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;
Knodler et al., 2002; Miao & Miller, 2000; Worley et al., 2000). Two effector proteins, ShrP 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; Deiwicks & Hensel, 1999; Deiwicks 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 (Deiwicks 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 Deiwicks 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. Furthermore, 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*::*Tn10* 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$_4$)$_2$SO$_4$, 0-5 mM K$_2$SO$_4$, 1 mM KH$_2$PO$_4$, 8 mM 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 mg ml$^{-1}$; kanamycin, 50 mg ml$^{-1}$; and tetracycline, 25 mg ml$^{-1}$. Bacteria were grown at 37°C overnight with aeration.

**Plasmids.** Plasmid pFVP25.1, carrying *gfpmut3A* under the control of a constitutive promoter, was introduced into bacterial strains by electroporation. pID835 is a derivative of pFVP25, a vector carrying a promoterless *gfpmut3A* (Valdivia & Falkow, 1997). 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-PrfI (5′-CCGGGATCTTTAATTGCGCAAGCTAAC-3′) and SIFA-Prrl (5′-CCGGGATCTTTAATTGCGCAAGCTAAC-3′). The 374 bp PCR product, containing terminal EcoRI and BamHI 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 SfBf-B (5′-GGATCTCTTGAAGGCTTGATG-3′) and SfBf-B (5′-GGATCTCTTGAAGGCTTGATG-3′) digested into pCR2.1 topo vector (Novagen). The 1-5 kb fragment was excised from this intermediate plasmid by EcoRI digestion and then digested with *DraI*, generating an *EcoRI*–*DraI* 375 bp fragment, which was ligated into *EcoRI*–*DraI*-digested pFP25, 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.

**Table 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>12023</td>
<td>Wild-type</td>
<td>NTCC</td>
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<tr>
<td>P3F4</td>
<td><em>ssrA::&lt;mTn5</em></td>
<td>Hensel et al. (1995)</td>
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<tr>
<td>HH102</td>
<td>ΔssrB::aphT*</td>
<td>Hensel et al. (1998)</td>
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<tr>
<td>HH182</td>
<td><em>ompR1009::Tn10</em></td>
<td>Beuzón et al. (2000)</td>
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<tr>
<td>HJG1</td>
<td><em>ssrA::mTn5, ompR1009::Tn10</em></td>
<td>This study</td>
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The transcript levels for specific genes were determined by Northern analysis of total RNA isolated from bacterial cultures 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). Bacteria were grown in 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$_4$)$_2$SO$_4$, 0-5 mM K$_2$SO$_4$, 1 mM KH$_2$PO$_4$, 8 mM 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 mg ml$^{-1}$; kanamycin, 50 mg ml$^{-1}$; and tetracycline, 25 mg ml$^{-1}$. Bacteria were grown at 37°C overnight with aeration.

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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 pFPV25.1 were used as negative and positive controls for fluorescence, respectively. For each sample, 10^7 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 sulphonyl 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% CO2.

Bacterial infection of HeLa cells. HeLa cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5 x 10^5 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% CO2. 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 with 0-1% saponin in PBS, once in PBS and once in H2O, 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 x 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 OD600 of 2-0. The cultures were diluted to an OD600 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% CO2. 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, 10^8 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 effectors 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_salH::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
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 ssaA or ompR single mutant strains, and ssaA 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 ssaA, ompR and ssaA ompR mutant strains (Fig. 2b). Expression of P_{sifB}::gfp was reduced more than tenfold in the ssaA mutant strain and twofold in the ompR mutant. The expression in the ssaA ompR double mutant strain was equivalent to that in the ssaA 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 MgCl2. Flow cytometry was carried out using wild-type bacteria harbouring either P_{sifA}::gfp or P_{sifB}::gfp fusions, after...
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 MgCl₂ (Fig. 3a, upper panel). Similarly, no significant differences were observed by immunoblotting either with an anti-GFP antibody using wild-type bacteria carrying either P₅sifA::gfp or Pₛₛ₄fB::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 Mg²⁺ inhibited the expression of ssaB, and this effect could be reverted by lowering the [PO₄³⁻]. However, in the presence of 8 μM Mg²⁺, ssaB expression was independent of [PO₄³⁻]. In agreement with these results, no significant differences were observed in P₅sifA::gfp or Pₛₛ₄fB::gfp expression in response to changes in [PO₄³⁻] in medium containing 8 μM Mg²⁺ (data not shown). However, under our assay conditions, no inhibitory effect of high [Mg²⁺] was observed (Fig. 3a, b). This prevented us from confirming a possible effect of PO₄³⁻ starvation in reverting an inhibitory effect of high [Mg²⁺].

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 [(NH₄)₂SO₄] 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 Mg²⁺, PO₄³⁻, glycerol and (NH₄)₂SO₄ have no significant effect on SPI-2 gene expression, after overnight growth in MgM medium.

The absence of Ca²⁺ leads to SPI-2 TTSS gene expression via SsrA–B

It has been shown previously that the absence of Ca²⁺ in Tris-buffered MgM medium induces expression from the SPI-2 promoters Pₛ₅saB and Pₛ₅saA (Deiwick et al., 1999). Consistent with these results, the fluorescence of wild-type bacteria carrying either Pₛ₅sifA::gfp or Pₛₛ₄fB::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 Ca²⁺ (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 Ca²⁺, but was detected after growth in MgM medium without Ca²⁺ (Fig. 4c).

To determine which regulatory system mediates the effect of low [Ca²⁺], we analysed Pₛ₅sifA::gfp expression in ssaA or ompR single mutant strains, and ssaA ompR double mutant.
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 \(P_{sifA}\) (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 \(sifB::gfp\) 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...
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 phospho-

rylated 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\(\text{sifA}::\text{gfp}\) or P\(\text{sifB}::\text{gfp}\) 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\(\text{sifA}::\text{gfp}\) and P\(\text{sifB}::\text{gfp}\) 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
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 ssaI, 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.
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 ssaA 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, ssaA and ssaB by an unknown iron-responsive regulatory system (Zaharik et al., 2002).

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, ssaA 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.

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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 ssaR ompR double mutant in all three conditions is identical to that of the ssaR single mutant. These results indicate that the effect of OmpR–EnvZ on the expression of SPI-2 genes, other than ssaRAB, is not a result of direct binding of OmpR to their promoters but is indirect, probably as a consequence of its binding to the ssaRAB 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 \textit{Bordetella pertussis} virulence regulon in response to diverse environmental signals. Transcription of \textit{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 \(\text{Mg}^{2+}\) and \(\text{Fe}^{3+}\) in the environment by a regulatory cascade of two-component regulatory systems, where the first one (PhoP–Q) senses \(\text{Mg}^{2+}\) and the second (PmrA–B) senses \(\text{Fe}^{3+}\) (Groisman, 2001). Supporting this notion, the expression of \textit{sfbB::gfp} in MgM medium in an \textit{ssaR} single or \textit{ssaB} \textit{ompR} double mutant strain remained eightfold higher than in LB, where \textit{sfbB::gfp} expression is severely repressed (data not shown), suggesting that additional regulatory system(s) contribute to \textit{sfb} 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). \textit{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 (\(\sigma^E\)). \textit{rpoE} mutant strains are highly attenuated in mice (Humphreys et al., 1999). Although able to invade both macrophage and epithelial cell lines normally, the \(\text{rpoE}\) 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

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**Fig. 7.** Model for \textit{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 \textit{ssrAB} promoter and activates the transcription of \textit{ssrA} and \textit{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.
gene expression? Using fluorescence lifetime imaging microscopy, it has been shown that inside macrophage lysosomes [Ca\textsuperscript{2+}] is at least five times lower (400 μM) than the concentration outside the cell (2 mM) (Christensen et al., 2002). Although the [Ca\textsuperscript{2+}] in the lumen of the SCV is unknown, the strong repression of SPI-2 TTSS gene expression observed \textit{in vitro} at a [Ca\textsuperscript{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 \textit{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 \textit{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 \textit{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 \textit{in vitro}. However, in infected macrophages, acidic pH has a clear effect on \(p_{\text{MFF}}\):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 \textit{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\textsuperscript{2+}], osmolarity and pH could account for SPI-2 TTSS intracellular expression \textit{in vivo}. These signals are predominantly transmitted through SsrA–B and may involve additional sensing proteins and regulators of the SPI-2 system.

**ACKNOWLEDGEMENTS**

We thank Kate Unsworth for the critical reading of the manuscript and Suzana Salcedo and Aaron Rae for their help with the flow cytometry. J. Garmendia is recipient of a long-term postdoctoral EMBO fellowship. This research was also supported by a grant from the Medical Research Council (UK).

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