From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of Enterococcus faecalis

Running title: E. faecalis pGpp

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ABSTRACT

The bacterial stringent response (SR) is a conserved stress tolerance mechanism that orchestrates physiological alterations to enhance cell survival. This response is mediated by the intracellular accumulation of the alarmones pppGpp and ppGpp, collectively (p)ppGpp. In Enterococcus faecalis, (p)ppGpp metabolism is carried out by the bifunctional synthetase/hydrolase RelEf and the small alarmone synthetase (SAS) RelQEf. Although Rel is the main enzyme responsible for SR activation in Firmicutes, there is emerging evidence that SASs can make important contributions to bacterial homeostasis. Here, we showed that RelQEf synthesizes ppGpp more efficiently than pppGpp without need of ribosomes, tRNA, or mRNA. In addition to (p)ppGpp synthesis from GDP and GTP, RelQEf also efficiently utilized GMP to form guanosine 5’-monophosphate, 3’-diphosphate (pGpp). Based on this observation, we sought to determine if pGpp exerts regulatory effects on cellular processes affected by (p)ppGpp. We found that pGpp, like (p)ppGpp, strongly inhibits the activity of E. faecalis enzymes involved in GTP biosynthesis and, to a lesser extent, transcription of rrnB by E. coli RNA polymerase. Activation of E. coli RelA synthetase activity was observed in the presence of both pGpp and ppGpp while RelQEf was activated only by ppGpp. Furthermore, enzymatic activity of RelQEf is insensitive to Relacin, a (p)ppGpp analog developed as an inhibitor of ‘long’ RSH enzymes. We conclude that pGpp can likely function as a bacterial alarmone with target-specific regulatory effects that are similar to what has been observed for (p)ppGpp.

IMPORTANCE

Accumulation of the nucleotide second messengers (p)ppGpp in bacteria is an important signal regulating genetic and physiological networks contributing to stress tolerance, antibiotic persistence, and virulence. Understanding the function and regulation of the enzymes involved in (p)ppGpp turnover is therefore critical for designing strategies to eliminate the protective effects of this molecule. While characterizing the (p)ppGpp synthetase RelQ of Enterococcus
faecalis (RelQ_{Ei}), we found that, in addition to (p)ppGpp, RelQ_{Ei} is an efficient producer of pGpp (guanosine 5'-monophosphate, 3'-diphosphate). *In vitro* analysis revealed that pGpp exerts complex, target-specific effects on processes known to be modulated by (p)ppGpp. These findings provide a new regulatory feature of RelQ_{Ei} and suggest that pGpp may represent a new member of the (pp)pGpp family of alarmones.
In order to survive under adverse environmental conditions, such as nutrient starvation, bacteria have evolved complex, interconnected regulatory networks that sense and integrate internal and external metabolic cues to activate cellular responses that enhance bacterial survival. One critical and highly conserved mechanism employed by bacteria to cope with nutritional as well as a variety of environmental and chemical stresses is the stringent response (SR). The SR is mediated by the accumulation of two guanine analogs derived by the addition of the β-γ phosphates from ATP to the ribose 3' hydroxyl of GTP or GDP generating guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp), abbreviated (p)ppGpp (1, 2). The intracellular accumulation of (p)ppGpp coordinates the remodeling of cellular physiology as it changes from a state of rapid growth to slow growth or stasis to ensure survival. These physiological adjustments occur at the levels of transcription, translation, DNA replication, and general metabolism (3, 4). During stress, the transcription of genes required for rapid growth, such as those encoding rRNAs, is limited by (p)ppGpp accumulation along with a concomitant activation of genes involved in amino acid biosynthesis, alternate nutrient transport, and general stress responses (3, 5). In addition, (p)ppGpp leads to rapid physiological adjustments by inhibiting enzymes involved in protein synthesis, DNA replication, and nucleotide synthesis, especially GTP (6-11).

The metabolism of (p)ppGpp is orchestrated by members of the RelA/SpoT Homolog (RSH) protein family that is divided into two categories - ‘long’ multi-domain RSHs and ‘short’ single-domain RSHs - with the latter category comprised of small alarmone synthetases (SAS) and small alarmone hydrolases (SAH) (12). In Gammaproteobacteria like Escherichia coli, the SR is mediated by two ‘long’ RSHs, RelA (1) and SpoT (13). RelA is a large monofunctional synthetase that is rapidly activated during amino acid starvation through interactions with stalled ribosomes containing deacylated tRNA bound to the A-site (14). SpoT is a similar sized bifunctional RelA homolog with strong hydrolase and weak synthetase activities. The balance of
SpoT activities is altered by other sources of stress (15-17). By far the most widespread 'long' RSH is the bifunctional synthetase/hydrolase Rel (also known as RSH) (12). The bifunctional Rel is responsible for the rapid accumulation of (p)ppGpp during amino acid starvation through what is believed to be interaction with stalled ribosomes by extension from work on the Mycobacterium tuberculosis Rel (RelMtb) enzyme and by the ability to provoke the SR using inhibitors of tRNA aminoacylation (18, 19). Regulation of the (p)ppGpp hydrolytic activity of Rel is less well understood. In vitro, the hydrolytic activity of RelMtb seems to be insensitive to ribosomal and tRNA signals (18), which can be interpreted as either hydrolytic activity is constitutive or that the regulatory partners are yet to be discovered. In the most thoroughly characterized Rel enzyme from Streptococcus equisimilis (RelSeq), the hydrolase and synthetase activities are reciprocally regulated with distinct conformations corresponding to hydrolase or synthase active forms (20). In this case, hydrolase activity is neither constitutive nor regulated in isolation of synthetase but instead linked to synthetase regulation.

Among Firmicutes, (p)ppGpp metabolism is mediated by Rel and one or two weak SASs, termed RelP and RelQ, that lack both the N-terminal hydrolase and C-terminal ribosome interaction domains of 'large' RSH enzymes (12, 19, 21, 22). There is emerging evidence that SASs make important contributions to bacterial physiology despite the dominant role of Rel in SR activation (23). For example, SASs were shown to be required for fully efficient SR induction, GTP homeostasis, growth rate control, tolerance to cell wall antibiotics, and virulence (10, 21, 24-29). Moreover, the stable genomic co-existence of the bifunctional Rel with one or two SASs in a large number of bacteria, indicates an evolutionary pressure that favors retention of the Rel-SAS (p)ppGpp synthetase redundancy. In principle, this might occur for a number of reasons. For example, SASs might respond to different internal or external cues than the Rel enzyme. In support, there is evidence of transcriptional induction of the Bacillus subtilis, Staphylococcus aureus, and Streptococcus mutans SASs under alkaline, cell wall, or oxygen stresses, respectively (22, 24, 30). Alternatively, they might differ from long RSH enzymes.
because their constitutive synthetase activity ensures persistent basal (p)ppGpp production (21, 28). A third possibility is they might catalyze synthesis of other nucleotides that provide unique regulatory properties not shared by (p)ppGpp. The goal of the work presented here is to explore these possibilities using E. faecalis, in which (p)ppGpp metabolism is carried out by the bifunctional RelEf (previously called RSH) and the SAS RelQEf (31). To accomplish this goal, we carried out the biochemical characterization of the RelQEf enzyme and found that in addition to catalyzing the synthesis of ppGpp and pppGpp from GDP and GTP, respectively, RelQEf is able to efficiently use GMP to synthesize guanosine 5'-monophosphate, 3'-diphosphate (pGpp). Furthermore, the availability of these compounds was exploited to demonstrate that pGpp has regulatory properties, which are capable of exerting control over several processes known to be altered by (p)ppGpp.

RESULTS

RelQEf prefers GDP over GTP as a substrate and is insensitive to high Mg2+ concentrations. We previously observed that in the absence of RelEf, constitutive (p)ppGpp synthesis by RelQEf lead to an approximately 4-fold increase in basal (p)ppGpp levels (28). Interestingly, this elevated basal level consisted primarily of ppGpp, whereas pppGpp predominates in wild type cells with both Rel and RelQEf (28). This result suggested that RelQEf prefers GDP over GTP as a substrate. However, there are other explanations for removal of the 5' γ-phosphate from pppGpp generating ppGpp such as activities of non-specific phosphohydrolases including polyphosphate phosphatase, NUDIX family hydrolases, or GTP requiring enzymes for protein synthesis such as EF-G or EF-Tu (32-35). A preference for GDP over GTP as an in vitro substrate has been observed for SASs from Mycobacterium smegmatis and S. aureus (24, 36). Of note, the ability of RelQEf to synthesize (p)ppGpp in vitro was previously documented but the relative efficiency of substrate utilization has not (35). Therefore, we determined the efficiency of GTP vs GDP as substrates for RelQEf synthesis of pppGpp and...
ppGpp (Table 1). The experiments were performed using varying guanine nucleotide substrate concentrations in the presence of constant amounts of either 100 μM pppGpp or ppGpp. The $k_{cat}$, $K_M$, and the specificity constant $k_{cat}/K_M$ were calculated to determine substrate preferences of RelQ Ef (37). RelQ Ef has a moderate preference towards GDP as a substrate over GTP, but the strength of this preference ($k_{cat}/K_M$) is dependent on the nature of the alarmone present. In the presence of ppGpp, RelQ Ef has an approximately 2-fold higher specificity for GDP ($k_{cat}/K_M$(GDPppGpp) of 2.08±0.82 mM⁻¹s⁻¹ vs $k_{cat}/K_M$(GTPppGpp) of 1.19±0.43 mM⁻¹s⁻¹), while pppGpp resulted in an approximately 4-fold higher specificity for GDP ($k_{cat}/K_M$(GDPpppGpp) of 3.90±1.28 mM⁻¹s⁻¹ vs $k_{cat}/K_M$(GTPpppGpp) of 0.87±0.30 mM⁻¹s⁻¹). Direct competition experiments, comparing (p)ppGpp production with individual substrates (Fig. S1A) to (p)ppGpp production when both GTP and GDP are present as substrates simultaneously, support the GDP preference (Fig. S1B).

Previous studies suggested that the GDP substrate preference of monofunctional synthetases is due to the presence of two charge reversals (underlined residues) in the catalytic domain of RelA (EXDD) compared to bifunctional Rel enzymes (RXKD) (38). This charge reversal also renders the monofunctional RelA from *E. coli* (RelAEc) insensitive to inhibition by Mg²⁺ ions in excess over the total purine nucleotide concentration, whereas RelMtb was inhibited at higher concentrations of Mg²⁺ ions (39). The RelQ Ef enzyme has an EERD motif in its catalytic domain carrying three negative residues similar to RelAEc but like RelMtb also bears a positive charge at the third residue of the catalytic motif. *In vitro* synthesis reactions with increasing concentrations of MgCl₂ revealed that RelQ Ef, like RelAEc, is effectively insensitive to excess Mg²⁺ (Fig. S2). Only under conditions where Mg²⁺ ions were in 10-fold or 20-fold excess over the total nucleotide concentration was a small reduction in ppGpp synthesis, 10% and 22% respectively, observed compared to optimal conditions where purine and MgCl₂ were in a 1:1 to 1:5 ratio. For comparison, synthetase capacity of the bifunctional Rel enzymes of *S. equisimilis* and *M. tuberculosis* dropped over 50% when Mg²⁺ is in 2-fold excess to the total purine.
concentration and activity is nearly absent once Mg\(^{2+}\) is in 3-fold excess (18, 40). Inhibition of
the *E. faecalis* Rel enzyme (Rel\(_{Ef}\)) synthetase activity at high concentrations of Mg\(^{2+}\) was slightly
stronger when compared to Rel\(_{Ef}\) but not as dramatically as previously described for either
Rel\(_{Mt}\) or Rel\(_{Seq}\) (Fig. S2). The relatively low Mg\(^{2+}\) sensitivity of Rel\(_{Ef}\) when compared to other Rel
enzymes (Rel\(_{Mt}\) and Rel\(_{Seq}\)) may result from biologically meaningful species differences or from
differences in the precise *in vitro* reaction conditions.

**pGpp is synthesized by Rel\(_{Ef}\) from GMP and ATP.** Previous *in vitro* characterization of the
catalytic products of the *B. subtilis* SAS YwaC (RelP) revealed the production of an unknown
compound from radiolabeled \([\gamma-^{32}P]\)ATP and unlabeled GTP or GDP that migrated almost
identically to GTP (22). We reasoned that this GTP-like product could be guanosine 5’-
monophosphate, 3’-diphosphate (pGpp) formed from GMP contamination in the GTP or GDP
substrates. Therefore, we decided to assess the ability of Rel\(_{Ef}\) to utilize GMP as a substrate
to synthesize pGpp. The incubation of Rel\(_{Ef}\) with labeled ATP and unlabeled GMP resulted in
the formation of a labeled product migrating as expected for a guanosine triphosphate, most
likely pGpp (Fig. 1A). Although pGpp co-migrates with GTP using 1.25 M KH\(_2\)PO\(_4\) for TLC
development in **Figure 1A**, the guanosine triphosphate produced by Rel\(_{Ef}\) was resolved from a
GTP standard using 0.75 M KH\(_2\)PO\(_4\) and visualization with UV light, which excludes the
possibility that the guanosine triphosphate product was GTP (Fig. 1B). Alternatively, the
observed guanosine triphosphate could be an isomer of pGpp, pGpp. These isomers differ with
respect to their susceptibility to hydrolysis in alkali: ppGp is stable and pGpp is alkali labile, with
hydrolysis products of pGp (which co-migrates with GDP) and inorganic phosphate (Pi) (41, 42).
We used 0.5 M NaOH with aliquots of *in vitro* synthesized guanosine triphosphate from GMP
and \([\gamma-^{32}P]\)ATP. The labeled product is degraded in alkali, which further supports that the
synthetic product is pGpp and not ppGp (Fig. 1C).
The substrate hierarchy of RelQEf is GDP > GMP ≥ GTP. We now turn to a comparison of the RelQEf enzymatic efficiency towards GMP as a substrate in comparison to GDP and GTP. Table 1 shows that GMP is used as a substrate about as efficiently as GTP in the presence of ppGpp ($k_{cat}/K_M(GMP)_{ppGpp}$) of 1.04±0.18 mM⁻¹s⁻¹ vs $k_{cat}/K_M(GTP)_{ppGpp}$ of 1.19±0.43 mM⁻¹s⁻¹). However, in the presence of pppGpp GMP is preferred over GTP ($k_{cat}/K_M(GMP)_{pppGpp}$) of 1.87±0.37 mM⁻¹s⁻¹ vs $k_{cat}/K_M(GTP)_{pppGpp}$ of 0.87±0.3 mM⁻¹s⁻¹), but is still utilized less efficiently than GDP ($k_{cat}/K_M(GDP)_{pppGpp}$) of 3.9±1.3 mM⁻¹s⁻¹).

We next estimated the efficiency with which RelQEf was able to synthesize pGpp relative to ppGpp and pppGpp with an equimolar total ratio of Mg²⁺ to total purine nucleotides present, 5 mM each. Equimolar amounts (2.5 mM each) of GMP and GDP or GTP incubated with RelQEf resulted in greater synthesis of pGpp than pppGpp but not ppGpp (Fig. S3A and B). When incubated with an equimolar (1.7 mM each) mix of all three pyrophosphate acceptors, RelQEf produced ppGpp in the greatest abundance, followed by pGpp, and, lastly, pppGpp (Fig. S3C). Collectively, these results revealed that the guanine nucleotide substrate preference of RelQEf is GDP > GMP > GTP. We will adopt the term (pp)pGpp when collectively referring to pGpp, ppGpp, and pppGpp, consistent with the commonly used (p)ppGpp for pppGpp and ppGpp.

Three SAS enzymes have different pGpp synthetase specific activities. To assess if the ability to synthesize pGpp is conserved among other SASs, we purified RelQ and RelP from S. mutans and assayed for pGpp synthetase activity. These assays show RelQ and RelP are active but show much weaker pGpp synthetase activities than RelQEf judging from incubation times required for the first appearance of pGpp (Fig. 1A). RelQEf converts more than half of the ATP to pGpp in 15 min and nearly all of the ATP to pGpp within 1 hour. In contrast RelQSm and RelPSm transfer ~5% or less, respectively, of the label to pGpp after 2 hours. Figure S4 shows results obtained with equivalent reactions containing GDP and GTP. It is evident that both RelQSm and RelPSm were able to produce ppGpp and pppGpp but, again, the S. mutans
enzymes showed weaker synthetase activities when compared to RelQef. We conclude that both RelQSm and RelPsm can synthesize pGpp but their \textit{in vitro} (pp)pGpp synthetic activities are markedly lower than RelQef.

\textbf{The \textit{E. faecalis} Rel enzyme has no detectable pGpp synthetase activity.} The full-length RelEf from \textit{E. faecalis} was included in the comparison shown in Figure 1 to ask if it too was able to synthesize pGpp \textit{in vitro} but, as expected, pGpp synthetase activity was undetectable. Figure S5 shows RelEf is able to synthesize pppGpp and, therefore, is catalytically active. Although, the activity for the enzyme preparation is very low. It is noteworthy that this low activity was found under assay conditions lacking ribosomes, uncharged tRNA, and mRNA. In contrast, efficient synthetase activity of full-length RelMtb was found to require the presence of the so-called Rel activation complex (RAC) composed of 70S ribosomes, mRNA, and uncharged tRNA (43). Nevertheless assays of RelEf performed in the presence of RAC containing \textit{E. faecalis} 70S ribosomes failed to enhance the overall synthetase efficiency of RelEf or enable pGpp production (Fig. S5B).

\textbf{RelEf hydrolyzes pGpp, ppGpp and pppGpp \textit{in vitro} with equal efficiency.} Based on previous studies in Firmicutes, Rel appears to be the primary enzyme responsible for degradation of ppGpp and pppGpp since inactivation of only the \textit{rel} gene results in either slow-growth phenotypes and elevated basal (p)ppGpp levels (21, 22, 28) or lethality unless the SASs are simultaneously inactivated (44, 45). Here, we asked whether RelEf could also hydrolyze pGpp. This is important because if RelEf lacks this activity and there is no other pGpp hydrolase, then pGpp synthesis tilts the balance in favor of accumulation amplifying its putative biological effects. Furthermore, non-specific or spontaneous degradation of pGpp may come into play, generating compounds such as pGp or Gpp, which might also have unique regulatory effects. Hydrolysis of pGpp by RelEf was tested by first using RelQef to synthesize radiolabeled pGpp.
and, as a control, ppGpp and pppGpp under conditions that completely consumed the labeled ATP pyrophosphate donor. The resulting products as well as a labeled GTP control were then incubated with RelE protein in the presence of Mn^{2+}. The breakdown of labeled pGpp, ppGpp and pppGpp showed that RelE is able to degrade all three substrate with virtually equivalent efficiency whereas the GTP control remained intact (Fig. 2).

\textbf{pGpp inhibits hypoxanthine phosphoribosyl transferase and guanylate kinase.} The evidence presented so far is taken to indicate that ppGp is formed and degraded by the same sorts of enzymes that are responsible for (p)ppGpp metabolism. Ideally, these features can be exploited to gather evidence that pGpp has regulatory functions in cells. However, we have been unable to observe pGpp accumulation in cellular extracts for reasons we suspect are due to technical difficulties (see Discussion). Therefore, in order to ask if pGpp might have physiologically relevant regulatory functions, a survey has been made of a selected set of diverse regulatory systems in which (p)ppGpp does have regulatory effects, although not necessarily only in \textit{Enterococcus faecalis}.

In \textit{B. subtilis}, (p)ppGpp was shown to be an important regulator of GTP homeostasis when guanine is present in the growth media by inhibiting activities of hypoxanthine-guanine phosphoribosyltransferase (HprT) and guanosine monophosphate kinase (Gmk) that convert guanine to GMP and GMP to GDP, respectively (10). In \textit{E. faecalis} pppGpp also strongly inhibits the activity of HprT (28). When pGpp was compared to ppGpp and pppGpp it was found that pGpp strongly inhibits the activity of HprT (IC\textsubscript{50} of 20.8 μM) (Fig 3A). The \textit{in vitro} activity of HprT was slightly less sensitive to ppGpp (IC\textsubscript{50} = 26 μM) and even less for pppGpp (IC\textsubscript{50} = 72.6 μM) (Fig 3A).

Comparing the relative biological effects of (p)ppGpp on \textit{E. faecalis} Gmk activity requires the consideration of two enzymes, since its genome encodes two Gmk paralogs unlike other Firmicutes. The Gmk-1 enzyme (EF2595) was extremely sensitive to pGpp inhibition with an
IC$_{50}$ of 0.90 μM (Fig 3B). Gmk-1 was also strongly inhibited by pppGpp (IC$_{50}$=8.9 ± 0.3 μM).

Previously, we showed that the Gmk-2 (EF3127) enzyme is only modestly, and non-specifically, inhibited by pppGpp with IC$_{50}$ value of 461.9 ± 106.4 μM (46). With pGpp, the inhibition is 3.3-fold more severe than pppGpp (IC$_{50}$ of 138.5 μM) (Fig. 3C) but not nearly as potent as with Gmk-1 (28).

pGpp inhibits *E. coli* RNA polymerase transcription of the *rrnB* P1 promoter for rRNA.

Many regulatory elements of SR in *E. coli* and several other Gammaproteobacteria are associated with accumulation of (p)ppGpp. Among them is a reduction in transcription initiating at rRNA promoters, such as *rrnB* P1. Many studies of this phenomenon indicate direct interactions of (p)ppGpp and DksA with RNA polymerase limit promoter activity (47-49).

Comparisons of individual regulatory contributions reveal ppGpp to be a more potent inhibitor than pppGpp with respect to promoter regulation in vitro, stable RNA accumulation in vivo, cellular growth rate control, RpoS induction, and other phenomena (50). Although pGpp has not been associated with Gammaproteobacteria, existing evidence of regulatory specificity for ppGpp vs pppGpp makes comparisons with pGpp a meaningful example of analog behavior using an additional member of a series of incremental 5’-phosphate additions (pGpp, ppGpp, and pppGpp). Therefore, *E. coli*-based in vitro transcription assays of *rrnBP1* promoter initiation were performed. The assays revealed that pGpp is a specific inhibitor (relative to GDP controls) but with far less potency than ppGpp and, by extrapolation with previous work (50), less than pppGpp (Fig. 4).

RelA$_{Ec}$ and RelQ$_{Ec}$ autocatalytic activities are differentially regulated by pGpp and ppGpp.

Recently, ppGpp was shown to stimulate the synthetase activity of *E. coli* RelA$_{Ec}$ (51).

Importantly, when high concentrations of RelA$_{Ec}$ were used in the system this product-mediated activation was masked by the *in situ* produced ppGpp. We have followed the kinetics of ppGpp...
synthesis by increasing concentrations of RelQ_{Ef}, both in the absence and in the presence of addition of 100 μM of ppGpp. In the absence of exogenous ppGpp, endogenous ppGpp production was nearly absent with 100 nM RelQ. At 150 nM RelQ_{Ef}, endogenous production was detected but with a pronounced lag. Both of these effects were absent at higher (250 nM) concentrations of the enzyme and activity became linear with time (Fig. 5A). Addition of ppGpp rendered all the three time courses linear and the kinetics of ppGpp production was proportional to concentration of RelQ_{Ef} (Fig. 5B). These results strongly indicate that ppGpp activates RelQ_{Ef}, in a manner similar to RelA_{Ec}.

We then followed the kinetics of ppGpp production by RelQ_{Ef} as well as RelA_{Ec} in the presence of increasing concentrations of pGpp or ppGpp. The experimental system was set up in such a way that in the absence of externally added guanosine alarmone, ppGpp production was detectable but showed a significant time lag. In the case of RelQ_{Ef}, addition of pGpp (up to 500 μM) had no effect on enzyme activity (Fig. 5C). On the other hand, ppGpp induced activation of RelQ_{Ef} with as little as 50 μM and had no inhibitory effects at concentrations up to 500 μM (Fig. 5D). As shown previously (51), ppGpp had a strong activating effect on RelA_{Ec} synthetase activity in a reconstituted translation system containing 70S ribosomes, model mRNA and deacylated tRNA. Interestingly, pGpp also had a strong activating effect on RelA_{Ec} activity, and different than ppGpp, did not inhibit the enzyme at higher concentrations (Fig. 5E and F). Activation of RelQ_{Ef} by ribosomes was also tested and, as expected for a single domain SAS, ribosome-dependent activation was not observed (data not shown).

Relacin does not inhibit RelQ_{Ef} activity. The SR has become a target for therapeutic intervention due to its established importance in pathogenesis and persister cell formation (3, 52-54). A RelA/Rel inhibitor and ppGpp mimic Relacin (55) represents an important stepping-stone toward the development of chemotherapeutics to block (p)ppGpp synthesis thereby interfering with long-term bacterial survival strategies. Relacin appears to block synthesis.
activity of long RSH enzymes (Rel/RelA) by inhibiting release from ribosomes as well as through a ribosome independent mechanism (55). Importantly, SASs have significant structural differences from larger RSH proteins apart from synthesis specificity since they lack the (p)ppGpp hydrolase domain as well as the regulatory C-terminal region necessary for ribosome interaction. To assess the ability of Relacin to inhibit the action of RelQ Ef, increasing concentrations of Relacin were added to in vitro ppGpp synthesis reactions containing RelQ Ef and, as a positive control, E. coli RelAEc. The addition of Relacin to reactions containing RelAEc produced the expected dose-dependent reduction in enzyme activity (Fig. 6), corroborating well with the original report (55). However, Relacin had no significant inhibitory effects on RelQ Ef activity even at 5 mM, which completely abolished the synthesis activity of RelAEc (Fig. 6).

DISCUSSION

In this study, the RelQ Ef SAS of E. faecalis, has been shown to synthesize pGpp, eliminating the otherwise likely possibility that this elusive nucleotide is but an intermediate in (p)ppGpp degradation. In vitro pGpp synthetase activity is appreciable when comparing the relative efficiency of (p)ppGpp production, more efficient than pppGpp but less efficient than ppGpp. This and other evidence that pGpp might qualify as a member of the (pp)pGpp alarmone family comes from a series of in vitro assays documented here comparing the regulatory effects of pGpp with (p)ppGpp. Previous observations that the cellular SR mediated by RelEf and RelQ Ef together is not as robust as when RelQ Ef is deleted (25) also suggest the possibility of unique regulatory roles for pGpp since the contribution of (p)ppGpp synthetase activity by RelQ is minor compared to RelEf in vivo. Clearly, cellular assays of pGpp abundance are needed for further study of these activities.

pGpp may represent a new member of the (p)ppGpp regulatory family. During the SR, (p)ppGpp was estimated to exist at 1-2 mM levels in E. coli (56). A report examining the
nucleotide pools of *B. subtilis* starved for isoleucine by treatment with O-methyltheronine found that pGpp accumulated to ~10% the level of (p)ppGpp (41). However, until now, the biological effects of pGpp were unknown. Given that pGpp has IC\textsubscript{50} values of 20 μM and 0.9 μM for HprT and Gmk-1, respectively, pGpp would be predicted to have physiologically significant effects even if its peak levels were 10% or less of levels achieved by (p)ppGpp during a fully developed SR. To this end, we attempted both two-dimensional TLC (2D-TLC) and HPLC approaches to measure pGpp abundance *in vivo* in order to establish it as a meaningful biological entity. This task has been historically complicated by the similar migration pattern and identical mass values of pGpp, ppGp, and GTP and equivocal assignments of enzymatic sources in natural systems. Despite exhaustive efforts to modify our 2D-TLC solvent system, including the same conditions used to resolve pGpp in *B. subtilis* (41), it proved insufficient to separate a pGpp standard from complex radiolabeled cell extracts. The separation of GTP from pGpp shown in Figure 1B was possible because it involves separation of pure substrates and products from a defined synthesis reaction. The use of an HPLC system employing a similar column and mobile phase combination to that described by Ooga et al. (33) overcame the hurdle of reliably separating pGpp standards from cell lysates. However, the selective loss of highly phosphorylated nucleotides (pGpp, ppGp, and pppGpp) during sample processing and column separation, coupled to the reduced sensitivity of UV detection greatly reduced our power to detect (pp)pGpp in *E. faecalis*. Work is underway to optimize sample processing and separation protocols to overcome the technical barriers preventing *in vivo* pGpp quantification.

Despite our unsuccessful attempts to detect pGpp *in vivo*, a recent study by Liu and colleagues strongly suggests that pGpp might reach biologically relevant concentrations because high GMP levels were measured during the SR in Firmicutes. Specifically, in *B. subtilis*, GMP pools were shown to dramatically increase from 10 μM during exponential growth to 0.3-0.5 mM, levels comparable or even exceeding GTP, which precipitously drops during amino acid downshift (46). Our preliminary HPLC analysis also indicated that GMP pools rise to
the mM level in starved cells of *E. faecalis* (Gaca *et al.*, unpublished). As GMP is used with approximately equal efficiency to GTP by RelQ<sub>ef</sub> (Table 1), it is a plausible that, after the GTP and GDP substrates are depleted, GMP could become the most abundant guanosine nucleotide and pGpp might then become the primary alarmone responsible for extending the duration of the SR. However, it is important to note that *E. faecalis* is unique in which two Gmk enzymes can be found. The two Gmk orthologs of *E. faecalis* show a large difference in sensitivity to (pp)pGpp analogs. Gmk-2 is not strongly inhibited by pppGpp or pGpp and this creates an interesting scenario. High Gmk-2 activity despite (pp)pGpp should abolish, or limit, (p)ppGpp-mediated inhibition of GMP to GDP conversion and, by extension, relieve inhibition of de novo GTP biosynthesis. However, our previous results showed that a slight 4-fold increase in basal (p)ppGpp resulted in a disproportionately large reduction of intracellular GTP pools (~40%) that are typically several orders of magnitude greater than levels estimated for basal (p)ppGpp (5, 28). Therefore, the ability of elevated (p)ppGpp to repress de novo GTP synthesis suggests that Gmk-2 inhibition by (p)ppGpp is not critical for regulating GTP levels in *E. faecalis* for unknown reasons. The relative intracellular abundance or relative activities of Gmk-1 and Gmk-2 could also impact the contribution of each enzyme to de novo GTP biosynthesis and (p)ppGpp-mediated control over GTP homeostasis.

Feedforward or autocatalytic stimulation of RelQ synthetase activity by ppGpp raises the possibility that RelQ or Rel products might serve as a signal amplifier for (pp)pGpp production in a manner homologous to the direct activation of RelA<sub>Ec</sub> by (p)ppGpp (51). Multiple attempts to test the activating effects of (pp)pGpp on Rel<sub>ef</sub> activity were unsuccessful due in part to the enzyme’s poor in vitro activity, even in the presence of ribosomes. Still, it is interesting that positive allosteric feedback regulation by ppGpp is present in a SAS, which lacks numerous sites conserved across the larger synthetases suggested to function in inter- and intra-molecular interactions such as those that might contact ppGpp to enhance synthetase efficiency in RelA<sub>Ec</sub> (57). The role of RelQ is further complicated because its synthetase activity shows
direct activation by ppGpp but not by pGpp (Fig. 5). Presently, it is unclear if activation occurs by separate mechanisms in RelA\textsubscript{Ec} and RelQ\textsubscript{Ef} or by the same catalytic center that binds ppGpp but not pGpp.

RelQ\textsubscript{Ef} appears to be a uniquely efficient source of pGpp synthesis. Over the past 3-4 decades there have been reports of several bacterial nucleotide analogs related to (p)ppGpp as potential candidates for involvement in stringent control; such as pGp, pGpp, ppGp (41, 58-60).

Although the source of many of these compounds was then unknown, there is conflicting evidence as to the involvement of RSH family enzymes. In \textit{B. subtilis}, it was noted that pGpp accumulation was eliminated in a probable Rel\textsubscript{Bs} deletion mutant (41) suggesting Rel as a source of pGpp other than a SAS. The caveat is that Rel deletions may be viable only if there are suppressor mutations inactivating “sister” SAS synthetases (45). Synthesis of pGpp \textit{in vitro} by a mutated Rel\textsubscript{Mtb} enzyme has been observed to generate pGpp but the route was not through use of GMP as a substrate but rather hydrolysis of the $\beta$ phosphate of ppGpp to yield pGpp (38). Although it has been shown that RelA\textsubscript{Ec} produced only trace amounts of pGpp in ribosome dependent assays (61), we also cannot rule out the potential importance of pGpp in Proteobacteria due to the presence of SAS enzymes in \textit{Vibrio} species, or as metabolic intermediate of (p)ppGpp degradation due to the presence of non-specific hydrolases, such as Nudix (33).

The large discrepancy in the relative ability of the SASs from \textit{S. mutans} to synthesize pGpp from GMP compared to RelQ\textsubscript{Ef} is intriguing considering the relatively high similarities of RelQ\textsubscript{Ef} with RelQ\textsubscript{Sm} ($\sim$80%) and RelP\textsubscript{Sm} ($\sim$60%). However, this appears to reflect overall catalysis, regardless of the guanine nucleotide substrate, because \textit{in vitro} activity of both \textit{S. mutans} enzymes was not as robust as RelQ\textsubscript{Ef} using either GDP or GTP as substrates (Fig. S4).

As discussed, during characterization of the (p)ppGpp synthetase activities of the \textit{B. subtilis} RelP\textsubscript{Bs} (YjbM) and RelQ\textsubscript{Bs} (YwaC) enzymes, an extra major spot with a migration pattern...
identical to that of GTP was identified in reactions containing RelQBs and either GDP or GTP as substrates (22). As pGpp is expected to co-migrate with GTP under the conditions used in this study, it is tempting to speculate that the GTP and GDP preparations might have been contaminated with GMP, which may have resulted in the synthesis of pGpp by RelQBs. Clearly additional studies are required to determine the degree of conservation and efficiency of pGpp synthesis among different SASs.

SAS enzymes may alter the efficiency of Relacin treatment. Relacin and its analogs are (p)ppGpp mimics designed to inhibit the function of RelA/Rel ‘long’ RSH enzymes (55, 62). If other SASs show similar insensitivity to Relacin as found here for RelQEf (Fig. 6), it could be argued that Relacin-resistant sources of (p)ppGpp may represent a major hurdle to the treatment of infections caused by organisms that encode SASs such as Firmicutes, Actinobacteria, and Vibrio species (12, 63, 64). More specifically, using E. faecalis as model organism, we showed that (p)ppGpp exerts a regulatory role in antibiotic persistence and virulence at concentrations that are much lower than those required to trigger the SR (28). In other words, low basal (pp)pGpp levels produced by RelQEf are sufficient for persistence and full virulence potential. On the other hand, Relacin was shown to reduce in vitro survival of wild-type B. subtilis and Streptococcus pyogenes, which harbor RelP and RelQ enzymes (55). It is conceivable that Relacin, by virtue of its ability to block ribosome dissociation, could also inhibit (p)ppGpp accumulation by tipping the balance of synthesis and hydrolysis to strongly favor hydrolysis thereby reducing (p)ppGpp accumulation from SAS enzymes. Further in vitro and in vivo testing of the effects of Relacin on stress tolerance, persistence, and virulence are necessary before making conclusions on the effectiveness of Relacin towards SAS-encoding bacteria.
Concluding remarks. The discovery and characterization of SASs are a relatively recent development compared to the longstanding and still ongoing research to understand the enzymatic and regulatory intricacies of 'long' RSHs. The results presented here reveal that RelQ$_{Ef}$ is directly activated by ppGpp and is able to synthesize pGpp. These biochemical properties may turn out to impart important biological functions to RelQ$_{Ef}$ and more generally to SAS enzymes. To our knowledge, this is the first study demonstrating regulatory effects of pGpp in vitro with specific regulatory targets shared by ppGpp and pppGpp. The inhibitory effects on Gmk, HprT, and RNAP activities, and the stimulatory effect on RelA$_{Ec}$ activity seem to qualify pGpp as a new member of the (pp)pGpp family of nucleotide second messengers. Judging from the history of (p)ppGpp, it will likely take considerable effort to pinpoint the targets of pGpp in vivo. We hope that others will be motivated by our findings to pursue the development of novel analytical tools that can be applied to study pGpp and possibly other related analogs that may be involved in bacterial regulatory circuits in addition to ppGpp and pppGpp.

MATERIALS AND METHODS

Bacterial strains and purification of (pp)pGpp metabolic enzymes. Escherichia coli strains (Table S1) were routinely grown in Bacto Luria Bertani (LB) broth (Becton, Dickinson, and Company) supplemented with 100 μg ml$^{-1}$ ampicillin at 37°C under normal atmosphere shaking at 200 rpm. For the overexpression of Rel and RelQ from E. faecalis or RelQ and RelP from S. mutans, coding regions were amplified by PCR using primers containing NheI and XhoI restriction sites (Table S2) and ligated into pET21a (EMD Biosciences, Madison, WI). The ligation mix containing pET21a expressing the (pp)pGpp metabolic enzymes were transformed into chemically competent E. coli BL21(λDE3) according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The resulting plasmids (Table S2) remove the native stop codon, fusing the coding region to a C-terminal His•Tag and place the coding region under dual control by a T7 promoter and lac operator. All E. coli cultures were grown in LB broth with antibiotic...
selective pressure until they reached an optical density (OD₆₀₀nm) of 0.5. Expression of the fusion protein was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. Cells were then harvested by centrifugation, washed once in chilled PBS buffer (pH 7), and cell pellets were lysed using a mini-bead beater (BioSpec Products, Bartlesville, OK). Proteins were purified under native conditions using Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA) and dialyzed into buffer containing 20 mM Tris base (pH 8), 500 mM NaCl, 1 mM EDTA, and 10% glycerol. Dialyzed protein was then separated into single use aliquots and stored at -80°C. In all cases, the N-terminal affinity tag was left intact.

**In vitro (pp)Gpp synthesis.** Unless otherwise indicated, in vitro synthesis of (pp)pGpp was carried out by adding 3 μM of purified Rel, RelQ or RelP to a reaction mix containing: 0.05 μCi μl⁻¹ of [γ-³²P] ATP, 25 mM Bis-Tris-propane (pH 9), 150 mM NaCl, and MgCl₂ at concentrations equivalent to the overall purine nucleotide concentration. Refer to figure legends for the concentration of individual purine nucleotides used in each experiment. For the Mg²⁺ inhibition assay, reactions contained the same components above with the addition of 0.5 mM ATP, 0.5 mM GTP, and MgCl₂ ranging from 0 to 20 mM. Reactions were incubated at 37°C for the indicated period of time at which (pp)pGpp synthesis was terminated by the addition of 1/4th volume 2 M formic acid (FoA). Control reactions were run under identical conditions for the duration of the experiment, including FoA acidification, but contained no enzyme. Acidified reactions were spotted onto polyethyleneimine (PEI) cellulose plates (J. T. Baker/Avantor Performance Materials, Center Valley, PA) and, unless otherwise noted, separated in 1.25 M KH₂PO₄ (pH 3.4). Reaction products were then visualized using a phosphorimager (Molecular Imager FX; Bio-Rad, Hercules, CA).

**Alkali hydrolysis.** Alkali hydrolysis reactions were carried out as described previously (65). Briefly, to an aliquot of in vitro synthesized [³²P]pGpp was added a solution containing 0.5 M
NaOH and 10 mM MgCl₂ and incubated at 37°C for the indicated time period. A control reaction lacking NaOH was used to monitor for inherent pGpp instability in the presence of divalent cations. Samples from the alkali hydrolysis reactions were stopped by with the addition of 1/4th volume 2 M FoA. Acidified reaction products were resolved by TLC, visualized, and quantitated as described above.

**Rel (pp)pGpp hydrolysis assays.** Labeled [³²P] pGpp, ppGpp, or pppGpp was synthesized from 200 µl reactions containing 2 mM GMP, GDP, or GTP as pyrophosphate acceptors and 10 µCi [γ-³²P]ATP. An excess of pyrophosphate donor (GMP, GDP, or GTP) was used to ensure that all [³²P]ATP was consumed and could not serve as a pyrophosphate donor for Rel mediated (pp)pGpp production during degradation experiments. Synthesis reaction products were visualized and quantified by phosphorimaging to confirm [³²P]ATP consumption and equalize the amount of labeled pGpp, ppGpp, and pppGpp product added to each degradation assay, respectively. The labeled (pp)pGpp was transferred into a reaction mix containing 150 nM Rel₂₁, 25 mM Bis-Tris-propane (pH 9), 150 mM NaCl, and 1 mM MnCl₂. Reactions were incubated at 37°C and, at the indicated time points, aliquots were removed and mixed with 1/4th volume 2 M FoA to halt (pp)pGpp degradation. Acidified aliquots were spotted onto PEI-cellulose TLC plates and resolved from the labeled degradation product inorganic pyrophosphate (PP₄) in either 4 M (NH₄)₂SO₄ for pGpp or 1.25 M KH₂PO₄ (pH 3.4) for (p)ppGpp. Reaction products were visualized and quantified using phosphorimaging as described above.

**(pp)pGpp preparations.** To make pure (pp)pGpp, (5 ml) RelQ₂₁ catalyzed in vitro synthesis reactions were prepared as described above with minor modifications including the removal of [γ-³²P]ATP and the addition of 1 mg ml⁻¹ bovine serum albumin (BSA) to enhance the long-term protein stability and activity. For ppGpp and pppGpp, reactions contained 6 mM unlabeled ATP and 4 mM GDP or GTP, respectively. For pGpp synthesis, reactions contained 4 mM ATP and 6
mM GMP. The reactions were incubated at 37°C and monitored for the complete consumption
of the limiting nucleotide by TLC. Reactions were then diluted 1:3 into 10:1 (mM) Tris:EDTA at
pH 7.5 and bound to DEAE-Sephadex A25. All of the following wash and elution buffers were
made in 10:1 Tris:EDTA (pH 7.5). The column was washed with 5 volumes of 0.1 M LiCl and
(pp)Gpp was eluted in 0.5 M LiCl by the successive addition of 0.25 ml aliquots that were
allowed to drip into 1 ml of absolute ethanol. Elutions were continued until the formation of white
precipitate was no longer observed. Tubes were then placed on ice for 30 minutes to complete
precipitation of (pp)pGpp. Purified (pp)pGpp was pelleted by centrifugation, washed 3 times with
absolute ethanol to remove LiCl, and dried in a fume hood. A small aliquot of (pp)pGpp was
resuspended in 0.5 M FoA, separated by TLC using 1.25 M KH₂PO₄, and visualized by
shortwave UV light to confirm the purity of each compound.

Inhibition of HprT and Gmk enzymes. Expression and purification of the *E. faecalis* HprT and
Gmk-2 enzymes have been previously published (28). The gene encoding Gmk-1 from *E.
faecalis* OG1RF was cloned into pLICTrPC-HA (66) using a ligation-independent cloning
technique, and the recombinant plasmids were transformed into *E. coli* BL21(DE3). Cells were
grown from a single colony at 37°C in LB supplemented with 100 µg ml⁻¹ carbenicillin to an
OD₆₀₀ of ~0.6, and IPTG was added to a final concentration of 1 mM. Cells were grown for
another 3 hours before harvest. Proteins were purified using Ni-NTA spin columns (Qiagen)
following the manufacturer’s instructions.

HprT reactions were performed at 25°C in a 100 µl reaction mix containing 100 mM Tris-
HCl (pH 7.4), 10 mM MgCl₂, 1 mM 5-phosphoribosyl 1-pyrophosphate (PRPP), 50 µM guanine,
20 nM purified HprT enzyme, and various (pp)pGpp concentrations. Reactions were initiated by
adding the enzyme and monitored for 10 min by measuring change of absorbance at 257 nm in
a temperature-controlled spectrophotometer (Shimadzu UV-2401PC). Gmk reactions were
performed at 25°C in a 100 µl mix containing 100 mM Tris pH 7.5, 100 mM KCl, 10 mM MgCl₂,

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4 mM ATP, 1.5 mM phospho(enol)pyruvic acid, 250 µM NADH, 2 U pyruvate kinase (from rabbit muscle, Sigma) and 2.64 U L-lactic dehydrogenase (from bovine heart, Sigma), and 10 nM *E. faecalis* Gmk-1 or Gmk-2. Reactions were initiated by adding either GMP or GMK and monitored for 5 min by measuring A340 in a temperature-controlled spectrophotometer. For inhibition curves, GMP was used at 50 µM and pGpp concentration was varied. Data fitting is done as previously described (10). For control experiments, pyruvate kinase was used at 0.02 U per 100 µl reaction and tested at 25°C. Reactions were initiated by adding GMP and monitored for 10 min by measuring the change in absorbance at 340 nm.

**Inhibition of rrnB transcription.** *E. coli* rrnB P1 promoter activity was measured by *in vitro* transcription essentially as described (49). Briefly, the reactions were carried out in the following buffer: 50 mM Tris-acetate (pH 8.0), 10 mM MgAc, 10 mM β-mercaptoethanol, 10 mg ml⁻¹ BSA, and 30 nM *E. coli* RNAP (Epicentre Technologies) was pre-incubated with the indicated concentrations of pGpp, ppGpp or GDP for 7 min at room temperature. Next, 10 nM linear DNA template, NTPs [100 µM ATP, CTP, GTP, and 10 µM UTP; 2 µCi per reaction α-³²P UTP (Perkin Elmer)], salt (90 mM potassium glutamate) and 300 nM DksA were added. The reactions were carried out at 30°C for 8 min, and terminated by the addition of an equal volume of the stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol). Samples were analyzed on 7 M urea, 6% polyacrylamide sequencing gels and quantified using a phosphorimager (GE Healthcare imaging system).

**Synthesis of Relacin.**
Relacin was prepared by modified method of Wexselblatt (58). First, commercially available diglycine (1) was esterified with benzylalcohol by refluxing in toluene in Dean-Stark apparatus under toluenesulfonic acid catalysis affording benzyl ester (2). Commercially available 2-N-isobutyryl-2-deoxyguanosine reacted with carbonyldiimidazole (CDI) and subsequently with benzyl ester 2 in DMF giving intermediate 4. Relacin was obtained by catalytic hydrogenation over palladium on charcoal in ethanol (removal of benzyl esters) and converted to its sodium salt by passing through a column of Dowex 50 in Na⁺ form.

**Regulation of RelAEc and RelQEf synthetase activity by pGpp, ppGpp, and Relacin.** The experiments were performed essentially as described in (51). If not stated otherwise, experiments were performed with 250 nM RelQ or 30 nM RelA, 0.5 μM *E. coli* 70S ribosomes, 0.3 mM ³H-labelled GDP (ARC), 1 mM ATP, 100 μM ppGpp, and 0.5 mg ml⁻¹ BSA. The reaction mixture was preincubated at 37°C for 2 min before the reaction was started by addition of ATP. Radiolabelled ppGpp product was separated from GDP by TLC and quantified using scintillation counting. All experiments were performed from three to five times.
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REFERENCES


Table 1. Kinetic constants for pGpp, ppGpp and pppGpp synthesis reaction catalyzed by *E. faecalis* SAS enzyme RelQ<sub>E</sub> in the presence of ppGpp or pppGpp.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cofactor</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(K_M) (mM)</th>
<th>(k_{\text{cat}}/K_M) (mM(^{-1})s(^{-1}))</th>
<th>Efficiency (X/GMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>ppGpp</td>
<td>0.58±0.07</td>
<td>0.49±0.17</td>
<td>1.19±0.43</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>ppGpp</td>
<td>0.74±0.1</td>
<td>0.35±0.27</td>
<td>0.87±0.3</td>
<td>0.87±0.43</td>
</tr>
<tr>
<td>GDP</td>
<td>ppGpp</td>
<td>1.34±0.2</td>
<td>0.65±0.24</td>
<td>2.08±0.82</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>ppGpp</td>
<td>0.81±0.07</td>
<td>0.21±0.07</td>
<td>3.9±1.28</td>
<td>3.9±1.28</td>
</tr>
<tr>
<td>GMP</td>
<td>ppGpp</td>
<td>1.28±0.1</td>
<td>1.23±0.19</td>
<td>1.04±0.18</td>
<td>1.04±0.18</td>
</tr>
<tr>
<td></td>
<td>ppGpp</td>
<td>1.6±0.12</td>
<td>0.85±0.15</td>
<td>1.87±0.37</td>
<td>1.87±0.37</td>
</tr>
</tbody>
</table>

All experiments were performed at 37ºC with 250 nM RelQ<sub>E</sub> in Polymix buffer (67) at 5 mM Mg\(^{2+}\) concentration in the presence of 0.5 mg ml\(^{-1}\) BSA and 100 µM ppGpp or pppGpp.
FIGURE LEGENDS

FIG 1. Small alarmone synthetases but not RelEl utilize GMP to synthesize pGpp. (A) TLC of an in vitro synthesis reaction containing 2.5 mM ATP, 2.5 mM GMP, and 3 μM of each enzyme. Control reactions (Ct) lack enzyme. The indicated times are in hours. (B) TLC separation of the purified pGpp product and a GTP standard. Nucleotides were resolved using 0.75 M KH2PO4 and visualized using shortwave UV light. (C) TLC of in vitro synthesized pGpp treated with 0.5 M NaOH and sampled at the indicated times in hours. The control (Ct) reaction was not treated with NaOH but incubated at 37°C for the full 2 hours. Ef, E. faecalis; Sm, S. mutans.

FIG 2. RelEl degrades pGpp and (pp)pGpp in vitro. (A) Time course of (pp)pGpp degradation. RelEl (150 nM) was added to pGpp, ppGpp, or pppGpp synthesized with [γ-32P]ATP to label the 3'-β phosphate on the ribose moiety of each nucleotide, or to a labeled GTP standard (negative control). Reactions were incubated at 37°C and sampled at the indicated time points in minutes. Percent remaining is the proportion of (pp)pGpp present at the indicated time point relative to the initial starting input, taken as 100%, for each respective nucleotide. Error bars represent the standard deviation from three independent experiments. (B) Representative TLC images of the in vitro degradation reactions. Only the residual substrate remaining after release of PP, is shown from three different chromatograms.

FIG 3. Inhibition of HprT and Gmk activities by (pp)pGpp. (A) HprT inhibition by increasing concentration of (pp)pGpp. GTP was used as a control for non-specific inhibition. Inhibition of (B) Gmk-1 and (C) Gmk-2 by increasing concentrations of (pp)pGpp. The inset for Gmk-1 shows the same data as the larger graph but with a narrower y-axis range (0-0.1) to highlight differential regulation by (pp)pGpp. Relative activity for Gmk-1 and Gmk-2 in the presence of exogenous pppGpp is reproduced from Liu et al. (46). Error bars represent the standard error from three independent experiments. In panel B (Gmk-1), the differences between pGpp and ppGpp and pGpp and pppGpp were statistically significance ($p \leq 0.05$, 2-tailed t-test assuming...
unequal variance) but the differences between ppGpp and pppGpp were not \( (p = 0.12) \). In panel C, the differences between pGpp and pppGpp were not statistically significant \( (p = 0.09) \).

**FIG 4.** Inhibition of rrnB1 transcription from the P1 promoter by pGpp and ppGpp. RNAP was preincubated with increasing concentration of pGpp, ppGpp, and GDP. *In vitro* transcription was started by the addition of DksA, DNA template, and NTP substrates. All reactions were incubated at 37°C for 8 minutes prior to transcript analysis by PAGE and quantitation by phosphorimaging. Error bars represent the standard deviation of three independent replicates. When compared to the GDP control, the differences observed with pGpp or ppGpp were statistically significance \( (p \leq 0.05, \text{2-tailed t-test assuming unequal variance}) \).

**FIG 5.** Effects of (p)ppGpp on the synthase activity of RelQEf and RelAEC. (A) Time course of ppGpp synthesis by RelQEf in the absence (A) or presence (B) ppGpp. (C) Time course of ppGpp synthesis by RelQEf (250 nM) in the presence of increasing concentrations of pGpp. (D) Time course of ppGpp synthesis by RelQEf (250 nM) with increasing concentrations of pppGpp. (E) ppGpp and (F) pGpp. Synthesis reactions involving RelAEC contain 0.5 μM *E. coli* 70S ribosomes, model mRNA and deacylated tRNA. All reaction mixtures were preincubated at 37°C and reactions were initiated by the addition of ATP. Panels (A) and (B) represent individual kinetic time series. For (C-F) error bars represent the standard deviation of at least three independent experiments. GDP to ppGpp conversion of 0 corresponds to 100% GDP and 1.0 to complete conversion of GDP into ppGpp.

**FIG 6.** RelQEf synthase activity is not inhibited by Relacin. *In vitro* ppGpp synthesis activities of *E. faecalis* RelQEf (500 nM, red circles) or *E. coli* RelAEC (100 nM, black circles) with increasing concentrations of the (p)ppGpp-analog Relacin. Reaction conditions are the same as those described in Fig. 5. Total ppGpp accumulation in the absence of Relacin is set to 1 and used to
calculate the relative enzyme activity. Error bars represent the standard deviation of at least three independent experiments.
**rrnB P1 Transcription**

**Relative Transcript Level**

- **Nucleotide (μM)**
  - 0
  - 100
  - 200
  - 300
  - 400
  - 500

- **pGpp**
- **ppGpp**
- **GDP**

*Graph showing the effect of different nucleotides on rrnB P1 transcription level.*