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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.

Abstract

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

Long-RSH genes

In E. coli the long-RSH gene $relA_{Ec}$ is under the control of four promoters, two σ^{70}-dependent promoters, relAP1 and relAP2, as well as the more recently discovered σ^{54}-dependent P3 and P4 promoters (Fig. 3) [18-20]. Transcription from relAP1 is constitutive throughout growth, and activity depends on an UP-element located 40 bp upstream of the start site [19]. relAP2 is located distally to relAP1 and transcription is induced at the transition from exponential to stationary phases [19]. This induction is regulated by CRP, H-NS and RpoS, implicating $RelA_{Ec}$ in responding to carbon, temperature and osmotic stresses [18, 19]. Transcription from relAP3 and relAP4 is activated by σ^{54} under nitrogen-starved conditions [20]. During nitrogen starvation, transcription of $relA_{Ec}$ is induced in an NtrC-dependent manner with the sensor kinase NtrB phosphorylating the response regulator NtrC, allowing it to bind enhancer-like elements upstream of the transcription start site and activate transcription from the σ^{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)ppGpp 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 persister 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 σ^{70}-RNAP through direct binding of the holoenzyme [24]. In cells without 6S RNA, transcription of $relA_{Ec}$ is slightly increased compared to wildtype during early stationary phase, however this is enough to increase cellular ppGpp levels, leading to characteristic stringent response-related changes in transcriptional profile [25]. Neusser et al. also observe 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 mupirocin, which inhibits the isoleucyl t-RNA synthetase and mimics amino acid stress, induces rel_{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, rel_{Mtb} is part of the σ^{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 MrpA. MrpA~P can then activate transcription of sigE, which has a positive effect of the transcription of rel_{Mtb} [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]. $relQ_{Bs}$ is mainly transcribed during exponential growth, with transcript levels dropping off as the cells enter stationary phase. This coincides with a massive induction of $relP_{Bs}$ transcription in late exponential phase that completely disappears in stationary phase. This differential expression ties in with observations that these proteins may have biologically distinct functions requiring temporal regulation. For instance the overexpression of $RelP_{Bs}$, but not $RelQ_{Bs}$, has been shown to result in increased 100S ribosome formation in B. subtilis [31].

$relP_{Bs}$ is part of the sigma factors σ^M and σ^W-induced regulons [32, 33]. Both of these σ factors are involved in response 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 σ factor in S. aureus is σ^S [37], but analysis of the $relP_{Sa}$ and $relQ_{Sa}$ promoters indicates they are regulated by the housekeeping σ 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 over-expression 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 $RelP_{Bs}$-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, however 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 $Rels_{eq}$ 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 [44, 45]. This is further complicated by the recent identification of an additional signalling molecule - pGpp. $RelA_{Ec}$ was initially shown to be able to synthesise this alarmone through the hydrolysis of the β phosphate of ppGpp, albeit in small quantities [41]. Subsequent reports have since demonstrated the ability of the SAS proteins $RelQ_{Ef}$ from E. faecalis and $RelS_{Cg}$ from Corynebacterium glutamicum to efficiently utilise GMP as a substrate to produce pGpp in vitro, although the presence of this small alarmone has yet to be conclusively demonstrated in vivo [51, 52].

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, $RelA_{Ec}$, 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 $RelA_{Ec}$ to increase activity. Presumably, the hydrolase activity of $SpoT_{Ec}$ 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 $RelQ_{Bs}$ 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].

An additional SAS in M. smegmatis, termed MS_RHII-RSD, has been shown to contain both a (p)ppGpp synthesis domain and a 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 fused to a functionally distinct enzyme. Alone each of the domains are inactive and a hexamer of full-length proteins is required for 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 destabilise 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 Rel_{Mtb} 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 Rel_{Mtb} 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 $RelA_{Ec}$ 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 $RelA_{Ec}$ interacting with charged tRNAs by steric exclusion. The hydrolase and synthetase domains of $RelA_{Ec}$ 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 $RelA_{Ec}$ 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 $SpoT_{Ec}$ (Fig. 4c) [68]. Deleting ObgE results in increased (p)ppGpp levels during 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 $SpoT_{Ec}$-dependent manner [71, 72]. $SpoT_{Ec}$ directly interacts 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 $SpoT_{Ec}$ and the holo form of ACP, and is required for (p)ppGpp accumulation during fatty acid starvation [75]. Later work by the authors suggests that this $SpoT_{Ec}$-ACP interaction is specific for the $SpoT_{Ec}$ 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 $SpoT_{Ec}$ 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, however 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 rRNA 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 becomes 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 utilised 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 no conflict of interest

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FIGURE LEGENDS

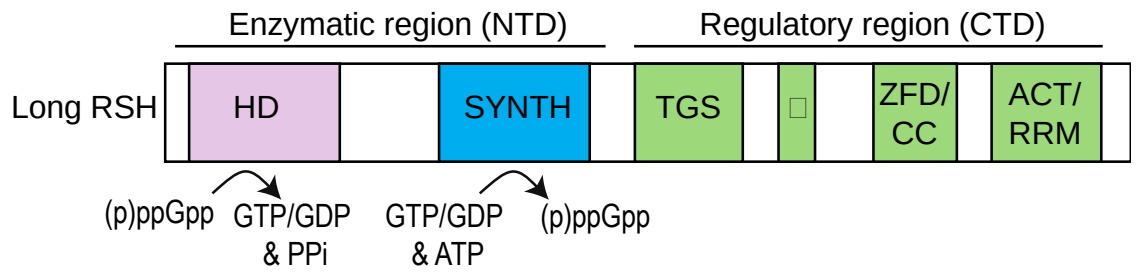
Fig. 1. Schematic representation of the RSH superfamily proteins. (a) Long-RSH proteins consist of an enzymatic N-terminal domain (NTD) and a regulatory C-terminal domain (CTD). The NTD comprises a hydrolase domain (HD; pink) that can degrade (p)ppGpp into GTP or GDP and PPi, and a synthetase domain (SYNTH; blue) that converts GTP/GDP and ATP in (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 tetramerisation. (c) Small alarmone hydrolase proteins (SAH) contain a single HD domain.

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 Vibrio genus ($RelV_{Vc}$). Gram-positive bacteria typically contain a bifunctional long-RSH and one or two SAS proteins.

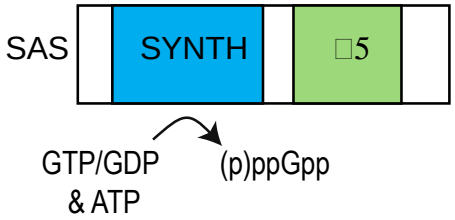
Fig. 3. Regulation of the four known $relA_{Ec}$ promoters. Transcription from P1 and P2 is σ^{70}-dependent, with P1 relying on an UP-element lying upstream. Transcription from P3 and P4 is activated by σ^{54} with the aid of NtrC during nitrogen starvation. Transcription from P2 is activated through 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 $relA_{Ec}$. Arrows and numbering represent the locations of the transcriptional start sites in relation to the start codon (solid – σ^{70}, dotted – σ^{54}).

Fig. 4. Summary of the types of regulation involved in RSH superfamily protein activity. (a) Transcriptional regulation: $relA_{Ec}$ 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 as indicated. (b) Ligand-mediated regulation: (p)pGpp increases the synthetase activity of $RelA_{Ec}$, 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 $SpoT_{Ec}$ to increase or reduce (p)ppGpp synthesis respectively. $RelA_{Ec}$ binding to a stalled ribosome increase (p)ppGpp production, while ComGA can bind to Rel_{Bs}, although the effect on SYNTH or HD activity is unclear.

a)


Enzymatic region (NTD)
Regulatory region (CTD)
Long RSH
HD
SYNTH
TGS
□
ZFD/
CC
ACT/
RRM
(p)ppGpp
GTP/GDP
& PPi
GTP/GDP
& ATP
(p)ppGpp

b)


SAS
SYNTH
□5
GTP/GDP
& ATP
(p)ppGpp

c)

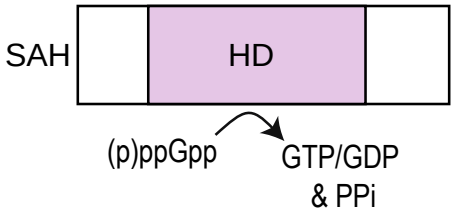

SAH
HD
(p)ppGpp
GTP/GDP
& PPi

Fig.1

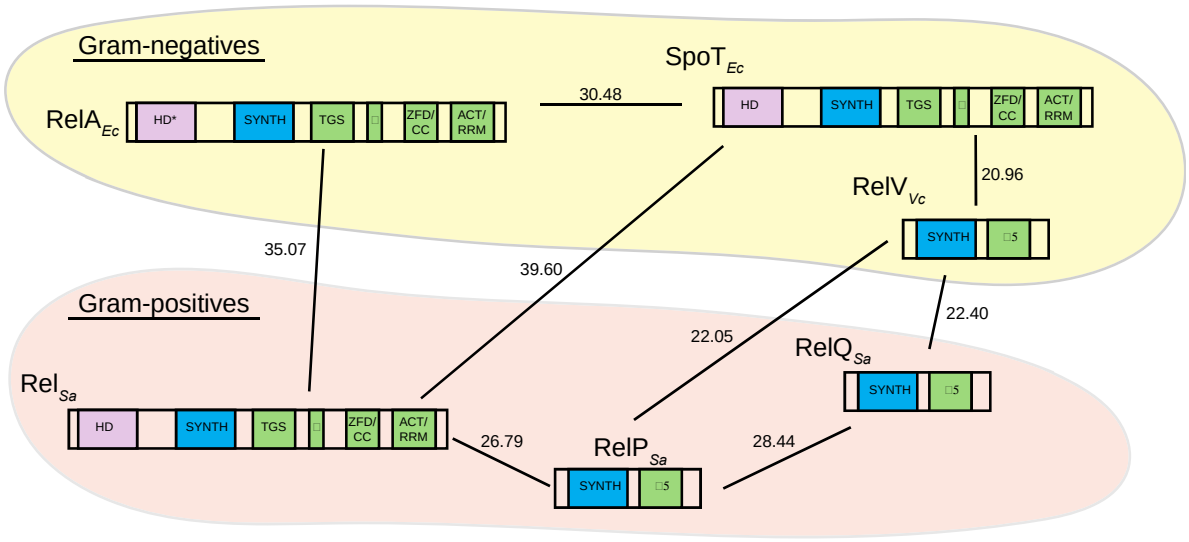

Gram-negatives
SpoT Ec
RelA Ec
HD*
SYNTH
TGS
ZFD/ CC
ACT/ RRM
30.48
HD
SYNTH
TGS
ZFD/ CC
ACT/ RRM
20.96
RelV Vc
SYNTH
5
35.07
39.60
Gram-positives
22.40
22.05
RelQ Sa
SYNTH
5
Rel Sa
HD
SYNTH
TGS
ZFD/ CC
ACT/ RRM
26.79
RelP Sa
28.44
SYNTH
5

Fig. 2

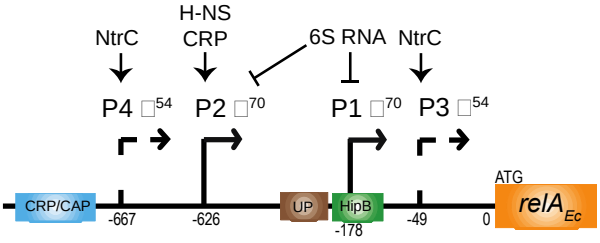

NtrC
H-NS
CRP
6S RNA
NtrC
P4 σ54
P2 σ70
P1 σ70
P3 σ54
ATG
CRP/CAP
UP
HipB
relA Ec
-667
-626
-178
-49
0

Fig. 3

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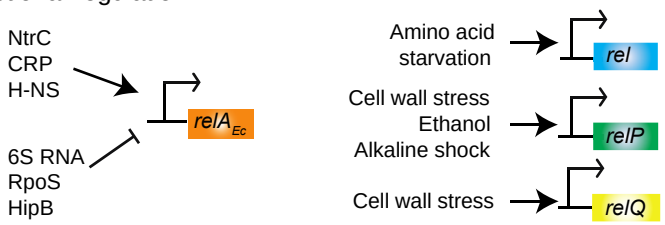
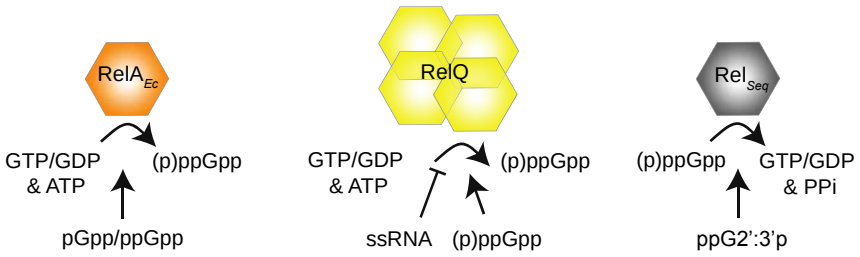
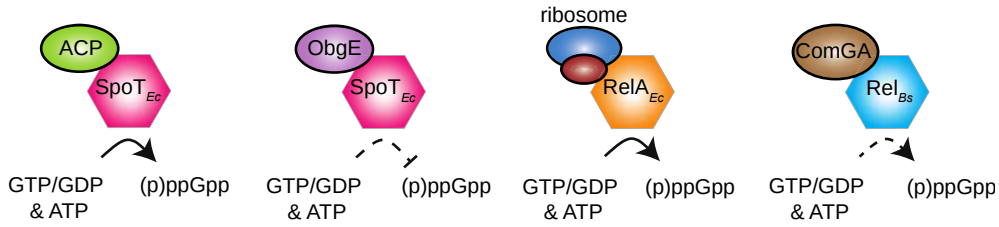
a) Transcriptional regulation



b) Ligand-mediated regulation



c) Heterologous protein interactions



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