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(p)ppGpp: Still Magical?*

Katarzyna Potrykus and Michael Cashel

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-2785; email: potrykuk@mail.nih.gov; mcashel@nih.gov

Annu. Rev. Microbiol. 2008. 62:35–51

First published online as a Review in Advance on May 2, 2008

The *Annual Review of Microbiology* is online at micro.annualreviews.org

This article's doi:
10.1146/annurev.micro.62.081307.162903

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0066-4227/08/1013-0035$20.00

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Key Words

stringent response, transcription, stress, pathogenesis, Rel/Spo homologs

Abstract

The fundamental details of how nutritional stress leads to elevating (p)ppGpp are questionable. By common usage, the meaning of the stringent response has evolved from the specific response to (p)ppGpp provoked by amino acid starvation to all responses caused by elevating (p)ppGpp by any means. Different responses have similar as well as dissimilar positive and negative effects on gene expression and metabolism. The different ways that different bacteria seem to exploit their capacities to form and respond to (p)ppGpp are already impressive despite an early stage of discovery. Apparently, (p)ppGpp can contribute to regulation of many aspects of microbial cell biology that are sensitive to changing nutrient availability: growth, adaptation, secondary metabolism, survival, persistence, cell division, motility, biofilms, development, competence, and virulence. Many basic questions still exist. This review tries to focus on some issues that linger even for the most widely characterized bacterial strains.

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(p)ppGpp: guanosine 5′-triphosphate, 3′-diphosphate; guanosine 5′-diphosphate, 3′-diphosphate

RSH proteins: proteins with Rel and Spo homology

INTRODUCTION

Nearly 40 years ago two spots appeared on autoradiograms, as if by magic, from extracts of *Escherichia coli* responding to the stress of amino acid starvation. This response provokes stringent inhibition of stable RNA (rRNA and tRNA) synthesis that is greatly relaxed in *relA* mutants. These spots, first called magic spots, were derivatives of GTP and GDP that differed only by the presence of a pyrophosphate esterified to the ribose 3′ carbon, abbreviated as pppGpp and ppGpp, respectively. Currently we know that (p)ppGpp signals nutritional stress, leading to adjustments of gene expression in most bacteria and plants. If magic can be defined as ignorance of how something happens and how it works, then much of the magic of (p)ppGpp is not lost. This is because fundamental details regarding (p)ppGpp remain uncertain in the best-studied bacterial strains, let alone the diverse bacteria that exploit this regulator in different ways. This is too broad a topic to review here; recent reviews are highly recommended (11, 40, 56, 72).

ALMOST A SINGLE SUPERFAMILY OF ENZYMES EXISTS FOR (p)ppGpp SYNTHESIS AND BREAKDOWN

The sequenced genomes of free-living eubacteria and plants contain one or more variants of *rsh* (Rel Spo homolog) genes. These genes encode large (∼750 amino acid) RSH proteins (**Figure 1**). The namesakes for RSH are the RelA and SpoT proteins of *E. coli*; two apparently similar RSH proteins exist among other beta- and gamma-proteobacteria, whereas most other bacteria have a single RSH protein, designated Rel with species names, such as Rel*Mtb*. RSH variants can have end extensions as well as insertions. Small fragments with weakly active synthase have been discovered in *Streptococcus mutans* and *Bacillus subtilis*, but their functions are unknown (39, 50). Similar sequences coexist generally in genomes of the class *Firmicutes* (e.g., bacilli, streptococci, staphylococci, *Listeria*, clostridia) together with a full-length RSH protein. Found among the first sequenced *Rickettsia* genomes are multiple *rsh* fragments whose activities are untested.

There is a small, secreted enzyme from *Streptomyces morookaensis* with no obvious homology to RSH proteins. Under special conditions this enzyme, once a commercial source for (p)ppGpp and (p)ppApp, transfers pyrophosphate residues indiscriminately to ribonucleoside 5′ mono-, di-, and triphosphates as well as synthesizes nucleotides with a 5′

polyphosphate, 2′,3′-cyclic monophosphate (46 and references therein). Such compounds unexpectedly are found in protein crystals and probably are biologically important.

(p)ppGpp Hydrolases and Synthases

The N-terminal half of generic RSH proteins contains catalytic activity domains for hydrolase and synthase. The RelA protein has only synthase activity; its hydrolase is inactive (**Figure 1*b***, left). RSH synthases in general have similarities to polymerases, such as DNA polymerase beta (30). RSH (p)ppGpp hydrolases are Mn^{2+}-dependent pyrophosphohydrolases with a conserved His-Asp (HD) motif (1, 30). For the SpoT protein, sequence variants limit its synthase activity, but not its hydrolase (**Figure 1*b***, right). Thus, RelA is viewed as specialized for synthesis because of an inactive HD domain sequence and SpoT is viewed as specialized for hydrolysis with a weak synthase. Separate engineered peptides for hydrolase and synthase are active, at least for Rel*Mtb* and Rel*Seq* (3, 30), despite an early report of overlapping functions that were deduced from behavior of progressive deletions in SpoT (24). Point mutants that define each domain can help predict activities of new RSH enzymes from their sequence (30).

Regulating the balance of the opposing activities of RSH enzymes is crucial. Equally active, unregulated hydrolase and synthase activities would catalyze a futile cycle of (p)ppGpp synthesis and hydrolysis (4, 43). Too much synthase elevates (p)ppGpp, which provokes a stringent response, inhibits growth and, in *E. coli*, adjusts gene expression to curtail unnecessary activities in nongrowing cells. Too little (p)ppGpp from excess hydrolase makes cells less able to respond appropriately to nutritional stress.

How Are RSH Activities Regulated?

Results from experiments with various RSH proteins indicate that both the N-terminal domain (NTD) and the C-terminal domain (CTD) can contribute to regulation. Synthase activation (RelA and bifunctional RSH enzymes) (**Figure 1*b*,*c***) seems to occur by a common signal. This involves sensing the inability of tRNA aminoacylation to keep up with the demands of protein synthesis, typically provoked in vivo by amino acid starvation or by adding inhibitors of aminoacyl tRNA synthases. Early in vitro experiments elegantly defined the ribosome idling reaction during elongation (27); this was verified for the Rel*Mtb* enzyme (4) with ribosomal activation components (RAC) by using puromycin-treated ribosomes, poly U, and uncharged Phe-tRNA.

The synthase catalytic sites of monofunctional (RelA-like) enzymes have a conserved acidic triad of residues (ExDD) that differs from the conserved basic (RxKD) triad found for bifunctional RSH proteins (64). The authors report that three crucial properties of the NTD synthases sort with the two sequences, even in chimerical enzymes: a mono/dual metal mechanism, a broad/sharp Mg^{2+} optimum for substrate binding, and a major helicity change. Accordingly, one must wonder whether the presence of an active hydrolase constrains synthase catalysis or vice versa. If so, then substituting the acidic triad of monofunctional enzymes for bifunctional RSH proteins (and the reverse) might alter cellular hydrolase or synthase regulatory properties.

Despite the availability of detailed ribosomal structures, little is known of the interactions between RelA or RSH and ribosomes, except that ribosomal mutants of the L11 protein (termed RelC) abolish activation. For RelA and Rel*Mtb*, point mutants in the CTD as well as CTD deletions abolish activation under RAC conditions, hinting an activation pathway from ribosome to CTD to NTD. An interesting regulatory role proposed for the CTD of RelA and Rel*Mtb* involves oligomerization (3, 25).

The conserved TGS region of the CTD (**Figure 1*a***) has now been implicated in the regulation of the strong hydrolase with a weak synthase of SpoT. The ability of SpoT to sense many sources of nutrient stress other

RelA: *E. coli* protein that activates (p)ppGpp synthesis during amino acid starvation

Rel*Mtb*: RSH enzyme from *M. tuberculosis*

SpoT: *E. coli* protein that mediates (p)ppGpp elevation during other nutrient stress

Stringent response: positive and negative effects on cells by elevated (p)ppGpp

RAC: ribosomal activation components

TGS: conserved domain on RSH CTD for uncharged ACP binding

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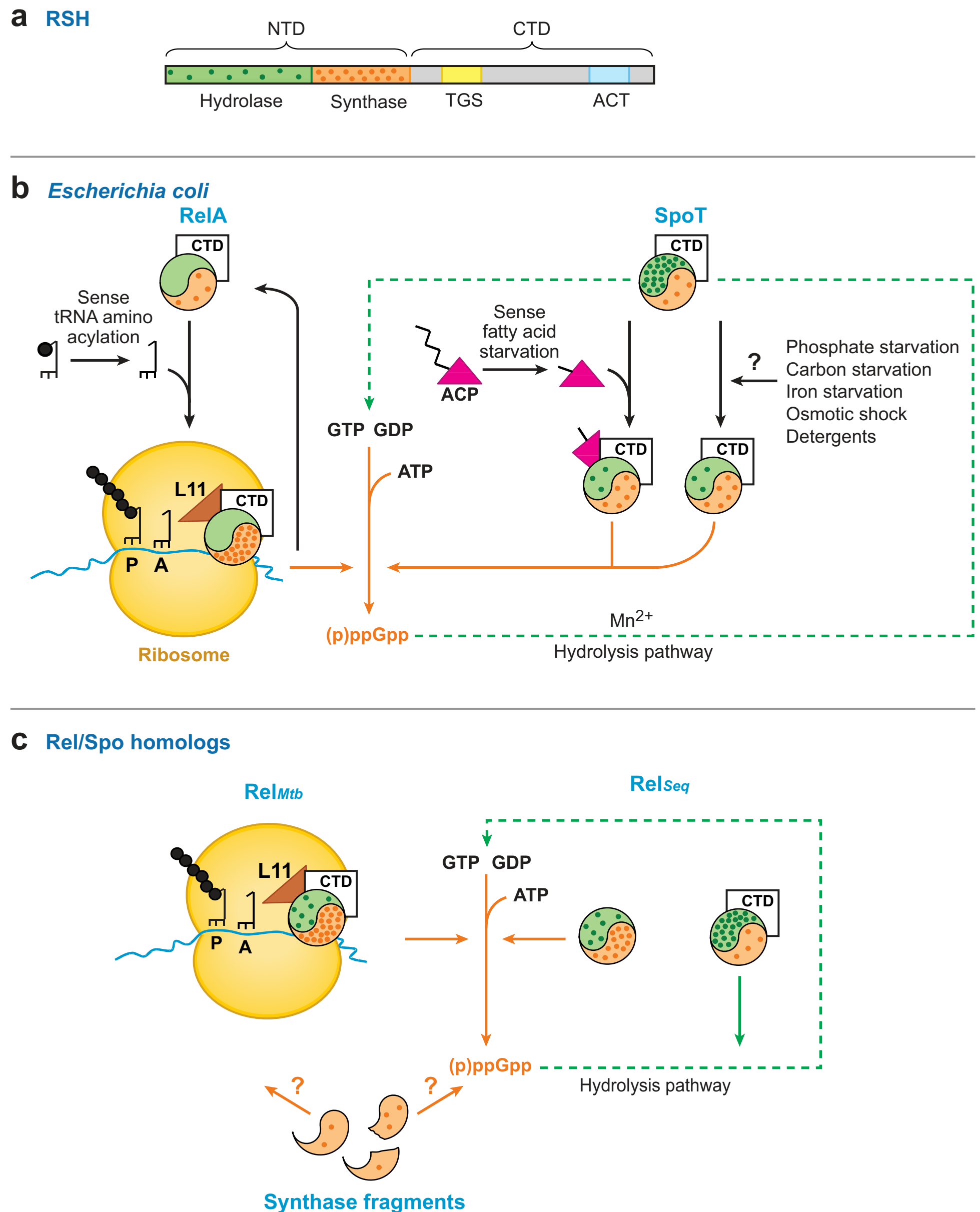
than amino acid starvation and to respond by limiting hydrolase has long been puzzling (**Figure 1*b***, middle). An exciting mechanism allowing SpoT to sense fatty acid synthesis limitation has been discovered (6). The acyl carrier protein (ACP) binds to the TGS domain of SpoT and this binding is probably influenced by the ratio of unacylated ACP to acylated ACP in the cell. Fatty acid starvation thus leads to a shift in the balance of the two SpoT activities in favor of synthesis. The authors point to parallels between SpoT and RelA sensing. They also raise the possibility that sensing uncharged (unacylated) ACP might explain SpoT-mediated (p)ppGpp accumulation during carbon source starvation because the expected metabolic



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consequences are to limit fatty acid synthesis as well as provoke RelA synthase activation (24). Sensing other nutritional stress may be complex. Phosphate starvation is sensed by SpoT hydrolase to elevate (p)ppGpp, which induces IraP, a RssB antiadaptor that antagonizes RssB activation of RpoS turnover, thereby inducing RpoS (10).

In vitro assays of Rel*Seq* individual activities reveal that a CTD deletion inhibits hydrolase and activates synthase (43) (**Figure 1*c***, right). However, a similar CTD deletion affects Rel*Mtb* without inhibiting hydrolase, although RAC-dependent activation is lost (3). In the absence of structures for a full-length RSH enzyme, notions of how the CTD alters the balance of hydrolase/synthase activity are speculative. The NTD-CTD boundary for both SpoT and Rel*Seq* is a solvent-accessible region that might be a hinge (43). Because the Rel*Seq* NTD structure shows the head of hydrolase neighbors the tail of synthase, this hinge could allow physical contact between the TGS region and hydrolase and/or synthase sequences. The structures suggest that avoidance of a (p)ppGpp futile cycle may be an intrinsic feature of the catalytic half of the protein. These crystals resolve two mutually exclusive active site conformers (hydrolase-OFF/synthase-ON versus hydrolase-ON/synthase-OFF). Substrate binding to either site is argued to induce the switch between the two conformations to affect catalytic sites 30 Å apart (30). The existence of hydrolase domain point mutants that reverse the synthase defect of some but not all synthase-defective alleles underscores the notion that there is cross-talk between sites (U. Mechold & M. Cashel, unpublished data). Altered CTD structure by ACP or RAC effectors might trigger an allosteric switch between the two NTD conformers either by physical contacts or by inducing a conformational cascade over the full length of the RSH protein. The net effect is an enzyme activity state that favors hydrolase or synthase, not both.

ACP: acyl carrier protein

EFFECTS OF (p)ppGpp ON BACTERIAL PHYSIOLOGY

The many effects of (p)ppGpp on metabolism and physiology are complex and seem to differ greatly among different organisms. Profiling and proteomic studies in different organisms consistent with this trend are beginning to appear but do not yet involve comparing effects of a complete absence of ppGpp with wild type (20, 51).

Figure 1

Cartoon of (p)ppGpp regulation. (*a*) Conserved domains of the N-terminal domain (NTD) and C-terminal domain (CTD) halves of a RSH protein. (*b*) Roles in *Escherichia coli* of RelA (*left*) and SpoT (*right*) displaying the NTD hydrolase/synthase (*yin and yang symbol*) and CTD (*square*), with the balance of hydrolase/synthase shown as the ratio of green to orange dots. The CTD contacts hydrolase and synthase, reflecting possible CTD regulation of each activity. (*Left*) Activation of RelA synthase requires cognate uncharged tRNA, a translating ribosome (*yellow*) with an empty A site paused for lack of cognate charged tRNA, and r-protein L11. Synthesis of (p)ppGpp from GTP (or GDP) involves pyrophosphoryl transfer from ATP and is accompanied by release of RelA. (*Right*) SpoT regulation is depicted in two ways. Acyl carrier protein (ACP) lacking acyl fatty acids (*purple triangle*) binds to the SpoT TGS region of the CTD, which shifts the activity balance to synthesis; this effect also requires other CTD functions. Other stress conditions provoke a similar shift of the activity balance by unknown mechanisms. Hydrolysis of (p)ppGpp regenerates GTP or GDP by an Mn^{2+}-dependent reaction releasing pyrophosphate. (*c*) Regulation of Rel*Mtb* activities from *Mycobacterium tuberculosis*; synthase is activated similar to RelA. Rel*Mtb* differs from RelA by the added presence of a hydrolase, and Rel*Mtb* also differs from SpoT in that the hydrolase is modestly inhibited when sensing stress. A related Rel*Seq* enzyme has strong hydrolase and weak synthase activities without ribosomal activation; removal of its CTD reverses the balance of activities, and structures have been resolved that reflect both activity states. Depicted at the bottom of panel *c* are small fragments with homology to RSH synthase recently discovered to coexist in members of the class *Firmicutes* with a full-length RSH protein. Physiological roles for these proteins are unknown.

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(p)ppGpp^0: a complete deficiency of (p)ppGpp; makes *E. coli* require several amino acids

Rapid Induction of (p)ppGpp Inhibits Growth, but How?

Induction of (p)ppGpp to high levels without starvation quickly inhibits growth and protein synthesis of exponentially growing *E. coli* (69). How might protein synthesis inhibition occur? It seems unlikely that inhibition of de novo rRNA and tRNA synthesis (via the stringent response) would so quickly block the activity of the pre-existing protein synthesis apparatus. Nor should rapid inhibition occur via (p)ppGpp induction of RpoS-dependent proteins that slow protein synthesis. Substrates for protein synthesis should not be limiting: Amino acid starvation does not occur under these conditions, and *E. coli* GTP levels drop only by half owing to inhibition of IMP dehydrogenase (GuaB). In contrast, more complete inhibition of GuaB in *B. subtilis* severely depletes GTP, leading to rRNA inhibition (36). Depleted GTP also leads to transcriptional regulation of about 200 genes by CodY, a GTP-binding protein (26). Although (p)ppGpp effects are largely indirect in *B. subtilis*, they contribute to the regulation of sporulation, competence, enzyme secretion, antibiotic production, and stress survival. The behavior of a (p)ppGpp-resistant GuaB protein would be interesting in both organisms.

Interactions of (p)ppGpp with protein synthesis elongation factors are generally reversed by equimolar (GTP)GDP (15). A recent report argues that ppGpp inhibits IF2-mediated fMet-Phe initiation dipeptide formation even at equimolar concentrations of (p)ppGpp and GTP, probably by interfering with 30S and 50S subunit interactions (44). This is intriguing because equimolar GTP and (p)ppGpp levels are reached physiologically during a stringent response. Inhibiting translation initiation would be an efficient pathway to limit excessive protein synthesis during nutritional impoverishment. Still, how (p)ppGpp might inhibit protein synthesis has been elusive historically, and independent verifications are in order.

The Extent of Growth Inhibition Differs for ppGpp and pppGpp

E. coli accumulates more ppGpp than pppGpp during amino acid starvation (15). Gratuitous (p)ppGpp induction inhibits growth about eightfold more severely with ppGpp than with pppGpp. This estimate comes from measuring growth rates while inducing only ppGpp or only pppGpp. This is accomplished using P*BAD* promoters and altering the abundance of the enzyme that converts pppGpp to ppGpp (GppA) as well as using (p)ppGpp synthases with different GTP or GDP affinities (U. Mechold & M. Cashel, unpublished data).

Basal Levels Control Growth by Regulating Ribosomal Number

Growth rate control is defined as the systematic variation of cellular RNA, DNA, and protein content as a function of rates of balanced growth. Basal level changes of (p)ppGpp over a 10- to 12-fold range are inversely correlated with growth rate and the number of ribosomes per cell (12). There is now a consensus that (p)ppGpp is a determinant of growth rate control rather than nucleoside triphosphate (NTP) substrate concentrations (65). Nevertheless, the existing literature remains confusing regarding the abolition of growth rate control in (p)ppGpp^0 strains. Different views on how (p)ppGpp curtails transcription of rRNA are discussed below. We argue that the growth-rate-determining role of basal levels of (p)ppGpp involves rRNA control and differs from the growth inhibitory effects of inducing large amounts of (p)ppGpp.

Inhibition of DNA Replication

Classical studies with *E. coli* concluded that amino acid starvation inhibited DNA replication at the initiation stage at *oriC*, most probably owing to the lack of the DnaA replication initiation protein. It was believed that replication arrest due to (p)ppGpp accumulation in

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B. subtilis was different and caused by the binding of an Rtp protein to specific sites about 100–200 kb away from *oriC* in both directions (for review see Reference 80). This view of *B. subtilis* behavior changed dramatically when DNA replication was studied with microarrays, revealing that inhibition of elongation in the presence of (p)ppGpp can take place throughout the chromosome, independent of Rtp and the proposed specific arrest sites (79). DNA primase (DnaG) was directly inhibited by (p)ppGpp. Unlike *E. coli*, *B. subtilis* accumulates more pppGpp than ppGpp; the more abundant nucleotide is a more-potent DnaG inhibitor. Replication forks were not disrupted, as they did not recruit RecA, thus maintaining genomic integrity. It is unknown whether *E. coli* behaves like *B. subtilis* in this respect.

ppGpp cocrystallizes with the *B. subtilis* Obg protein, which belongs to the conserved, small GTPase protein family (14). Obg interacts with several regulators (RsbT, RsbW, RsbX) necessary for the stress activation of σ^B, the global controller of a general stress regulon in *B. subtilis* (66). The *E. coli* ObgE protein (also known as CgtA) stabilizes arrested replication forks, and an *obgE* depletion causes disruption of cell cycle events, leading to filamentation and polyploidy (22, 23). CgtA is also associated with ribosomes and SpoT and is argued to alter SpoT basal activities (31). However, in a different study, SpoT was not detected when high-salt-washed ribosomes immobilized with a HA-tagged L1 protein were employed (H. Murphy & M. Cashel, unpublished data). In the same study, RelA binding to ribosomes is stoichiometric. It remains possible that SpoT could bind to ribosomes but that L1-tag interferes.

Effects on Phage Replication and Development

A truncated form of IF2 (IF2-2) was recently identified as the *E. coli* factor necessary to promote assembly of the *E. coli* replication restart proteins, PriA, PriC, DnaT, and DnaB-DnaC complex, at the phage Mu replication fork (52). This allows the DNA polymerase III complex to be recruited. Ordinarily, PriA, PriC, and DnaT promote the assembly of a replisome without the initiator protein DnaA and the *oriC* and play an essential role in restarting replication after stalling of the replication fork (52). The authors mention their unpublished results, in which premixing IF2-2 with high levels of GTP diminished Mu replication in vitro by the PriA-PriC pathway, whereas premixing with ppGpp stimulated the reaction. They further speculate that ppGpp might activate the PriA-PriC restart pathway to ensure that chromosomal replication is completed when ppGpp shuts down the initiation at *oriC*. Extensive studies have shown that resolution of arrested replication forks has requirements for (p)ppGpp that can be satisfied by RNA polymerase (RNAP) M+ (p)ppGpp^0 suppressor mutants (discussed below) (75).

RNAP: RNA polymerase

The (p)ppGpp levels of the host seem to act as a sensor for phage lambda development, primarily affecting transcription. Modest ppGpp levels inhibit pR and activate pE, pI, and paQ promoters in vivo (67) and have effects in vitro (61, 62) that seem to favor lysogeny. In contrast, absent or high concentrations of (p)ppGpp favor lysis. This unusual concentration dependence similarly affects the switch between lytic and lysogenic growth through regulation of HflB (alias FtsH), a protease responsible for degradation of CII, a lysogeny-promoting phage protein. Again, modest ppGpp levels favor lysogeny by leading to low HflB levels. When ppGpp is either absent or high, HflB protease levels are high; this leads to lower CII levels and favors lysis (67).

(p)ppGpp^0 Physiology

Apart from phage growth, a complete absence of (p)ppGpp generates its own unique phenotypic features in *E. coli*. These include multiple amino acid requirements, poor survival of aged cultures, aberrant cell division, morphology, and immotility, as well as being locked in a growth mode during entry into starvation (41, 82). The multiple amino acid requirements are of special interest for two reasons: (*a*) They

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M+ mutant: suppresses (p)ppGpp^0 phenotypes; *E. coli* M+ grows without added amino acids

DnaK suppressor (DksA): protein that reverses thermolability of a *dnaK* mutant

reflect positive regulation by (p)ppGpp at the transcriptional level, and (*b*) they allow isolation of spontaneous mutants growing on minimal glucose medium. These (p)ppGpp^0 phenotypic suppressors are called M+ mutants, which so far map exclusively within RNAP *rpoB*, *rpoC*, and *rpoD* subunit genes (15, 47).

(p)ppGpp ACTS AT THE LEVEL OF TRANSCRIPTION

Inhibition of rRNA synthesis is the classical feature of the stringent response; numerous hypotheses have been made to explain this event (see recommended reviews above). Understanding (p)ppGpp regulation of transcription currently seems based on three key features: (*a*) shared characteristics of promoters affected by (p)ppGpp, (*b*) genetic and structural evidence that RNAP is the target of (p)ppGpp, and (*c*) the DksA protein augments (p)ppGpp regulation. Because early in vitro studies showed no differences between the effect of ppGpp and pppGpp on transcription, most studies use ppGpp exclusively.

Characteristics of Affected Promoters

One of the key elements of promoters inhibited by (p)ppGpp is the presence of a GC-rich discriminator, defined as a region between TATA-box (–10 box) and +1 nt (where +1 is the transcription start site) (76). In addition, the discriminator's activity depends on –35 and –10 sequences, as well as the length of the linker, i.e., the region between them (53). Promoters negatively regulated by ppGpp have a 16-bp linker, in contrast with the 17-bp consensus. Promoters activated by ppGpp seem to have an AT-rich discriminator and longer linkers (for example, the *his* promoter linker is 18 bp). There is also evidence that sensitivity to supercoiling influences ppGpp responses (21).

RNAP Is the Target

Although it is plausible that transcription of rRNA should be regulated by (p)ppGpp during the stringent response, for many years attempts to verify this hypothesis in vitro with pure RNAP were plagued by irreproducibility. Genetic evidence suggesting that RNAP was the target of (p)ppGpp came from the discovery that M+ mutants (also called stringent RNAP mutants) display in vitro and in vivo mimicry of physiology and transcription regulation conferred by (p)ppGpp, even in its absence. Cross-linking ppGpp to RNAP reinforced this notion, although different contacts were deduced (16, 74). Structural details of an association between ppGpp and RNAP came from the analysis of cocrystals that positioned ppGpp in the secondary channel of RNAP near the catalytic center (2). This channel provides access to the catalytic center for NTP substrates during polymerization as well as an entry point for derailed backtracked, nascent RNA in RNAP arrested during elongation. The ppGpp target could be defined by direct contacts with appropriate RpoB and RpoC residues. However, sequence changes in M+ mutants, chemical cross-linking, and cocrystallization provide a different target locations.

DksA Augments Regulation

DksA has many regulatory functions in addition to its ability to restore thermotolerance to a *dnaK* mutant when overexpressed (34). Among these functions was a need for DksA and (p)ppGpp to stimulate the accumulation of RpoS during early stationary phase of growth (13). The regulatory interrelationship between (p)ppGpp and DksA was clarified when DksA was found to be necessary for the stringent response. This finding was followed by discoveries that DksA potentiated (p)ppGpp regulation generally in vitro and in vivo, based on studies of inhibition of a rRNA promoter or activation of selected amino acid biosynthetic promoters (54, 55).

Determining the structure of DksA was a major contribution toward understanding its regulatory properties—the 17-kDa protein is structurally similar to GreA and GreB, which is not evident from sequences (57). Both GreA

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and GreB are well-characterized transcriptional elongation factors (9). They bind directly to RNAP rather than DNA and act by inserting their N-terminal coiled-coil finger domain through the RNAP secondary channel. Two conserved acidic residues at the tip of the finger domain are necessary to induce RNAP's intrinsic ability to cleave backtracked RNA, whose 3′ end then comes near the catalytic center and is available for polymerization, functionally rescuing the arrested enzyme. Binding of the GreA/B factors to RNAP is thought to occur by contacts between the C-terminal globular domain of Gre and RpoC residues 645–703 at the entrance of the secondary channel that form a coiled-coil (77). DksA also possesses two acidic residues at its finger tip, but it does not induce nucleolytic cleavage activity. Instead, these residues are proposed to stabilize ppGpp binding to RNAP by mutual coordination of an Mg^{2+} ion that is crucial for polymerization (57).

Evidently, GreA/B and DksA are structural homologs with different activities. This diversity has been extended by showing that GreA exhibits antagonistic effects to DksA at the *rrnB* P1 promoter, independent of ppGpp, and acts at an earlier initiation step than DksA does (60). Another example is that GreB, when overproduced, might mimic DksA (63). Two additional factors predicted to have shapes similar to GreB and GreA have been investigated: Gfh1 (37, 70) and TraR (8). It is exciting that these proteins possess different functions, none of which involves specific DNA-binding properties of more common regulators of specific promoters. However, *Pseudomonas aeruginosa* DksA is reported to bind to DNA (58).

Because DksA enhances ppGpp's effect, whether inhibition or activation, it was termed a ppGpp cofactor. Yet, in $ppGpp^0$ strains DksA overproduction can completely compensate for positive and negative regulation with respect to amino acid auxotrophy, cell-cell aggregation, motility, filamentation, stationary-phase morphology, and stimulating RpoS accumulation (41). DksA and (p)ppGpp can also have opposing roles on cellular adhesion (41). This implies that DksA is not only present to stabilize ppGpp's interaction with RNAP but that each regulator can have different modes of action. These epistatic relationships hint that DksA might function downstream of (p)ppGpp.

The above RNAP crystal studies were performed with RNAP from *Thermus thermophilus*. This organism produces ppGpp upon amino acid starvation, but like *B. subtilis* its rRNA levels in vivo respond to the availability of GTP rather than ppGpp directly (35). On the other hand, ppGpp inhibition of *Tth* rRNA promoters in vitro with *Tth* RNAP was observed but required higher ppGpp concentrations than the measured intracellular pool (35). Perhaps this is because no DksA homolog has been identified in *T. thermophilus* to date. A yet unidentified protein might be required for ppGpp to exert its full effect in vitro. However, the same statement raises concerns over the predicted DksA-RNAP interaction model, in which *E. coli* DksA was docked into the structure of *Tth* RNAP bound with ppGpp. This issue might be resolved by constructing RNAP mutants in the residues predicted to be involved in ppGpp and DksA interactions. However, because these residues are in proximity to the catalytic center, such mutations might alter RNAP properties as well as a mode of action other than the one intended.

Transcription Inhibition

A consensus is building that ppGpp directly inhibits transcription from ribosomal promoters. There are several models of how this might occur (**Figure 2*a***). One model relies on the fact that ppGpp and DksA together and independently decrease the stability of the open complexes formed on DNA by RNAP (5, 54, 55). If decreasing open complex stability is the major role of ppGpp and DksA, the model suggests that only intrinsically unstable promoters, such as *rrn* promoters, would be inhibited, whereas those that form relatively stable complexes would be activated. Although appealing in its simplicity, this model does not explain all the instances of inhibition, such as the lambda

pR promoter that forms stable open complexes yet is inhibited by ppGpp (61). The lambda pR study suggested that although ppGpp affects many steps of transcription initiation, the first phosphodiester bond formation might be the key target for pR. Similar proposals were made for *rrnB* P1 and *rrnD* P1 promoters in which substrate competition between ppGpp and NTPs was implied (33).

A different model was proposed for an M+ mutant with deletion of four residues of the RNAP rudder sequence (*rpoC* Δ312–315). Transcription of the P*argT* tRNA promoter with RNAP from these mutants formed

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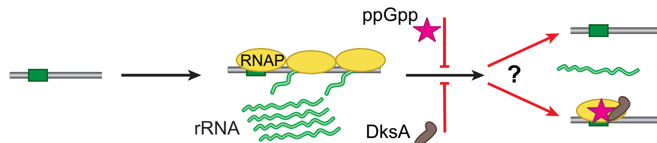
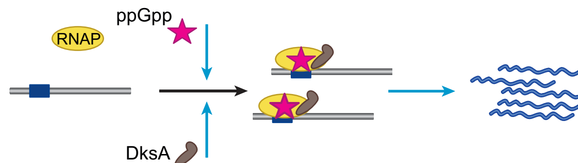
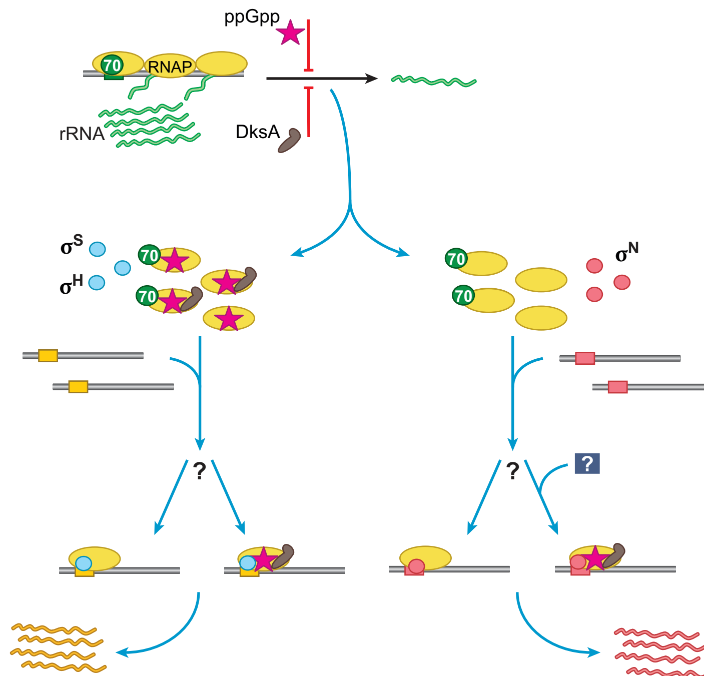
a Direct inhibition: ribosomal promoters



b Direct activation: amino acid biosynthesis promoters



c Models of indirect activation: alternative σ factors (σ^H, σ^S, σ^N)



dead-end promoter complexes with features similar to those of stable closed complexes (42). These observations are in agreement with a trapping mechanism, previously proposed to explain ppGpp action (28). In this model, RNAP is trapped by ppGpp in closed complexes and is unable to initiate transcription. Studies with *rrnB* P1 promoters employing ppGpp and DksA also seem to indicate formation of such dead-end complexes (60). Thus, ppGpp seems to act at many levels, and the mechanism of its action is a complex outcome of several factors, intrinsic promoter properties not being the least of them.

Activation of Transcription

Many models calling for direct, passive, or indirect mechanisms had been proposed for activation of transcription by ppGpp; many of the mechanisms are not mutually exclusive (for comprehensive reviews, see References 40 and 72). This is further complicated by the difficulty to distinguish between direct and indirect effects of interconnected cellular processes. To simplify definitions, we propose to use the following criteria. Direct activation occurs when RNAP interacts with effectors, such as ppGpp, DksA, or both, to increase transcription from a given promoter (**Figure 2*b***). Activation of a promoter in a pure in vitro system is proof of direct activation. Indirect activation by these effectors of one promoter relies on inhibition of other (strong) promoters, leading to increased availability of RNAP that indirectly activates transcription initiation (**Figure 2*c***).

Direct Activation

Historically, models in which ppGpp bound to RNAP would directly activate transcription have not been favored simply because such activation was not demonstrable in vitro. The first promising report implying such a possibility came from coupled transcription-translation system with the use of cellular extracts, demonstrating activation of *hisG* promoter (17). Later, such an effect was demonstrated in a defined in vitro transcription system for lambda paQ promoter (62). However, the in vitro demonstration that certain amino acid biosynthesis promoters (P*argI*, P*thrABC*, P*livJ*, and P*hisG*) are activated by combining ppGpp and purified DksA provides convincing evidence of direct activation according to our definition (36). The precise mechanism is still unknown, although it has been suggested that ppGpp and DksA stimulate the rate of an isomerization step on the pathway to open complex formation (55). It is probable that the other σ^{70}-dependent promoters activated by ppGpp are also affected through a direct mechanism.

Indirect Activation

Transcription directed by the alternative sigma factors σ^{S}, σ^{H}, σ^{N} (40), and σ^{E} (18) also requires (p)ppGpp for activation in vivo. Attempts to verify this dependence in vitro have failed, with the exception of σ^{E}, which was recently demonstrated (19). Therefore, a sigma competition model was proposed whereby ppGpp alters the affinity of the housekeeping σ^{70} to

Figure 2

Models of how ppGpp inhibits and activates RNA polymerase (RNAP). (*a*) ppGpp and DksA inhibit transcription from ribosomal promoters by interacting directly with RNAP; this may occur either by destabilizing open complexes formed by RNAP on DNA or by trapping RNAP at the promoter site. (*b*) ppGpp and DksA directly activate transcription from certain amino acid biosynthesis promoters and possibly other σ^{70} promoters. (*c*) Inhibition of transcription from ribosomal promoters by ppGpp and DksA liberates core RNAP that can now be engaged in transcription dependent on alternative sigma factors. (*Left*) In the case of σ^{H} and σ^{S} it is proposed that ppGpp, and possibly DksA, aids these factors in competing for core RNAP; it is uncertain whether ppGpp together with DksA might further promote transcription at the initiation stage. (*Right*) Neither ppGpp nor DksA aids σ^{N} in competing for core RNAP or enhances transcription in in vitro assays. It is possible that a yet unidentified factor is necessary to observe a direct activation in vitro.

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SPI: *Salmonella* pathogenicity island

core RNAP, allowing other sigma factors to bind (32, 40, 72). This requires increased core RNAP availability, attained through inhibition of strong promoters, such as *rrn* (**Figure 2*c***), that account for most transcripts occurring under normal growth conditions. However, not all RNAPs in the cell are involved in active transcription, but they reside at chromosomal sites; the dynamics of their release and how they can contribute to the competition is unclear (78).

The sigma competition model was mainly based on in vivo observations indicating that σ^S and σ^H promoters lose their dependence on ppGpp for activity when σ^{70} levels are depleted or when σ^{70} is mutated, lowering its affinity for core RNAP (32). ppGpp could reduce σ^{70} competitiveness for core RNAP in vitro when assayed together with σ^H (32). Moreover, some of the ppGpp^0 *rpoD* (σ^{70}) M+ suppressor mutants behaved as if the mutant σ^{70} had less affinity to core RNAP (29, 71). Thus, ppGpp and/or DksA would be expected not only to increase the availability of free RNAP by inhibiting strong σ^{70}-dependent promoters, but also to make core RNAP more available by disturbing σ^{70}-core RNAP interactions. Whether this disturbance happens at the level of association or dissociation with core RNAP is uncertain. Also, in vitro transcription studies employing DksA together with ppGpp have not been reported with σ^S and σ^H factors to date, so the direct activation model cannot be completely excluded.

Similar in vivo observations were made for σ^N (38). Recently, DksA was also found to be required for σ^N-dependent transcription of the *Pseudomonas* Po promoter in vivo (using *E. coli* system), but not in vitro because neither ppGpp nor DksA, added together or separately, could mimic this dependence (7). Competition between σ^{70} and σ^N by in vitro assay was also unaffected. Certain *rpoB* or *rpoC* M+ mutants can stimulate the same σ^N-dependent Po promoter in vivo. However, purified M+ RNAP did not alter transcription initiation from the Po promoter enough to account for the observed high activity in vivo. Nevertheless, it could be verified that the M+ mutant RNAP bound σ^{70} more poorly than σ^N (71). Either the ability of the M+ mutant RNAP to bypass requirements for ppGpp and DksA for rRNA promoters is not the same for the Po promoter or this promoter has a requirement for an additional, so far unidentified factor present in vivo (**Figure 2*c***).

PATHOGENESIS AND (p)ppGpp

A growing number of studies report involvement of (p)ppGpp in processes related to growth, stress, starvation, and survival that affect pathogenicity. A frequent scenario is that when (p)ppGpp is absent, pathogenicity is compromised for reasons that vary with the organism studied. Inhibitory effects can also occur on host interactions that enhance pathogen survival, invasiveness, or persistence. Examples include *Vibrio cholerae*, *Salmonella typhimurium*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Brucella abortus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Borrelia burgdorferi* (see Reference 11).

The enterobacterial pathogen *S. typhimurium* accumulates (p)ppGpp in stationary phase to induce *hilA*, a master regulator of *Salmonella* pathogenicity island 1 (SPI 1) and SPI 2 virulence genes (73). The transcriptional basis of regulation is unexplored, although the SPI sequences are AT rich. The SPI 1 genes are involved in host cell invasion and SPI 2 genes are required for replication within the host cell. Deleting *relA* and *spoT* genes, but not *relA* alone, gave a (p)ppGpp^0 state that resulted in strong attenuation in mice and noninvasiveness in vitro (59, 68, 73). Vaccine tests reveal that 30 days after single immunization with the (p)ppGpp^0 strain, mice were protected from challenge with wild-type *Salmonella* at a dose 10^6-fold above the established LD_{50} (48). It is also intriguing that this requirement for (p)ppGpp depends on SpoT function rather than RelA. For another enteric bacterium, enterohemorrhagic *E. coli* (EHEC), adherence capacity, as well as expression in the enterocyte effacement pathogenicity island locus, depends on *relA*, *spoT*, and *dksA* (49).

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Another example of the diversity of stress inducing (p)ppGpp is *Helicobacter pylori*, a pathogen with a single RSH enzyme. Although originally found to be unresponsive to amino acid starvation, accumulation of ppGpp seems required for survival, specifically during aerobic shock and acid exposure (45, 81). These conditions are likely to be encountered during the course of infection and transmission.

SUMMARY POINTS

1. Virtually all bacteria and plants synthesize ppGpp and pppGpp, which are regulatory analogs of GDP and GTP.
2. *E. coli* (and all beta- and gamma-proteobacteria) synthesizes (p)ppGpp with RelA and SpoT. Other bacteria contain a single Rel/Spo homolog gene. Members of the class *Firmicutes* have additional small fragments with synthetic activity.
3. (p)ppGpp signals different kinds of environmental stress and leads to adjustments of gene expression and physiology. In *E. coli*, complete elimination of (p)ppGpp seems to lock cells in a growth mode unperturbed by environmental changes.
4. Diverse bacteria exploit (p)ppGpp in fundamentally different ways that can alter many aspects of cell biology.
5. Many basic questions as to how (p)ppGpp is made and how it works remain unanswered.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Daniel Vinella, Agnieszka Szalewska-Palasz, Rajendran Harinarayanan, Helen Murphy, and the Friday Seminar group for many discussions. We apologize to coauthors and colleagues whose important citations are absent owing to space limitations. This work was supported by the NICHD intramural program of the NIH.

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27. The first and still the best demonstration of RelA activation.

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