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Activation of RelA by pppGpp as the basis for its differential toxicity over ppGpp in *Escherichia coli*

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The nucleotide derivatives (p)ppGpp, comprising ppGpp and pppGpp, are important signalling molecules that control various facets of gene regulation and protein synthesis in *Escherichia coli*. Their synthesis is catalysed by RelA (in response to amino acid limitation) and by SpoT (in response to the limitation of carbon source or fatty acids). SpoT is also a hydrolase for degradation of both ppGpp and pppGpp, while GppA catalyses the conversion of pppGpp to ppGpp. Here we provide evidence to show that pppGpp exerts heightened toxicity compared to that by ppGpp. Thus, *gppA spoT* double mutants exhibited lethality under conditions in which the single mutants were viable. The extent of RelA-catalysed (p)ppGpp accumulation in the *gppA spoT* strain was substantially greater than that in its isogenic *gppA*$^{+}$ derivative. The data is interpreted in terms of a model in which toxicity of pppGpp in the *gppA spoT* mutants is mediated by its activation of RelA so as to result in a vicious cycle of (p)ppGpp synthesis.

Keywords. (p)ppGpp; GppA; SpoT; RelA; stringent response

1. Introduction

Nucleotide-based signalling molecules play a key role in the physiology of organisms by coordinating cellular processes with extracellular or intracellular signals (Pesavento and Hengge 2009). In eubacteria, the signalling molecules ppGpp and pppGpp [collectively referred to as (p)ppGpp] accumulate during starvation, switching the balance of metabolism from growth and cell division to survival and stress response (Chatterji and Kumar Ojha 2001; Braeken *et al.* 2006; Potrykus and Cashel 2008; Hauryliuk *et al.* 2015). The pair of molecules are synthesised by the transfer of a pyrophosphate moiety from ATP to GDP or GTP, respectively. The role of (p)ppGpp in bacterial physiology is well-studied in the gram-negative model bacterium *Escherichia coli*. In β- and γ-proteobacteria, (p)ppGpp metabolism is driven by two proteins, RelA and SpoT, which are members of the Rel/Spo homolog (RSH) family and share similar domain architecture (Mittenhuber 2001; Atkinson *et al.* 2011). The N-terminal half has the catalytic domain with the (p)ppGpp synthase and hydrolase functions in the case of SpoT and only synthase function in case of RelA. The C-terminal half of the proteins have the regulatory domains important for sensing stress and starvation signals. RelA is a ribosome-bound protein that is activated by the 'hungry' codons that appear following amino acid starvation and the consequent increase in the concentration of uncharged tRNA [Wendrich *et al.* (2002) and references there in]. Recent cryo-electron microscopy studies have provided important insights into the structural basis for RelA activation by the entry of uncharged tRNA into the A-site of an elongating ribosome (Arenz *et al.* 2016; Brown *et al.* 2016; Loveland *et al.* 2016).

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SpoT has a weak synthase and a strong (p)ppGpp hydrolase activity and unlike *relA* is an essential gene (An *et al.* 1979; Xiao *et al.* 1991). It regulates the basal (p)ppGpp levels in the cell (Sarubbi *et al.* 1988) and elicits stringent response following carbon (Xiao *et al.* 1991), fatty acid (Seyfzadeh *et al.* 1993), and iron (Vinella *et al.* 2005) limitation. The SpoT hydrolase activity was reported to be inhibited in the presence of uncharged t-RNA and ribosomes (Richter 1980), conditions that mimic amino acid starvation. Interaction of SpoT with other factors regulate the balance between its synthase and hydrolase functions. The acyl carrier protein was reported to interact with SpoT and promote its synthase activity during fatty acid starvation (Battesti and Bouveret 2006). Interaction of the GTPase, CgtA/ObgE with SpoT was proposed to regulate the hydrolase activity during exponential growth (Jiang *et al.* 2007).

Hydrolysis of pppGpp is accomplished also by a second enzyme GppA (guanosine pentaphosphate phosphohydrolase), in addition to SpoT. GppA converts pppGpp to ppGpp (Somerville and Ahmed 1979; Harat and Sy 1983; Keasling *et al.* 1993). The physiological relevance of this reaction has been unclear, but ppGpp being the predominant stringent nucleotide in the cell was considered the principal effector of stringent response in gram-negative bacteria (Mechold *et al.* 2013).

Genetic evidence had suggested that SpoT is the primary (p)ppGpp hydrolase in the cell, and its activity supported growth by preventing the accumulation of (p)ppGpp (Xiao *et al.* 1991). However, conditional SpoT depletion to demonstrate (p)ppGpp accumulation have not been carried out so far. Further, if there was accumulation, it would be of interest to study the relative proportion of the two nucleotides as compared to that seen during stringent response. We have addressed these by monitoring the (p)ppGpp accumulation and growth response associated with SpoT depletion in isolation or together with the inactivation of the GppA hydrolase. Our results have shown that the loss of GppA exacerbates the sickness associated with SpoT deficiency and there was a disproportionate increase in the (p)ppGpp level under these conditions. These data have been interpreted in terms of a model in which RelA was activated by pppGpp under these conditions.

2. Materials and methods

2.1 *Growth conditions*

Cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), MOPS buffered minimal medium (Neidhardt *et al.* 1974) or minimal A medium (Miller 1992). The minimal medium was supplemented with 0.5% glucose, unless mentioned otherwise, and when indicated, 20 amino acids each at a final concentration of 40 μg ml^{-1}. In plates, glucose and casamino acids were each supplemented at 0.2% final concentration. Antibiotics and their final concentration in the growth medium are ampicillin (Amp) 50 μg ml^{-1}, kanamycin (Kan) 25 μg ml^{-1}, tetracycline (Tet) 10 μg ml^{-1} and chloramphenicol (Cm) 15 μg ml^{-1}. Isopropyl β-D-thiogalactopyranoside (IPTG) and 5-Bromo-4-chloro 3-indolyl-β-D-thiogalactoside (X-gal) were used at a final concentration of 1 mM and 50 μg ml^{-1}, respectively.

2.2 *Construction of strains and plasmids*

All strains used in this study are derivatives of the *E. coli* K-12 strain MG1655. Strains, plasmids, and primers used in this study are listed in supplementary table 1. Mutations were introduced into the chromosome by phage P1 mediated transduction using standard protocols. Gene deletions have been sourced from the Keio collection (Baba *et al.* 2006), and when required, the kanamycin resistance cassette was removed using the FLP recombinase expressed from the pCP20 plasmid (Cherepanov and Wackernagel 1995). The plasmid construct referred here as P_{lac}-*spoT*$^+$ was constructed using the vector pRC7 (Bernhardt and De Boer 2004) and has been referred to as pRCspoT previously (Nazir and Harinarayanan 2016). The *spoT* gene in P_{lac}-*spoT*$^+$ is under the *lac* promoter and has the native RBS and the TTG start codon. Transposon mutagenesis was carried out using the phage λNK1316 and λNK1098 (Miller 1992) and the transposon insertions *relA*::Tn*10*dTet and *rlmD*::Tn*10*dKan were mapped by inverse PCR (Higashitani *et al.* 1994). The β-galactosidase reporter fusions *relA'-lac* and *rlmD'-lac* within the *relA* and *rlmD* genes, respectively, were constructed using the knockout alleles available in the Keio collection and plasmid pKG137 using a published protocol (Ellermeier *et al.* 2002). The new junctions generated were verified by sequencing.

2.3 *Depletion of SpoT using the plasmid P_{lac}-spoT$^+$*

In strains carrying the single-copy plasmid P_{lac}-*spoT*$^+$, the chromosomal *spoT* gene was replaced with the *ΔspoT::*Cm (*spoT207*) or the Δ*spoT* (*spoT212*) allele. Since SpoT expression in the plasmid, P_{lac}-*spoT*$^+$ was from the *lac* promoter, and the plasmid also carried the

lacI gene coding for the repressor protein and the *lacI*^{q} mutation that increases the expression of the repressor, SpoT expression can be inhibited by the withdrawal of IPTG from the growth medium. Cultures, grown in the presence of ampicillin and IPTG (1 mM) in LB or MOPS medium containing only glucose or glucose and 20 amino acids, were washed with minimal medium to remove IPTG. The cultures were then diluted 100-fold (unless mentioned otherwise) into appropriate growth medium lacking IPTG or serially diluted and spotted on LB agar plates with reduced IPTG concentration or no IPTG.

2.4 *Plasmid segregation assay*

The assay is based on the principle that an essential gene function when provided from an unstable plasmid, would stabilise the plasmid. In this assay, referred here as the 'blue–white assay', the ability of strains to grow following the loss of the unstable, single-copy, amp^r-plasmid P_{lac}-*spoT*$^+$ was assessed in the Δ*lacZYAI::FRT* genetic background. Since the plasmid carries the *lacZ* gene, blue and white colonies represent the retention and loss of the plasmid, respectively. The stabilization of the plasmid (no white colonies) would indicate that the plasmid encoded function was essential for growth. Strains containing the plasmid P_{lac}-*spoT*$^+$ were grown overnight in LB broth containing ampicillin and IPTG; the latter was used at a final concentration of 1 mM, unless mentioned otherwise. Cultures were washed with minimal medium to remove IPTG and serially diluted. Appropriate dilutions were spread on plates containing IPTG and X-gal in order to obtain ∼300 colonies per plate. Plates were incubated at 37°C for 24 hours in the case of LB medium or minimal A medium containing glucose and casaminoacids and 48 hours in the case of minimal A medium with glucose. Incubation was extended to 72 hrs whenever white colonies were not evident after 24 or 48 hrs of incubation. The percentage of white colonies was calculated from the ratio of white colonies over the total number of colonies scored (white + blue).

2.5 *β-Galactosidase assay*

Cultures were grown overnight in the appropriate medium and diluted 100-fold in the same medium. At mid-log phase (A_{600}—0.4 to 0.6), cultures were assayed for β-galactosidase activity (Miller 1992). The values, reported as Miller units, are the means from three or more independent experiments.

2.6 *(p)ppGpp estimation by thin layer chromatography*

Cultures grown to saturation were diluted 100-fold, except in the case of RS206, which was diluted 20-fold due to the severe growth inhibition seen in the absence of IPTG, and allowed to grow in MOPS medium containing appropriate carbon source. When the cultures reached an A_{600} of ∼ 0.4 to 0.5, the cultures were diluted 10-fold into the pre-warmed low phosphate medium (0.4 mM K_2HPO_4) and allowed to undergo at least two doublings in the presence of 100–200 μCi ml^{-1} of ^{32}P-H_3PO_4 before sample collection at ∼ 0.2–0.3 A_{600}. An unlabelled culture was used to monitor A_{600}. Samples were collected in tubes containing an equal volume of 2 N HCOOH, kept chilled on ice. These were subjected to three cycles of freeze–thaw and centrifuged at 10000 rpm for 5 minutes at 4°C. The supernatant was applied on PEI cellulose sheets and resolved using 1.5 M KH_2PO_4, pH 3.4. The nucleotide spots were imaged using phosphorimager (Typhoon FLA 9500). The intensity of the spots was quantified by densitometry after subtracting background using the multi-gauge V3.0 software (Fujifilm).

2.7 *Elicitation of isoleucine, glucose, fatty acid or glucose + fatty acid starvation*

Isoleucine starvation was induced by the addition of valine to a final concentration of 100 μg ml^{-1} to early log phase cultures cultivated in MOPS medium containing 0.2% glucose. Starvation for glucose was induced by growing cells in a medium containing 0.02% glucose. Fatty acid starvation was induced by the addition of the antibiotic cerulenin to a final concentration of 200 μg ml^{-1} to an early log phase culture cultivated in MOPS medium containing 0.2% glucose. Combined starvation for glucose and fatty acid was achieved by the addition of cerulenin to the culture grown in MOPS medium containing 0.02% glucose.

2.8 *Isolation and mapping of the hypomorphic relA alleles that suppressed the loss of SpoT function*

Following transposon mediated mutagenesis (Kleckner *et al.* 1991) of the Δ*spoT::*Cm/P_{lac}-*spoT*$^+$ strain, mutants that survived the loss of P_{lac}-*spoT*$^+$ were identified as white colonies in LB IPTG X-gal plates. After confirming the transposon insertions were

sufficient for the suppression of the SpoT requirement, those insertions that supported the growth of the Δ*spoT* strain in minimal glucose medium and as well as in the same medium containing amino acids serine, methionine, and glycine (a plate test for RelA functionality) were mapped by sequencing the transposon–chromosome junctions. One insertion was at the 3′-end of the *rlmD* ORF that is immediately upstream of *relA* and was designated *rlmD*::Tn*10*dKan. The second insertion was within the *relA* ORF and created a TAA stop codon immediately after the 496th codon; this was designated as *relA496*Δ::Tn*10*dTet. We also constructed the *relA*496Δ::Kan allele, wherein all codons after 496 were deleted and replaced with a TAG stop codon and the kanamycin cassette from pKD13 (Datsenko and Wanner 2000) by recombineering (Yu *et al.* 2000) using the primer pair JGOrelA496aaPS4 and JGOrelAPS1. The construct was verified by sequencing using primers JGOrelA+882 and K1. A cartoon showing the location of the transposon insertions and the other *relA* alleles used in this study is shown in supplementary figure 2.

3. Results

3.1 *Approaches employed to differentially modulate intracellular levels of ppGpp and pppGpp*

Since RelA and SpoT catalyse the synthesis of both ppGpp and pppGpp (from GDP and GTP, respectively) most of the earlier studies have treated these two molecules without distinction by referring to them by the combined designation of (p)ppGpp. In this study, we have attempted to exploit the differences in the catalytic function of the two hydrolases SpoT and GppA that degrade (p)ppGpp to achieve differential accumulation of the two molecules in the cells. SpoT hydrolyses both ppGpp and pppGpp, whereas, GppA converts pppGpp to ppGpp. Thus, whereas the simultaneous loss of both SpoT and GppA function may be expected to increase levels of both ppGpp and pppGpp, loss of SpoT alone in comparison would increase ppGpp preferentially (since any pppGpp that is synthesised will be converted to ppGpp by GppA) and of GppA alone to have minimal effect.

A Δ*gppA* mutant is viable (Somerville and Ahmed 1979; Baba *et al.* 2006), but the loss of SpoT function in an otherwise wild-type strain is lethal (Xiao *et al.* 1991). Hence, the abrogation of SpoT hydrolase function (in the presence or the absence of GppA) was achieved by one of the following means: (i) by using *spoT1*, which encodes a hydrolase deficient allele of SpoT but is still viable; (ii) by using *relA* mutations, which act to reduce (p)ppGpp synthesis and therefore are viable with Δ*spoT;* or (iii) by using a Δ*lac* Δ*spoT* strain bearing an unstable single-copy-number plasmid with $lacI^q$ and P_{lac}-$spoT^+$ $lacZ^+$, so that SpoT depletion could be achieved either by withdrawal of the inducer IPTG from the growth medium or by identification of cells that had spontaneously lost the plasmid during cell division. This plasmid is referred to below as P_{lac}-$spoT^+$. In these genetic backgrounds, we monitored the (p)ppGpp levels, both under basal conditions and after inducing isoleucine starvation (by addition of valine) in defined medium.

3.2 *Synthetic phenotype upon combined loss of GppA and SpoT hydrolase activities*

We obtained several lines of genetic evidence that suggested extreme sickness or lethality of *E. coli* cells upon the combined loss of SpoT and GppA hydrolase activities. (i) Thus, whereas the *spoT1* and *gppA* single mutants were viable, the double mutant was lethal; (ii) Similarly, whereas Δ*spoT* was viable in the presence of either of two *relA* mutations (RelA-↓ or *relA496*Δ::Kan, which is described below) the introduction of the Δ*gppA* allele into these derivatives rendered them inviable. The synthetic lethality was demonstrated with the plasmid segregation assay that works on the principle that an essential gene function when provided from an unstable plasmid would stabilise the plasmid (see methods for details). This assay was performed after introducing the plasmid construct with P_{lac}-$spoT^+$ into strains lacking the chromosomal *lacZYAI* genes. When propagated without selection, the retention and loss of the plasmid can then be scored as blue and white colonies, respectively, on growth medium containing IPTG and X-gal.

This ‘blue–white’ assay showed that, unlike the parental *spoT1* strain, the *spoT1* Δ*gppA*::Kan strain was inviable in minimal glucose medium with or without casamino acids and distinctly slow growing on LB medium (figure 1, compare panels i, iv and vii with panels ii, v and viii). The slow growing colonies obtained on the LB plate were genetically unstable (-supplementary figure 1). A *relA* deletion suppressed the *spoT1* Δ*gppA*::Kan synthetic growth defect (figure 1, panels iii, vi and ix). The hydrolase deficient *spoT* allele, *spoT202* (Sarubbi *et al.* 1989) also exhibited synthetic lethality with the Δ*gppA*::Kan allele and the growth defect appeared to be more severe than in

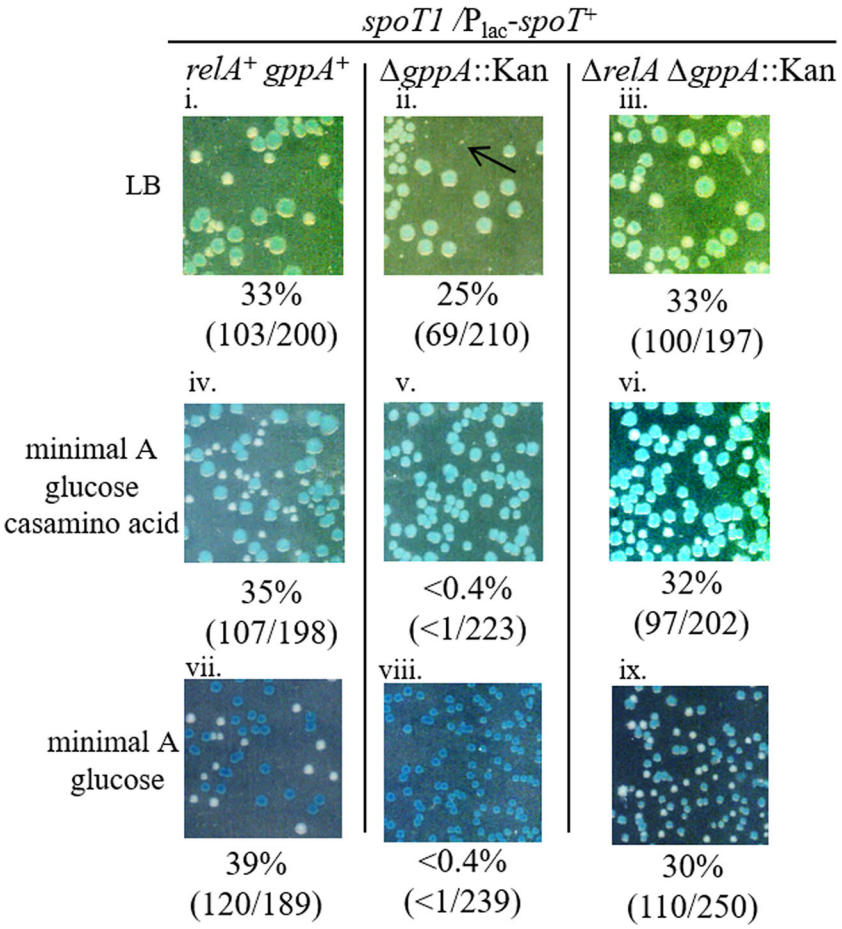

Figure 1. The *spoT1* allele and the Δ*gppA* mutation exhibit RelA-dependent synthetic lethality. The loss or retention of the unstable plasmid P_{lac}-*spoT*$^{+}$ was assayed in plates containing IPTG and X-gal as described in the methods. Relevant genotype of the strain is indicated above each image showing the section of a plate. The percentage of white colonies and the total number of colonies (blue + white) used to calculate the ratio are indicated below the image. Blue and white colonies from multiple plates were counted to estimate the percentage of white colonies. A representative picture of blue and white colonies from a section of the plate is shown. The arrow points to a small (slow growing) white colony in the image. Strains used are RS40 in panels i, iv and vii; RS461 in panels ii, v and viii; and RS486 in panels iii, vi and ix.

the *spoT1* Δ*gppA*::Kan strain as no white colonies were recovered on minimal medium or LB medium (data not shown). These results suggested that elevated basal pppGpp and lowered *spoT* hydrolase activity induced RelA-dependent growth inhibition.

Two transposon insertions that rescued the growth of a Δ*spoT* strain were identified from a genetic screen (Methods), that were, respectively, a Kan^{R} insertion in *rlmD* (immediately upstream of *relA*) (supplementary figure 2, ii) and a Tet^{R} insertion after codon 496 within *relA* (supplementary figure 2, iii). Since a reduction in RelA function caused by the transposon insertion in the upstream *rlmD* gene (*rlmD*::Tn*10*dKan) cannot be due to any alteration in the RelA polypeptide sequence, we infer that it is because of reduced expression of an intact unaltered RelA protein. In support, we found also a non-polar deletion of *rlmD* (derived from the Δ*rlmD*::Kan allele of the Keio collection by FLP mediated excision of the Kan^{R} determinant) (supplementary figure 2, iv), mimicked the growth phenotypes of the transposon insertion *rlmD*::Tn*10*dKan. Consistent with the loss of *relA* promoters located within the *rlmD* ORF (Metzger *et al.* 1988; Nakagawa *et al.* 2006; Brown *et al.* 2014), the Δ*rlmD* deletion reduced the expression of *relA* ~75-fold (supplementary figure 3). The Δ*rlmD*::FRT allele would be referred to as RelA-↓ from here on.

We then constructed a pair of isogenic *gppA*$^{+}$ and Δ*gppA* derivatives of a RelA-↓ Δ*spoT* strain carrying the P_{lac}-*spoT*$^{+}$ shelter plasmid. The *relA*$^{+}$ Δ*spoT* strain with the same plasmid was used as a control. Using the 'blue–white' assay, we found, as expected, that the

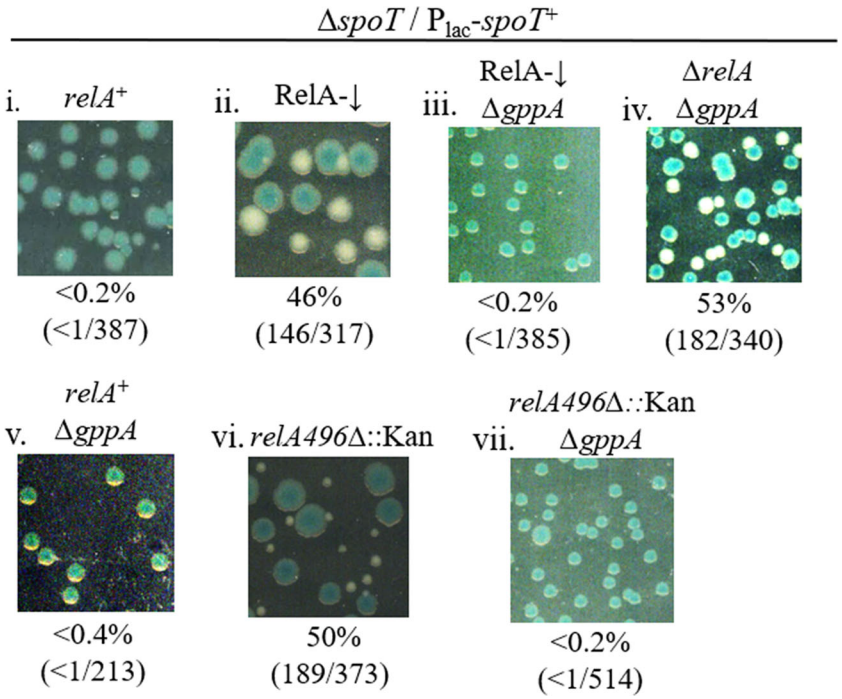

Figure 2. Hypomorphic *relA* alleles that support growth in the absence of SpoT function require the GppA hydrolase activity. Segregation of the unstable plasmid P_{lac}-*spoT*$^{+}$ was assayed in LB IPTG X-gal plates as described in the methods. Relevant genotype of the strain is indicated above each image showing the section of a plate. The percentage of white colonies and the total number of colonies (blue + white) used to calculate the ratios are indicated below the image. Blue and white colonies from multiple plates were counted to estimate the percentage of white colonies. A representative picture of blue and white colonies from a section of the plate is shown. Strains used are, RS14—panel i, RS361—panel ii, RS415—panel iii, RS344—panel iv, RS478—panel v, RS420—panel vi and RS359—panel vii.

Δ*spoT* strain was inviable while the RelA-↓ Δ*spoT* was viable (figure 2, panels i and ii). However, the RelA-↓ Δ*spoT* Δ*gppA*::Kan strain was inviable in LB (figure 2, panels iii) or in minimal glucose medium with or without amino acids (data not shown), which indicated that the GppA hydrolase was required for the growth of the RelA-↓ Δ*spoT* mutant. The inability of the RelA-↓ Δ*spoT* Δ*gppA*::Kan strain to grow was due to the residual RelA function, because, the Δ*relA* Δ*spoT* Δ*gppA*::Kan strain was viable (panel iv). As expected, the essentiality of the SpoT function was continued to be seen in the *relA*$^{+}$ *gppA* background (panel v). These results indicated that the function of the RelA-↓ allele was modulated by GppA function. Experiments described below showed that RelA-mediated (p)ppGpp synthesis was enhanced under GppA-deficient conditions.

The location of the *relA496*::Tn*10*dTet insertion identified in the genetic screen indicated that the mutant *relA* allele could encode a truncated RelA polypeptide with the N-terminal 496 amino acids. To ask if the truncated RelA polypeptide was sufficient to support growth in the absence of SpoT function, we engineered the *relA496*Δ::Kan deletion (see methods, supplementary figure 2, v) that would result in the synthesis of an identical polypeptide. The viability of the *relA496*Δ::Kan Δ*spoT* strain (figure 2, panel vi) indicated that the RelA truncation, leading to the loss of the C-terminal ribosome binding domain, supported growth in the absence of SpoT function. However, the *relA496*Δ::Kan Δ*spoT* Δ*gppA* strain was inviable in LB (figure 2, panel vii) and as well as in minimal glucose medium with or without amino acids (data not shown).

Altogether, the above results showed that the RelA-dependent synthetic lethality was observed in the *spoT1* Δ*gppA relA*$^{+}$, Δ*spoT* Δ*gppA* RelA-↓, and Δ*spoT* Δ*gppA relA496*Δ::Kan strains, but not in the isogenic *gppA*$^{+}$ strains. This is consistent with the expectation that RelA activity contributes to growth inhibition from (p)ppGpp accumulation following the loss of GppA activity. This view is supported by the suppression of the Δ*spoT* Δ*gppA* synthetic lethality by the Δ*relA* allele (figure 2, panel iv). The *relA1* allele was similarly a suppressor of this lethality (data not shown).

When we compared the growth of isogenic *gppA*$^{+}$ and Δ*gppA* derivatives of the Δ*spoT*/P_{lac}-*spoT*$^{+}$ strain

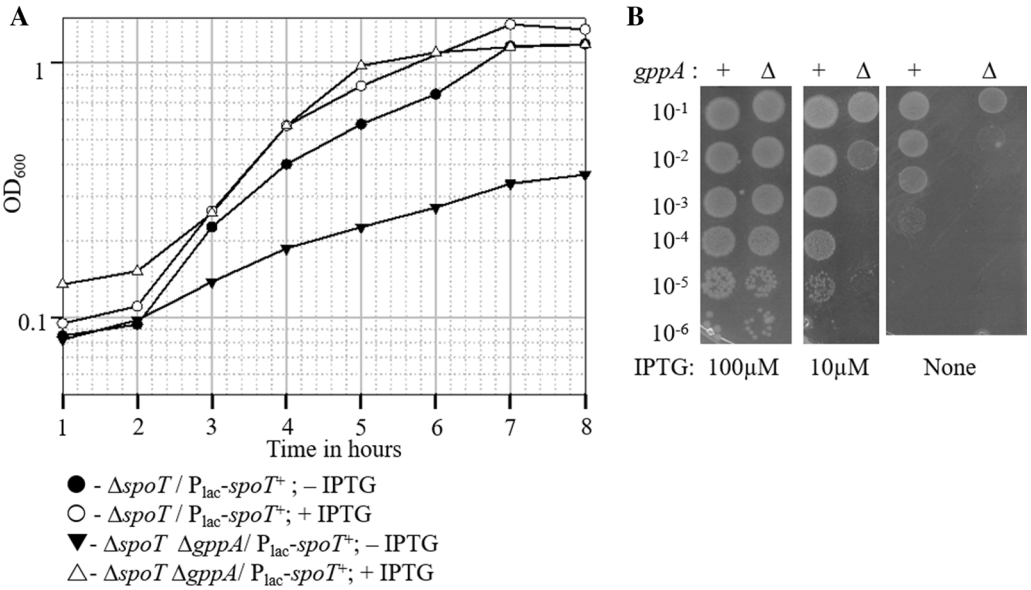

Figure 3. SpoT depletion in the *gppA* mutant was associated with more severe growth inhibition than in the *gppA*$^+$ mutant. (**A**) Strains Δ*spoT*/ P_{lac}-*spoT*$^+$ (RS260) and Δ*spoT* Δ*gppA*/ P_{lac}-*spoT*$^+$ (RS478) cultured in MOPS medium containing glucose, 20 amino acids, ampicillin and 1mM IPTG were washed and sub-cultured in the same medium with (○,Δ) or without (●,▼) IPTG. The growth inhibition following IPTG withdrawal in the Δ*spoT* /P_{lac}-*spoT*$^+$ strain was not evident here; this becomes evident after a longer period of growth in the absence of IPTG (supplementary figure 4). A representative growth curve from an experiment performed multiple times is shown. (**B**) Δ*spoT*/P_{lac}-*spoT*$^+$ (RS14) and Δ*spoT* Δ*gppA*/ P_{lac}-*spoT*$^+$ (RS206) strains cultured in LB containing ampicillin and 1mM IPTG were washed, serially diluted and spotted on LB agar plates containing Amp and either 100 μM or 10 μM or no IPTG. A representative picture of the colony forming units (CFU) following serial dilution and 16 hrs of incubation at 37°C is shown.

following IPTG withdrawal, we observed more severe inhibition of growth for the latter (figure 3A); the two strains behaved identically in the presence of IPTG. In a plating efficiency experiment carried out on media with different IPTG concentration, growth of the Δ*gppA* strain was once again more severely affected as compared to the isogenic *gppA*$^+$ strain only on the plate with limiting IPTG (figure 3B). Growth inhibition following IPTG-withdrawal became evident in the Δ*spoT*/P_{lac}-*spoT*$^+$ strain when it was allowed to undergo more rounds of cell division (supplementary figure 4).

3.3 *Disproportionate increase in (p)ppGpp accumulation in the combined absence of SpoT and GppA hydrolases*

Since GppA acts only to convert pppGpp to ppGpp, one would expect that the sum total of ppGpp and pppGpp concentration in a Δ*gppA* mutant would be unchanged from that in the isogenic *gppA*$^+$ strain. However, this was not the case when the pair of strains was also deficient for SpoT hydrolase, as described below.

Thus, following SpoT depletion (by IPTG withdrawal) in isogenic *spoT1*/P_{lac}-*spoT*$^+$ derivatives, significant accumulation of (p)ppGpp, with ppGpp being greater than pppGpp, was observed in Δ*gppA* but not the isogenic *gppA*$^+$ strain (figure 4A, compare lanes 1 and 5, supplementary table 2). When the pair of strains was Δ*spoT* (instead of *spoT1*), there was no difference between the strains in IPTG-supplemented cultures (figure 4B, compare lanes 2 and 4), but when the (p)ppGpp accumulation upon SpoT depletion was compared between the *gppA*$^+$ and the Δ*gppA* strains, two features could be recognised (figure 4B, compare lanes 3 and 5). First, relative to the intensity of the GTP spot, the pppGpp level in the Δ*gppA* strain was substantially higher than in the former (supplementary table 2); this may be attributable to the lack of GppA hydrolase in the latter. Second, once again, relative to the intensity of the GTP spot, there was a vastly greater accumulation of ppGpp as well in the Δ*gppA* mutant compared to that in the *gppA*$^+$ strain (supplementary table 2). These results suggested that combined loss of GppA and SpoT functions may be associated with a disproportionate increase in (p)ppGpp level, in comparison with the loss of SpoT function alone. These results are discussed further

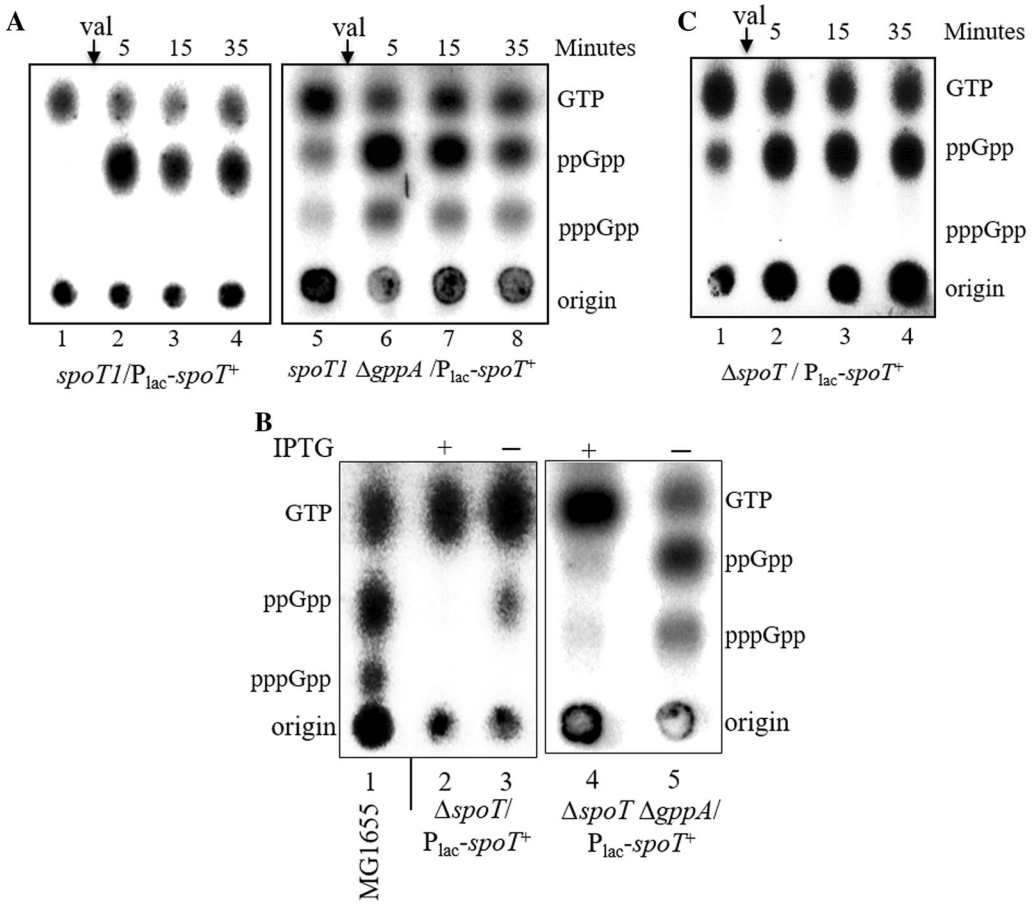

Figure 4. ppGpp and pppGpp levels in *gppA*$^+$ and Δ*gppA* strains with reduced SpoT function. Strains *spoT1*/ P_{lac}-*spoT*$^+$ (RS40), *spoT1* Δ*gppA* / P_{lac}-*spoT*$^+$ (RS194) and *ΔspoT* / P_{lac}-*spoT*$^+$ (RS260) cultured in MOPS glucose medium containing ampicillin and 1 mM IPTG were washed and diluted in the same medium but lacking IPTG (**A**, **C**). Strains *ΔspoT*/ P_{lac}-*spoT*$^+$ (RS14) and *ΔspoT* Δ*gppA*/ P_{lac}-*spoT*$^+$ (RS206), cultured in MOPS glucose 20 amino acid medium containing ampicillin and 1 mM IPTG, were washed and diluted 100-fold in the same medium either in the presence (+) or the absence of IPTG (−); due to the severe growth inhibition seen in the absence of IPTG, RS206 was diluted 20-fold (**B**). The wild type strain MG1655 was cultured in MOPS glucose medium and stringent response was induced by the addition of valine at an A_{600} of ∼ 0.2—0.3. The nucleotides were labelled and analysed by TLC as described in the methods. Samples were collected just before the addition of valine (indicated by an arrow) and at the times indicated after the addition of valine. In 4B, the ppGpp/GTP and pppGpp/GTP ratios before and after IPTG withdrawal was calculated from the quantification of radioactive material in the spots. Lane 2, ppGpp/GTP = 0.09, pppGpp/GTP = 0.04; Lane 3, ppGpp/GTP = 0.32, pppGpp/GTP = 0.06; Lane 4, ppGpp/GTP = 0.14, pppGpp/GTP = 0.08; Lane 5, ppGpp/GTP = 1.71, pppGpp/GTP = 0.79. Data presented is representative of experiments done many times.

below in the context of a model that pppGpp itself is a stimulator of RelA activity.

We also studied (p)ppGpp accumulation after amino acid starvation in strains with reduced SpoT hydrolase activity. When isoleucine starvation was induced (by valine addition) after SpoT depletion (by IPTG withdrawal) in the *gppA*$^+$ derivatives of either *spoT1* or the Δ*spoT* strain, accumulation of ppGpp alone was observed (figure 4A, lanes 2 to 4 and figure 4C, lanes 2 to 4 respectively); similar data have been reported earlier for the *spoT1* strain (Laffler and Gallant 1974; Fiil *et al.* 1977). On the other hand, in the Δ*gppA* *spoT1* mutant, there was an accumulation of both pppGpp and ppGpp, with the increase of ppGpp being more marked than that of pppGpp (figure 4A, lanes 6 to 8, supplementary table 2). Models to explain the disproportionately increased levels of ppGpp relative to pppGpp in Δ*gppA* strains following SpoT depletion are discussed below.

Data from the Δ*spoT* strains carrying hypomorphic *relA* alleles also provided support to the interpretation that pppGpp could stimulate RelA activity. After SpoT depletion in RelA-↓ Δ*spoT* / P_{lac}-*spoT*$^+$ strain (by IPTG withdrawal), the Δ*gppA* derivative accumulated

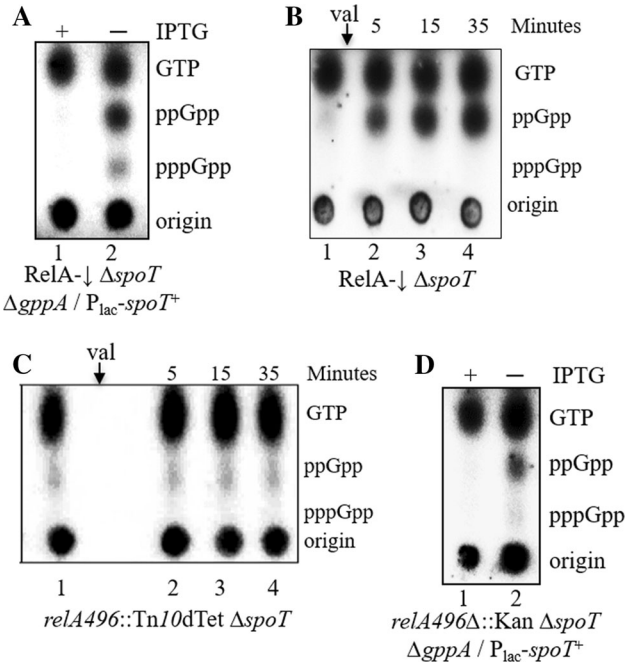

Figure 5. Disproportionate (p)ppGpp accumulation following SpoT depletion in the Δ*gppA* strain carrying hypomorphic *relA* alleles. (p)ppGpp accumulation as a consequence of SpoT depletion was studied in the RelA-↓ Δ*spoT Δgppa* / P_{lac}-*spoT*$^+$ (RS415) (**A**), RelA-↓ Δ*spoT* (RS361, white colony) (**B**), *relA496*::Tn*10*dTet Δ*spoT* (RS17, white colony) (**C**) and *relA496Δ*::Kan Δ*spoT Δgppa* / P_{lac}-*spoT*$^+$ (RS359) (**D**) strains. Cultures grown in MOPS medium containing glucose, 20 amino acids, ampicillin and 1 mM IPTG were washed and sub-cultured in the same medium with (+) or without (−) IPTG (**A**, **D**). Cultures grown in MOPS medium containing glucose was sub-cultured in the same medium and amino acid starvation was induced by the addition of valine (arrow) and sampled at the time points indicated (**B**, **C**). The nucleotides were labelled and analysed by TLC as described in the methods. Data presented is representative of experiments done many times.

pppGpp and ppGpp (figure 5A, lane 2), whereas in the *gppA*$^+$ strain only a small amount of ppGpp was detectable (figure 5B, lane 1). When subjected to isoleucine starvation (by valine addition), the RelA-↓ Δ*spoT* (*gppA*$^+$) strain accumulated ppGpp but not pppGpp (figure 5B, lanes 2 to 4) and, consistent with the lowered expression of *relA*, the rate of such accumulation was slow (ppGpp continued to increase for up to 35 min).

In the *relA496*Δ::Tn*10*dTet Δ*spoT gppA*$^+$ strain, consistent with the absence of SpoT activity, we observed a faint ppGpp spot before isoleucine starvation (figure 5C, lane 1), and as expected (due to the absence of RelA-CTD) there was no further accumulation of (p)ppGpp following amino acid starvation (figure 5C, lanes 2 to 4). On the other hand, in the absence of GppA hydrolase, significant accumulation of ppGpp and a smaller amount of pppGpp was observed after SpoT depletion by IPTG withdrawal (figure 5D, compare lanes 1 and 2, supplementary table 2).

3.4 *The relative proportions of pppGpp to ppGpp are different between RelA-mediated stringent response and SpoT-mediated stringent response*

The experiments above had been undertaken in the context of SpoT depletion (by IPTG withdrawal from P_{lac}-*spoT*$^+$ carrying strains). We next compared the effect of GppA inactivation in *spoT*$^+$ strains on the stringent response elicited by amino acid limitation (that is, RelA-mediated) or by carbon/fatty acid limitation (which is SpoT-mediated).

As described above and consistent with an earlier report, after amino acid starvation (where the stringent response is RelA-mediated), the ratio of pppGpp to ppGpp was < 1 in the wild type and > 1 in the *gppA* mutant (Somerville and Ahmed 1979) (figure 6A, compare lanes 2 to 4 with lanes 6 to 8, supplementary table 2). On the other hand, with carbon or fatty acid starvation separately imposed or in combination, this ratio was < 1 even for the *gppA* mutant (figure 6B lanes 7 and 8; figure 6C lanes 2, 3, 5 and 6; supplementary table 2). Without starvation, as expected, an increase in GTP and basal (p)ppGpp levels was observed with the increasing optical density of the culture (figure 6B, lanes 1 to 6). The comparison between stringent responses elicited by RelA versus that by SpoT in the wild type strain also showed that pppGpp accumulation was far less in the latter than in the former (compare supplementary figure 5A, lanes 6 to 8 and supplementary figure 5B, lanes 2, 3, 5, 6 with figure 6A lanes 2 to 4). Without starvation, as expected, an increase in GTP and basal (p)ppGpp levels was observed with the increasing optical density of the culture (supplementary figure 5A, lanes 1 to 4).

4. Discussion

In this study, by using genetic approaches to alter the SpoT and GppA hydrolase activities, we attempted to change the relative levels of the stringent nucleotides ppGpp and pppGpp in order to study its consequences on bacterial growth. Our results show that strains compromised for the SpoT and GppA hydrolase

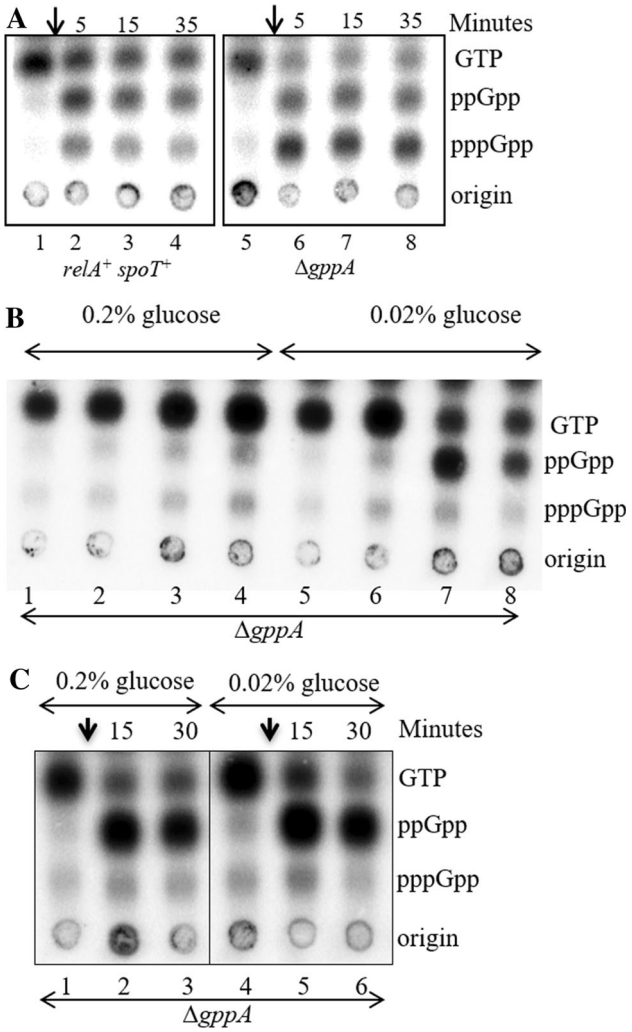

Figure 6. Disproportionate accumulation of (p)ppGpp during SpoT-dependent stringent response in the absence of the GppA hydrolase. (**A**) The *relA*$^+$ *spoT*$^+$ strain RS1 and the isogenic Δ*gppA* mutant (RS307, white colony), were subjected to isoleucine starvation by the addition of valine (arrow) and samples were collected just before valine addition and after valine addition, at the time points indicated. The ratio of intensity of pppGpp spot to that of ppGpp was 0.61, 0.61 and 0.63 for lanes 2, 3 and 4, respectively, and 1.25, 1.20 and 1.23 for lanes 6, 7 and 8, respectively. (**B**) The Δ*gppA* strain (RS307, white colony), cultured overnight in MOPS medium containing 0.2% glucose was diluted 100-fold in the same medium and subsequently at mid-log phase diluted 10-fold into low phosphate $^{32}P\text{-}H_3PO_4$ medium with either 0.2% glucose or 0.02% glucose (to induce glucose starvation). A parallel culture without $^{32}P\text{-}H_3PO_4$ was used to follow A_{600} and the onset of starvation. The ratio of intensity of pppGpp spot to that of ppGpp was 0.22 and 0.27 in lanes 7 and 8, respectively. (**C**) Fatty acid starvation or simultaneous starvation for glucose and fatty acid was induced in the Δ*gppA* strain (RS307, white colony) by the addition of cerulenin (arrow) to cultures grown with 0.2% glucose and 0.02% glucose, respectively, and samples were collected at the time points indicated. The nucleotides were labelled as described in the method and quantified (supplementary table 2). The ratio of intensity of pppGpp spot to that of ppGpp was 0.09, 0.06, 0.1 and 0.05 in lanes 2, 3, 5 and 6, respectively. Data presented is representative of experiments done many times.

activities in combination are growth inhibited, ostensibly due to the accumulation of pppGpp. As discussed below, our results are consistent with the model that pppGpp accumulation can activate RelA to set up a vicious cycle of (p)ppGpp synthesis, and that this is prevented in presence of either the SpoT or the GppA hydrolases. Our results also show that a decrease in the SpoT hydrolase activity leads to an increase in the proportion of ppGpp, relative to pppGpp within the cells. This adjustment is important for the physiology of the cell, because, perturbing this by the inactivation of GppA results in growth inhibition.

4.1 *Activation of RelA by pppGpp as a possible mechanism for the disproportionate increase of (p)ppGpp level in the combined absence of SpoT and GppA hydrolases*

The physiological significance of the GppA hydrolase in *E. coli* is not fully understood, since the loss of its function does not significantly affect growth. On the other hand, growth of the strains with partially defective SpoT hydrolase activity, as well as those of strains completely lacking SpoT but rendered viable as a result of weakened RelA function, was dependent upon GppA (figures 1, 2, 3). Furthermore, the GppA function becomes obligatory for the growth of these strains only when there is a functional RelA allele (including partially functional alleles). Conditional depletion of SpoT (using P_{lac}-*spoT*$^+$) in strains lacking GppA resulted in a disproportionate increase in (p)ppGpp which was correlated with growth inhibition figure 4A, lane 5; figure 4B, lane 5; figures 5A lane 2 and 5D lane 2). While an increase in pppGpp may be attributed to the loss of GppA function, the increase in ppGpp is likely to be from increased RelA activity.

Putting these observations together, we propose a model that pppGpp, but not ppGpp, can activate RelA in the absence of specific starvation signals to set up a vicious cycle of (p)ppGpp synthesis. We think that RelA activation is a feature unique to pppGpp, because, the inactivation of both SpoT and GppA, but not of

SpoT alone, was necessary to elicit the response. Therefore, an increase in the concentration of pppGpp, the substrate for both the hydrolases and not ppGpp, which is the substrate only for SpoT, is necessary for the activation of RelA.

The molecular basis for the postulated activation of RelA by pppGpp needs to be addressed. We wish to point out that accumulation of (p)ppGpp, and growth inhibition, was observed upon SpoT depletion even in the *relA496*Δ::Kan Δ*gppA* strain (figure 2 panel vii and figure 5D), in which the RelA protein is missing its CTD; this suggests that the activation of RelA is possible even when the protein is not ribosome-associated.

Using purified components, a stimulatory effect of pppGpp on the RelA-mediated conversion of GDP to ppGpp has been noted (Kudrin *et al.* 2018) and the stimulatory effect was also seen in the absence of the ribosomes. Our results bring out the biological significance of this finding and the possible role of GppA in the negative regulation of RelA activity. To our knowledge, the role of GppA in the physiology of the cell has not been clear thus far. Recent studies have shown RelA-like small alarmone synthases (SAS) in bacteria are allosterically activated by pppGpp (Steinchen *et al.* 2015; Syal *et al.* 2015; Beljantseva *et al.* 2017) *in vitro,* and that these proteins have the synthase domain and lack the regulatory CTD.

Finally, we would like to mention that the *relA1* Δ*spoT* Δ*gppA* strain did not exhibit any growth defect (data not shown). The *relA1* allele, naturally selected in the laboratory strains of *E. coli*, is believed to be capable of weak (p)ppGpp synthesis – the *relA1* Δ*spoT* strain (unlike the Δ*relA* Δ*spoT* counterpart) is able to grow in minimal medium. Thus, every mutant allele of *relA* capable of (p)ppGpp synthesis in the absence of SpoT function may not be subjected to pppGpp mediated activation.

ppGpp is more potent than pppGpp in the regulation of growth rate, RNA/DNA ratio, ribosomal RNA transcription, threonine operon and RpoS induction (Mechold *et al.* 2013); this study was carried out in a *spoT*$^+$ genetic background. Our results indicate that, only in the SpoT hydrolase deficient strains, an increase in pppGpp sets up vicious synthesis of (p)ppGpp through RelA and therefore is not in conflict with the previous study. Novel (p)ppGpp degrading enzymes have been identified from proteome wide screen using the DRACALA assay (Zhang *et al.* 2018); but apparently, these are insufficient to prevent (p)ppGpp accumulation in the absence of SpoT and GppA. Notably, SpoT and GppA were not picked up in this screen.

4.2 *A model for the increase in the relative proportion of ppGpp to pppGpp following decrease in SpoT hydrolase activity*

Our results indicate that reduction in SpoT hydrolase activity leads to an increase in the proportion of ppGpp relative to pppGpp, both in the presence, as well as the absence, of the GppA hydrolase. This is not the case when the stringent response by amino acid starvation is elicited in *spoT*$^+$ strains. Based on the literature, we have considered possible mechanisms to explain these observations and if they are supported by data from this study.

(i) One possibility is that there is a preference for RelA-mediated synthesis of ppGpp over that of pppGpp. This idea is not supported by our data, because, the decrease in GTP level after isoleucine starvation in the SpoT depleted strains or in the *spoT1* background (figure 4A, lanes 2 to 4 and figure 4C, lanes 2 to 4) suggested the conversion of GTP into pppGpp. However, it is also possible, pppGpp synthesis was affected because the SpoT-hydrolase defect reduced the (p)ppGpp turnover, leading to a reduction in GTP pool. *E. coli* RelA preferred GDP as a substrate over GTP when presented in equal concentration (Sajish *et. al.* 2009).

(ii) Reduced SpoT-mediated synthesis of pppGpp: Although SpoT is a bi-functional protein capable of (p)ppGpp synthesis and degradation, there is no evidence for SpoT-dependent (p)ppGpp synthesis following amino acid starvation—the complete absence of (p)ppGpp accumulation in the *relA* mutant subjected to amino acid starvation supports this view. This data suggests that the reduced synthesis of pppGpp by SpoT may not be responsible for the increase in the proportion of ppGpp to pppGpp.

(iii) Increase in the GppA hydrolase activity following SpoT depletion: Our data shows the decrease in pppGpp level was independent of GppA hydrolase. The relative proportion of ppGpp to pppGpp remained high after SpoT depletion in the *gppA* mutant than in the *gppA spoT*$^+$ strain (compare figure 4A, lanes 6 to 8 and figure 6A lanes 6 to 8). Therefore, the phenotype has to be independent of the GppA hydrolase.

(iv) Since none of the mechanisms discussed above appear to provide a satisfactory explanation for the increase in the proportion of ppGpp to pppGpp upon reduction in SpoT hydrolase

activity, we wish to propose that a novel pppGpp hydrolase could be involved in lowering the pppGpp level when the SpoT hydrolase activity is reduced. Such activity may also explain the increased proportion of ppGpp to pppGpp observed during the SpoT-mediated stringent response in the wild type strain and the *gppA* mutant, as compared to the RelA-mediated stringent response in these strains (figure 6 and supplementary figure 5).

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