Transcriptional and Post-Transcriptional Modulation of SPI1 and SPI2 Expression by ppGpp, RpoS and DksA in *Salmonella enterica* sv Typhimurium

Christopher J. Rice¹*, Vinoy K. Ramachandran², Neil Shearer¹, Arthur Thompson¹*

¹ Institute of Food Research, Norwich, NR4 7UA, United Kingdom, ² Department of Plant Sciences, University of Oxford, Oxford, OX1 3RB, United Kingdom

* Current address: Campden BRI, Surrey, RH1 4HY, United Kingdom

* arthur.thompson@ifr.ac.uk

Abstract

The expression of genes within *Salmonella* Pathogenicity Islands 1 and 2 (SPI1, SPI2) is required to facilitate invasion and intracellular replication respectively of *S*. Typhimurium in host cell lines. Control of their expression is complex and occurs via a variety of factors operating at transcriptional and post-transcriptional levels in response to the environmental stimuli found within the host. Several of the factors that modulate SPI1 and SPI2 expression are involved in the redistribution or modification of RNA polymerase (RNAP) specificity. These factors include the bacterial alarmone, ppGpp, the alternative sigma factor, RpoS, and the RNAP accessory protein, DksA. In this report we show not only how these three factors modulate SPI1 and SPI2 expression but also how they contribute to the ‘phased’ expression of SPI1 and SPI2 during progress through late-log and stationary phase in aerobic rich broth culture conditions. In addition, we demonstrate that the expression of at least one SPI1-encoded protein, SipC is subject to DksA-dependent post-transcriptional control.

Introduction

Pathogenic serovars of *Salmonella* infect both humans and animals, causing in humans either a self-limited gastroenteritis (e.g. *S*. Typhimurium, *S*. Enteriditis), or potentially fatal systemic infections (e.g. *S*. Typhi, *S*. Paratyphi). *Salmonella* is the second most reported zoonotic infection in humans and the most frequent cause of food borne outbreaks in the EU [1]. During infection, *Salmonella* invades epithelial cells lining the small intestine, mediated by *Salmonella* Pathogenicity Island 1 (SPI1), encoding a type 3 secretion system (T3SS). SPI1 triggers the injection of effector proteins into the host cell to facilitate uptake of bacteria during the process of invasion. Intracellular *Salmonella* employ a second T3SS encoded within SPI2, which modifies the initial membrane-bound compartment or phagosome to form the ‘*Salmonella* containing vacuole’ (SCV) [2]. The SCV avoids fusion with lysosomes, enabling *Salmonella* to evade the
antimicrobial compounds that form part of the host immune response. In systemic infections, *Salmonella* passes through the gut wall and is phagocytosed by macrophages which can transport and disseminate the pathogen throughout the host [3,4].

One of the major regulators of virulence gene expression in *Salmonella* is the bacterial alarmone guanosine tetraphosphate (ppGpp) [5]. Using both microarray-based and differential RNA sequencing (dRNA-seq) approaches, it has been shown that ppGpp is required for the expression of nearly all of the genes within SPI1 and SPI2 as well as many other *Salmonella*-virulence related genes [5–8]. In addition, next-generation transcriptomics has revealed that ppGpp is involved in regulating several of the virulence-related, non-coding RNAs discovered thus far in *S. Typhimurium* [7]. Guanosine tetraphosphate is synthesised by the RelA and SpoT enzymes in all beta- and gammaproteobacteria and, whereas RelA only has ppGpp synthetic function, SpoT is able to both synthesise and hydrolyse ppGpp (for reviews see [9–11]). In addition to *Salmonella*, it has also been shown that ppGpp plays a key role in coupling virulence to metabolic status in several pathogenic bacteria including *Mycobacterium tuberculosis* [12,13], *Listeria monocytogenes* [14], *Legionella pneumophilia* [15,16], *Vibrio cholera* [17] and *Pseudomonas aeruginosa* [18].

DksA is a small 151 amino acid protein found in most bacterial species, including *S. Typhimurium* and *Escherichia coli*. DksA was originally discovered as a dose dependent suppressor in a *dnaK* deletion mutant. Subsequently, DksA was found to play a physiologically pleiotropic role including mediating chaperonin function, cell division, amino acid biosynthesis, phage sensitivity, quorum sensing, responses to envelope stress and virulence [19,20]. DksA is thought to mediate these effects via directly binding to RNA polymerase (RNAP). As a consequence of this mechanism of DksA binding, RNAP is sensitive to changes in ppGpp concentration (and the initial NTP of the transcript), resulting in the reduction or inhibition of rRNA transcription at low steady state growth rates and during entry into stationary phase [20]. In addition to inhibiting some promoters, ppGpp and DksA can also activate promoters through a direct and/or indirect mechanism [21–25]. Indirect activation may occur via liberation of RNAP from tRNA operons, thereby increasing its availability to lower affinity promoters or promoters that are able to make higher-stability complexes with RNAP. DksA and ppGpp also indirectly regulate several promoters that are transcribed by alternative sigma factors (e.g. σ^54 and σ^S). This regulation has been suggested to occur either as a result of competition for RNAP, by alternative sigma factors, or through some other mechanism [26,27]. As well as the above, it has been shown that the zinc finger motif of DksA can serve as a thiol switch to sense oxidative and nitrosative stress, which may suggest one reason why *S. Typhimurium dksA* mutants are attenuated in mouse infection models [28,29]. Finally, in addition to *Salmonella*, virulence regulation has been attributed to DksA in *P. aeruginosa*, *S. flexneri*, and *E. coli*, and to a DksA-like protein in *C. jejuni* [22,25,30–33].

The alternative sigma factor, RpoS (σ^S, σ^38) is involved in the general stress response, and is induced during entry into stationary phase (for review, see [34]). Production of RpoS occurs very rapidly upon entry into stationary phase but protein concentrations are maintained at very low levels in exponentially growing cells. Regulation of RpoS occurs at multiple levels—transcription, translation, degradation and activity; the large number of stresses that are transduced via RpoS occur at one or more of these regulatory levels. RpoS is involved in the virulence mechanisms of many bacterial pathogens; however its effect on virulence appears to be variable. RpoS has been found to be necessary for virulence in certain pathogens including *Salmonella enterica*, *Vibrio cholerae*, *Burkholderia plantarii*, and *Serratia entomophila* but less important in other pathogens [35–40], reviewed in [41]. In this study, we determine the roles of three RNAP modulatory elements, ppGpp, RpoS and DksA, in the control of SPI1 and SPI2 expression during stationary phase in aerated rich broth culture. Whereas ppGpp activates SPI1
and SPI2 expression at different points during stationary phase, RpoS reduces their expression, and DksA can act as both a repressor and an activator of SPI1 and SPI2 encoded genes respectively. The disparate effects of ppGpp, RpoS and DksA on SPI1 and SPI2 expression suggests they may play a role in controlling the often mutually exclusive expression of these pathogenicity islands during invasion or intracellular growth [42].

Results

ppGpp and RpoS contribute to the modulation of SPI1 and SPI2 transcript levels during stationary phase

During progress through late-log and stationary phase, batch cultures of S. Typhimurium growing aerobically in Luria-Bertani (LB) medium express SPI1 and SPI2 encoded genes (Fig 1A and 1E) [5, 6, 43]. S. Typhimurium cultures at the late-log stage of growth are frequently used to promote invasion of epithelial cell lines to determine intracellular replication rates in tissue-culture based gentamicin protection assays. According to microarray-based transcriptomic analyses, SPI1 gene expression increases and is maximal at an OD_{600} of 2.3 to 3.0, thereafter expression declines, a finding in accordance with previous work [43]. In the present study, during early stationary phase (ESP; OD_{600} = 2.3), the most highly expressed SPI1 genes were found to be sipC, sipB and sicA (Fig 1A). During later stationary phase (OD_{600} = 4.2), microarray (Fig 1E, [6]), ChIP-chip data (Fig 2B) and dRNA-seq data [43] show that SPI2 expression increases. SPI1 and SPI2 expression is complex and controlled by a number of different factors that operate at the transcriptional and post-transcriptional levels, and that respond to environmental cues. However, one of the major signals for the induction of SPI1 and SPI2 expression in response to environmental conditions, both in vitro and in vivo is the bacterial alarmone, ppGpp [5–8,44]. Both microarray and dRNA-seq data showed that in the absence
of ppGpp (ΔrelAΔspoT), SPI1 and SPI2 transcript levels are extremely low compared to the parent strain (Fig 1B and 1F, S2 Table, [7,43]). Since ppGpp acts primarily to redistribute RNA polymerase, the very low levels of SPI1 and SPI2 transcription in the absence of ppGpp strongly suggested that there was a lack of RNAP recruitment at SPI1 and SPI2 sites in the ΔrelAΔspoT strain. A ChIP-chip analysis using an antibody to the beta subunit of E. coli RNAP verified that this was indeed the case (Fig 2A and 2B).

Interestingly, the expression of a few SPI2 genes increased in the ΔrelAΔspoT compared to the parent strain, suggesting they are ppGpp-repressed; these were orf319 (4.4-fold) and sseA (10.3-fold), (S2 Table, Fig 1F).

In addition to SPI1 and SPI2, the alternative sigma factor, RpoS is known to be highly expressed during stationary phase in S. Typhimurium and has been shown to be required for the successful infection of mice. We therefore decided to investigate the effect of RpoS on SPI1 and SPI2 expression. Using a strain of S. Typhimurium in which the rpoS gene had been deleted, we found that the transcript levels of SPI1 encoded genes at late log phase (LLP) and ESP were very similar (less than 2–fold) compared to the parent strain (S2 Table). However, expression of the sicA operon (consisting of the virulence factors sicA, sipB, sipC, sipD, and sipA) was significantly elevated by up to 8-fold at mid- and late-stationary phases; MSP and LSP (Fig 1B, S2 Table); in addition sicP, STM2880 and hilA were significantly elevated > 2-fold at LSP (S2 Table). We also observed a similar elevation of SPI2 transcript levels in the ΔrpoS compared to the parent strain at MSP and LSP, but not at LLP and ESP (Fig 1F). The SPI2 encoded genes significantly elevated by > 2-fold at both MSP and LSP were ssaCGHIJKLNO, ssaAB, sseCDEG and the SPI2 regulators, ssaAB; additionally, ssaBJMR and ssaT were also significantly elevated between 2 and 4.2-fold at LSP (S2 Table).

Using sipC as an example of a SPI1 encoded gene exhibiting high transcript levels at MSP and LSP in the ΔrpoS compared to the parent strain (Fig 1A, S2 Table), we performed β-galactosidase assays on a sipC::lacZ construct in the ΔrpoS and parent strains to verify the elevated sipC transcript levels (Fig 3). This result showed that expression of a sipC::lacZ fusion increased during stationary phase, peaking at 3-fold higher activity levels in the ΔrpoS strain compared to the parent strain after 6h growth, corresponding to the mid to late stationary phase of growth. Interestingly, despite the observations of elevated sipC transcript levels in the ΔrpoS relative to
the parent strain (5.7 and 7.0-fold respectively at MSP and LSP), and elevated sipC::lacZ expression in the ΔrpoS strain, western blots revealed little difference in intracellular or culture supernatant SipC protein levels in the ΔrpoS strain relative to the parent strain during stationary phase (Fig 4A and 4B). In addition, the relatively small changes in expression levels of the sipC::lacZ fusion in the parent strain during stationary phase (Fig 3), when compared to the significantly elevated sipC transcript levels observed in the parent strain at ESP and MSP (7.4 and 2.2-fold respectively, Fig 1A, S2 Table) is suggestive of post-transcriptional control of SipC stability. Despite our observation that sipC::lacZ activity increased 3-fold in the ΔrpoS strain through early and late stationary phase (Fig 3), controlled over-expression of rpoS from an inducible promoter resulted in a considerable decrease of sipC::lacZ activity compared to the control during mid- and late stationary phase (Fig 5). One explanation for this observation is that RpoS is able to compete for RNAP availability to reduce the ppGpp-dependent recruitment of RNAP and thus reduce sipC transcription; this would be consistent with the sigma factor competition model of RNAP distribution [44].

Fig 3. Expression of an S. Typhimurium sipC::lacZ fusion in parental, ΔrelAΔspoT (ppGpp0), ΔdksA and ΔrpoS backgrounds during growth in aerobic LB batch cultures. Data is from 3 biological replicate experiments.

doi:10.1371/journal.pone.0127523.g003

Fig 4. (A) western blot showing intracellular levels of RpoS, DksA, SipC and GroEL (loading control) at LLP, ESP, MSP and LSP in parental (SL1344), ΔrpoS, ΔdksA and ppGpp0 (ΔrelAΔspoT) strains. (B) western blot showing SipC levels in culture supernatants from parental, ΔrpoS, ΔdksA and ΔrelAΔspoT strains during mid stationary phase (MSP) and late stationary phase (LSP).

doi:10.1371/journal.pone.0127523.g004
One prediction of the above model would be that the presence or absence of RpoS in a ppGpp\(^0\) (\(\Delta\)relA\(\Delta\)spoT) background would make little difference to SPI1 or SPI2 transcript levels, since RNAP could not be efficiently recruited to SPI promoters in the absence of ppGpp. In accordance with this, we found that the significantly elevated expression (> 2-fold) of SPI1 and SPI2 genes observed in the \(\Delta\)rpoS strain relative to the parent did not occur in a comparison with the \(\Delta\)relA\(\Delta\)spoT\(\Delta\)rpoS strain, and in fact the expression of the majority of SPI1 and SPI2 genes in the latter strain decreased to the similar levels observed in the \(\Delta\)relA\(\Delta\)spoT strain (Fig 1D and 1H, S2 Table). A few exceptions where transcript levels were higher in the \(\Delta\)relA\(\Delta\)spoT strain compared to the \(\Delta\)relA\(\Delta\)spoT\(\Delta\)rpoS strain included invF (4.7-fold), prgI (4.0-fold), invC (2.1-fold) and invJ (2.0-fold), (S2 Table, Fig 1D). In a similar manner to SPI1, we found that within SPI2, the expression of only 4 genes showed significantly higher expression in the \(\Delta\)relA\(\Delta\)spoT strain compared to the \(\Delta\)relA\(\Delta\)spoT\(\Delta\)rpoS strain; these were orf319 (22.2-fold), sseA (6.1-fold), ssaI (2.1-fold) and ssaG (2.1-fold), (S2 Table, Fig 1H). Expression of the latter genes may therefore be RpoS as well as ppGpp-dependent.

Finally, in E. coli it has been shown that RpoS levels are positively controlled by ppGpp [45], however, in S. Typhimurium, RpoS levels appear to be unaffected by the absence of ppGpp [46]. Our data verified this observation in S. Typhimurium (Fig 4A). Whether these differences in control of RpoS by ppGpp in S. Typhimurium compared to E. coli represents an adaptation to optimising virulence gene expression relative to other RpoS-dependent regulatory requirements in S. Typhimurium remains to be seen.

Regulation of SPI1 and SPI2 transcription by DksA during stationary phase

DksA is an RNAP accessory protein and has been shown to potentiate the effects of ppGpp [20,23,47]. Previous work has also demonstrated that DksA is required for virulence of S.
Typhimurium in a murine infection model [32]. In order to investigate the role of DksA in SPI1 and SPI2 expression, we constructed an S. Typhimurium ΔdksA strain and showed that an early stationary phase culture of the mutant strain was attenuated by 3.6-fold for invasion in a HeLa cell infection model when compared to the parent strain (S4 Fig). We also performed a microarray-based transcriptomic analysis of the ΔdksA strain and showed that the transcript levels of 20 and 25 SPI1 encoded genes were significantly increased between 2 and 29-fold at MSP and LSP respectively compared to the parent strain (Fig 1C, S2 Table). The transcripts elevated in the ΔdksA strain at both MSP and LSP were prgHIJK, invBCEFGHIJ, spaPR, iagB, STM2870, STM2891, orgA and the SPI1 regulator, hilD (S2 Table). Consistent with the elevated expression of many SPI1 genes, a ChIP-chip analysis revealed an enhanced recruitment of RNAP to SPI1 promoter sites in the ΔdksA mutant compared to the parent strain at LSP (Fig 2A). In contrast to the elevated transcript levels of a majority of the SPI1 genes at MSP and LSP, the transcript levels of a subset of SPI1 genes were reduced at LLP and ESP by 2- to 5-fold (Fig 1C); these genes included all of those within the sicA operon (sicA, sipBCDA). The increased SPI1 transcript levels observed in the ΔdksA strain at LSP were found to be ppGpp-dependent since a ΔrelAΔspoTΔdksA showed no significant increase of SPI1 transcript levels compared to the ΔdksA strain apart from orgA which was significantly reduced by 5.9-fold in the ΔrelAΔspoTΔdksA compared to the ΔrelAΔspoT strain (S2 Table, Fig 1D). The latter data is consistent with a scenario where, in the absence of DksA (or a DksA-dependent transcription factor), recruitment of RNAP to SPI1 sites is ppGpp-dependently increased at LSP relative to the parent strain; this is corroborated by the ChIP-chip data which showed increased RNAP recruitment to SPI1 sites in the ΔdksA compared to the parent strain (Fig 2A). In the absence of ppGpp, recruitment of RNAP to SPI1 cannot efficiently occur (as the ChIP-chip data shows, Fig 2A), therefore the proposed effect of DksA (or a DksA-dependent transcription factor) in reducing recruitment or activity of RNAP is lost and SPI1 transcript levels in the ΔrelAΔspoTΔdksA mutant become comparable to those in the ΔrelAΔspoT strain (Fig 1D). The observation that there was no discernible difference in DksA protein levels in the presence or absence of ppGpp is consistent with the above scenario (Fig 4A).

Although we observed elevated SPI1 transcript levels in the ΔdksA strain at MSP and LSP, we found that SPI2 transcript levels were reduced at all points sampled during late-log and stationary phase by up to 40-fold (Fig 1G, S2 Table). The observed reduction in SPI2 transcript levels in the ΔdksA strain during stationary phase correlated with a decreased recruitment of RNAP to SPI2 sites in the ΔdksA strain compared to the parent at ESP and LSP (Fig 2B). As expected, the distribution of RNAP to SPI2 genomic sites in the ΔrelAΔspoT strain was also reduced compared to the parent strain at both ESP and LSP, in accordance with the reduced SPI2 transcript levels observed in the former compared to the latter strain (Figs 1F and 2B). SPI2 transcript levels were also found to be further reduced in the ΔrelAΔspoTΔdksA strain compared to the ΔdksA strain suggesting that DksA-dependent activation of their transcription requires ppGpp (Fig 1H, S2 Table). Interestingly, DksA therefore seems to have opposite effects on SPI1 and SPI2 transcription—on the one hand ppGpp-dependently repressing SPI1 transcription at MSP and LSP, (Fig 1C), whilst at the same time activating SPI2 expression (Fig 1G). The ChIP-chip data indicates these effects are likely to occur by modulating RNAP distribution (Fig 2).

SipC levels are post-transcriptionally regulated by DksA

The transcript level of the SPI1 effector gene, sipC was found to be very high at ESP in the parent strain (7.4-fold compared to LLP, Fig 1A, S2 Table), and was also considerably elevated in the ΔdksA strain at LSP (4.9-fold compared to the parent strain, Fig 1C, S2 Table). We
therefore decided to use western blotting to determine whether the level of SipC also changed in accordance with its transcript level in the ΔdksA mutant. When we tested the effect of loss of DksA on the intracellular and secreted levels of SipC, we were surprised to discover that intracellular SipC was undetectable at all of the time points sampled during late-log and stationary phase, despite the elevated sipC transcript levels observed at MSP and LSP (Fig 4A, S2 Fig). We were also unable to detect SipC in the ΔrelA ΔspoT strain, however, this appears to be unrelated to the absence of SipC in the ΔdksA strain since DksA levels were unaffected in the ΔrelA ΔspoT strain compared to the parent strain (Fig 4A). Despite the complete absence of intracellular SipC in the ΔdksA and ΔrelA ΔspoT strains, SipC was detectable in culture medium, although at much lower amounts compared to the parent strain (Fig 4B); this may represent SipC accumulated from earlier growth phases. The observations that intracellular SipC was undetectable by western blotting, yet sipC transcripts were elevated at MSP and LSP according to the microarray and ChIP-chip data, and measurement of sipC::lacZ activity revealed only a slight decrease in the ΔdksA strain compared to the parent strain (Fig 3), suggests that the stability of SipC is regulated by a DksA-dependent post-transcriptional mechanism during late-log/stationary phase.

**RpoS and DksA dependent expression levels of known SPI1 and SPI2 regulators at ESP and LSP**

The regulatory networks involved in the control of SPI1 and SPI2 genes are complex and operate at several levels [48]. So far, at least 65 and 23 regulators have been shown to be involved in the control of genes within SPI1 and SPI2, respectively [48,49]. To determine whether any of these regulators were transcriptionally RpoS and/or DksA-dependent, and therefore potentially involved in the regulation of SPI1 and SPI2 by RpoS and DksA, (in addition or instead of the proposed effects of sigma factor competition), we used microarrays to determine their expression levels in the ΔrpoS and ΔdksA mutants relative to the parent strains at ESP and LSP (Fig 6, S3 Table). A comparison of the known regulators of SPI1 at LSP compared to ESP showed significantly increased expression (> 2-fold) of several SPI1 activators in the ΔrpoS strain (Fig 6, S3 Table). The rfaH gene showed the greatest increase in expression between LSP and ESP in the ΔrpoS compared to the parent strain (7.1-fold and 1.3-fold at LSP and ESP respectively, Fig 6, S3 Table). An rfaH null mutation has been correlated with a 4-fold decrease in hilA expression under SPI1 inducing conditions in LB [48]. RfaH has also previously been shown to be modulated by RpoS in S. Typhi; in S. Typhimurium, the rfaH promoter also contains a predicted RpoS consensus sequence [7,50]. RfaH encodes a DNA-binding antiterminator, and is involved in the expression of distal genes in long, horizontally-acquired operons [51]. Its role in the regulation of SPI2 genes under conditions where SPI2 is expressed has not yet been determined; however, impaired intracellular replication within macrophages and mice has been demonstrated in an RfaH-deficient strain. This phenotype has been previously attributed to truncated LPS in S. Typhimurium [52]. Other RpoS-dependent activators which have been shown to increase both SPI1 and SPI2 expression and were significantly elevated > 2-fold at LSP compared to ESP in the ΔrpoS relative to the parent strain included hupA, hupB, corA, rtsA, trkH, ydgP and STM2303 (S3 Table, Fig 6). Although hilA expression was increased by 2.4-fold at LSP in the ΔrpoS relative to the parent strain, it was not significant at p < 0.05 (S3 Table). Of the SPI2 activators, ssrA, ssrB and hupB were the most highly differentially expressed genes in the ΔrpoS compared to the parent strain at LSP compared to ESP. Expression of the ssrA, ssrB and hupB genes were significantly increased by 2.0, 2.9 and 5.3-fold respectively in the ΔrpoS compared to the parent strain at LSP, whereas at ESP their expression levels were 0.7, 1.0 and 2.0 respectively (Fig 6, S3 Table). The transcript levels of the slyA and hupA gene
were also significantly overexpressed by 2.4, 2.8 fold respectively in the ΔrpoS compared to the parent strain at LSP (S3 Table), however, their transcript levels at ESP were 1.5 and 1.8-fold respectively. Therefore ssrA, ssrB and hupB displayed the highest ratio of transcript levels at LSP compared to ESP (Fig 6, S3 Table).

In the ΔdksA strain, SPI1 expression was elevated at LSP compared to ESP. A comparison of transcript levels for the known SPI1 regulators in the ΔdksA compared to the parent strain at LSP vs. ESP revealed significant differences in several known SPI1 activators. The activator showing the largest increase in expression at LSP compared to ESP in the ΔdksA relative to the parent strain was rtsA, (9.4-fold at LSP, S3 Table, Fig 6). RtsA is a major regulator of both hilA and hilD expression and forms part of a feed-forward loop for activation of SPI1 expression [53]. In addition, the expression of hilD was also significantly increased in the ΔdksA compared to the parent strain at LSP compared to ESP (6.9 and 1.7-fold respectively). Other significantly upregulated activators at LSP compared to ESP in the ΔdksA relative to the parent strain included fis, hupB and the genes encoding the flagellar regulators, flhC, flhD and fliZ (S3 Table, Fig 6).SPI2 gene transcript levels were reduced in the ΔdksA compared to the parent strain at all points sampled during late-log/stationary phase (Fig 1G). Of the known SPI2 activators, the expression of the major SPI2 activators ssrA and ssrB were significantly repressed between 2.2 and 6.1-fold in the ΔdksA relative to the parent strain at the four growth phases sampled (S3 Table). In addition, expression of phoP was reduced by 2.7-fold at both LLP and ESP and phoQ by 2.3 and 2.2 at LLP and MSP respectively in the ΔdksA relative to the parent strain (S3 Table). Expression of the SPI2 repressor, ydgT was also significantly increased by 2.3 and
2.7-fold at ESP and LSP in the ΔdksA compared to the parent strain (S3 Table). Which of these regulators are responsible for the RpoS and DksA-dependent alterations in SPI1 and SPI2 expression is the subject of future work.

Discussion

In this study we show that RpoS, DksA and ppGpp play both antagonistic and complementary roles resulting in the modulation of SPI1 and SPI2 transcription during late-log and stationary phase growth in aerobic LB broth cultures. Whereas the absence of ppGpp resulted in decreased SPI1 encoded gene transcript levels at LLP, ESP, MSP and to a lesser extent at LSP (Fig 1B), in the ΔrpoS and ΔdksA strains, the transcript levels of most SPI1 encoded genes were increased at MSP and LSP (Fig 1B and 1C). The SPI1 genes showing the greatest increase in transcript levels in the ΔrpoS and ΔdksA compared to the parent strain at MSP and LSP were members of the sicA operon. The SPI1 expression data for the ΔrpoS, ΔdksA and ΔrelAΔspoT strains therefore indicates that in the parent strain, ppGpp would be expected to activate the expression of most of the SPI1 encoded genes throughout stationary phase, (and to a lesser extent at LSP), whereas RpoS and DksA would have an antagonistic effect to ppGpp at MSP, and particularly at LSP, suggesting that the net result would be a modulation of SPI1 transcript levels such that their transcript levels were repressed during LSP compared to ESP and MSP. The loss of ppGpp did not reduce SPI2 transcript levels as highly compared to SPI1 transcript levels during LLP, ESP and MSP; however, at LSP, SPI2 transcript levels in the ΔdksA strain were considerably reduced by up to 15-fold relative to the parent strain (Fig 1F). Similarly to SPI1, the absence of RpoS resulted in an increase in the majority of SPI2 transcript levels at MSP and LSP (Fig 1F); despite this, the differential effects of loss of ppGpp and RpoS on SPI2 transcript levels at LSP suggests that, in the parent strain, ppGpp is likely to play a significant role in elevating SPI2 transcript levels at LSP (Fig 1E). In a broader context, the effect of RpoS in reducing SPI1 and SPI2 transcript levels may partially contribute to the reason for the prevalence of rpoS mutations found in several S. Typhi field isolates, where they might be expected to enhance virulence traits [54]. In contrast to the marked growth phase specific effects of loss of ppGpp and RpoS on SPI2 gene transcript levels, the effect of loss of DksA was less obvious, and SPI2 transcript levels were reduced at LLP, ESP, MSP and LSP in the ΔdksA compared to the parent strain, suggesting DksA is required for SPI2 transcription throughout late-log/stationary phase (Fig 1G). The changing growth phase-dependent levels and/or activities of RpoS, ppGpp and DksA may also potentially be expected to have a significant impact upon the ‘timing’ of SPI1 and SPI2 expression during infection, since it is established that SPI1 and SPI2 genes tend to be expressed under conditions conducive to either invasion and intracellular replication respectively, although some overlap has been found [5].

It is of interest that, although DksA is synthesised constitutively throughout growth (Fig 4A, [20]), deletion of dksA resulted in opposing effects on SPI1 and SPI2 transcript levels during stationary phase (Fig 1C and 1G). The simultaneous activating and repressive effects of DksA on gene expression is not unprecedented [20,23,47,55]. DksA binds to RNAP and greatly enhances direct effects of ppGpp on the negative control of E. coli rRNA promoters [20]; in addition, DksA has also been shown to potentiate the direct activation of amino acid promoters by ppGpp [23]. DksA also has disparate effects on the expression of virulence determinants in E. coli O157; although both ppGpp and DksA were required for activation of the LEE1 promoter during entry into stationary phase, their effect was different at late stationary phase and LEE1 promoter activity was increased in the ΔdksA strain [22]. These results and our own data indicate that DksA can have both positive and negative effects on the expression of different virulence genes in both E. coli O157 and S. Typhimurium; the exact mechanism by which this
occurs remains to be clarified. However, recently a 5-bp AT rich discriminator region (\(P_{\text{disc}}\), AAGGA), located immediately downstream of the -10 element has been shown to be critical for positive control of the \(E.\ coli\ uspA\) promoter by ppGpp/DksA \[56\]. SPI2 encoded genes are under the positive control of the major regulators SsrA/B, OmpR/EnvZ and SirA/BarA \[57\]. Neither of the two published transcriptional start sites (TSS’s) for the SPI2 ssrA promoters have a proximal upstream AAGGA motif, however the discriminator region immediately upstream of the \(sirA\) TSS contains an AAGGA motif: TAAGGAG\(_G\), where \(G\) is the annotated TSS at genomic position 1996515 \[43\]. The \(sirA\) gene was found to be 2-fold repressed in the \(\Delta dksA\) strain compared to ESP (S3 Table), and therefore suggests a possible mechanism by which SPI2 may be indirectly activated by DksA. The \(ssrA\) promoter does however contain AT rich discriminator regions: ATTCTAT\(_G\) at genomic position 1436617 and TGTTGTT\_G at genomic position 1436769 (where \(T\) represents the TSS; \[43,58\]). It remains to be seen whether these discriminator regions are directly involved in positive control by DksA/ppGpp.

The opposing effects of ppGpp and RpoS in respectively activating and reducing transcript levels of SPI1 and SPI2 genes during stationary phase and the observation that SPI1 and SPI2 gene transcript levels were not elevated in the \(\Delta relA\Delta spoT\Delta rpoS\) strain compared to the \(\Delta rpoS\) strain, yet remained at the same reduced levels observed in the \(\Delta relA\Delta spoT\) strain (Fig 1D and 1H) is in agreement with the ‘sigma factor competition’ model whereby ppGpp is required to facilitate competition between the sigma 70 ‘housekeeping’ factor and alternative sigma factors, based on their relative intracellular ratios \[59\]. In this model, ppGpp would be required for the recruitment of RNAP to SPI1 and SPI2 sites, whereas it would also facilitate competition between sigma 70 and RpoS, which would result in loss of RNAP from SPI1 and SPI2 promoter sites, perhaps contributing to the decreased SPI1 expression observed in the parent strain at LSP (Fig 1A). For SPI2, although RpoS is competing for recruitment of RNAP, ppGpp or ppGpp-dependent activating factors appear to play a role to facilitate the elevation of SPI2 transcript at LSP compared to earlier time points (Fig 1E). The sigma factor competition model would predict that overexpression of RpoS may result in reduced expression of the SPI1 effector gene \(sipC\), due to the inferred redistribution of RNAP to RpoS-dependent promoter sites rather than SPI1 promoter sites and consistent with this, we confirmed that ectopic induction of RpoS resulted in severely reduced \(sipC::lacZ\) activity compared to the parent strain during stationary phase (Fig 5). Although the absence of SipC can be correlated with lack of \(sipC\) transcripts in the \(\Delta relA\Delta spoT\) strain, \(sipC\) transcript levels were increased in the \(\Delta rpoS\) compared to the parent strain at MSP and LSP by 5.7 and 7.0 fold respectively (Fig 1B, S2 Table). In addition, activity of a \(sipC::lacZ\) fusion was increased 3-fold in the \(\Delta rpoS\) background compared to the parent strain (Fig 3). Despite these observations, the elevated transcript levels appear to be modulated post-transcriptionally, resulting in little overall change in either the intracellular or secreted SipC protein levels (Fig 4A and 4B). This may suggest a mechanism whereby \(sipC\) and possibly other SPI1 encoded gene expression could remain ‘buffered’ against rapid changes in RNAP distribution caused by alternative sigma factors such as RpoS, thus potentially optimising a balance between the RpoS-dependent stationary phase stress response and invasion. The latter mechanism may also represent an adaptation by which limited RNAP availability can be efficiently managed to balance stress and virulence \[59\].

Similarly to RpoS, the absence of DksA also resulted in increased transcript levels of many SPI1 genes including \(sipC\) at MSP and LSP compared to the parent strain (Fig 1C). Deletion of \(dksA\) resulted in a slight reduction in RpoS (Fig 4A); however, since deletion of \(rpoS\) had no effect on SipC levels (Fig 4A), it seems unlikely that DksA is acting via RpoS to reduce parental \(sipC\) transcript levels. Despite the elevated \(sipC\) (and other SPI1) transcript levels in the \(\Delta dksA\) strain at MSP and LSP, intracellular SipC protein remained completely undetectable throughout stationary phase in the \(\Delta dksA\) mutant (Fig 4A). The latter result may explain the...
discrepancy between the observed elevation of SPI1 transcript levels in the ΔdksA strain and the invasion defect of the ΔdksA strain in HeLa cells invasion assays (S4 Fig). SipC was however present in the culture supernatants of both the ΔdksA and ΔrelAΔspoT strains, although at considerably reduced levels compared to the parent strain (Fig 4B); this may reflect SipC accumulated prior to late-log/stationary phase. The small reduction in overall activity of the sipC::lacZ fusion in the ΔdksA compared to the parent strain throughout stationary phase (Fig 3) and complete absence of SipC protein in the ΔdksA relative to the parent strain (Fig 4A) suggests that DksA is directly or indirectly required to stabilise SipC. Further experiments are in progress to determine the basis for the post-transcriptional regulation of the SipC effector protein by DksA.

Finally, a transcriptional analysis was performed to determine whether any of the known SPI1 and SPI2 activators could play a role in the observed differences in the transcript levels of SPI1 and SPI2 encoded genes at LSP in the ΔrpoS and ΔdksA mutants compared to the parent strain, as well or instead of any effects caused by potential sigma factor competition. We found that the most highly differentially expressed SPI1 activators at LSP compared to ESP were rfaH and rtsA. The transcript levels of rfaH in the ΔrpoS strain were increased by 5.5-fold at LSP compared to ESP, and rtsA transcript levels were increased by 15.9 fold in the ΔdksA strain at LSP compared to ESP (S3 Table). This may suggest that RpoS and DksA act via different regulatory pathways to efficiently repress SPI1 expression under the environmental conditions studied here. For SPI2, the transcript levels of the major SPI2 regulators ssrA and ssrB were the most highly differentially expressed genes in the ΔrpoS versus the parent strain at LSP compared to ESP, suggesting they may play a role in the observed elevation of SPI2 transcript levels at LSP (Fig 6).

Materials and Methods

Strains and culture conditions

A full list of strain details used in this study is described in S1 Table. Salmonella enterica sv. Typhimurium SL1344 parent and isogenic ΔrelAΔspoT, ΔrpoS, ΔdksA, ΔrelAΔspoTΔrpoS and ΔrelAΔspoTΔdksA strains were routinely grown in Luria-Bertani (LB) medium at 37°C, shaking at 250 rpm. For growth experiments, single colonies grown on LB agar were added to 5 mL LB and grown overnight, before inoculation into liquid LB medium (1:100). Strains were grown to optical densities (OD’s) measured at 600 nm corresponding to late-log phase (LLP, OD≈1.0), early stationary phase (ESP, OD≈2.3), mid-stationary phase (MSP, OD≈3.2) and late stationary phase (LSP, OD≈3.6), and samples taken at these points for further analysis. Optical density growth curves (OD600) and CFUs of sampling points are shown in S1 and S2 Figs. For experiments, requiring selection of strains or plasmids, antibiotics were added at the following concentrations: ampicillin (100 μg ml⁻¹), chloramphenicol (20 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and tetracycline (20 μg ml⁻¹).

For experiments involving the controlled overexpression of rpoS, a DNA fragment containing the rpoS open reading frame (ORF) and EcoRI and BamHI sites was cloned into the high copy number vector, pASK75 [58]. The rpoS ORF was amplified by PCR. The forward and reverse primer sequences (5’ to 3’), containing the restriction sites were TAGAGCGAATTC TAGGAGCCACCTTTATGAGTC and CACCTTGGATCCCAAGGGTACTTACTCGCGGA respectively. After digestion with EcoRI and BamHI, the fragment was ligated into the high copy number, inducible tetA⁺, Amp⁺ vector, pASK75, which was transformed into four strains of S. Typhimurium (parent, ΔrpoS, ΔdksA and ΔrelAΔspoT) by electroporation. The plasmid was maintained by addition of 100 μg ml⁻¹ ampicillin to the culture medium and expression from the tetA promoter was induced by the addition of 0.2 μg ml⁻¹
anhydrotetracycline (ATC) (Fluka, 37919) as per the method outlined [60], and confirmed by western blotting.

**β-galactosidase assay**

A sipC::Tn5lacZY transcriptional fusion from *S. Typhimurium* strain SA29 [61] was transduced by electroporation into four *S. Typhimurium* SL1344 genetic backgrounds (parent strain, ΔrpoS, ΔdksA, ΔrelAΔspoT) and used to assay sipC promoter activity. Strains containing the sipC::Tn5lacZ fusion were grown in batch cultures and the culture was sampled at 2h, 3h, 5h, 6h and 8h. For clarity, the growth curves and sipC::Tn5lacZ expression levels are shown in S3 Fig.

The β-galactosidase assay was performed at 28°C according to [62]. Briefly, 0.2 ml of cell culture was diluted in 0.8 ml Z-Buffer and 40 μl chloroform and 20 μl 0.1% sodium dodecyl sulphate added to permeabilise the cells. The reaction was initiated by the addition of o-nitrophenyl-β-D-galactoside (ONPG; 4 mg/ml). Once the reaction began to turn yellow, it was quenched by the addition of 0.5 ml 1M sodium carbonate. β-galactosidase activity was then measured spectrophotometrically at 550 nm and 420 nm, and the cell culture optical density measured at 600 nm. The data were expressed in Miller Units, according to the following equation: Miller Units = 1000 x [(OD420 – 1.75 x OD550) / (T x V x OD600)], where T = time of the reaction (minutes) and V = volume of culture used in the assay (ml).

**Microarray analysis**

Microarray analysis was performed as described previously [43]. *S. Typhimurium* SL1344 parent and mutant strains were grown to LLP, ESP, MSP and LSP as described under Strains and culture conditions. Total RNA was extracted from the strains as described above. The RNA was labelled and hybridised to IFR SALSA2 microarrays (www.ifr.ac.uk/Safety/Microarrays/default.html#protocols), and data processed and analysed using GeneSpring (Agilent). The data was from 3 biological replicates, statistically filtered (p = 0.05) and a 2-fold cut off applied. The microarray data discussed in this publication are MIAME compliant and have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO accession number GSE63715.

**ChIP-chip analysis**

Strains SL1344 parent and isogenic ΔrelAΔspoT and ΔdksA strains were grown in LB broth under normal aeration at 37°C to LLP, ESP, MSP and LSP, as described under Strains and culture conditions. Co-immunoprecipitation was carried out using monoclonal antibody raised against the beta subunit of *E. coli* RNA polymerase (Neoclone, W0002) which has 100% sequence identity to *S. Typhimurium* RNAP. The CoIP protocol is described in [63]. Microarrays used for the ChIP-on-chip experiments were designed and produced by Oxford Gene Technology (Kidlington, UK). They consisted of approximately 44,000 60-mer oligonucleotides tiled throughout the *S. Typhimurium* SL1344 NCTC13347 genome and 636 control oligonucleotides giving a 100 nt resolution. Microarray hybridisations were carried out according to the manufacturer’s instructions. Further descriptions of the microarray and protocols used for generating and analysing the data are associated with the dataset deposited in the GEO data repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE63715. In order to identify peaks, the microarray data was loaded onto ChiPOTle Visual Basic for applications macro [64] and then visualized in the Integrated Genome Browser (IGB) Affymetrix (version 8.1.8), [65]. Each ChIP-chip experiment for each strain was performed in triplicate.
Western blotting

To visualise intracellular bacterial protein production, parent and mutant strains were grown to LLP, MSP, ESP and LSP as described under Strains and culture conditions, harvested by centrifugation (7000×g, 4°C, 10 minutes) and re-suspended in 1× NuPAGE protein loading buffer (Life Technologies, NP0007), containing 50mM freshly-added dithiothreitol (DTT). For each 0.1 OD unit, 10 μl sample buffer was added. Lysis and solubilisation was carried out by boiling the samples for 10 minutes and centrifuging the lysates for 30 minutes (> 10,000×g). Lysates were diluted 1:10 and subjected to size separation by SDS-PAGE on 12% Bis-Tris Protein Gels (Life Technologies, NP0342PK2). Growth curves and optical densities at which samples were removed for western blotting are shown for parent, ΔrpoS, ΔdksA and ΔrelAΔspoT strains in S1 and S2 Figs.

Analysis of proteins in the cultures supernatant was performed using the method of [66]. Briefly, cell cultures were centrifuged to remove intact cells, and supernatants were passed through a 0.22μm filter. To 1 mL of supernatants, 0.3 ml ice-cold trichloroacetic acid was added and the samples left on ice for 15 min to precipitate the proteins. Samples were centrifuged at 10,000×g for 15 minutes, washed twice with acetone and the final protein pellet was dissolved in loading buffer and separated by SDS-PAGE.

After gel electrophoresis, proteins were transferred to a methanol-treated PVDF membrane using semi-dry transfer apparatus (Bio-Rad; 1h, 0.25A) and the membrane blocked using a solution of PBST containing 10% skimmed milk powder (Marvel). Antibodies to specific proteins (mouse α-RpoS (Neoclone, W0009; 1:1000), chicken α-DksA (1:2500), mouse α-SipC (1μg ml⁻¹), rabbit α-GroEL (Sigma, G6532; 1:40000)) were added to binding solution (PBST + 0.5% Marvel) and incubated at room temperature, with vigorous agitation for 2 hours (primary antibody) or 1 hour (secondary antibodies). Detection of protein was performed using Goat IgG secondary antibodies, conjugated to horseradish peroxidase. Peroxidase activity was identified using Pierce SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080) and bands were visualised using the FluorChem E System (ProteinSimple). In order to ensure consistent loading, all blots were subsequently stripped according to the manufacturer’s instructions and re-probed with antibody against GroEL.

Invasion assays

Invasion assays in HeLa epithelial cells (obtained from American Type Culture Collection, Rockville, MD) were performed according to [67]. Briefly, HeLa cells were grown in DMEM medium (Sigma, D5546) containing 1 g/L glucose and supplemented with 10% fetal bovine serum (Sigma), 2 mM L glutamine (Sigma) and 20 mM HEPES buffer (Sigma). Between 1 and 3 ×10⁵ HeLa cells were seeded into each well of a 6- or 12-well cell culture plate and infected with S. Typhimurium SL1344 and mutant strains at an MOI of 10:1. Prior to infection, the S. Typhimurium strains had been grown to an OD₆₀₀ of 2.3 to allow expression of the SPI1 Type-3 secretion system. To increase the uptake of Salmonella, plates were centrifuged at 1000 g for 5 min, and this was defined as time 0 h. After 1 h of infection, extracellular bacteria were killed with 30 μg.ml⁻¹ gentamicin. The media was replaced after 1 h with medium containing 5 μg.ml⁻¹ gentamicin. Incubations were continued for 2 h and 6 h. To estimate the amount of intracellular bacteria at each time point, cells were lysed using 0.1% SDS, and samples were taken for viable counts. Statistical significances were assessed by using Student’s unpaired t-test, and a p value ≤ 0.05 was considered significant.
Supporting Information

S1 Fig. Growth phenotypes of SL1344 parental strain and ΔrpoS, ΔrelAΔspoT and ΔdksA strains in LB media and grown aerobically, with shaking (250 rpm) at 37°C. (DOCX)

S2 Fig. Optical density and equivalent CFU’s at which samples were taken for protein extraction and western blot analysis, shown in Fig 4. (DOCX)

S3 Fig. Growth characteristics and LacZ activities for samples assayed for sipC promoter activity shown in Fig 3. (DOCX)

S4 Fig. Invasion assay of S. Typhimurium SL1344 parental and ΔdksA strain in HeLa cells. (DOCX)

S1 Table. Strains and plasmids used in this study. (DOCX)

S2 Table. Expression levels and statistical analysis of SPI1 and SPI2 encoded genes in parent and mutant strains used in the construction of Fig 1. (XLSX)

S3 Table. Expression levels and statistical analysis of known SPI1 and SPI2 activators and repressors in parent and mutant strains used in the construction of Fig 6. (XLSX)

Acknowledgments

The authors would like to thank the following for kind gifts of reagents and advice: Prof. Mark Stevens (The Roslin Institute, University of Edinburgh) and Prof. David Holden (Imperial College, London) for antibodies, Dr. Francis Mulholland (IFR, Norwich) for helpful advice relating to western blots and Prof. Arne Skerra (Technical University of Munich, Germany) for plasmid pASK75.

Author Contributions

Conceived and designed the experiments: AT. Performed the experiments: CJR VKR NS. Analyzed the data: CJR VKR NS AT. Contributed reagents/materials/analysis tools: AT. Wrote the paper: CJR AT.

References

1. European Food Safety Authority (2014) The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal 12: 312.
2. Haraga A, Ohlson MB, Miller SI (2008) Salmonellae interplay with host cells. Nat Rev Microbiol 6: 53–66. PMID: 18026123
3. Vazquez-Torres A, Jones-Carson J, Baumler AJ, Falkow S, Valdivia R, et al. (1999) Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature 401: 804–808. PMID: 10548107
4. Muller AJ, Kaiser P, Dittrmar KE, Weber TC, Haueter S, et al. (2012) Salmonella gut invasion involves TTSS-2-dependent epithelial traversal, basolateral exit, and uptake by epithelium-sampling lamina propria phagocytes. Cell Host Microbe 11: 19–32. doi: 10.1016/j.chom.2011.11.013 PMID: 22264510
5. Song M, Kim HJ, Kim EY, Shin M, Lee HC, Hong Y, et al. (2004) ppGpp-dependent stationary phase induction of genes on Salmonella pathogenicity island 1. J Biol Chem, 279(33): 34183–90. PMID: 15161921
Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, et al. (2000) The stringent response of

Dalebroux ZD, Swanson MS (2012) ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol 10:

Klinkenberg LG, Lee JH, Bishai WR, Karakousis PC (2010) The stringent response is required for full

Taylor CM, Beresford M, Epton HA, Sigee DC, Shama G, et al. (2002) Hammer BK, Tateda ES, Swanson MS (2002) A two-component regulator induces the transmission

Kang PJ, Craig EA (1990) Identification and characterization of a new

Haralalka S, Nandi S, Bhadra RK (2003) Mutation in the

Zusman T, Gal-Mor O, Segal G (2002) Characterization of a

Paul BJ, Barker MM, Ross W, Schneider DA, Webb C, et al. (2004) DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell 118: 311–322. PMID: 15294157

Costanzo A, Nicoloff H, Barchinger SE, Banta AB, Gourse RL, et al. (2008) ppGpp and DksA likely regulate the activity of the extracytoplasmic stress factor sigmaE in Escherichia coli by both direct and indirect mechanisms. Mol Microbiol 67: 619–632. PMID: 18086212

Nakanishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, et al. (2006) ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic Escherichia coli through activation of two virulence regulatory genes. Mol Microbiol 61: 194–205. PMID: 16824105

Paul BJ, Berkmen MB, Gourse RL (2005) DksA potentiates direct activation of amino acid promoters by ppGpp. Proc Natl Acad Sci U S A 102: 7823–7828. PMID: 15899978

Paul BJ, Ross W, Gaal T, Gourse RL (2004) rRNA transcription in Escherichia coli. Annu Rev Genet 38: 749–770. PMID: 15568992

Sharma AK, Payne SM (2006) Induction of expression of hfq by DksA is essential for Shigella flexneri virulence. Mol Microbiol 62: 469–478. PMID: 17020583

Gourse RL, Ross W, Rutherford ST (2006) General pathway for turning on promoters transcribed by RNA polymerases containing alternative sigma factors. J Bacteriol 188: 4589–4591. PMID: 16788165

Magnusson LU, Farewell A, Nystrom T (2005) ppGpp: a global regulator in Escherichia coli. Trends Microbiol 13: 236–242. PMID: 15866041

6. Song M, Kim HJ, Ryu S, Yoon H, Yun J, Choy HE (2010) ppGpp-mediated stationary phase induction of the genes encoded by horizontally acquired pathogenicity islands and cob/pdu locus in Salmonella enterica serovar Typhimurium. J Microbiol, 48(1): 89–95. doi: 10.1007/s12275-009-0179-6 PMID: 20221735

7. Ramachandran VK, Shearer N, Jacob JJ, Sharma CM, Thompson A (2012) The architecture and ppGpp-dependent expression of the primary transcriptome of Salmonella Typhimurium during invasion gene expression. BMC Genomics 13: 25. doi: 10.1186/1471-2164-13-25 PMID: 22251276

8. Thompson A, Rolfe MD, Lucchini S, Schwerk P, Hinton JC, et al. (2006) The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of Salmonella. J Biol Chem 281: 30112–30121. PMID: 16905537

9. Potrykus K, Cashel M (2008) (p)ppGpp: still magical? Annu Rev Microbiol 62: 35–46. doi: 10.1146/annurev.micro.62.081307.162903 PMID: 18454629

10. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS (2010) ppGpp conjures bacterial virulence. Microbiol Mol Biol Rev 74: 171–199. doi: 10.1128/MMBR.00046-09 PMID: 20508246

11. Dalebroux ZD, Swanson MS (2012) ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol 10: 203–212. doi: 10.1038/nrmicro2720 PMID: 22337166

12. Klinkenberg LG, Lee JH, Bishal WR, Karakousis PC (2010) The stringent response is required for full virulence of Mycobacterium tuberculosis in guinea pigs. J Infect Dis 202: 1397–1404. doi: 10.1086/656524 PMID: 20863231

13. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, et al. (2000) The stringent response of Mycobacterium tuberculosis is required for long-term survival. J Bacteriol 182: 4889–4898. PMID: 10940033

14. Taylor CM, Beresford M, Epton HA, Sigee DC, Shama G, et al. (2002) Listeria monocytogenes relA and hot mutants are impaired in surface-attached growth and virulence. J Bacteriol 184: 621–628. PMID: 11790730

15. Hammer BK, Tateda ES, Swanson MS (2002) A two-component regulator induces the transmission phenotype of stationary-phase Legionella pneumophila. Mol Microbiol 44: 107–118. PMID: 11967072

16. Zusman T, Gal-Mor O, Segal G (2002) Characterization of a Legionella pneumophila relA insertion mutant and roles of RelA and RpoS in virulence gene expression. J Bacteriol 184: 67–75. PMID: 11741845

17. Haralaka S, Nandi S, Bhadra RK (2003) Mutation in the relA gene of Vibrio cholerae affects in vitro and in vivo expression of virulence factors. J Bacteriol 185: 4672–4682. PMID: 12896985

18. Erickson DL, Lines JL, Pesce EC, Venturi V, Storey DG (2004) Pseudomonas aeruginosa relA contributes to virulence in Drosophila melanogaster. Infect Immun 72: 5638–5645. PMID: 15385461

19. Kang PJ, Craig EA (1990) Identification and characterization of a new Escherichia coli gene that is a dosage-dependent suppressor of a dnaK deletion mutation. J Bacteriol 172: 2055–2064. PMID: 2180916

20. Paul BJ, Barker MM, Ross W, Schneider DA, Webb C, et al. (2004) DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell 118: 311–322. PMID: 15294157

21. Costanzo A, Nicoloff H, Barchinger SE, Banta AB, Gourse RL, et al. (2008) ppGpp and DksA likely regulate the activity of the extracytoplasmic stress factor sigmaE in Escherichia coli by both direct and indirect mechanisms. Mol Microbiol 67: 619–632. PMID: 18086212

22. Nakaniishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, et al. (2006) ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic Escherichia coli through activation of two virulence regulatory genes. Mol Microbiol 61: 194–205. PMID: 16824105
28. Henard CA, Tapscott T, Crawford MA, Husain M, Doulas PT, et al. (2014) The 4-cysteine zinc-finger motif of the RNA polymerase regulator DksA serves as a thiol switch for sensing oxidative and nitrosative stress. Mol Microbiol 91: 790–804. doi: 10.1111/mmi.12498 PMID: 24354846

29. Henard CA, Vazquez-Torres A (2012) DksA-dependent resistance of Salmonella enterica serovar Typhimurium against the antimicrobial activity of inducible nitric oxide synthase. Infect Immun 80: 1373–1380. doi: 10.1128/IAI.006316-11 PMID: 22311927

30. Jude F, Kohler T, Branny P, Perron K, Mayer MP, et al. (2003) Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in Pseudomonas aeruginosa. J Bacteriol 185: 3558–3566. PMID: 12775693

31. Mogull SA, Runyen-Janecky LJ, Hong M, Payne SM (2001) dksA is required for intercellular spread of Shigella flexneri via an RpoS-independent mechanism. Infect Immun 69: 5742–5751. PMID: 11500451

32. Webb C, Moreno M, Wilmes-Riesenberg M, Curtiss R 3rd, Foster JW (1999) Effects of DksA and ClpP protease on sigma S production and virulence in Salmonella typhimurium. Mol Microbiol 34: 112–123. PMID: 10540290

33. Yun J, Jeon B, Barton YW, Plummer P, Zhang Q, et al. (2008) Role of the DksA-like protein in the pathogenesis and diverse metabolic activity of Campylobacter jejuni. J Bacteriol 190: 4512–4520. doi: 10.1128/JB.00105-08 PMID: 18456813

34. Battesti A, Majdalani N, Gottesman S (2011) The RpoS-mediated general stress response in Escherichia coli. Annu Rev Microbiol 65: 189–213. doi: 10.1146/annurev-micro-090110-102946 PMID: 21639793

35. Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, et al. (1992) The alternative sigma factor KatF (rpoS) regulates Salmonella virulence. Proc Natl Acad Sci U S A 89: 11978–11982. PMID: 1465428

36. Giddens SR, Tormo A, Mahanty HK (2000) Expression of the antifeeding gene anfA1 in Serratia entomophila. Appl Environ Microbiol 66: 1711–1714. PMID: 10742266

37. Kowarz L, Coynault C, Robbe-Saule V, Norel F (1994) The Salmonella typhimurium katF (rpoS) gene: cloning, nucleotide sequence, and regulation of spvR and spvABCD virulence plasmid genes. J Bacteriol 176: 6852–6860. PMID: 7961444

38. Nielsen AT, Dolganov NA, Otto G, Miller MC, Wu CY, et al. (2006) RpoS controls the Vibrio cholerae mucosal escape response. PLoS Pathog 2: e109. PMID: 17054394

39. Solis R, Bertani I, Degrassi G, Devescovi G, Venturi V (2006) Involvement of quorum sensing and RpoS in rice seedling blight caused by Burkholderia plantarii. FEMS Microbiol Lett 256: 109–112. PMID: 16684109

40. Yildiz FH, Schoolnik GK (1998) Role of rpoS in stress survival and virulence of Vibrio cholerae. J Bacteriol 180: 773–784. PMID: 9473029

41. Dong T, Schellhorn HE (2010) Role of RpoS in virulence of pathogens. Infect Immun 78: 887–897. doi: 10.1128/IAI.00882-09 PMID: 19948835

42. Moest TP, Meresse S (2013) Salmonella T3SSs: successful mission of the secret(ion) agents. Curr Opin Microbiol 16: 38–44. doi: 10.1016/j.mib.2012.11.006 PMID: 23295139

43. Ramachandran VK, Shearer N, Thompson A (2014) The Primary Transcriptome of Salmonella enterica Serovar Typhimurium and Its Dependence on ppGpp during Late Stationary Phase. PLoS One 9: e92690. doi: 10.1371/journal.pone.0092690 PMID: 24664308

44. Jishage M, Kvint K, Shingler V, Nystrom T (2002) Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev 16: 1260–1270. PMID: 12023304

45. Gentry DR, Hernandez VJ, Nguyen LH, Jensen DB, Cashel M (1993) Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. J Bacteriol 175: 7982–7989. PMID: 8253685

46. Pizarro-Cerda J, Tedin K (2004) The bacterial signal molecule, ppGpp, regulates Salmonella virulence gene expression. Mol Microbiol 52: 1827–1844. PMID: 15186428

47. Magnusson LU, Gummesson B, Joksimovic P, Farewell A, Nystrom T (2007) Identical, independent, and opposing roles of ppGpp and DksA in Escherichia coli. J Bacteriol 189: 5193–5202. PMID: 17496080

48. Golubeva YA, Sadik AY, Ellemieer JR, Slauch JM (2012) Integrating global regulatory input into the Salmonella pathogenicity island 1 type III secretion system. Genetics 190: 79–90. doi: 10.1534/genetics.111.132779 PMID: 22021388

49. Fábrega A, Vila J (2013) Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev 26: 308–341. doi: 10.1128/CMR.00066-12 PMID: 23554419
50. Bittner M, Saldias S, Altamirano F, Valvano MA, Contreras I (2004) RpoS and RpoN are involved in the growth-dependent regulation of rfaH transcription and O antigen expression in Salmonella enterica serovar Typhi. Microb Pathog 36: 19–24. PMID: 14646366

51. Bailey MJ, Hughes C, Koronakis V (1996) Increased distal gene transcription by the elongation factor RfaH, a specialized homologue of NusG. Mol Microbiol 22: 729–737. PMID: 8951819

52. Nagy G, Danino V, Dobrindt U, Pallen M, Chaudhuri R, et al. (2006) Down-regulation of key virulence factors makes the Salmonella enterica serovar Typhimurium rfaH mutant a promising live-attenuated vaccine candidate. Infect Immun 74: 5914–5925. PMID: 16988271

53. Ellermeier CD, Ellermeier JR, Slauch JM (2005) HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol Microbiol 57: 691–705. PMID: 16045614

54. Martinez LC, Yakhnin H, Camacho M, Georgellis D, Babitzke P, et al. (2011) Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the Salmonella SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol 80: 1637–1656. doi: 10.1111/j.1365-2958.2011.07674.x PMID: 21518393

55. Aberg A, Fernandez-Vazquez J, Rouilhac I, Norel F (2003) Characterization of the RpoS Status of Clinical Isolates of Salmonella enterica. Appl Environ Microbiol 69: 4352–4358. PMID: 12902215

56. Miller JH (1992) A Short Course in Bacterial Genetics. Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. NY: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.