

OmpR Regulates the Two-Component System SsrA-SsrB in *Salmonella* Pathogenicity Island 2

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***Salmonella* pathogenicity island 2 (SPI-2) encodes a putative, two-component regulatory system, SsrA-SsrB, which regulates a type III secretion system needed for replication inside macrophages and systemic infection in mice. The sensor and regulator homologs, *ssrAB* (*spiR*), and genes within the secretion system, including the structural gene *ssaH*, are transcribed after *Salmonella* enters host cells. We have studied the transcriptional regulation of *ssrAB* and the secretion system by using *gfp* fusions to the *ssrA* and *ssaH* promoters. We found that early transcription of *ssrA*, after entry into macrophages, is most efficient in the presence of OmpR. An *ompR* mutant strain does not exhibit replication within cultured macrophages. Furthermore, footprint analysis shows that purified OmpR protein binds directly to the *ssrA* promoter region. We also show that minimal medium, pH 4.5, induces SPI-2 gene expression in wild-type but not *ompR* mutant strains. We conclude that the type III secretion system of SPI-2 is regulated by OmpR, which activates expression of *ssrA* soon after *Salmonella* enters the macrophage.**

Salmonella enterica serovar Typhimurium is a facultative intracellular bacterium that generally causes gastroenteritis in humans and a typhoid fever-like disease in mice. During the course of infection in mice, serovar Typhimurium colonizes many different organs, including the Peyer's patches of the small intestine, mesenteric lymph nodes, spleen, and liver, where it is found in both extracellular and intracellular locations (23, 45, 71). The ability of *Salmonella* to replicate inside cultured cell lines has been linked to virulence in mice (22, 49). *Salmonella* flourishes inside host macrophages in specialized vacuolar compartments and resists oxygen radicals, defensins, and cationic antimicrobial peptides. *Salmonella*-containing vacuoles (also called phagolysosomes) have an acidic pH and low concentrations of Fe²⁺ and Mg²⁺ (28, 29, 69, 70). Once inside the host cell, *Salmonella* induces expression of over 30 proteins as determined by two-dimensional gel electrophoresis (1, 9). In vitro studies that mimic the vacuolar environment reveal that *Salmonella* has a network of coordinately regulated genes to counter the hostile intracellular environment (4, 34, 55).

Pleiotropic regulators of *Salmonella* virulence genes include *rpoS*, *phoP*, and *ompR*; mutations in any of these render *Salmonella* avirulent (14, 19, 21, 57). *rpoS* encodes an alternative sigma factor that regulates genes expressed during stationary phase in response to nutrient deprivation (53, 65). RpoS regulates the *spv* genes carried on a plasmid essential for *Salmonella* virulence in mice (21, 47, 63). However, *Salmonella rpoS* mutants are reported to replicate normally inside macrophages (10, 62). Both *phoP* and *ompR* encode elements of bacterial two-component regulatory systems (also called sensor-kinase systems), PhoP-PhoQ and OmpR-EnvZ, respectively. PhoP-PhoQ regulates over 40 genes in *Salmonella*; only a subset of these is involved in virulence (30, 56, 58). The PhoP-PhoQ system responds to environmental Mg²⁺ concentrations (29). A *phoP* mutant strain of *Salmonella* is defective for survival

inside macrophages (9, 32, 57). OmpR-EnvZ has been studied primarily in *Escherichia coli*, where it was first discovered to respond to changes in osmolarity and to regulate expression of the porin proteins OmpF and OmpC (2, 68). Acidified medium also affects transcription of *ompC* and *ompF*, independent of osmolarity (38, 77). *Salmonella* also has the *ompR* and *envZ* operon (50, 51). In *Salmonella*, the OmpR-EnvZ system regulates *ompC* and *ompF*, as well as the genes encoding tripeptide permease (*tppB*), *Salmonella*-induced filaments (*sifA*) in HeLa cells, and 2-acylglycerolphosphoethanolamine acyltransferase (*aas*), which is induced under acidic conditions and inside macrophages (31, 60, 80, 81). Mutations in *ompF*, *ompC*, *tppB*, or *aas* have no effect on virulence, whereas *ompF ompC* double mutants or *sif* mutants are partially attenuated for virulence in mice (11, 19, 76; R. H. Valdivia, M. Rathman, and S. Falkow, unpublished data). In addition, a *Salmonella ompR* mutant does not kill macrophages in vitro (52).

Serovar Typhimurium has two pathogenicity islands (SPI-1 and SPI-2) that code for type III secretion systems (25, 59, 64, 74). (For reviews of type III secretion systems, see references 43 and 54.) SPI-1 mutants are defective for virulence in mice when the bacteria are administered orally, not intraperitoneally, suggesting that SPI-1 has a role early in infection (26). SPI-1 promotes bacterial invasion of the M cells in the small intestine and is essential for apoptosis (13, 46, 59, 61). InvF and HilA are transcriptional regulators of SPI-1 genes that are encoded within SPI-1; *hilA* itself is regulated by several factors, including PhoP-PhoQ, SirA, HilC, and HilD (3–5, 20, 37, 44, 66, 72).

SPI-2 genes are required for systemic infection, since SPI-2 mutants are avirulent in mice infected orally, intraperitoneally, or intravenously (35, 64, 74). SPI-2 has been characterized by several groups as essential for replication inside macrophages (12, 36, 64). It has been suggested that changes in Mg²⁺ concentration and pH in growth media also affect Spi2 gene expression (6, 16). Little is known about the genetic regulation of SPI-2 except that expression of genes encoding the type III secretion system of SPI-2 is dependent on a sensor-kinase system, SsrA-SsrB (also called SpiR) (12, 16, 36, 64). The *ssrAB* operon is also encoded within SPI-2 (64, 74). *ssrA* is the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Serovar Typhimurium		
SL1344	<i>xyl hisG rpsL</i>	40
P3F4	SL1344 <i>ssrA</i> ::miniTn5	35
PhoP	SL1344 <i>phoP</i> ::Tn10	70
SL1344K	SL1344 <i>rpoS</i> :: <i>kan</i>	14
CJD359	SL1344 <i>ompR1009</i> ::Tn10	19
P3F4/CJD359	SL1344 <i>ssrA</i> ::miniTn5 <i>ompR1009</i> ::Tn10	This work
BRD454	SL1344 <i>ompC396</i> ::Tn10	19
BRD456	SL1344 <i>ompF1006</i> ::Tn10	19
CJD372	SL1344 <i>ompC396</i> ::Tn10 <i>ompF1006</i> ::Mu dl-8	11
CJD408	SL1344 <i>tpxB83</i> ::MudJ	11
Plasmids		
pFPV25	<i>colE1 mob bla gfp</i> ; promoterless <i>gfp</i> ; no GFP expression	80
pFPV25.1	pFPV25 with promoter region for <i>rpsM</i> , a gene encoding the ribosomal protein S13; constitutive GFP expression	80
pMIC-10C32	2,273-bp fragment spanning promoter region of <i>ssrA</i> and part of the <i>ssaBCD</i> operon cloned into pFPV25	12
pFMI10	Promoter fragment of <i>ssaH</i> cloned in pFPV25	81
pFMI10-cam	pFMI10 with chloramphenicol gene cloned into <i>EcoRV</i> site	This work
pANT30.2-cam	pANT30.2 with chloramphenicol gene cloned into <i>EcoRV</i> site	This work
pANT30.1	645-bp fragment spanning promoter of <i>ssrA</i> and part of <i>ssaB</i> cloned into pFPV25	This work
pANT30.2	335-bp fragment spanning promoter of <i>ssrA</i> cloned into pFPV25	This work
pANT30.3	192-bp fragment upstream of <i>ssrA</i> ATG site cloned into pFPV25	This work

first gene of the operon, and its product is a sensor homolog belonging to the BvgS family of cytoplasmic-membrane sensor proteins (79). *ssrB* is a response regulator with a receiver domain and a helix-turn-helix DNA binding domain (16).

Plasmid-encoded promoter fusions to the gene for green fluorescent protein (GFP) have shown that transcription of SPI-2 genes, including *ssrA* and *ssaH*, is induced inside macrophages (12). *ssaH* encodes a structural gene of the type III secretion system (81). Transcription from the *ssaH* promoter, but not the *ssrA* promoter, is abolished in an *ssrA* mutant strain after entry into cultured macrophages (12). The activation of the *ssrA* promoter in an *ssrA* mutant strain suggests that the two-component regulatory system of the type III secretion system itself is induced by a different regulator. We report here that *OmpR* positively regulates the SsrA-SsrB two-component regulatory system by directly binding to the *ssrA* promoter region. These studies show that in *Salmonella*, the global regulator, *OmpR*, plays a major role in coordinating gene expression upon entry into the host cell.

MATERIALS AND METHODS

Growth conditions of bacterial strains and cells. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown standing at 37°C overnight in Luria-Bertani (LB) broth (optical density at 600 nm [OD₆₀₀], ~0.4) unless otherwise stated. The LB broth and M9 (without CaCl₂) recipes have been published elsewhere (2a). M9 minimal medium was supplemented with 0.004% histidine and 0.01 M glucose; the pH was adjusted with concentrated HCl. Antibiotics were used as needed at the following concentrations: ampicillin, 50 µg/ml; streptomycin, 200 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 30 µg/ml. Plasmids were moved into serovar Typhimurium by electroporation or P22-HT-mediated transduction.

RAW264.7 cells were routinely maintained in Dulbecco modified Eagle medium containing sodium pyruvate and 10% fetal bovine serum (Gibco) at 37°C and 5% CO₂.

Construction of pANT30 plasmids. Standard molecular cloning techniques were used to clone fragments of the *ssrA* promoter region upstream of *gfp* in plasmid pFPV25 (Table 1). The following oligonucleotides with restriction sites were generated: reverse primer *3ssrA* (5'-ATTAGGTACCGATCCGCTGATTAATAAGATGTTTGC3') includes *KpnI* and *BamHI* sites; forward primer *5ssrA.1* (5'-ATACGGATCCGAATTCGTCGACGGCAAGACAAGGCTTAGGTAAGC3') includes *BamHI*, *EcoRI*, and *SalI* sites and was used to clone pANT30.1; forward primer *5ssrA.2* (5'-ATACGGATCCGAATTCGTAGTCAT

CGACTGGG3'), with *BamHI* and *EcoRI* sites, was used to clone pANT30.2; and forward primer *5ssrA.3* (5'-ATACGGATCCGAATTCGTAGTCATCGACTGGG3'), with *BamHI* and *EcoRI* sites, was used to clone pANT30.3. Standard PCR and molecular biology protocols were used to amplify these fragments from SL1344, and fragments were cloned directionally into plasmid pFPV25 in either *E. coli* DH5α or DH10β before the plasmids were moved into serovar Typhimurium.

Gentamicin protection assay. RAW264.7 cells were seeded at 2.5 × 10⁵ per well in a 24-well tissue culture plate. Salmonellae were grown in LB broth on a rotating wheel and opsonized with 50% normal mouse serum for 15 min at 37°C. The cultures were diluted in phosphate-buffered saline (PBS) to allow a multiplicity of infection of 10. Infected cells were centrifuged at 1,800 × g for 5 min and incubated for 1 h at 37°C and 5% CO₂. The cells were washed two times with medium and incubated with 100 µg of gentamicin/ml for 90 min. The cells were lysed with 1% Triton X-100, and intracellular bacteria were counted as CFU, or the cells were allowed to incubate an additional 20 h with 10 µg of gentamicin/ml, and intracellular counts were determined as stated above.

Assays for induction of gene expression inside macrophages. RAW264.7 cells were seeded to confluency in 24-well tissue culture plates. Bacteria were grown standing at 37°C overnight and diluted for a multiplicity of infection of ~50, and the cells were infected without centrifugation. After incubation for 30 min (37°C; 5% CO₂), the medium was replaced and the bacteria were allowed to incubate for various times. Supernatant containing extracellular bacteria was set aside for analysis. The cells were washed three times with medium and lysed with Triton X-100 to release intracellular bacteria. A total of 10,000 intracellular or extracellular bacterium-size particles were measured with the FACScalibur (Becton Dickinson) cytometer for fluorescence intensity as previously described (12, 80, 81). All induction experiments were repeated at least twice.

In vitro gene induction. Bacteria were grown standing in standard LB broth at neutral pH overnight at 37°C, and 1 ml of liquid culture was pelleted and washed two times in PBS. The bacteria were incubated for 3 h in the following media: LB broth, pH 7.0; LB broth, pH 4.5; M9 minimal medium, pH 7.0, with 1, 10, or 100 µM or 1 mM MgSO₄; or M9 minimal medium, pH 4.5, with 1, 10, or 100 µM or 1 mM MgSO₄. When appropriate, 0.5 M NaCl or 20% sucrose was added to the M9 medium. The bacteria were also tested in 1% ethanol, 0.5% sodium dodecyl sulfate, or 0.1 µg of polymyxin B/µl for 2 h. Subcultured bacteria were diluted in PBS and analyzed by flow cytometry.

Flow cytometry. Flow cytometry experiments were performed with a FACScalibur (Becton Dickinson) cytometer. The bacteria were detected as described previously (80). Briefly, 10,000 bacterium-size particles were collected for each experiment, and the fluorescence intensity of each particle was detected by the FACScalibur machine. CELLQUEST (Becton Dickinson) software was used for data analyses and histogram production. The area under the curve of the histograms represents the total number of detected particles. The fluorescence intensity for each particle is reported on the x axis. A peak representing background machine noise is always present in the negative fluorescent population and cannot be avoided because of the small size of bacteria. Peak fluorescence, the fluorescence value of the bacterial majority, was determined with CELLQUEST

software and is reported where appropriate. Induction ratios (IR) were calculated by dividing the peak fluorescence induction of bacteria in the inducing condition (i.e., inside macrophages or in M9 medium) by the peak fluorescence induction of bacteria in noninducing conditions (i.e., outside macrophages or in LB broth). GFP fluorescence for induced and uninduced bacteria was compared within each individual experiment, not between experiments, because the nature of flow cytometry analysis only allows data interpretation relative to internal positive and negative controls on a per-experiment basis. Each flow cytometry experiment included a negative control, SL1344 carrying plasmid pFPV25 (promoterless GFP), and a positive control, SL1344 carrying plasmid pFPV25.1 (constitutive GFP expression), to calibrate the relative fluorescence for each experiment. The negative and positive controls do not change fluorescence in response to the macrophage environment or to changes in the media tested (reference 80 and data not shown).

DNase protection assays. DNase protection assays were carried out as described previously (41) with the following modifications. The forward PCR oligonucleotide, 5*ssrA*, was labeled with [γ - 32 P]ATP by T4 polynucleotide kinase forward reaction (New England Biolabs). The radioactive oligonucleotide and a nonradioactive reverse primer, 3*ssrA*, were used in a PCR to generate radioactive target DNA for subsequent binding and sequencing reactions. Excess radioactive label from the kinase reaction and unincorporated deoxynucleoside triphosphates from the PCR were removed by using Qiaquick (Qiagen) columns according to the manufacturer's instructions. Target DNA equivalent to 2.3×10^5 cpm was bound to 1.76 μ g of purified OmpR protein or 2.5 μ g of MBP-EnvZ in the appropriate buffers and conditions as described previously (41). DNase I reactions were carried out by the method of Huang and Igo, as was the parallel A+G Maxam-Gilbert sequencing reaction (41). Samples were separated by electrophoresis on a 7.5 M urea-6% polyacrylamide gel, and detected by autoradiography.

RESULTS

OmpR is important for inducing *ssrA* transcription. *ssrA* transcription is induced inside macrophages, presumably in response to the vacuolar environment (12). Global regulators often modulate bacterial gene expression in response to environmental changes, so we chose to examine the effects of mutations in three known global regulators, *phoP*, *rpoS*, and *ompR*, on *ssrA* gene expression because mutations in these genes are known to decrease *Salmonella* virulence (14, 19, 21, 57). We tested the abilities of strains with mutations in these genes to induce the *ssrA* promoter inside RAW264.7 murine macrophage-like cells. Plasmid pMIC-10C32, which contains a transcriptional fusion of the *ssrA* promoter region to *gfp*, was used to measure transcriptional activity of the promoter. RAW264.7 macrophages were infected with either SL1344 (wild type), P3F4 (*ssrA* mutant), PhoP (*phoP* mutant), SL1344K (*rpoS* mutant), or CJD359 (*ompR* mutant) carrying plasmid pMIC-10C32 (Table 1 and Fig. 1). The bacteria were released from the cells 2 h postinfection, and GFP expression from extracellular and intracellular bacteria was compared as described in Materials and Methods. The IR was calculated by dividing the peak fluorescence of intracellular bacteria by the peak fluorescence of extracellular bacteria (Fig. 1). Intracellular wild-type and *ssrA* mutant bacteria harboring the plasmid reporter construct induced GFP expression (IR, 13 and 11, respectively), as did *phoP* (IR = 9) and *rpoS* (IR = 10) strains (Fig. 1a). In contrast, *gfp* expression was reduced fourfold for intracellular bacteria in an *ompR* mutant background (IR = 1.6) compared to the wild type (IR = 6.5) (Fig. 1b). This result suggested that either OmpR or an OmpR-dependent event induced *ssrA* transcription.

We also tested the transcription of *ssaH* in an *ompR* mutant strain. Surprisingly, we found that the *ompR* mutant carrying plasmid pFMI10 (*ssaH* promoter fusion to *gfp*) still induced inside macrophages (IR = 55.5) (Fig. 1b). This result suggested that some SsrA might be present at 2 h postinfection, since *ssaH* transcription requires SsrA (81), and might be sufficient for induction of *ssaH*. We addressed this phenomenon by comparing the kinetics of *ssrA* and *ssaH* transcription in wild-type and *ompR* mutant backgrounds.

We measured the relative amounts of GFP expression from

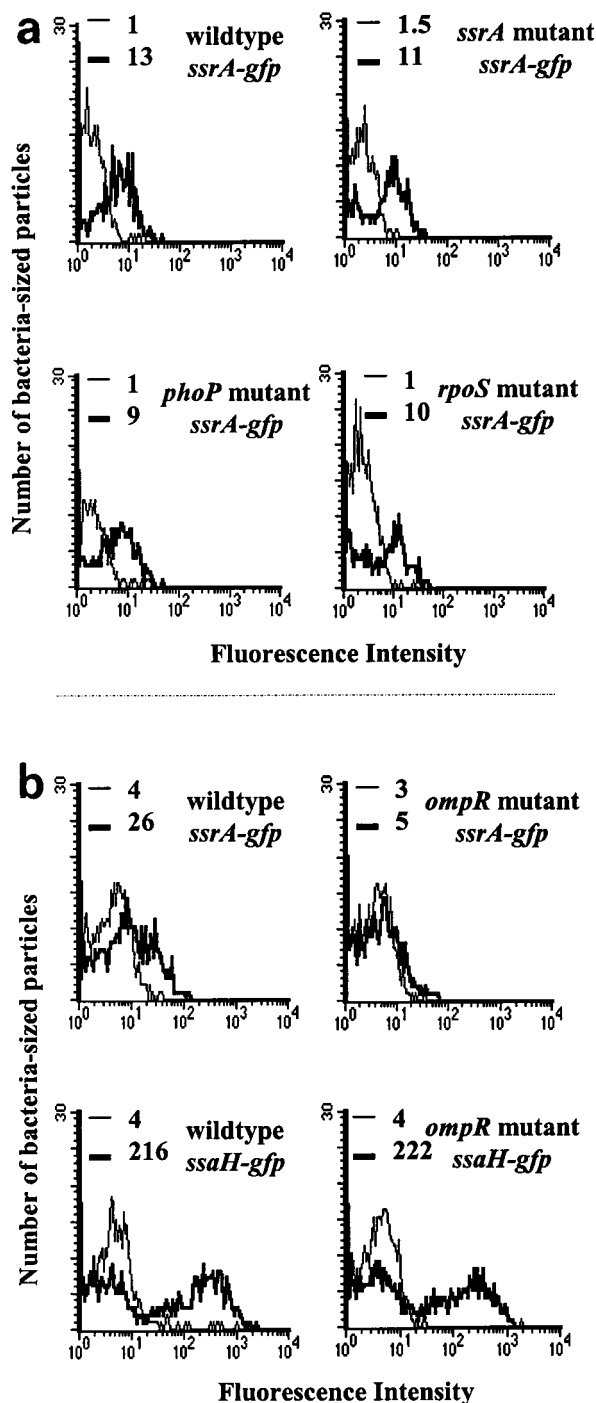


FIG. 1. *ssrA* gene expression is reduced significantly in an *ompR* mutant background. RAW264.7 cells were infected for 2 h with strains bearing reporter plasmids. The histograms show GFP expression from extracellular bacteria (thin lines) and intracellular bacteria (thick lines). Peak fluorescence intensity is indicated for each sample. (a) Macrophages were infected with the following strains: SL1344 (wild type) with pMIC-10C32 (*ssrA-gfp*), P3F4 (*ssrA* mutant) with pMIC-10C32, PhoP (*phoP* mutant) with pMIC-10C32, and SL1344K (*rpoS* mutant) with pMIC-10C32. (b) Macrophages were infected with SL1344 with pMIC-10C32, CJD359 (*ompR* mutant) with pMIC-10C32, SL1344 with pFMI10 (*ssaH-gfp*), and CJD359 with pFMI10. The experiments shown in panels a and b were performed on different days.

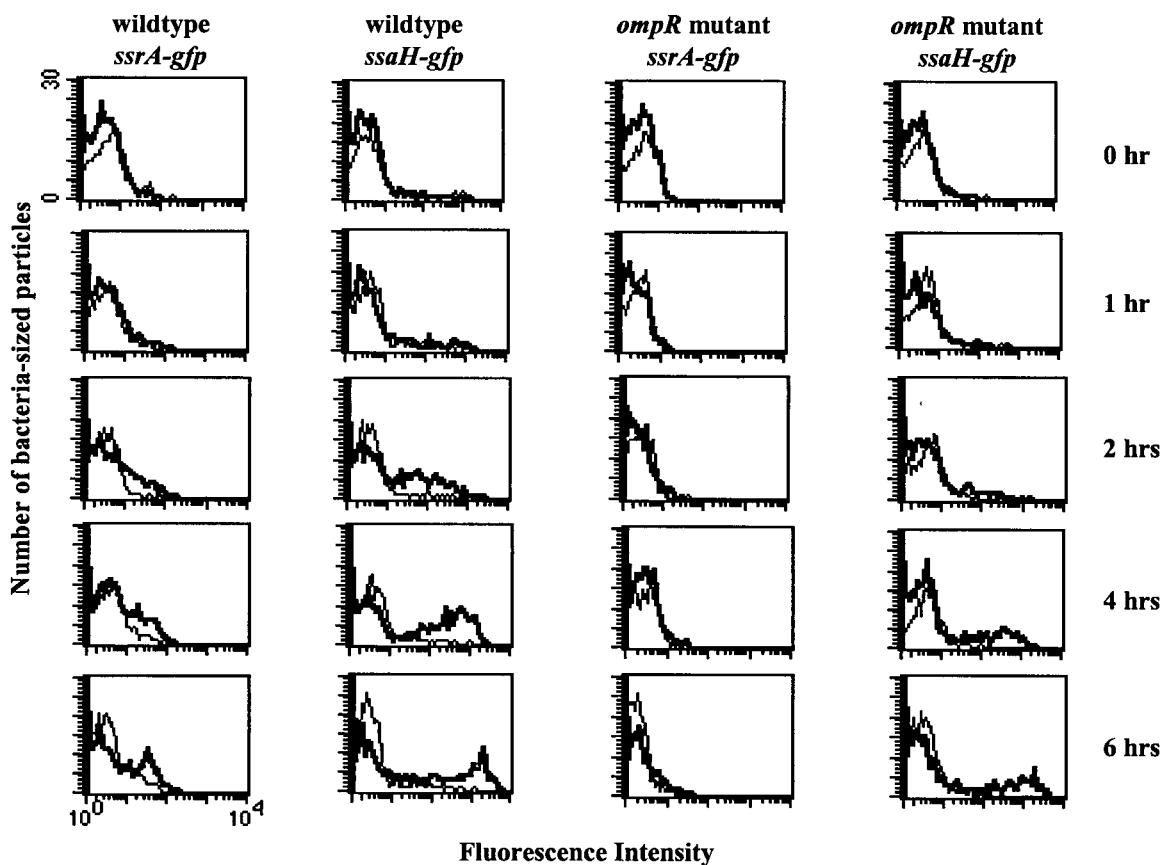


FIG. 2. Kinetics of induction for *ssrA*- and *ssaH*-*gfp* fusions inside macrophages. RAW264.7 macrophages were infected with the following bacteria: SL1344 (wild type) with pMIC-10C32 (*ssrA-gfp*), SL1344 with pFMI10 (*ssaH-gfp*), CJD359 (*ompR* mutant) with pMIC-10C32, or CJD359 with pFMI10. The histograms show GFP expression from extracellular bacteria (thin lines) and intracellular bacteria (thick lines) at each time point postinfection. The axes for all histograms are identical.

strains carrying plasmid pMIC-10C32 (*ssrA-gfp*) or pFMI10 (*ssaH-gfp*) over time to determine when *ssrA* and *ssaH* were transcribed in the absence of OmpR inside macrophages. For this time course, we monitored transcription of either *ssrA* or *ssaH* by watching for the appearance of an inducing population of intracellular bacteria over time. In a wild-type background, *ssrA* transcription was evident by 2 h and strong by 6 h (Fig. 2, column 1). In an *ompR* mutant background, *ssrA* induction was barely detectable even at 6 h postinfection, indicating that *ompR* is important for *ssrA* induction (Fig. 2, column 3). The absence of OmpR delayed but did not eliminate induction of the *ssaH* reporter (Fig. 2, columns 2 and 4). Therefore, *ssrA* expression may not be completely abolished in the *ompR* mutant, and a small but undetectable (by our assay) level of SsrA probably accumulates in the *ompR* mutant. We conclude that OmpR is important for early transcription of *ssrA* inside macrophages.

SPI-2 induction is independent of OmpC and OmpF porin defects. It was possible that the reduced *ssrA* expression in an *ompR* mutant strain was an indirect effect of defects from other OmpR-regulated genes, specifically, *ompC* and *ompF*. In vitro, OmpR regulates the proportion of OmpF and OmpC in an osmolarity-dependent fashion, and the amount of OmpC and OmpF is reduced in an *ompR* mutant of *Salmonella* (51). Is the *ompR* mutant strain defective for SPI-2 expression because the absence of porin expression prevents *Salmonella* from sensing an environmental cue inside macrophages? We tested the induction of *ssrA* and *ssaH* promoter-*gfp* fusions in *ompF*, *ompC*

and *ompC ompF* mutant backgrounds and compared the induction inside macrophages to that of wild-type bacteria. A chloramphenicol gene was cloned into plasmid pANT30.2 to make pANT30.2-cam and into pFMI10 to make pFMI10-cam for these experiments. Wild-type, *ompC*, *ompF*, and *ompC ompF* strains all induced *ssrA* and *ssaH* transcription to the same level (IR, ~5 and ~60, respectively) inside macrophages. We also tested plasmids pANT30.2-cam and pFMI10-cam in a *tpxB* mutant strain; this strain also induced *ssrA* and *ssaH* transcription at wild-type levels inside macrophages. These results indicate that *ssrAB* is controlled independently of the OmpR-regulated genes, *ompC*, *ompF*, and *tpxB*.

An *ompR* mutant strain of *Salmonella* does not survive inside macrophages. In wild-type serovar Typhimurium, the type III secretion system in SPI-2 and its two-component regulatory system, SsrA-SsrB, are essential for replication inside macrophages (12, 36, 64, 73). We established that OmpR has a marked effect on induction of *ssrA* and next investigated whether *ompR* mutants, like *ssrA* mutants, fail to replicate inside macrophages by using a gentamicin protection assay. Wild-type *Salmonella* replicated while a *phoP* mutant negative control did not survive inside macrophages (Fig. 3). Strain P3F4 (*ssrA* mutant) did not replicate after 20 h inside macrophages, as expected (12, 36). We found that serovar Typhimurium carrying an *ompR::Tn10* insertion did not replicate inside macrophages, in contrast to what was reported previously (52). While strain differences might account for this discrepancy, it is most likely due to the differences in the way

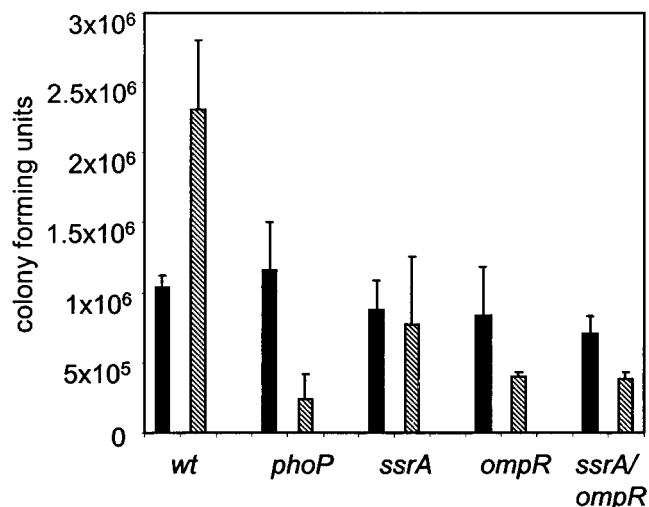


FIG. 3. Comparison of survival and replication in RAW264.7 macrophages. RAW264.7 macrophages were infected with either SL1344 (wild type), PhoP (*phoP*), P3F4 (*ssrA*), CJD359 (*ompR*), or P3F4-CJD359 double mutant (*ssrA/ompR*) for 1 h before the addition of gentamicin. Gentamicin-protected CFU were determined at 3 (solid bars) and 23 (hatched bars) h postinfection.

Salmonella was grown. Lindgren et al. used bacterial cultures grown with reduced aeration, which optimizes for bacterial invasion and bacterium-induced host cell apoptosis (48, 61). Thus, in their experiments, wild-type bacteria would cause apoptosis by 2 to 6 h postinfection, releasing bacteria from the macrophages into gentamicin-containing medium. This would result in an underrepresentation of CFU for wild-type bacteria, which may obscure the difference in the actual number of intracellular bacteria between wild-type and *ompR* mutant *Salmonella*. We circumvented the apoptosis effect by growing the bacteria to stationary phase with aeration and using complement-mediated phagocytosis. Bacteria grown in this manner do not induce apoptosis (61), allowing differences in intracellular replication to be detected.

It has been proposed that the SPI-2 secretion system is needed for bacterial replication, but not survival, in vivo (73). Our data support this view, since the CFU for the *ssrA* mutant remain similar at both time points postinfection, in contrast to the *phoP* mutant, which dies inside macrophages (Fig. 3). Finally, a strain with mutations in both *ssrA* and *ompR* also did not replicate inside macrophages.

OmpR protein binds directly to the *ssrA* promoter region. We asked if OmpR interacts directly with the *ssrA* promoter region to activate *ssrA* gene expression. Studies of *E. coli* have not revealed a clear consensus for an OmpR binding sequence; however, a common feature of OmpR binding regions is the abundance of adenines and thymines (24, 41, 67). The sequence of the region upstream of the translational start for the *ssrA* gene is rich in adenines and thymines, suggesting a possible binding site for OmpR protein.

A DNase I protection assay was used to determine if OmpR binds directly to the *ssrA* promoter region. *E. coli* OmpR protein is 99% identical at the amino acid level to *Salmonella* OmpR protein. MBP-EnvZ, the sensor component for OmpR and a protein that does not bind DNA, was included as a negative control. Purified OmpR from *E. coli* was incubated with a 335-bp DNA fragment that included 51 bp downstream of the ATG start codon and 284 bp upstream of the ATG. The samples were treated with DNase I and separated on a 6% acrylamide gel. OmpR protein binds between -207 and -231

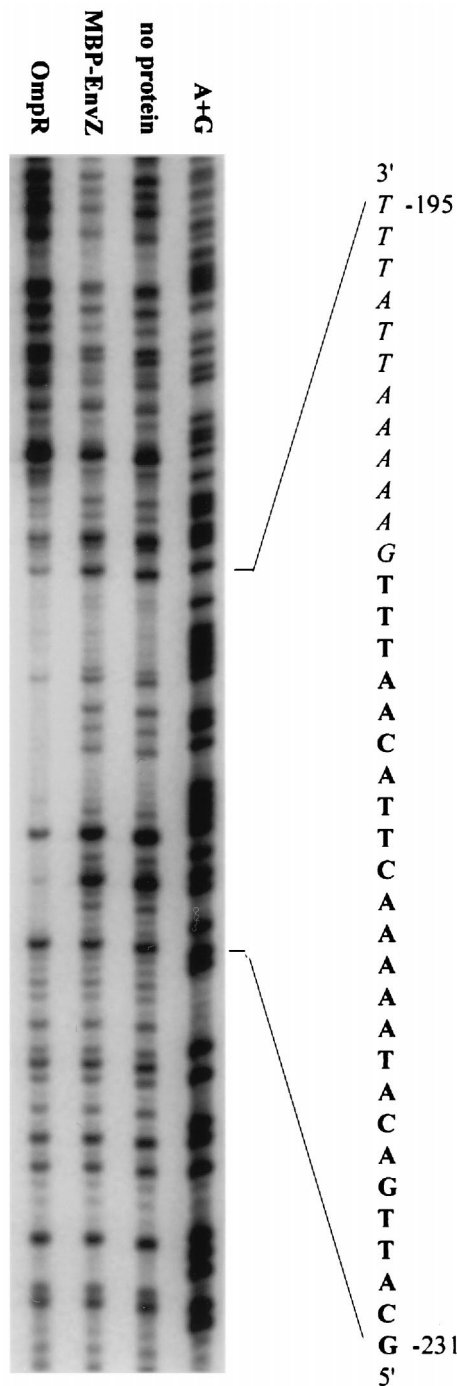


FIG. 4. OmpR protein binds the *ssrA* promoter region. A 335-bp fragment spanning the *ssrA* promoter region was the target in a DNase protection assay. Purified OmpR protein binds the region -207 through -231 from the ATG of the *ssrA* gene. Each lane contained target DNA plus the indicated proteins. The exact sequence of the binding region is indicated, with protected sequences in boldface. The italicized letters indicate a potential OmpR binding half-site that cannot be determined from this experiment.

from the ATG start codon (Fig. 4). Based on the OmpF studies, a single OmpR binding site is 18 bp (41). Approximately 24 bp are protected here, so we conclude that there are at least one and a half, probably two, binding sites for OmpR. An additional 12-bp area from -195 to -206 may also be bound

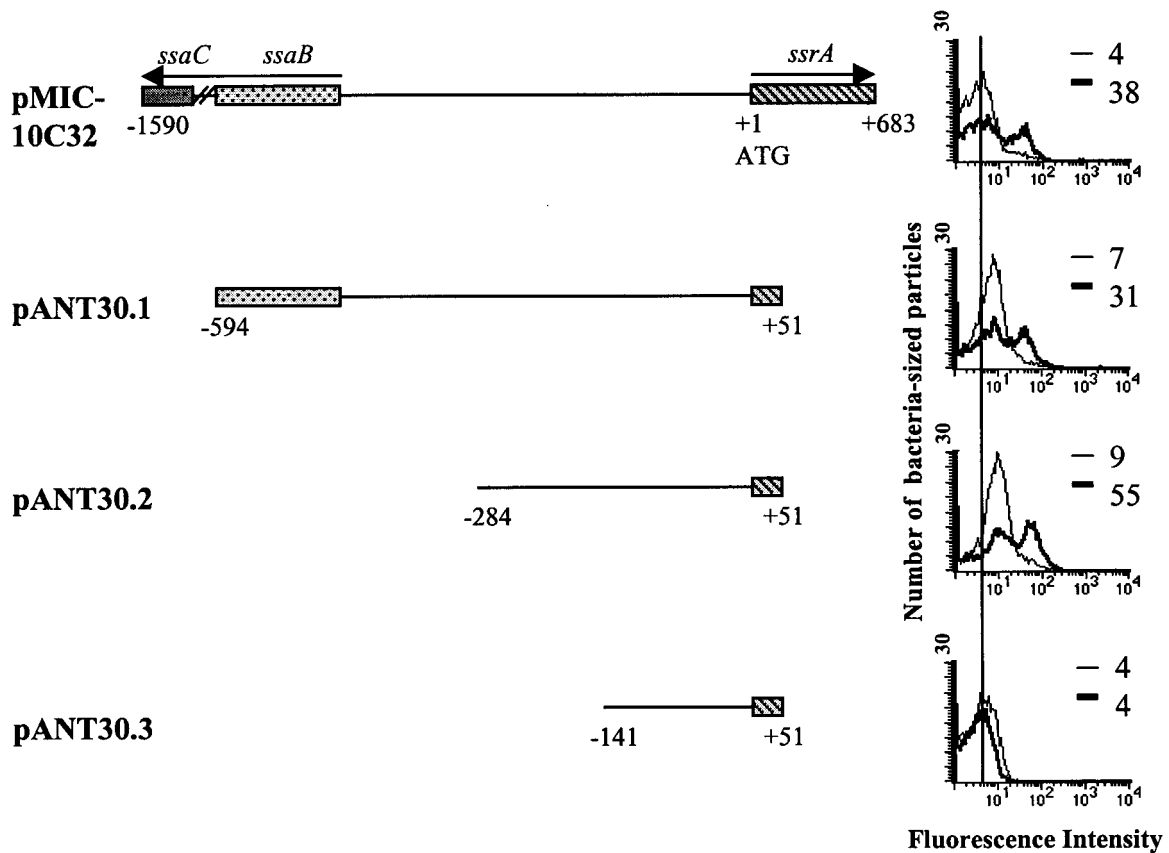


FIG. 5. Effects of *ssrA* promoter region deletions on expression inside macrophages. Different fragments of the *ssrA* promoter region cloned upstream of *gfp* in plasmid pFPV25 are shown. RAW264.7 cells were infected with wild-type bacteria harboring the indicated plasmids. The histograms show GFP expression from extracellular bacteria (thin lines) and intracellular bacteria (thick lines). The vertical line drawn through the histograms shows the peak fluorescence of extracellular bacteria from constructs pMIC-10C32 and pANT30.3. Peak fluorescences are indicated for each sample. Maps are not drawn to scale.

by OmpR protein, but that cannot be determined by this assay because DNase I enzyme does not cleave efficiently in this region. The sequence of the *ssrA* promoter region that is protected by OmpR is rich in adenines and thymines but has no exact homology with published OmpR binding sites for the OmpR-regulated genes *ompC* and *ompF* (24, 41, 67).

Deletion analysis of *ssrA* promoter region. We analyzed the intergenic region between *ssrA* and *ssaB* (*spiC*) to define the promoter region of *ssrA*. In accordance with the universal nomenclature for type III secretion systems, we will refer to *spiC* and *spiA* genes as *ssaB* and *ssaC* (8, 36). The pMIC-10C32 plasmid carries a 2.3-kb fragment that includes much of the coding region for *ssaB* and *ssaC* (12, 64) as well as a 683-bp coding region of *ssrA* (Fig. 5). In plasmid pANT30.1, a 645-bp fragment cloned upstream of promoterless *gfp* in plasmid pFPV25 contains 194 bp of the *ssaB* coding region and 51 bp of the *ssrA* coding region. This fragment induces GFP inside macrophages as well as the original pMIC-C32 clone. A 335-bp fragment cloned in plasmid pANT30.2 also induces GFP inside macrophages. However, an additional 143-bp deletion in plasmid pANT30.3 does not induce GFP inside macrophages. These analyses show that the region between -141 and -284 is necessary for transcription of *ssrA*. The induction profiles inside macrophages for pANT30.1 and pANT30.2 in strains CJD359 (*ompR* mutant) and P3F4 (*ssrA* mutant) were similar to that of plasmid pMIC-10C32, while pANT30.3 was never induced in any strain.

While serovar Typhimurium carrying plasmids pANT30.1

and pANT30.2 induced GFP inside macrophages, the peak fluorescence of extracellular bacteria increased for both deletions (Fig. 5). This shift was consistently reproduced in several independent experiments. The increase in extracellular GFP expression was not observed in pANT30.3, which contains only 141 bp upstream of the *ssrA* ATG, and also does not induce *ssrA* inside macrophages. These results imply that additional regulatory mechanisms which repress *ssrA* expression exist and exert their effect in the intergenic region between *ssaB* and *ssrA* or the region downstream of +51. Our induction experiments cannot determine if there is direct binding from a repressor protein or if repression is due to DNA conformational changes. We addressed the first possibility by creating a random MudJ insertional library in which each clone carried the reporter plasmid pMIC-10C32. We failed to generate any clones that resulted in constitutive GFP expression, although such a mutation could be lethal to *Salmonella* grown on LB agar.

***ssrA* and *ssaH* are induced in minimal medium, pH 4.5.** We tested several in vitro growth conditions for induction of *ssrA* and *ssaH* transcription to learn what *Salmonella* might be detecting inside the host cell. The wild-type and the *ssrA* and *ompR* mutant strains carrying pMIC-10C32 (*ssrA-gfp*) or pFMI10 (*ssaH-gfp*) plasmids were grown standing overnight in LB broth at 37°C (OD₆₀₀, ~0.4) and then subcultured for 3 h in either LB broth at pH 7.0, LB broth at pH 4.5, or minimal media at pH 7.0 and 4.5 and high (1 mM) or low (1 μM) Mg²⁺. Fluorescence profiles in all media tested remained unchanged

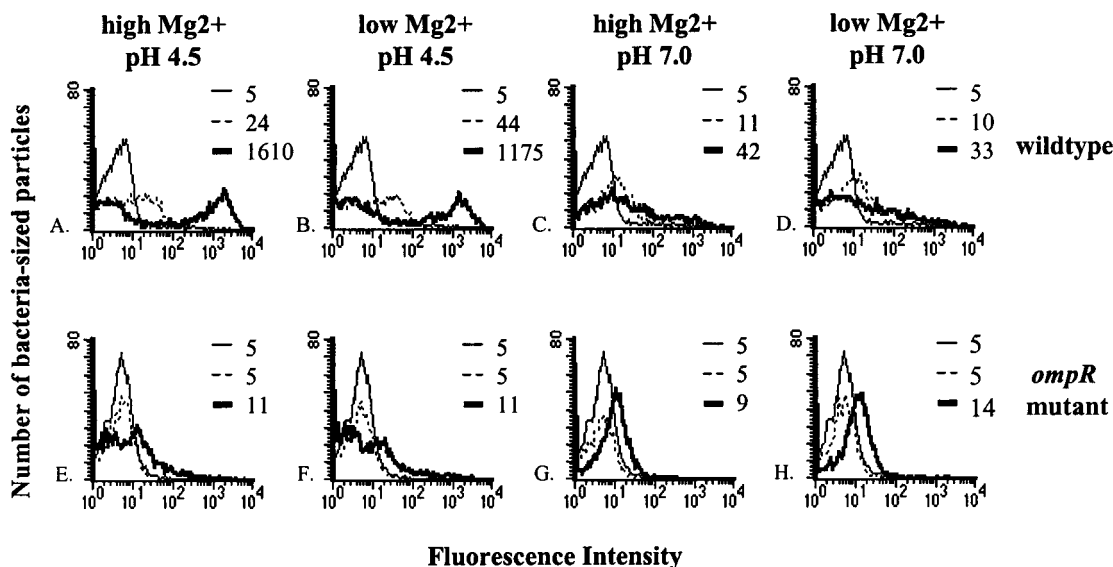


FIG. 6. Minimal medium at low pH is optimal for in vitro induction of *ssrA* and *ssaH* transcription. *Salmonella* cells were grown in LB broth overnight and subcultured in the indicated minimal media for 3 h. The histograms show GFP expression from bacteria carrying plasmids pFMI10 (*ssaH-gfp*) subcultured in LB broth (thin lines), pMIC-10C32 (*ssrA-gfp*) subcultured in minimal medium (dotted lines), or pFMI10 subcultured in minimal medium (thick lines). Peak fluorescences are indicated for each population. The histograms for bacteria with pMIC-10C32 subcultured in LB broth are superimposed on histograms from bacteria carrying plasmid pFMI10 subcultured in LB broth (data not shown).

for SL1344 carrying plasmid pFPV25 or pFPV25.1 (GFP-negative and -positive control reporters, respectively, included in all experiments) (reference 80 and data not shown). Only subcultures in minimal medium, pH 4.5, with either high or low Mg²⁺, induced *ssrA* (IR = 4.8) and *ssaH* (IR = 180) transcription in a wild-type strain (Fig. 6A to D). Consistent with our macrophage data, *ssrA* and *ssaH* induction was reduced significantly in an *ompR* mutant background at low pH with either high or low Mg²⁺ (Fig. 6E to H). We observed slight induction in minimal medium at pH 7.0 (IR = 2.2 for *ssrA* and 8.4 for *ssaH*) but not in LB broth at pH 7.0 (IR = 1) or 4.5 (IR = 1), indicating that SPI-2 genes are transcribed to some extent in response to other signals in minimal medium itself, independent of acidity. We also titrated the pH of minimal medium from pH 7.0 to 4.5 at 0.5-unit intervals and tested the induction of our reporter genes. We found that induction of *ssrA* and *ssaH* increased as acidity increased; maximum induction occurred from pH 5.5 to 4.5 (data not shown). We were able to detect the putative effector proteins, SseA and SseD, by immunoblot analysis from cultures induced in minimal medium at pH 4.5, indicating that components of the SPI-2 type III secretion system are made during our assays.

Our results agree with the recent report that acidic medium induces SPI-2 expression (6) but are in contrast to the report that SPI-2 genes are induced by Mg²⁺ deprivation, not acidity (16). The differences in our results might be attributed to differences in the growth phase of the bacteria (Deiwick et al. used bacteria grown to stationary phase with aeration, while we used bacteria grown with reduced aeration [OD₆₀₀, ~0.4]). Deiwick et al. also reported that SPI-2 gene expression is induced during Mg²⁺ deprivation and that this induction is dependent on the PhoP-PhoQ two-component regulatory system. We tested the induction of *ssaH*-promoter fusions in wild-type and *phoP* mutant strains in minimal medium over 10-fold dilutions of Mg²⁺ concentration (1 mM through 1 μM) and at pH 7.0 or 4.5. Consistent with the results presented in Fig. 6, wild-type bacteria induced high levels of *ssrA* and *ssaH*

transcription at low pH, regardless of Mg²⁺ concentration (data not shown). In contrast, a *phoP* mutant strain showed reduced *ssaH* induction at 1 and 10 μM Mg²⁺ but wild-type levels of induction at 100 μM and 1 mM Mg²⁺ at pH 4.5. We conclude that PhoP-PhoQ has an effect on SPI-2 induction in minimal medium only when the combination of low pH and an Mg²⁺ concentration below 10 μM is present. However, we also found that a *phoP* mutant strain inside macrophages is capable of inducing *ssrA* (Fig. 1) and *ssaH* (data not shown and reference 81) expression to the levels of wild-type bacteria. It is possible that inside a macrophage vacuole, the concentration of Mg²⁺ is above 10 μM, allowing induction of SPI-2 in the absence of *phoP* (28). An alternative explanation is that minimal-medium systems do not accurately mimic the conditions inside the phagosome.

Increasing osmolarity of minimal medium, pH 4.5, represses SPI-2 expression. OmpR-EnvZ is thought to sense differences in osmolarity; therefore, we hypothesized that changing the osmolarity of our in vitro inducing conditions might affect SPI-2 expression. Figure 7 shows that increasing the osmolarity of our in vitro inducing medium, minimal medium at pH 4.5, had a marked effect on SPI-2 expression. We added either 0.5 M NaCl (final concentration of Na²⁺, 1.0 M) or 20% sucrose (data not shown) to minimal medium at pH 4.5. We then compared the inductions of *ssrA-gfp* and *ssaH-gfp* fusions in the original minimal medium at pH 4.5 or in the minimal medium at pH 4.5 with raised osmolarity (Fig. 7). In a wild-type background, *ssrA* and *ssaH* transcription is induced in minimal medium at pH 4.5 (peak fluorescence, 10 and 1,676, respectively). However, when the osmolarity is increased, wild-type strains have reduced *ssrA* (peak fluorescence, 2) and *ssaH* (peak fluorescence, 1) transcription. Fluorescence profiles for the GFP control reporters, pFPV25 and pFPV25.1, remained the same under all conditions. Consistent with the results shown in Fig. 6, induction is abrogated in an *ompR* mutant background. We conclude from these data that high osmolarity

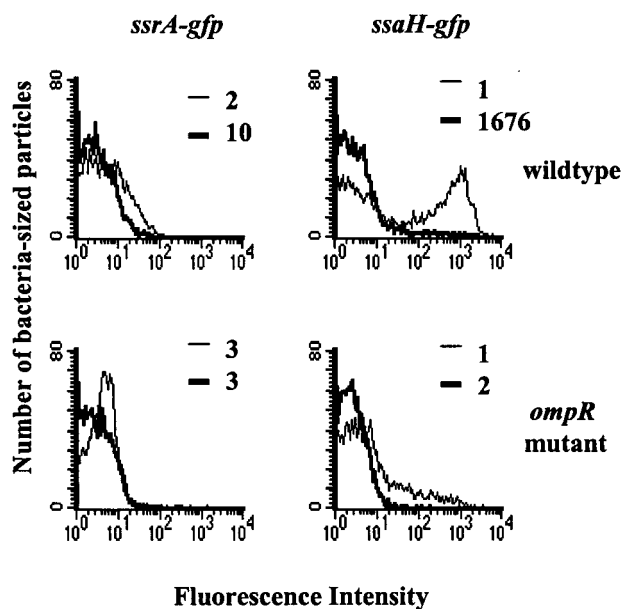


FIG. 7. Increasing the osmolarity of minimal medium at pH 4.5 in an *OmpR*⁺ strain represses *ssrA* and *ssaH* expression. *Salmonella* was grown in LB broth overnight and subcultured in minimal medium at pH 4.5 (thin lines) or minimal medium at pH 4.5 plus 0.5 M NaCl (thick lines). The histograms show GFP expression from the indicated strains subcultured in either low (thin lines)- or high (thick lines)-osmolarity media. Peak fluorescences are indicated for each population. Bacteria subcultured in LB broth did not express significant levels of GFP (data not shown).

represses, whereas low pH induces, SPI-2 expression and that *OmpR* is required for responding to both conditions.

DISCUSSION

We have established that *OmpR* regulates transcription of the two-component regulatory system SsrA-SsrB in serovar Typhimurium. While an *ompR* mutant has been shown to be avirulent in mice, no known *OmpR*-regulated genes have been assigned a clear role in virulence during *Salmonella* infection (reviewed in reference 33). Our study suggests that the avirulent phenotype of the *ompR* strain is at least partially a result of *OmpR* regulating *ssrA* and thereby the genes of the type III secretion system encoded on SPI-2, which are essential for virulence in mice.

The DNase I protection assays show that the *OmpR* protein binds to the *ssrA* promoter region (Fig. 4). This is only the second example of biochemical data showing a response regulator protein directly binding to the promoter region of a different two-component regulatory system. In *Bacillus subtilis*, the PhoP protein of the PhoP-PhoR two-component system directly binds to the promoter region of the *resD* gene of the ResD-ResE sensor-kinase system, which regulates cytochrome *c* biogenesis (7). There is also genetic evidence of other two-component regulatory system cascades. For example, the PhoP-PhoQ system in *Salmonella* controls the expression of the two-component system PmrA-PmrB, which in turn controls expression of a subset of PhoP-activated genes (75). Furthermore, Soncini and Groisman reported that all PhoP-activated genes are induced by Mg²⁺ limitation, but those that are PmrA-PmrB dependent also respond to acid, independent of PhoP-PhoQ. Thus, multiple signals can affect different steps in two-component regulatory cascades.

What is *Salmonella* sensing before and after host cell inva-

sion? In vitro, the invasion genes encoded on SPI-1 are induced at pH 8 (the approximate pH of the gut lumen) and repressed by low pH and low osmolarity (conditions of the phagosome) (4, 15, 28). After *Salmonella* enters the macrophage, the *Salmonella*-containing vacuole acidifies to pH 4.0 to 5.0 within 30 min and *ssrA* and *ssaH* transcription increases (70) (Fig. 2). Phagosome acidification is required for Spi2 gene induction, and low-pH minimal medium induces SPI-2 genes in vitro (6, 12) (Fig. 6). Thus, SPI-1 and SPI-2 appear to be reciprocally regulated. High pH and high osmolarity induce SPI-1 and repress SPI-2, whereas low pH and low osmolarity repress SPI-1 and induce SPI-2.

In addition to *OmpR*-dependent positive regulation, repression of *ssrA* gene expression by unknown factors is evident. Successive deletions of the region upstream of the *ssrA* ATG in plasmids pANT30.1 and pANT30.2 resulted in extracellular bacteria expressing increasing amounts of GFP, indicating that we may have deleted regions necessary for repression of *ssrA* transcription (Fig. 5). Many different things could repress *ssrA* expression. For example, environmental changes might affect DNA conformation and result in altered *ssrA* expression. Changes in DNA topology have been shown to affect expression of a variety of genes (17, 18, 27, 39). *OmpR* protein itself might be an activator as well as a repressor of *ssrA* transcription. In *E. coli*, *OmpR* acts as an activator of *ompF* transcription in low-osmolarity conditions but acts as a repressor of *ompF* transcription in high-osmolarity conditions (reviewed in reference 68). Another possibility is that SsrB or another protein binds to the *ssrAB* promoter region, with or without *OmpR*, to activate or repress transcription. Further experiments are needed to confirm or reject these hypotheses.

We showed that timely induction of SPI-2 inside macrophages is dependent upon *OmpR* (Fig. 2). However, if the SsrA-SsrB two-component regulatory system can eventually detect the vacuolar environment in the absence of *OmpR* and turn on the SPI-2 type III secretion system, then why is an *ompR* mutant still deficient for survival inside macrophages upon complement-mediated entry (Fig. 3)? We speculate that the timing of environmental sensing and response is critical for *Salmonella*. *Salmonella* must respond immediately to the host intracellular environment in order to turn on the appropriate genes to avoid host defenses. The replication assay, which measures bacterial growth within macrophages at 23 h postinfection, cannot determine when after invasion SPI-2 genes function, but at least one SPI-2 gene, SsaB (SpiC), is known to act within hours of invasion (78). In the absence of *OmpR*, low levels of SsrA accumulate enough to allow *ssaH* expression eventually but not enough to allow *Salmonella* to survive and replicate inside the host cell (Fig. 3). This indicates either that low levels of Spi2 are not sufficient to support replication or that *OmpR* regulates other unidentified genes necessary for survival and replication inside macrophages.

OmpR was originally identified as the response regulator for the osmolarity-sensing gene *EnvZ* in *E. coli* (2, 68). By analogy with *E. coli*, we presumed that *EnvZ* is the sensor through which *OmpR* is responding in order to regulate *ssrAB* in *Salmonella*. Our model predicts that there are at least two tiers of *ssrA* regulation: *EnvZ* senses the low pH and low osmolarity of the vacuole, and SsrA is a second sensor, perhaps for later signals in the vacuolar compartment. We do not know what the environmental stimulus for SsrA is at this point. It has been suggested that phosphate starvation may be another signal *Salmonella* is sensing inside the vacuole, though we have not tested this (16). Collaboration between the *OmpR*-*EnvZ* and SsrA-SsrB systems would yield efficient detection of and response to the intracellular environment (Fig. 8).

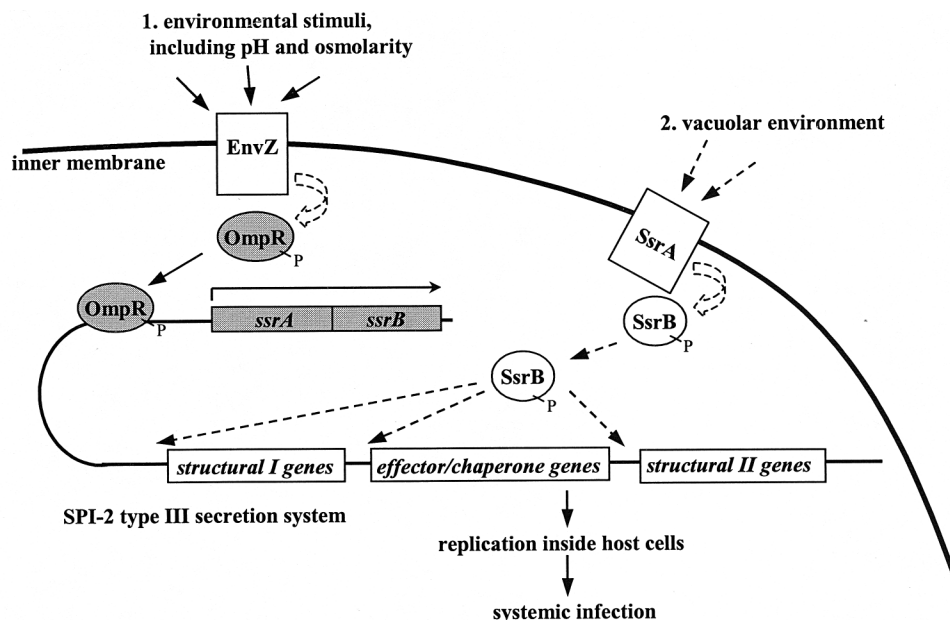


FIG. 8. Model for *Salmonella* SPI-2 regulation inside host macrophages. The OmpR-EnvZ system responds to the intracellular environment, possibly stimulated by the acidic pH and low osmolarity of the phagosome. OmpR binds to the *ssrA* promoter region to activate transcription of the *ssrAB* genes. Later, SsrB detects a different environmental stimulus in the vacuole. SsrB activates expression of the type III secretion system encoded within SPI-2, which then allows for replication inside cells and systemic infection in mice. The solid arrows indicate supporting data from this study and others (12, 35, 36, 64, 74). OmpR and SsrB are hypothesized to be phosphorylated (as indicated by "P") in this model. The shaded proteins and genes represent those studied here. The dashed arrows indicate speculative function based on protein homology, not experimental evidence.

The survival of *Salmonella* demands that the bacterium respond immediately to its surroundings, especially the host cell vacuole. It must turn on the appropriate genes to counter the potentially fatal intracellular host environment. We established here that the OmpR protein of the global two-component regulatory system OmpR-EnvZ regulates the SPI-2 two-component regulatory system, SsrA-SsrB, which in turn activates the type III secretion system in SPI-2. As more information on coordinate gene regulation in *Salmonella* is discovered, we will gain a greater understanding of how pathogens use complex networks of genetic regulation both to exert control and to maintain flexibility in response to the host milieu.

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