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Diversity in (p)ppGpp metabolism and effectors

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Abstract

Bacteria produce guanosine tetraphosphate and pentaphosphate, collectively named (p)ppGpp, in response to a variety of environmental stimuli. These two remarkable molecules regulate many cellular processes, including the central dogma processes and metabolism, to ensure survival and adaptation. Work in *Escherichia coli* laid the foundation for understanding the molecular details of (p)ppGpp and its cellular functions. As recent studies expand to other species, it is apparent that there exists considerable variation, with respect to not only (p)ppGpp metabolism, but also to its mechanism of action. From an evolutionary standpoint, this diversification is an elegant example of how different species adapt a particular regulatory network to their diverse lifestyles.

Introduction

Since their discovery over 40 years ago, the signaling nucleotides guanosine pentaphosphate and guanosine tetraphosphate, collectively named (p)ppGpp, have been shown to be critical for bacterial stress responses [1]. (p)ppGpp was first identified as a key inhibitor of stable RNA synthesis during amino acid starvation, called the stringent response. Later work expanded the role of (p)ppGpp beyond the starvation response, showing that (p)ppGpp is induced by diverse stresses, regulates many cellular targets, and exerts its influence even at much lower concentrations than those induced during the stringent response [2,3**]. Although our understanding of (p)ppGpp is becoming deeper, the mechanisms of its metabolism and action remain unclear. Much of this confusion is due to diversity in the synthesis and the action of (p)ppGpp in different bacteria and to the numerous (p)ppGpp targets even within the same bacterium. Here we summarize new mechanistic insights regarding (p)ppGpp production and regulation, in addition to the broad physiological effects and diverse targets of (p)ppGpp.

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(p)ppGpp metabolism

Central to (p)ppGpp metabolism are enzymes that synthesize and degrade (p)ppGpp, which can be divided into three major groups: long RelA/SpoT homologue proteins (RSH) bearing both the synthetase and hydrolase domains, and small alarmone synthetases (SAS) and hydrolases (SAH), containing only the synthetase domain or the hydrolase domain, respectively [4]. These enzymes are widely distributed in bacteria and can coexist within one species in various combinations. For example, *E. coli* has two RSHs: RelA and SpoT, whereas *Bacillus subtilis* has one RSH and two SASs: RelA, RelP (YwaC), and RelQ (YjbM). Interestingly, genes encoding (p)ppGpp hydrolases have also been discovered in metazoans, and the gene (*Mesh1*) in *Drosophila melanogaster* was found to be important for starvation resistance [5], raising the possibility that (p)ppGpp might also function in higher organisms. An exception to the canonical RSH and SAS is a dual-function protein with both (p)ppGpp synthetase and RNase HII activities, recently identified in *Mycobacterium smegmatis*, suggesting crosstalk between RNA metabolism and stress signaling [6].

Differential regulation of these enzymes allows bacteria to sense various stresses. The ribosome-associated *E. coli* RelA senses amino acid scarcity by synthesizing (p)ppGpp, when an uncharged tRNA binds to the A-site of the ribosome. This signal is thought to be transduced by direct contact between the uncharged tRNA and RelA [7**]. Activation of RelA to synthesize (p)ppGpp may also depend on the identity of uncharged tRNAs, as those with higher affinity for the ribosome stimulate more (p)ppGpp synthesis *in vitro* [8]. Moreover, (p)ppGpp was shown to stimulate RelA activity *in vitro*, as a novel example of positive allosteric feedback [9].

In contrast, SASs seem to be activated transcriptionally, and canonical examples include RelP and RelQ, which are widely conserved in Gram-positive bacteria. The *ywaC* gene encoding RelP in *B. subtilis* is a member of the σ^M regulon [10] and is strongly induced by many antibiotics that target cell wall synthesis [11]. Likewise, expression of *relP* and *relQ* in *Staphylococcus aureus* is also induced by vancomycin and ampicillin [12].

Regulation of cellular processes by (p)ppGpp

(p)ppGpp has profound influence on bacterial physiology by directly or indirectly regulating many critical cellular processes, such as replication, transcription, translation, and metabolism. Here we focus on the molecular mechanisms by which (p)ppGpp adjusts these cellular activities to adapt cells to stresses.

Regulation of transcription initiation

(p)ppGpp has been known to regulate transcription since its discovery in *E. coli* [1]. Its impact on transcription was further demonstrated in recent transcriptomic analyses of several Gram-positive and Gram-negative species. These microarray-based studies showed that hundreds to thousands of genes exhibit (p)ppGpp-dependent changes [13–18]. (p)ppGpp can regulate transcription both directly and indirectly and the underlying mechanisms can vary between species. This section will center on how (p)ppGpp regulates transcription

initiation of genes directing protein synthesis (downregulated) and amino acid biosynthesis (upregulated), as they are the mostly extensively studied and best understood regulations.

In the proteobacterium *E. coli*, (p)ppGpp targets RNA polymerase (RNAP) to directly regulate transcription initiation [1,19,20] (Figure 1A). It binds to an interface between the β′ and ω subunits and acts as an allosteric regulator [21**,22*,23*]. The transcription factor DksA binds the secondary channel and sensitizes RNAP to (p)ppGpp at many promoters [24,25]. Binding of (p)ppGpp and DksA to RNAP destabilizes all of the promoter open complexes examined to date, but the transcriptional outcome varies: it inhibits transcription from promoters controlling synthesis of stable RNA (rRNA and tRNA), ribosomal proteins, fatty acids, and flagella, but activates transcription from promoters governing amino acid biosynthesis [19,26–28]. A strong correlation exists between negatively regulated promoters and the short lifetime of their open complexes [19].

The tripartite interaction between (p)ppGpp, DksA, and RNAP is central to transcriptional regulation not only in *E. coli* but also very likely in species evolutionarily close to *E. coli*. Many proteobacteria have the *E. coli* ppGpp binding site well-conserved on their RNAPs [21**]. Moreover, putative *dksA* genes have been found in many proteobacterial genomes [29], and those of *Pseudomonas aeruginosa* and *Rhodobacter sphaeroides* have been experimentally validated [29,30]. These observations suggest that the *E. coli* model may be widely shared among proteobacteria.

In the distantly-related firmicute *B. subtilis*, (p)ppGpp does not regulate transcription initiation directly, but rather by an indirect mechanism that strongly relies on modulating intracellular GTP levels (Figure 1B). Strong (p)ppGpp induction under stresses (e.g. amino acid starvation) drastically reduces GTP levels by two mechanisms: consumption of GTP during pppGpp synthesis and inhibition of GTP biosynthesis. (p)ppGpp directly inhibits multiple enzymes in the GTP biosynthesis pathways, IMP dehydrogenase (IMPDH) [31], hypoxanthine-guanine phosphoribosyltransferase (HprT), and guanylate kinase (GMK) [3**] (Figure 1B). Lowering GTP levels affects transcription initiation directly and indirectly, depending on the promoters examined. Direct effects are observed on promoters of stable RNA synthesis, whose initiating nucleoside triphosphate is GTP [32]. These promoters display GTP concentration-dependent activities *in vitro* and are sensitive to intracellular GTP levels *in vivo* [32]. On the other hand, transcription of amino acid biosynthesis genes is indirectly regulated by GTP via several mechanisms. First, branched chain amino acid (BCAA) biosynthesis genes are repressed by the transcription factor CodY in its GTP bound form [33,34], and lowering GTP inactivates CodY and thus upregulates amino acid biosynthesis [17]. Second, decreasing GTP levels is often accompanied by a concomitant increase in ATP, which enhances transcription from BCAA promoters as they initiate with ATP and are sensitive to ATP levels [35,36]. Finally, decrease of GTP could also lead to the redistribution of RNAP from GTP-initiating promoters (e.g. those of stable RNA genes), which could contribute to the enhanced transcription of amino acid biosynthesis genes [17].

The *B. subtilis* mechanism appears to be conserved in *Firmicutes*, since many species within this group contain a (p)ppGpp-sensitive GMK [37] and they also have a CodY homologue

[38]. Interestingly, species from *Actinobacteria* and *Deinoccous-Thermus*, two distantly related phyla, also exploit (p)ppGpp to inhibit GMK activity [37]. Although CodY does not appear to be conserved [38], modulation of GTP levels by (p)ppGpp may still play an important role in transcriptional regulation. In *Thermus thermophilus*, (p)ppGpp does not affect RNAP [39] and instead is proposed to regulate rRNA transcription by controlling GTP levels via IMPDH [40] and GMK [37].

Regulation beyond transcription initiation

In addition to regulating transcriptional initiation, (p)ppGpp also controls many other processes, which allows it to function as a master regulator to adjust cellular physiology and to facilitate stress survival/adaptation (Figure 2).

Translation—(p)ppGpp regulates translation indirectly through inhibiting transcription of ribosomal RNA and protein genes, thus curtailing production of the building blocks for ribosome assembly. In *E. coli*, (p)ppGpp also directly binds the translation initiation factor 2 (IF2), elongation factor G (EF-G), and the ribosome assembly factor ObgE, to regulate not only translation initiation and elongation, but also ribosome maturation during stresses [41,42].

Replication—In *E. coli*, (p)ppGpp indirectly inhibits replication initiation, possibly through a DNA methylation-dependent and SeqA-dependent mechanism [43]. In addition, (p)ppGpp directly binds the replication enzyme primase of *S. aureus* at a position overlapping the active site and interferes with its activity [44*] to slow or halt replication elongation in response to diverse stresses [45,46].

Conflicts between central dogma processes—DNA replication and transcription occur simultaneously on the same template, leading to potential conflicts between the machineries. Furthermore, translation is coupled to transcription via active ribosomes on the nascent mRNA. Amino acid starvation has the potential to exacerbate conflicts between these processes by uncoupling transcription and translation, leading to stalled transcription complexes that form barriers to replication. The (p)ppGpp cofactor DksA alleviates starvation-induced replication-transcription conflicts by preventing transcription stalling [47,48]. Intriguingly, lack of full-length IF2, a ppGpp target, sensitizes cells to DNA damaging agents, which is counteracted by increasing (p)ppGpp levels or mutations in RNAP [49]. In addition, (p)ppGpp inhibits replication, slows down transcription elongation [50], and prevents the formation of arrays of stalled RNAP [51], which may facilitate transcription-translation coupling and/or minimize transcription-replication conflicts.

Cellular metabolism—(p)ppGpp co-crystallized with the inducible lysine decarboxylase (LcdI) of *E. coli* and inhibits its activity *in vitro* and *in vivo*, thus regulating lysine metabolism during acid stress [52]. (p)ppGpp also inhibits exopolyphosphatase (PPX) activity to regulate metabolism of polyphosphate [53], which mediates antibiotic tolerance [54**], oxidative stress responses and general stress responses (Gray and Jakob, in this issue). Moreover, (p)ppGpp directly regulates intracellular purine nucleotide pools. Both *E. coli* and *B. subtilis* HprT and IMPDH are inhibited by (p)ppGpp [3**,55], although only the

B. subtilis, but not the *E. coli*, GMK is sensitive to (p)ppGpp [37]. Lastly, in *B. subtilis* (p)ppGpp inhibits the activity of YybT, a phosphodiesterase that hydrolyzes cyclic di-AMP and cyclic di-GMP [56], suggesting crosstalk between the (p)ppGpp and c-di-AMP (or c-di-GMP) signaling pathways.

Physiological importance of (p)ppGpp

Since (p)ppGpp is involved in regulating so many essential cellular processes, it is perhaps not surprising that cells lacking (p)ppGpp, although viable, exhibit severe and pleiotropic defects during stresses.

Survival of and adaptation to amino acid starvation

The canonical phenotype of cells lacking (p)ppGpp, at least in *E. coli* and *B. subtilis*, is polyauxotrophy for amino acids [17,57,58]. In both organisms, the transcriptional regulation by (p)ppGpp is important for adapting to amino acid starvation [3**,21**]. Moreover, it appears that survival of starvation also involves a tradeoff with growth rate, at least in *B. subtilis* [59]. In addition, (p)ppGpp production is negatively correlated with growth rate in *E. coli* [57]. This suggests that in both organisms (p)ppGpp first acts as a brake on cellular processes during stresses, which could require inhibition of replication, translation, transcription of rRNA, and/or cellular metabolism. Cells would then adapt to the new conditions by modulating transcription of stress response, amino acid biosynthesis and other genes required for growth [57].

Antibiotic tolerance and resistance

Lack of (p)ppGpp often leads to impaired ability to survive antibiotic insult, suggesting a critical role of (p)ppGpp in antibiotic tolerance/resistance (for an in-depth review see [60]). In addition, strong induction of (p)ppGpp by starvation or increased basal levels of (p)ppGpp through mutation lead to enhanced antibiotic tolerance in *P. aeruginosa*, *Enterococcus faecalis*, and *B. subtilis* [61–64]. However, the molecular basis for tolerance/resistance in many bacterial species is still underexplored. The best understood mechanism was reported by Maisonneuve and colleagues in *E. coli*, who found that (p)ppGpp stochastically induces persistence through toxin-antitoxin (TA) modules [54**]. In their model, (p)ppGpp inhibits the activity of PPX to activate the Lon protease, which indirectly activates the toxin by degrading the antitoxin and thus induces persistence [54**]. Intriguingly, many toxins target translation, and at least one toxin, HipA in *E. coli*, increases (p)ppGpp levels by inhibiting the activity of a glutamyl-tRNA synthetase, which creates uncharged $tRNA^{Glu}$ that activate RelA [65**]. Thus the stochastic production of (p)ppGpp may reinforce its own synthesis through the activation of TA systems. In the closely related bacterium *Salmonella* Typhimurium, persistence induced by macrophage uptake also requires (p)ppGpp, Lon, and TA modules, although Lon appears to be dispensable for persistence under laboratory cultivation [66].

In addition to their implication in antibiotic tolerance, (p)ppGpp is also shown to mediate antibiotic resistance, at least in *S. aureus*, known for its ability to acquire resistance to multiple antibiotics. Isolates displaying high levels of methicillin resistance have point

mutations in *relA* that lead to increased levels of (p)ppGpp [67*]. Inducing (p)ppGpp production with mupirocin also increases *S. aureus* resistance to β-lactam antibiotics [68,69].

Because (p)ppGpp protects cells against antibiotics, compounds that target (p)ppGpp metabolism may be viable antimicrobials when used in combination with traditional antibiotics. Recently, Relacin, a 2′-deoxyguanosine-based analogue of ppGpp, was shown to inhibit (p)ppGpp synthesis to decrease cell survival and impede biofilm formation [70*]. The peptide 1018 potently inhibits biofilm formation by directly interacting with ppGpp and promoting its degradation [71]. These studies thus may serve as models for future antimicrobial design.

Conclusions

Recent studies have highlighted conservation and divergence in (p)ppGpp metabolism and regulation. Its direct and indirect effects on multiple cellular processes and in coordinating these processes adapt cells to various stresses and maintain homeostatic growth. However, the challenge of discerning the physiologically relevant targets of (p)ppGpp under different growth and stress conditions remains.

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Highlights

- (p)ppGpp metabolic enzymes sense diverse stresses
- (p)ppGpp regulates transcription initiation by targeting RNAP or GTP levels
- (p)ppGpp controls replication, translation, and metabolism to allow stress survival
- (p)ppGpp coordinates central dogma processes to prevent conflict during stress
- (p)ppGpp contributes to antibiotic tolerance and resistance

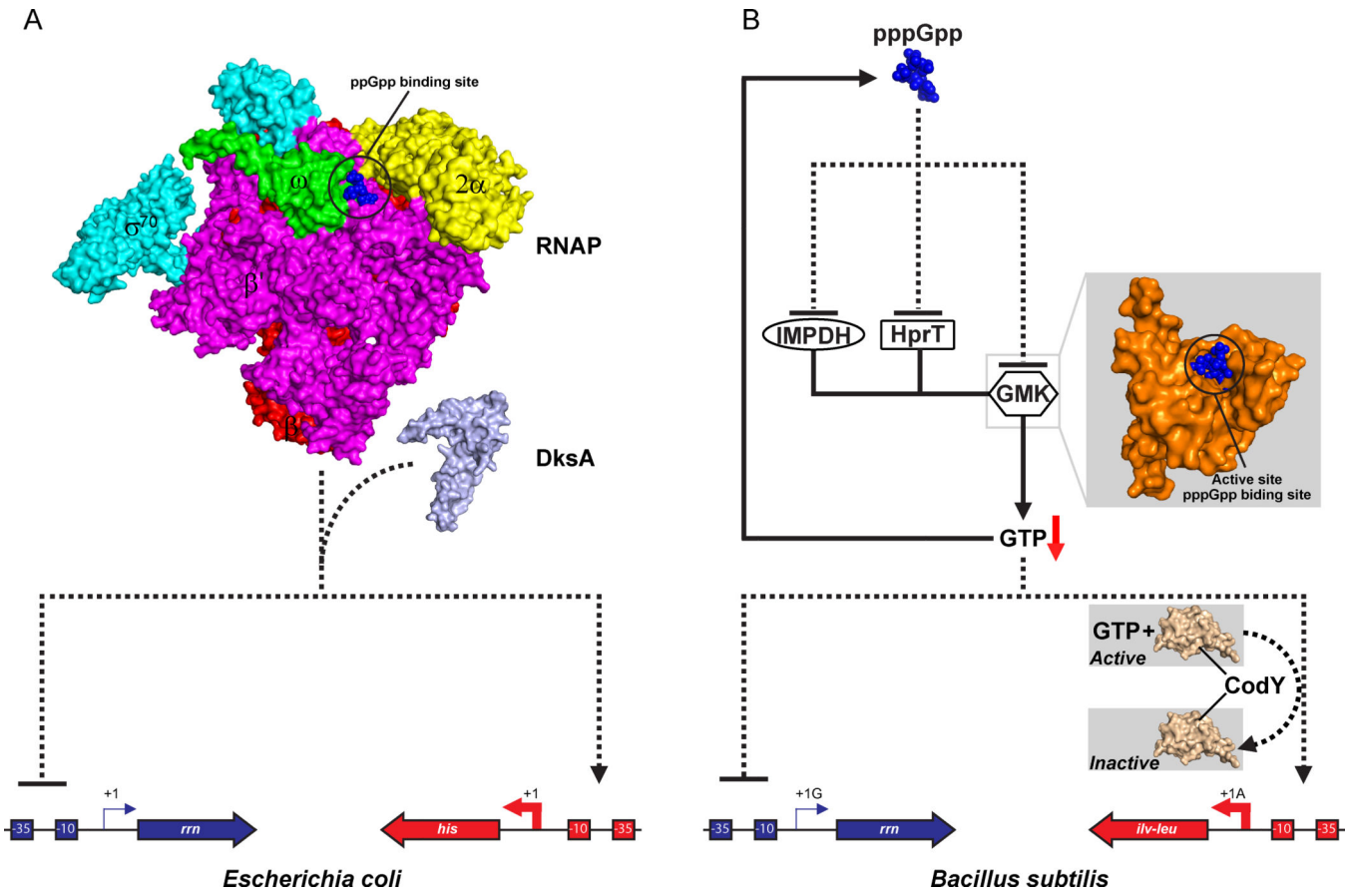

Figure 1. Divergent mechanisms of transcription initiation regulation by (p)ppGpp in *E. coli* and *B. subtilis*

(A) In *E. coli*, this regulation is mediated by the tripartite interaction between RNAP, (p)ppGpp, and DksA. Cross-linking and crystallography suggest that ppGpp (blue spheres) binds to an interface between the β′ (magenta) and ω (green) subunits of RNA polymerase [21**,22*,23*]. This binding site is 28 Å away from the active site located between the β and β′ subunits (not visible under current view), which makes (p)ppGpp an allosteric regulator. DksA binds to the secondary channel of RNAP [24]. Classic examples of negatively and positively regulated promoters are those that direct ribosomal RNA and histidine biosynthesis, respectively. Structures of RNAP (PDB: 4JKR) and DksA (PDB: 1TJL) were used for figure preparation [22*,72]. **(B)** In *B. subtilis*, (p)ppGpp regulates GTP levels by directly inhibiting IMPDH, GMK, and HprT [3**,31], and by passively consuming GTP (GDP) during (p)ppGpp synthesis. pppGpp (blue spheres) binds the GMK active site and thus acts as a competitive inhibitor [37]. Lowering GTP levels (the downward red arrow) decreases transcription from ribosomal RNA promoters, which initiate with GTP [32], but activates transcription from amino acid biosynthesis promoters (e.g. the *ilv-leu* operon), in part through inactivating CodY [17,38]. The C-terminal domain of *B. subtilis* CodY (PDB: 2B0L) was used for figure preparation [73]. Solid lines indicate biosynthesis pathways, whereas dotted lines indicate regulation. Negatively and positively regulated promoters are colored in blue and red, respectively.

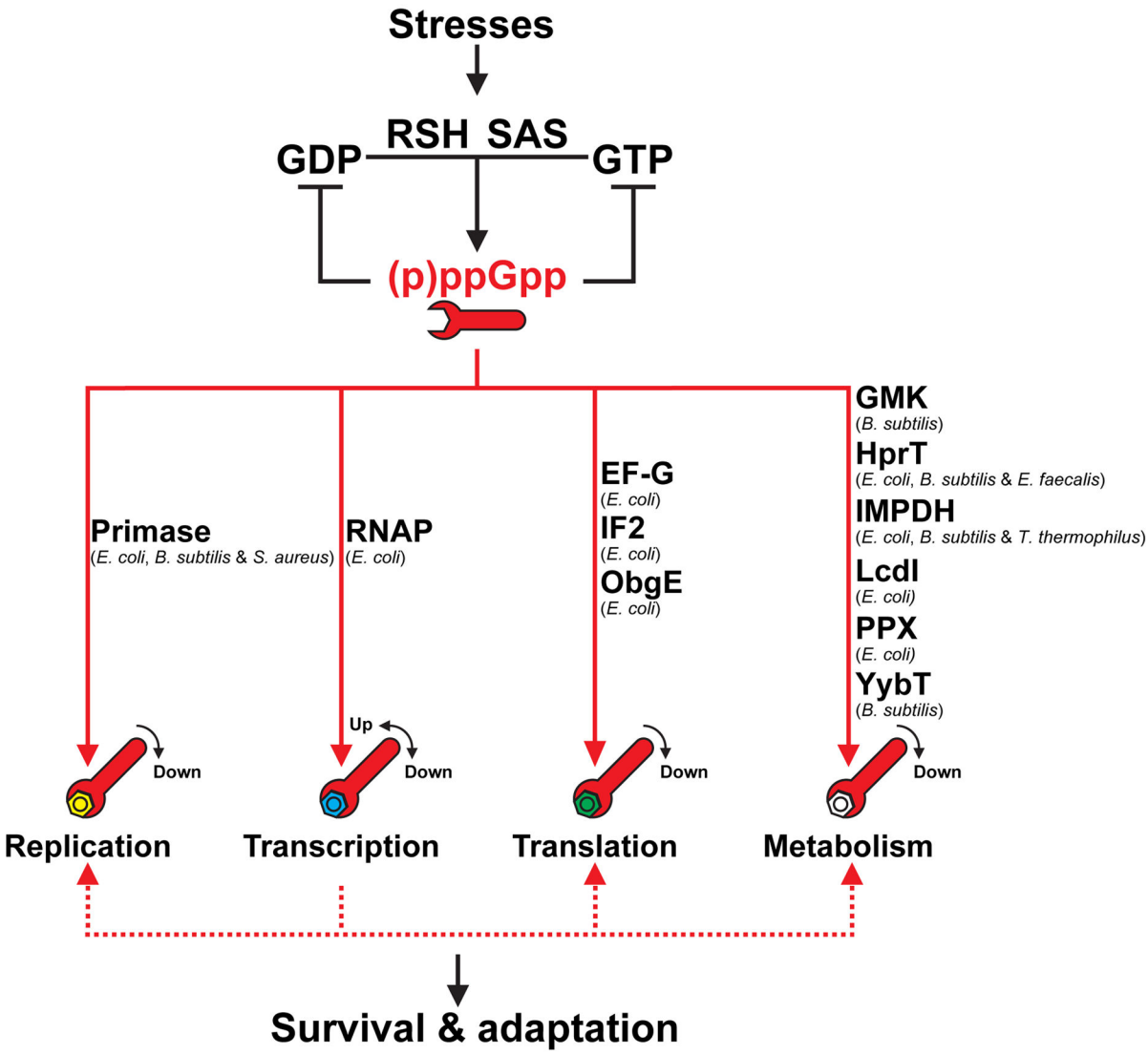

Figure 2. (p)ppGpp regulates various important cellular processes to allow survival and adaptation to stresses

Stresses, such as amino acid starvation and antibiotic treatment activate/upregulate (p)ppGpp synthetases (RSH and SAS) to produce (p)ppGpp (the wrench). In *B. subtilis*, by directly inhibiting several GTP biosynthesis enzymes, (p)ppGpp curtails production of GDP/GTP, the substrates for (p)ppGpp synthesis, thus constituting a negative feedback regulation and maintaining homeostasis of guanylate nucleotide pools [3**]. Importantly, through direct interaction with its targets, (p)ppGpp regulates replication, transcription, translation, and metabolism (differently colored bolts) to adjust the cellular physiology to survive and adapt to adverse conditions. The dotted lines indicate that (p)ppGpp also indirectly modulates replication, translation, and metabolism through its effects on transcription.