Research article

**RpoE fine tunes expression of a subset of SsrB-regulated virulence factors in *Salmonella enterica* serovar Typhimurium**

Suzanne E Osborne¹ and Brian K Coombes*¹,²

Address: ¹Michael G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada and ²Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, ON, Canada

Email: Suzanne E Osborne - osborns@mcmaster.ca; Brian K Coombes* - coombes@mcmaster.ca

* Corresponding author

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Abstract

**Background:** The survival of *Salmonella enterica* within the intracellular host niche requires highly co-ordinated expression of virulence effectors predominantly regulated by the SsrAB two-component regulatory system. *S. enterica* serovar Typhimurium mutants lacking the ssrAB genes are avirulent in mice, highlighting the importance of this regulatory system in vivo. Mutants lacking the gene encoding the alternative sigma factor σE (rpoE) are also highly attenuated for intracellular survival, pointing to a potential connection with the SsrAB regulatory system.

**Results:** In this study we demonstrate that RpoE is involved in fine-tuning the expression of a subset of SsrB-regulated genes found in the *Salmonella* pathogenicity island-2 (SPI-2) genetic locus that encodes a horizontally acquired type III secretion system, and unlinked genes integrated into this regulon that are required for virulence in host animals.

**Conclusion:** These data point to a potential connection between the virulence phenotype of strains lacking ssrB and rpoE, and highlight new transcriptional regulation that might be essential for appropriate temporal and spatial control of the virulence-associated type III secretion system during host infection.

**Background**

*Salmonella enterica* are enteric pathogens that acquired a type III secretion system (T3SS) through horizontal gene transfer of a genomic island termed *Salmonella* Pathogenicity Island 2 (SPI-2) [1,2]. The SPI-2-encoded T3SS and its translocated effectors modify the intracellular host niche for *Salmonella* replication [3-5]. SPI-2 also has genes, ssrA and ssrB, which code for SsrAB, a two-component regulatory system needed for expression of the T3SS [6,7]. SsrB regulates the expression of SPI-2 encoded substrate effectors including ssaB, as well as several integrated virulence effectors such as sseL [8] and srfN [9] that are encoded elsewhere on the chromosome but that have integrated into the SsrB regulon. Mutants lacking ssrAB are unable to survive within macrophages and are avirulent in mice [1].

Alternative sigma factors coordinate gene expression in response to environmental cues sensed by the bacterium. Sigma factors have a specific recognition motif at the -35 and -10 positions and function to concentrate RNA polymerase at a subset of promoters [10]. One alternative sigma factor, RpoE (σE) responds to envelope stress at the cell surface. Release of σE from its inner membrane anchored anti-sigma factor, RseA, leads to induction of genes required to maintain cell envelope integrity [11].
SsrB-regulated translocated effectors protect *S. Typhimurium* against host cell defences such as oxidative stress and antimicrobial peptides that perturb bacterial membrane integrity and provide a stimulus for σ^E^ release [4,12-15]. Although proficient at cellular invasion, *rpoE* or *ssrB* mutants are highly attenuated for intracellular survival in both cultured cells and animal hosts [16]. In addition, the expression of *rpoE* and *ssrB* is up-regulated within macrophages [17]. Links between RpoE and virulence gene expression is evident in other bacterial systems as well. Deletion of *rseA* in *Yersinia pseudotuberculosis* causes elevated virulence effector synthesis and secretion [18], establishing links between alternative sigma factors and virulence-specific regulators. Taken together, a connection between σ^E^ and SsrB is suggested from the available literature, however the role of σ^E^ in activating SsrB-regulated genes has not been studied.

We tested the hypothesis that RpoE is involved in expression of genes that use the SsrB response regulator for activation. By testing six promoters representing four classes of SsrB-regulated promoters ((i) two type III secretion structural operons in SPI-2, (ii) the effector operon in SPI-2, (iii) two effector genes unlinked with SPI-2, and (iv) an integrated virulence gene unlinked with SPI-2) we demonstrate that RpoE elicits an effect on a subset of SsrB-regulated genes. This effect was bidirectional depending on the promoter and was downstream of *ssrB* expression itself, since deletion of *rpoE* had no effect on SsrB levels in the mutant cells. These data help unite the virulence phenotypes of strains lacking SsrB and RpoE, and highlight new transcriptional regulation that might be essential for appropriate temporal and spatial control of the virulence-associated type III secretion system during host infection.

**Results**

**Deletion of rpoE affects a subset of SsrB-regulated virulence genes**

*Salmonella* virulence gene expression is coordinated in *vivo* and may be regulated, in part, by alternative sigma factor(s) in order to quickly respond to the host environment. To date, no sigma factor has been identified as regulating SsrB-dependent virulence genes. To start, we first screened four alternative sigma factor mutants of *S. Typhimurium* (*rpoS*, *rpoN*, *rpoE*, *rpoH*) for their ability to express a key virulence gene, *sseB*, that requires SsrB for expression and whose gene product is essential for intracellular pathogenesis. For an *rpoH* deletion, this strain was only viable at temperatures below 30 °C. Since SPI-2 gene activation is integrated into a thermosensing circuit [19] we were unable to test the role of σ^H^ in this study (data not shown). In this screen, *rpoS* deletion resulted in a slight increase in SseB levels (Figure 1A) indicating a role for RpoS in the repression of SPI-2. Both *rpoE* and *rpoN* deletions resulted in decreased SseB levels with a more pronounced effect in the *rpoE* deletion. Since we were predominantly interested in sigma factors that activate SPI-2 and which could be linked to the previous observation that *rpoE* mutants are highly attenuated in *vivo*, we choose to focus on RpoE in the current study, which had the most influence on SseB levels in the cell.

An unmarked in-frame deletion of *rpoE* was then generated in *S. Typhimurium* strain SL1344 and we verified that this in-frame deletion had the same effect on SseB as...
the rpoE::cat mutant used previously (Figure 1B). A low-copy plasmid containing full-length rpoE and the three endogenous promoters that can drive its expression [20] was able to restore wild type levels of SseB to ΔrpoE cells (Figure 1B) demonstrating that the results were specific to the rpoE deletion. In these complementation experiments, attempts were made to examine the levels of SseB secreted into the culture supernatant [21], however consistent with previous observations [22,23] perturbations to the rpoE pathway increased cell lysis resulting in contamination of secreted fractions with cytosolic proteins which precluded accurate interpretation (data not shown).

In order to examine the effect of σE (rpoE) on the expression of a broad range of SsrB-regulated virulence genes, we tested whether or not the effect of rpoE deletion was specific to sseB or if it extended to other SsrB-regulated genes. To do this we examined the levels of SseL-2HA, SifA-2HA and SifN-2HA expressed from their endogenous promoters under SPI-2 inducing conditions (Figure 1C). Consistent with the results for SseB, there was a decrease in SifA-2HA levels in ΔrpoE compared to wild type, although deletion of rpoE did not have an effect on SseL-2HA. Relative to its expression in wild type cells, the level of SifN-2HA was reproducibly increased in the ΔrpoE cells, suggesting a role for σE in the repression of SifN, although it is unlikely that this is through a direct mechanism.

**RpoE is involved in transcriptional activity of a subset of virulence genes**

In order to confirm the effect of σE on the expression of a broad range of SsrB-regulated virulence genes, we used wild type and ΔrpoE cells and integrated into the chromosome individually six single-copy transcriptional fusions representing promoters for four classes of SsrB-dependent genes or operons ((i) type III secretion effector operon (sseA); (ii) structural operon I (ssaB); (iii) structural operon II (ssaG); (iv and v) effectors encoded outside of SPI-1 (sseL and sifA); and (vi) integrated virulence gene promoters linked to SPI-2 (srfN) [9]. Transcriptional fusions to lacZ of the sseA, ssaB, ssaG, sseL, sifA and srfN promoters (mapped previously [9, 24]) were inserted into the chromosome of wild type and ΔrpoE cells and then grown in the SsrB-activating medium LPM. The activity of each promoter was measured using a β-galactosidase assay during exponential growth. Although ΔrpoE was observed to have a slightly prolonged lag phase relative to wild type cells under these experimental conditions, at later time points the mutant grew similarly to wild type. To account for any differences in growth kinetics of the cultures, all data was normalized to the optical density at 600 nm of the culture, which permitted direct comparisons.

In wild type cells, promoter activity from all the transcriptional fusions was high, as expected, because LPM medium is highly inducing for SsrB activity [21]. In contrast, promoter activity for sseA, ssaB, and sifA decreased in the rpoE mutant compared to wild type cells (Figure 2A, B and 2D), whereas promoter activity from the ssaG and srfN reporters was upregulated in the rpoE mutant (Figure 2C and 2F). β-galactosidase activity observed from the sseL reporter was unaltered in the rpoE deletion compared to that in wild type cells (Figure 2E). These data are consistent with the protein levels detected for these gene products. Together, these data indicate that σE can have a variable and bidirectional effect on SsrB-regulated virulence genes.

**The effect of RpoE on virulence genes is downstream of ssrB expression**

The variable effects of rpoE deletion on SsrB-regulated effectors suggested that RpoE might direct transcription downstream of ssrB expression. To test this, we replaced the ssrB gene in ΔrpoE and wild type cells with an ssrB::FLAG allele [19] and examined the levels of SsrB protein in the strains by western blot. There was no change in the levels of SsrB-FLAG between wild type and ΔrpoE cells (Figure 3), indicating that the effects of RpoE on the four classes of virulence gene promoters examined here was not mediated through changes to SsrB protein levels. Together these data establish a role for RpoE in the fine-tuning of virulence gene expression in S. Typhimurium.

**Discussion**

In this work we found that the alternative sigma factor, σE, is involved in fine tuning the expressing of a subset of SsrB-regulated virulence genes required for Salmonella pathogenesis. Although the effect of rpoE deletion on promoter activity in some cases was mild, we have previously shown that gene regulators providing only modest transcriptional input have a profound influence on bacterial
Figure 2

The transcriptional activity of SsrB-regulated virulence genes is affected by an rpoE deletion. Wild type and ΔrpoE cells carrying single-copy chromosomal transcriptional reporters of (A) P_{sseA}:pP_{sseA}-lacZ, (B) P_{ssaB}:pP_{ssaB}-lacZ, (C) P_{ssaG}:pP_{ssaG}-lacZ, (D) P_{sifA}:pP_{sifA}-lacZ, (E) P_{siseL}:pP_{siseL}-lacZ and (F) P_{srfN}:pP_{srfN}-lacZ were grown in LPM (pH 5.8). At the indicated time β-galactosidase activity was measured and expressed as relative light units (RLU) normalized to optical density of the culture. Wild type and ΔrpoE cells lacking the transcriptional reporters were used as controls in each experiment. Data are the means with standard error from triplicate determinations from three independent experiments.
fitness in a host animal [25]. In cases where the regulator is deleted, the loss of genetic fine-tuning causes incongruous changes in the timing and magnitude of virulence gene expression, leading to fitness loss and strong attenuation. We predict that RpoE confers a similar fine-tuning effect on Salmonella virulence gene expression that is required for optimal within-host fitness during infection.

When we examined the -10 and -35 positions of the promoters studied here relative to the transcriptional start sites identified previously [24], these promoters did not appear to contain σE consensus sequences. Instead they appeared to have consensus sites for σ70. Although a bioinformatics screen identified σ70 consensus sequences upstream of the SPI-2 genes ssaU, ssaJ, sscA and sscC [26], these genes were not tested for σ70-dependence in the present study because the identified consensus sites are in coding sequence within operons, and as a result may not be directly relevant. Due to the high degree of conservation in σ factor binding sequences, σE may not be directly regulating SsrB-dependent promoters. The lack of a canonical σE sequence at these promoters suggests that another regulatory gene may be epistatic to σE or that these promoters encode functional, but non-canonical σE-binding sites due to their horizontal acquisition and gradual integration into the σE regulatory network. This integration may help Salmonella coordinate expression of the virulence-associated T3SS in response to host factors that compromise bacterial membrane integrity (Figure 4). This mechanism would activate a restorative σE pathway, which is consistent with the enhanced susceptibility of rpoE mutants to oxidative stress and antimicrobial peptides [13,15,16], both of which perturb membrane integrity in vivo. Although there is no evidence that σE can directly repress transcription, the negative effect on two promoters observed here might be due to an intermediate RpoE-regulated repressor or compensatory effect where loss of rpoE increases the relative abundance of another sigma factor that can directly activate the ssaG and srfN promoters. Future work will be required to resolve these possibilities.

Our results indicate that rpoE deletion has no effect on SsrB levels under SPI-2 inducing conditions suggesting that the σE pathway regulates effector expression downstream of ssrAB transcription. Unlinking ssrAB transcription from the σE regulon would be advantageous to the cell to prevent commitment to a virulence gene expression program in response to envelope stress not associated with infection. The results from this study demonstrate that σE has the ability to affect expression of SsrB-regulated virulence genes and offers potential insight into the virulence attenuation of rpoE mutants. Although when considered individually, each promoter was modestly affected by deletion of rpoE, the cumulative effects of mild rewired inputs on multiple virulence promoters has been shown to severely compromise in-host fitness and virulence ability [25].

Conclusion

Based on these and other data [4,12-15], the genetic interaction between σE and a subset of SsrB-regulated genes may serve to coordinate the spatial and temporal activation of virulence genes in a host setting, likely in response to membrane damage resulting from oxidative antimicrobial systems and membrane-targeted host defence peptides.

Methods

Strains and Growth Conditions

Bacteria were propagated at 37°C with aeration in Luria-Bertani (LB) broth. S. enterica serovar Typhimurium (S. Typhimurium) strain 14028s with inactivating mutations in rpoE, rpoS, rpoN and rpoH were provided by Ferric Fang (University of Washington, Seattle, WA) [27]. ΔrpoH was grown at 30°C and ΔrpoN was supplemented with 2 mM L-glutamine. An unmarked, in-frame deletion of rpoE was
made in S. Typhimurium strain SL1344 by λ Red recombination [28] using primers BKC187 and BKC188. Mutants were screened for loss of rpoE using primers BKC193 and BKC194. To generate an ssrB::FLAG allele in ΔrpoE, the ssrB::FLAG allele from wild type SL1344 [19] was transduced into ΔrpoE by P22-mediated transduction. All plasmids and strains used in this work are described in Table 1. Primer sequences for mutant and plasmid construction are listed in Table 2.

**Plasmid Construction**

A complementation construct for rpoE was generated that included the three endogenous promoters (rpoEp1, rpoEp2, rpoEp3) identified in Salmonella [20] plus the wild type rpoE gene. This construct was cloned into the low-copy plasmid pWSK29 using primers SEO095 and SEO096 as a Sall and XbaI fragment. Constructs were verified by sequencing and transformed into S. Typhimurium SL1344 ΔrpoE and selected on LB agar with appropriate antibiotics. The promoters for ssaB (SEO005 and SEO006), ssaG (SEO011 and SEO012), sifA (SEO205 and SEO206), sseL (BKC185 and BKC186) and srfN (BKC183 and BKC184) were cloned into pIVET5n [29] to generate single-copy transcriptional fusions to lacZ. Reporters were transformed into E. coli SM10 λpir, conjugated into SL1344 and merodiploid cells were selected on LB agar with appropriate antibiotics. Transcriptional fusions, including a previously constructed reporter for the sseA promoter [30], were integrated into the chromosome of wild type and ΔrpoE cells using homologous recombination. The promoters we chose use the SsrB response regulator for expression of the downstream gene or operon, and include both SPI-2-encoded and non-SPI-2-encoded virulence effectors representing structural apparatus genes and effector substrates of the type III secretion system [8,30-35].

**Chemiluminescent β-galactosidase Assay**

Reporters strains were inoculated from an overnight culture into culture medium (LPM pH 5.8) that induces SsrB
Table 1: Strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference |
|-------------------|-------------------------|-----------|
| **Plasmids**      |                         |           |
| pKD46             | repA101ts, oriR101, araC-PmarB-gam-bet-exo, bla | [28]      |
| pCP20             | FLP*, λ cI857* λ, pR Rep*; AmpR, CmR | [28]      |
| pKD3              | pANTSy derivative, FRT-flanked cat from pSC140, CmR | [28]      |
| prpoE             | rpoE with endogenous promoters in pWSK29, AmpR | This work |
| psrfN-2HA         | srfN with endogenous promoter and C-term. tandem HA fusion in pWSK129, KanR | Our collection |
| pssel2-2HA        | ssel with endogenous promoter and C-term. tandem HA fusion in pWSK129, KanR | [8]       |
| pIVET5n           | tnpR-lacZ, sacB, R6K ori, bla, AmpR | [29]      |
| pPsseA-lacZ       | sseA promoter fused to tnpR-lacZ in pIVET5n, AmpR | [29]      |
| pPsseL-lacZ       | sseA promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| psrN-lacZ         | srfN promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| psfA-lacZ         | sifA promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| pSseB-lacZ        | ssaB promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| pPsseA-lacZ       | sseA promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| pPsseL-lacZ       | sseA promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| pPssfA-lacZ       | sifA promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| pPssgA-lacZ       | ssaG promoter fused to tnpR-lacZ in pIVET5n, AmpR | This work |
| **Strains**       |                         |           |
| SL1344            | wild type S. enterica sv. Typhimurium, SmR | [38]      |
| 14028s            | wild type S. enterica sv. Typhimurium | [27]      |
| 14028s rpoE::cat  | rpoE deletion, CmR | [27]      |
| 14028s rpoS::Tn10dCm | rpoS deletion, CmR | [27]      |
| 14028s rpoN::Ap   | rpoN deletion, AmpR | [27]      |
| 14028s rpoH::PCN  | rpoH deletion, temperature sensitive, AmpR | [27]      |
| SL1344 ΔrpoE      | Unmarked, in-frame deletion of rpoE, SmR | This work |
| SL1344 ssrB::3FLAG | ssrB-FLAG replacement allele on chromosome | This work |
| SL1344 ΔrpoE PsseA::pPsseA-lacZ | Merodiploid containing integrated PsseA-lacZ reporter | This work |
| SL1344 ΔrpoE PsseL::pPsseL-lacZ | Merodiploid containing integrated PsseL-lacZ reporter | This work |
| SL1344 ΔrpoE PsrN::pPsrfN-lacZ | Merodiploid containing integrated PsrN-lacZ reporter | This work |
| SL1344 ΔrpoE PsfA::pPsifA-lacZ | Merodiploid containing integrated PsfA-lacZ reporter | This work |
| SL1344 ΔrpoE PssgA::pPssgA-lacZ | Merodiploid containing integrated PssgA-lacZ reporter | This work |

*aSm, streptomycin; Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin
dependent gene expression [21,36]. Cultures were propagated at 37°C for 7 hours and samples were taken hourly to measure β-galactosidase activity using a chemiluminescence assay described previously [25]. Data was expressed as relative light units (RLU) and was normalized to the optical density (OD600 nm) of the parent culture.

**Immunoblotting**

To examine the protein levels of SseB, SseL, SrfN and SifA under SPI-2 inducing conditions, we used plasmids pssfA-2HA, ssfL-2HA and ssfN-2HA that were published previously (Table 1) [8,37]. These constructs express the given gene under the control of the endogenous promoter. Wild type and ΔrpoE cells were transformed with these plasmids and grown in LPM pH 5.8 at 37°C for 6 hours. Whole cell lysates were collected and analyzed by immunoblotting using anti-SseB (1:1000) [21] and anti-HA (1:1000, Covance) antibodies. Blots were probed for DnaK (1:3500, Stressgen) as a control.

**Authors’ contributions**

SEO designed and performed research, interpreted data and wrote the paper. BKC designed and interpreted research and wrote the paper. Both authors read and approved the final manuscript.

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