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# The stringent response and physiological roles of (pp)pGpp in bacteria

**Sophie E. Irving, Naznin R. Choudhury and Rebecca M. Corrigan***

The Florey Institute, Department of Molecular Biology and Biotechnology,

University of Sheffield, Sheffield, United Kingdom

* Correspondence: r.corrigan@sheffield.ac.uk

**The stringent response is a stress signalling system mediated by the alarmones (pp)pGpp in response to nutrient deprivation. Research into the stringent response has exploded in recent years, with new insights highlighting the complexity and broad range of functions that these alarmones control. This review will provide an update on our current understanding of the enzymes involved in (pp)pGpp nucleotide turnover, including those shown to produce pGpp and its analogue (pp)pApp. Many studies to date have examined the impact of (p)ppGpp interacting with the RNAP to alter cellular processes. Here, we will describe a broader range of target pathways controlled by (pp)pGpp in the bacterial cell and the impact of this on multiple cellular processes, including DNA replication, transcription, nucleotide synthesis, ribosome biogenesis and function, as well as lipid metabolism. Finally, we will review the role of (p)ppGpp in bacterial pathogenesis, providing examples of how these nucleotides are involved in regulating many aspects of virulence and chronic infection.**

Bacteria encounter numerous environmental stresses during their lifecycle and need to respond quickly and efficiently in order to survive. There are several stress signalling pathways that enable bacterial adaptation, including some that rely on small nucleotide messengers. The stringent response is a ubiquitous stress signalling pathway that allows bacteria to respond to nutrient starvation[1,2]. During the stringent response, there is an accumulation of the alarmones guanosine tetra- (ppGpp) and pentaphosphate (pppGpp). These guanosine-based nucleotides are produced by members of the RSH (RelA/SpoT homologue) protein superfamily[3] and are formed from GDP or GTP, respectively. More recently, the presence of a third member of this family, pGpp, has been confirmed in numerous species[4-8]. When discussing alarmone synthesis and

functions known to involve pGpp, we will refer to all three nucleotides as (pp)pGpp, otherwise (p)ppGpp will be used for ppGpp and pppGpp. (pp)pGpp have many intracellular targets including both protein and RNA molecules, allowing several aspects of bacterial metabolism and physiology to be activated or inhibited during the stringent response. Some (pp)pGpp-binding targets are common across the Bacteria, whereas others are specific to the lifestyle and niche of a species.

As (pp)pGpp is such a widespread signalling nucleotide, it has been the focus of intense study in recent years. In this review, we discuss the latest advances in (pp)pGpp signalling. An update on our current understanding of the enzymes that synthesise and degrade all three alarmones, as well as the similar nucleotide (pp)pApp, is provided. We then highlight the broad range of metabolic pathways that (pp)pGpp regulates. This includes, but extends beyond, well-known interactions with the RNA polymerase (RNAP), to emphasise the importance of these alarmones in regulating diverse metabolic processes. (pp)pGpp is also an important player in bacterial pathogenicity. Here, we examine the contribution of these alarmones to virulence and chronic infection, using selected examples from different pathogens. Although a number of signals that trigger alarmone synthesis are referred to throughout, the mechanisms of how most of the stress signals are sensed by RSH enzymes have been comprehensively reviewed elsewhere[9,10] and so will not be discussed in detail.

## Overview of (pp)pGpp synthesising enzymes

The discovery of phosphorylated nucleotides is by no means recent, with both (p)ppGpp and (p)ppApp discovered over 50 years ago[11-13]. In the case of (p)ppGpp, work by Cashel and Gallant identified two unusual phosphorylated compounds after depriving *Escherichia coli* of amino acids[11]. These nucleotides controlled a decrease in

synthesis of stable RNAs such as tRNA and rRNA during starvation, commencing research into the stringent response. It is now well known that enzymes from the RSH superfamily are responsible for the synthesis and hydrolysis of (pp)pGpp[3,4,14]. Synthesis requires the transfer of a pyrophosphate (PPi) group from ATP to the 3′-OH group of the ribose moiety of GTP, GDP or GMP, achieved by nucleophilic attack of the β-phosphate of ATP by the 3′-OH of GTP/GDP/GMP[15] (FIG. 1a). RSH enzymes with catalytically active hydrolase (HD) domains are also able to hydrolyse (pp)pGpp by removing a $PP_i$ group to produce GTP/GDP/GMP[16]. The RSH superfamily is divided into three groups: long RSHs; small alarmone synthetases (SASs); and small alarmone hydrolases (SAHs)[3].

*Long RSH enzymes*

The first RSH enzyme characterised was a long RSH termed RelA from *E. coli*, so named as *relA* mutant strains display a 'relaxed' phenotype, with stable RNA synthesis continuing in the absence of amino acids required for growth[17]. Long RSH proteins have now been identified from across the Bacteria, green algae and in plant chloroplasts[3,14]. These enzymes contain multiple domains divided between an enzymatic N-terminal region and a regulatory C-terminal region (FIG. 1b). The HD (Pfam accession PF13328) and synthetase domains (SYNTH: PF04607) comprise the enzymatic region. The SYNTH and HD domains work in concert to maintain an optimum level of (pp)pGpp depending on the environmental conditions[18-20], with a functional HD domain required to avoid toxic accumulation of (pp)pGpp[21]. The switch between enzymatic activities is controlled by binding of the C-terminal region with interaction partners such as the starved ribosome[22-26], as well as by substrate interactions[27]. Here, the binding of GDP and ATP to the SYNTH domain opens the

structure of the enzyme, activating synthetase activity and inhibiting hydrolase activity[27]. The binding of ppGpp to the HD domain, on the other hand, induces conformational changes that occlude the SYNTH domain and permit hydrolysis[27]. The identity of the domains present in the C-terminal region of long RSH proteins is described differently throughout the literature but generally are considered to comprise a TGS region (ThrRS, GTPase and SpoT: PF02824), a ZFD or CC domain (zinc finger domain/conserved cysteine), an alpha-helical domain and an ACT or RRM domain (aspartate kinase, chorismate and TyrA/RNA recognition motif: PF13291) (FIG. 1b).

Using a Hidden Markov Model-based approach, 24,072 genomes were searched for RSH enzymes based on the presence of a SYNTH or a HD domain and classified into subfamilies based on phylogeny[14]. Long RSH enzymes were classified into 13 different subfamilies, with most species possessing one long bifunctional enzyme, in addition to one other enzyme (monofunctional long RSH or SAS)[3,14]. *E. coli*, like most organisms in the Beta- and Gammaproteobacteria, contains two long RSH enzymes: the monofunctional RelA, which has a catalytically inactive pseudo-HD domain due to active site mutations; and the bifunctional enzyme SpoT[28]. Interestingly, the pseudo-HD domain is structurally and evolutionarily conserved in the Beta- and Gammaproteobacteria, suggesting an additional role in stability or regulation of enzymatic activity[3]. Aside from the pseudo-HD domain in RelA, there are also differences in the catalytic sites of the SYNTH domains between RelA and SpoT from *E. coli*. RelA contains the acidic residues EFDD, whereas SpoT has basic residues RFKD[29]. This difference may be responsible for the preference of RelA for GDP and of SpoT for GTP as a substrate, as the motif is located near the GDP/GTP binding pocket[30]. It could also explain why SpoT has weaker synthetase activity than RelA[31,32]. *E. coli* also encodes a third enzyme involved in (p)ppGpp metabolism called GppA[33]

114 (FIG. 1a). Although it does not have a HD or SYNTH domain (and is therefore not a
115 member of the RSH superfamily), it does convert pppGpp to ppGpp through its
116 guanosine pentaphosphate phosphohydrolase activity so that ppGpp is the dominant
117 nucleotide produced in *E. coli*[33]. Most bacteria outside of the Beta- and
118 Gammaproteobacteria contain one bifunctional long RSH protein termed Rel, which
119 can be accompanied by one or two SAS proteins[3,14].

120 Several interaction partners have been shown to regulate the enzymatic activity
121 of long RSH proteins. For example, SpoT from *E. coli* interacts with the uncharged acyl
122 carrier protein (ACP)[34] and YtfK[35] to respond to fatty acid starvation, as well as the *E.*
123 *coli* $\sigma^{70}$-binding protein Rsd to sense carbon starvation[36], while Rel/RelA proteins are
124 regulated by stalled ribosomes[37-39] (for recent reviews on regulation of synthetase
125 activity see references[9,10]). The way in which Rel and RelA sense starved ribosomes
126 has long been disputed, with theories suggesting that RelA from *E. coli* could 'hop'
127 between different ribosomes to sense the charged status of tRNAs[38] or that (p)ppGpp
128 was produced following dissociation of active RelA from the ribosome[40], as opposed
129 to only when bound[41]. In recent years, much of how long RSH enzymes sense amino
130 acid starvation was clarified, with publications of biochemical studies and a number of
131 cryo-EM structures of RelA from *E. coli* in complex with the stalled ribosome[22-25] (FIG.
132 1c). When not bound to the ribosome, RelA/Rel enzymes adopt a closed conformation
133 that favours (p)ppGpp hydrolysis[25,42]. Cryo-EM structures reveal that upon ribosome
134 binding, RelA adopts an open conformation where (p)ppGpp synthesis is favoured[23-25].
135 Uncharged tRNA is not required for this initial RelA/Rel binding event, but it does
136 stabilise the interaction and promotes synthesis[22,25,26]. When bound, the TGS,
137 ACT/RRM and ZFD/CC domains of the C-terminal region of RelA interact with the A-
138 site finger element and the uncharged tRNA, while the enzymatic region extends away

139 from the ribosome producing (p)ppGpp[23-26] (FIG. 1c). pppGpp can also allosterically
140 bind to the N-terminal domain and positively influence its own synthesis, ensuring that
141 production is fully induced in response to amino acid starvation[22,43].
142
143 *Small alarmone synthetases*
144 SAS proteins only contain SYNTH domains and divide into 30 subfamilies spread
145 across a diverse range of bacteria[14]. The best characterised of these are the RelP and
146 RelQ families, which are found in the Firmicutes and share approximately 30%
147 sequence identity[10]. Both RelP and RelQ lack regulatory sensory domains and
148 expression is controlled at the transcriptional level, where alkaline shock[44,45], ethanol
149 stress[46] and exposure to cell wall-targeting antibiotics[44,47] can increase transcription (for
150 recent reviews on regulation of synthetase activity see references[9,10]). Aside from RelP
151 and RelQ, a number of additional SAS enzymes have now been characterised, such as
152 RelV from the Proteobacterium *Vibrio cholerae*[48], and RelS and RelZ from the actRel
153 subgroup present in the Actinobacteria[7]. RelZ, is a unique SAS, in that it is the only
154 RSH superfamily member characterised thus far that contains an additional enzymatic
155 domain not involved in the metabolism of (pp)pGpp[49]. The enzyme, found in
156 *Mycobacterium smegmatis*, has a SYNTH domain fused to an RNase HII domain that
157 is involved in separating RNA-DNA hybrid structures termed R-loops, suggesting that
158 (pp)pGpp has a role in resolving DNA damage[49,50].
159
160 *Small alarmone hydrolases*
161 The presence of active HD-domain-containing SAH enzymes in bacteria was
162 confirmed as recently as 2018[51] and 11 subfamilies have now been predicted based on
163 phylogenetic analysis[14]. RelH from the Actinobacterium *Corynebacterium glutamicum*

is a member of the Mesh1-L (metazoan SpoT homologue-1 L) subgroup and is capable of hydrolysing (pp)pGpp *in vitro*, in an $Mn^{2+}$ and pH-dependent manner[51]. An SAH protein called MESH1 is present in eukaryotes such as humans and *Drosophila melanogaster*, with both homologues able to hydrolyse (p)ppGpp and ppApp[14,52]. Mesh1-deficient *Drosophila* displayed slowed body growth and impaired revival upon amino acid depletion, suggesting a role in starvation responses[52]. However, these organisms do not appear to have homologues of (pp)pGpp synthetases[52]. Biochemical analysis has demonstrated that human MESH1 is instead, a cytosolic NADPH phosphatase that is able to cleave NADPH to NADH and inorganic phosphate to control cellular ferroptosis[53]. Structurally, NADPH has similarity to ppGpp, which together with the ability of MESH1 to degrade ppApp[14], suggests that this enzyme has broad substrate specificity and is not specifically involved in alarmone signalling[53].

**Expanding the nucleotide pool: pGpp and (pp)pApp**

*pGpp*

Although first identified in Actinobacteria and *Bacillus subtilis* cells in response to amino acid starvation in the 1970s[12,54], the characterisation of pGpp and its role in the stringent response is a topic of recent investigation. In 2015, the SAS RelQ from *E. faecalis* was shown to produce pGpp from GMP and ATP[4], which could then be degraded by the HD domain of long RSH enzymes[6]. Like both pppGpp and ppGpp, pGpp from *E. faecalis* is capable of inhibiting enzymes involved in the GTP synthesis pathway (outlined below), as well as the transcription of *rrnB* by the *E. coli* RNAP, suggesting that it is a third nucleotide that functions in modulating the stringent response[4]. pGpp synthesis has now been detected from multiple long and short RSH

188 enzymes, including *M. smegmatis*[6], *Staphylococcus aureus*[5] and *C. glutamicum*[7], as
189 well as from RelA in *E. coli*[30].

190 Interestingly, in *B. subtilis* and *Bacillus anthracis* cells, pGpp is also
191 synthesised by the enzymatic degradation of pppGpp and ppGpp by a NuDiX
192 (nucleoside diphosphate linked moiety X) hydrolase termed NahA[8] (FIG. 1a). Here,
193 NahA can hydrolyse between the 5′-α and 5′-β phosphate groups to produce pGpp both
194 *in vitro* and *in vivo*. Similarly to (p)ppGpp, pGpp is capable of binding to a number of
195 enzymes in the purine synthesis pathway, but unlike (p)ppGpp, does not interact with
196 ribosome-associated GTPases[8]. NahA is not the only NuDiX hydrolase capable of
197 cleaving (p)ppGpp, with MutT, NudG and RppH from *E. coli*[55,56], and Ndx8 from
198 *Thermus thermophilis*[57], able to degrade (p)ppGpp to pGp. This highlights that the
199 regulation of the alarmone pool composition is much more complex than previously
200 appreciated.

201

202 *(pp)pApp*

203 Phosphorylated purine molecules are not limited to guanosine variants. Enzymes
204 capable of synthesising (p)ppApp have been identified in the actinomycetes[12] and high
205 levels of (p)ppApp accumulate in sporulating *B. subtilis* cells in a ribosome-dependent
206 fashion[13]. A putative function for this nucleotide was identified in the 1970s, where
207 ppApp was shown to positively affect transcription of rRNA *in vitro*, in contrast to the
208 actions of (pp)pGpp[58]. In the last few years there has been a resurgence in interest in
209 (p)ppApp. A crystal structure of ppApp in complex with the RNAP from *E. coli* has
210 been solved, revealing that the nucleotide binds at a site distinct from the two (p)ppGpp
211 binding sites that is near the catalytic centre[59]. This work also observed that *in vitro*

(p)ppApp functions in an opposite way to (pp)pGpp and activates transcription of *rrn* promoters by enhancing promoter binding and stabilising open complex formation[59].

Importantly, a number of RSH enzymes have now been shown capable of producing (pp)pApp *in vivo*. Of the 30 identified SAS subtypes, 5 are encoded in bicistronic toxin-antitoxin-like operons (toxSAS) that are widespread throughout the Bacteria[14]. While expression of members of the well-known RelP and RelQ groups in *E. coli* were nontoxic, expression of SAS enzymes from these 5 subfamilies inhibited cell growth unless co-expressed with their cognate antitoxin[14]. Inhibition of bacterial growth was mediated by the production of ppApp and ppGpp, and resulted in a downregulation of transcription, translation and DNA replication[14]. In each case, toxin activity could be controlled by the production of a proteinaceous antitoxin that binds to and inactivates the toxin, as for type II toxin-antitoxin systems. The activity of one tested toxin was also inhibited by a second antitoxin. This antitoxin had hydrolase activity (SAH) and cleaved the produced alarmones, similar to type IV toxin-antitoxin systems[14].

*Pseudomonas aeruginosa* encodes an enzyme that is structurally similar to RSH enzymes, however it cannot synthesise (p)ppGpp, instead producing (pp)pApp[60]. This enzyme, Tas1, acts as an interbacterial toxin, as it is injected by a type VI secretion system into neighbouring cells where it produces (pp)pApp[60]. The net result of this is a rapid depletion of cellular ATP, leading to wide-reaching rearrangements in metabolic processes. In addition, (pp)pApp is able to bind to (pp)pGpp target proteins such as PurF and directly inhibit purine biosynthesis[60]. Tas1 and toxSAS enzymes are by no means alone in their synthesis of alarmones as toxins. Genes encoding (p)ppGpp synthetases have been identified in a number of prophages, with suggested roles in viral

defence[61-63]. Here, (p)ppGpp is produced to shut down protein synthesis and arrest growth, thereby impeding lytic phage attack.

It is interesting to note that unchecked production of (pp)pApp is toxic, just like unchecked production of (pp)pGpp in mutants without any hydrolase activity[47]. It may be that in addition to acting as a toxin, (pp)pApp is also a regulatory molecule and can positively influence transcription in certain species, as the *in vitro* data suggests[58,59]. This function would require the presence of a dedicated hydrolase and presumably a mechanism for controlling the rate of synthesis. As discussed above, there is a non-toxic accumulation of (p)ppApp in sporulating *B. subtilis* cells[13] and the role this plays in successful sporulation is unclear. Further research into regulation by (pp)pApp and its connection with the (pp)pGpp regulon will be essential for a complete view of nucleotide signalling in bacteria.

**Diverse metabolic pathways are controlled by (pp)pGpp**

The accumulation of (pp)pGpp via RSH enzymes has a diverse range of consequences in the cell by binding to a plethora of targets. Advances in binding-target identification techniques, including the use of DRaCALA-based interaction screens[8,55,64] and biotinylated capture-compounds[65], have revealed the widespread range of (pp)pGpp-interacting targets. This diversity of function allows bacteria to respond to stress in different ways that are relevant to their life cycles or niches. Interestingly, there does not seem to be a consensus (pp)pGpp binding site shared by targets, with this nucleotide capable of interacting with members from varied protein families to both activate or inhibit enzyme function. This promiscuity may arise from a degree of conformational flexibility that (pp)pGpp possesses, where the 5′ and 3′ phosphate moieties can adopt either a ring-like or elongated conformation[66].

*DNA replication*

DnaG is a DNA primase that synthesises the RNA primers needed for DNA replication[67]. In *E. coli* and *B. subtilis*, the priming activity of DnaG is inhibited by direct binding of (p)ppGpp to the enzyme active site, reducing DNA replication[68-70]. Interestingly, ppGpp is a more potent inhibitor of DnaG in *E. coli*, whilst in *B. subtilis* pppGpp is the more potent inhibitor[68,69]. This may reflect the higher ratio of ppGpp to pppGpp in *E. coli*, while the opposite occurs in *B. subtilis*[71-73].

The stringent response is also responsible for decreasing the initiation of DNA replication in *E. coli*, with replication rates inversely correlated with ppGpp levels[74,75]. This is due, in part, to a reduction in the transcription of the replication initiation ATPase DnaA during the stringent response[76], with expression of DnaA from a ppGpp-insensitive promoter sufficient to overcome inhibition[77]. Interestingly, over-producing DnaA prior to (p)ppGpp accumulation does not rescue initiation[78], whereas concurrent production does[77], suggesting it is vital that DnaA is in the ATP-bound active state in order to compensate for the effects of (p)ppGpp. (p)ppGpp further impacts DNA replication by indirectly controlling the binding of DnaA to *oriC* via the lowering of negative supercoiling[78]. During normal growth, transcription induces negative supercoils in the *oriC* origin, thus promoting replication initiation[79]. When produced, (p)ppGpp binds to the RNAP and decreases cellular transcription, resulting in fewer negative supercoils near *oriC,* thereby decreasing the occupancy of DnaA[78]. It is currently unclear whether it is a general reduction in transcription or of specific key genes, for instance the DNA gyrase gene *gyrA* or topoisomerase IV, which acts to lower negative supercoiling[78,80]. However, the combined effect on negative supercoiling

285 together with the decrease in *dnaA* transcription, allows for more nuanced control of
286 DNA replication.

287 Control of DNA replication by (p)ppGpp is by no means limited to *E. coli*. In
288 the Alphaproteobacterium *Caulobacter crescentus*, the differentiation of motile,
289 scavenger swarmer cells into stalk cells requires the activation of DNA replication,
290 which is inhibited in the swarmer cells[81]. This is reciprocally regulated by DnaA and
291 the cell cycle transcriptional regulator CtrA, which controls promoter reprogramming
292 during growth transitions[82,83]. The production of (p)ppGpp by this organism impacts
293 cell cycle progression by activating the degradation of DnaA, stopping the synthesis of
294 DnaA and inhibiting the proteolysis of CtrA[84-86], all of which ensures that DNA
295 replication does not occur. This prevents a premature switch from swarmer to stalk cell
296 before the swarmer cell has found a nutrient rich environment.

297

298 *Nucleotide synthesis*

299 As (pp)pGpp accumulates in the cell, the GTP/GDP/GMP pools are lowered following
300 substrate depletion[87]. However, it is now well established that (pp)pGpp also actively
301 inhibits the synthesis of purine nucleotides[88] (FIG. 2a), and that this regulation is
302 essential for the survival of *B. subtilis* cells during starvation[88,89]. Here, synthesis begins
303 with 5-phosphoribosyl-1-pyrophosphate (PRPP) leading to the production of inosine
304 5′-monophosphate (IMP), which serves as the branch point between adenosine and
305 guanosine nucleotide synthesis. In *E. coli*, both ppGpp and (p)ppApp can inhibit PurF,
306 responsible for the first step in the conversion of PRPP to IMP, thus halting *de novo*
307 biosynthesis of all purine nucleotides[60,65]. Two molecules of ppGpp bind to the PurF
308 tetramer at an allosteric site, disrupting the formation of the catalytic centre and
309 competitively inhibiting enzyme activity[65] (FIG. 2b). (p)ppGpp can also inhibit the

ATP biosynthesis enzyme PurA, which converts IMP to adenylosuccinate[8,65,90] (FIG. 2a). Crystallisation of PurA soaked with ppGpp revealed guanosine 5′-diphosphate 2′:3′-cyclic monophosphate (ppG2′:3′p) in the binding pocket (FIG. 2b), suggesting that the enzyme either has cyclase activity, or was able to extract the cyclic derivative from the soaking buffer[91]. The inhibition of these enzymes leads to a 65% decrease in ATP synthesis in the cell, a decrease which is balanced by less ATP being consumed in cells upon induction of the stringent response[65].

In addition to PurF, a number of enzymes specifically involved in the GTP biosynthetic pathway are targeted by (pp)pGpp (FIG. 2a), including the IMP dehydrogenase GuaB and the guanylate kinase Gmk[8,88,92]. GuaB is common to both the *de novo* and salvage nucleotide synthesis pathways and catalyses the formation of xanthosine monophosphate (XMP) from IMP, a reaction that is weakly inhibited by (p)ppGpp in *B. subtilis*[88]. In the same pathway, Gmk converts GMP to GDP, which is strongly inhibited by all three alarmones[4,64,88]. This inhibition is not conserved across bacteria however, as it does not occur in most Proteobacteria[93,94]. This is due to the lid domain of Gmk either being in a closed conformation (ppGpp insensitive – *E. coli*) or an open conformation (ppGpp sensitive – *S. aureus*) upon dimerisation of Gmk (FIG. 2b)[94,95].

Components of the salvage nucleotide synthesis pathway are also inhibited by (pp)pGpp. Both HprT and XprT use PRPP as a phosphoribose donor to convert the purine bases hypoxanthine and guanine to IMP and GMP (HprT) or xanthine to XMP (XprT) (FIG. 2a)[4,64,88,96]. XprT is most potently inhibited by pGpp and ppGpp, which bind at the PRPP binding site (FIG. 2b) and protect cells from excess environmental xanthine[96]. The (pp)pGpp-bound form of HprT exists as an apo-tetramer (FIG. 2b), where it prevents dissociation of HprT into two active PRPP-bound dimers[95]. (pp)pGpp

binding not only competitively blocks the PRPP binding site but also the interaction at the dimer-dimer interface, which further potentiates (pp)pGpp inhibition[95]. Inhibition of HprT appears to be widespread across bacteria[95], suggesting that the regulation of this enzyme by (pp)pGpp is important for GTP homeostasis as well as a stress response mechanism.

Interestingly, PpnN, the enzyme which catalyses the reverse reactions to HprT and XprT is activated by (p)ppGpp (FIG. 2a)[97]. PpnN exists as a tetramer and ppGpp binds to allosteric sites at the monomer interfaces (FIG. 2b)[55,97]. This binding results in a conformational change that opens up the active site, thus increasing PpnN activity[97] and further directs metabolism away from GTP biosynthesis upon induction of the stringent response.

*Transcription*

One of the key consequences of (pp)pGpp accumulation during the stringent response is a change in transcriptional profile. There is general repression of rRNA synthesis and genes involved in metabolism of macromolecules such as DNA and phospholipids, with a concurrent increase in transcription of amino acid biosynthesis-encoding enzymes and nutrient transporters to overcome nutrient limitations. The transcriptional changes that occur during the stringent response are key to producing the slow growing phenotype characteristic of this response.

In Alpha-, Beta-, Gamma-, Delta- and Epsilonproteobacteria, (p)ppGpp binds directly to RNAP to alter the transcription of genes both positively and negatively, with ppGpp being a more potent effector nucleotide than pppGpp[94,98]. There are two ppGpp binding sites on RNAP (FIG. 3a), and these are generally conserved across Proteobacteria[99]. Site 1 is found at the interface between the ω and β′ subunits[100] (FIG.

3b, 3c). Site 2 is located at the interface between the β′ subunit and the transcription factor DksA[101] (FIG. 3b, 3d), where the binding of ppGpp is thought to allosterically potentiate the inhibition of transcription by DksA[102]. The ability of RNAP from a given species to bind to (p)ppGpp at site 1 can be predicted by the presence of a MAR motif at the N-terminal end of the ω subunit[103]. The MAR motif is conserved in Alpha-, Beta-, Gamma-, Deltaproteobacteria but is absent in other classes[103]. In *E. coli*, when (p)ppGpp binds to site 1, transcription is inhibited approximately 2-fold, whereas when both sites are bound, along with DksA, there is 20-fold inhibition[101,104,105].

A recent study has revealed the extent to which transcription is modulated upon activation of the stringent response in *E. coli*, with altered expression of 757 genes 5 minutes, and 1224 genes 10 minutes after inducing expression of RelA from a plasmid[106]. Intriguingly, in an *E. coli* strain expressing a mutant RNAP that cannot bind to ppGpp, there were almost no changes in transcription following ppGpp accumulation[106]. This suggests that in *E. coli*, there are few, if any, genes that are regulated by ppGpp in an RNAP-independent manner. Comparing the transcriptional profiles through accumulation of ppGpp by recombinant expression of RelA[106], rather than by nutritional limitation[107], reveals large differences. 75% of the genes that are differentially expressed through recombinant expression of RelA are different to those seen upon stringent induction by serine hydroxamate[106]. This is likely due to metabolic changes or other stress responses being triggered during starvation conditions with confounding effects.

In Firmicutes, Actinobacteria and Deinococcus-Thermus, (p)ppGpp does not interact with the RNAP[94,108], and has no effect on the stability of the DNA-RNAP open complex. Therefore, any transcriptional changes observed during the stringent response are indirect[108] or by (p)ppGpp interacting with riboswitches[109] (BOX 1). During the

stringent response the level of GTP in the cell decreases due to its consumption in the production of pppGpp and through the direct inhibition of GTP biosynthesis enzymes by (pp)pGpp as described above[8,21,88,95,96,108]. This has an impact on transcription through two mechanisms. Firstly, in *B. subtilis*, $\sigma^A$-dependent promoters that are sensitive to iNTP levels and have a GTP as the initiating nucleotide at the +1 position, e.g. rRNA promoters, are transcribed at a lower frequency due to a slower initiation rate, while those that begin with an ATP tend to be upregulated[108,110]. Indeed, GTP nucleotides at positions +1 to +4 play a role in gene expression in *S. aureus* during the stringent response, suggesting that initial mRNA elongation is also sensitive to nucleotide levels in some species[111]. Secondly, GTP along with branched chain amino acids (BCAAs), are cofactors of the transcription repressor CodY, which is present in low G+C Gram-positive bacteria[112]. When GTP levels are low, CodY repression is relieved, allowing transcription of a variety of genes including those involved in amino acid biosynthesis and transport[113]. In *S. aureus*, 150 genes are upregulated when the stringent response is activated by leucine and valine starvation, with 143 of these increases due to CodY derepression[114]. On the other hand, 161 genes are downregulated independently of CodY, highlighting that CodY is an important factor for gene upregulation but not downregulation during the stringent response in *S. aureus*.

Box 1: Riboswitches

The repertoire of (p)ppGpp binding targets has now been expanded with the discovery that (p)ppGpp can target RNA molecules through interacting with riboswitches. Riboswitches are non-coding sections of mRNAs that bind to various ligands to allow regulation of gene expression. (p)ppGpp selectively binds to subtype 2a variants of the *ykkC* riboswitch, where it increases transcription of downstream genes[109]. Although not

410 widespread, this riboswitch is associated with BCAA biosynthesis and transporter
411 genes, as well as ABC transporters and glutamate synthases and is mostly present in a
412 subset of organisms within the Firmicutes[109]. Interestingly, ~40% of (p)ppGpp
413 riboswitches in the 5′ untranslated region of BCAA biosynthesis genes are
414 accompanied by a leucine T box RNA[109]. This element regulates gene expression by
415 binding to uncharged leucine tRNA[115]. This suggests that when (p)ppGpp riboswitches
416 and leucine T box RNA are found together, the presence of both (p)ppGpp and
417 uncharged leucine tRNA is required for full gene expression[109]. This adds further
418 complexity to the regulation of BCAA biosynthesis genes during the stringent response
419 beyond CodY. Furthermore, there are no examples of a canonical CodY binding site
420 consensus sequence[116] upstream of a (p)ppGpp riboswitch, despite the fact that most
421 species with (p)ppGpp riboswitches do have a *codY* gene, so the interplay between these
422 regulatory mechanisms is unknown[109].
423
424 *Ribosome maturation and function*
425 In addition to the effect on rRNA transcription, the stringent response also plays a role
426 in inhibiting ribosome maturation and protein translation. Upon stringent response
427 induction, it is generally accepted that mature ribosomes have a reduced rate of
428 translation due to the inhibitory action of (p)ppGpp on several key enzymes (FIG. 4a).
429 The initiation factor IF2 for instance, is inhibited by ppGpp, preventing the formation
430 of the 30S initiation complex (30S IC), thus reducing translation[117]. However,
431 translation of a subset of proteins is still required during the stringent response. Recent
432 work indicates that 30S-bound IF2 has different tolerances for ppGpp depending on the
433 mRNA present in the 30S pre-IC[118] (FIG. 4b). Two consecutive hairpins known as a
434 structured enhancer of translation initiation (SETI) next to the translation initiation

region mediate ppGpp tolerance for *tufA* and *rnr* mRNA (encoding elongation factor EF-Tu and RNase R, respectively) in *E. coli*[118]. Binding of SETI-containing mRNA to the pre-IC complexed with ppGpp allows for the exchange of ppGpp for GTP and the continuation of translation. pppGpp regulates translation slightly differently when bound to IF2, where it permits 30S IC formation in *E. coli*, but requires higher concentrations of IF2 to do so[118]. Whilst the utilisation of this mechanism throughout the Bacteria has yet to be demonstrated, in *E. coli* this allows permissive synthesis of a subset of proteins[118]. In addition to IF2, (p)ppGpp also acts to halt translation through inhibition of the elongation factors EF-Tu and EF-G (FIG. 4a), which are required for charged tRNA delivery and translocation of the peptide chain during synthesis[119]. During termination, RF3, which is required for recycling RF1 and RF2 from the ribosome, is inhibited by ppGpp[120], whilst the inhibition of EF-G could also impact the recycling of the post-termination complex (FIG. 4a).

In addition to halting translation, (p)ppGpp also inhibits active 70S formation in a number of ways. (p)ppGpp reduces mature 50S and 30S formation by inhibiting small GTPases involved in ribosome maturation[64,121]. Here, (p)ppGpp binds to the GTPases RsgA, RbgA, Era and Obg in a number of species[8,55,64,122] and inhibits their GTPase activities[64,122] as a way of reducing the 70S ribosome pool. Once formed, (p)ppGpp can also promote the sequestration of ribosomes. Overexpression of the synthetase RelP in a strain of *B. subtilis* that does not produce (p)ppGpp results in the formation of inactive 70S ribosome dimers termed 100S ribosomes, in a hibernation promoting factor (Hpf)-dependent manner[123]. (p)ppGpp also induces the transcription of *hpf*, *rmf* (ribosome modulation factor) and *raiA* (ribosome-associated inhibitor), all factors important for ribosome inactivation and dimerisation in *E. coli*[124,125]. As such, the stringent response controls 100S ribosome formation through transcriptional

460 regulation in multiple species[123-125]. Furthermore, the GTPase HflX in *S. aureus* can
461 dissociate 100S ribosomes in a GTPase-dependent manner[126]. This activity is inhibited
462 by (p)ppGpp, thus maintaining the inactive 100S ribosomes[126]. In *E. coli*, the
463 transcription of *hflX* is under the control of a heat sensitive promoter[127], implying that
464 HflX functions during stress as a ribosome splitting factor and not in subunit
465 maturation. However, it is unlikely that HflX is the main splitting factor in bacteria[128]
466 and so it will be interesting to see if novel splitting factors are also regulated by
467 (p)ppGpp. From all of the above, it is clear that (p)ppGpp can act to halt protein
468 production at several key stages, ensuring slowed growth under stress.
469
470 *Lipid metabolism*
471 Fatty acid starvation is a well-known trigger of the stringent response[129]. (p)ppGpp
472 production during fatty acid starvation inhibits many metabolic activities, ensuring that
473 the cell volume does not outstrip the cell envelope capacity, thus maintaining the
474 integrity of the envelope[130]. In *E. coli*, ACP is charged with a fatty acid chain during
475 fatty acid biosynthesis. When fatty acid levels are low, an uncharged ACP binds to the
476 TGS/RRM domain of SpoT, inducing (p)ppGpp production and thus triggering the
477 stringent response[34,131,132]. (p)ppGpp synthesis by RelA is also triggered during fatty
478 acid starvation, and to a greater extent than SpoT[133]. When fatty acid levels are low
479 there is a reduction in cellular lysine, resulting in an accumulation of uncharged lysine
480 tRNA that directly activates RelA[133]. This reduction in lysine is likely the result of a
481 depletion of its precursor pyruvate, brought about during fatty acid starvation[133]. The
482 interaction between ACP and the long RSH does not occur with the Rel enzymes from
483 *B. subtilis* or *Streptococcus pneumoniae* due to differences in the isoelectric points[134].
484 However, Rel from *B. subtilis* is still required for surviving fatty acid starvation, which

485 may be more related to cellular GTP/ATP levels, rather than to production of
486 (p)ppGpp[130,135]. It is possible that fatty acid starvation triggers the stringent response in
487 the Firmicutes through depletion of lysine or other amino acids which use pyruvate as
488 a precursor (such as valine, isoleucine and leucine) but this has not yet been
489 investigated.

490 A second protein that can trigger the stringent response during fatty acid or
491 phosphate depletion is YtfK[35]. YtfK is a protein of unknown function present in
492 Gammaproteobacteria, where it interacts with the N-terminal region of SpoT to trigger
493 (p)ppGpp production and cell survival[35]. Internal imbalances in central metabolism can
494 also trigger the stringent response. Repression of *plsC, lptA* and *lpxA* through CRISPRi
495 results in (p)ppGpp accumulation in *E. coli*[136]. PlsC is involved in phospholipid
496 biosynthesis, whilst *lptA* and *lpxA* encode key components of the outer membrane
497 biogenesis pathway. Disrupting these aspects of lipid and cell envelope metabolism in
498 a (p)ppGpp-null mutant causes unregulated growth and cell lysis[136]. Phospholipid
499 metabolism is also regulated by (p)ppGpp binding to and inhibiting the enzymes
500 responsible for the first and second steps of lipid and phospholipid biosynthesis (PlsB
501 and PgsA, respectively)[137,138].

502 Additionally, (p)ppGpp inhibits the activity of several enzymes involved in the
503 bacterial type-II fatty acid biosynthesis (FAS-II) pathway[139]. AccA and AccD are
504 protein subunits of the heterotetrameric acetyl-CoA carboxytransferase which transfers
505 a carbonyl group to acetyl-CoA, forming malonyl-CoA. The activity of the
506 carboxytransferase complex ($AccA_2AccD_2$) is inhibited by (p)ppGpp[139]. Furthermore,
507 FabA and FabZ, homologous β-hydroxyacyl-ACP dehydratases involved in fatty acid
508 elongation, are inhibited by ppGpp at biologically significant levels[140], highlighting that
509 (p)ppGpp impacts lipid metabolism in multiple ways.

## Impact of the stringent response on bacterial pathogenicity

The impact of the stringent response on virulence and pathogenesis is most commonly mediated through changes in transcriptional profile rather than through direct binding of (p)ppGpp to an effector protein. Even horizontally acquired virulence genes can be incorporated into the native stringent regulon, demonstrating the elasticity of the response[141]. While also impacting steady-state growth (BOX 2), the stringent response has been implicated in each stage of an infection, including adhesion, invasion, immune evasion, dissemination, biofilm formation and chronic infection, by many pathogens. While reviewed extensively by Dalebroux *et al.,*[142,143], here we use examples from various pathogens, including *E. coli*, *Salmonella* species and *S. aureus*, to illustrate the role of the stringent response throughout an infection.

### Box 2: (p)ppGpp – indirect controller of steady-state growth rates in *E. coli*

Altered cell growth is perhaps one of the most important aspects of bacterial survival and adaption to new environments. During the stringent response, (p)ppGpp is central to reorganising cellular processes, the effect of which is a reduction in growth[2]. Indeed, *E. coli* (p)ppGpp mutant strains lack growth rate control[144], highlighting the important contribution (p)ppGpp makes to growth rate through its inhibition of numerous cellular processes, including DNA replication, ribosome synthesis and translation.

During exponential growth of *E. coli*, the initiation of DNA replication increases, resulting in a high *ori*/*ter* ratio. In the absence of (p)ppGpp however, cells contain a constantly high *ori*/*ter* ratio despite changes in growth rate[80], illustrating how (p)ppGpp-mediated inhibition of DNA replication can impact growth. The effects of (p)ppGpp on steady-state growth of *E. coli* were quantitatively explored by altering the

535 expression of either the RelA synthetase from *E. coli* or the Mesh1 hydrolase from *D.*
536 *melanogaster* in amino acid-free minimal media[145]. Increasing (p)ppGpp levels during
537 steady-state growth decreased the number of ribosomes in the cell, while lowering
538 (p)ppGpp levels had the opposite affect[145]. A global resource allocation model[146] was
539 utilised, wherein the proteome was divided into three fractions: metabolic proteins and
540 those constitutively expressed; ribosome-associated proteins; and growth rate-
541 independent proteins. This model revealed that increased levels of (p)ppGpp decreased
542 growth by reducing ribosome-associated protein expression[145]. Interestingly, decreased
543 amounts of (p)ppGpp also reduced growth as a result of lower expression of metabolic
544 proteins. From this, it was apparent that (p)ppGpp indirectly impacts growth through
545 finely balancing resource allocation to match the cellular growth rate.
546
547 *Adhesion*
548 As enterohemorrhagic *E. coli* (EHEC) moves from the nutrient-rich upper intestine to
549 the nutrient-poor lower intestine, the expression of the horizontally-acquired locus of
550 enterocyte effacement (LEE) pathogenicity island is triggered. This locus encodes
551 factors involved in attachment and colonisation of the gut, including the type III
552 secretion system (T3SS) and effector proteins needed for intimate adhesion[147]. In
553 addition to a number of other transcriptional regulators, the expression of this locus is
554 triggered by an accumulation of (p)ppGpp via RelA, resulting in an increase in
555 adherence to epithelial cells during the stringent response[141] (FIG. 5). Together with
556 DksA, (p)ppGpp is essential for the activation of *ler* and *pch* transcription, two
557 regulators required for the expression of the LEE regulon[141]. (p)ppGpp is also required
558 for the downregulation of the flagella regulon in *E. coli*. Here, (p)ppGpp can inhibit the
559 transcription of *fhlDC*, a master regulator of flagella synthesis genes[106,148].

560 Fimbriae are crucial factors for bacterial attachment to host cells. The expression
561 of type 1 fimbrial genes in uropathogenic *E. coli* (UPEC) is activated by (p)ppGpp
562 through transcriptional activation of the *fimB* gene[149]. FimB is a recombinase that flips
563 the promoter of the *fimAICDFGH* operon into the 'on' orientation, allowing
564 transcription of these fimbriae-encoding genes. (p)ppGpp-null UPEC mutants display
565 no fimbriae on their surface[149]. In contrast to this, mutants of the transcription factor
566 *dksA* are hyperfimbriated, revealing that DksA and (p)ppGpp can function
567 independently, as well as codependently[150]. Control of fimbriae expression by
568 (p)ppGpp is also observed in *Bordetella pertussis*, where the expression of *fim3*, which
569 encodes a subunit of the long filamentous structure, was decreased in a Δ*relA* Δ*spoT*
570 double mutant[151].
571
572 *Invasion*
573 During an infection, many bacterial species use the stringent response to aid in invading
574 host tissues. A (p)ppGpp-null mutant of *Salmonella enterica* serovar Typhimurium
575 shows reduced invasion of intestinal epithelial cells and is attenuated in the BALB/c
576 mouse model (FIG. 5)[152]. The (p)ppGpp mutant strain had reduced expression of *hilA*
577 and *invF*, two transcriptional activators of the *Salmonella* pathogenicity island 1
578 (SPI1)[152]. SPI1 encodes a T3SS which secretes factors enabling bacterial uptake and
579 invasion upon contact with specialised M cells in the intestinal epithelium[153].
580 Furthermore, the SlyA transcriptional activator in *S.* Typhimurium directly binds to
581 (p)ppGpp, allowing the formation of SlyA dimers[154]. These dimers then bind to
582 promoter DNA resulting in the transcription of many virulence genes[154], such as *pagC*
583 which encodes a membrane surface protein that aids survival in serum[155].

584 The use of an iron overload murine model for *S.* Typhi infection revealed that
585 mice infected with (p)ppGpp-null mutants were able to outlive their wild-type-infected
586 counterparts, with decreased numbers of the mutant recovered from visceral organs,
587 highlighting a role for (p)ppGpp in systemic infection[156]. (p)ppGpp was crucial for the
588 production of flagella, with the null strain displaying decreased adhesion to, and uptake
589 by, THP-1 phagocytes *in vitro*. (p)ppGpp mutants were reduced in their ability to
590 invade epithelial cells, to survive in human serum and to survive within
591 macrophages[156]. All of this ties in with observations from *S.* Typhimurium, where
592 strains with C-terminal domain mutants of SpoT had defects in producing (p)ppGpp in
593 response to acid stress, resulting in reduced metal cation uptake and reduced activation
594 of SPI2 genes, producing an attenuated phenotype in a mouse model[157]. The
595 requirement of (p)ppGpp for invasion of host cells is by no means limited to *Salmonella*
596 species, with the stringent response important for the invasion and virulence of a
597 number of intracellular pathogens, including *Campylobacter jejuni*[158], *Streptococcus*
598 *suis*[159], *Legionella pneumophila*[160] and *E. faecalis*[161].
599
600 *Immune evasion and dissemination*
601 Many pathogens enter host cells during an infection in order to evade the immune
602 system, requiring adaptation to a different environment. In *S. aureus*, the stringent
603 response is induced following uptake by human polymorphonuclear neutrophils
604 (PMNs), resulting in Rel-dependent induction of the cytotoxic phenol soluble modulins
605 *psm*α1-4 and *psm*β1-2[114]. These cytotoxins lyse neutrophils and promote escape[162,163],
606 making the stringent response crucial for *S. aureus* survival after phagocytosis[114] (FIG.
607 5).

608 Biofilms are complex communities of microorganisms held together by an
609 extracellular matrix that can form on many different surfaces. This matrix enables
610 resistance to opsonisation, complement deposition, phagocytosis by macrophages and
611 ultimately immune evasion and subsequent bacterial dissemination[164]. A number of
612 reports suggest a role for (p)ppGpp in regulating biofilm formation, however whether
613 it is required for the formation or for the dispersal of biofilms appears to differ between
614 species, with the precise mechanisms not yet fully understood. *E. faecalis*, for instance,
615 can cause biofilm-mediated catheter-associated urinary tract infections (CAUTI),
616 which are impacted by both (p)ppGpp and CodY[165] (FIG. 5). (p)ppGpp-null and Δ*codY*
617 single mutants of *E. faecalis* show reduced biofilm formation and reduced colonisation
618 of bladders and catheters *in vivo*[165]. However, the biofilm formation and colonisation
619 of a (p)ppGpp-null Δ*codY* double mutant was similar to wild-type, although this strain
620 was unable to cause kidney infection, suggesting decreased virulence[165]. These findings
621 agree with research in *Listeria monocytogenes* demonstrating that *codY* inactivation re-
622 establishes virulence to a certain extent in a (p)ppGpp mutant, as there is no repression
623 of the CodY regulon (mimicking the stringent response)[165,166]. Biofilm formation is also
624 regulated by the stringent response in species which do not encode *codY,* for example
625 *B. pertussis*[151], *E. coli*[167] *and Helicobacter pylori*[168].

626 In contrast to the above examples, increased biofilm formation in the absence of
627 a functional stringent response has been demonstrated in a number of species, including
628 *Porphorymonas gingivalis*[169] and *Actinobacillus pleuropneumoniae*[170]. In
629 *Pseudomonas putida*, (p)ppGpp is required for biofilm dispersal by inhibiting the
630 synthesis of the adhesin LapA, while simultaneously promoting its proteolysis[171,172]
631 (FIG. 5). In *S. epidermidis*, the β-PSMs are responsible for cell detachment and
632 dissemination of cells from biofilms[173], and as mentioned above, these toxins are

upregulated by (p)ppGpp in staphylococcal species[114]. These contradictions between species highlights the complexity and non-uniformity of the stringent response across bacteria, with much about how (p)ppGpp mechanistically controls biofilm formation and immune evasion remaining to be clarified.

*The role of (p)ppGpp in chronic infections*

Tolerance to cyclic antibiotics by a bacterial population has been found to enable the emergence of resistance[174]. These populations often contain small colony variants (SCVs), which are characterised as slow growing cells, with small colony sizes and mutations resulting in decreased virulence and metabolism[175]. In the laboratory, (p)ppGpp mutants are artificially generated in order to examine their effect on growth and virulence. However, an MRSA strain from a chronically infected patient harboured a mutation affecting (p)ppGpp production[176]. (p)ppGpp overexpression occurred due to a F128Y substitution in the hydrolase domain of Rel, constitutively activating the stringent response[176]. The transcription profile of the clinical isolate was similar to that of a strain in which the stringent response had been induced by the antibiotic mupirocin. This strain also had an upregulation of the global regulator-encoding *agr* locus, as well as substitutions in RpoB, in the DNA topoisomerase IV ParC and in the ribosomal methyltransferase RlmN, resulting in rifampicin, ciprofloxacin and linezolid resistance[176]. Notably, linezolid is a last resort antibiotic in the treatment of resistant infections. Altogether, these four mutations sequentially allowed the emergence of a resistant SCV within a chronic infection[176]. This is similar to findings by Mwangi and colleagues, who showed high levels of (p)ppGpp in a strain highly resistant to methicillin[177] and demonstrates in a clinical environment how bacteria evolve to adapt to stressful conditions.

## Conclusions and outlook

In this review, we have examined some of the more recent discoveries relating to the synthesis and function of (pp)pGpp in bacteria. Although first identified many years ago, the recent characterisation of pGpp production and the use of (p)ppApp as a toxin, has stressed that there is still much to learn about these signalling systems. Excitingly, this also extends beyond the identification of new regulatory nucleotides, as an increasing number of pathways that are controlled by these nucleotides are being identified. Here, we have touched upon a number of metabolic processes that are regulated by (pp)pGpp, including priming for DNA replication, synchronising the production of both GTP and ATP to match cellular needs, controlling protein production at multiple points and adjusting fatty acid production. As discussed, all of the above processes are vital for bacterial survival in a host, explaining why these alarmones are intertwined with virulence and bacterial survival.

The reallocation of cellular processes during times of stress is an incredibly complex process. Despite (p)ppGpp having been identified in the 1960s[11], many unknowns about this signalling system remain. Until recently the vast majority of research had been conducted in *E. coli*. However, we now understand that while this system is ubiquitous throughout the Bacteria, the mechanisms of stress sensing, alarmone production and alarmone function are far more diverse than previously anticipated. Of the subfamilies of RSH enzymes identified[3,14], only a small number have been characterised. Work to characterise the toxSAS subfamilies[14] demonstrates that there may be functions for (pp)pGpp beyond the classical stringent response. Furthermore, other unidentified enzymes without SYNTH or HD domains involved in (pp)pGpp turnover may exist. Prime examples of this are the phosphohydrolase GppA[33]

683 or the NuDiX hydrolase NahA[8]. In addition, many other HD/SYNTH enzymes with
684 functional domain fusions, like RelZ, could exist in order to adapt the stringent response
685 to respond to stimuli specific for the survival of a given species. Extending from this,
686 (pp)pGpp synthesis enzymes are present in the chloroplasts of plants and in green
687 algae[178-181], suggesting a fairly understudied role for these alarmones beyond the
688 prokaryotes.

689 New tools are continually being developed to aid our understanding of this
690 signalling pathway. For example, the use of a DRaCALA (pp)pGpp binding screen in
691 *S. aureus*[64], *E. coli*[55] and *B. anthracis*[8], as well as the development of a (p)ppGpp
692 capture-compound[65] have significantly broadened the repertoire of (pp)pGpp targets.
693 Combining that, with numerous "omic" techniques such as RNA-seq that provide
694 interesting insights into processes controlled by (pp)pGpp[106], has led to a much greater
695 understanding of this system. These tools now need to be combined with *in vivo* work
696 to fully explore how (pp)pGpp impacts bacterial pathogenicity. Much is still to be
697 clarified on the role of (pp)pGpp in chronic infection, and the development of methods
698 to detect the production of (pp)pGpp *in vivo* and monitor the triggers of the stringent
699 response in host cells, will greatly improve our understanding of this essential
700 signalling system in the future.
701

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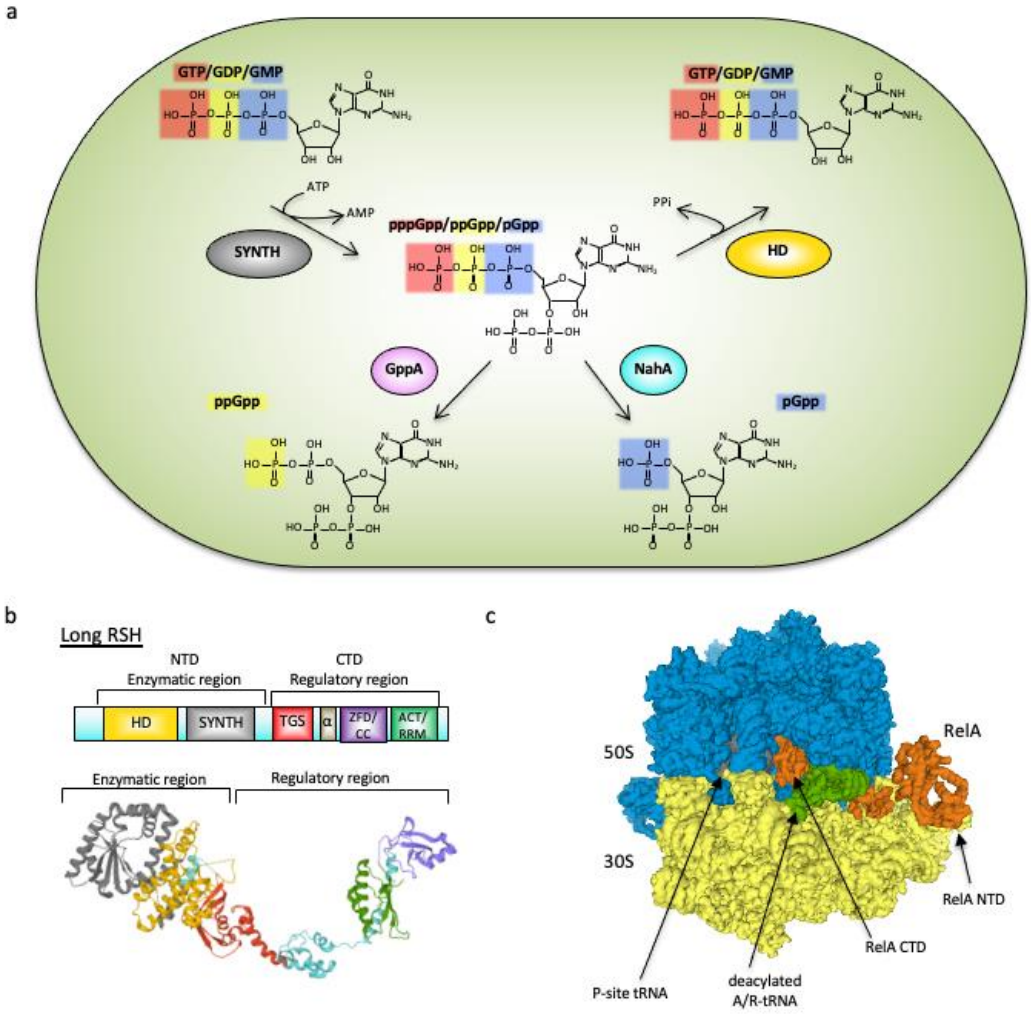


Figure 1. **Synthesis and hydrolysis of (pp)pGpp by RSH enzymes. a)** The synthetase domain (SYNTH: grey) of RSH enzymes catalyses the transfer of a pyrophosphate group from ATP to the ribose moiety of GTP/GDP/GMP to produce pppGpp, ppGpp or pGpp, respectively. This reaction also generates a molecule of AMP. Conversely, the hydrolase domain (HD: orange) is responsible for removing the pyrophosphate group to recover GTP/GDP/GMP. Enzymes outside of the RSH superfamily are also involved in (pp)pGpp metabolism, with GppA (pink) hydrolysing pppGpp to ppGpp[33], and NahA (blue) hydrolysing (p)ppGpp to pGpp[8]. **b)** Domain architecture and structure of the long RSH enzyme RelA from *E. coli* (PDB: 5KPX), consisting of an N-terminal domain (NTD) enzymatic region and a C-terminal domain (CTD) regulatory region.

The enzymatic region consists of the HD (orange – inactive in RelA) and SYNTH (grey) domains. The regulatory region contains four domains: the TGS region (ThrRS, GTPase and SpoT: red), an alpha-helical domain (tan), the ZFD/CC domain (zinc finger domain/conserved cysteine: purple) and the ACT/RRM domain (aspartate kinase, chorismate and TyrA/RNA recognition motif: green). **c)** Structure of RelA from *E. coli* (orange) bound to the ribosome (PDB: 5L3P). An uncharged tRNA is located in the A-site (green) and P-tRNA in the P-site (grey). The 50S (blue) and 30S (yellow) subunits of the ribosome are shown.

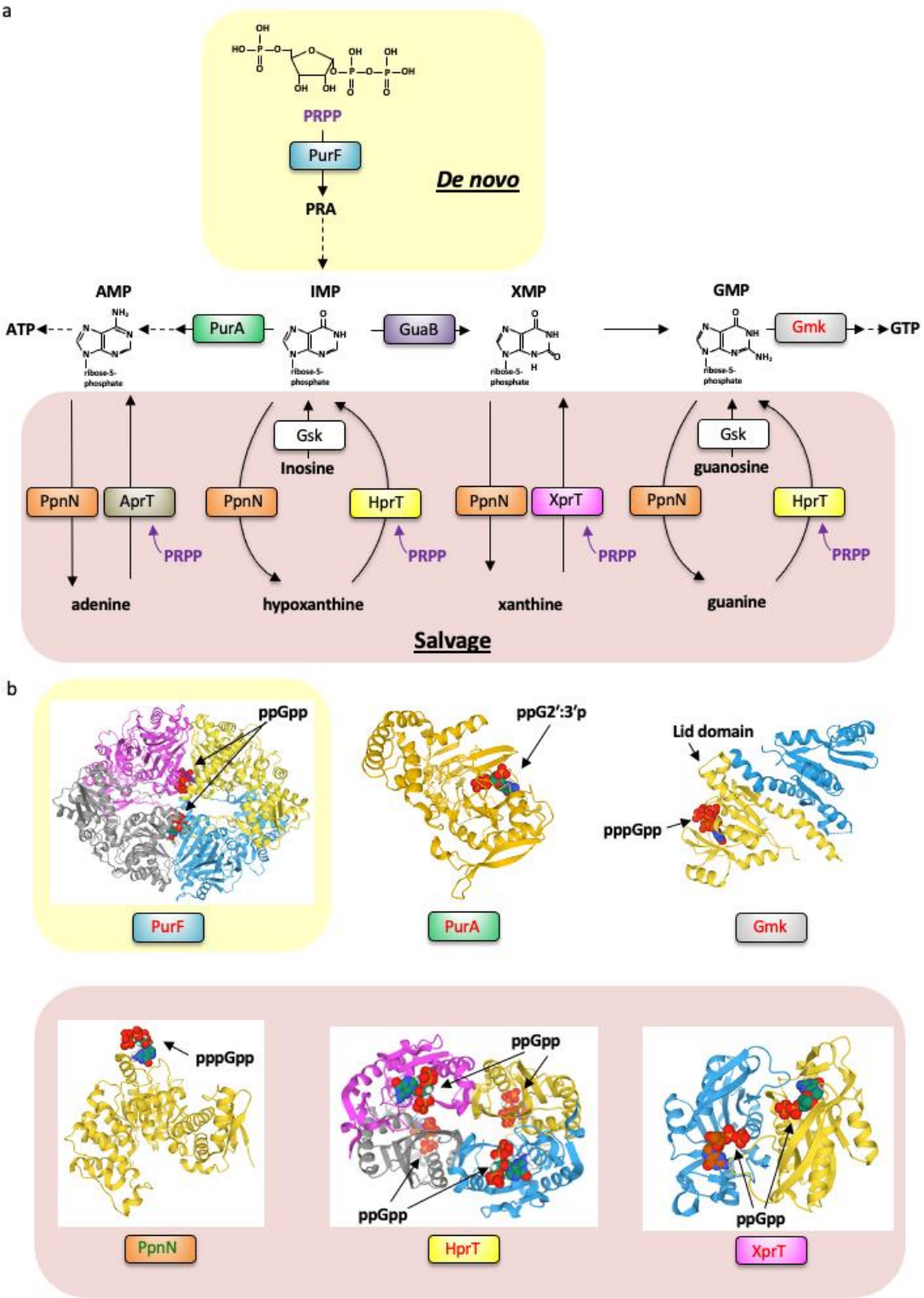


Figure 2. **(p)ppGpp-mediated inhibition of purine nucleotide synthesis. a)** The first step of *de novo* purine synthesis (yellow box) begins with 5-phosphoribosyl-1-pyrophosphate (PRPP) which is converted to 5-phosphoribosylamine (PRA) by PurF,

an amidophosphoribosyltransferase (blue). Through a complex series of reactions, PRA is ultimately modified to inosine 5′-monophosphate (IMP), which is the precursor for both ATP and GTP. The salvage pathway (pale red box) on the other hand, begins with the conversion of purine bases such as hypoxanthine, xanthine and guanine to IMP, xanthosine monophosphate (XMP) and guanosine monophosphate (GMP) respectively. Conversions to IMP and GMP are catalysed by hypoxanthine phosphoribosyltransferase (HprT: yellow), while XMP is formed by xanthine phosphoribosyltransferase (XprT: pink), which all utilise PRPP as a phosphoribose donor. Adenine is modified to AMP by adenine phosphoribosyltransferase (AprT) (brown). PpnN, pyrimidine/purine nucleotide 5′-monophosphate nucleosidase (orange) catalyses the reverse reaction using AMP, IMP, XMP and GMP as substrates. For GTP synthesis, the IMP dehydrogenase GuaB (purple) converts IMP to XMP, which is utilised by GuaA to form GMP. Guanylate kinase (Gmk: grey) converts GMP to GDP. For ATP synthesis, the adenylosuccinate synthetase PurA (green) produces adenylosuccinate (AMP-S) from IMP, which is a precursor for ATP. All enzyme names written in red are inhibited by (pp)pGpp[8,65,88,90,92,96], while those in green (PpnN) are activated[97]. AprT, displayed as a fainter red, is weakly inhibited by (p)ppGpp in *E. coli*[55]. **b)** Crystal structures of purine nucleotide synthesis enzymes shown in cartoon representation in complex with ppGpp, pppGpp or ppG2′:3′p (spacefilled). Monomers (PurA from *E. coli* – PDB: 1CH8 & PpnN from *E. coli* – PDB: 6GFM) are coloured yellow. Dimers (Gmk from *S. aureus* – PDB: 4QRH & XprT from *B. subtilis* – PDB: 1Y0B) in yellow and blue, while tetramers (PurF from *E. coli* – PDB: 6CZF & HprT from *B. anthracis* – PDB 6D9S) are coloured yellow, blue, pink and grey. Structures from the *de novo* pathway was boxed in yellow and salvage in pale red.

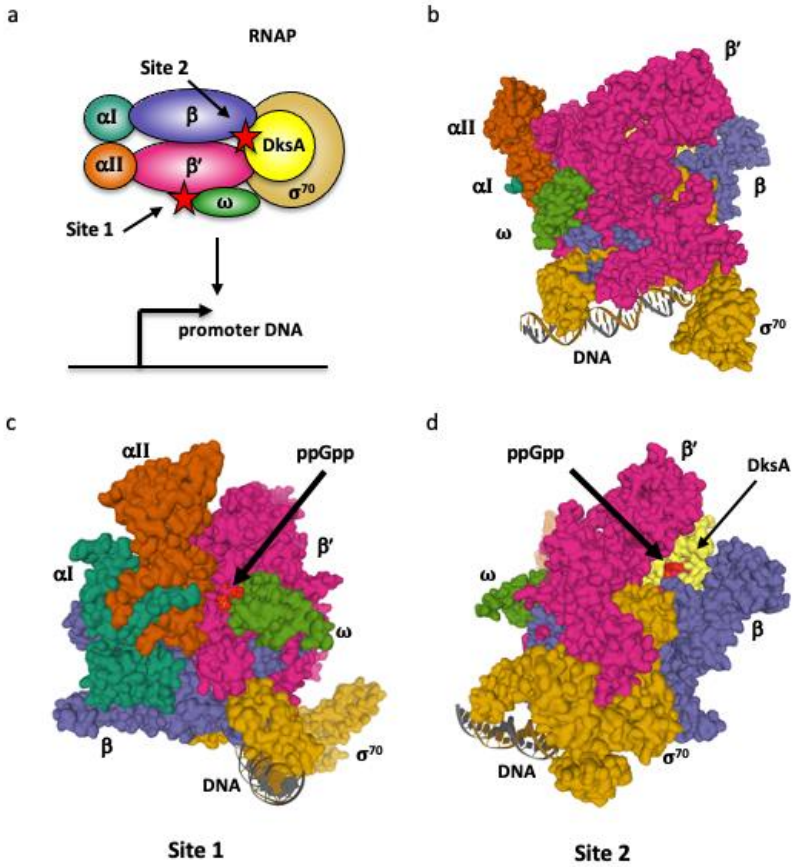


Figure 3. **ppGpp binding to the RNAP. a)** Schematic of the holoenzyme of RNAP bound to two molecules of ppGpp (red) and DksA (yellow). Individual RNAP subunits are coloured: β – purple, β′ – pink, αI – teal, αII - orange, ω - green and $\sigma^{70}$ – brown. **b-d)** Crystal structure of the RNAP holoenzyme from *E. coli* in complex with ppGpp, DksA and the *rrnBP1* promoter (PDB: 6WRD). Coloured as in (a). **b)** Face-on view of the RNAP bound to the *rrnBP1* DNA. **c)** Rotation of the RNAP 90º to visualise ppGpp (red) bound to site 1 between the β′ (pink) and ω (green) subunits. **d)** Rotation to visualise ppGpp (red) bound to site 2 between the β′ subunit (pink) and DksA (yellow).

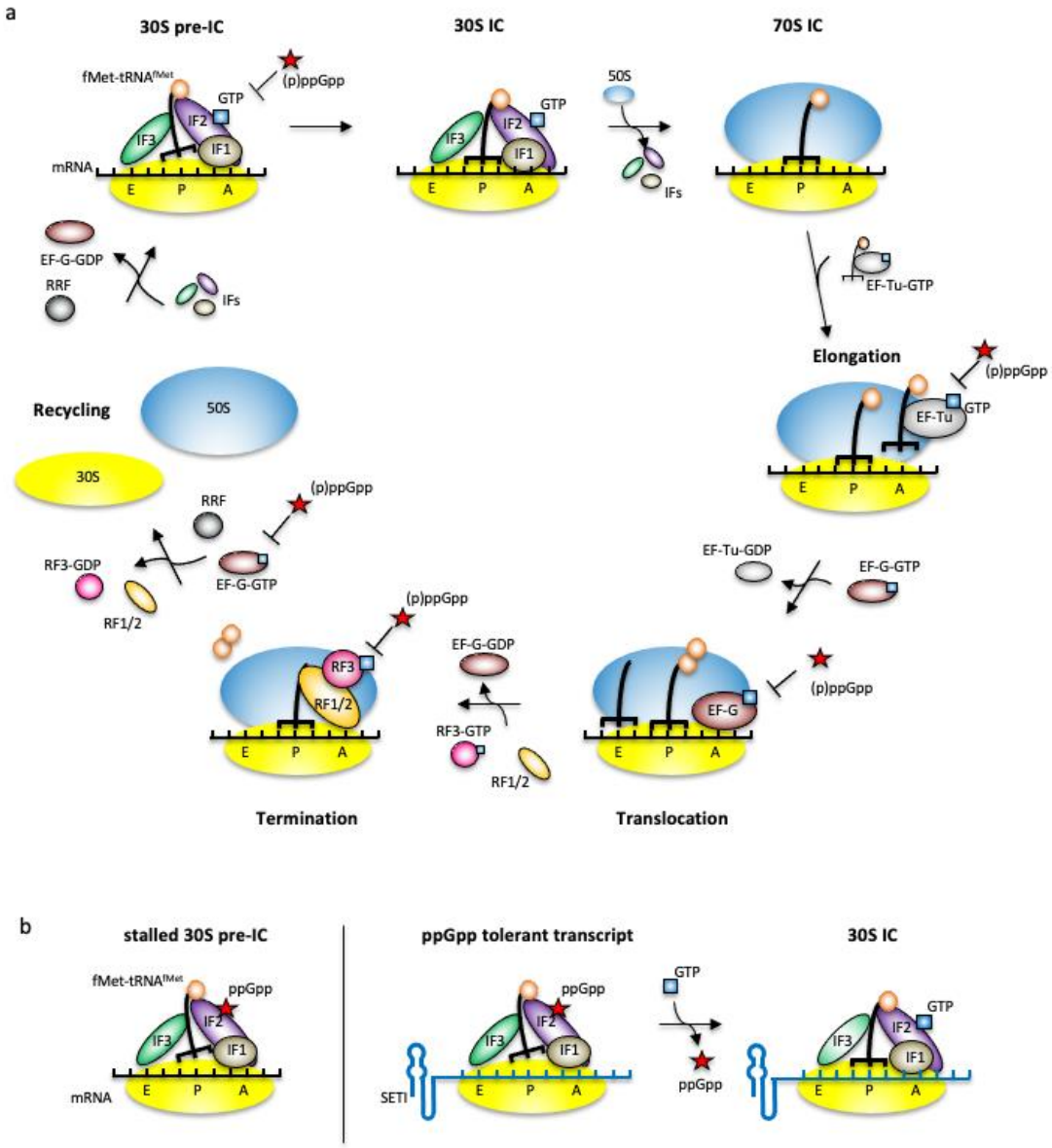


Figure 4. **Control of protein translation by (p)ppGpp. a)** (p)ppGpp directly binds to and inhibits a number of proteins responsible for the different stages of translation. Through association with GTP, the prokaryotic initiation factor IF2 acts to position the initiating fMet-tRNA$^{fMet}$ in the 30S pre-IC and promote the association of the 50S subunit, a process which is inhibited by (p)ppGpp[117,118]. Elongation and translocation processes are also targets of (p)ppGpp. (p)ppGpp-mediated inhibition of the elongation factor EF-Tu prevents the binding of an aminoacyl-tRNA to the A site of the 70S

ribosome[119]. Inhibiting EF-G, a translocase, stops the translocation of aminoacyl-tRNA through the ribosome[119]. (p)ppGpp also inhibits the release factor RF3, therefore preventing the recycling of RF1/2 from the ribosome[120]. The post-termination complex is recycled by RRF and EF-G, allowing the cycle to start again, a process that may also be inhibited by (p)ppGpp interacting with EF-G. **b)** ppGpp binding to IF2 inhibits start codon recognition. The pre-IC can then exchange a non-compatible transcript for one that contains a structured enhancer of translation initiation (SETI) element that is tolerated by ppGpp e.g. *tufA*, allowing the exchange of ppGpp for GTP and the continuation of translation[118].

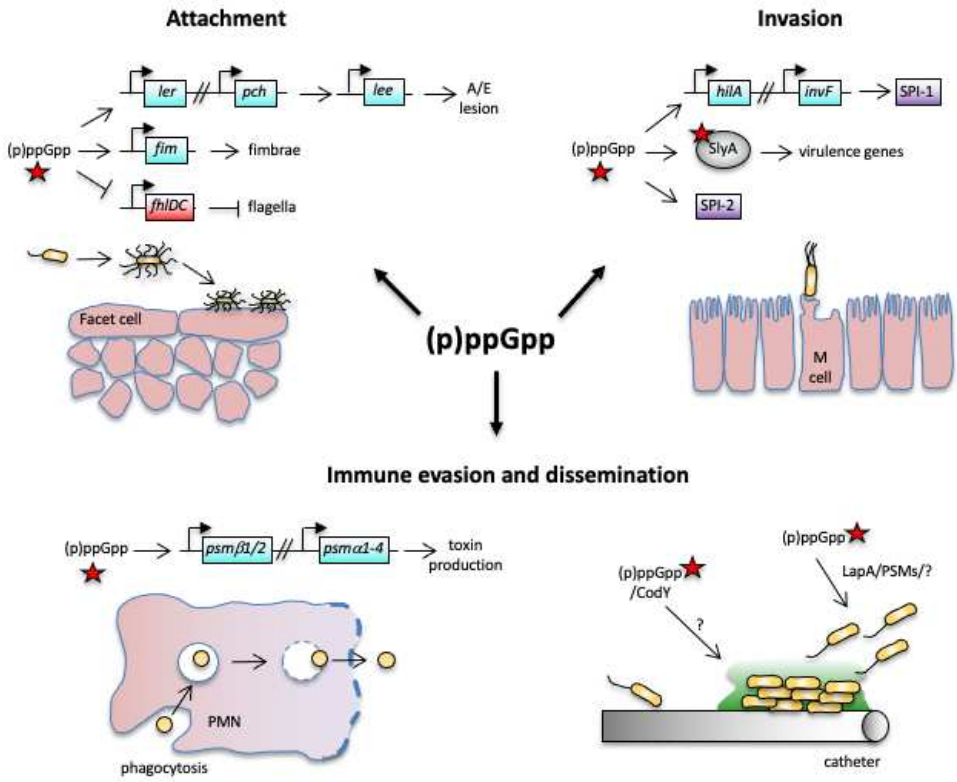


Figure 5. **(p)ppGpp affects bacterial pathogenicity in multiple ways.** (p)ppGpp (red star) can regulate genes to promote pathogenicity, including the downregulation of flagella and the upregulation of surface proteins and factors that promote increased attachment to host cell surfaces[141,147-150]. (p)ppGpp can promote invasion into host cells by upregulating toxins and pathogenicity islands, and avoidance of cellular defences by controlling the production of cytolytic toxins[152,153,155]. (p)ppGpp also has a role to play in immune evasion and bacterial dissemination via toxin production[114,162] and the regulation of biofilm formation. Both (p)ppGpp and the transcription factor CodY have been implicated in the formation of biofilms[165,166,168], while the promotion of biofilm dispersion through various (p)ppGpp-mediated mechanisms has also been reported[170-172].