Environmental regulation of *Salmonella* pathogenicity island 2 gene expression

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Summary
The enteric pathogen *Salmonella typhimurium* co-ordinates the expression of virulence determinants in response to environmental cues from the host organism. *S. typhimurium* possesses *Salmonella* pathogenicity island 2 (SPI2), a large virulence locus encoding a type III secretion system for virulence determinants required for systemic infections and accumulation inside host cells. We have generated transcriptional fusions to SPI2 genes to analyse expression and used antibodies against recombinant SPI2 proteins to monitor levels of SPI2 proteins under various conditions. Here, we demonstrate that SPI2 gene expression is induced by Mg$^{2+}$ deprivation and phosphate starvation. These conditions are likely to represent the environmental cues encountered by *S. typhimurium* inside the phagosome of infected host cells. The induction of SPI2 gene expression is modulated by the global regulatory system PhoPQ and is dependent on SsrAB, a two-component regulatory system encoded by SPI2.

Introduction
The enteric pathogen *Salmonella* spp. is one of the leading causes of gastrointestinal infections ranging from mild, self-limiting inflammation of the intestinal mucosa to typhoid fever, a life-threatening systemic infection. During the complex multistage pathogenesis, *Salmonella* spp. have to respond to a variety of environmental signals (such as deprivation of nutrients), stress conditions (osmolarity, temperature) and the antimicrobial defence mechanisms of the host (Foster and Spector, 1995; Slauch et al., 1995). To successfully colonize the host organism and to avoid clearance by the host immune system, a large number of general stress response systems as well as specific virulence factors are required (Groisman and Ochman, 1997).

The expression of these sets of genes is co-ordinately regulated in a temporal and spatial fashion (Mekalanos, 1992). Often, the expression of virulence genes is regulated by two-component regulatory systems composed of the membrane-bound sensor and a transcriptional regulator of gene expression (Parkinson and Kofoid, 1992). Although some of the two-component regulatory systems are shared between non-pathogenic bacteria like *Escherichia coli* K-12 and pathogens like *S. typhimurium*, additional systems can be found in the pathogen. The two-component regulatory system PhoPQ is present in both species, but represents an important global regulator of *Salmonella* virulence. The expression of more than 40 loci is under the control of PhoPQ (Soncini et al., 1996), and it has been demonstrated that mutants defective in PhoP or PhoQ, and also a mutant exhibiting a hyperactive state of the PhoQ kinase, are dramatically attenuated for virulence (Miller et al., 1989; Miller and Mekalanos, 1990). Garcia Vescovi et al. (1996) demonstrated that the PhoPQ system senses the concentration of the divalent cations Mg$^{2+}$ and Ca$^{2+}$. PhoPQ has been proposed to be a regulatory system for genes which allows *Salmonella* to survive and replicate within macrophages (Miller, 1991; Garcia Vescovi et al., 1994).

An important virulence trait of *S. typhimurium* is the ability to invade host cells (Galan, 1996). The invasion phenotype is determined by a large cluster of genes present in *Salmonella* pathogenicity island 1 (SPI1), encoding a complex type III secretion system for virulence determinants (Mills et al., 1995). Expression of SPI1 genes is regulated in a co-ordinated fashion in response to environmental signals (Bajaj et al., 1996).

A large cluster of virulence genes located within *Salmonella* pathogenicity island 2 (SPI2) has been identified (Hensel et al., 1995; Ochman et al., 1996; Shea et al., 1996). It has been shown that this locus encodes a second type III secretion system (Hensel et al., 1997a; 1997b), secreted effector proteins and their specific chaperones (Cirillo et al., 1998; Hensel et al., 1998), as well as a two-component regulatory system (Ochman et al., 1996; Shea et al., 1996). Inactivation of these genes by transposon insertions or introduction of antibiotic resistance cassettes results in a dramatic attenuation of virulence in the model system of murine salmonellosis. *In vitro* analyses showed that SPI2 mutations cause pleiotropic
effects, including effects on the expression of SPI1 genes (Deiwick et al., 1998). It has been suggested that SPI2 is important for the survival of Salmonella inside macrophages because SPI2 mutant strains fail to accumulate in macrophages (Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998). Recently, Valdivia and Falkow (1997) reported that a green fluorescent protein (GFP) reporter gene fusion to a promoter of SPI2 was highly induced inside macrophages and epithelial cells. However, the chemical or physical nature of the signals inducing the gene expression of SPI2 was not investigated.

In this study, we set out to characterize the environmental cues affecting the expression of SPI2 genes. Furthermore, the modulation of SPI2 gene expression by regulatory systems in S. typhimurium was analysed. Our data allowed us to define signals inducing SPI2 expression in vitro and indicate that conditions encountered by the pathogen inside the intracellular compartment of the host cell specifically induce the expression of SPI2 genes.

Results

Generation of reporter gene fusions to SPI2 promoters and antibodies against recombinant SPI2 proteins

For the analysis of the expression of SPI2 virulence genes, transcriptional reporter gene fusions of SPI2 genes to firefly luciferase (luc) were generated (Fig. 1). To avoid artefacts by increased copy numbers of promoter elements, fusions were integrated into the S. typhimurium chromosome using luc suicide vectors (Gunn and Miller, 1996). As a second approach, the amount of proteins encoded by SPI2 genes was monitored using antibodies raised against recombinant SPI2 proteins rSsaP and rSscA. SsaP is encoded by a gene of the ssaK/U operon, encoding structural components of the type III secretion system (Hensel et al., 1997b). In contrast to other components of the SPI2 type III secretion system, SsaP has no significant similarity to components of other type III secretion systems, including the SPI1 secretion system of S. typhimurium. SscA is encoded by the third gene of a cluster of nine genes encoding putative effector proteins of SPI2 and their chaperones, and has low sequence similarity to specific chaperones of other type III secretion systems (Hensel et al., 1998). Detection with antisera raised against rSsaP and rSscA allowed us to monitor protein levels of different classes of SPI2 proteins (Fig. 2B).

Effect of environmental parameters on expression of SPI2 genes

The amounts of SPI2 proteins SsaP and SscA under various growth conditions were monitored by Western blot analysis (Fig. 2B), and the expression was analysed

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**Fig. 1. Genetic organization of SPI2, position of reporter gene fusions, plasmids and mutations used in this study.**

A. Genes encoding the type III secretion system apparatus (ssa, open arrows), putative effector proteins (sse, filled arrows), specific chaperones (ssc, shaded arrows) and the two-component regulatory system (ssr, hatched arrows) of SPI2 are indicated. The position of transcriptional fusions to luc of strains MvP127, MvP131 and MvP266, as well as the mTn5 insertion of mutant strain P8G12 is shown. B. The positions of restriction sites (B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; Sa, SalI; Sm, SmaI; Sp, SphI; V, EcoRV), and the position of inserts of plasmids used in this study is indicated.

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using single-copy luc reporter gene fusions to SPI2 genes (Fig. 2A). No significant expression of SPI2 genes was detectable after growth in LB, tryptic soy broth, or tissue culture media such as RPMI-1640 and DMEM, either with or without the addition of 5% fetal calf serum. This observation is in accordance with previous work of Valdivia and Falkow (1997), who observed that expression of a plasmid-borne fusion of the reporter gene GFP to ssaH was highly induced in S. typhimurium residing in the intracellular compartment of infected host cells but repressed during growth in LB or tissue culture media. Expression of ssaP was detected in cells grown in certain batches of BHI (brain–heart infusion) medium, however use of other batches did not induce SPI2 gene expression. In minimal media of various formulations, only low levels of SPI2 gene expression were detected (Fig. 2A). To simulate conditions experienced by S. typhimurium during intracellular episodes, such as the low pH of the intracellular compartment of infected host cells, no significant expression of SPI2 genes was detectable after growth in LB, tryptic soy broth, or tissue culture media such as RPMI-1640 and DMEM, either with or without the addition of 5% fetal calf serum. This observation is in accordance with previous work of Valdivia and Falkow (1997), who observed that expression of a plasmid-borne fusion of the reporter gene GFP to ssaH was highly induced in S. typhimurium residing in the intracellular compartment of infected host cells but repressed during growth in LB or tissue culture media. Expression of ssaP was detected in cells grown in certain batches of BHI (brain–heart infusion) medium, however use of other batches did not induce SPI2 gene expression. In minimal media of various formulations, only low levels of SPI2 gene expression were detected (Fig. 2A). To simulate conditions experienced by S. typhimurium during intracellular episodes, such as the low pH of the

Fig. 2. Expression of SPI2 genes and levels of SPI2 proteins under various growth conditions. S. typhimurium wild-type strain and strain MvP131 harbouring a ssaB::luc fusion were grown overnight in various media. In detail, the following conditions were used: BHI, brain–heart infusion broth; E salts, Vogel–Bonner minimal medium containing 38 mM glycerol and 0.1% casamino acids; NCE, non-citrate Vogel–Bonner minimal medium containing 1 mM MgCl₂, 38 mM glycerol and 0.1% casamino acids; M9, M9 minimal medium containing 0.2% glucose (Miller, 1992); ISM, intracellular salt medium (Headley and Payne, 1990); RPMI, RPMI-1640; PCN, MOPS-buffered minimal medium (Neidhardt et al., 1974); -N, -P, -C, starvation conditions for phosphate, carbon, and nitrogen according to Spector and Cubitt (1992); PCN anaerobic, medium according to Stewart and Parales (1988); PCN-P 8 μM MgCl₂, PCN medium containing 8 μM MgCl₂ and 113 μM phosphate; N salts (100 mM Bis/Tris-HCl, pH 7.0, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 38 mM glycerol, 0.1% casamino acids) containing 10 mM MgCl₂ or 8 μM MgCl₂ as indicated.

A. The Luc activities of lysates of strain MvP131 were determined. Error bars indicate standard deviation from mean.

B. Lysates of the S. typhimurium wild type were subjected to SDS-PAGE, protein was transferred onto nitrocellulose membranes, and SsaP and SscA were detected using antibodies raised against rSsaP and rSscA respectively. Equal amounts of cells as adjusted by OD at 600 nm (OD₆₀₀) were analysed. The positions of SsaP and SscA are indicated. We observed that SscA appears as diffuse double bands in Western analyses. Total cell fractions of IPTG-induced cultures of E. coli BL21(DE3) harbouring pET21(+) (negative control), p5-4-T7 (T7 control for SscA, 50-fold diluted), and p7-26-T7 (T7 control for SsaP, 500-fold diluted) were analysed.

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phagolysosomal compartment (Garcia del Portillo et al., 1992; Rathman et al., 1996), the effect of exposure of bacteria to media at various pHs on SPI2 gene expression was assayed. During growth in LB, no effects of lowered pH on SPI2 gene expression were observed, whereas a two- to fivefold increase in expression was observed if the pH of E or NCE minimal medium was lowered from 7.0 to 5.0 (data not shown). However, only low rates of expression were observed.

Bacteria inside the phagolysosomal compartment of host cells also experience deprivations of nutrients and a low concentration of divalent cations (Garcia del Portillo et al., 1992). The minimal media formulation of Neidhardt et al. (1974) was used to simulate effects of starvation for phosphate, carbon or nitrogen. High levels of expression were only observed after starvation for phosphate (Fig. 2). Next, we analysed the effect of various concentrations of Mg$^{2+}$ or Ca$^{2+}$ on SPI2 gene expression (Fig. 3). Although expression of SPI2 genes was very low after growth in minimal media containing high amounts of Mg$^{2+}$ (10 mM) or Ca$^{2+}$ (2 mM), reporter gene fusions to SPI2 genes were highly induced after growth in minimal media containing Mg$^{2+}$ or Ca$^{2+}$ at a minimal concentration of 8 μM. This effect of divalent cation concentration on SPI2 gene expression was observed in various minimal media such as N, M9, E or NCE, with or without additional nitrogen sources (casamino acids) and with carbon sources such as glycerol, ethanol or glucose (data not shown). As the sole exposure to acidic pH did not induce SPI2 gene expression, the combined exposure of bacteria to limiting concentrations of Mg$^{2+}$ and to acidic pH was analysed. However, no additive effect of both conditions on SPI2 gene expression was observed.

We also analysed the effects of other stress conditions known to activate the expression of Salmonella virulence genes according to Abshire and Neidhardt (1993). The addition of a cationic peptide (2.4 μg ml$^{-1}$ polymyxin B) or oxidizing agents (195 μM paraquat; 125 μM or 300 μM

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H$_2$O$_2$) did not induce the expression of SPI2 genes (data not shown). Increasing the osmolarity of medium with minimal amounts of Mg$^{2+}$ resulted in the reduction of SPI2 gene expression (Table 1). Changes in osmolarity resulting from various concentrations of divalent cations had no effect on SPI2 expression. A reduced osmolarity did not result in increased expression of the ssaB::luc fusion, however evaluation of media of very low osmolarity was limited by the buffer component required (data not shown). Western blot analysis revealed that the levels of proteins encoded by SPI2 genes are not affected by the growth temperature of cultures.

### Effects of divalent cations on SPI2 gene expression

To analyse the effect of divalent cations in more detail, expression of reporter fusions was assayed after growth in minimal media containing various concentrations of divalent cations (Fig. 3). The effect of the Mg$^{2+}$ concentration on SPI2 gene expression was analysed using the Tris-buffered N salts medium formulation as described previously (Hmielet al., 1986; Garcia Vescovi et al., 1996). SPI2 gene expression was highly induced in media containing less than 30 mM Mg$^{2+}$, but not induced in media of very low osmolarity.

### Table 1. Expression of ssaB in media of various osmolarities.

<table>
<thead>
<tr>
<th>Growth conditions (% NaCl)</th>
<th>Relative Luc activity ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>816.141 ± 228.305</td>
</tr>
<tr>
<td>1</td>
<td>808.144 ± 241.541</td>
</tr>
<tr>
<td>2</td>
<td>505.080 ± 188.106</td>
</tr>
<tr>
<td>3</td>
<td>526.63 ± 158.42</td>
</tr>
</tbody>
</table>

Strain MvP131 harbouring a ssaB::luc fusion was grown in N salts minimal medium containing 8 $\mu$M Mg$^{2+}$ and various concentrations of NaCl as indicated, and Luc activities were assayed.
media with higher concentrations of Mg \(^{2+}\). A similar effect
was observed if Mg \(^{2+}\) was replaced by Ca \(^{2+}\); however,
expression of reporter fusions to \(ssaB\) and \(sseA\) was
observed at concentrations equal to or below 100 \(\mu\)M
Ca \(^{2+}\) (data not shown). We observed that the pH of this
medium dropped below 5 after growth of cultures for 14–16 h. Additional experiments were performed with
Bis/Tris-buffered N salts medium to ensure medium pH
was constant over the entire period of growth (Fig. 3B).
Under these conditions, highest induction of SPI2
expression was observed at about 50 \(\mu\)M Mg \(^{2+}\) (Fig.
3B). In contrast, analysis of a reporter gene fusion to
\(mgtB\), a previously defined PhoPQ-regulated gene (Hmiel
\textit{et al.}, 1989), indicated highest induction at 8 \(\mu\)M Mg \(^{2+}\),
without effects of the buffer capacity of the medium (data
not shown).

Further experiments were performed to determine
whether the expression of SPI2 genes could be induced
by withdrawal of divalent cations. (Fig. 4). After growth
in minimal medium containing 8 \(\mu\)M Mg \(^{2+}\) and 2 mM
Ca \(^{2+}\), the expression of reporter gene fusions to SPI2
genes was induced after chelation of Ca \(^{2+}\) by addition of
EGTA (ethylene glycol-bis[\(\beta\)-aminoethyl ether]-\(N,N,N',N'\)-tetraacetic acid) (Fig. 4A). A similar result was obtained
after growth of cultures in minimal media containing
1 mM Mg \(^{2+}\), washing of cells, and shifting cultures to a
medium containing 8 \(\mu\)M Mg \(^{2+}\) (Fig. 4B). This effect on
SPI2 gene expression was observed for reporter gene
fusions to \(ssaB\) and \(ssaj\) (Fig. 4B), and correlated with
the levels of SsaP and SscA detected in Western blots
(Fig. 4C).

**Fig. 5.** Effect of phosphate starvation on SPI2 gene expression.
Strain MvP131 was grown in minimal medium according to O’Neal
\textit{et al.} (1994) containing 25 mM or 110 \(\mu\)M PO\(_4^{3-}\) and various
concentrations of Mg \(^{2+}\) (filled circles, 8 \(\mu\)M Mg \(^{2+}\); open circles
100 \(\mu\)M Mg \(^{2+}\); triangles, 10 mM Mg \(^{2+}\)) as indicated. Cultures were
grown at 37°C with aeration, samples were withdrawn at various
time points, and the Luc activity of bacterial lysates was
determined.

**Effects of starvation for phosphate on SPI2 gene expression**

The effect of phosphate starvation on SPI2 expression
was further investigated by growing cultures in media
containing saturating or limiting amounts of phosphate
and various concentrations of Mg \(^{2+}\), and subsequent ana-
lysis of the expression of a \(ssaB::luc\) fusion (Fig. 5). In the
presence of high amounts of phosphate, this fusion was
expressed in media with low Mg \(^{2+}\) concentrations. In con-
trast, under phosphate starvation conditions, the \(ssa-
B::luc\) fusion was highly induced, but the expression was
not dependent on Mg \(^{2+}\) deprivation. This observation
suggests that phosphate starvation may represent a
further environmental cue stimulating the expression of
SPI2 genes.

**Identification of the transcriptional regulator SsrB**

The partial sequence of the gene of the sensor component
\(SsrA\) (or \(SpiR\)) of a two-component regulatory system in
SPI2 has been reported previously (Ochman \textit{et al.}, 1996).

As part of our effort to characterize the SPI2 locus, cloning
and sequence analysis of DNA downstream of the \(ssrA\)
gene was performed. The insert of a clone of a \(\lambda\) library of
\(S.\ typhimurium\) genomic DNA harbourted the 3’ end of
\(ssrA\) as well as the region downstream of \(ssrA\). The
\(ssrA\) gene encodes a protein of 920 amino-acid residues
and a theoretical molecular weight of 103.6 kDa. Sequence
analysis revealed an open reading frame with coding
capacity for a 24.3 kDa protein downstream of \(ssrA\). The
predicted protein shares significant sequence similarity
with a family of transcriptional activators such as DegU
of \(Bacillus subtilis\), NarL and UvrY of \(E. coli\), SirA of \(S.
typhimurium\) and BvgA of \(Bordetella pertussis\) (Fig. 6A).
Therefore, it is likely that the protein acts as the regulatory
component of the \(ssr\) system and the gene was desig-
nated \(ssrB\). The size of proteins identified after expression
of \(ssrAB\) under control of the T7 promoter was in accor-
dance with the predicted molecular weight (Fig. 6B).
Further characterization of the SPI2 locus indicated that
ORF 242 and ORF 319 downstream of \(ssrB\) are not
required for \(S.\ typhimurium\) virulence (Hensel \textit{et al.},
1999).
We next analysed the effect of mutations in the ssrAB system on the levels of SPI2 proteins. Characterization of the mTn5 insertion of mutant strain P8G12 initially identified by signature-tagged mutagenesis (Hensel et al., 1995) indicated that ssrB was inactivated in this mutant strain. Virulence properties and effects on SPI2 gene expression of a mutant strain in which ssrB had been inactivated by insertion of a aphT gene cassette lacking transcriptional terminators were similar to those of mutant strain P8G12, indicating that the phenotype of the latter is not the result of polar effects on downstream genes (data not shown). Complementation of SPI2 expression in mutant strain P8G12 was possible by introduction of plasmid pssrAB harbouring ssrAB (data not shown). Presence of pssrAB resulted in decreased effects of environmental signals on the amounts of SPI2 proteins present in the cell (Fig. 8). High levels of SsaP and SscA were observed in the wild type and ssrB⁺ mutant strain harbouring Q1999 Blackwell Science Ltd, Molecular Microbiology, 31, 1759–1773

Fig. 6. Alignment of SsrB of S. typhimurium to various bacterial transcriptional regulators. A. The deduced amino-acid sequence of SsrB was aligned to SirA of S. typhimurium (Swiss-Prot accession number P96058), UvrY of E. coli (P07027), DegU of Bacillus subtilis (P13800), NarL of E. coli (P10957) and BvgA of Bordetella pertussis (P16574) using the ClustalW module of MacVector version 6.0. Similar residues are boxed, identical residues are indicated by shading.

B. Expression of ssrB under control of the T7 promoter according to Studier et al. (1990). E. coli BL21(DE3) harbouring pWSK29 (lane 1) or pssrAB (lane 2) were grown to mid-log phase, 1 mM IPTG and 20 μCi [35S]-methionine/cysteine (Promix, Amersham) were added and labelling was performed for 5 min. Bacteria were pelleted, boiled for 5 min in SDS–PAGE sample buffer, and aliquots corresponding to 100 μl culture were analysed by SDS–PAGE on 12% gels and subsequent autoradiography.

Effects of SsrAB, PhoPQ and further regulatory systems on SPI2

We next analysed the effect of mutations in the ssrAB system on the levels of SPI2 proteins. Characterization of the mTn5 insertion of mutant strain P8G12 initially identified by signature-tagged mutagenesis (Hensel et al., 1995) indicated that ssrB was inactivated in this mutant strain. Virulence properties and effects on SPI2 gene expression of a mutant strain in which ssrB had been inactivated by insertion of a aphT gene cassette lacking transcriptional terminators were similar to those of mutant strain P8G12, indicating that the phenotype of the latter is not the result of polar effects on downstream genes (data not shown). Analysis of luc fusions in the strain backgrounds of mutants P3F4 (ssrA::mTn5; Hensel et al., 1998) and P8G12 (ssrB::mTn5; Hensel et al., 1998; Fig. 9) resulted in a strong reduction of the expression of SPI2 genes. Furthermore, very low levels of SscA were detected in the ssrB⁺ background after growth in media containing limiting amounts of Mg²⁺ or phosphate (Figs 7 and 8). Complementation of SPI2 expression in mutant strain P8G12 was possible by introduction of plasmid pssrAB harbouring ssrAB (data not shown). Presence of pssrAB resulted in decreased effects of environmental signals on the amounts of SPI2 proteins present in the cell (Fig. 8). High levels of SsaP and SscA were observed in the wild type and ssrB⁺ mutant strain harbouring
Fig. 7. Role of SsrAB and PhoPQ. S. typhimurium wild type (wild type), strain P8G12 harbouring a mTn5 insertion in ssrB (ssrB−), and strains CS022 (pho-24) and CS015 (phoP−) were analysed. Bacterial cultures were grown in minimal medium containing 10 mM MgCl2 to mid-log phase (OD600 about 0.5), pelleted, washed three times in minimal medium with 8 μM MgCl2 and resuspended in fresh minimal media containing 10 mM MgCl2. Cultures were incubated at 37°C and samples were withdrawn at various time points as indicated. Total protein from equal amounts of bacterial cells as adjusted by OD600 was separated by SDS–PAGE, transferred onto membranes and SscA was detected using antibodies raised against the recombinant protein.

The induction of SPI2 gene expression in minimal media containing micromolar amounts of Mg2+ or Ca2+ is similar to the characteristics of a subset of genes whose expression is controlled by the global regulatory system PhoPQ (Miller et al., 1989; Garcia Vescovi et al., 1996; Soncini et al., 1996). These loci are referred to as ‘PhoP-activated genes’ or pag. To determine whether SPI2 represents a pag locus or whether exposure of S. typhimurium to conditions of low cation concentrations is sensed by a regulatory system independent from PhoPQ, luc fusions to SPI2 genes were moved into strains CS015 (phoP−) and CS022 (pho-24) by P22-mediated transduction. It was observed before that phoP− strains show growth defects in minimal media with limiting concentrations of divalent cations (Miller and Mekalanos, 1990). To analyse the effect of the PhoPQ system on SPI2 expression under similar conditions, cells were grown to mid-log phase in media with 10 mM Mg2+, washed, then transferred to media containing 8 μM or 10 mM Mg2+. Under these assay conditions, all strains had similar growth rates for up to 2.5 h after transfer to low-Mg2+ media. After this time point, no further growth of CS015 was observed. Levels of SscA were analysed at various time points after the shift using antibodies raised against rSscA (Fig. 7). In the wild type and pho-24 background, the expression of ssscA was induced after shift of cultures to media containing 8 μM Mg2+. However, levels of SscA were highly reduced in the phoP− strain. This observation was confirmed by analysis of the expression of reporter gene fusions in the genetic background of wild-type, pho-24 and phoP− strains. After shifting cultures to minimal media containing 8 μM Mg2+, no expression of the luc fusions to SPI2 genes was observed in the phoP− background (data not shown).

The role of other regulatory systems affecting Salmonella virulence gene expression on SPI2 gene expression was analysed. Reporter gene fusions were transduced in strains harbouring mutations in hilA, the local transcriptional activator of SPI1 gene expression (Bajaj et al., 1995; 1996), sirA, a regulator affecting the expression of SPI1 (Johnston et al., 1996), and rpoS, encoding an alternative sigma factor important for the regulation of spv genes of the Salmonella virulence plasmid (Swords et al., 1997; Wilmes Riesenberg et al., 1997). Expression of luc fusions to SPI2 genes was assayed after growth in minimal media containing various concentrations of MgCl2. The induction of SPI2 gene expression was similar in the wild-type background and in strain backgrounds with mutations in hilA, sirA and rpoS (data not shown). Under the in vitro conditions defined in this study, SPI2 gene expression is not affected by these regulators.

Regulation of SPI1 and SPI2 gene expression

We studied the expression of genes for the SPI1 and SPI2 type III secretion systems under conditions previously described to be inducing for SPI1 genes (Bajaj et al., 1996), and defined here to be inducing for SPI2 genes (Fig. 9). Growth in media with high osmolarity and O2 limitation induced SPI1 gene expression, but not SPI2 gene expression. As previously shown (Bajaj et al., 1995; 1996), our experiments confirm that the expression of SPI1 genes is dependent on the function of the local...
transcriptional activator HilA. However, under these conditions only low levels of expression of sipC were detected in the ssrB¹ background, indicating that SsrB is required for the expression of sipC. The effect of mutations in ssrB and other SPI2 genes on the expression of SPI1 genes has been reported in detail (Deiwik et al., 1998). The molecular basis of these effects of SPI2 mutations has not yet been elucidated. In contrast, under conditions inducing SPI2 gene expression, no significant expression of SPI1 genes was observed. Inactivation of HilA had no effect on the expression of the ssaB::luc fusion.

SPI2 gene expression of intracellular Salmonella

To investigate whether in vitro conditions that induce SPI2 reflect the conditions encountered by S. typhimurium during infection of host cells, the expression of reporter gene fusions by bacteria localized within macrophages was analysed (Fig. 10). Intracellular location of S. typhimurium resulted in a strong induction of the reporter gene fusion ssaB::luc and ssaJ::luc. No significant expression was observed in the ssrB⁻ background, indicating that SsrB is required for the expression of sipC. The effect of mutations in ssrB and other SPI2 genes on the expression of SPI1 genes has been reported in detail (Deiwik et al., 1998). The molecular basis of these effects of SPI2 mutations has not yet been elucidated. In contrast, under conditions inducing SPI2 gene expression, no significant expression of SPI1 genes was observed. Inactivation of HilA had no effect on the expression of the ssaB::luc fusion.

Fig. 9. Inverse regulation of expression of genes in SPI1 and SPI2. The expression of reporter gene fusions in SPI2 (ssaB::luc, open bars) and SPI1 (sipC::lacZ) were analysed in the background of S. typhimurium wild-type strain, and ssrB and hilA mutant strains. Strains were grown under conditions previously described as highly inducing for SPI1 gene expression (Bajaj et al., 1996) or defined here as inducing for SPI2 gene expression. Cultures were grown for about 16 h in LB containing 1% NaCl for induction of SPI1, or in N salts minimal medium containing 8 μM Mg²⁺ for the induction of SPI2. The Luc activities of lysates of strains harbouring the ssaB::luc fusion (open columns) and β-galactosidase activities of strains harbouring the sipC::lacZ fusion (solid columns) were determined.

Discussion

Induction of SPI2 gene expression by environmental signals

SPI2 plays a pivotal role in the virulence of S. typhimurium in the model of murine salmonellosis (Ochman et al., 1996; Shea et al., 1996), and it has been shown that SPI2 contributes to the intracellular proliferation of S. typhimurium within infected host cells (Cirillo et al., 1998; Hensel et al., 1998). Recently, a fusion of a SPI2 gene to GFP was identified which was highly induced in bacteria inside macrophages (Valdivia and Falkow, 1997), indicating that the presence within an intracellular compartment specifically induces the expression of SPI2 genes. We identified environmental signals inducing SPI2 gene expression by analysing SPI2 gene expression under in vitro conditions. These experiments indicated that the exposure of S. typhimurium to media with limiting concentrations of divalent cations was highly inducing for the expression of various SPI2 genes. Furthermore, starvation of S. typhimurium for phosphate resulted in the induction of the expression of SPI2 genes. Similarities between protein patterns of S. typhimurium grown under phosphate starvation in vitro and bacteria exposed to the intracellular environment were observed using proteome analysis (Abshire and Neidhardt, 1993). Recently, a GFP fusion to a gene encoding a phosphate transport
protein has been identified as intracellularly induced (Valdivia and Falkow, 1997). These observations suggest that phosphate limitation is an important environmental cue within intracellular compartments. Our in vitro analyses did not identify further inducing SPI2 gene expression. It has been suggested that exposure to acidic pH is an important signal for the induction of S. typhimurium virulence genes (Alpuche Aranda et al., 1992; Rathman et al., 1996). However, the environmental stimulus ‘acidic pH’ did not significantly induce SPI2 expression. These observations may indicate that the acidification of the phagosome is a prerequisite for subsequent changes in the phagosomal milieu that affects gene expression of intracellular Salmonella, or that there are subsets of genes whose expression is induced by low pH, and others whose expression is induced by other signals such as nutrient starvation or low concentrations of divalent cations.

### Role of two-component regulatory systems in SPI2 gene expression

The expression of SPI2 virulence genes is dependent on the function of the two-component regulatory system SsrAB, encoded by SPI2. Inactivation of SsrA or SsrB resulted in a strong attenuation of virulence (Shea et al., 1996; Valdivia and Falkow, 1997). SPI2 gene expression was not induced in the srbB background in vitro (this study) or in intracellular bacteria (Valdivia and Falkow, 1997). The induction of reporter gene fusions in intracellular bacteria indicated that environmental signals received and transmitted by SsrAB are present during growth in vitro as well as within infected host cells.

A strong correlation between the expression of various SPI2 genes and the amount of Mg$^{2+}$ present in the growth medium was observed. Furthermore, only very low levels of SPI2 proteins were detected in the phoP$^+$ background. However, levels of SPI2 proteins were dependent on the Mg$^{2+}$ concentration of the growth medium in the background of the pho-24 mutation. The observation that the pho-24 allele did not result in a constitutive expression of SPI2 genes may indicate that SPI2 gene expression is not directly regulated by but modulated via the PhoPQ system. The effect of PhoPQ on the expression of SPI2 genes described here is in contrast to the observations of Valdivia and Falkow (1997), who reported that the intramacrophage induction of a GFP fusion to a SPI2 promoter was independent of PhoP. Possible explanations of this difference may be the type of reporter fusion used (single-copy chromosomal fusions in this study compared with plasmid-encoded fusions) or the existence of signals specific for the intracellular environment that compensate the PhoP$^+$ phenotype. It is worth noting that a phoP$^+$ background results in attenuation of virulence (Miller et al., 1989), defects in intramacrophage accumulation (Miller et al., 1989; Hensel et al., 1998), as well as in growth defects in minimal media containing limiting amounts of Mg$^{2+}$ (Garcia Vescovi et al., 1996; Soncini et al., 1996). An alternative explanation to the modulation of SPI2 expression via PhoPQ is a direct sensing of divalent cation concentrations by SsrA, or the sensing of changes to the cell envelope under conditions of Mg$^{2+}$ or phosphate starvation. Further work has to be carried out to identify the signal integrated by the SsrAB system.

### Inverse regulation of SPI1 and SPI2

Two structurally similar type III secretion systems for virulence determinants are present in S. typhimurium. This observation raised the question of how the secretion of target proteins can be directed via either of the secretion systems. Our results indicate that the expression of the type III secretion systems of SPI1 and SPI2 is induced by different environmental conditions. It has been demonstrated that certain SPI1 genes are PhoPQ repressed (Pegues et al., 1995), and it was assumed that expression of SPI1 genes is not required after entry into cells or penetration of the epithelial layer. In contrast, the expression of SPI2 genes is positively modulated by PhoPQ and induced by the intracellular location of bacteria. We have not been able to define media or growth conditions under which the expression of both type III secretion systems of SPI1 and SPI2 is induced. The comparison of in vitro conditions activating SPI1 and SPI2 gene expression indicates that environmental conditions that result in the simultaneous induction of both SPI are not likely to occur in vivo.

### Co-ordinated regulation of SPI2 gene expression

Our data indicate that SPI2 represents a virulence locus whose expression is co-ordinately regulated and activated in a specific phase of the pathogenesis. S. typhimurium possesses sensory systems to detect stress conditions and situations of nutritional deprivation present in the vacuolar environment of a host cell. Previous reports indicate that nutritional deprivation within the vacuole results in the induction of various stress response systems and high-affinity uptake systems for ions and nutrients (Buchmeier and Heffron, 1990; Garcia del Portillo et al., 1992; Burns-Kellher et al., 1998). Mutants in PhoP or the transport systems MgtCB are unable to proliferate within the intracellular environment of infected macrophages. These mutants have been rescued by addition of high amounts of extracellular Mg$^{2+}$ (Blanc-Potard and Groisman, 1997), however the physiological significance of these observations is controversial (Moncrief and Maguire, 1998). We suggest that the environmental stimuli ‘low-Mg$^{2+}$}'

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concentration’ and ‘phosphate starvation’ signal to *Salmonella* that it is within a vacuole of the host cell. One of the specific responses is the induction of SPI2 gene expression. Proteins translocated by the SPI2-encoded type III secretion system may then influence the intracellular trafficking of the *Salmonella*-containing vacuole and contribute to the survival and accumulation of *Salmonella* inside the host cell. The regulation by environmental signals reported here further support the role of SPI2 during intracellular life.

The regulatory cascade postulated in this work also reflects an important event in the evolution of the *Salmonella* virulence. *ssrAB*, the local regulatory system for the expression of SPI2 genes, was probably introduced together with genes encoding the type III secretion system of SPI2 in the same event of horizontal gene transfer. However, the bacterial cell gained control over this virulence factor by modulation of SPI2 expression by the PhoPQ system. A similar proposal has been made for the *pmr* locus (Gunn and Miller, 1996; Gunn et al., 1996), and for the *mgtCB* genes within SPI3 which are also under the control of PhoPQ (Blanc-Potard and Groisman, 1997). Our results extend the role of the PhoPQ system as a global regulator of *Salmonella* virulence.

There are several questions arising from the environmental regulation of SPI2 reported here. Further work is needed to reveal how SPI2 gene expression is modulated by PhoPQ, and how the regulatory hierarchy of SPI2 genes is organized. Because the two-component regulatory system SsrAB is absolutely required for the expression of SPI2 genes, identification of the signal acting on the sensory component SsrA will be crucial for the understanding of SPI2 function and intracellular pathogenesis of *S. typhimurium*.

### Table 2. Bacterial strains, phages and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Description</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5a</td>
<td>See reference</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>CC118 λpir</td>
<td>λpir lysogen</td>
<td>de Lorenzo and Timmis (1994)</td>
</tr>
<tr>
<td>S17-1 λpir</td>
<td>λpir lysogen</td>
<td>de Lorenzo and Timmis (1994)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
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<td>Novagen</td>
</tr>
<tr>
<td>M15</td>
<td>pREP</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>S. typhimurium strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC12023</td>
<td>Wild type</td>
<td>NCTC</td>
</tr>
<tr>
<td>LT2a</td>
<td>Wild type</td>
<td>SGSC</td>
</tr>
<tr>
<td>CS015</td>
<td><em>phoP-102::Tn10d-Cm, PhoP-</em></td>
<td>Miller et al. (1989)</td>
</tr>
<tr>
<td>CS022</td>
<td><em>pho-24</em></td>
<td>Miller and Mekalanos (1990)</td>
</tr>
<tr>
<td>EE638</td>
<td><em>sipC::lacZY</em></td>
<td>Hueck et al. (1995)</td>
</tr>
<tr>
<td>P6G12</td>
<td><em>ssaB::mTn5</em></td>
<td>Shea et al. (1996)</td>
</tr>
<tr>
<td>MvP127</td>
<td><em>ssaA::lac</em></td>
<td>Hensel et al. (1998)</td>
</tr>
<tr>
<td>MvP131</td>
<td><em>ssaB::lac</em></td>
<td>This study</td>
</tr>
<tr>
<td>MvP239</td>
<td>EE638 in NCTC12023</td>
<td>This study</td>
</tr>
<tr>
<td>MvP244</td>
<td><em>ssaB::lac ssrB::mTn5</em></td>
<td>This study</td>
</tr>
<tr>
<td>MvP266</td>
<td><em>ssaA::lac</em></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
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<td>λ1, 2, 5, 7</td>
<td>Clones from a library of <em>S. typhimurium</em> genomic DNA in λ1059</td>
<td>Shea et al. (1996)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
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<td>pUC18</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, high-copy vector</td>
<td>Gibco-BRL</td>
</tr>
<tr>
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<td>Amp&lt;sup&gt;+&lt;/sup&gt;, high-copy vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pWSK29</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, low-copy vector</td>
<td>Wang and Kushner (1991)</td>
</tr>
<tr>
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<td>Novagen</td>
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<td>Gunn and Miller (1996)</td>
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<td>pGPL01</td>
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<td>Gunn and Miller (1996)</td>
</tr>
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<td>Amp&lt;sup&gt;+&lt;/sup&gt;, His tag expression vector</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE32</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, His tag expression vector</td>
<td>Qiagen</td>
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<td>p1-4</td>
<td>7 kb <em>PstI</em> fragment of <em>λ1</em> in pT7-Blue</td>
<td>This study</td>
</tr>
<tr>
<td>p2-2</td>
<td>5.7 kb <em>BamHI</em> fragment of <em>λ2</em> in pUC18</td>
<td>This study</td>
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<tr>
<td>p5-2</td>
<td>5.7 kb <em>EcoRI</em> fragment of <em>λ5</em> in pKS+</td>
<td>Hensel et al. (1998)</td>
</tr>
<tr>
<td>p5-4</td>
<td>5.5 kb <em>HindIII</em> fragment of <em>λ5</em> in pKS+</td>
<td>Hensel et al. (1998)</td>
</tr>
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<td>p7-26</td>
<td>7 kb <em>NsiI</em> fragment of <em>λ7</em> in pSK+</td>
<td>This study</td>
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<tr>
<td>p5-4-17</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, p5-4 insert in pET21(+)</td>
<td>This study</td>
</tr>
<tr>
<td>p7-26-T7</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, p7-26 insert in pET21(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pssrAB</td>
<td>5.9 kb <em>KpnI/PvuI</em> fragment of SPI2 in pWSK29</td>
<td>This study</td>
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<td>pJD11</td>
<td><em>ssaP</em> in pQE30</td>
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</tr>
<tr>
<td>pJD12</td>
<td><em>sscA</em> in pQE32</td>
<td>This study</td>
</tr>
</tbody>
</table>

Experimental procedures

Bacterial strains and growth conditions

Strains used in this study are listed in Table 2. E. coli and S. typhimurium were routinely cultivated in Luria broth (LB) or on LB agar (Miller, 1992). Minimal media were used as follows: M9, E salts (Vogel-Bonner minimal salts); NCE salts (citrate-free minimal salts) (Maloy et al., 1996) containing 1 mM MgCl₂ and intracellular salt medium (ISM) (Headley and Payne, 1990). N salts minimal medium containing 0.1% casamino acids (Difco) and 38 mM glycerol (Hmiel et al., 1986) with various concentrations of divalent cations added as indicated. For buffering of N salts medium over the entire time of cultural growth, 100 mM Tris-HCl (pH 7.4) used in the original formulation was replaced by 100 mM MOPS-NaOH (pH 7.0) or 100 mM Bis/Tris-HCl (pH 7.0). For the analysis of starvation conditions, MOPS-buffered salts minimal medium (Neidhardt et al., 1974) was used with modifications for anaerobic culture conditions (Stewart and Parales, 1988) and carbon, nitrogen or phosphate starvation (Spector and Cubitt, 1992). The basal MOPS salts containing micronutrients, 15 mM NH₄Cl, and 0.4% glucose has been used with 25 mM K₂HPO₄/KH₂PO₄ (pH 7.4) for high-phosphate and 0.113 mM K₂HPO₄/KH₂PO₄ for phosphate starvation conditions (O'Neal et al., 1994). Other growth media were used as brain–heart infusion medium (BHI, Difco), RPMI-1640 and DMEM. Routinely, bacterial cultures of 3 ml volume were grown aerobically in glass test tubes in a roller drum (New Brunswick) with agitation at 50 r.p.m. Antibiotics were used in the following concentrations: 50 µg ml⁻¹ carbenicillin, 50 µg ml⁻¹ kanamycin, 50 µg ml⁻¹ chloramphenicol, 100 µg ml⁻¹ nalidixic acid, 20 µg ml⁻¹ tetracycline.

The pho-24 allele of strain CS022 and its derivatives was confirmed by streaking on LB agar containing 40 µg ml⁻¹ X-phosphate and a selection of deep-blue colonies (Belden and Miller, 1994).

DNA manipulations were performed according to standard procedures (Sambrook et al., 1989). For the sequencing and cloning of ssrB and the 3’ end of ssrA, a PetI fragment of a clone λ1 (Shea et al., 1996) harbouring a portion of SPI2 was subcloned to generate p1-4. Sequence analysis was performed by a primer-walking strategy using the dye terminator chemistry on an ABI 373 sequencer. The DNA sequence is available under EMBL accession number 295891.

Plasmids for expression of SPI2 genes under control of the T7 promoter were generated by subcloning the insert of plasmids p5-4 and p7-26 in pET21 (+) to generate p5-4-T7 and p7-26-T7 respectively. E. coli BL21(p5-4-T7) and BL21(p7-26-T7) expressed sscA and ssrA, respectively, after induction by IPTG. A plasmid for complementation and expression of ssrAB under control of the T7 promoter was constructed by subcloning a 3.7 kb BamHI/KpnI fragment of p2-2 into p1-4. The resulting construct was digested by PvuI, blunt ended by treatment with the Klenow fragment of DNA polymerase I, followed by digestion with KpnI. A 5.9 kb fragment was recovered and ligated to KpnI/EcoRV-digested pWSK29 to generate pssrAB.

For the generation of recombinant SsaP (rSsaP), a 374 bp PCR fragment was generated using primers SsaP-For (5'-CTCGGATCCAAATGAGGGAAG-3') and SsaP-Rev (5'-GTGAAGCTTCTCATCCTGATTTGTA-3'); and for rSscA, a 475 bp PCR fragment was generated using SscA-For (5'-CTCGGATCCCGACCCTAACAAGC-3') and SscA-Rev (5'-GTGAAGCTTCTCATCCTGATTTGTA-3') introducing BamHI and HindIII sites as indicated by underlining. After digestion with BamHI and HindIII, fragments were ligated to pQE30 and pQE32 vectors to generate pJD11 and pJD12 for the expression of rSsaP and rSscA, respectively, introducing N-terminal His-tag fusions.

Recombinant SPI2 proteins, generation of antibodies and Western blotting

Recombinant proteins rSsaP and rSscA were expressed according to standard protocols (Qiagen), and purified by metal-chelating chromatography using HiTrap columns according to the manufacturer (Pharmacia). Antigens were administered to rabbits by subcutaneous injection of purified protein (about 1 mg) and emulsified with complete or incomplete Freund’s adjuvant for initial or booster immunisations respectively (Harlow and Lane, 1988). Antibody titres were checked by Western blots using the purified antigen and total cell fractions of E. coli expression SPI2 genes under the control of the T7 promoter.

For Western blot analysis, total bacterial cells were resuspended in SDS–PAGE sample buffer (Schägger and von Jagow, 1987), boiled for 5 min and protein was separated on 12% SDS–PAGE gels using the tricine buffer system (Schägger and von Jagow, 1987). Protein was transferred into nitrocellulose membranes (Schleicher and Schuell) using a semidybind blotting apparatus (Bio-Rad) with a buffer system according to Kyhle-Andersen (1984). Detection of antibodies was performed using the ECL system according to the manufacturer’s protocol (Amersham).

Generation of reporter gene fusions

Fusions of the reporter gene firefly luciferase to various genes in SPI2 were obtained using the suicide vectors pLB02 and pGPL01 (Gunn and Miller, 1996), kindly provided by Drs Gunn and Miller (Seattle). For the generation of a fusion to ssaB, a 831 bp EcoRV fragment of p2-2 was subcloned in EcoRV-digested pSK1 (Schaegger and von Jagow, 1987), boiled for 5 min and protein was separated on 12% SDS–PAGE gels using the tricine buffer system (Schägger and von Jagow, 1987). Protein was transferred into nitrocellulose membranes (Schleicher and Schuell) using a semidybind blotting apparatus (Bio-Rad) with a buffer system according to Kyhle-Andersen (1984). Detection of antibodies was performed using the ECL system according to the manufacturer’s protocol (Amersham).
of *S. typhimurium* was confirmed by Southern analysis. Fusions were then moved into a mouse-passaged strain of *S. typhimurium* 12023 by P22 transduction according to standard procedures (Maloy et al., 1996).

Assay of reporter genes

β-Galactosidase activities of reporter gene fusions were determined according to standard procedures (Miller, 1992).

Luciferase activities of bacterial cultures were determined using the Promega (Heidelberg) luciferase assay kit or custom-made reagents accordingly. Briefly, bacteria were pelleted by centrifugation for 5 min at 20,000 × g at 4°C and resuspended in lysis buffer (100 mM KH₂PO₄–KOH, pH 7.8, 2 mM EDTA, 1% Triton X-100, 5 mg ml⁻¹ bovine serum albumin, 1 mM DTT, 5 mg ml⁻¹ lysozyme). Lysates were incubated for 15 min at room temperature with repeated agitation and subjected to a freeze–thaw cycle. Aliquots of the lysates (25 μl) were transferred into microtiter plates (Microfluor, Dynatech) and immediately assayed after addition of 50 μl of luciferase reagent [20 mM Tricine-HCl, pH 7.8, 1.07 mM (MgCO₃)₄Mg(OH)₂, 100 μM EDTA, 33.3 mM DTT, 270 μM Li₃-coenzyme A, 470 μM d(-)-luciferin, 530 μM Mg-ATP] for photon emission using the TriLux MicroBeta luminometer (Wallac). All assays were carried out in triplicate and repeated on independent occasions. Luciferase activities of equal amounts of cells as adjusted by OD₆₀₀ are shown.

Cell culture and infection assays

The macrophage-like cell line J774.A1 was maintained in DMEM with 10% fetal calf serum at 37°C in an atmosphere containing 5% CO₂. J774.A1 cells were seeded at 10⁵ cells per well in 24-well tissue culture dishes and incubated overnight. Bacterial strains were grown in LB broth to stationery phase. Bacterial strains were added to the cell monolayer at a multiplicity of infection (m.o.i.) of 10:1 and centrifuged for 5 min at 3000 rpm at RT and frozen immediately at −20°C. The frozen samples were thawed and 25 μl used for the Luc assay. The luciferase activity per recovered bacteria was calculated.

Acknowledgements

This work was supported by DFG grant 1964/2 and the BMBF grant 01KI 9609 ‘Molekulare Pathogenese der Salmonellen’. We would like to thank Drs S. I. Miller, J. Gunn, C. J. Hueck, and M. E. Maguire for providing bacterial strains and plasmids. Critical reading of the manuscript by Drs W.-D. Hardt and D. W. Holden is acknowledged. We are indebted to Drs E. Riedel and J. Heesemann for generous support of this work and stimulating discussions.

References


