Regulation of the *spvR* gene of the *Salmonella typhimurium* virulence plasmid during exponential-phase growth in intracellular salts medium and at stationary phase in L broth

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The authors previously showed that the SpvR-regulated *spvABCD* operon of the *Salmonella typhimurium* virulence plasmid is highly induced during exponential-phase growth by salmonellae intracellularly in mammalian cells and in a medium designed to mimic the intracellular environment of mammalian cells, intracellular salts medium (ISM), as well as at stationary phase in L broth (LB). The most relevant signal(s) for *spv* gene expression *in vivo* is not known. To elucidate the means by which salmonellae regulate the *spv* genes in response to the environment during the disease process, expression of the *spvR* gene, encoding the positive regulatory protein SpvR, was examined under these same growth conditions by using RNase-protection analysis. *spvR* was expressed at a low, basal level during exponential growth in LB but was induced during exponential growth in ISM and during stationary phase in LB, the same conditions that increased expression of the *spvABCD* operon. Basal expression of *spvR* during exponential growth in LB was independent of both SpvR and the alternative sigma factor RpoS, whereas maximal induction of *spvR* was dependent on both SpvR and RpoS. In an RpoS[−] background, *spvR* message was decreased in stationary phase, whereas *spvR* exhibited residual RpoS-independent induction during exponential growth in ISM. Deletion of *spvA* from the virulence plasmid of *S. typhimurium* increased expression of *spvR* during stationary phase in LB, but not during exponential growth in ISM. These results suggest that expression of *spvR* is controlled by different regulatory factors, depending on the growth conditions encountered by the salmonellae.

Keywords: *Salmonella typhimurium*, virulence, regulation, expression

INTRODUCTION

The plasmid-encoded *spv* virulence genes of *S. typhimurium* and other non-typhoidal serovars of *Salmonella enterica* have been correlated with systemic salmonellosis in humans (Fang & Fierer, 1991; Montenegro *et al.*, 1991; Fierer *et al.*, 1992; Gulig *et al.*, 1993) and are essential for systemic infection in orally inoculated mice (Gulig & Curtiss, 1987; Gulig *et al.*, 1993). The *spv*

genes increase the growth rate of the salmonellae within host cells (Gulig & Doyle, 1993), specifically macrophages (Gulig *et al.*, 1998). However, the exact mechanism(s) by which the *spv* genes increase the growth rate within host cells is not known. The *spv* genes consist of *spvABCD*, which are transcribed as an operon (Krause *et al.*, 1992) and *spvR*, which encodes the positive regulatory protein for the *spv* genes (Caldwell & Gulig, 1991; Fang *et al.*, 1991; Matsui *et al.*, 1991; Taira *et al.*, 1991). SpvR is a member of the LysR family of positive activators and regulates the transcription of the *spv* genes by binding to sequences upstream of *spvA* and *spvR* (Grob & Guiney, 1996; Grob *et al.*, 1997). However, analysis of the DNA and

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Abbreviations: ISM, intracellular salts medium; LB, L broth; MU, Miller units.

deduced amino acid sequences of *spvABCD* has not aided in elucidating their function.

We previously examined the expression of the *spvABCD* operon by measuring expression of *spvA* under different growth conditions *in vitro*, as well as intracellularly in cell culture and in infected mice (Wilson *et al.*, 1997). It has been widely reported that the *spv* genes are induced during the stationary phase of growth (Fang *et al.*, 1991; Coynault *et al.*, 1992; Valone *et al.*, 1993; O'Byrne & Dorman, 1994a, b; Wilson *et al.*, 1997). However, we also found that *spvA* was significantly induced when *S. typhimurium* was replicating with a generation time of 60 min in intracellular salts medium (ISM) (Wilson *et al.*, 1997), a defined medium designed to mimic the intracellular environment of mammalian cells (Headley & Payne, 1990). Furthermore, *spvA* was also induced when the salmonellae were replicating with a generation time of approximately 2 h within cultured macrophage-like cells and epithelial cells. These results raised the question of what type of signal or growth condition is most relevant for inducing expression of the *spv* genes *in vivo* in infected animal hosts.

To gain a better understanding of the mechanism of regulation of *spv* gene expression, we extended the analysis of *spv* regulation to *spvR*. We considered the possibilities that regulation of the *spvABCD* operon was governed primarily by altering the expression of the *spvR* regulatory gene, or that a more complex system regulates the transcription at the *spvA* promoter in conjunction with regulation of *spvR*. Most previous studies examining regulation of *spvR* employed *lacZ* fusions to *spvR* on multicopy plasmids and over-expression of cloned *spvR* from strong promoters on multicopy plasmids. By using RNase-protection analysis of native *spvR* on the virulence plasmid, we found that alterations in levels of *spvR* mRNA were considerably lower than those previously reported using *lacZ* fusions. Relevant issues involved with these studies were whether expression of *spvR* is qualitatively similar to that of *spvA*, and whether *spvR* is similarly regulated in response to the dual signals of ISM and stationary-phase growth conditions.

We report here that, similar to *spvA* (Wilson *et al.*, 1997), *spvR* is induced in both exponential growth in ISM and at the stationary phase of growth in L broth (LB). Like *spvA*, stationary-phase induction of *spvR* expression is dependent on RpoS and SpvR, as was previously reported (Abe *et al.*, 1994; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994; O'Byrne & Dorman, 1994b; Chen *et al.*, 1995). However, ISM induction of *spvR*, although at a lower level, still occurs in an RpoS⁻ background, whereas stationary-phase expression of *spvR* decreases in an RpoS⁻ background. Furthermore, SpvA represses *spvR*, but only in relation to stationary-phase induction. Together, these data suggest that most of the regulation of the *spvABCD* operon can be accounted for at the level of expression of *spvR* and that the ISM and stationary-phase inductions of the *spv* genes exhibit genetically separable components or functions.

These results were previously reported at the 94th and 95th general meetings of the American Society for Microbiology (Rogers & Gulig, 1994; Rogers *et al.*, 1995).

METHODS

Bacterial strains and growth conditions. The *S. typhimurium* strains used in this study are listed in Table 1. Wild-type, virulence-plasmid-containing *S. typhimurium* SR-11 strains χ 3181 and χ 3306 (*gyrA1816*) and isogenic virulence-plasmid-cured χ 3337 (*gyrA1816*) have been described by Gulig & Curtiss (1987). The deletions of *spvR*, *spvA* and *spvB* are represented in Fig. 1(a). All *spv* mutations were recombined into the virulence plasmid of χ 3306 using the allelic-exchange suicide vector pCVD442 (Donnenberg & Kaper, 1991; Wilson *et al.*, 1997).

Unless indicated otherwise, bacteria were grown at 37 °C with aeration in LB (Lennox, 1955) or ISM (Headley & Payne, 1990). L agar medium was made by adding 1.5% (w/v) agar (Difco) to LB. When appropriate, media were supplemented with antibiotics at the following concentrations: ampicillin, 100 µg ml⁻¹; chloramphenicol, 30 µg ml⁻¹; nalidixic acid, 25 µg ml⁻¹; and tetracycline, 12.5 µg ml⁻¹.

Plasmid constructs used in this study are described in Table 2. An internal, in-frame *Scal*-*DraI* deletion in the *spvR* coding sequence, which removes the last two-thirds of the proposed helix-turn-helix DNA-binding domain of *spvR* (Caldwell & Gulig, 1991), was constructed as follows, to yield strain UF082. The deletion was first constructed by ligating the 575 bp *Clal*-*Scal* fragment and the 670 bp *DraI*-*SstII* fragment of *spvR* into *Clal*-*SstII*-digested pBluescript KS(-), yielding pGTR344. A 1.2 kb *Sall*-*SstI* fragment containing the deletion and adjacent *spvR* sequences was then subcloned into pCVD442 (Donnenberg & Kaper, 1991). The resulting suicide vector, pGTR345, was transformed into *Escherichia coli* S17-1 λ pir (Simon *et al.*, 1983) to support plasmid replication and enable the plasmid to be mobilized into wild-type *S. typhimurium* χ 3306 by filter conjugation. The conjugation mixture was plated on L agar containing ampicillin and nalidixic acid, to select for integration of the entire suicide plasmid into the salmonella virulence plasmid. The cointegrate strain, UF080, was then grown in the absence of ampicillin selection to enable the second recombinational event to occur. Salmonellae that had excised the suicide vector sequences were selected by plating the bacteria on sucrose plates as described by Donnenberg & Kaper (1991). PCR analysis was performed to determine which colonies contained the deletion and which had reverted back to the wild-type. The LPS profile of each mutant was examined by silver staining LPS in SDS-polyacrylamide gels by the method of Hitchcock & Brown (1983) to confirm that the mutants had retained smooth LPS.

A deletion of the entire *spvA* open reading frame, leaving the promoter for *spvABCD* intact, was similarly constructed. The 0.8 kb *PstI* fragment encoding *spvA* was deleted from a recombinant plasmid encoding the *spv* genes, and the sequences flanking the Δ *PstI* mutation were subcloned into pCVD442. The deletion was recombined into the virulence plasmid of χ 3306, as described above, and the resulting strain was named UF104.

An internal, in-frame deletion of most of the *spvB* open reading frame was constructed by inverse PCR, using oligonucleotide primers PG69 (5'-CCCTCGAGCAGGAAAGGG-GGAGTGATCAGCGC-3') and PG70 (5'-CCCTCGAGGG-ATCCCAAGACTTTGCAAGCCAG-3'), leaving amino acids

Table 1. Bacterial strains used in this study.

Strain	Relevant genotype	Comments/reference
<i>S. typhimurium</i>		
χ3181	pStSR100 ⁺	Virulence plasmid positive; Gulig & Curtiss (1987)
χ3306	<i>gyrA1816</i> pStSR100 ⁺	Virulence plasmid positive, Nal ^r ; Gulig & Curtiss (1987)
χ3337	<i>gyrA1816</i> pStSR100 ⁻	Virulence plasmid cured derivative of χ3306, Nal ^r ; Gulig & Curtiss (1987)
SF1005	<i>rpoS</i> ::pRR10	Ap ^r , source of RpoS insertion; Fang <i>et al.</i> (1992)
UF005	pStSR100(<i>spvA9</i> ::Tn5)	Tn5 mutant of χ3181, Kan ^r ; Gulig <i>et al.</i> (1992)
UF051	<i>gyrA1816</i> pStSR100(<i>spvBA30</i>)	1.3 kb, PCR-generated in-frame deletion within the coding region of <i>spvB</i> , derivative of χ3306, Nal ^r ; H. Matsui, T. J. Doyle, C. M. Bacot & P. A. Gulig (unpublished)
UF064	pStSR100 ⁺ <i>rpoS</i> ::pRR10	RpoS ⁻ derivative of χ3181 made by P22 transduction from SF1005; Wilson <i>et al.</i> (1997)
UF080	<i>gyrA1816</i> pStSR100::pGTR345	Nal ^r Ap ^r derivative of χ3306 made by conjugation of pGTR345 from S17-1λpir(pGTR345) resulting in integration of pGTR345 into the virulence plasmid
UF082	<i>gyrA1816</i> pStSR100(<i>spvRA50</i>)	Resolution of pGTR345 from UF080, generating an in-frame deletion of the <i>ScaI</i> - <i>DraI</i> fragment within the coding region of <i>spvR</i> , Nal ^r
UF104	<i>gyrA1816</i> pStSR100(<i>spvAA20</i>)	Deletion of the <i>PstI</i> fragment encoding the entire <i>spvA</i> open reading frame, leaving the promoter for the <i>spvABCD</i> operon intact. Nal ^r ; H. Matsui, T. J. Doyle, C. M. Bacot & P. A. Gulig (unpublished)
<i>Escherichia coli</i>		
χ6060	Δ <i>lacX74 rpoB hsdR4 F'(traD36 proA⁺ proB⁺ lacI^a lacZΔM15 Tn5)</i>	Recipient for transcription vector plasmids, enables α-complementation; Wilson <i>et al.</i> (1997)
S17-1λpir	RP4-2-Tc::Mu-Km::Tn7 λpir	Host for Pir-requiring suicide plasmids, provides mobilization for suicide plasmid, Str ^r ; Simon <i>et al.</i> (1983)

1–20 and 556–592 of *SpvB* intact, with a leucine–glutamate linker from an engineered *XhoI* site. The deletion was subcloned into pCVD442 and was recombined into the virulence plasmid of χ3306 as described above. The resulting strain was named UF051.

S. typhimurium UF064 is strain SR-11 with the chromosomal *rpoS*::pRR10 mutation of *S. typhimurium* SF1005 (Fang *et al.*, 1992) constructed by P22HTint-mediated generalized transduction as described by Wilson *et al.* (1997).

Construction of *lacZ* operon fusion plasmids. All DNA manipulations were performed by standard genetic and molecular techniques (Maniatis *et al.*, 1982; Ausubel *et al.*, 1993). *lacZ* operon fusion plasmids were constructed essentially as described by Caldwell & Gulig (1991) and are depicted in Fig. 1(b).

pGTR322 was constructed from pGTR315, which consists of sequences from the *SalI* site upstream of *spvR* to the *EcoRI* site downstream of *spvR*. The *lacZ*,*cat* cartridge on a blunt-ended *Bam*HI fragment from pSGMU32 was inserted into the *HpaI* site of pGTR315, yielding an *spvR*–*lacZ* operon fusion that contained 1180 bp of upstream nontranslated *spvR* sequence.

Restriction analysis was used to determine the orientation of the cassette relative to the *spv* gene in which it was cloned. Plasmids containing *lacZ* operon fusions were transformed or electroporated into the desired *S. typhimurium* strains for analysis of gene expression.

Construction of *spvR* transcription vectors. The construction of the *in vitro* transcription vectors used in this study and the sizes of the cloned *spv* regions in each construct are summarized in Table 2. The regions of the probes containing *spvR* sequences are represented in Fig. 1(b). The *spv* sequences were directionally cloned in the antisense orientation into the pBluescript KS(–) transcription vector (Stratagene), and the ligation reactions were transformed into *E. coli* χ6060 (*lacI^a*, *lacZΔM15*). Plasmid constructs were verified by sequence analysis and were purified by caesium chloride centrifugation (Maniatis *et al.*, 1982).

pGTR334 and pGTR335 were designed to measure *spvR* mRNA that was within the coding region. pGTR334 contains the *ScaI*–*DraI* fragment of *spvR* cloned into the *SmaI* site of pBluescript KS(–). pGTR335 consists of the *DraI*–*EcoRV* fragment of *spvR* cloned into the *SmaI* site of pBluescript KS(–). *NotI* digestion of the DNA templates created the 3' ends of the antisense-RNA transcripts in the T3 run-off transcription reactions.

Measurement of β-galactosidase activity. This was performed as described by Miller (1972), except that cell debris was removed by centrifugation prior to determination of the absorbance at 420 nm. To examine equivalent amounts of bacterial cells and ensure equal cell lysis from exponential and stationary-phase cultures, 0.5 ml of exponential-phase cells or 0.5 ml of a 1:10 dilution of stationary-phase cells was used in the reactions. Enzyme activity normalized to bacterial cell

Fig. 1. (a) Location of the *spv* deletion mutations used to examine the effects of SpvR, SpvA and SpvB on *spvR* mRNA. All mutations are in the virulence plasmid of *S. typhimurium* χ 3306. Deletions were introduced into the virulence plasmid using the allelic-exchange suicide vector, pCVD442, as described in Methods. Restriction enzyme sites: B, *Bam*HI; D, *Dra*I; E, *Eco*RI; P, *Pst*I; Sc, *Sc*I; S, *Sal*I. (b) Physical and genetic maps of the probes used to detect *spvR* mRNA using the RNase-protection assay and *lacZ* operon fusion plasmid pGTR322 used to examine *spvR* expression. The *spvR* gene of *S. typhimurium* is depicted as the arrow in the open box. The solid bars depict the regions of the ^{32} P-labelled antisense RNA probes R1 and R2, that are complementary to *spvR* mRNA. The orientation and insertion site of the *lacZ,cat* cassette is indicated by the solid arrow (not drawn to scale) at the *Hpa*I site. Restriction enzyme sites: B, *Bst*EII; C, *Cl*aI; D, *Dra*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hpa*I; Sc, *Sc*I; S, *Sal*I.

number is presented as Miller units (MU) $\pm$ SD. The values are reported as the mean $\pm$ SD of triplicate wells. Each experiment was performed at least three times with similar results.

Isolation of bacterial RNA. *Salmonella* strains were grown overnight at 37 °C as static cultures supplemented with the appropriate antibiotics. The overnight cultures were diluted to 1×10^6 c.f.u. ml $^{-1}$ into fresh medium without antibiotics and shaken at 37 °C. Exponential-phase cultures were harvested when the cell density reached an OD $_{600}$ of 0.3–0.4; stationary-phase cultures were harvested 4–5 h after exponential growth ceased. Total bacterial RNA was isolated using the step-gradient caesium chloride density centrifugation method of Deretic *et al.* (1987) as modified by Wilson *et al.* (1997). Relative quantification of RNA was confirmed by examining rRNA bands on ethidium-bromide-stained agarose-formaldehyde gels (Ausubel *et al.*, 1993).

Quantification of *spvR* mRNA using RNase-protection analysis. RNase-protection analysis was performed as previously described (Ausubel *et al.*, 1993; Wilson *et al.*, 1997) with ^{32} P-labelled antisense RNA probes generated by T3 run-off transcription using an RNA transcription kit (Stratagene) and [α - ^{32} P]CTP [specific activity 3000 Ci mmol $^{-1}$ (111 TBq mmol $^{-1}$); Amersham] according to the manufacturer's instructions. One hundred micrograms of either total bacterial RNA (for quantification of mRNA) or yeast tRNA (Boehringer Mannheim Biochemicals) (as a negative control) were hybridized with ^{32} P-labelled antisense probe RNA. Following digestion of unhybridized probe and resolution on a 5% (w/v) polyacrylamide, 7 M urea sequencing gel, the amount of residual radiolabelled antisense probe RNA protected by *spvR* mRNA was quantified on a PhosphorImager (Molecular Dynamics) at the University of Florida Interdisciplinary Center for Biotechnology Research DNA Synthesis Facility. Preliminary results demonstrated that the RNase-protection

assay yielded a linear dose-response curve with the levels of probe and mRNA used in this study (data not shown).

Infection of mice. Oral infection of BALB/c mice and enumeration of splenic bacteria were performed as described by Gulig & Doyle (1993). Briefly, 7–11-week-old female BALB/c mice (Charles River) were deprived of food and water for at least 4 h before administration of 50 μ l 10% (w/v) sodium bicarbonate followed by 20 μ l exponential-phase salmonellae suspended in buffered saline containing 0.01% (w/v) gelatin. The total inoculum was approximately 10^8 c.f.u. per mouse. Food and water were restored 30 min later. Five days later, the mice were killed by carbon dioxide asphyxiation, spleens were removed and homogenized in buffered saline-gelatin, and the homogenates were plated on media containing the appropriate selective antibiotics. Data are reported as mean $\pm$ SD of log $_{10}$ c.f.u.

RESULTS AND DISCUSSION

spvR is induced during exponential growth in ISM as well as stationary phase in LB

We previously identified exponential growth of *S. typhimurium* in ISM, along with stationary phase in LB, as growth conditions which strongly induce expression of *spvA* (Wilson *et al.*, 1997). Since exponential growth in ISM with a generation time of 60 min is very different from stationary phase in rich media, it is conceivable that the mechanisms of regulation could be different under these conditions. We therefore examined the expression of the *spvR* positive regulatory gene from the virulence plasmid of wild-type *S. typhimurium* χ 3181 grown under these two conditions. Equivalent amounts of RNA were analysed by RNase-protection analysis

Table 2. Plasmids used in this study

Plasmid designation	Description/construction
pACYC184	Medium copy cloning vector; Chang & Cohen (1978)
pBluescript KS(–)	T7/T3 transcription vector; Stratagene
pCVD442	Positive selection allelic exchange suicide vector; Donnenberg & Kaper (1991)
pSGMU32	Promoterless <i>lacZ</i> , <i>cat</i> cassette in pUC19 used for construction of <i>lacZ</i> operon fusions; Errington (1986)
pYA2204	Low-copy cloning vector with pUC9 multiple cloning site; Galan <i>et al.</i> (1988)
pGTR079	1.2 kb <i>Bst</i> EII– <i>Eco</i> RI fragment of <i>spvR</i> cloned into pYA2204 so that <i>spvR</i> is in the same orientation as the <i>lacUV5</i> promoter; Caldwell & Gulig (1991)
pGTR315	2.3 kb <i>Sal</i> I/ <i>Eco</i> RI (blunt-ended) fragment of <i>spvR</i> cloned into <i>Sal</i> I– <i>Eco</i> RV-digested pACYC184
pGTR316	1.6 kb blunt-ended <i>Cla</i> I– <i>Eco</i> RI fragment of <i>spvR</i> cloned into <i>Eco</i> RV site of pACYC184
pGTR322	<i>spvR</i> – <i>lacZ</i> operon fusion plasmid created by subcloning the <i>lacZ</i> cartridge of pSGMU32 on a blunt-ended <i>Bam</i> HI fragment into the <i>Hpa</i> I site of pGTR315
pGTR334	Transcription vector created by subcloning the 240 bp <i>Sca</i> I– <i>Dra</i> I fragment of pGTR316 into the <i>Sma</i> I site of pBluescript KS(–)
pGTR335	Transcription vector created by subcloning the 230 bp <i>Dra</i> I– <i>Eco</i> RV fragment of pGTR316 into the <i>Sma</i> I site of pBluescript KS(–)
pGTR344	Contains a 240 bp <i>Sca</i> I– <i>Dra</i> I in-frame deletion in <i>spvR</i> generated by triple ligation with the <i>Cla</i> I– <i>Sca</i> I and <i>Dra</i> I– <i>Sst</i> II fragments of pGTR316 and <i>Cla</i> I– <i>Sst</i> II-digested pBluescript KS(–)
pGTR345	Suicide vector plasmid used to delete the 240 bp <i>Sca</i> I– <i>Dra</i> I fragment of <i>spvR</i> on the virulence plasmid. A 1.2 kb <i>Sal</i> I– <i>Sst</i> I fragment containing the <i>Sca</i> I– <i>Dra</i> I deletion on pGTR344 was subcloned into pCVD442 cut with the same enzymes

Fig. 2. The steady-state levels of *spvR* mRNA are increased during exponential growth in ISM and during stationary-phase growth in LB. RNA was isolated from wild-type *S. typhimurium* χ 3181 growing exponentially in LB (L) or ISM (I), or at stationary phase in LB (S). *spvR* mRNA was analysed using RNase-protection analysis and the antisense coding region probe R1 (Fig. 1b). Yeast tRNA (T) was included as a negative control. RNA was resolved on a 5% polyacrylamide, 7 M urea sequencing gel, visualized by autoradiography and quantified on a PhosphorImager.

using the ^{32} P-labelled antisense RNA probe R1 (Fig. 1b). No protected fragment corresponding to *spvR* mRNA was detected in negative-control samples containing yeast tRNA in the place of bacterial RNA (Fig. 2, lane T). In addition, no protected fragments were observed

upon analysis of RNA from virulence plasmid-cured *S. typhimurium* χ 3337 with *spvR* probes (data not shown). Similar to what was observed for the *spvA* gene (Wilson *et al.*, 1997), we detected a low level of *spvR* mRNA from salmonellae growing exponentially in LB (Fig. 2, lane L); this is henceforth referred to as basal expression of *spvR*. When the bacteria were grown to stationary phase in LB (Fig. 2, lane S), *spvR* was induced twofold relative to exponential phase in LB. As was previously seen for *spvA* mRNA (Wilson *et al.*, 1997), the greatest amount of induction of *spvR* occurred when the salmonellae were growing exponentially in ISM (Fig. 2, lane I), 4.5-fold relative to exponential phase in LB. However, relative to exponential growth in ISM, the amount of *spvR* mRNA decreased at stationary phase in ISM (data not shown). We previously reported a similar result for *spvA* mRNA under the same conditions (Wilson *et al.*, 1997). These results demonstrate that a cessation of growth does not always lead to induction of the *spv* genes. When we examined *spvR* mRNA using the second internal antisense RNA probe R2 (Fig. 1b), we obtained quantitatively similar results as with probe R1 (data not shown). Since the probes used in these assays were designed to detect internal fragments of *spvR* mRNA, the protected fragments which migrated

with sizes shorter than the full length most likely represent degradation products of mRNA. When these bands were included in quantitative analysis of mRNA expression, similar results were obtained as for the longest, fully protected fragment.

The differential expression of *spvA* at stationary phase in LB and exponential-phase growth in ISM (Wilson *et al.*, 1997) can therefore be accounted for by a qualitatively similar differential expression of the gene encoding the positive regulator, *spvR*. However, the order of magnitude of induction was much lower for *spvR* than for *spvA*. For example, we reported that *spvA* mRNA was induced 65-fold in ISM and 27-fold at stationary phase in LB (Wilson *et al.*, 1997), whereas we report here that *spvR* mRNA was increased no more than 3.7-fold during stationary phase in LB and 8-fold in exponential phase in ISM. This result is consistent with *spvR* encoding a positive regulator, where amplification of the signal at the regulated *spvABCD* operon is likely to occur. Additionally, levels of the SpvR protein could be further increased by higher rates of translation or increased protein stability as part of overall *spv* gene regulation.

SpvR is autostimulatory during stationary phase and in ISM

It has been reported that SpvR activates its own expression during the stationary phase of growth (Abe *et al.*, 1994; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994; O'Byrne & Dorman, 1994b; Spink *et al.*, 1994; Chen *et al.*, 1995; Taira *et al.*, 1995). Those studies examined the expression of reporter genes in transcriptional or translational fusions to *spvR*, where multicopy vectors were used to express either the reporter fusion or the cloned *spvR* gene. Furthermore, SpvR was usually expressed from a strong promoter. We initially performed similar studies to examine whether SpvR is autoregulatory during exponential growth in ISM by measuring expression of an *spvR-lacZ* operon fusion from a recombinant plasmid, pGTR322 (Fig. 1b), in virulence-plasmid-cured *S. typhimurium* χ 3337. pGTR322 contains 1180 bp of sequence upstream of the *spvR* open reading frame, which contains regulatory sequences involved in both the stationary phase and ISM inductions of *spvR* (Rogers & Gulig, 1994). SpvR was provided from the low-copy recombinant plasmid pGTR079, which constitutively expresses *spvR* from a *lacUV5* promoter (Caldwell & Gulig, 1991). In the SpvR⁺ background of pGTR079, expression of *spvR-lacZ* from pGTR322 during exponential-phase growth in LB, stationary phase in LB and exponential-phase growth in ISM was 11.2 ± 0.2 MU, 1190 ± 17 MU and 260 ± 0.1 MU, respectively. Expression of *spvR-lacZ* in χ 3337(pGTR079, pGTR322) was therefore induced 106-fold at stationary phase in LB and 23-fold during exponential growth in ISM relative to exponential growth in LB. When the pYA2204 vector for pGTR079 was used as an SpvR⁻ control, expression of *spvR-lacZ* from pGTR322 at exponential-phase growth in LB, stationary phase in LB and exponential-phase

growth in ISM was 2.4 ± 0.1 MU, 24.8 ± 0.6 MU and 33.8 ± 0.1 MU, respectively. Comparison of the β -galactosidase activity from χ 3337(pGTR079, pGTR322) and χ 3337(pYA2204, pGTR322) showed that expression of SpvR from pGTR079 increased the expression of *spvR-lacZ* 5-fold during exponential growth in LB, 48-fold during stationary phase and 8-fold during exponential growth in ISM, relative to exponential-phase growth in LB.

Upon comparing the results generated using the *spvR-lacZ* reporter fusion with the data generated from measuring *spvR* mRNA from the virulence plasmid of wild-type *S. typhimurium* (Fig. 2), two inconsistencies were noted. First, the expression of *spvR-lacZ* was considerably higher from χ 3337(pGTR079, pGTR322) during stationary phase in LB than in ISM, but expression of *spvR* mRNA from the virulence plasmid as measured by RNase protection was higher in ISM than at stationary phase in LB. Second, the inductions of *spvR* examined using the reporter fusion (106-fold at stationary phase in LB and 23-fold during exponential-phase growth in ISM, relative to exponential phase in LB) were much higher than those observed by analysing mRNA from the virulence plasmid in wild-type salmonellae (2-fold at stationary phase in LB and 4.5-fold during exponential-phase growth in ISM). It should be noted that the direct measurement of mRNA by RNase-protection analysis is not affected by the copy number of recombinant plasmids, stability of fusion-containing mRNAs, or stability of the β -galactosidase reporter protein.

Since we identified qualitative and quantitative differences in measuring *spvR* expression, depending on whether we used *lacZ* reporter fusions or RNase-protection analysis for *spvR* mRNA, we performed RNase-protection analysis to determine whether the positive autoregulation we detected using *lacZ* fusions occurs when *spvR* is expressed from the native virulence plasmid. We constructed strain UF082 with an internal in-frame deletion of the DNA-binding region of SpvR, *spvRA50*, which rendered the bacteria SpvR⁻ while leaving *spvR* mRNA sequences which could be measured by using RNase-protection analysis (Fig. 1a, b). *spvR* mRNA was measured from wild-type χ 3306 and *spvRA50* UF082 growing exponentially in LB and ISM or at stationary phase in LB. Similar to the results in Fig. 2, the steady-state levels of *spvR* mRNA from wild-type salmonellae increased when the bacteria were at stationary phase in LB (Fig. 3a, lane S) and when the bacteria were growing exponentially in ISM (Fig. 3b, lane I) relative to basal expression in LB (Fig. 3, lane L). The *spvRA50* mutation did not affect basal expression of *spvR* during exponential growth in LB. In contrast, in strain UF082, the steady-state levels of *spvR* mRNA decreased when the salmonellae were grown to stationary phase in LB (Fig. 3a, lane S) and *spvR* was no longer induced during exponential growth in ISM (Fig. 3b, lane I). These results demonstrate that SpvR is essential for both the stationary phase and ISM inductions of *spvR*, but not basal expression of *spvR*.

Fig. 3. Effect of SpvR on (a) stationary phase and (b) ISM inductions of *spvR*. RNA isolated from *S. typhimurium* χ 3306 (wild-type) and UF082 (*spvR* Δ 50) growing exponentially in LB (L) or ISM (I), or at stationary phase in LB (S), or yeast tRNA (T) was analysed using RNAse-protection analysis with probe R2 (Fig. 1b) as described in the legend for Fig. 2.

Our results detailed above confirm those of others (Abe *et al.*, 1994; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994; O'Byrne & Dorman, 1994b; Chen *et al.*, 1995) that SpvR is autostimulatory during stationary phase, and we extend this observation of autostimulation to exponential-phase growth in ISM. Consistent with an autoregulatory role for SpvR, DNA sequences upstream of *spvR* and *spvA* which can be bound by the SpvR protein have been identified (Matsui *et al.*, 1993; Krause *et al.*, 1995; Grob & Guiney, 1996; Grob *et al.*, 1997). By using RNAse-protection analysis and a deletion mutation in *spvR* on the virulence plasmid of *S. typhimurium*, we found that the low basal expression of *spvR* during exponential-phase growth in LB is independent of SpvR. However, functional SpvR was necessary for the induction of both *spvR* (Fig. 3) and *spvA* (Wilson *et al.*, 1997) at stationary phase in LB and during exponential-phase growth in ISM. In contrast, Chen *et al.* (1995) reported that an *spvR-lacZ* fusion in *S. dublin* was induced in stationary phase in the absence of expressed SpvR. This difference could be explained by the fact that Taira *et al.* (1995) reported a functional polymorphism in the *spvR* genes between *S. typhimurium* and *S. dublin*, with *S. typhimurium* having a defect in the ability of *spvR* to induce itself, and subsequently *spvA*. Although we did not perform similar comparative experiments between salmonella serovars in our study, our experiments with *S. typhimurium* *spvR* shown above and previous expression for *spvA* (Wilson *et al.*, 1997) appear to contradict Taira *et al.* (1995), in that SpvR induced both *spvR* and *spvA* both at stationary phase in LB and during exponential-phase growth in ISM.

RpoS differentially affects the steady-state levels of *spvR* mRNA at stationary phase in LB and during exponential phase in ISM

RpoS is essential for stationary-phase induction of both the *spvABCD* operon (Fang *et al.*, 1992; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994; Chen *et al.*, 1995; Wilson *et al.*, 1997) and *spvR* (Abe *et al.*, 1994; Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994; Abe & Kawahara, 1995; Chen *et al.*, 1995). Most studies of RpoS have concentrated on stationary-phase growth; however, dif-

ferent conditions affecting levels of RpoS during exponential growth are being identified (Hengge-Aronis, 1996; Jishage *et al.*, 1996; Muffler *et al.*, 1997). We therefore considered the possibility that induction of *spvR* during exponential-phase growth in ISM might be independent of or differentially regulated by RpoS, relative to stationary phase in LB.

To determine whether RpoS is essential for regulation of *spvR* during exponential-phase growth in ISM compared with expression at stationary phase in LB, we examined RNA from wild-type *S. typhimurium* χ 3181 and RpoS⁻ UF064. *spvR* was measured using RNAse-protection analysis with the internal *Scal*-*Dra*I antisense RNA probe R1 (Fig. 1b). The *rpoS* mutation did not affect the basal expression of *spvR* from salmonellae growing exponentially in LB (Fig. 4, lane L). The basal expression of *spvR* is therefore independent of both SpvR and RpoS, suggesting that σ^{70} can act at an *spvR* promoter independently of the SpvR protein. This result is consistent with the conclusions of others (Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994, 1996; Chen *et al.*, 1995). In wild-type, RpoS⁺ salmonellae, *spvR* was induced 2.3-fold at stationary phase (Fig. 4, lane S) relative to exponential phase in LB (Fig. 4, lane L). In contrast, the steady-state level of *spvR* mRNA from RpoS⁻ salmonellae decreased by 40% in stationary phase relative to exponential-phase growth in LB, demonstrating that RpoS is essential not only for the stationary-phase induction of *spvR*, but maintenance of even the basal level of expression at stationary phase.

When *spvR* mRNA was measured from wild-type *S. typhimurium* growing exponentially in ISM, *spvR* was induced 5.2-fold relative to basal expression in LB (Fig. 4b). Although the level of *spvR* mRNA produced by RpoS⁻ UF064 during exponential growth in ISM was reduced compared to χ 3181 under the same growth conditions, *spvR* was still induced 2.6-fold in RpoS⁻ salmonellae growing exponentially in ISM (Fig. 4b, lane I) relative to LB (Fig. 4b, lane L). This result demonstrated that both RpoS-dependent and RpoS-independent regulatory factors are involved in the induction of *spvR* in ISM. However, this RpoS-independent induction of *spvR* was not in itself sufficient to significantly increase levels of expression of *spvABCD* in ISM

Fig. 4. Effect of RpoS on (a) stationary phase and (b) ISM inductions of *spvR*. RNA isolated from *S. typhimurium* χ 3306 (wild-type) and UF064 (*RpoS*⁻) growing exponentially in LB (L) or ISM (I), or at stationary phase in LB (S), or yeast tRNA (T) was analysed using RNAse-protection analysis with probe R1 (Fig. 1b) as described in the legend for Fig. 2.

(Wilson *et al.*, 1997). The divergent patterns of expression of *spvR* in *RpoS*⁻ *S. typhimurium* during exponential growth in ISM and stationary phase in LB suggest that regulation of *spv* genes under these growth conditions possesses genetically separable components.

Effect of deleting *spvA* or *spvB* on the stationary-phase and ISM inductions of *spvR*

The results shown above demonstrated that both SpvR and RpoS are necessary for maximal induction of *spvR* at stationary phase in LB and during exponential-phase growth in ISM. To determine whether any of the other *spv* genes were involved in the regulation of *spvR*, we examined expression of *spvR* mRNA from *S. typhimurium* UF005, which contains a Tn5 insertion in *spvA* with polar effects on the downstream *spv* genes (Gulig *et al.*, 1992). Comparison of *spvR* mRNA expressed from UF005 relative to wild-type *S. typhimurium* was performed using RNAse-protection analysis on RNA isolated from salmonellae growing exponentially and at stationary phase in LB. UF005 exhibited higher levels of *spvR* mRNA than wild-type salmonellae during stationary phase growth in LB (data not shown), indicating that other *spv* genes were involved in the regulation of *spvR*. While these studies were being performed, Abe *et al.* (1994) reported that overexpression of either SpvA or SpvB gave negative regulation of a cloned *S. choleraesuis* *spvR-lacZ* translational fusion in *E. coli*. Spink *et al.* (1994) reported that SpvA, but not SpvB, was involved in the regulation of an *spvR-lacZ* fusion in *S. typhimurium*. Both groups reported that SpvC had no effect on *spvR*. Therefore, to determine whether either SpvA or SpvB contribute to the regulation of *spvR* expressed from the native virulence plasmid of *S. typhimurium* under our growth conditions, we compared the expression of *spvR* mRNA from wild-type χ 3306, Δ *spvA* UF104 and Δ *spvB* UF051 growing exponentially and at stationary phase in LB, or during exponential growth in ISM. *spvR* mRNA was quantified using RNAse-protection analysis with the antisense RNA probe con-

taining the internal *ScaI*-*DraI* fragment of *spvR* (Fig. 1b, probe R1).

Wild-type *S. typhimurium* χ 3306 growing exponentially in LB exhibited a low basal level of *spvR* mRNA (Fig. 5, lane L). *spvR* was induced 3.7-fold when the bacteria were grown to stationary phase in LB (Fig. 5a, lane S) and 8-fold during exponential growth in ISM (Fig. 5b, lane I). The amount of *spvR* mRNA expressed during exponential growth in LB, at stationary phase in LB, or during exponential growth in ISM was not significantly different between Δ *spvB* and wild-type salmonellae (Fig. 5), demonstrating that SpvB does not regulate the expression of *spvR* under these conditions. This result confirms those of Spink *et al.* (1994) and conflicts with Abe *et al.* (1994), who used *spvR-lacZ* fusions to measure *spvR* expression during stationary phase in *S. typhimurium* and *S. choleraesuis*, respectively.

When *spvR* mRNA was measured in Δ *spvA* salmonellae, the steady-state level of *spvR* mRNA during exponential growth in LB increased slightly (1.5-fold) over basal expression in wild-type salmonellae (Fig. 5a). At stationary phase in LB, the steady-state level of *spvR* mRNA was 3.5-fold higher in the Δ *spvA* strain relative to the wild-type parent strain (Fig. 5a, lane S), indicating that SpvA suppresses *spvR* expression during the stationary phase of growth in LB. In contrast, the level of *spvR* mRNA from the Δ *spvA* strain was not significantly different from the wild-type parent during exponential growth in ISM (Fig. 5b). This result indicated that, as opposed to stationary-phase induction in LB, SpvA does not regulate the expression of *spvR* during exponential growth in ISM.

SpvA therefore has the potential to regulate *spvR* expression in a negative-feedback mechanism under specific conditions of stationary phase in rich media. Since SpvA does not contain any DNA- or RNA-binding motifs, the mechanism by which it could act to negatively regulate *spvR* is unknown and may require specific protein-protein interactions. Furthermore, El-Gedaily *et al.* (1997b) recently demonstrated that SpvA

Fig. 5. Effect of Δ SpvA and Δ SpvB on (a) stationary phase and (b) ISM inductions of *spvR*. RNA isolated from *S. typhimurium* χ 3306 (wild-type), UF104 (Δ SpvA), or UF051 (Δ SpvB) growing exponentially in LB (L) or ISM (I), or at stationary phase in LB (S), or yeast tRNA (T) was analysed using RNase-protection analysis and probe R1 (Fig. 1b) as described in the legend for Fig. 2.

is localized primarily to the outer membrane of *S. dublin*, a site not consistent with a direct regulatory function for SpvA.

We next wanted to determine whether possible negative regulation of *spvR* by SpvA is involved with pathogenesis. Roudier *et al.* (1992) addressed the role of *spvA* in virulence of *S. dublin* by using a translational termination mutation in *spvA* and reported no effect on virulence. However, they inoculated mice intraperitoneally; in *S. typhimurium* the *spv* genes are of less importance after intraperitoneal inoculation compared with oral inoculation (Gulig & Curtiss, 1987). Therefore, we orally inoculated BALB/c mice with wild-type *S. typhimurium* χ 3306 or the isogenic Δ spvA UF104. Five days post-inoculation the $\log_{10}$ splenic c.f.u. were 4.8 ± 0.7 for χ 3306 and 4.7 ± 0.3 for UF104, demonstrating that SpvA has no detectable role in virulence. In comparison, infection of mice with virulence plasmid-cured *S. typhimurium* resulted in 300-fold fewer c.f.u. per spleen, demonstrating the necessity of Spv-mediated replication to achieve the levels of infection observed for χ 3306 and UF104. Therefore, although SpvA can act as a regulatory factor of *spvR* under stationary-phase growth conditions (but not during exponential growth in ISM), this SpvA-mediated regulation of *spvR* is not essential for pathogenesis in the murine model.

Overall regulation of the *spv* genes

Our results (Wilson *et al.*, 1997) (Fig. 4) and those of others (Heiskanen *et al.*, 1994; Kowarz *et al.*, 1994, 1996; O'Byrne & Dorman, 1994b; Chen *et al.*, 1995) showed that RpoS is essential for maximal induction of both *spvR* and the *spvABCD* operon. However, these data were not able to differentiate whether RpoS regulates the *spv* genes solely through its effects on the positive regulatory gene, *spvR*, or whether it also acts directly at the *spvABCD* promoter. The lack of induction of *spvA* mRNA during exponential growth in ISM in an RpoS⁻ strain (Wilson *et al.*, 1997) even though transcription of *spvR* is still increased (Fig. 4) argues that RpoS is also necessary for transcription at the *spvA* promoter. Using *spv-lacZ* reporter fusions *in trans* to cloned *spvR* and *rpoS* genes that were under the control of exogenous promoters, two other laboratories addressed this question in *S. dublin* (Chen *et al.*, 1995)

and *S. typhimurium* (Heiskanen *et al.*, 1994). Both studies concluded that RpoS acted at both the *spvR* and the *spvABCD* promoters to achieve maximal expression of the *spv* genes during stationary-phase growth. It is also possible that RpoS indirectly influences the transcription of the *spv* genes by affecting either the concentration of a co-inducer molecule or another regulatory protein that is necessary for induction of the *spv* genes. Candidates for other regulatory proteins include *hns* (O'Byrne & Dorman, 1994a), *cya/crp* (O'Byrne & Dorman, 1994b) and *phoP/phoQ* (Heithoff *et al.*, 1997), although their direct relation to RpoS is uncharacterized.

Since the *spv* genes are induced from a low, basal level under specific growth conditions and overexpression is detrimental to the salmonellae (P. A. Gulig & J. A. Wilson, unpublished), expression of the *spv* genes should be tightly controlled. To account for the rapid induction of *spvR* under specific growth conditions, we agree with the model proposed by Chen *et al.* (1995). Briefly, the basal expression of the *spv* genes during exponential-phase growth in rich media is the result of transcription from RNA polymerase containing the sigma factor σ^{70} and is not dependent on the plasmid-encoded positive regulatory protein, SpvR. When the salmonellae attain stationary phase or are growing in the intracellular environment of an animal host, they synthesize more RpoS molecules, which compete with σ^{70} for core RNA polymerase. The σ^s -holoenzyme then interacts with the basal levels of SpvR in the cell to amplify expression of *spvR* and to induce the expression of the *spvABCD* genes. Since SpvR is autostimulatory, this positive regulatory loop would enable the *spv* genes to be rapidly induced from a low level of expression in response to a specific signal encountered in the host. Therefore, to prevent overexpression of the *spv* genes, repression of *spvR* would be beneficial. Down-regulation of *spvR* could be mediated by decreasing the concentration of RpoS in the cell, by a ligand which alters the conformation of the SpvR regulatory protein, or by negative regulation by another gene product. Ligands which could affect SpvR function include short chain fatty acids (El-Gedaily *et al.*, 1997a) and iron (Spink *et al.*, 1994). However, the relevance of these potential regulatory molecules to induction of *spv* genes during exponential phase in ISM and at stationary phase

in LB is unclear, since ISM does not contain short-chain fatty acids and neither medium is iron-depleted.

Conclusions

Our results reported here continue to demonstrate that two very different types of inductions, stationary phase in rich media and exponential-phase growth in ISM, can regulate *spv* gene expression through similar, yet genetically separable, mechanisms. We are interested in determining which, if any, of the these inducing and regulatory mechanisms is relevant for pathogenesis *in vivo* in infected animals. Since the *spv* genes increase the intracellular replication rate of salmonellae *in vivo* (Gulig & Doyle, 1993), it is conceivable that the *spv* genes could be induced *in vivo* by a cessation of growth, which would then enable the bacteria to resume replication in the host. Alternatively, the salmonellae may induce *spv* expression by sensing an appropriate signal during exponential growth in an intracellular environment, thus ensuring continued intracellular growth of the salmonellae in the host before cessation of growth occurs. Identification of the relevant signal(s) involved in inducing the *spv* genes *in vivo* will aid in elucidation of the intracellular environment in which the salmonellae reside and the eventual elucidation of the mechanism by which the *spv* genes sustain systemic infection in the host.

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