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Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria

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Abbreviations: HD, hydrolase domain; PPi, pyrophosphate; SYNTH, synthetase domain; RSH, RelA/SpoT homologue; SAS, small alarmone synthetase; SAH, small alarmone hydrolase; NTD, N-terminal domain; CTD, C-terminal domain; TGS, ThrRS, GTPase and SpoT; CC, conserved cysteine; ZFD, zinc finger domain; ACT, aspartate kinase, chorismate and TyrA; RRM, RNA recognition motif; PVC, *Planctomycetes*, *Verrucomicrobia* and *Chlamydiae*; *Ec*, *Escherichia coli*; *Mtb*, *Mycobacterium tuberculosis*; *Seq*, *Streptococcus equisimilis*; *Ef*, *Enterococcus faecalis*; *Cg*, *Cornybacterium glutamicum*; *Sa*, *Staphylococcus aureus; Bs*, *Bacillus subtilis*; *Vc*, *Vibrio cholerae*; RAC, ribosome activating complex; ACP, acyl carrier protein; CRP, cAMP receptor protein; H-NS, histone-like nucleoid-structuring protein; RNAP, RNA polymerase.

32 **Abstract**

33 The stringent response is a conserved bacterial stress response mechanism that allows bacteria
34 to respond to nutritional challenges. It is mediated by the alarmones pppGpp and ppGpp,
35 nucleotides that are synthesised and hydrolysed by members of the RSH superfamily. Whilst
36 there are key differences in the binding targets for (p)ppGpp between Gram-negative and
37 Gram-positive bacterial species, the transient accumulation of (p)ppGpp caused by nutritional
38 stresses results in a global change in gene expression in all species. The RSH superfamily of
39 enzymes is ubiquitous throughout the bacterial kingdom, and can be split into three main
40 groups: the long-RSH enzymes; the small alarmone synthetases (SAS); and the small alarmone
41 hydrolases (SAH). Despite the prevalence of these enzymes, there are however, important
42 differences in the way in which they are regulated on a transcriptional and post-translational
43 level. Here we provide an overview of the diverse regulatory mechanisms that are involved in
44 governing this crucial signalling network. Understanding how the RSH superfamily members
45 are regulated gives insights to the varied important biological roles for this signalling pathway
46 across the bacteria.

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, utilised 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 and pyrophosphate (PPi) by hydrolase domain (HD)-containing enzymes (PF13328). 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) comprised of 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$_{Ec}$) 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$_{Ec}$ and SpoT$_{Ec}$), which are homologous enzymes believed to have arisen following a gene duplication event (Fig. 2) [6]. The hydrolysis domain of RelA$_{Ec}$ is inactive due to the absence of a conserved HDXXED motif in the active site, making it monofunctional [7]. SpoT$_{Ec}$, 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 Firmicutes phylum, 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 that *E. coli*, which contains two long-RSH enzymes and no SAS/SAH proteins, should not be used as the sole model organism for characterising 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 now been linked to many bacterial functions such as environmental adaptation, persister formation, virulence, motility, cell division, biofilm formation and development (reviewed by [15]). The mechanisms by which (p)ppGpp alter cellular physiology once synthesised has 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 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

115 **Long-RSH genes**

116 In *E. coli* the long-RSH gene *relA*$_{Ec}$ is under the control of four promoters, two σ^{70}-dependent
117 promoters, *relA*P1 and *relA*P2, as well as the more recently discovered σ^{54}-dependent P3 and
118 P4 promoters (Fig. 3) [18-20]. Transcription from *relA*P1 is constitutive throughout growth,
119 and activity depends on an UP-element located 40 bp upstream of the start site [19]. *relA*P2 is
120 located distally to *relA*P1 and transcription is induced at the transition from exponential to
121 stationary phases [19]. This induction is regulated by CRP, H-NS and RpoS, implicating
122 RelA$_{Ec}$ in responding to carbon, temperature and osmotic stresses [18, 19]. Transcription from
123 *relA*P3 and *relA*P4 is activated by σ^{54} under nitrogen-starved conditions [20]. During nitrogen
124 starvation, transcription of *relA*$_{Ec}$ is induced in an NtrC-dependent manner with the sensor
125 kinase NtrB phosphorylating the response regulator NtrC, allowing it to bind enhancer-like
126 elements upstream of the transcription start site and activate transcription from the σ^{54}-RNAP
127 complex (Fig. 3) [20, 21]. Interestingly, RNAP binds to the promoter element of *spoT*$_{Ec}$ less
128 efficiently during nitrogen starvation, presumably allowing for quicker accumulation of
129 (p)ppGpp without the hydrolase activity of SpoT$_{Ec}$ [20]. NtrC is considered to be the master
130 regulator of the nitrogen starvation response and its coupling with the stringent response
131 highlights the intricacies of bacterial transcriptional regulation.

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

143 Very little is known about the transcriptional regulation of the long-RSH genes outside
144 of *E. coli*. The antibiotic mupirocin, which inhibits the isoleucyl t-RNA synthetase and mimics
145 amino acid stress, induces *rel*$_{Sa}$ transcription in *S. aureus* (Fig. 4a) [27, 28]. However no effect
146 was noted on the homologous transcript from *S. mutans* when grown in chemically-defined
147 media depleted of amino acids [29]. In *Mycobacterium tuberculosis*, *rel*$_{Mtb}$ is part of the σ^{E}
148 regulon, which is indirectly activated by polyphosphate chains. Polyphosphate can act as a

149 phosphate donor for the sensor histidine kinase MprB, which in turn phosphorylates MrpA.
150 MrpA~P can then activate transcription of *sigE,* which has a positive effect of the transcription
151 of rel_{Mtb} [30].
152

153 **SAS genes**

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

164 $relP_{Bs}$ is part of the sigma factors σ^{M} and σ^{W}-induced regulons [32, 33]. Both of these
165 σ factors are involved in response to a number of different cell wall stresses such as LL-37,
166 vancomycin and alkaline shock, suggesting a role for SAS proteins in responding to cell wall
167 stress (Fig. 4a) [34-36]. The homologous σ factor in *S. aureus* is σ^{S} [37], but analysis of the
168 $relP_{Sa}$ and $relQ_{Sa}$ promoters indicates they are regulated by the housekeeping σ factor A [12].
169 However, transcription of $relP_{Sa}$ and $relQ_{Sa}$ is induced upon cell wall stress caused by
170 vancomycin, indicating that homologous SAS enzymes do have similar functions [12].

171 Additional stresses such as exposure to ethanol or alkaline conditions have been shown
172 to affect the transcription of SAS genes. During ethanol-induced stress the transcription of
173 $relP_{Sa}$ increases >20 fold. This over-expression leads to slower cell growth and allows cells to
174 survive ethanol stress [38]. In the Firmicutes, alkaline shock also causes an accumulation of
175 (p)ppGpp [10, 39, 40]. Whilst the mechanism behind this in *S. aureus* and *Enterococcus*
176 *faecalis* is unclear, in *B. subtilis* it seems to be $RelP_{Bs}$-mediated [10]. The differences in
177 synthetase gene transcription between different species highlighted here, again hint at a
178 currently overlooked functional nuance to RSH superfamily members.
179

180 **LIGAND-MEDIATED REGULATION OF ENZYME ACTIVITY**

181 **Substrate stimulation**

182 Once produced, RSH superfamily enzymes use both GTP and GDP as substrates, however
183 different enzymes display a preference for either substrate, resulting in differential production
184 of pppGpp and ppGpp. $RelA_{Ec}$ favours GDP *in vitro*, while $SpoT_{Ec}$, Rel_{Mtb} and Rel_{Seq} prefer
185 GTP [41-43]. These differences in specificity are due to a charge reversal in a conserved motif
186 present in the substrate binding pocket, with EXDD and RXKD motifs conferring a preference
187 for GDP and GTP respectively [41, 43]. There is evidence to suggest that pppGpp and ppGpp
188 may have differing potencies as signalling nucleotides, with ppGpp acting as a stronger
189 regulator of growth rate, RNA/DNA ratios, and transcription in *E. coli* [44], whereas
190 experiments performed with the DNA primase from *B. subtilis* suggest that pppGpp is the more
191 potent inhibitor of this enzyme [45]. These substrate preferences may explain the different
192 ppGpp/pppGpp ratios seen across bacteria. It appears that in response to amino acid deprivation
193 ppGpp is predominantly produced by the Gram-negative *E. coli* [46], whereas Gram-positive
194 organisms favour pppGpp production [47-49]. However, the presence of a pppGpp
195 pyrophosphatase termed GppA in *E. coli* that is capable of degrading pppGpp to ppGpp, blurs
196 the relationship between intracellular alarmone ratio and synthetase preference [50]. It follows
197 that nucleotide production and enzyme specificity may provide an interesting intricacy to the
198 stringent response and its regulation [44, 45]. This is further complicated by the recent
199 identification of an additional signalling molecule - pGpp. $RelA_{Ec}$ was initially shown to be
200 able to synthesise this alarmone through the hydrolysis of the β phosphate of ppGpp, albeit in
201 small quantities [41]. Subsequent reports have since demonstrated the ability of the SAS
202 proteins $RelQ_{Ef}$ from *E. faecalis* and $RelS_{Cg}$ from *Corynebacterium glutamicum* to efficiently
203 utilise GMP as a substrate to produce pGpp *in vitro,* although the presence of this small
204 alarmone has yet to be conclusively demonstrated *in vivo* [51, 52].
205

206 **Product-induced activation**

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

Other members of the RSH superfamily are also regulated by the stringent alarmones. The *B. subtilis* SAS RelQ$_{Bs}$ is positively regulated by pppGpp but not ppGpp (Fig. 4b) [54]. Crystallisation studies in the presence of ATP and GTP revealed that RelQ$_{Bs}$ 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 synthesis of both ppGpp and pppGpp *in vitro*. An altered allosteric binding site is also present in RelP$_{Bs}$, however this negatively charged site would not promote the binding of pppGpp and may be regulated by an alternative effector. Unlike RelQ$_{Bs}$, the homologous SAS enzyme from *E. faecalis*, RelQ$_{Ef}$, is positively activated by ppGpp. 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 tetrameric 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 on the activity of RelA$_{Ec}$ [55]. A provisional consensus binding sequence for RelQ$_{Ef}$ was determined as GGAGG, with consecutive GG motifs deemed important. The similarity to the core Shine-Dalgarno sequence is striking [56], however 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 mupirocin treatment [48]. This effect is RSH-dependent, but c-di-AMP does not directly bind to Rel$_{Sa}$, nor is there an increase in *rel*$_{Sa}$ 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 ppGpp 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 hydrolase-ON/synthetase-OFF form, ppG2′:3′p was found bound to the hydrolase domain, locking the conformation of the enzyme. However it is not currently known whether ppG2′:3′p is synthesised *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 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 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 oligomerisation on (p)ppGpp production

Oligomerisation of long-RSH enzymes is believed to have a regulatory effect on synthetase activity. In *E. coli*, $RelA_{Ec}$ forms a dimer through 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 oligomerisation had a positive effect on (p)ppGpp synthesis, implicating oligomerisation as an important regulatory control point [61]. In *M. tuberculosis*, the full-length Rel_{Mtb} forms trimers. An N-terminal fragment, $Rel_{Mtb1\text{-}394}$, 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 oligomerisation 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 oligomerisation plays in 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 tetramerisation [54, 55]. The allosteric pppGpp binding sites of $RelQ_{Bs}$ are only present in the tetramer, and when oligomerisation is disrupted the enzymatic activity of $RelQ_{Ef}$ is lost [55]. Tetramerisation of $RelQ_{Bs}$ also leads to high positive cooperativity of (p)ppGpp synthesis [54].

284 An additional SAS in *M. smegmatis*, termed MS_RHII-RSD, has been shown to contain
285 both a (p)ppGpp synthesis domain and a RNase HII domain involved in the resolving of RNA-
286 DNA hybrid structures known as R-loops [63]. This enzyme is the only example to date of a
287 (p)ppGpp synthetase domain fused to a functionally distinct enzyme. Alone each of the
288 domains are inactive and a hexamer of full-length proteins is required for activity of either [63,
289 64]. This coupling hints at a link between R-loop removal and the stringent response. The
290 joining of these domains would allow for the production of (p)ppGpp near an RNA polymerase
291 stalled at an R-loop, where (p)ppGpp may then help to destabilise the stalled polymerase [64].
292
293 **Heterologous interaction partners**
294 Since the 1970s it has been understood that RelA-mediated synthesis of (p)ppGpp is activated
295 by the presence of an uncharged tRNA in the acceptor site of the ribosome [65]. The synthetase
296 activity of Rel_{Mtb} was shown to be activated by a complex of uncharged tRNA, ribosomes and
297 mRNA, now termed the ribosome activating complex (RAC: Fig. 4c) [66]. The RAC
298 simultaneously decreases the activity of the Rel_{Mtb} hydrolase domain, resulting in a switch-like
299 mechanism of regulation. Recent work has provided a detailed insight into the interaction of
300 RelA with the ribosome [3-5]. Cryo-electron microscopy images of $RelA_{Ec}$ bound to a stalled
301 ribosome show that the CTD wraps around the uncharged tRNA in the 30S A site [3-5]. The
302 3’ -OH of the uncharged amino acid acceptor stem lies against the β5 strand of the TGS/RRM
303 domain. This prevents $RelA_{Ec}$ interacting with charged tRNAs by steric exclusion. The
304 hydrolase and synthetase domains of $RelA_{Ec}$ have very few contacts with the ribosome,
305 suggesting that RelA activation is not direct but could be through release of the auto-inhibitory
306 effect of the CTD [3-5]. Another possible explanation is that binding to the ribosome prevents
307 the auto-inhibitory effect of $RelA_{Ec}$ homodimers [60, 61, 67].
308 In addition to the ribosome, a number of protein binding partners for the synthetases
309 have now been identified. The Obg family GTPase ObgE (CgtA, YhbZ) binds to $SpoT_{Ec}$ (Fig.
310 4c) [68]. Deleting ObgE results in increased (p)ppGpp levels during exponential phase
311 suggesting that ObgE ensures a low basal (p)ppGpp level during bacterial growth [69]. Whilst
312 an ObgE deletion mutant has no effect on (p)ppGpp levels during amino acid starvation [69],
313 it does result in a higher ratio of pppGpp to ppGpp [70]. Interestingly the GTPase activity of
314 ObgE is inhibited by ppGpp at physiological levels but the biological function of this is unclear
315 [70].
316 During fatty acid limitation, *E. coli* accumulates (p)ppGpp in a $SpoT_{Ec}$-dependent
317 manner [71, 72]. $SpoT_{Ec}$ directly interacts with a central cofactor of fatty acid synthesis, the

318 acyl carrier protein (ACP: Fig. 4c) [73, 74]. This interaction is between the TGS/RRM domain
319 of $SpoT_{Ec}$ and the holo form of ACP, and is required for (p)ppGpp accumulation during fatty
320 acid starvation [75]. Later work by the authors suggests that this $SpoT_{Ec}$-ACP interaction is
321 specific for the $SpoT_{Ec}$ long-RSH, and is only found in bacteria with two long-RSH proteins
322 (RelA and SpoT). Organisms with only one long-RSH, such as *B. subtilis*, have no ACP-
323 synthetase interaction, despite the presence of a TGS/RRM domain [76]. This could be due to
324 the basic pI of $SpoT_{Ec}$ compared to other long-RSH proteins, which allows binding to the acidic
325 ACP. Whilst no mechanism of activation has been elucidated, the long-RSH-dependent
326 stringent response is still important for fatty acid limitation survival in *B. subtilis*, however it
327 may be dependent on (p)ppGpp regulation of intracellular GTP/ATP levels, as no (p)ppGpp
328 accumulation was observed [77].
329 Whilst the long-RSH protein from *B. subtilis* does not bind ACP, it has been shown to
330 interact with ComGA, a protein conserved in naturally competent bacteria (Fig. 4c) [78].
331 ComGA is involved in achieving a growth-arrested state known as the K state, partly by
332 causing a decrease in transcription of the rRNA gene *rrnB*. In a mutant that cannot produce
333 (p)ppGpp, overproduction of ComGA does not lead to the usual decrease in *rrnB* transcription,
334 showing that this aspect of the K state is (p)ppGpp-dependent.
335
336 **CONCLUSION**
337 In conclusion, as we piece together a picture of the stringent response in Gram-positive
338 bacteria, it becomes clear that there are major differences compared to this signalling pathway
339 in Gram-negative organisms. The types of synthetase enzymes present are different, as is the
340 way in which these enzymes are transcriptionally and post-transcriptionally regulated.
341 Understanding the environmental signals that trigger the stringent response will allow us to
342 comprehend how it is utilised by bacteria in order to survive. As the stringent response is
343 important for the pathogenicity of bacteria [79, 80], understanding the regulation of (p)ppGpp
344 synthetases, and other factors, could provide information on useful therapeutic targets.
345
346

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351

352 Conflicts of interest

353 The authors declare no conflict of interest

354

355 **REFERENCES**

356 1. **Cashel M**. The control of ribonucleic acid synthesis in *Escherichia coli*. IV. Relevance
357 of unusual phosphorylated compounds from amino acid-starved stringent strains. *J Biol Chem*
358 1969;244(12):3133-3141.
359 2. **Atkinson GC, Tenson T, Hauryliuk V**. The RelA/SpoT homolog (RSH) superfamily:
360 distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of
361 life. *PLoS One* 2011;6(8):e23479.
362 3. **Brown A, Fernandez IS, Gordiyenko Y, Ramakrishnan V**. Ribosome-dependent
363 activation of stringent control. *Nature* 2016;534(7606):277-280.
364 4. **Loveland AB, Bah E, Madireddy R, Zhang Y, Brilot AF et al.** Ribosome*RelA
365 structures reveal the mechanism of stringent response activation. *Elife* 2016;5.
366 5. **Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL et al.** The stringent
367 factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis.
368 *Nucleic Acids Res* 2016;44(13):6471-6481.
369 6. **Mittenhuber G**. Comparative genomics and evolution of genes encoding bacterial
370 (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J Mol Microbiol*
371 *Biotechnol* 2001;3(4):585-600.
372 7. **Aravind L, Koonin EV**. The HD domain defines a new superfamily of metal-
373 dependent phosphohydrolases. *Trends Biochem Sci* 1998;23(12):469-472.
374 8. **Das B, Pal RR, Bag S, Bhadra RK**. Stringent response in *Vibrio cholerae*: genetic
375 analysis of *spoT* gene function and identification of a novel (p)ppGpp synthetase gene. *Mol*
376 *Microbiol* 2009;72(2):380-398.
377 9. **Lemos JA, Lin VK, Nascimento MM, Abranches J, Burne RA**. Three gene products
378 govern (p)ppGpp production by *Streptococcus mutans*. *Mol Microbiol* 2007;65(6):1568-1581.
379 10. **Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T et al.** Identification and
380 functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. *Mol Microbiol*
381 2008;67(2):291-304.
382 11. **Srivatsan A, Han Y, Peng J, Tehranchi AK, Gibbs R et al.** High-precision, whole-
383 genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genet*
384 2008;4(8):e1000139.
385 12. **Geiger T, Kastle B, Gratani FL, Goerke C, Wolz C**. Two small (p)ppGpp synthases
386 in *Staphylococcus aureus* mediate tolerance against cell envelope stress conditions. *J Bacteriol*
387 2014;196(4):894-902.
388 13. **Sun D, Lee G, Lee JH, Kim HY, Rhee HW et al.** A metazoan ortholog of SpoT
389 hydrolyzes ppGpp and functions in starvation responses. *Nat Struct Mol Biol*
390 2010;17(10):1188-1194.
391 14. **Potrykus K, Cashel M**. (p)ppGpp: still magical? *Annu Rev Microbiol* 2008;62:35-51.
392 15. **Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K**. Recent functional
393 insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol* 2015;13(5):298-
394 309.
395 16. **Liu K, Bittner AN, Wang JD**. Diversity in (p)ppGpp metabolism and effectors. *Curr*
396 *Opin Microbiol* 2015;24:72-79.
397 17. **Steinchen W, Bange G**. The magic dance of the alarmones (p)ppGpp. *Mol Microbiol*
398 2016;101(4):531-544.
399 18. **Metzger S, Dror IB, Aizenman E, Schreiber G, Toone M et al.** The nucleotide
400 sequence and characterization of the *relA* gene of *Escherichia coli*. *J Biol Chem*
401 1988;263(30):15699-15704.
402 19. **Nakagawa A, Oshima T, Mori H**. Identification and characterization of a second,
403 inducible promoter of *relA* in *Escherichia coli*. *Genes Genet Syst* 2006;81(5):299-310.

404 20. **Brown DR, Barton G, Pan Z, Buck M, Wigneshweraraj S**. Nitrogen stress response
405 and stringent response are coupled in *Escherichia coli*. *Nat Commun* 2014;5:4115.
406 21. **Villadsen IS, Michelsen O**. Regulation of PRPP and nucleoside tri and tetraphosphate
407 pools in *Escherichia coli* under conditions of nitrogen starvation. *J Bacteriol* 1977;130(1):136-
408 143.
409 22. **Lin CY, Awano N, Masuda H, Park JH, Inouye M**. Transcriptional repressor HipB
410 regulates the multiple promoters in *Escherichia coli*. *J Mol Microbiol Biotechnol*
411 2013;23(6):440-447.
412 23. **Maisonneuve E, Castro-Camargo M, Gerdes K**. (p)ppGpp controls bacterial
413 persistence by stochastic induction of toxin-antitoxin activity. *Cell* 2013;154(5):1140-1150.
414 24. **Wassarman KM, Storz G**. 6S RNA regulates *E. coli* RNA polymerase activity. *Cell*
415 2000;101(6):613-623.
416 25. **Cavanagh AT, Chandrangsu P, Wassarman KM**. 6S RNA regulation of *relA* alters
417 ppGpp levels in early stationary phase. *Microbiology* 2010;156(Pt 12):3791-3800.
418 26. **Neusser T, Polen T, Geissen R, Wagner R**. Depletion of the non-coding regulatory
419 6S RNA in *E. coli* causes a surprising reduction in the expression of the translation machinery.
420 *BMC Genomics* 2010;11:165.
421 27. **Reiss S, Pane-Farre J, Fuchs S, Francois P, Liebeke M et al.** Global analysis of the
422 *Staphylococcus aureus* response to mupirocin. *Antimicrob Agents Chemother* 2012;56(2):787-
423 804.
424 28. **Anderson KL, Roberts C, Disz T, Vonstein V, Hwang K et al.** Characterization of
425 the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their
426 effects on log-phase mRNA turnover. *J Bacteriol* 2006;188(19):6739-6756.
427 29. **Lemos JA, Brown TA, Jr., Burne RA**. Effects of RelA on key virulence properties of
428 planktonic and biofilm populations of *Streptococcus mutans*. *Infect Immun* 2004;72(3):1431-
429 1440.
430 30. **Sureka K, Dey S, Datta P, Singh AK, Dasgupta A et al.** Polyphosphate kinase is
431 involved in stress-induced *mprAB-sigE-rel* signalling in mycobacteria. *Mol Microbiol*
432 2007;65(2):261-276.
433 31. **Tagami K, Nanamiya H, Kazo Y, Maehashi M, Suzuki S et al.** Expression of a small
434 (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-
435 dependent dimerization of ribosome. *Microbiologyopen* 2012;1(2):115-134.
436 32. **Eiamphungporn W, Helmann JD**. The Bacillus subtilis *sigma(M)* regulon and its
437 contribution to cell envelope stress responses. *Mol Microbiol* 2008;67(4):830-848.
438 33. **Cao M, Kobel PA, Morshedi MM, Wu MF, Paddon C et al.** Defining the *Bacillus*
439 *subtilis sigma(W)* regulon: a comparative analysis of promoter consensus search, run-off
440 transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. *J Mol*
441 *Biol* 2002;316(3):443-457.
442 34. **Pietiainen M, Gardemeister M, Mecklin M, Leskela S, Sarvas M et al.** Cationic
443 antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-
444 type sigma factors and two-component signal transduction systems. *Microbiology* 2005;151(Pt
445 5):1577-1592.
446 35. **Thackray PD, Moir A**. SigM, an extracytoplasmic function sigma factor of *Bacillus*
447 *subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide
448 stress. *J Bacteriol* 2003;185(12):3491-3498.
449 36. **Wiegert T, Homuth G, Versteeg S, Schumann W**. Alkaline shock induces the
450 Bacillus subtilis *sigma(W)* regulon. *Mol Microbiol* 2001;41(1):59-71.
451 37. **Miller HK, Carroll RK, Burda WN, Krute CN, Davenport JE et al.** The
452 extracytoplasmic function sigma factor *sigmaS* protects against both intracellular and
453 extracytoplasmic stresses in *Staphylococcus aureus*. *J Bacteriol* 2012;194(16):4342-4354.

454 38. **Pando JM, Pfeltz RF, Cuaron JA, Nagarajan V, Mishra MN et al.** Ethanol-induced
455 stress response of *Staphylococcus aureus*. *Can J Microbiol* 2017;63(9):745-757.
456 39. **Abranches J, Martinez AR, Kajfasz JK, Chavez V, Garsin DA et al.** The molecular
457 alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in
458 *Enterococcus faecalis*. *J Bacteriol* 2009;191(7):2248-2256.
459 40. **Anderson KL, Roux CM, Olson MW, Luong TT, Lee CY et al.** Characterizing the
460 Effects of Inorganic Acid and Alkaline Shock on the *Staphylococcus aureus* Transcriptome
461 and Messenger RNA Turnover. *FEMS Immunol Med Microbiol* 2010;60(3):208-250.
462 41. **Sajish M, Kalayil S, Verma SK, Nandicoori VK, Prakash B**. The significance of
463 EXDD and RXKD motif conservation in Rel proteins. *J Biol Chem* 2009;284(14):9115-9123.
464 42. **Mechold U, Murphy H, Brown L, Cashel M**. Intramolecular regulation of the
465 opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from *Streptococcus*
466 *equisimilis*. *J Bacteriol* 2002;184(11):2878-2888.
467 43. **Sajish M, Tiwari D, Rananaware D, Nandicoori VK, Prakash B**. A charge reversal
468 differentiates (p)ppGpp synthesis by monofunctional and bifunctional Rel proteins. *J Biol*
469 *Chem* 2007;282(48):34977-34983.
470 44. **Mechold U, Potrykus K, Murphy H, Murakami KS, Cashel M**. Differential
471 regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Res* 2013;41(12):6175-
472 6189.
473 45. **Wang JD, Sanders GM, Grossman AD**. Nutritional control of elongation of DNA
474 replication by (p)ppGpp. *Cell* 2007;128(5):865-875.
475 46. **Cashel M, Gentry DR, Hernandez VJ, Vinella D (editors)**. *The stringent response*.
476 Washington DC: ASM Press; 1996.
477 47. **Gaca AO, Abranches J, Kajfasz JK, Lemos JA**. Global transcriptional analysis of
478 the stringent response in *Enterococcus faecalis*. *Microbiology* 2012;158(Pt 8):1994-2004.
479 48. **Corrigan RM, Bowman L, Willis AR, Kaever V, Grundling A**. Cross-talk between
480 two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem* 2015;290(9):5826-
481 5839.
482 49. **Samarrai W, Liu DX, White AM, Studamire B, Edelstein J et al.** Differential
483 responses of *Bacillus subtilis* rRNA promoters to nutritional stress. *J Bacteriol*
484 2011;193(3):723-733.
485 50. **Somerville CR, Ahmed A**. Mutants of *Escherichia coli* defective in the degradation of
486 guanosine 5'-triphosphate, 3'-diphosphate (pppGpp). *Mol Gen Genet* 1979;169(3):315-323.
487 51. **Gaca AO, Kudrin P, Colomer-Winter C, Beljantseva J, Liu K et al.** From (p)ppGpp
488 to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small
489 Alarmone Synthetase of *Enterococcus faecalis*. *J Bacteriol* 2015;197(18):2908-2919.
490 52. **Ruwe M, Kalinowski J, Persicke M**. Identification and Functional Characterization
491 of Small Alarmone Synthetases in *Corynebacterium glutamicum*. *Front Microbiol*
492 2017;8:1601.
493 53. **Shyp V, Tankov S, Ermakov A, Kudrin P, English BP et al.** Positive allosteric
494 feedback regulation of the stringent response enzyme RelA by its product. *EMBO Rep*
495 2012;13(9):835-839.
496 54. **Steinchen W, Schuhmacher JS, Altegoer F, Fage CD, Srinivasan V et al.** Catalytic
497 mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone.
498 *Proc Natl Acad Sci U S A* 2015;112(43):13348-13353.
499 55. **Beljantseva J, Kudrin P, Andresen L, Shingler V, Atkinson GC et al.** Negative
500 allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-
501 stranded RNA. *Proc Natl Acad Sci U S A* 2017;114(14):3726-3731.

502 56. **Schurr T, Nadir E, Margalit H**. Identification and characterization of *E.coli*
503 ribosomal binding sites by free energy computation. *Nucleic Acids Res* 1993;21(17):4019-
504 4023.
505 57. **Rao F, See RY, Zhang D, Toh DC, Ji Q et al.** YybT is a signaling protein that contains
506 a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity.
507 *J Biol Chem* 2010;285(1):473-482.
508 58. **Whiteley AT, Pollock AJ, Portnoy DA**. The PAMP c-di-AMP Is Essential for *Listeria*
509 *monocytogenes* Growth in Rich but Not Minimal Media due to a Toxic Increase in (p)ppGpp.
510 [corrected]. *Cell Host Microbe* 2015;17(6):788-798.
511 59. **Hogg T, Mechold U, Malke H, Cashel M, Hilgenfeld R**. Conformational antagonism
512 between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp
513 metabolism during the stringent response [corrected]. *Cell* 2004;117(1):57-68.
514 60. **Yang X, Ishiguro EE**. Dimerization of the RelA protein of *Escherichia coli*. *Biochem*
515 *Cell Biol* 2001;79(6):729-736.
516 61. **Gropp M, Strausz Y, Gross M, Glaser G**. Regulation of *Escherichia coli* RelA
517 requires oligomerization of the C-terminal domain. *J Bacteriol* 2001;183(2):570-579.
518 62. **Avarbock A, Avarbock D, Teh JS, Buckstein M, Wang ZM et al.** Functional
519 regulation of the opposing (p)ppGpp synthetase/hydrolase activities of RelMtb from
520 *Mycobacterium tuberculosis*. *Biochemistry* 2005;44(29):9913-9923.
521 63. **Murdeshwar MS, Chatterji D**. MS_RHII-RSD, a dual-function RNase HII-(p)ppGpp
522 synthetase from *Mycobacterium smegmatis*. *J Bacteriol* 2012;194(15):4003-4014.
523 64. **Krishnan S, Petchiappan A, Singh A, Bhatt A, Chatterji D**. R-loop induced stress
524 response by second (p)ppGpp synthetase in *Mycobacterium smegmatis*: functional and domain
525 interdependence. *Mol Microbiol* 2016;102(1):168-182.
526 65. **Haseltine WA, Block R**. Synthesis of Guanosine Tetra- and Pentaphosphate Requires
527 the Presence of a Codon-Specific, Uncharged Transfer Ribonucleic Acid in the Acceptor Site
528 of Ribosomes. *Proc Natl Acad Sci U S A* 1973;70(5):1564-1568.
529 66. **Avarbock D, Avarbock A, Rubin H**. Differential regulation of opposing RelMtb
530 activities by the aminoacylation state of a tRNA.ribosome.mRNA.RelMtb complex.
531 *Biochemistry* 2000;39(38):11640-11648.
532 67. **English BP, Hauryliuk V, Sanamrad A, Tankov S, Dekker NH et al.** Single-
533 molecule investigations of the stringent response machinery in living bacterial cells. *Proc Natl*
534 *Acad Sci U S A* 2011;108(31):E365-373.
535 68. **Wout P, Pu K, Sullivan SM, Reese V, Zhou S et al.** The *Escherichia coli* GTPase
536 CgtAE cofractionates with the 50S ribosomal subunit and interacts with SpoT, a ppGpp
537 synthetase/hydrolase. *J Bacteriol* 2004;186(16):5249-5257.
538 69. **Jiang M, Sullivan SM, Wout PK, Maddock JR**. G-protein control of the ribosome-
539 associated stress response protein SpoT. *J Bacteriol* 2007;189(17):6140-6147.
540 70. **Persky NS, Ferullo DJ, Cooper DL, Moore HR, Lovett ST**. The ObgE/CgtA GTPase
541 influences the stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol*
542 2009;73(2):253-266.
543 71. **Seyfzadeh M, Keener J, Nomura M**. *spoT*-dependent accumulation of guanosine
544 tetraphosphate in response to fatty acid starvation in *Escherichia coli*. *Proc Natl Acad Sci U S*
545 *A* 1993;90(23):11004-11008.
546 72. **Gong L, Takayama K, Kjelleberg S**. Role of *spoT*-dependent ppGpp accumulation in
547 the survival of light-exposed starved bacteria. *Microbiology* 2002;148(Pt 2):559-570.
548 73. **Butland G, Peregrin-Alvarez JM, Li J, Yang W, Yang X et al.** Interaction network
549 containing conserved and essential protein complexes in *Escherichia coli*. *Nature*
550 2005;433(7025):531-537.

551 74. **Gully D, Moinier D, Loiseau L, Bouveret E**. New partners of acyl carrier protein
552 detected in *Escherichia coli* by tandem affinity purification. *FEBS Lett* 2003;548(1-3):90-96.
553 75. **Battesti A, Bouveret E**. Acyl carrier protein/SpoT interaction, the switch linking
554 SpoT-dependent stress response to fatty acid metabolism. *Mol Microbiol* 2006;62(4):1048-
555 1063.
556 76. **Battesti A, Bouveret E**. Bacteria possessing two RelA/SpoT-like proteins have
557 evolved a specific stringent response involving the acyl carrier protein-SpoT interaction. *J*
558 *Bacteriol* 2009;191(2):616-624.
559 77. **Pulschen AA, Sastre DE, Machinandiarena F, Crotta Asis A, Albanesi D et al.** The
560 stringent response plays a key role in *Bacillus subtilis* survival of fatty acid starvation. *Mol*
561 *Microbiol* 2017;103(4):698-712.
562 78. **Hahn J, Tanner AW, Carabetta VJ, Cristea IM, Dubnau D**. ComGA-RelA
563 interaction and persistence in the *Bacillus subtilis* K-state. *Mol Microbiol* 2015;97(3):454-471.
564 79. **Pizarro-Cerda J, Tedin K**. The bacterial signal molecule, ppGpp, regulates
565 *Salmonella* virulence gene expression. *Mol Microbiol* 2004;52(6):1827-1844.
566 80. **Haralalka S, Nandi S, Bhadra RK**. Mutation in the *relA* gene of *Vibrio cholerae*
567 affects *in vitro* and *in vivo* expression of virulence factors. *J Bacteriol* 2003;185(16):4672-
568 4682.
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571 **FIGURE LEGENDS**

572 **Fig. 1.** Schematic representation of the RSH superfamily proteins. (a) Long-RSH proteins
573 consist of an enzymatic N-terminal domain (NTD) and a regulatory C-terminal domain (CTD).
574 The NTD comprises a hydrolase domain (HD; pink) that can degrade (p)ppGpp into GTP or
575 GDP and PPi, and a synthetase domain (SYNTH; blue) that converts GTP/GDP and ATP in
576 (p)ppGpp. The CTD regulatory region (green) contains a ThrRS, GTPase and SpoT domain
577 (TGS), a conserved alpha helical domain (α), a zinc finger or conserved cysteine domain
578 (ZFD/CC), and an RNA recognition motif or aspartate kinase, chorismate and TyrA domain
579 (RRM/ACT). (b) Small alarmone synthetase enzymes (SAS) contain a single SYNTH domain
580 and a C-terminal alpha helix (α5) which is required for SAS tetramerisation. (c) Small alarmone
581 hydrolase proteins (SAH) contain a single HD domain.

582

583 **Fig. 2.** Example of the distribution of RSH superfamily proteins in Gram-negative and Gram-
584 positive bacteria. The alignment scores between RSH superfamily proteins from *E. coli*, *V.*
585 *cholera*, and *S. aureus* as determined by ClustalW are shown. Gram-negative bacteria can
586 contain one or two long-RSH proteins but frequently do not express SAS proteins, with the
587 exception of the *Vibrio* genus ($RelV_{Vc}$). Gram-positive bacteria typically contain a bifunctional
588 long-RSH and one or two SAS proteins.

589

590 **Fig. 3.** Regulation of the four known $relA_{Ec}$ promoters. Transcription from P1 and P2 is σ^{70}-
591 dependent, with P1 relying on an UP-element lying upstream. Transcription from P3 and P4 is
592 activated by σ^{54} with the aid of NtrC during nitrogen starvation. Transcription from P2 is
593 activated through CRP binding to the CRP/CAP site, as well as by H-NS. 6S RNA
594 downregulates transcription from both P1 and P2, while HipB binding to the HipB palindromic
595 sequence inhibits transcription of $relA_{Ec}$. Arrows and numbering represent the locations of the
596 transcriptional start sites in relation to the start codon (solid – σ^{70}, dotted – σ^{54}).

597

598 **Fig. 4.** Summary of the types of regulation involved in RSH superfamily protein activity. (a)
599 Transcriptional regulation: $relA_{Ec}$ is upregulated by NtrC, CRP and HNS and inhibited by 6S
600 RNA, RpoS and HipB. The transcription of *rel*, *relP* or *relQ* is induced by various conditions
601 as indicated. (b) Ligand-mediated regulation: (p)pGpp increases the synthetase activity of
602 $RelA_{Ec}$, while RelQ is regulated by two ligands: (p)ppGpp which augments synthetase activity
603 and ssRNA which inhibits synthetase activity. ppG2′:3′p binds to Rel from *S. equisimilis*,
604 causing a conformational change that favours (p)ppGpp hydrolysis. (c) Heterologous protein
605 interactions: ACP and ObgE both bind to $SpoT_{Ec}$ to increase or reduce (p)ppGpp synthesis
606 respectively. $RelA_{Ec}$ binding to a stalled ribosome increase (p)ppGpp production, while
607 ComGA can bind to Rel_{Bs}, although the effect on SYNTH or HD activity is unclear.

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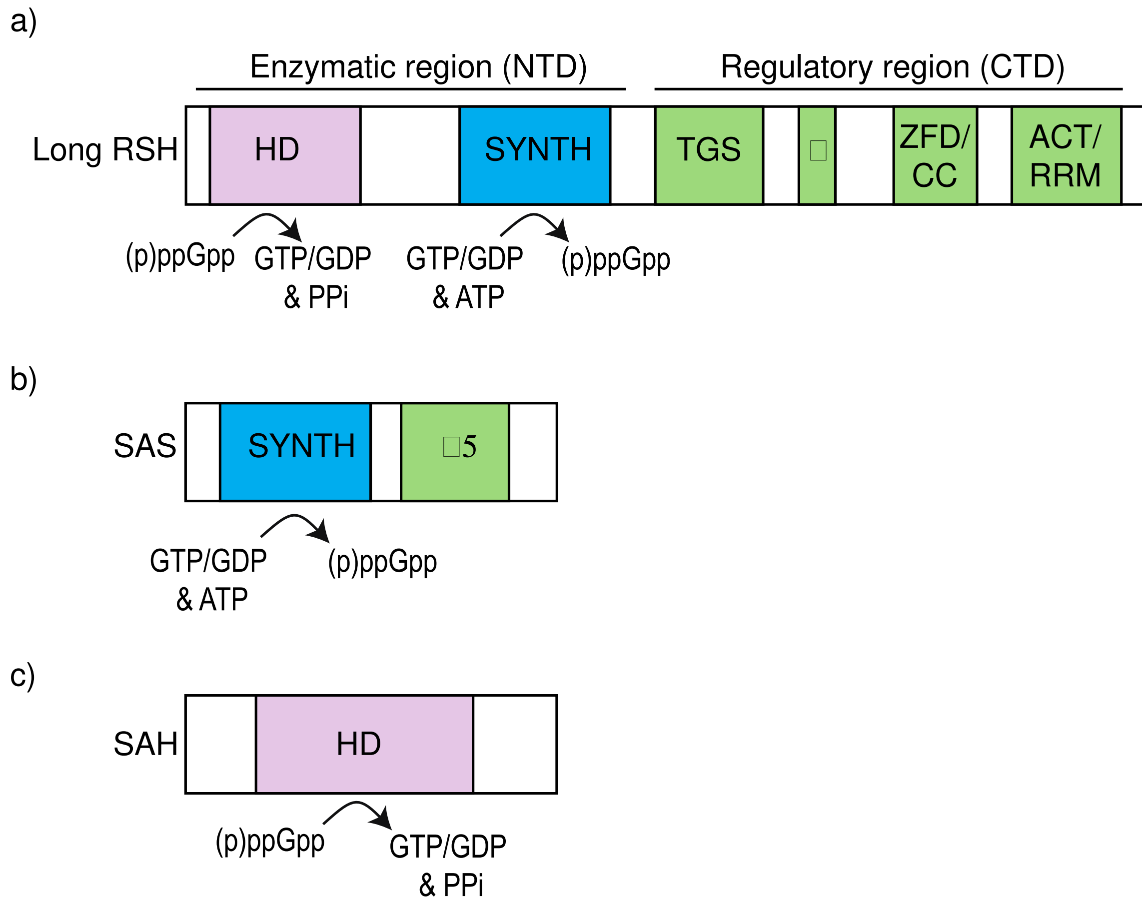

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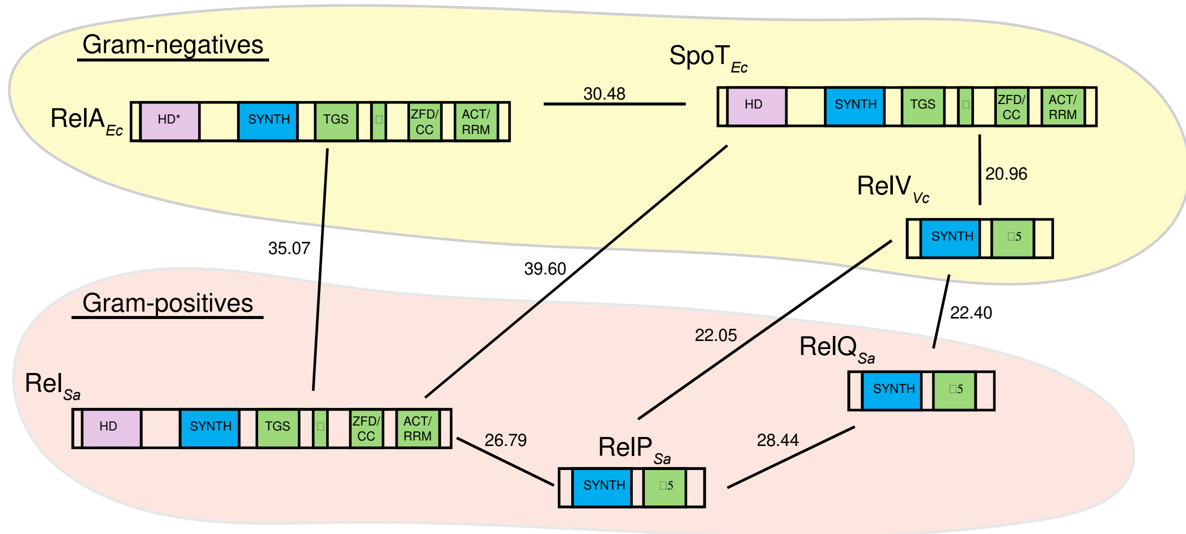

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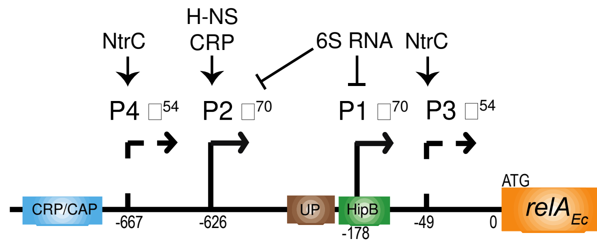

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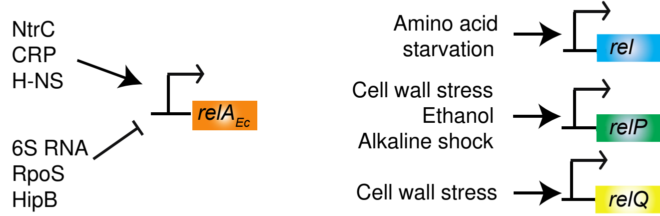

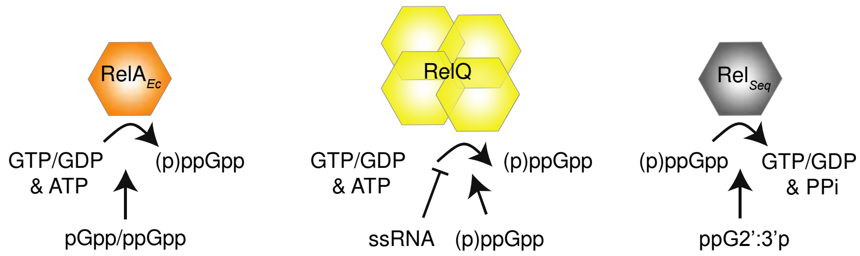

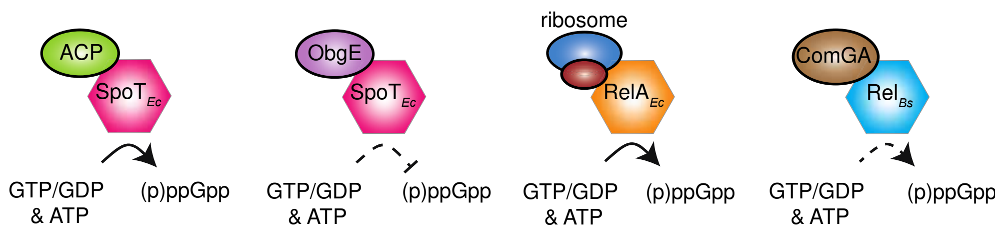

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