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Regulatory Themes and Variations by the Stress-Signaling Nucleotide Alarmones (p)ppGpp in Bacteria

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Keywords

pGpp, (p)ppGpp, (p)ppApp, purine biosynthesis, allosteric regulation, protein evolution

Abstract

Bacterial stress-signaling alarmones are important components of a protective network against diverse stresses such as nutrient starvation and antibiotic assault. pppGpp and ppGpp, collectively (p)ppGpp, have well-documented regulatory roles in gene expression and protein translation. Recent work has highlighted another key function of (p)ppGpp: inducing rapid and coordinated changes in cellular metabolism by regulating enzymatic activities, especially those involved in purine nucleotide synthesis. Failure of metabolic regulation by (p)ppGpp results in the loss of coordination between metabolic and macromolecular processes, leading to cellular toxicity. In this review, we document how (p)ppGpp and newly characterized nucleotides pGpp and (p)ppApp directly regulate these enzymatic targets for metabolic remodeling. We examine targets' common determinants for alarmone interaction as well as their evolutionary diversification. We highlight classical and emerging themes in nucleotide signaling, including oligomerization and allostery along with metabolic interconversion and crosstalk, illustrating how they allow optimized bacterial adaptation to their environmental niches.

1. INTRODUCTION

(p)ppGpp is a collective term for the conserved signaling nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), which are critical to cellular homeostasis and stress survival in bacteria. (p)ppGpp is synthesized from the pyrophosphorylation of guanosine diphosphate (GDP) or guanosine triphosphate (GTP) (**Figure 1*a***), and its intracellular concentration can vary from micromolar to millimolar levels in response to environmental fluctuations. Such dramatic changes in (p)ppGpp levels are dictated by different (p)ppGpp synthetases and hydrolases that respond to distinct environmental and cellular cues (70). These enzymes are broadly classified into long multidomain enzymes known as the RelA-SpoT homologs (RSHs), or single-domain enzymes known as small alarmone synthetases (SASs) or hydrolases (SAHs) (**Figure 1*b***).

While (p)ppGpp metabolizing enzymes are prevalent in the bacterial domain (7, 41), RSH orthologs have been found in plant chloroplasts and are associated with plant growth and development (87). *Drosophila*, *Caenorhabditis elegans*, and humans also contain a SAH homolog, Mesh-1, that is capable of hydrolyzing (p)ppGpp (39, 54, 88). Although (p)ppGpp can be detected in cultured cells (39), a (p)ppGpp synthetase in animals has not been identified. In addition, (p)ppGpp appears to be detrimental at high levels in eukaryotic cells (36, 39); thus, whether (p)ppGpp is an authentic signaling molecule in animals remains unclear.

In bacteria, (p)ppGpp is not only required for adaptation to amino acid starvation (66) but also critical for tolerating many other stresses (34), notably antibiotics (20) and host defenses (19, 38). In addition, (p)ppGpp has been linked to developmental processes such as sporulation (53, 56, 81), motility (1), and biofilm formation (15, 31). Due to the widespread conservation and physiological importance of (p)ppGpp in bacteria, it is an attractive target for the development of new-generation antibacterial agents (62).

The discovery of (p)ppGpp as a so-called magic spot on a thin-layer chromatography plate of radiolabeled cellular extract from starved *Escherichia coli* more than 50 years ago (13) has developed into a major topic of nucleotide signaling. Accumulated research has established canonical themes of (p)ppGpp regulation but also opened new opportunities to understand how (p)ppGpp regulation has evolved to co-opt into different cellular processes. Furthermore, new findings have begun to uncover new layers of (p)ppGpp signaling, such as self-stimulation and crosstalk, as well as an expansion of (p)ppGpp analogs and derivatives that appear to serve distinct regulatory roles. In this review, we highlight and discuss these new developments with a focus on purine synthesis, which is the heart of metabolic control by (p)ppGpp. For in-depth discussion of other specific aspects of (p)ppGpp signaling, such as (p)ppGpp synthesis and degradation (70), transcription control (32), or bacterial virulence (49), we refer readers to recent reviews on these topics.

2. COMMON THEMES AND DIVERSE STRATEGIES OF (p)ppGpp REGULATION ACROSS BACTERIAL SPECIES

In essence, (p)ppGpp across bacterial species share some common regulatory roles: reprogramming transcription, inhibiting purine metabolism, and regulating ribosome synthesis and assembly (**Figure 2**) to maintain cellular homeostasis at basal levels (4, 26, 29, 67), and, at high levels, inhibiting growth to promote survival under stress (26). However, regulation by (p)ppGpp is mediated by its direct interactions with a repertoire of protein targets (85) or specific riboswitches (75) that often differ between different bacterial species.

In many bacteria, common features of (p)ppGpp-mediated transcription changes include downregulation of ribosomal RNA expression and upregulation of amino acid biosynthesis genes (46, 73). However, (p)ppGpp achieves these same regulatory themes in different bacterial species by affecting different molecular targets. In Proteobacteria, (p)ppGpp binds directly to the RNA

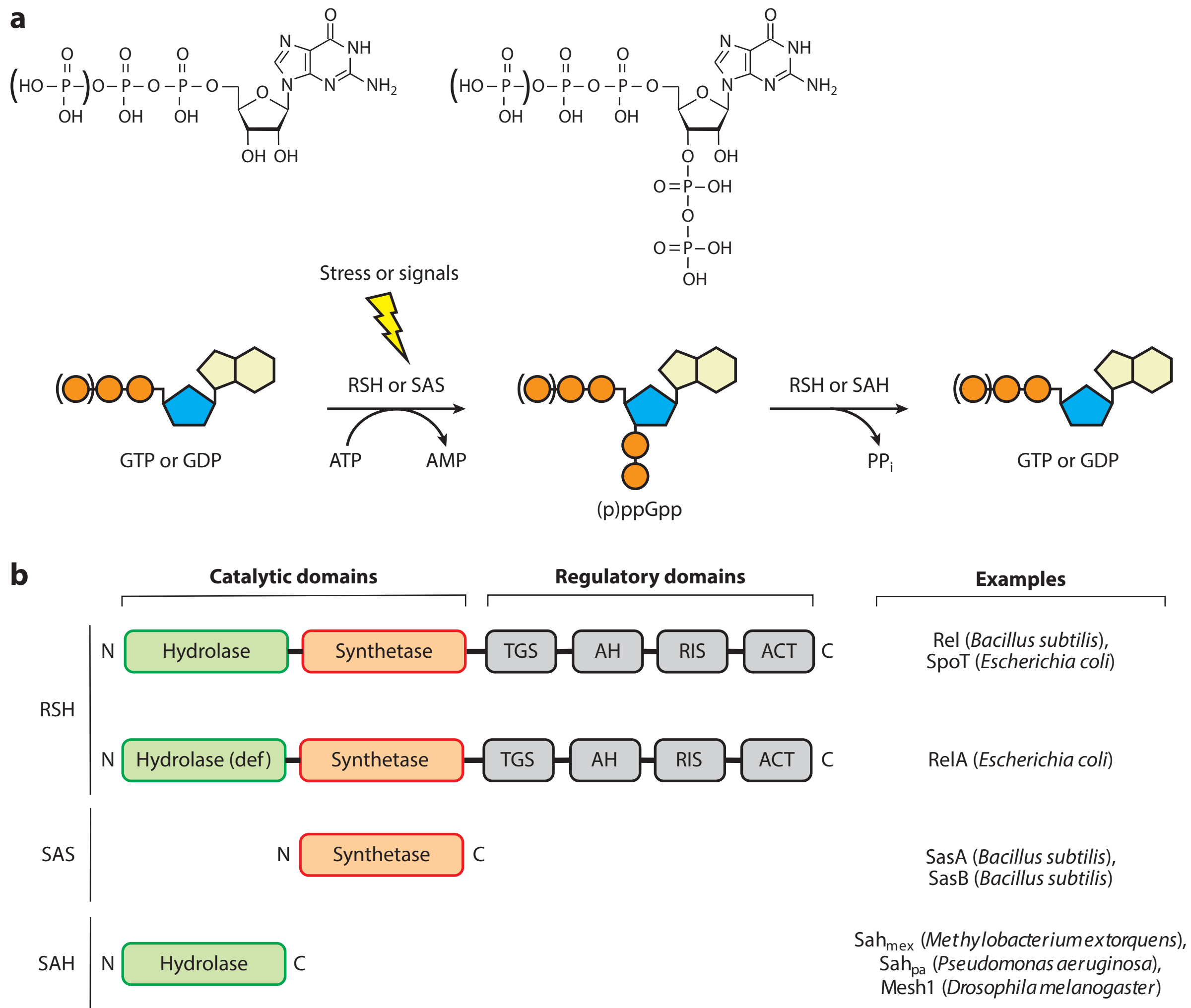

Figure 1

Synthesis and hydrolysis of (p)ppGpp. (*a*) Under stress or other cellular signals, (p)ppGpp is produced by the transfer of the pyrophosphate group from ATP to the 3′-OH of GDP or GTP. This reaction is catalyzed by different (p)ppGpp synthetases broadly classified into multidomain enzymes known as RSHs and single-domain enzymes known as SASs. Hydrolysis of (p)ppGpp is catalyzed by some RSH enzymes, as well as single-domain SAHs. PP_i and GDP or GTP are released as products of hydrolysis. Parentheses indicate the additional 5′-γ phosphate in pppGpp versus ppGpp. (*b*) Examples of RSHs, SASs, and SAHs. RSH typically contains both N-terminal (p)ppGpp synthetase and hydrolase domains, as well as various regulatory C-terminal domains. Many RSH proteins are bifunctional (p)ppGpp synthetase and hydrolase, but in some cases one of the catalytic domains is nonfunctional, which renders the RSH protein monofunctional. In contrast, SASs and SAHs contain only either a synthetase or hydrolase domain and are strict monofunctional enzymes. Abbreviations: ACT, aspartate kinase, chorismate mutase, TyrA domain; AH, alpha helical domain; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; PP_i, inorganic pyrophosphate; RIS, ribosome intersubunit domain; RSH, RelA-SpoT homolog; SAH, small alarmone hydrolase; SAS, small alarmone synthetase; TGS, ThrRS, GTPase, SpoT domain.

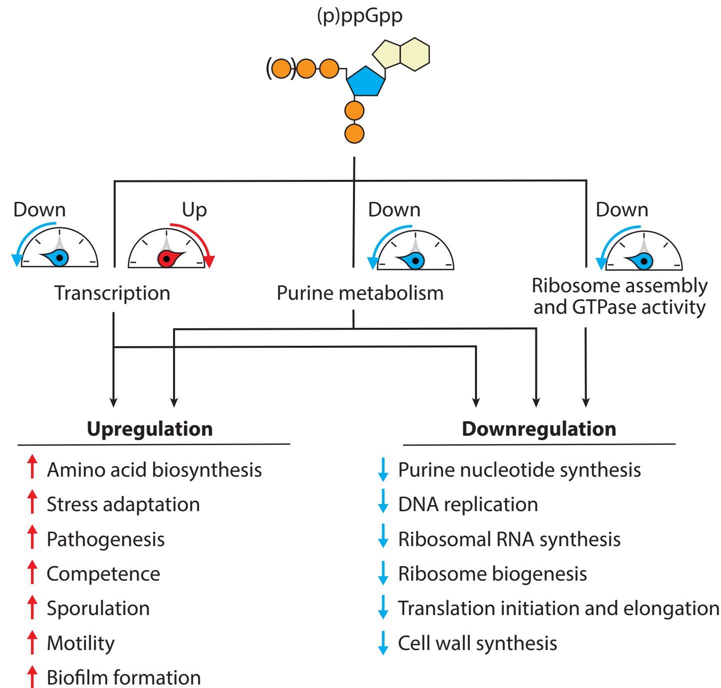

Figure 2

Themes of (p)ppGpp regulation. In bacteria, (p)ppGpp broadly regulates transcription, purine nucleotide synthesis, and macromolecule synthesis. (p)ppGpp modulates global transcription directly through RNA polymerase regulation or indirectly through global or specific transcription regulators such as CodY or PurR. In addition, (p)ppGpp downregulates purine nucleotide synthesis through direct regulation of various purine pathway enzymes, as well as slowing down macromolecular synthesis through interactions with different GTPases. It is important to note that different (p)ppGpp regulations often function in a coordinated manner to achieve the same regulatory goals. At basal levels, (p)ppGpp modulates multiple central metabolic processes to maintain optimal growth. When (p)ppGpp accumulates to high levels, such as under stress, it prepares cells for stress survival by attenuating growth and replicative processes while activating amino acid biosynthesis, stress adaptation and different developmental processes.

polymerase core enzyme complex and to the transcription factor DksA to globally regulate transcription (32, 73). In contrast, in Firmicutes, (p)ppGpp indirectly elicits similar global changes in transcription by regulating intracellular levels of GTP (45, 46, 82). (p)ppGpp also directly affects specific transcription regulators such as the purine transcription regulator PurR in Firmicutes (5), the MglA/SspA complex in *Francisella tularensis* (18), and (p)ppGpp-specific riboswitches in *Thermosediminibacter oceani* and *Desulfitobacterium hafniense* (75). These transcriptional changes lead to downregulation of macromolecular biosynthesis and upregulation of stress adaptation (**Figure 2**).

Purine metabolism is another conserved process diversely regulated by (p)ppGpp in many bacteria. (p)ppGpp downregulates the synthesis of the purine nucleotide GTP. In Firmicutes, (p)ppGpp regulates purine biosynthesis by interacting with de novo and salvage pathway enzymes, including guanylate kinase (GMK) (52), hypoxanthine phosphoribosyltransferase (HPRT) (4), and xanthine phosphoribosyltransferase (XPRT) (3), and downregulating de novo purine synthesis genes by interacting with the purine transcription regulator PurR (5). In Proteobacteria such as *E. coli*, (p)ppGpp instead inhibits the activity of the de novo purine biosynthesis enzyme

glutamine phosphoribosyl diphosphate amidotransferase (PurF) (90), purine salvage enzyme inosine-guanosine kinase (Gsk) (91), and the nucleotidase PpnN (95).

Furthermore, (p)ppGpp downregulates many key macromolecular synthesis processes, most noticeably protein translation. These can largely be classified into (*a*) downregulating the transcription of ribosomal RNA and transfer RNA, (*b*) inhibiting translation initiation and elongation through interactions with various initiation and elongation factors (9, 23, 33, 51, 55, 57, 69), and (*c*) regulating ribosome assembly by targeting GTPases in the ribosome biogenesis pathways (8, 10, 12, 16, 21, 25).

Finally, other types of macromolecular synthesis, including DNA replication (92) and cell wall synthesis, are also inhibited by (p)ppGpp (89, 93), although these processes are less well characterized.

Regulation of different processes by (p)ppGpp is intricately interconnected (**Figure 2**). For example, in Firmicutes, (p)ppGpp robustly inhibits GTP and purine biosynthesis (3–5, 52). (p)ppGpp also directly inhibits various GTPases through competition with GTP (10, 72). As a result, the lowering of GTP by (p)ppGpp synergizes its inhibition of GTPases. This duality of regulation enables synergistic and coordinated regulation of purine and macromolecule synthesis. Since the regulation of purine metabolism and GTPase activities by (p)ppGpp is highly conserved, similar modes of synergism also likely exist in many bacteria.

3. MOLECULAR DETERMINANTS OF (p)ppGpp–PROTEIN INTERACTIONS

How did different bacterial species evolve this remarkable diversity of (p)ppGpp targets underlying bacterial stress adaptation? What is their molecular basis of (p)ppGpp recognition? Recent work has elucidated and characterized the network of (p)ppGpp targets, providing insight into the mechanisms underlying (p)ppGpp's ability to mediate bacterial stress survival. Proteome-wide screens have cataloged over 50 (p)ppGpp effectors across bacterial species, including *Staphylococcus aureus*, *E. coli*, and *Bacillus anthracis* (16, 43, 90, 94, 96). However, while (p)ppGpp is ubiquitous in bacteria, there has been limited analysis of how it evolved to bind diverse proteins across bacteria.

(p)ppGpp interacts with proteins of diverse structure and function, from RNA polymerase to metabolic enzymes and translational control GTPases to transcription factors. Structural knowledge of many of these interactions has yielded themes underlying the ability of (p)ppGpp to interact with such a diverse set of proteins. As shown in **Table 1**, there are now 34 structures of (p)ppGpp-bound proteins. While not all these structures have been biologically validated, they provide insight into how this hyperphosphorylated alarmone interacts with protein targets.

3.1. (p)ppGpp-Binding Motifs and Classes of (p)ppGpp-Binding Proteins

Overall, (p)ppGpp interacts with a wide set of protein targets using diverse mechanisms instead of a single motif. Recent structures of (p)ppGpp–protein interactions have revealed trends in (p)ppGpp-binding motifs, showing that the phosphates of (p)ppGpp tend to be coordinated by the side chains of lysine, arginine, and tyrosine residues. While there are plenty of exceptions (discussed in Section 4), two classes of common binding pockets are found in many (p)ppGpp–protein interactions: (*a*) (p)ppGpp mimicking GTP to bind a GTP-binding pocket and (*b*) (p)ppGpp mimicking phosphoribosyl pyrophosphate (PRPP) to bind a PRPP-binding pocket. These two classes are responsible for the majority of characterized (p)ppGpp interactions with proteins from Gram-positive bacteria. In this section, we review how these two pockets recognize and bind (p)ppGpp.

Table 1 (p)ppGpp protein structures

PDB ID	Protein	Organism	Ligand	Binding site	ppGpp conformation
1CH8	PurA	*Escherichia coli*	ppG2′:3′p	Noninterface, active site	N/A
1LNZ	Obg	*Bacillus subtilis*	ppGpp	Noninterface, active site	Elongated
1SMY	RNA polymerase	*Thermus thermophilus*	ppGpp	Noninterface, active site	Elongated
1VJ7	Rel N-terminal domain	*Streptococcus equisimilis*	ppG2′:3′p	Noninterface, active site	N/A
2J4R	GppA	*Aquifex aeolicus*	ppGpp	Noninterface, active site	Elongated
3N75	LdcI	*E. coli*	ppGpp	Interface, allosteric	Ring-like
3VR1	Release factor 3	*E. coli*	ppGpp	Noninterface, active site	Elongated
4EDT	DnaG	*Staphylococcus aureus*	ppGpp	Noninterface, active site	Elongated
4EDV	DnaG	*S. aureus*	pppGpp	Noninterface, active site	Elongated
4HNX	NatA	*Saccharomyces cerevisiae*	ppGpp	Interface	Ring-like
4JK1	RNA polymerase (holo)	*E. coli*	ppGpp	Interface, allosteric	Elongated
4JK2	RNA polymerase (holo)	*E. coli*	pppGpp	Interface, allosteric	Elongated
4JKR	RNA polymerase	*E. coli*	ppGpp	Interface, allosteric	Ring-like
4QRH	GMK	*S. aureus*	pppGpp	Noninterface, active site	Ring-like
4XPD	NatE	*S. cerevisiae*	ppGpp	Interface	Ring-like
4Y49	NatE	*S. cerevisiae*	ppGpp	Interface	Ring-like
4ZCM	BipA	*E. coli*	ppGpp	Noninterface, active site	Ring-like
5A9Y	BipA	*E. coli*	ppGpp	Noninterface, active site	Ring-like
5DED	SasB (YjbM)	*B. subtilis*	pppGpp	Interface, allosteric	Ring-like
5U51	MglA-SspA	*Francisella tularensis*	ppGpp	Interface, allosteric	Ring-like
5VOG	Hypothetical	*Neisseria gonorrhoeae*	ppGpp	Noninterface	Elongated
5VSW	RNA polymerase–DksA	*E. coli*	ppGpp	Interface, allosteric	Ring-like
6CZF	PurF	*E. coli*	ppGpp	Interface, allosteric	Ring-like
6D9S	Hpt-1	*Bacillus anthracis*	ppGpp	Noninterface, active site	Elongated
6G14	RbgA	*S. aureus*	ppGpp	Noninterface, active site	Elongated
6G15	RbgA	*S. aureus*	pppGpp	Noninterface, active site	Elongated
6GFM	PpnN	*E. coli*	pppGpp	Interface, allosteric	Elongated
6GTM	SmbA	*Caulobacter vibrioides*	ppGpp	Noninterface	Ring-like
6PC1	GppA	*Helicobacter pylori*	ppGpp	Noninterface	Elongated
6EXO	RelP (SAS2)	*S. aureus*	pppGpp	Noninterface, active site	Ring-like
6VCL	RppH	*E. coli*	pppGpp	Noninterface, active site	Elongated
6VWP	Gsk	*E. coli*	ppGpp	Interface, allosteric	Elongated
6W1I	XPRT	*B. subtilis*	ppGpp	Interface, active site	Elongated
7RMW	PurR	*B. subtilis*	ppGpp	Noninterface, allosteric	Elongated

Abbreviations: GMK, guanylate kinase; Gsk, inosine-guanosine kinase; Hpt-1, hypoxanthine phosphoribosyltransferase; LdcI, lysine decarboxylase; PBD ID, Protein Data Bank identification; PurF, phosphoribosyl diphosphate amidotransferase; SAS, small alarmone synthetase; XPRT, xanthine phosphoribosyltransferase.

3.1.1. GTP-binding motif. Given the structural similarity between (p)ppGpp and GTP, it is perhaps unsurprising that numerous GTP-binding proteins have been identified as (p)ppGpp targets (16, 43, 90, 94, 96). These include GTPases involved in translation and ribosome assembly, of which there are four ppGpp-bound structures: *Bacillus subtilis* Obg, *E. coli* release factor 3 (RF3), *B. subtilis* RbgA, and *E. coli* BipA (12, 25, 44, 63). *B. subtilis* DNA primase, another (p)ppGpp target with a ppGpp-bound structure, also binds GTP as a substrate (72).

(p)ppGpp mimics GTP to bind GTPases at their active sites, and it interacts with a motif common to GTPases: the phosphate-binding loop (P-loop or Walker A motif). The P-loop is found in ATP- and GTP-binding proteins and coordinates the binding of the nucleotide's 5′-phosphates (74). Positively charged backbone amides of the P-loop interact with the negative charges of the phosphates. The P-loop also extends into an α-helix, and the positive dipole of the α-helix likely also helps coordinate the phosphates. The 5′-phosphates of (p)ppGpp interact with the P-loop just like the 5′-phosphates of the GTP substrate (12, 25, 44, 63). (p)ppGpp also contains 3′-phosphates that GTP lacks. In all (p)ppGpp-GTPase structures, these 3′-phosphates do not form significant interactions with the protein, and in many cases they are pointed outward away from the protein. In the case of Obg, a Mg^{2+} ion is situated between the 5′- and 3′-phosphates, helping to coordinate the 3′-phosphates (12).

The P-loop is also found in the (p)ppGpp target GMK (52). Instead of binding GTP, GMK uses the P-loop to bind ATP as a substrate. Interestingly, the P-loop in GMK binds the 3′-phosphates of pppGpp rather than its 5′-phosphates (52).

3.1.2. PRPP-binding motif. We recently identified a (p)ppGpp-binding motif in three key regulatory targets of (p)ppGpp: the GTP synthesis enzymes HPRT and XPRT and the nucleotide transcription repressor PurR (3–5) (**Figure 3**). (p)ppGpp shares this binding motif with PRPP, which is a substrate of HPRT and XPRT and an inducer of PurR. These three proteins are part of the phosphoribosyltransferase protein family and share a similar overall architecture to their

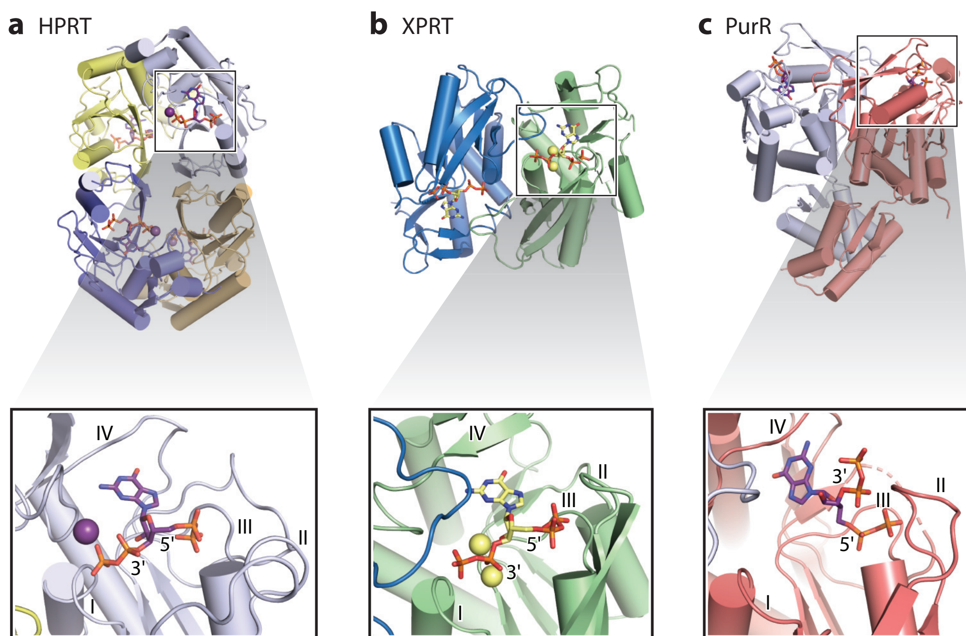

Figure 3

A shared (p)ppGpp-binding pocket. ppGpp crystallized with (*a*) *Bacillus anthracis* Hpt-1 (HPRT), (*b*) *Bacillus subtilis* XPRT, and (*c*) *B. subtilis* PurR. ppGpp binds the same binding pocket on all three proteins. I–IV refer to the loops that comprise the binding pocket. The purple sphere represents Mg^{2+}, and the yellow spheres represent Na^{+}. Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; PurR, purine biosynthesis regulator; XPRT, xanthine phosphoribosyltransferase.

PRPP/(p)ppGpp-binding pocket. The pocket comprises four loops (loops I–IV) (79). Loops I and III frame the pocket on each side. Loop III contains a previously annotated PRPP-binding motif that leads into an α-helix. Loop IV forms a hood above the binding pocket, and loop II is flexible and important for catalysis in HPRT and XPRT.

HPRT, XPRT, and PurR share common determinants that are essential for their interactions with (p)ppGpp. First and foremost, all three proteins share a PRPP-binding motif in loop III that also interacts with (p)ppGpp. This motif is able to interact with both PRPP and (p)ppGpp due to the ligands' shared ribose and 5′-phosphate moieties. The motif starts with two acidic residues that are positioned under the ribose ring and that coordinate the ribose's hydroxyls in both (p)ppGpp and PRPP. For HPRT and XPRT, one of the two acidic residues coordinates a metal ion that in turn binds the hydroxyls of (p)ppGpp's ribose. Changing these acidic residues to an alanine weakens (p)ppGpp binding to HPRT (4). The remainder of loop III extends around the 5′-phosphates of (p)ppGpp (**Figure 3**). The backbone amides along loop III form a positively charged pocket to coordinate the 5′-phosphates. Interestingly, loop III structurally resembles the phosphate-binding P-loop seen in GTPases, though not in amino acid sequence (74). Loop III also extends into the positive dipole of an α-helix, just like the P-loop. It is possible that other PRPP-binding proteins with this motif are also regulated by (p)ppGpp. For example, the interaction between (p)ppGpp and *E. coli* xanthine-guanine phosphoribosyltransferase (XGPRT) has not been structurally characterized, but (p)ppGpp likely binds XGPRT using this motif.

A second determinant common to (p)ppGpp's interaction with this class of proteins is aromatic π-stacking interactions with the guanine ring of ppGpp. For HPRT, a phenylalanine on loop IV extends above ppGpp. For XPRT and PurR, a phenylalanine forms π-stacking interactions from loop III below the guanine ring. Interestingly, in the PurR–ppGpp interaction, a tyrosine also extends out from loop IV, sandwiching ppGpp's guanine ring between a tyrosine and a phenylalanine.

Despite the shared overall architecture between the three proteins, there are also differences in how they interact with (p)ppGpp, in particular the different roles of loop II. The phosphates of (p)ppGpp extend across the binding pocket between loops I and III in HPRT and XPRT (**Figure 3*a*,*b***). But in PurR, the 5′-phosphates interact with loop III while the 3′-phosphates are pointed out and away from the binding pocket where they interact with loop II (**Figure 3*c***). Loop II in XPRT also compresses the 5′-phosphate-binding pocket, making it more difficult for pppGpp to bind compared to ppGpp and pGpp (3). Accordingly, XPRT binds pGpp and ppGpp more tightly than pppGpp (3). Loop II in HPRT does not interact with (p)ppGpp in most bacterial homologs since it is sequestered at a dimer–dimer interface and held away from the (p)ppGpp-binding pocket (4).

3.2. Allosteric Protein–Protein Interfaces as a Secondary Determinant of (p)ppGpp–Protein Interactions

In addition to the (p)ppGpp-binding pocket, allosteric interactions can also make targets sensitive or insensitive to (p)ppGpp regulation. (p)ppGpp binds the enzymes HPRT and GMK at conserved active sites (4, 52). This conservation is likely due to (p)ppGpp's near-complete overlap with substrates for binding this site—any changes in the active site residues would be detrimental to enzyme activity. Surprisingly, however, (p)ppGpp does not regulate all HPRTs and GMKs. This is due to oligomeric interactions in the HPRT and GMK enzymes altering the architecture of the (p)ppGpp-binding pocket from a distance. For HPRT, a dimer–dimer interface holds a flexible loop away from the binding pocket, and, in GMK, a lid domain that covers the binding pocket interacts with an adjoining monomer. Remarkably, HPRTs and GMKs that lack regulation by

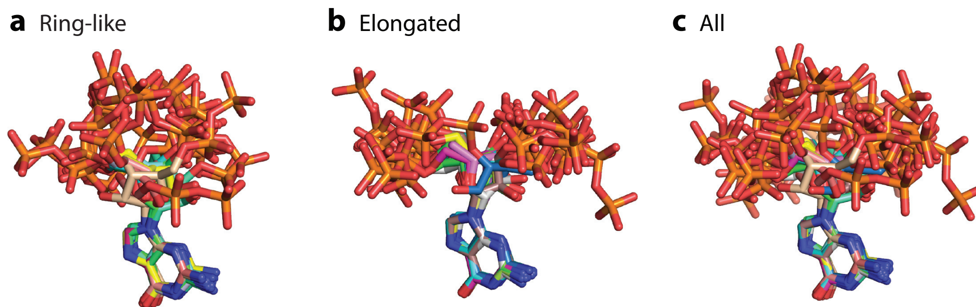

Figure 4

An alignment of elongated and ring-like conformations of ppGpp and pppGpp molecules crystallized with proteins. These molecules take two major forms: (*a*) ring-like and (*b*) elongated. In panel *a*, 14 molecules are aligned; in panel *b*, 14; and in panel *c*, 28. ppG2′:3′p molecules (PDB IDs 1CH8 and 1VJ7) and ppGpp molecules with multiple conformations in the same crystal (PDB IDs 3N75 and 6CZF) were excluded. See **Table 1** for more information.

(p)ppGpp have evolved residues at these interfaces to allosterically influence (p)ppGpp binding instead of evolving the binding pocket itself (4).

These results suggest that evolving oligomeric interactions can be a mechanism for evolving (p)ppGpp–protein interactions. Importantly, (p)ppGpp does not bind HPRT or GMK at an interface binding site, yet altering interface residues can control (p)ppGpp binding. Evolving interfaces to allosterically alter (p)ppGpp binding may allow proteins to sample more functional space while retaining enzymatic function. Active sites, and ligand binding sites in general, undergo slower evolution due to the constraints placed on their functions (24, 59). Interfaces are more rapidly evolvable, especially when one amino acid change results in two changes at a symmetrical interface (22, 24). Importantly, this mechanism is extendable to other ligand–protein interactions beyond (p)ppGpp.

3.3. (p)ppGpp Adopts Flexible Conformations in Binding Diverse Pockets

(p)ppGpp takes two major conformations when interacting with proteins (83). In one conformation, its phosphate arms can form a compact Y shape in a so-called ring-like conformation (**Figure 4*a***). Otherwise, the phosphate arms can extend away from one another in a T shape, or elongated, conformation (**Figure 4*b***). Of the (p)ppGpp conformations in current crystal structures, about half are ring-like and half are elongated (**Table 1**).

The main theme arising from (p)ppGpp's conformations is that it is highly flexible. The phosphate arms can be compact or extended, and rotation around the C1′–N9 bond connecting the ribose and guanine ring further increases the number of possible (p)ppGpp conformations. Accordingly, aligning 26 (p)ppGpp molecules by their guanine ring and ribose reveals a veritable tree-like diversity in the conformations of the phosphate arms (**Figure 4*c***). These observations were recently supported by molecular dynamics analyses, which suggested that the two linear arms of (p)ppGpp make it much more flexible than other signaling nucleotides (50). (p)ppGpp's ability to interact with a diversity of proteins in such diverse conformations tells us that there is likely much more to learn about its breadth of interaction partners.

It has been observed that the ring-like conformation of (p)ppGpp is associated with higher affinity interactions (83). For example, (p)ppGpp has inhibition or binding constants <10 μM for lysine decarboxylase (LdcI) and GMK (42, 52). On the other hand, (p)ppGpp has inhibition

constants >30 μM for the primase DnaG, RF3, and the GTPase BipA (25, 44, 72). Ring-like (p)ppGpp structures tend to coordinate a metal ion (e.g., Mg^{2+} or Mn^{2+}) between the phosphate arms, possibly explaining the increased coordination and binding in the ring-like form. However, not all elongated conformations of (p)ppGpp bind weakly: (p)ppGpp binds HPRT and XPRT in an elongated conformation with a binding constant around 1 μM (3, 4).

4. (p)ppGpp REGULATION BEYOND THE CATALYTIC SITE: (p)ppGpp AS AN ALLOSTERIC EFFECTOR AND OLIGOMERIZATION AS A MECHANISM CONTROLLING (p)ppGpp BINDING

(p)ppGpp inhibits many enzymes by mimicking substrates to compete with them for binding the active site. This includes enzymes such as translational GTPases and their substrates guanosine diphosphate (GDP), GMK and its substrate guanosine monophosphate (GMP), and HPRT and XPRT and their substrates of PRPP and nucleobases. However, (p)ppGpp control of enzyme activity and protein function extends beyond the active site. Here we review how (p)ppGpp can act as an allosteric effector by binding sites away from the active site. We also consider the underappreciated factor of protein oligomerization controlling (p)ppGpp binding.

4.1. (p)ppGpp as an Allosteric Effector

(p)ppGpp regulates many proteins allosterically by binding protein–protein interfaces. Like classic allosteric regulation of protein activity, oligomerization allows for signal transduction from the (p)ppGpp-binding site. (p)ppGpp allosterically regulates enzyme activity by binding at allosteric sites distant from the active site. In some cases, (p)ppGpp allosterically activates enzyme activity by changing the conformation of the active site to increase affinity for substrate binding. This has been demonstrated for the small alarmone synthetase SAS1 in Firmicutes and the nucleosidase PpnN in *E. coli* (84, 95). In other cases, (p)ppGpp allosterically inhibits enzyme activity by reducing substrate binding to the active site. PurF, LdcI, and Gsk are allosterically inhibited by (p)ppGpp in this fashion (42, 90, 91).

(p)ppGpp also binds interface-binding pockets to allosterically affect transcription. For example, (p)ppGpp binds the *E. coli* RNA polymerase at two interfaces: the ω and β′ and the β′ and DksA interfaces (58, 71). By binding the heterooligomeric β′ and DksA interface, (p)ppGpp augments transcription depending on the promoter sequence (58, 71). (p)ppGpp can also promote heterooligomerization to affect transcription. In *F. tularensis*, (p)ppGpp binds the interface of MglA and SspA, promoting their association and recruiting the transcription factor PigR. This activates the expression of a pathogenicity island (14, 18).

4.2. Protein Oligomerization and (p)ppGpp Binding

As reviewed in the previous section, there is now evidence that protein oligomeric interfaces have evolved to affect (p)ppGpp binding. This phenomenon likely allows for greater flexibility in (p)ppGpp binding to its targets across bacteria. In some cases, this flexibility creates allosteric linkages between (p)ppGpp and protein function. In other cases, it can be the difference between strong and weak ligand binding. There are two broad mechanisms underlying this phenomenon. First, (p)ppGpp binds at a pocket at the protein interface, linking (p)ppGpp binding to the oligomerization of the protein. Second, oligomerization can allosterically influence the conformation of (p)ppGpp-binding pockets not at protein interfaces.

By binding protein interfaces, (p)ppGpp can promote oligomeric interactions. It enables the heterooligomerization of MglA and SspA in *F. tularensis* and DksA and RNA polymerase in

E. coli (18, 71). (p)ppGpp also promotes homooligomerization of Gsk in *E. coli* and XPRT in *B. subtilis*. (p)ppGpp binding to Gsk promotes the association of two dimers to form a homotetramer (91). It restructures the N-terminal and C-terminal sequences in a homodimer to create a new tetramerization interface. Altering residues at this new homotetrameric interface results in a protein that cannot tetramerize and is less sensitive to ppGpp inhibition (91). For XPRT in *B. subtilis*, (p)ppGpp links two monomers together to form a stable dimer by binding residues across the monomer–monomer interface (3).

While in nearly all cases (p)ppGpp binds interface sites as an allosteric effector, the (p)ppGpp target *B. subtilis* XPRT is a unique target where (p)ppGpp binds the enzyme's active site but its binding also requires oligomeric interface residues (3). Two (p)ppGpp-binding pockets face one another across an XPRT monomer–monomer interface (**Figure 3*b***). A bridging loop extends across this interface, and (p)ppGpp binding relies on interactions with this bridging loop. (p)ppGpp completes a network of electrostatic interactions bridging the monomer–monomer interface. Accordingly, (p)ppGpp promotes the dimerization of XPRT monomers, and making substitutions at key residues in the bridging loop that interact with (p)ppGpp completely abolishes (p)ppGpp binding. (p)ppGpp's interaction with the bridging loop at the interface makes (p)ppGpp binding to XPRT cooperative. This is likely due to (p)ppGpp strengthening interactions across the monomer–monomer interface, which promotes monomer–monomer association and (p)ppGpp binding to the second subunit.

Perhaps most strikingly, the oligomerization of HPRT, another PRT that is a (p)ppGpp target, dictates its sensitivity to (p)ppGpp through an indirect allosteric mechanism (4). HPRT tetramerization sequesters the flexible loop II away from the (p)ppGpp-binding pocket, allowing (p)ppGpp to bind and inhibit at low concentrations (**Figure 3*a***) (4). Disrupting the dimer–dimer interface at residues distant from the active site results in weakened (p)ppGpp binding, likely due to loop II blocking (p)ppGpp access to the binding pocket. Some bacterial HPRTs have evolved distinct residues at the dimer–dimer interface, causing them to lose the ability to tetramerize. The structures of these HPRTs demonstrate that loop II is shifted over the (p)ppGpp-binding pocket. The sequestration of loop II at a dimer–dimer interface is not seen in XPRT or in *B. subtilis* PurR (3, 5). Both proteins appear to function as dimers, meaning that loop II is not at a protein interface (**Figure 3*b*,*c***) (6, 78). Therefore, even these three related proteins that bind both (p)ppGpp and PRPP have evolved different mechanisms to interact with (p)ppGpp for controlling their function.

(p)ppGpp binding to its targets HPRT and XPRT exemplifies the diversity of ways that protein oligomerization affects (p)ppGpp binding. While HPRT and XPRT use a common motif and a binding pocket with similar overall architecture to interact with (p)ppGpp, there are distinct differences in how (p)ppGpp binds and regulates each protein.

4.3. (p)ppGpp Synthesis Control Through Oligomerization

The oligomerization of (p)ppGpp-interacting proteins not only led to the diversification of (p)ppGpp regulation but also the control of (p)ppGpp synthesis itself (**Figure 5*a***). Although (p)ppGpp synthesis is typically activated in response to stresses, it is now known that (p)ppGpp itself can also stimulate its own synthesis. For example, it was revealed that the small alarmone synthetase SasB (YjbM) is a tetramer with two intersubunit allosteric sites that, when bound by pppGpp, cooperatively stimulate ppGpp synthesis activity (84). It has also been reported that (p)ppGpp synthesis by RSH protein RelA can be stimulated by pppGpp in vitro in *E. coli* (48, 77). In the case of SasB, the concentration of pppGpp that leads to synthetase activation is roughly ~10 to 50 μM with a Hill coefficient of ~3.0 (84), suggesting cooperativity in (p)ppGpp synthesis. While the physiological role of cooperative (p)ppGpp synthesis remains largely unexplored, our

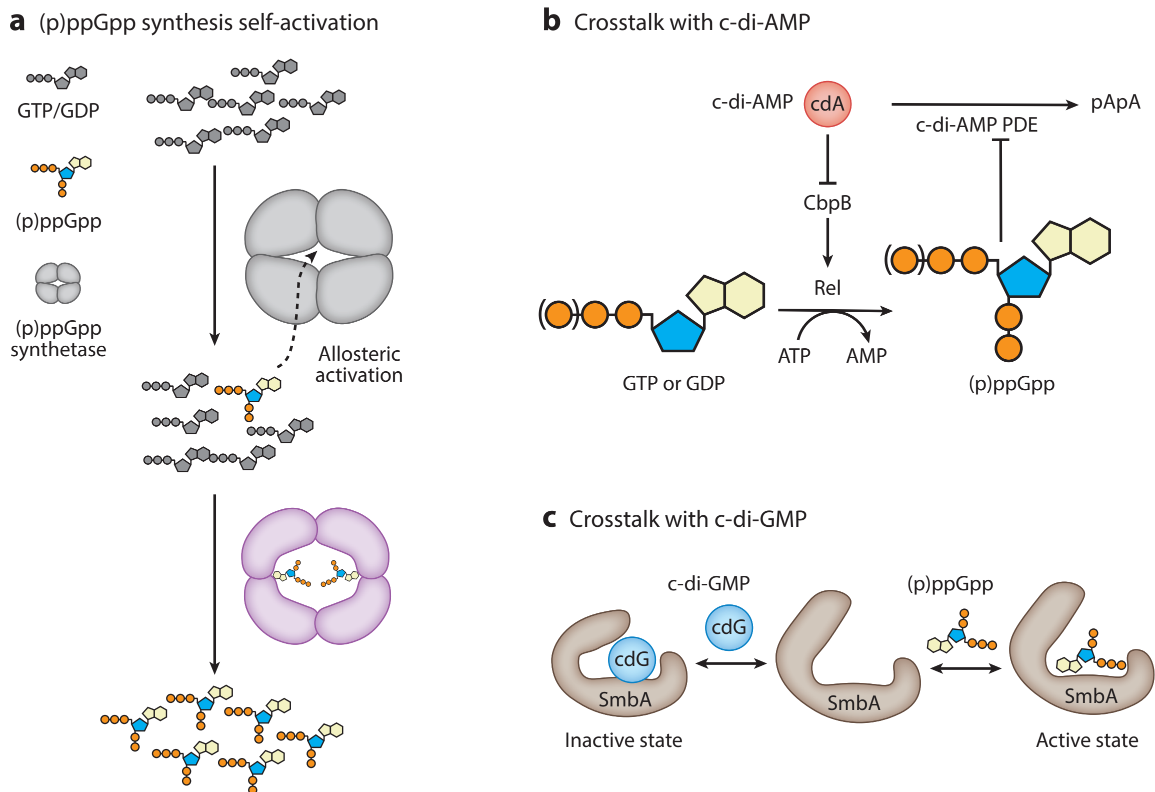

Figure 5

Additional layers of (p)ppGpp regulation. (*a*) Self-activation of (p)ppGpp synthesis. (p)ppGpp can allosterically bind to specific (p)ppGpp synthetases, such as SasB in *Bacillus subtilis* or *Staphylococcus aureus*, to stimulate its (p)ppGpp synthesis. This self-activation allows the rapid amplification of (p)ppGpp and potentially cell-to-cell heterogeneity in (p)ppGpp levels. (*b*) Crosstalk between (p)ppGpp and c-di-AMP signaling. In *Listeria monocytogenes*, c-di-AMP can modulate (p)ppGpp synthesis by Rel through interaction with CbpB. Additionally, (p)ppGpp can inhibit the degradation of c-di-AMP through the inhibition of specific c-di-AMP PDE in *L. monocytogenes* and *S. aureus*. (*c*) Crosstalk between (p)ppGpp and c-di-GMP. In *Caulobacter*, both (p)ppGpp and c-di-GMP compete for the same binding pocket of SmbA, which determines the activity states of the protein. Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; c-di-AMP, cyclic di-AMP; c-di-GMP, cyclic di-GMP; GDP, guanosine diphosphate, GMP, guanosine monophosphate; GTP, guanosine triphosphate; PDE, phosphodiesterase.

recent study suggested that it contributes to the generation of (p)ppGpp heterogeneity in single cells to promote survival against antibiotic assault (27).

5. ADDITIONAL LAYERS OF (p)ppGpp REGULATION: CROSSTALK BETWEEN DIFFERENT NUCLEOTIDE MESSENGERS AND (p)ppGpp-RELATED ALARMONES

5.1. Crosstalk Between (p)ppGpp and Cyclic Second Messenger Nucleotides

Recent studies on (p)ppGpp regulation also extend to the interfaces of (p)ppGpp and other signaling nucleotides. In addition to (p)ppGpp, bacteria utilize signaling nucleotides such as cyclic AMP (c-AMP), cyclic di-AMP (c-di-AMP), and cyclic di-GMP (c-di-GMP) (35, 40, 86) as second messengers. Intriguingly, (p)ppGpp and cyclic dinucleotides can crosstalk to modulate their synthesis and hydrolysis (**Figure 5*b***) or function as competitors for target regulation (**Figure 5*c***). In

Firmicutes, (p)ppGpp inhibits c-di-AMP phosphodiesterases in *S. aureus* (17) and *Listeria monocytogenes* (37). Additionally, c-di-AMP can prevent (p)ppGpp synthesis by binding to the (p)ppGpp synthetase RelA activator CbpB in *L. monocytogenes* (65) and *B. subtilis* (47). These two regulatory interactions result in an overall negative feedback to complete a homeostatic regulatory circuit between c-di-AMP and (p)ppGpp (65). In contrast, it has been shown that c-di-AMP can increase (p)ppGpp synthesis by RelA through an unknown mechanism in *S. aureus* (17), thus potentially forming a positive feedback circuit in this organism.

Another type of crosstalk was recently discovered in *Caulobacter crescentus* where (p)ppGpp competes with c-di-GMP for the same binding site in SmbA, and they respectively toggle the active and inactive states of the protein (76). Thus, the interactions of different signaling systems likely allow flexible coordination of cellular processes.

5.2. Expanding the Alarmone Vocabulary: pGpp, pApp, ppApp, and pppApp

While ppGpp and pppGpp are well-established alarmones, it has been long reported that bacteria can also produce other nucleotide derivatives, such as pGpp (60) and (p)ppApp (60, 68). However, due to limited understanding of their metabolism and regulatory functions, whether these nucleotides are bona fide alarmones remained an open question until recent breakthroughs, which are discussed below.

5.2.1. (pp)pApp. Recent findings revealed that bacterial alarmone synthetases can produce (p)ppGpp analogs such as pGpp, ppApp, and pppApp with key functions (**Figure 6*a*,*b***). The *Pseudomonas* type VI toxin Tas1 is a potent synthetase of pApp, ppApp, and pppApp using cellular ATP as the substrate (2). Tas1 is secreted to a target bacterial cell where it produces (pp)pApp massively to deplete cellular ATP pools and inhibit growth in target cells. A recent genome survey on alarmone synthetases uncovered toxin-antitoxin pairs in other species of bacteria, such as the FaRel toxin from *Cellulomonas marina*, that can also synthesize ppApp (41).

Although (pp)pApp's best-recognized role is a toxin product, evidence suggests that (pp)pApp may also be produced and function as a bona fide nucleotide alarmone. ppApp and pppApp can be synthesized not only by toxins but also by the endogenous bacterial alarmone synthetases. In an early study, Rhaese et al. (68) reported that *B. subtilis* ribosome preparations (presumably containing the RSH protein RelA) harvested during the early stage of sporulation synthesize (p)ppApp instead of (p)ppGpp, and recent studies showed that the small alarmone synthetase SasA from firmicutes can synthesize not only ppGpp but also ppApp in vitro (Gert Bange, personal communication) and in vivo (28). Similarly, RSH protein in *Methylobacterium extorquens* can synthesize both (p)ppGpp and pppApp (80). Second, (p)ppApp has regulatory targets similar to those of (p)ppGpp. (p)ppApp binds and inhibits PurF in *E. coli* in a similar fashion as (p)ppGpp (2), suggesting an overlapping function between the two alarmones. In addition, (p)ppApp interacts with RNA polymerase at a location distinct from that of (p)ppGpp in *E. coli* (11). The same study also found that (p)ppApp activates transcription from the *rrnB* P1 promoter in contrast to inhibition by (p)ppGpp (11), suggesting that (p)ppApp can share the same target as (p)ppGpp but exhibit a different regulatory effect.

5.2.2. pGpp. Recently, pGpp, a small analog of (p)ppGpp with a reduced number of 5′-phosphates (**Figure 6*a***), has emerged as a bona fide alarmone relevant for cellular physiology in certain bacterial phyla, including Firmicutes (30). pGpp can be detected consistently by liquid chromatography–mass spectrometry (LC-MS) in *B. subtilis* cells both during homeostatic

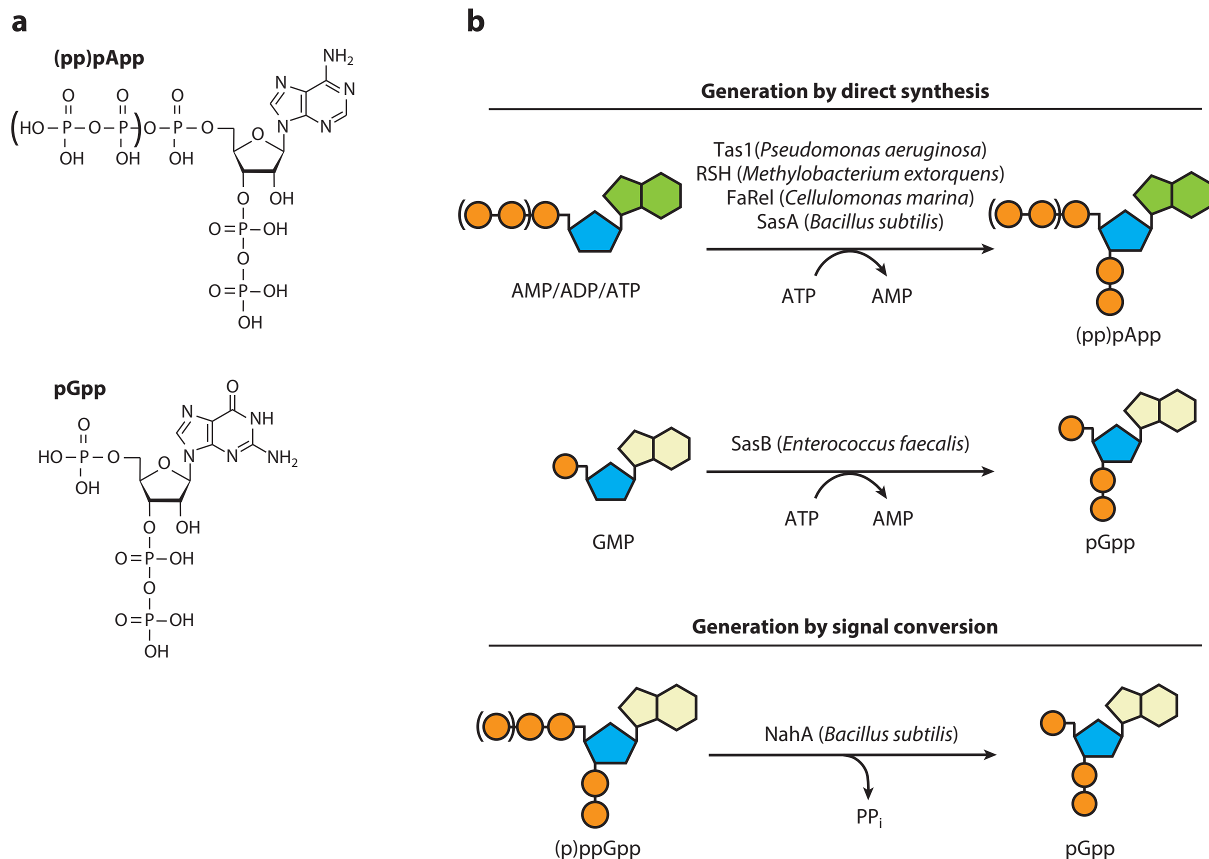

Figure 6

Emerging (p)ppGpp-related alarmones. (*a*) Chemical structures of (pp)pApp and pGpp. Parentheses indicate the additional 5′-γ phosphates in pppApp versus ppApp or pApp. (*b*) Synthesis mechanisms of (p)ppGpp-related alarmones. pApp, ppApp, and pppApp [collectively (pp)pApp] synthesis is catalyzed by some (p)ppGpp synthetases from adenosine nucleotides AMP, ADP, or ATP using ATP as the pyrophosphate donor. Similarly, pGpp can be directly synthesized from GMP and ATP, such as by SasB in *Enterococcus faecalis*. Alternatively, pGpp can also be produced from (p)ppGpp by Nudix hydrolases such as NahA in *Bacillus subtilis*. Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GMP, guanosine monophosphate; PP_i, inorganic pyrophosphate; RSH, RelA-SpoT homolog.

growth and upon starvation (94). pGpp can be produced via two pathways: It can be directly produced by certain (p)ppGpp synthetases using GMP and ATP as substrates (30, 64) and, alternatively, by enzymatic conversion from (p)ppGpp by the Nudix hydrolase NahA in *B. subtilis* (94). NahA efficiently produces pGpp by hydrolyzing (p)ppGpp, thus modulating alarmone composition and function. Importantly, proteomic screens to identify proteins that interact with pGpp in *B. anthracis* revealed that, contrary to ppGpp and pppGpp, which interact both with purine biosynthesis proteins and with GTPases that control translation, pGpp interacts with purine biosynthesis proteins but not with the GTPases. These results, together with prior work in *Enterococcus faecalis* (30) and *Thermus thermophilus* (61), support the existence and physiological relevance of pGpp as a third alarmone, with functions that can be distinct from those of (p)ppGpp, to fine-tune bacterial stress responses.

6. FUTURE DIRECTIONS

The recent findings that different nucleotide alarmones signal stresses by regulating diverse targets, enabling allostery and cooperativity, and crosstalking with one another, implicates a broader involvement of nucleotide signaling metabolic remodeling in stress responses. In recent years, a wealth of information regarding both the nucleotide signaling network and the proteomic network has been obtained. However, there are extensive unidentified interactions between the two networks that are important for physiological regulation. They form rapid, accurate, and efficient signal processing feedback circuitries as the first response, before long-term adaptation takes place through gene regulation. Future endeavors to map these interactions and to delineate the expanded stress networks will eventually provide ammunition against pathogens through compromising their signaling.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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