INTRODUCTION

Bacteria have evolved numerous strategies to cope with environmental stress, including the use of nucleotide signalling pathways to ensure a rapid cellular response. The stringent response is one such signalling pathway, and is utilized by the vast majority of bacterial species to deal with nutritional deficiencies. The effectors of this signalling pathway are the alarmone nucleotides guanosine tetra- and pentaphosphate, collectively termed (p)ppGpp. (p)ppGpp is produced from ATP and either GTP (pppGpp) or GDP (ppGpp) by the action of synthetase enzymes containing a SYNTH domain (PF04607), and is degraded to GTP/GDP (ppGpp) by the action of synthetase enzymes containing a (p)ppGpp. (p)ppGpp is produced from ATP and phosphates, collectively termed (p)ppGpp. (p)ppGpp is produced from ATP and either GTP or GDP by the action of synthetase enzymes containing a SYNTH domain (PF04607). These enzymes are all members of the RSH superfamily (RelA/SpoT homologue), so named after the RelA and SpoT enzymes in Escherichia coli, where these nucleotides were first discovered [1].

There are three main groups of enzymes in the RSH superfamily that are responsible for the controlling the cellular levels of these alarmones: long-RSH enzymes; small alarmone synthetases (SAS); and small alarmone hydrolases (SAH) (Fig. 1) [2]. Long-RSH proteins typically have a hydrolase and synthetase domain in their N-terminal domain (NTD), and a regulatory C-terminal domain (CTD) comprising TGS (ThrRS, GTPase and SpoT: PF02824), helical, CC (conserved cysteine) and ACT (aspartate kinase, chorismate and TyrA: PF13291) domains. Recent cryo-electron microscopy images of RelA from E. coli (RelA<sub>Ec</sub>) in complex with the ribosome, however, suggest that the ACT domain fold is actually more similar to an RNA recognition motif (RRM), and also show an unpredicted zinc-finger domain (ZFD) lying upstream of the ACT/RRM domain (Fig. 1a) [3–5].

Gram-negative bacteria, like E. coli, generally contain two long-RSH synthetases (RelA<sub>Ec</sub> and SpoT<sub>Ec</sub>), which are homologous enzymes believed to have arisen following a gene duplication event (Fig. 2) [6]. The hydrolysis domain of RelA<sub>Ec</sub> is inactive due to the absence of a conserved HDXED motif in the active site, making it monofunctional [7]. SpoT<sub>Ec</sub> on the other hand, is bifunctional, containing both active synthetase and hydrolase domains. The presence of functional SAS or SAH proteins in Gram-negative bacteria is relatively rare, although there is a conserved SAS, RelV, in the Vibrio genus (Fig. 2) [8]. Gram-positive bacteria in...
the phylum *Firmicutes*, such as *Streptococcus mutans* [9], *Bacillus subtilis* [10, 11] and *Staphylococcus aureus* [12], typically contain one long bifunctional RSH protein, and two SAS proteins, RelP and RelQ, that contain synthetase domains only (Fig. 2). The long-RSH enzymes in the *Firmicutes* have been referred to as both Rel and Rsh in the literature, but we will stick with the Rel nomenclature for the purposes of this review. SAH proteins such as Mesh-1 have been identified in eukaryotes, including humans and fruit flies. The function of these enzymes is ambiguous, given the lack of synthetase enzymes in these organisms [2, 13]. SAH enzymes have also been predicted in many bacterial clades, such as the *Firmicutes*, but whether or not these are functional hydrolases has not been investigated [2]. The majority of bacterial species contain at least one protein from the RSH superfamily, with the exception of those in the PVC (*Planctomycetes*, *Verrucomicrobia* and *Chlamydiae*) superphylum, and those that inhabit stable microenvironments [2]. Whilst an analysis of 928 complete bacterial genome sequences revealed that 92% contain genes encoding for a long RSH, only 44% of those encode for long RSH proteins without additional SAS- or SAH-encoding genes [2]. This highlights the fact that *E. coli*, which contains two long-RSH enzymes and no SAS/SAH proteins, should not be used as the sole model organism for characterizing the stringent response in bacteria.

Upon activation of the stringent response, characteristic changes occur within the cell, with an increase in the (p)ppGpp pool and a concurrent decrease in GTP levels [14]. This leads to a decrease in the overall levels of cellular transcription, specifically of genes involved in the biosynthesis of macromolecules, such as phospholipids, ribosomes and amino acids, until conditions become more favourable [14]. Together, these changes contribute to the slow-growth phenotype associated with the stringent response, which has

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**Fig. 1.** Schematic representation of the RSH superfamily proteins. (a) Long RSH proteins consist of an enzymatic N-terminal domain (NTD) and a regulatory C-terminal domain (CTD). The NTD comprises a hydrolase domain (HD; pink) that can degrade (p)ppGpp into GTP or GDP and PPI, and a synthetase domain (SYNTH; blue) that converts GTP/GDP and ATP into (p)ppGpp. The CTD regulatory region (green) contains a ThrRS, GTPase and SpoT domain (TGS), a conserved alpha helical domain (α), a zinc-finger or conserved cysteine domain (ZFD/CC) and an RNA recognition motif or aspartate kinase, chorismate and TyrA domain (RRM/ACT). (b) Small alarmone synthetase enzymes (SAS) contain a single SYNTH domain and a C-terminal alpha helix (α5), which is required for SAS tetramerization. (c) Small alarmone hydrolase proteins (SAH) contain a single HD domain.
now been linked to many bacterial functions, such as environmental adaptation, persister formation, virulence, motility, cell division, biofilm formation and development (reviewed in [15]). The mechanisms by which (p)ppGpp alters cellular physiology once synthesized have recently been reviewed and will not be covered here [15–17].

Bacteria inhabit a diverse range of niches and it follows that a diverse range of environmental cues should trigger the stringent response. As with most aspects of this signalling pathway, more is known about the conditions that trigger it in Gram-negatives than in Gram-positive species. Indeed, the ‘magic spots’ of (p)ppGpp themselves were discovered when investigating the effects of amino acid starvation on *E. coli* cells [1]. Since then it has become clear that different organisms encode various combinations of RSH superfamily proteins that are also regulated differently. When discussing induction of the stringent response it is important to remember that (p)ppGpp accumulation can occur through different routes upon detection of a stress: increased transcription from the synthetase genes, increased activity of the synthetase domains and/or reduced activity of the hydrolase domains. These regulation points of synthetase activity will often work in unison to ensure rapid adaptation when needed and are the focuses of this review.

**TRANSCRIPTIONAL REGULATION OF THE SYNTHETASE GENES**

**Long-RSH genes**

In *E. coli* the long-RSH gene *relA*<sub>Ec</sub> is under the control of four promoters, two σ<sup>70</sup>-dependent promoters, *relA*<sub>P1</sub> and *relA*<sub>P2</sub>, as well as the more recently discovered σ<sup>54</sup>-dependent P3 and P4 promoters (Fig. 3) [18–20]. Transcription from *relA*<sub>P1</sub> is constitutive throughout growth, and activity depends on an UP element located 40 bp upstream of the start site [19]. *relA*<sub>P2</sub> is located distally to *relA*<sub>P1</sub> and transcription is induced at the transition from exponential to stationary phases [19]. This induction is regulated by CRP, H-NS and RpoS, implicating *RelA*<sub>Ec</sub> in responding to carbon, temperature and osmotic stresses [18, 19]. Transcription from *relA*<sub>P3* and *relA*<sub>P4* is activated by σ<sup>54</sup> under nitrogen-starved conditions [20]. During nitrogen starvation, transcription of *relA*<sub>Ec</sub> is induced in an NtrC-dependent manner, with the sensor kinase NtrB

![Figure 2](image2.png)

*Fig. 2.* Example of the distribution of RSH superfamily proteins in Gram-negative and Gram-positive bacteria. The alignment scores between RSH superfamily proteins from *E. coli*, *V. cholera* and *S. aureus* as determined by CLUSTALW are shown. Gram-negative bacteria can contain one or two long RSH proteins, but frequently do not express SAS proteins, with the exception of the genus *Vibrio* (*RelV*<sub>Vc</sub>). Gram-positive bacteria typically contain a bifunctional long RSH and one or two SAS proteins.

![Figure 3](image3.png)

*Fig. 3.* Regulation of the four known *relA*<sub>Ec</sub> promoters. Transcription from *P1* and *P2* is σ<sup>70</sup>-dependent, with *P1* relying on an UP element lying upstream. Transcription from *P3* and *P4* is activated by σ<sup>54</sup> with the aid of NtrC during nitrogen starvation. Transcription from *P2* is activated through CRP binding to the CRP/CAP site, as well as by H-NS. 6S RNA downregulates transcription from both *P1* and *P2*, while HipB binding to the HipB palindromic sequence inhibits transcription of *relA*<sub>Ec</sub>. The arrows and numbering represent the locations of the transcriptional start sites in relation to the start codon (solid, σ<sup>70</sup>; dotted, σ<sup>54</sup>).
phosphorylating the response regulator NtrC, allowing it to bind enhancer-like elements upstream of the transcription start site and activate transcription from the $\sigma^{54}$-RNAP complex (Fig. 3) [20, 21]. Interestingly, RNAP binds to the promoter element of spoT$_{Ec}$ less efficiently during nitrogen starvation, presumably allowing for quicker accumulation of (p)pGpp without the hydrolase activity of SpoT$_{Ec}$ [20]. NtrC is considered to be the master regulator of the nitrogen starvation response and its coupling with the stringent response highlights the intricacies of bacterial transcriptional regulation.

Additional levels of transcriptional regulation of relA$_{Ec}$ occur through HipB and 6S RNA. Transcription is negatively regulated by HipB, the anti-toxin component of the type II toxin–antitoxin module HipAB that is involved in persistor formation in E. coli [22, 23]. HipB binds to a palindromic sequence upstream of the P3 promoter, binding that is potentiated by HipA (Fig. 3). 6S RNA is a small non-coding RNA that downregulates transcription by $\sigma^{70}$-RNAP through direct binding of the holoenzyme [24]. In cells without 6S RNA, transcription of relA$_{Ec}$ is slightly increased compared to wild-type during the early stationary phase, although this is sufficient to increase cellular ppGpp levels, leading to characteristic stringent response-related changes in the transcriptional profile [25]. Neusser et al. also observed this ppGpp accumulation in strains lacking 6S RNA, but both in the presence and absence of RelA$_{Ec}$, suggesting SpoT$_{Ec}$ involvement [26].

Very little is known about the transcriptional regulation of the long-RSH genes outside of E. coli. The antibiotic muripiricin, which inhibits the isoleucyl tRNA synthetase and mimics amino acid stress, induces relS$_{Sa}$ transcription in S. aureus (Fig. 4a) [27, 28]. However, no effect was noted on the homologous transcript from S. mutans when grown in chemically defined media depleted of amino acids [29]. In Mycobacterium tuberculosis, relM$_{Bs}$ is part of the $\sigma^{E}$ regulon, which is indirectly activated by polyphosphate chains. Polyphosphate can act as a phosphate donor for the sensor histidine kinase MprB, which in turn phosphorylates MprA. MprA–P can then activate transcription of sigE, which has a positive effect of the transcription of relM$_{Bs}$ [30].

**SAS genes**

Since the discovery of SAS enzymes over a decade ago [9–11], researchers have been interested in elucidating the regulatory mechanisms and environmental cues to which these proteins respond. Under unstressed conditions the SAS genes from B. subtilis are differentially expressed during growth phases [10]. relP$_{Bs}$ is mainly transcribed during exponential growth, with transcript levels dropping off as the cells enter the stationary phase. This coincides with a massive induction of relP$_{Bs}$ transcription in the late exponential phase that completely disappears in the stationary phase. This differential expression ties in with observations that these proteins may have biologically distinct functions that require temporal regulation. For instance, the overexpression of RelP$_{Bo}$ but not RelQ$_{Bo}$ has been shown to result in increased 100S ribosome formation in B. subtilis [31].

relP$_{Bo}$ is part of the sigma factor $\sigma^M$- and $\sigma^W$-induced regulons [32, 33]. Both of these $\sigma$ factors are involved in responses to a number of different cell wall stresses, such as LL-37, vancomycin and alkaline shock, suggesting a role for SAS proteins in responding to cell wall stress (Fig. 4a) [34–36]. The homologous $\sigma$ factor in S. aureus is $\sigma^E$ [37], but analysis of the relP$_{Sa}$ and relQ$_{Sa}$ promoters indicates they are regulated by the housekeeping $\sigma$ factor A [12]. However, transcription of relP$_{Sa}$ and relQ$_{Sa}$ is induced upon cell wall stress caused by vancomycin, indicating that homologous SAS enzymes do have similar functions [12].

Additional stresses, such as exposure to ethanol or alkaline conditions, have been shown to affect the transcription of SAS genes. During ethanol-induced stress the transcription of relP$_{Sa}$ increases >20-fold. This overexpression leads to slower cell growth and allows cells to survive ethanol stress [38]. In the Firmicutes, alkaline shock also causes an accumulation of (p)ppGpp [10, 39, 40]. Whilst the mechanism behind this in S. aureus and Enterococcus faecalis is unclear, in B. subtilis it seems to be RelP$_{Bo}$-mediated [10]. The differences in synthetase gene transcription between different species highlighted here again hint at a currently overlooked functional nuance to RSH superfamily members.

**LIGAND-MEDIATED REGULATION OF ENZYME ACTIVITY**

**Substrate stimulation**

Once produced, RSH superfamily enzymes use both GTP and GDP as substrates, but different enzymes display a preference for either substrate, resulting in differential production of pppGpp and ppGpp. RelA$_{Ec}$ favours GDP in vitro, while SpoT$_{Ec}$, Rel$_{Mtb}$ and Rel$_{Seq}$ prefer GTP [41–43]. These differences in specificity are due to a charge reversal in a conserved motif present in the substrate-binding pocket, with EXDD and RXKD motifs conferring a preference for GDP and GTP, respectively [41, 43]. There is evidence to suggest that pppGpp and ppGpp may have differing potencies as signalling nucleotides, with ppGpp acting as a stronger regulator of growth rate, RNA/DNA ratios and transcription in E. coli [44], whereas experiments performed with the DNA primase from B. subtilis suggest that pppGpp is the more potent inhibitor of this enzyme [45]. These substrate preferences may explain the different ppGpp/pppGpp ratios seen across bacteria. It appears that in response to amino acid deprivation ppGpp is predominantly produced by the Gram-negative E. coli [46], whereas Gram-positive organisms favour pppGpp production [47–49]. However, the presence of a pppGpp pyrophosphatase termed GppA in E. coli that is capable of degrading pppGpp to ppGpp blurs the relationship between intracellular alarmone ratio and synthetase preference [50]. It follows that nucleotide production and enzyme specificity may provide an interesting intricacy to the stringent response and its regulation.
Product-induced activation

Positive regulation of an enzyme by its product is rare, but allows rapid amplification of a signal that is much quicker than a transcription-dependent feedback loop. In *E. coli*, RelA<sub>Ec</sub>, in complex with 70S ribosomes, was demonstrated to be positively regulated by ppGpp at physiologically relevant levels (Fig. 4b) [53]. The mechanism of regulation has not yet been determined, but it is likely that ppGpp binds allosterically to RelA<sub>Ec</sub> to increase activity. Presumably, the hydrolase activity of SpoT<sub>Ec</sub> maintains ppGpp levels below a threshold level required for signal amplification during non-stringent conditions. Once amino acids become plentiful, the reduction in deacetylated tRNA levels reduces ppGpp accumulation and thus the stringent response.

Other members of the RSH superfamily are also regulated by the stringent alarmones. The *B. subtilis* SAS RelQ<sub>Bs</sub> is positively regulated by pppGpp but not ppGpp (Fig. 4b) [54]. Crystallization studies in the presence of ATP and GTP revealed that RelQ<sub>Bs</sub> forms a tetramer, with two molecules of pppGpp bound to allosteric binding sites created by the association of the four monomers. This causes a 10-fold increase in the synthesis of both ppGpp and pppGpp *in vitro*. An altered allosteric binding site is also present in

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**Fig. 4.** Summary of the types of regulation involved in RSH superfamily protein activity. (a) Transcriptional regulation: relA<sub>Ec</sub> is upregulated by NtrC, CRP and HNS, and inhibited by 6S RNA, RpoS and HipB. The transcription of rel, relP or relQ is induced by various conditions including amino acid starvation, cell wall stress, ethanol and alkaline shock. (b) Ligand-mediated regulation: (p)pGpp increases the synthetase activity of RelA<sub>Ec</sub>, while RelQ is regulated by two ligands: (p)ppGpp, which augments synthetase activity, and ssRNA, which inhibits synthetase activity. ppG2′:3′p binds to Rel from *S. equisimilis*, causing a conformational change that favours (p)ppGpp hydrolysis. (c) Heterologous protein interactions: ACP and ObgE both bind to SpoT<sub>Ec</sub> to increase or reduce (p)ppGpp synthesis, respectively. RelA<sub>Ec</sub> binding to a stalled ribosome increases (p)ppGpp production, while ComGA can bind to Rel<sub>Bs</sub>, although the effect on SYNTH or HD activity is unclear.
although this negatively charged site would not promote the binding of pppGpp and may be regulated by an alternative effector. Unlike RelQ_{Es}, the homologous SAS enzyme from *E. faecalis*, RelQ_{Ef} is positively activated by pppGpp. However, it is not affected by the recently discovered pGpp, which has been shown to positively affect the activity of RelA_{Ec} [51].

**Induction by a heterologous nucleotide**

Unusually, RelQ_{Ef} is also regulated by another ligand, single-stranded RNA (ssRNA: Fig. 4b) [55]. When ssRNA, such as mRNA, binds to the tetramer RelQ_{Ef}, it severely inhibits (p)ppGpp synthesis, an effect that is mitigated in the presence of (p)ppGpp. This phenomenon appears to be specific for SAS enzymes, as no inhibition was observed for the activity of RelA_{Ec} [55]. A provisional consensus binding sequence for RelQ_{Ef} was determined as GGAGG, with consecutive GG motifs being deemed important. The similarity to the core Shine–Dalgarno sequence is striking [56], but it is as yet unclear whether RelQ binds to the ribosome-binding site of mRNA and what biological function this may have.

The (p)ppGpp signalling pathway is also involved in cross-talk with other secondary messenger signalling molecules. For instance, high levels of the cyclic dinucleotide c-di-AMP have been shown to amplify the production of (p)ppGpp in *S. aureus* following mucopein treatment [48]. This effect is RelC-dependent, but c-di-AMP does not bind directly to RelC, nor is there an increase in relC transcription when c-di-AMP levels are high, indicating some unknown mechanism of regulation. The cross-talk between these two nucleotide signalling systems is also bi-directional, with pppGpp inhibiting the hydrolysis of c-di-AMP by the phosphodiesterase enzyme GdpP, leading to an increase in c-di-AMP concentration [57]. Indeed, studies with *Listeria monocytogenes* have revealed that deletion of the c-di-AMP cyclase enzymes was only possible in strains lacking (p)ppGpp [58], suggesting that both systems are linked in responding to stress signals.

Additional cross-talk occurs between the unusual nucleotide GDP-2′,3′′-cyclic monophosphate (ppG2′,3′′p) and (p)ppGpp (Fig. 4b). In *Streptococcus equisimilis*, the crystal structure of the N-terminal catalytic fragment of the long-RSH, Rel_{seq}, was solved, revealing two differing enzyme conformations with opposite activities [59]. In the hydrolyase-ON/synthetase-OFF form, ppG2′,3′p was found bound to the hydrolyase domain, locking the conformation of the enzyme. However, it is not currently known whether ppG2′,3′p is synthesized in *vivo*, casting doubt on whether this is a physiologically relevant interaction.

**PROTEIN–PROTEIN INTERACTION AS A MECHANISM FOR REGULATION**

**Intramolecular regulation**

In bifunctional long-RSH enzymes (e.g. SpoT_{Ec}) there must be careful regulation of competing (p)ppGpp synthesis and hydrolysis domains to avoid a futile production cycle. One way this is achieved is through the self-regulation of enzyme activity by the CTD. This was nicely demonstrated using Rel_{seq} where the synthetase activity of a truncated Rel_{seq} protein lacking the CTD was found to be 12-fold higher than that of the full-length protein, while conversely the hydrolase activity was 150-fold lower [42]. This intrinsic regulation makes the regulation of Rel_{seq} more switch-like, allowing sharp (p)ppGpp accumulation when required.

**The impact of oligomerization on (p)ppGpp production**

Oligomerization of long-RSH enzymes is believed to have a regulatory effect on synthetase activity. In *E. coli*, RelA_{Ec} forms a dimer through the interactions of amino acids 455–538 and 550–682 in monomer CTDs [60, 61]. The usual increase in (p)ppGpp levels upon amino acid starvation is reduced when the CTD is overexpressed in relA^{+} strains, while the disruption of oligomerization had a positive effect on (p)ppGpp synthesis, implicating oligomerization as an important regulatory control point [61]. In *M. tuberculosis*, the full-length Rel_{Mtb} forms trimers. An N-terminal fragment, Rel_{Mtb-1-394}, forms both monomers and trimers, and isolation of each fraction revealed that the trimer form is less catalytically active and dissociates when incubated with substrate (GTP and ATP) or product (pppGpp) [62]. Taken together, these data suggest that oligomerization is involved in regulating long-RSH enzyme activity, where the higher ordered state is less active or indeed inactive.

It is becoming clear that the role oligomerization plays in the regulation of RSH family enzymes is important, and this is not solely confined to long-RSH proteins. Indeed, as mentioned above, the positive and negative regulation of RelQ enzymes by (p)ppGpp and RNA, respectively, is dependent on tetramerization [54, 55]. The allosteric (p)ppGpp-binding sites of RelQ_{Bs} are only present in the tetramer, and when oligomerization is disrupted the enzymatic activity of RelQ_{Ef} is lost [55]. The tetramerization of RelQ_{Bs} also leads to high positive cooperativity of (p)ppGpp synthesis [54].

An additional SAS in *M. smegmatis*, termed MS_RH III-RSD, has been shown to contain both a (p)ppGpp synthesis domain and an RNase HII domain involved in the resolving of RNA–DNA hybrid structures known as R-loops [63]. This enzyme is the only example to date of a (p)ppGpp synthetase domain being fused to a functionally distinct enzyme. Alone, each of the domains are inactive and a hexamer of full-length proteins is required for the activity of either [63, 64]. This coupling hints at a link between R-loop removal and the stringent response. The joining of these domains would allow for the production of (p)ppGpp near an RNA polymerase stalled at an R-loop, where (p)ppGpp may then help to destabilize the stalled polymerase [64].

**Heterologous interaction partners**

Since the 1970s it has been understood that RelA-mediated synthesis of (p)ppGpp is activated by the presence of an uncharged tRNA in the acceptor site of the ribosome [65].
The synthetase activity of RelMtb was shown to be activated by a complex of uncharged tRNA, ribosomes and mRNA, now termed the ribosome-activating complex (RAC; Fig. 4c) [66]. The RAC simultaneously decreases the activity of the RelMtb hydrolase domain, resulting in a switch-like mechanism of regulation. Recent work has provided a detailed insight into the interaction of RelA with the ribosome [3–5]. Cryo-electron microscopy images of RelAEc bound to a stalled ribosome show that the CTD wraps around the uncharged tRNA in the 30S A site [3–5]. The 3’-OH of the uncharged amino acid acceptor stem lies against the β5 strand of the TGS/RRM domain. This prevents RelAEc from interacting with charged tRNAs by steric exclusion. The hydrolase and synthetase domains of RelAEc have very few contacts with the ribosome, suggesting that RelA activation is not direct but could be through release of the auto-inhibitory effect of the CTD [3–5]. Another possible explanation is that binding to the ribosome prevents the auto-inhibitory effect of RelAEc homodimers [60, 61, 67].

In addition to the ribosome, a number of protein-binding partners for the synthetases have now been identified. The Obg family GTPase ObgE (CgtA, YhbZ) binds to SpoTEc (Fig. 4c) [68]. Deleting ObgE results in increased (p)ppGpp levels during the exponential phase, suggesting that ObgE ensures a low basal (p)ppGpp level during bacterial growth [69]. Whilst an ObgE deletion mutant has no effect on (p)ppGpp levels during amino acid starvation [69], it does result in a higher ratio of pppGpp to ppGpp [70]. Interestingly, the GTPase activity of ObgE is inhibited by ppGpp at physiological levels, but the biological function of this is unclear [70].

During fatty acid limitation, E. coli accumulates (p)ppGpp in a SpoTEc-dependent manner [71, 72]. SpoTEc interacts directly with a central cofactor of fatty acid synthesis, the acyl carrier protein (ACP; Fig. 4c) [73, 74]. This interaction is between the TGS/RRM domain of SpoTEc and the holo form of ACP, and is required for (p)ppGpp accumulation during fatty acid starvation [75]. Later work by the authors suggested that this SpoTEc-ACP interaction is specific for the SpoTEc long-RSH, and is only found in bacteria with two long-RSH proteins (RelA and SpoT). Organisms with only one long-RSH, such as B. subtilis, have no ACP/synthetase interaction, despite the presence of a TGS/RRM domain [76]. This could be due to the basic pI of SpoTEc compared to other long-RSH proteins, which allows binding to the acidic ACP. Whilst no mechanism of activation has been elucidated, the long-RSH-dependent stringent response is still important for fatty acid limitation survival in B. subtilis, although it may be dependent on (p)ppGpp regulation of intracellular GTP/ATP levels, as no (p)ppGpp accumulation was observed [77].

Whilst the long-RSH protein from B. subtilis does not bind ACP, it has been shown to interact with ComGA, a protein conserved in naturally competent bacteria (Fig. 4c) [78]. ComGA is involved in achieving a growth-arrested state known as the K state, partly by causing a decrease in transcription of the RNA gene rrnB. In a mutant that cannot produce (p)ppGpp, overproduction of ComGA does not lead to the usual decrease in rrnB transcription, showing that this aspect of the K state is (p)ppGpp-dependent.

**CONCLUSION**

In conclusion, as we piece together a picture of the stringent response in Gram-positive bacteria, it is becoming clear that there are major differences compared to this signalling pathway in Gram-negative organisms. The types of synthetase enzymes present are different, as is the way in which these enzymes are transcriptionally and post-transcriptionally regulated. Understanding the environmental signals that trigger the stringent response will allow us to comprehend how it is utilized by bacteria in order to survive. As the stringent response is important for the pathogenicity of bacteria [79, 80], understanding the regulation of (p)ppGpp synthetases, and other factors, could provide information on useful therapeutic targets.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

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