

Fig. 2. Flow cytometric analysis of *Salmonella* strains carrying P_{sifA}::*gfp* (a) or P_{sifB}::*gfp* (b) transcriptional fusions, grown overnight at 37 °C in MgM medium. Lines depict GFP expression in *S. typhimurium* wild-type (blue), *ssrA* (blue dashed), *ompR* (red) and *ssrA ompR* (red dashed) mutant strains. The black line shows a non-fluorescent negative control (*S. typhimurium* wild-type not carrying any transcriptional fusion). In each individual experiment 10^5 bacterial-sized particles were analysed. In the upper panels, the fluorescence intensity of each particle is reported on the *x* axis and the number of bacterial-sized particles is shown on the *y* axis. Lower panels show the fold increase in expression detected by flow cytometry, calculated as the geometric mean ($n=3$) of the fluorescence of the wild-type strain carrying the corresponding plasmids divided by the fluorescence of each mutant strain carrying the same plasmid.

overnight growth in MgM medium at pH 7·5. No significant differences in fluorescence were detected for either fusion after growth in 8 μM or 200 μM $MgCl_2$ (Fig. 3a, upper panel). Similarly, no significant differences were observed by immunoblotting either with an anti-GFP antibody using wild-type bacteria carrying either P_{sifA}::*gfp* or P_{sifB}::*gfp* fusions (Fig. 3a, lower panel), or with an anti-SseB antibody using wild-type bacteria (Fig. 3b).

Deiwick *et al.* (1999) reported that, after growth in MOPS-salts medium (O'Neal *et al.*, 1994), the presence of 200 μM Mg^{2+} inhibited the expression of *ssaB*, and this effect could be reverted by lowering the [PO_4^{3-}]. However, in the presence of 8 μM Mg^{2+}, *ssaB* expression was independent of [PO_4^{3-}]. In agreement with these results, no significant differences were observed in P_{sifA}::*gfp* or P_{sifB}::*gfp* expression in response to changes in [PO_4^{3-}] in medium containing 8 μM Mg^{2+} (data not shown). However, under our assay conditions, no inhibitory effect of high [Mg^{2+}] was observed (Fig. 3a, b). This prevented us from confirming a possible effect of PO_4^{3-} starvation in reverting an inhibitory effect of high [Mg^{2+}].

Removal of 38 mM glycerol from the medium had no significant effect on *sifA*::*gfp* or *sifB*::*gfp* expression, when analysed by flow cytometry (data not shown). Similarly, reducing [$(NH_4)_2SO_4$] in the growth medium by 10- or 100-fold did not have a significant effect on *sifA*::*gfp* or *sifB*::*gfp* expression (data not shown).

Together, these results indicate that changes in the concentrations of Mg^{2+}, PO_4^{3-}, glycerol and $(NH_4)_2SO_4$ have no significant effect on SPI-2 gene expression, after overnight growth in MgM medium.

The absence of Ca^{2+} leads to SPI-2 TTSS gene expression via SsrA–B

It has been shown previously that the absence of Ca^{2+} in Tris-buffered MgM medium induces expression from the SPI-2 promoters P_{ssaB} and P_{sseA} (Deiwick *et al.*, 1999). Consistent with these results, the fluorescence of wild-type bacteria carrying either P_{sifA}::*gfp* or P_{sifB}::*gfp* increased by six- to eightfold and eightfold, respectively, after growth in MgM medium, compared to the expression after growth in MgM medium containing 2 mM Ca^{2+} (data not shown and Fig. 4b). Similarly, SseB was undetectable in wild-type bacteria grown overnight in MgM medium in the presence of 2 mM Ca^{2+}, but was detected after growth in MgM medium without Ca^{2+} (Fig. 4c).

To determine which regulatory system mediates the effect of low [Ca^{2+}], we analysed P_{sifA}::*gfp* expression in *ssrA* or *ompR* single mutant strains, and *ssrA ompR* double mutant

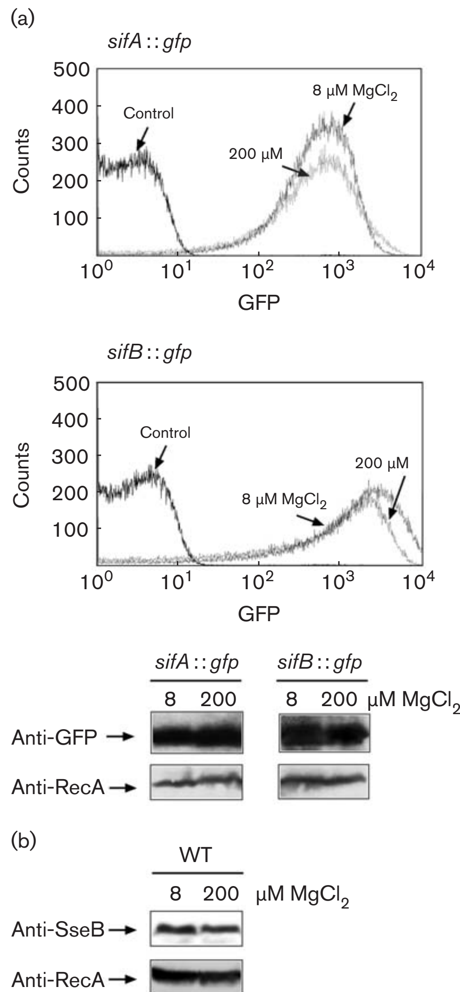

Fig. 3. (a) P_{sifA} : : *gfp* and P_{sifB} : : *gfp* expression by wild-type *S. typhimurium* carrying the corresponding plasmid grown in MgM medium or in MgM containing 200 μM Mg^{2+}, overnight at 37 °C. GFP expression from P_{sifA} : : *gfp* and P_{sifB} : : *gfp* was detected by immunoblot analysis with mouse monoclonal anti-GFP and anti-mouse horseradish-peroxidase-conjugated antibodies (lower panel) and by flow cytometry (upper two panels). (b) Expression of SseB in response to changes in [Mg^{2+}]. Immunoblot analysis of *S. typhimurium* wild-type 12023 grown in MgM medium or in MgM with 200 μM Mg^{2+}, overnight at 37 °C. SseB was detected with rabbit polyclonal anti-SseB and anti-rabbit horseradish-peroxidase-conjugated antibodies (upper panel). The presence of equal amounts of protein in each sample was confirmed by immunoblot analysis using rabbit polyclonal anti-RecA and anti-rabbit horseradish-peroxidase-conjugated antibodies (lower panel).

strains, after growth in MgM medium with or without 2 mM Ca^{2+}. Since expression of P_{sifA} : : *gfp* in the mutant strains was below the level of detection by flow cytometry, the protein levels were determined by immunoblot analysis. The higher level of *sifA* : : *gfp* expression in low [Ca^{2+}] was completely abolished in the *ssrA* single mutant and the *ssrA ompR* double mutant strains (Fig. 4a). This indicates that induction of *sifA* expression in response to the absence of Ca^{2+} is dependent on the presence of SsrA–B. In the *ompR* mutant *sifA* : : *gfp* expression levels were higher in low [Ca^{2+}] (Fig. 4a), although it was not possible to determine if the level of induction was equivalent to that of the wild-type strain. Similarly, when analysed by flow cytometry, the effect of 2 mM Ca^{2+} on *sifB* : : *gfp* expression was completely abolished in both the *ssrA* single mutant and the *ssrA ompR* double mutant strains, whereas in the *ompR* mutant *sifB* : : *gfp* expression was decreased 3·5-fold (Fig. 4b). Together, these results indicate that the response to low [Ca^{2+}] is mediated mainly through SsrA–B. Although not as important as SsrA–B, OmpR–EnvZ is necessary for full induction of SPI 2 TTSS gene expression in response to low [Ca^{2+}].

Low osmolarity induces SPI-2 TTSS gene expression via SsrA–B

It has been shown previously that high osmolarity (generated by the presence of 20 % sucrose or 0·5 M NaCl) in MgM medium represses expression from the SPI-2 promoter P_{ssaH} (Lee *et al.*, 2000). *S. typhimurium* is not able to use sucrose as a carbon source, but the presence of Casamino acids allows normal growth of bacterial cells in MgM medium containing 20 % sucrose. Expression of P_{sifA} : : *gfp* and P_{sifB} : : *gfp* was eight- and tenfold higher, respectively, in MgM medium than in the same medium containing 20 % sucrose (Fig. 5a, upper panel, and 5b). The addition of the osmoprotectant glycine betaine partially prevented the inhibitory effect of 20 % sucrose on both P_{sifA} : : *gfp* and P_{sifB} : : *gfp* expression, showing that the effect of sucrose is mainly osmotic (Fig. 5a, upper panel, and 5b). SseB levels were also higher in the absence than in the presence of 20 % sucrose (Fig. 5c).

To determine which regulatory system mediates the osmotic effects of sucrose, the *ssrA* or *ompR* single mutant, and *ssrA ompR* double mutant strains carrying P_{sifA} : : *gfp* were grown in MgM medium with or without 20 % sucrose and then analysed by immunoblotting with the anti-GFP antibody. The higher level of *sifA* : : *gfp* expression in low osmolarity was completely abolished in the *ssrA* single mutant and the *ssrA ompR* double mutant strains (Fig. 5a, lower panel). However, the decrease in P_{sifA} : : *gfp* expression in the presence of 20 % sucrose could still be observed in an *ompR* mutant (Fig. 5a, lower panel). Flow cytometry of single and double mutant strains carrying P_{sifB} : : *gfp* showed that the higher level of P_{sifB} : : *gfp* expression at low osmolarity was abolished in the *ssrA* single mutant and *ssrA ompR* double mutant strains. However, in the *ompR* mutant strain, the osmotic effect of sucrose accounted for a sixfold difference in gene expression (Fig. 5b). These results indicate that the effect of osmolarity on the expression from *sifA* and *sifB* promoters is mediated predominantly through SsrA–B, and that OmpR–EnvZ plays a minor role in sensing this signal.

It has been shown that *S. typhimurium* reacts to a sudden

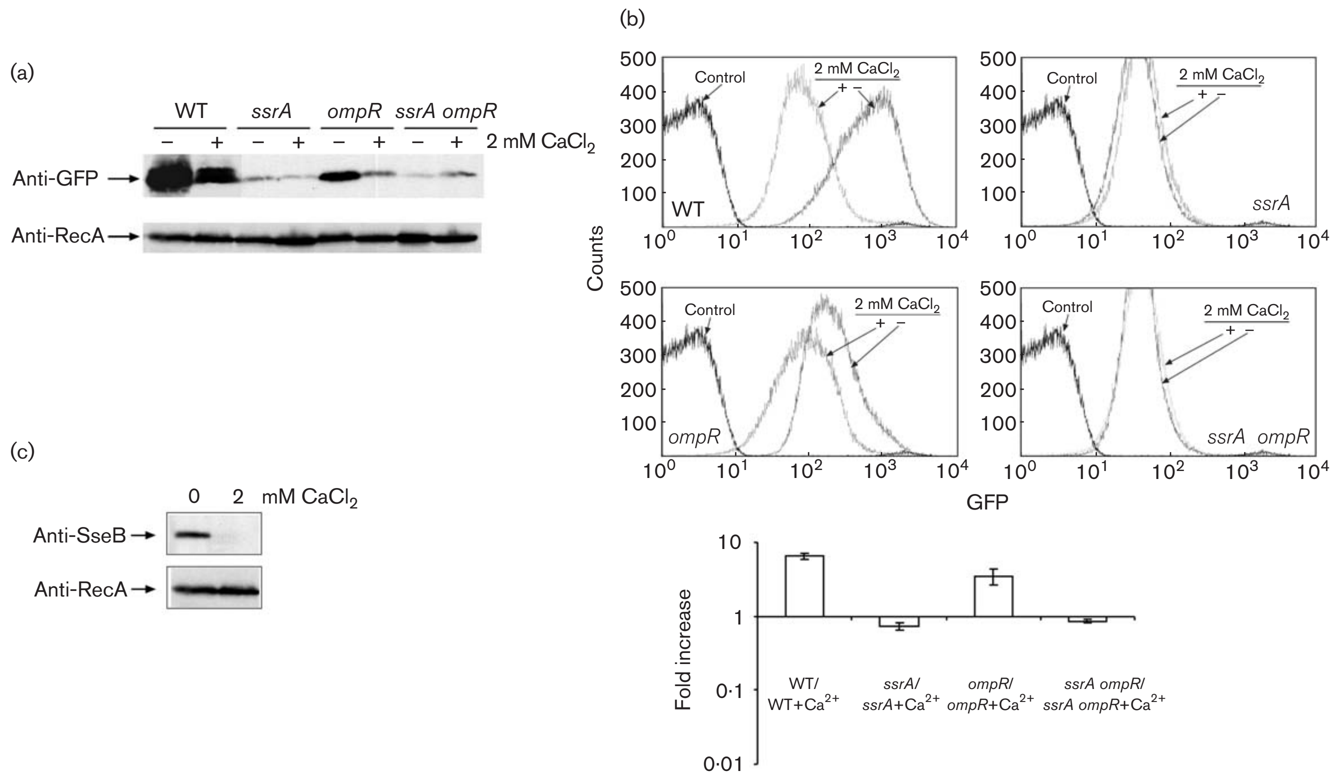

Fig. 4. (a) P_{sifA}::*gfp* expression in *S. typhimurium* wild-type, *ssrA*, *ompR*, or *ssrA ompR* mutant strains grown in MgM medium or in MgM with 2 mM Ca^{2+}, overnight at 37 °C. GFP expression from P_{sifA}::*gfp* was detected by immunoblot analysis using mouse monoclonal anti-GFP and anti-mouse horseradish-peroxidase-conjugated antibodies. The presence of equal amounts of protein in each sample was confirmed as described in Fig. 3(a). (b) P_{sifB}::*gfp* expression in *S. typhimurium* wild-type, *ssrA*, *ompR*, and *ssrA ompR* mutant strains grown in MgM medium or in MgM with 2 mM Ca^{2+}, overnight at 37 °C. GFP expression for P_{sifB}::*gfp* was detected by flow cytometry. The lower panel shows the fold increase in expression detected, calculated as the geometric mean ($n=3$) of the fluorescence of the wild-type and mutant strains carrying P_{sifB}::*gfp*, grown in MgM, divided by that of the corresponding strain grown in MgM with 2 mM Ca^{2+}. (c) Expression of SseB in response to changes in [Ca^{2+}]. Immunoblot analysis of *S. typhimurium* 12023 grown in MgM medium or in MgM with 2 mM Ca^{2+}, overnight at 37 °C. SseB was detected with rabbit polyclonal anti-SseB and anti-rabbit horseradish-peroxidase-conjugated antibodies.

increase in osmolarity by taking up large amounts of K^+ from the environment (Kempf & Bremer, 1998). Jung *et al.* (2001) showed that the EnvZ-autokinase activity of purified and reconstituted EnvZ is stimulated in the presence of increasing [KCl] and the amount of phosphorylated OmpR in the reconstituted signal cascade increases over time in the presence of KCl, suggesting that [K^+] in the medium could act as the signal detected by OmpR–EnvZ when changes in osmolarity occur. Although the [K^+] in the SCV is not known, [K^+] in *Staphylococcus aureus*-containing vacuoles in neutrophils has been reported to be in the 200–300 mM range (Reeves *et al.*, 2002). Therefore, we investigated if changes in [K^+] affect the expression of *sifA*, *sifB* and *sseB*. However, no effects comparable to those caused by changes in osmolarity were detected. The increase of [KCl] from 5 mM to 200 mM caused only a minor increase in the level of SseB detected by immunoblotting (data not shown). No significant differences were observed in P_{sifA}::*gfp* or P_{sifB}::*gfp* expression either by flow cytometry or by immunoblotting using an anti-GFP antibody, at 5 mM or 200 mM KCl (data not shown). Despite these results, we cannot rule out a role for [K^+] in mediating osmolarity-dependent activation of OmpR–EnvZ, since the extracellular increase of [K^+] might not result in an increase in its uptake into the periplasm.

SsrA–B and OmpR–EnvZ are both required to mediate the effect of acidic pH on SPI-2 TTSS gene expression

Flow cytometry and immunoblot experiments were performed to analyse the effect of pH on P_{sifA}::*gfp* and P_{sifB}::*gfp* expression, by comparing the expression of each gene in bacterial cultures grown in MgM medium at pH 7·5 or at pH 4·5. However, the results obtained were too variable to allow any conclusions to be drawn. On the

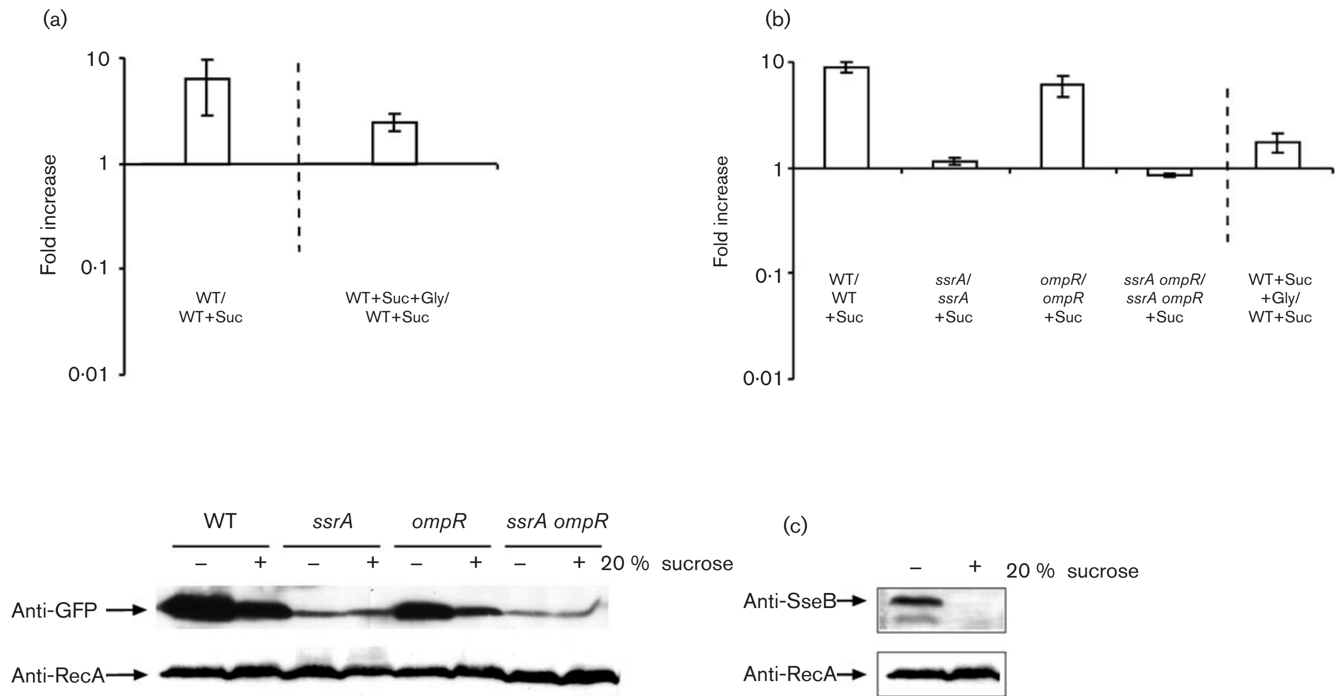

Fig. 5. (a) P_{sifA}: : *gfp* expression by *S. typhimurium* wild-type, *ssrA*, *ompR*, and *ssrA ompR* mutant strains grown in MgM medium or in MgM with 20 % sucrose, overnight at 37 °C. GFP expression from P_{sifA}: : *gfp* was detected by flow cytometry (wild-type strain in upper panel) and by immunoblot analysis using mouse monoclonal anti-GFP and anti-mouse horseradish-peroxidase-conjugated antibodies (lower panel). The upper panel shows the fold increase in expression detected by flow cytometry, calculated as the geometric mean ($n=3$) of the fluorescence of the wild-type strain carrying P_{sifA}: : *gfp* fusion grown in MgM (left column) or in MgM with 20 % sucrose and glycine betaine (right column) divided by the fluorescence of the same strain grown in MgM with 20 % sucrose. (b) P_{sifB}: : *gfp* expression in *S. typhimurium* wild-type, *ssrA*, *ompR*, and *ssrA ompR* mutant strains grown in MgM medium or in MgM with 20 % sucrose, overnight at 37 °C. P_{sifB}: : *gfp* expression was detected by flow cytometry. The fold increase in expression was calculated as the geometric mean ($n=3$) of the fluorescence of the wild-type and mutant strains carrying P_{sifB}: : *gfp* grown in MgM divided by that of the corresponding strain grown in MgM with 20 % sucrose. The right-hand column shows the fluorescence of the wild-type strain carrying P_{sifB}: : *gfp* grown in MgM with 20 % sucrose and glycine betaine divided by the fluorescence of the wild-type strain grown in MgM with 20 % sucrose. (c) Expression of SseB in response to changes in osmolarity. Immunoblot analysis of *S. typhimurium* wild-type 12023 grown in MgM medium or in MgM with 20 % sucrose, overnight at 37 °C. SseB was detected with rabbit polyclonal anti-SseB and anti-rabbit horseradish-peroxidase-conjugated antibodies (upper panel).

other hand, levels of SseB were consistently higher in MgM medium at pH 4·5 than at pH 7·5 (Fig. 6c). The effect of pH on the expression of *sifA*: : *gfp* and *sifB*: : *gfp* was then analysed in infected macrophages, where acidification of the SCV can be prevented by treatment with bafilomycin A1, a specific inhibitor of vacuolar-type H^{+}-ATPase, which acidifies endosomal and lysosomal compartments (Cirillo *et al.*, 1998). P_{sifA}: : *gfp* and P_{sifB}: : *gfp* expression by intracellular wild-type bacteria was strongly inhibited in macrophages pre-treated with bafilomycin A1, as shown by immunofluorescence and flow cytometry, respectively (Fig. 6a, b). Flow cytometric analysis showed that the expression of P_{sifB}: : *gfp* was twofold higher in intracellular bacteria inside non-treated macrophages than inside those treated with bafilomycin A1 (Fig. 6b). These results are in agreement with those obtained by Cirillo *et al.* (1998) for vacuole acidification-dependent induction of *ssaJ*, *sscB* and *spiA* (*ssaC*) expression. The absence of detectable P_{sifA}: : *gfp* expression by both microscopy and flow cytometry when the fusion construct was carried by intracellular *ssrA*, *ompR*, and *ssrA ompR* mutant bacteria prevented further analysis of this promoter.

Flow cytometric analysis showed that P_{sifB}: : *gfp* expression in *ssrA* and *ompR* mutant bacteria within non-treated macrophages was, respectively, 1·2-fold and 1·5-fold higher than that displayed by the same bacterial strains within bafilomycin A1-treated macrophages (Fig. 6b). The effect of bafilomycin A1 was completely abolished only in the *ssrA ompR* double mutant strain. These results suggest that the induction of *sifB* expression by acidic pH inside the vacuole is mediated by both SsrA–B and OmpR–EnvZ.

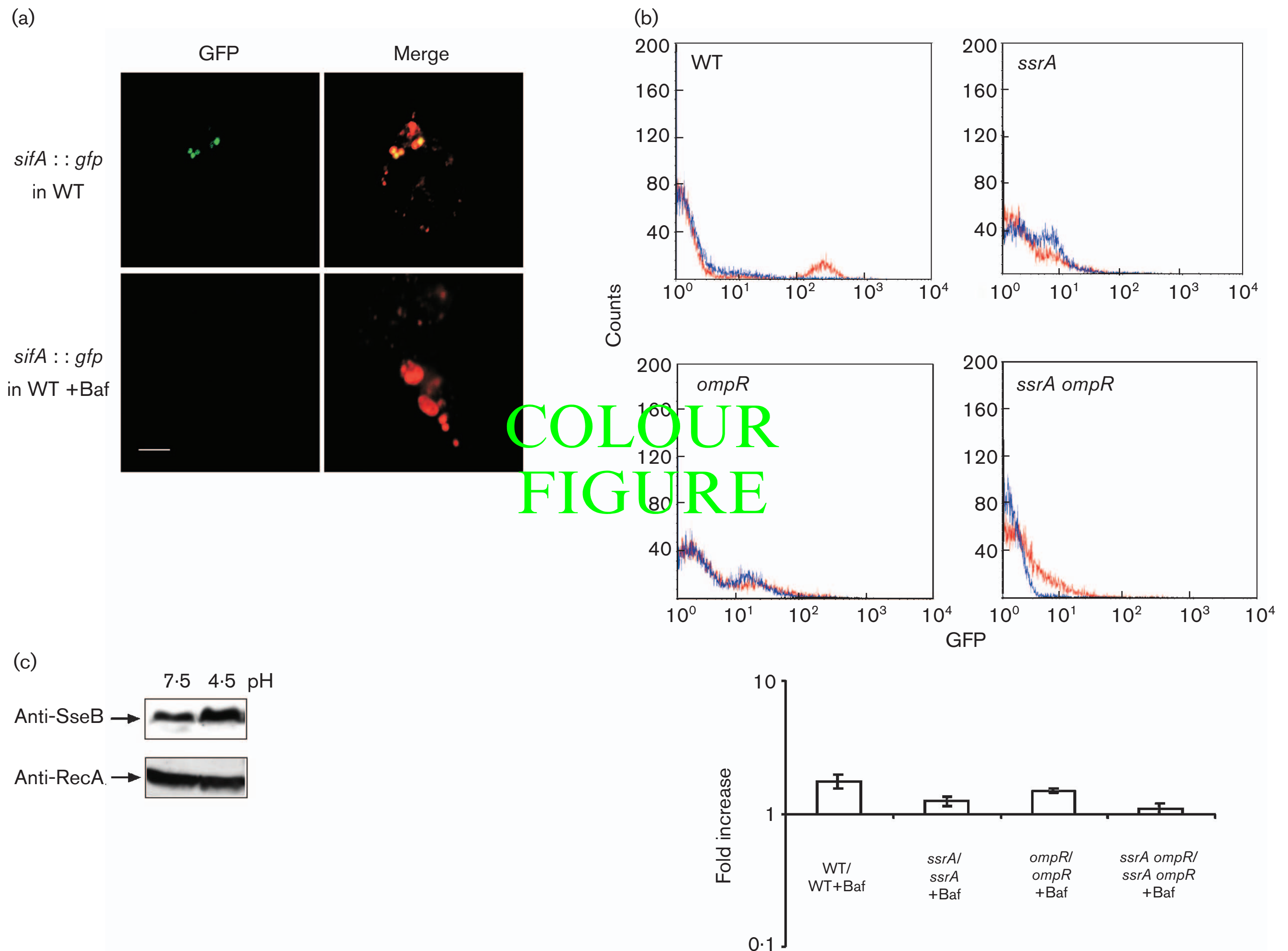

Fig. 6. (a) P_{sifA} : : *gfp* expression in bafilomycin A1-treated or untreated macrophages. Bafilomycin A1-treated or untreated macrophages were infected with *S. typhimurium* wild-type strain 12023 carrying the P_{sifA} : : *gfp* transcriptional fusion and *gfp* expression was analysed by confocal immunofluorescence microscopy of macrophages 2 h after uptake. *S. typhimurium* was detected with goat anti-*Salmonella* and TRSC-conjugated donkey anti-goat antibodies (red). Scale bar, 5 µm. (b) P_{sifB} : : *gfp* expression in bafilomycin A1-treated (blue lines) or untreated (red lines) macrophages. Bafilomycin A1-treated or untreated macrophages were infected with *S. typhimurium* wild-type, *ssrA*, *ompR*, *ssrA ompR* mutant strains carrying the P_{sifB} : : *gfp* transcriptional fusion. At 2 h after uptake, intracellular bacteria were released by treating the samples with 1 % Triton X-100 for 10 min and their fluorescence was measured by flow cytometry. The fold increase in expression was calculated as the geometric mean ($n=3$) of the fluorescence of the wild-type and mutant strains carrying P_{sifB} : : *gfp* from untreated cells divided by that of the corresponding strain released from bafilomycin A1-treated macrophages. (c) Expression of SseB in response to changes in pH. Immunoblot analysis of *S. typhimurium* 12023 grown in MgM medium (pH 7·5) or in MgM pH 4·5 overnight at 37 °C. SseB was detected with rabbit polyclonal anti-SseB and anti-rabbit horseradish-peroxidase-conjugated antibodies.

DISCUSSION

In this study, we have attempted to clarify the nature of the environmental signals that induce SPI-2 TTSS gene expression, and to determine whether these signals are transmitted through the SsrA–B or the OmpR–EnvZ regulatory system. We found that low osmolarity and absence of Ca^{2+} are the major signals affecting SPI-2 TTSS gene expression when bacteria are grown in MgM medium. Our results support those previously reported by Miao *et al.* (2002) and Lee *et al.* (2000) but differ from those of Deiwick *et al.* (1999), who reported that in MgM medium buffered with Tris/HCl or Bistris/HCl, [Mg^{2+}] has an effect on *sseA* and *ssaB* expression. Also, we cannot exclude the possibility that other environmental signals affect SPI-2 gene expression. For example, [Fe^{2+}] appears to have a role in down-regulating the expression of *ssrA*, *sseA* and *sseJ* by an unknown iron-responsive regulatory system (Zaharik *et al.*, 2002).

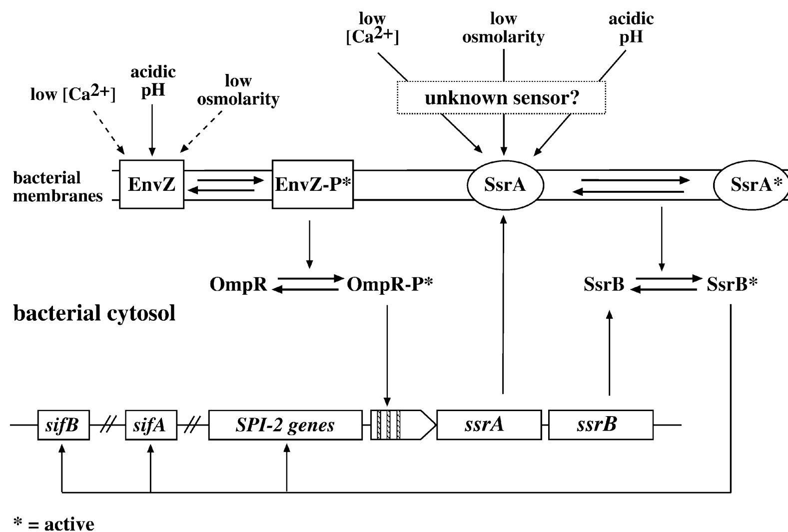

Fig. 7. Model for *Salmonella* SPI-2 TTSS gene expression regulation inside host cells. The OmpR–EnvZ two-component regulatory system has a minor role in mediating the response to the absence of Ca^{2+} and low osmolarity (dashed lines), but a greater role in mediating the response to acidic pH (solid line). The activated form of OmpR (OmpR*) binds to the *ssrAB* promoter and activates the transcription of *ssrA* and *ssrB* genes. SsrA, the sensor element of the SsrA–B two-component system, detects the absence of Ca^{2+}, acidic pH and low osmolarity inside the vacuole directly or via unknown sensor(s), and activates SsrB. SsrB activates the expression of SPI-2-related genes, both inside and outside the pathogenicity island.

The main finding of this work is that the effects on SPI-2 gene expression caused by the absence of Ca^{2+} and low osmolarity are transmitted predominantly through SsrA–B, whereas acidic pH is sensed by both SsrA–B and OmpR–EnvZ (Fig. 7). Lee *et al.* (2000) reported that the effect of osmolarity and acidic pH on *ssaH* transcription was decreased in an *ompR* mutant. In agreement with these results, we found that OmpR–EnvZ is required for the full expression of SPI-2 genes in response to these signals. Furthermore, this effect fully depends on a functional SsrA–B system, since the phenotype of an *ssrA ompR* double mutant in all three conditions is identical to that of the *ssrA* single mutant. These results indicate that the effect of OmpR–EnvZ on the expression of SPI-2 genes, other than *ssrAB*, is not a result of direct binding of OmpR to their promoters but is indirect, probably as a consequence of its binding to the *ssrAB* promoter (Lee *et al.*, 2000).

Our results indicate that SsrA–B, a single two-component regulatory system, can mediate the effect of three physiologically disparate environmental signals: acidic pH, low osmolarity and absence of Ca^{2+}. This is reminiscent of the BvgAS two-component regulatory system, which controls the *Bordetella pertussis* virulence regulon in response to diverse environmental signals. Transcription of *bvgAS*-activated genes is almost completely abolished by a variety of environmental factors, which include the levels of sulfate anion and nicotinic acid, and the temperature of the growth medium (Miller *et al.*, 1992; Uhl & Miller, 1994). Sequence similarity searches of the protein domain databases reveal that SsrA, like BvgS (Beier *et al.*, 1995), is an unorthodox two-component sensor protein, since it contains a C-terminal region similar to the response regulator receiver domain (represented by the Interpro database entry IPR001789), usually found only in the response regulators.

Whether SsrA can sense different environmental signals directly is an open question. It is formally possible that SsrB could be activated independently from SsrA. It is also possible that at least one of the signals is sensed by another sensor–regulator and that this is then linked through SsrA–B. This is the case for the PmrA–B-regulated genes, whose expression is modulated by the [Mg^{2+}] and [Fe^{3+}] in the environment by a regulatory cascade of two-component regulatory systems, where the first one (PhoP–Q) senses [Mg^{2+}] and the second (PmrA–B) senses [Fe^{3+}] (Groisman, 2001). Supporting this notion, the expression of *sifB*: : *gfp* in MgM medium in an *ssrA* single or *ssrA ompR* double mutant strain remained eightfold higher than in LB, where *sifB*: : *gfp* expression is severely repressed (data not shown), suggesting that additional regulatory system(s) contribute to *sifB* expression. It was initially proposed that PhoP–Q (Groisman, 2001), required for intra-macrophage replication and systemic growth within the mouse (Fields *et al.*, 1986), could be involved in the control of SPI-2 gene expression (Deiwick *et al.*, 1999; Worley *et al.*, 2000). However, it has been recently shown that PhoP–Q and the SPI-2 TTSS are functionally independent (Beuzón *et al.*, 2001; Miao *et al.*, 2002). One possible candidate is the transcriptional regulator SlyA, which is required for virulence and survival in macrophages (Libby *et al.*, 1994). *slyA* mutants are sensitive to oxidative products of the respiratory burst (Buchmeier & Libby, 1997). Another candidate for the additional regulation of SPI-2 TTSS genes is the alternative sigma factor RpoE (σ^E). *rpoE* mutant strains are highly attenuated in mice (Humphreys *et al.*, 1999). Although able to invade both macrophage and epithelial cell lines normally, the *rpoE* mutant is defective in its ability to survive and proliferate in both cell lines (Humphreys *et al.*, 1999), and has also an increased sensitivity to the respiratory burst (Testerman *et al.*, 2002).

What is the physiological significance of the roles of Ca^{2+}, low osmolarity and acidic pH in regulating SPI-2 TTSS

gene expression? Using fluorescence lifetime imaging microscopy, it has been shown that inside macrophage lysosomes [Ca^{2+}] is at least five times lower (400 μM) than the concentration outside the cell (2 mM) (Christensen *et al.*, 2002). Although the [Ca^{2+}] in the lumen of the SCV is unknown, the strong repression of SPI-2 TTSS gene expression observed *in vitro* at a [Ca^{2+}] equivalent to that found outside the cell (2 mM) could explain the absence of SPI-2 gene expression in extracellular bacteria (Beuzón *et al.*, 2000). Low osmolarity represses SPI-1 TTSS gene expression (Bajaj *et al.*, 1996), whereas it induces SPI-2 TTSS gene expression *in vitro* (this work; Lee *et al.*, 2000). An opposite regulation by osmolarity on SPI-1 TTSS gene expression (expressed extracellularly and required for invasion) and SPI-2 TTSS (expressed intracellularly and required for intracellular replication) could help to ensure that these functionally distinct systems are expressed independently and only when and where they are required during the infection process. It has also been shown that the majority of vacuoles containing *S. typhimurium* acidify from pH 6·0 to between pH 4·0 and 5·0 within 60 min after formation (Rathman *et al.*, 1996). Although it is clear that acidic pH induces SPI-2 TTSS-mediated secretion *in vitro*, the effect of acidic pH on inducing SPI-2 TTSS gene expression has been controversial (Beuzón *et al.*, 1999; Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*, 2002). In this study, we found it impossible to ascertain whether acidic pH has a significant effect on SPI-2 TTSS gene expression *in vitro*. However, in infected macrophages, acidic pH has a clear effect on P_{sifB}::*gfp* expression (Fig. 6), confirming the requirement of vacuolar acidification for SPI-2 gene expression reported by Cirillo *et al.* (1998). If the effect of pH on SPI-2 TTSS expression is not as strong as its effect on secretion, or the effective range of action is very narrow, it is possible that, even in pH-controlled *in vitro* conditions, undetectable variations of pH from experiment to experiment could result in a lack of reproducibility which might explain the different results obtained (Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*, 2002). In conclusion, [Ca^{2+}], osmolarity and pH could account for SPI-2 TTSS intracellular expression *in vivo*. These signals are predominantly transmitted through SsrA–B and may involve additional sensing proteins and regulators of the SPI-2 system.

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