RelZ-Mediated Stress Response in *Mycobacterium smegmatis*: pGpp Synthesis and Its Regulation

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ABSTRACT Stringent response is a conserved stress response mechanism in which bacteria employ the second messengers guanosine tetraphosphate and guanosine pentaphosphate (collectively termed (p)pGpp) to reprogram their cellular processes under stress. In mycobacteria, these alarmones govern a multitude of cellular phenotypes, such as cell division, biofilm formation, antibiotic tolerance, and long-term survival. *Mycobacterium smegmatis* possesses the bifunctional RelMsm as a (p)pGpp synthetase and hydrolase. In addition, it contains a short alarmone synthetase MS_RHII-RSD (renamed RelZ), which contains an RNase H domain in tandem with the (p)pGpp synthetase domain. The physiological functions of RelMsm have been well documented, but there is no clear picture about the cellular functions of RelZ in *M. smegmatis*. RelZ has been implicated in R-loop induced stress response due to its unique domain architecture. In this study, we elucidate the differential substrate utilization pattern of RelZ compared to that of RelMsm. We unveil the ability of RelZ to use GMP as a substrate to synthesize pGpp, thereby expanding the repertoire of second messengers known in mycobacteria. We have demonstrated that the pGpp synthesis activity of RelZ is negatively regulated by RNA and pppGpp. Furthermore, we investigated its role in biofilm formation and antibiotic tolerance. Our findings highlight the complex role played by the RelZ in cellular physiology of *M. smegmatis* and sheds light upon its functions distinct from those of RelMsm.

IMPORTANCE Bacteria utilize nucleotide messengers to survive the hostile environmental conditions and the onslaught of attacks within the host. The second messengers guanosine tetraphosphate and pentaphosphate [(p)pGpp] have a profound impact on the long-term survival, biofilm formation, antibiotic tolerance, virulence, and pathogenesis of bacteria. Therefore, understanding the stress response mechanism regulated by (p)pGpp is essential for discovering inhibitors of stress response and potential drug targets. *Mycobacterium smegmatis* contains two (p)pGpp synthetases: RelMsm and RelZ. Our study unravels the novel regulatory mechanisms of RelZ activity and its role in mediating antibiotic tolerance. We further reveal its ability to synthesize novel second messenger pGpp, which may have regulatory roles in mycobacteria.


THE SURVIVAL OF BACTERIA depends on their ability to sense and adapt to rapidly fluctuating environmental conditions. A key survival mechanism used by bacteria during hostile conditions is the stringent response, which is orchestrated by the nucleotide second messengers guanosine tetraphosphate and pentaphosphate [collectively named (p)pGpp] (1). Bacteria trigger the stringent response upon encountering any environmental stress or starvation and reprogram their cellular machinery accordingly. (p)pGpp regulates replication, transcription, and translation (2–4). In addition, (p)pGpp influences bacterial physiology by binding to several cellular targets...
such as RNA polymerase and modulating pathways related to metabolism, cell division, and antibiotic resistance, among others. The mechanism and triggers for (p)ppGpp-mediated stress response vary across bacterial species (4). In Gram-positive bacteria, (p)ppGpp has a profound impact on GTP homeostasis and thereby stress survival (5).

The synthesis and hydrolysis of (p)ppGpp are catalyzed by the proteins of the RelA-SpoT homologue (RSH) family (4, 6). Most Gram-negative bacteria such as Escherichia coli possess two RSH proteins: RelA, which can only synthesize (p)ppGpp, and SpoT, which is a (p)ppGpp hydrolase and a weak synthetase (7). In Gram-positive bacteria, including Actinobacteria, the main enzyme responsible for (p)ppGpp turnover is the multidomain Rel, which can both synthesize and hydrolyze (p)ppGpp (8, 9). In addition to these classical RSHs, several single-domain monofunctional RSH enzymes called "short alarmone synthetases" (SASs) have been identified in several bacterial species, such as Bacillus subtilis, Staphylococcus aureus, and Enterococcus faecalis (4). Although the multidomain RSHs have been well studied, little is known about the physiological relevance of SASs. In addition to maintaining basal (p)ppGpp levels, these have been implicated in specific stress responses such as cell wall stress and alkaline stress (10, 11).

In mycobacteria, (p)ppGpp affects long-term survival, virulence, cell division, and antibiotic tolerance (8, 9, 12–14). M. smegmatis contains a bifunctional RelMsm for (p)ppGpp synthesis and hydrolysis. Deletion of RelMsm leads to changes in cellular morphology, growth, cell wall metabolism, antibiotic tolerance, and biofilm formation (14, 15). The genome of M. smegmatis also encodes a unique SAS, MS_RHII-RSD (here termed RelZ), which contains an RNase H domain (RHII) in tandem with the (p)ppGpp synthesis domain (RSD) (16). RelZ can make but not hydrolyze (p)ppGpp. Similarly, the pathogen Mycobacterium tuberculosis contains a bifunctional Rel (RelMtb) and SAS homolog Rv1366 (17). Interestingly, Rv1366 lacks the RHII domain and is incapable of synthesizing (p)ppGpp in vitro (18). No discernible phenotype could be attributed to the protein, so the presence of SAS homologs such as RelZ and Rv1366 in mycobacterial species remains a mystery (19). In our previous work, we demonstrated the ability of RelZ to cleave RNA/DNA hybrids and hydrolyze triple-stranded structures called R-loops (16, 20). We further revealed the inability of the individual domains to function separately and the necessity of the hexameric oligomerization for its activity. However, it is unclear what other cellular phenotypes are regulated by RelZ.

Dysregulation in intracellular (p)ppGpp levels impacts important cellular pathways and is detrimental to bacteria (19). Therefore, it is imperative for M. smegmatis to regulate the synthesis activity of both RelZ and RelMsm during different growth conditions for maintaining (p)ppGpp homeostasis. The activity of RelMsm is regulated at the transcription level and through feedback regulation by (p)ppGpp (21–24). Recent studies have uncovered evidence for the transcriptional and allosteric regulation of SAS in organisms such as B. subtilis and E. faecalis (10, 25, 26). SASs and Rel may also have different substrate specificities (27). Therefore, SASs may have distinct physiological functions from that of Rel, and together their activities are coordinated to generate a cellular response. Our investigation was prompted with the aim to decipher the functions of RelZ in modulating the cellular physiology of M. smegmatis. We have constructed strains devoid of RelZ and RelMsm and characterized the role of RelZ in biofilm formation, cell surface-associated phenotypes, and antibiotic tolerance. We have elucidated the substrate utilization preference for RelZ in comparison to RelMsm and explored how the activity of RelZ is regulated. We reveal the ability of RelZ to synthesize pGpp using GMP as a substrate and discuss the potential implications of pGpp as a second messenger in mycobacteria.

RESULTS

RelZ but not RelMsm can synthesize pGpp. It has been shown in our earlier work that RelZ can synthesize (p)ppGpp by catalyzing the transfer of a pyrophosphate moiety from the 5′-riboyl of ATP to the 3′-riboyl OH of GDP and GTP (16). When resolving the ppGpp synthesis reaction mixtures, we often observed the presence of an
additional spot on our chromatograms with a similar migration profile as that of GTP. We hypothesized that GMP is present as a contaminant with GDP and being utilized as a substrate by RelZ. The ability of E. faecalis RelQEf to consume GMP as the substrate further bolstered our hypothesis (27). To examine this possibility, we incubated RelZ and ATP with GMP and observed the appearance of this spot (Fig. 1A). Mass spectrometry revealed the mass of the compound to be 523 Da, which is the same as that of GTP (Fig. 1B). Therefore, there were three possibilities for the identity of this compound: 5′-pppG (GTP), 5′-pppGp, or 5′-pppGpp. All of these compounds could be formed by the transfer of the radiolabeled phosphate from ATP to GMP. The migration of this compound differed to that of GTP when 0.75 M KH₂PO₄ is used as a solvent for thin-layer chromatography (TLC), indicating that the compound is not GTP (Fig. 1C) (27). To further distinguish if the compound is pGpp or ppGp, we treated the reaction mixture with NaOH since only pGpp is susceptible to alkali hydrolysis. The degradation of the compound in the presence of alkali validated the identity of the compound to be pGpp (Fig. 1D).

Subsequently, we probed the ability of RelMsm to synthesize pGpp. No significant pGpp formation was observed when RelMsm was allowed to react with ATP and GMP for an hour (Fig. 1E and F). During the same time, RelZ could utilize more than 75% of the ATP substrate provided. This suggested that RelZ, unlike RelMsm, is an efficient pGpp synthetase in M. smegmatis.

**RelZ and Rel have different substrate specificity.** To understand the significance of this pGpp synthesis by RelZ, we further assessed the preference of RelZ for different guanine nucleotides as the substrates. Our previous work demonstrated that RelZ prefers GDP as a substrate over GTP, whereas RelMsm prefers GTP as a substrate over GDP (16). We evaluated the kinetic parameters of RelZ using GMP as a substrate and found that though the $K_m$ of RelZ for GMP was similar to that of GTP and GDP, there was a marked difference in the $k_{cat}$ value ($k_{cat}$ [GMP]) is 2.98 s⁻¹ and $k_{cat}$ [GDP] is 1.78 s⁻¹ (Table 1). The $k_{cat}/K_m$ value indicated that RelZ preferred GMP (2.63 mM⁻¹ s⁻¹) as a substrate over GDP (1.81 mM⁻¹ s⁻¹) and GTP (0.35 mM⁻¹ s⁻¹). In a reaction mixture containing equimolar concentrations of GDP, GTP, and GMP, it was evident that RelZ and RelMsm have contrasting substrate utilization patterns since pGpp and pppGpp synthesis was highest in the cases of RelZ and RelMsm respectively (Fig. 2A). In conclusion, the substrate hierarchy for RelZ is GMP > GDP > GTP, and for RelMsm it is GTP > GDP.

**Mutation in DDRD site in RelZ alters its substrate utilization.** We next investigated the underlying reason behind this differential substrate specificity. Differential preference for GTP and GDP as the substrate in RSHs has been attributed to a charged motif that affects interaction with Mg²⁺ ion (28, 29). Monofunctional Rel such as that in E. coli (RelAₑᶜ) has an acidic EXDD motif that leads to a preference for GDP in comparison to GTP and insensitivity to an increase in Mg²⁺ concentration. On the other hand, bifunctional Rel, such as RelMₑᵗ, contains a basic RXKD motif, prefers GTP, and has a lower activity at high Mg²⁺ concentration. RelMₘₑᵗ contains an RFKD motif, whereas the RelZ contains an acidic DDRD motif. To elucidate whether the difference in substrate specificity is indeed due to the charged motif, we generated a mutant of RelZ with RFKD as the motif instead of DDRD and analyzed its substrate specificity (Fig. 2B). In comparison to RelZ, the RKFD mutant showed enhanced (p)pppGpp synthesis. This mutant showed a marked increase in its ability to synthesize ppGpp (2.6-fold higher) and pppGpp (1.5-fold higher) (Fig. 2C). The pGpp synthesis by RelZ RKFD mutant was not significantly affected. However, we did not see a reversal of substrate specificity to that of RelMₑᵗ. When a similar mutation was made in RelAₑᶜ, the mutant had increased (p)pppGpp synthesis activity and also had a substrate preference opposite to that of RelAₑᶜ (29). RelZ possesses an acidic motif, but it contains a basic residue at the third site similar to RelMₑᵗ (RFKD) and RelQₑᶜ (ERKD) (Fig. 2D). Interestingly, mutating the RXKD motif in RelMₑᵗ to EXDD leads to an ability to synthesize pGpp since the mutant can hydrolyze the Pₐ-O-Pₐ bond of GTP/GDP (28). This is unlike the case in RelZ, which
FIG 1 Synthesis of pGpp by RelZ. (A) TLC of the reaction mixture containing 1 μM RelZ with 1 mM ATP and 1 mM GMP, GDP, or GTP. The reaction was stopped by adding formic acid, and the nucleotides were resolved on PEI-cellulose using 1.5M KH₂PO₄ as a solvent. (B) Mass spectrometry analysis of the product of the RelZ reaction mixture containing ATP and GMP as the substrates. 522 Da and 544 Da represent the masses of the compound and sodiated compound, respectively. (C) Differential migration of pGpp and GTP. Pure radiolabeled GTP and pGpp were resolved using one-dimensional TLC in 0.75M KH₂PO₄. (D) TLC of the reaction mixture treated with 0.5M NaOH for 2 h at 37°C. The control reaction mixture (Ct) was incubated for 2 h in the absence of NaOH. (E) Time course of pGpp synthesis. The ATP spot density on the chromatogram was analyzed by densitometry and plotted for all time points by taking the ATP spot density at beginning to be 100%. Error bars indicate the standard deviations from three independent experiments. (F) TLC showing pGpp synthesis assay with 1 mM ATP, 1 mM GMP containing 1 μM RelZ, or RelMsm and sampled at different time intervals.
can directly use GMP as a substrate. The altered activity of the RFKD mutant is not due to a change in the oligomerization and secondary structure as SEC-MALS and circular dichroism analysis did not reveal any deviations from RelZ (data not shown). Together, these results hint at either a difference in catalytic mechanism or the side chain interactions for RelZ.

RelMsm can hydrolyze pGpp. For pGpp-mediated signaling to occur in M. smegmatis, there must be a protein capable of hydrolyzing it once the stress is removed. We assessed the ability of both RelMsm and RelZ to hydrolyze pGpp. RelMsm showed a remarkable efficiency for pGpp hydrolysis as seen by the almost complete hydrolysis within 15 min (Fig. 3). RelMsm hydrolyzes pGpp to GMP and pyrophosphate, as evidenced by the comigration of the radiolabeled product with purified pyrophosphate. In contrast to RelMsm, 10% of pGpp was hydrolyzed by RelZ to yield a phosphate moiety, which could be considered a weak hydrolysis. The genome of M. smegmatis also encodes other nucleotidyl phosphohydrolases, but their ability to degrade (pp)pGpp is not yet reported.

Single-stranded RNA inhibits pGpp synthesis by RelZ. RelZ is a bifunctional protein, so the next question we addressed was how the RNase H and (pp)pGpp synthesis activities are regulated. Mutation in the active site of one domain of RelZ does not inactivate the other domain and only the full-length protein is active (20). We first explored whether a regulatory mechanism for RelZ exists. The RHII domain of RelZ hydrolyzes R-loops to release the double-stranded DNA (dsDNA) and single-stranded RNA, so we speculated that the presence of the R-loops and their components modulates the pGpp synthesis activity. We performed the pGpp synthesis assay for RelZ in the presence of R-loops but did not observe any difference in the activity (Fig. 4A and B). Interestingly, we discovered that the presence of 4 μM RNA inhibited ≈40% of pGpp synthesis. Addition of the same concentration of dsDNA did not affect the pGpp synthesis activity, proving that the inhibition is RNA specific. Single-stranded DNA (ssDNA) did not inhibit RelZ to the same extent as RNA. The reason for the inhibitory effect in the presence of RNA but not in the presence of R-loops is unclear. The E. faecalis RelQef has a regulatory mechanism in which the (p)ppGpp synthesis activity is inhibited by the presence of single-stranded RNA, but the biological significance remains unknown (26). The evolution of an RHII domain in association with an RSD domain in RelZ hints at a direct link to a potential source of regulatory RNA.

pGpp has a moderate effect on the RNase H activity of RelZ. We subsequently examined whether the RNase H activity of RelZ is affected by the product of the RSD activity, pGpp. We used a poly(rA)-poly(dT) hybrid (AH) in which dabsyl quenches the fluorescence of the fluorescein. RelZ cleaves AH, leading to an increase in fluorescence, as a measure of RNase H activity. In the presence of 4 μM pGpp, we observed a minor decrease in fluorescence intensity, indicating a reduction in RNase H activity (Fig. 4C). This suggests that intracellular pGpp could modulate the RNase H activity of RelZ, but the relevance of this mode of regulation is unknown.

A high pppGpp concentration affects the pGpp synthesis activity of RelZ. Once the environmental conditions become favorable for growth, the cells recover by inhibiting the stringent response. For this recovery, (pp)pGpp synthesis by RelZ has to be halted. We sought to determine whether RelZ possesses a feedback inhibition mechanism in which (pp)pGpp downregulates its own synthesis. Therefore, we carried

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**TABLE 1** Kinetic constants for (pp)pGpp synthesis by RelZ

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>Mean $K_m$ (mM) ± SEM</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
<th>Efficiency (relative to GMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>2.98</td>
<td>1.13 ± 0.03</td>
<td>2.63</td>
<td>1</td>
</tr>
<tr>
<td>GDP</td>
<td>1.78</td>
<td>0.98 ± 0.03</td>
<td>1.81</td>
<td>0.69</td>
</tr>
<tr>
<td>GTP</td>
<td>0.34</td>
<td>0.96 ± 0.06</td>
<td>0.35</td>
<td>0.13</td>
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$^a$The kinetic parameters for GMP as the substrate were estimated using reaction conditions described earlier (16). The kinetic constants for GTP and GDP used for comparison were taken from the same study.
out pGpp synthesis assay for RelZ in the presence of 1 mM pGpp, ppGpp, or pppGpp (Fig. 5). Our data revealed that pppGpp inhibited the pGpp synthesis activity of RelZ to <80%. However, ppGpp and pGpp did not affect the activity significantly. This suggests that a high concentration of pppGpp is a prerequisite for inhibiting RelZ-mediated synthesis.
Previously, it has been shown that RelMsm has an autoregulatory feedback mechanism (23, 24). We explored the possibility that pGpp could modulate the activity of RelMsm. We assessed the pppGpp synthesis assay for RelMsm in the presence of 1 mM pGpp, ppGpp, or pppGpp and observed the expected inhibition of the pppGpp synthesis by (p)ppGpp. Furthermore, we observed that pGpp caused a significant inhibition (almost 40%) of RelMsm activity, although it was not as potent as pppGpp. Thus, RelMsm is a potential cellular target of pGpp.

RelZ affects the antibiotic tolerance of M. smegmatis. The evidence presented so far demonstrates that RelZ is an efficient pGpp synthetase, which raises an important question as to its regulatory roles in vivo. To gain a deeper insight into how the stress response is modulated by the two RSH proteins with differing substrate specificities and to understand the (p)pGpp phenotype of M. smegmatis, we generated strains devoid of RelMsm and RelZ (Δrel ΔrelZ). To our surprise, we found that the strain with deletions of RelMsm and RelZ (Δrel ΔrelZ) has residual (p)ppGpp, indicating the presence of another synthetase (see Fig. S1 in the supplemental material).

Since antibiotic tolerance is one of the most important phenotypes linked with (p)pGpp, we analyzed the effect of RelZ deletion on antibiotic susceptibility in M. smegmatis. In contrast to our expectations, we had previously observed the Δrel strain to have a higher tolerance to most antibiotics (15). To examine the role of RelZ in antibiotic tolerance, we carried out phenotype microarray analysis with the strains to test their sensitivity against 240 different antibiotics/chemicals. The growth of the strains is inferred from the dye reduction values plotted as a function of time, and the phenotypemicroarray (PM) software compares the area under the curve for each strain. Figure 6 depicts the growth of the wild-type, knockout, and complementation strains in the presence of the antimicrobials bleomycin, ofloxacin, and rifampin. Table S3 provides a list of antimicrobials in which the area under the curve (AUC) difference between the strains was >4,000. In contrast to the Δrel strain, the Δrel ΔrelZ strain displayed sensitivity to most antibiotics. Several of these chemicals were genotoxic in nature (e.g., ofloxacin and bleomycin). Moreover, a complementation strain (Δrel ΔrelZ + pRelZ) containing a copy of the relZ gene under the control of a constitutive promoter led to the reversal of this phenotype, confirming that the antibiotic sensitivity is indeed due to relZ deletion and not due to polar effects. MIC analysis of the strains for the antibiotics using a resazurin microtiter plate assay (REMA), as well as CFU analysis, reflected a similar trend (see Table S4 and Fig. S3 in the supplemental material). The Δrel ΔrelZ strain showed a similar tolerance level or decreased tolerance compared to the Δrel

**FIG 3 In vitro pGpp hydrolysis assay for RelMsm and RelZ.** (A) Representative TLC results depicting hydrolysis of pGpp by RelMsm and RelZ. First, 1 mM purified pGpp was incubated with 1 μM RelMsm or RelZ. The reaction mix was sampled at different time points and resolved on PEI-cellulose using 1.5 M KH₂PO₄. The control reaction mix (Ct) lacked an enzyme and was incubated for 60 min. Pure pyrophosphate (C2) was used as a standard. RelMsm shows potent pGpp hydrolase activity, whereas RelZ is a weak hydrolase. (B) The time course of pGpp hydrolysis by RelMsm and RelZ is plotted. The radioagam was visualized by densitometry and plotted by considering the pGpp density at the starting time to be 100%. Error bars indicate standard deviations from three independent experiments.
strain. The ∆rel+pRelZ strain, in which RelZ is expressed in the ∆rel strain, showed reduced survival compared to the ∆rel strain though the susceptibility was not identical to the wild-type strain in most cases. This indicated that the (pp)pGpp synthesis by RelZ could reverse the ∆rel phenotype to some extent. The antibiotics and chemicals in which RelZ deletion affected growth belong to multiple classes with differing mechanisms of action. It could be hypothesized that the antibiotic influx/efflux is affected in these strains due to the altered (pp)pGpp homeostasis.

The ∆rel ΔrelZ strain is defective in biofilm formation and has altered cell surface properties. Biofilm formation is a major stress-associated phenotype governed
by (p)pGpp. To understand the effect of RelZ deletion, we assessed the biofilm formation of all the strains. The $\Delta$rel strain is defective in biofilm formation (15) and the $\Delta$relZ strain mirrored the phenotype (Fig. 7A). The $\Delta$relZ strain did not show robust biofilm formation, unlike the wild-type strain, but the effects were not as drastic as that of the $\Delta$rel strain. The biofilm did not have a thick and wrinkled appearance like that of the wild-type strain. This suggests that RelZ does not impact biofilm formation significantly and that the biofilm formation is more substantially affected by RelMsm. Biofilm quantification using the crystal violet method estimated a significant reduction (almost 50%) in the biofilm formation ability of the $\Delta$relZ strain, thus supporting our conclusion that it is impaired in biofilm formation (Fig. 7B).

The deletion of RelMsm leads to altered surface phenotypes with respect to colony morphology, sliding motility, aggregation, and cell wall hydrophobicity (15). We examined these cell surface properties in cells devoid of RelZ. We noticed that the stationary-phase cultures of the $\Delta$relZ strain often settle faster than the wild-type cultures. Moreover, we found that the promoter of RelZ is constitutive in nature, but its activity is elevated during stationary phase, further emphasizing the significance of RelZ under normal growth conditions, as well as during starvation (Fig. S2). The $\Delta$rel $\Delta$relZ strain

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**FIG 5** Regulation of RelZ by (p)pGpp. (A) TLC showing in vitro pGpp synthesis by RelZ and in vitro pppGpp synthesis by RelMsm separately. RelZ and RelMsm were incubated with 1 mM ATP and 1 mM GMP or GTP, respectively, in the presence of 1 mM pGpp, ppGpp, or pppGpp. The control reaction (Ct) lacked an enzyme. (B) The pGpp synthesis activity of RelZ and ppGpp synthesis activity of RelMsm was plotted using densitometry analysis of the pGpp/pppGpp spot intensity on the TLC plate. The pGpp/pppGpp intensity in a reaction devoid of the nucleotide inhibitor was taken to be 100%. Error bars indicate the standard deviations from three independent experiments.
cultures have a high tendency to form clumps and settle down quickly. We quantified this by calculating the percentage of aggregated cells in stationary-phase cultures of these strains (Fig. 7C). As expected, the ΔrelZ (>40% aggregation) and Δrel ΔrelZ (>80% aggregation) strains showed significantly higher aggregation compared to the wild type. RelZ complementation could rescue the phenotype in the ΔrelZ+pRelZ strain validating that the change in phenotype is due to the deletion of relZ. Both Δrel and Δrel ΔrelZ strains aggregate more than the ΔrelZ strains and complementation of RelZ in a Δrel strain did not recover the phenotype to that of the wild-type strain. This suggested that the deletion of RelMsm has a more profound effect on these phenotypes.
A similar trend was reflected in the case of Congo red binding assay (used as a measure of cell wall hydrophobicity) and sliding motility (30) (Fig. S4). Taken together, our findings indicate that RelZ has a role in cell wall metabolism, as seen by the altered cell surface properties of the ΔrelZ strain. The Δrel ΔrelZ strain is similar to the Δrel strain in the macroscopic properties. It has increased cell wall hydrophobicity, which is most likely due to the change in the lipid composition of the cell wall. We can conclude that lowering the alarmone levels severely affects the cell envelope in *M. smegmatis*.

**DISCUSSION**

In our study, we have attempted to decode the role of the RelZ in *M. smegmatis* stress response. In contrast to the SAS known from other bacteria, RelZ also contains an RNase H domain, which led us to decipher its role in R-loop induced stress response (16, 20). In our current work, we reveal yet another function of RelZ: the synthesis of pGpp. Importantly, GMP is the most preferred substrate for RelZ. On the other hand, RelMsm prefers GTP as the substrate over GDP (16, 28). To our knowledge, this is the first reported mycobacterial SAS with the ability to synthesize pGpp, and it raises an interesting question about its significance *in vivo*.

Recently, the SAS from *E. faecalis* and *Corynebacterium glutamicum* were shown to be capable of synthesizing pGpp (27, 31). The authors of those studies speculated the involvement of pGpp in prolonging the stringent response after the depletion of the cellular pools of GTP and GDP. The triggering of stringent response involves utilization of GTP and GDP for synthesis of (p)ppGpp, which further inhibit purine biosynthesis (5). We hypothesize that an SAS, like RelZ, capable of utilizing GMP would be beneficial to *M. smegmatis* under such circumstances. The cellular concentration of GTP is known to be much higher than GMP in bacteria, so it remains a question whether the intracellular levels of GMP increase to such levels that pGpp synthesis by RelZ is likely. Previous studies on this subject lend credence to our hypothesis that pGpp synthesis by RelZ could have biological relevance under specific conditions. A substantial increase in GMP levels during amino acid deprivation has been documented in *B. subtilis* (32). In addition, amino acid-starved cultures of *B. subtilis* have been previously shown to contain pGpp, albeit at a much lower concentration than (p)ppGpp (33). We have been unsuccessful in our attempts to detect the presence of pGpp in nucleotide extracts of *M. smegmatis* using two-dimensional TLC, high-pressure liquid chromatography (HPLC), and mass spectrometry due to the identical masses and migration patterns of pGpp and GTP. The presence of pGpp-binding proteins in *E. faecalis* (Gmk and Hpt) and in *M. smegmatis* (RelMsm) hints at putative regulatory roles for pGpp as a second messenger (27).

Perturbation of purine nucleotide levels by (p)ppGpp affects bacterial fitness and antibiotic sensitivity (34). Tipping the balance of (p)ppGpp toward synthesis or hydrolysis affects mycobacteria, and the importance of this homeostasis is highlighted by the effect of a hydrolysis-defective Rel mutant on the growth and pathogenesis of *M. tuberculosis* (19). Our data indicate that the pGpp synthesis by RelZ is elegantly modulated via two mechanisms: RNA and pppGpp. The inhibition of the SAS RelQEF by RNA is sequence specific and counteracted by (p)ppGpp (26). RelZ is involved in hydrolyzing RNA/DNA hybrids and R-loops, so once they have been degraded by the RHiII domain, the alarmone synthesis would no longer be required. Hence, it could be envisaged that the hydrolyzed RNA would inhibit the pGpp synthesis by RelZ. However, direct evidence for this conjecture is lacking. Alternatively, the RNA arises from the cellular pool of mRNA, and any sequence specificity could imply potential cellular targets. More structure-function insights are needed to understand the catalytic and regulatory mechanism of the SAS RelZ.

We speculate that the inhibition of RelZ by pppGpp could help modulate the overall alarmone levels within the cell, and it could be beneficial when the cells no longer require any alarmone synthesis.

To elucidate the (p)ppGpp<sup>8</sup> phenotype in *M. smegmatis*, we characterized the strain devoid of both its alarmone synthetases. We found that the Δrel ΔrelZ strain is capable...
of synthesizing ppGpp indicative of the presence of another (pp)pGpp synthetase. A recent study by Njire et al. demonstrated the weak (pp)pGpp synthesis activity of *M. tuberculosis* Rv2783 encoding a PNPase (35). We suspect that the *M. smegmatis* PNPase homolog (MSMEG_2656) could have a weak (pp)pGpp synthesis activity, but the presence of any other protein with such an activity cannot be ruled out.

Decreased (pp)pGpp levels can lead to decreased fitness, especially under nutrient-limiting conditions, and have an effect on GTP homeostasis (4, 5, 7, 36–39). *M. smegmatis* *rel* cells are longer, multiseptate, and multinucleate and show an altered lipid profile and defective biofilm formation (14, 15). Unlike the case with RelMsm, the deletion of RelZ increases sensitivity to many antibiotics. The reason for the antibiotic sensitivity is difficult to comprehend since it could either be a pleiotropic effect of (pp)pGpp or due to the RNase H activity, and it is difficult to delineate the role of each domain. It is tempting to speculate that the RNase H activity of RelZ affects the tolerance of DNA-damaging agents. The role of SASs in antibiotic resistance tends to be species specific. In *S. aureus*, the SASs RelP and RelQ are upregulated during cell wall stress and provide tolerance against cell wall active antibiotics (11). In *E. faecalis*, the order of vancomycin sensitivity for the various strains was found to be as follows: ∆relA ∆relQ∗ strain < ∆relQ− strain < wild type < ∆relA strain (40). Higher basal levels correlated with higher antibiotic tolerance in *E. faecalis*. The constitutive (pp)pGpp synthesis by RelZ is therefore significant in this scenario. The deletion of RelMsm leads to elevated levels of polar lipids, making the cell wall more hydrophobic and hindering the uptake of several antibiotics (15). The important consequence of lowering (pp)pGpp levels is reflected in the profound alterations in the cell surface properties of the ∆rel ∆relZ strain. The complementation by RelZ rescued the phenotypes of ∆rel strain to a certain extent, thus underscoring the importance of alarmone synthesis by this SAS despite the prominent role played by RelMsm. Deletion of RelMsm also causes an upregulation of several genes linked with antibiotic resistance (14). We argue that the ∆rel ∆relZ strain also would have an altered transcriptome that impacts this phenotype.

We believe that the (pp)pGpp levels are fine-tuned during the different growth conditions in *M. smegmatis*, and the SAS RelZ plays a significant role in this regard in association with RelMsm. Knowledge of the (pp)pGpp levels is essential to conceive a clear picture about the significance of (pp)pGpp on antimicrobial susceptibility.

Realization of the importance of SASs has led to exciting discoveries, and we expect there will be more interesting findings related to their cellular functions and downstream effectors. More extensive research is needed in the future with the hope of developing inhibitors of stress response in bacteria.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *E. coli* strains DH5α and BL21(DE3) were grown in Luria-Bertani broth at 37°C with shaking at 180 rpm. The cultures were supplemented with ampicillin at 100 µg ml⁻¹. *M. smegmatis* cultures were grown in Middlebrook 7H9 broth (MB7H9; Difco) containing 2% glucose and 0.05% Tween 80 at 37°C. Hygromycin and apramycin were used at a concentration of 50 µg ml⁻¹. The list of strains and plasmids is in Table S1 in the supplemental material.

Plasmid pRelZ was generated by cloning the *relZ* gene in pMV261 vector (containing constitutive promoter) using the primers listed in Table S2 in the supplemental material. Plasmid pRelZ was transformed into the *M. smegmatis* ∆relZ and ∆rel strains to generate the ∆relZ::pRelZ and ∆rel::pRelZ strains, respectively. The clones were confirmed by sequencing and the RelZ expression in the strains were analyzed by SDS-PAGE. The cultures were grown in cultures supplemented with kanamycin at 25 µg ml⁻¹.

Site-directed mutagenesis. The RFKD mutant of RelZ was generated using pET-MS, RHII-RSD as the template. The primers incorporating the desired mutations are listed in Table S2 in the supplemental material. The products were phosphorylated, ligated, and transformed into *E. coli* DH5α, and the mutation was confirmed by sequencing.

The sequences of the proteins were retrieved from UniProt, and the multiple sequence alignment was done using Clustal Omega with default parameters.

Protein expression and purification. RelMsm, RelZ, and its RFKD mutant were expressed from pET 21b and purified using nickel affinity chromatography as described earlier (16). Briefly, the cultures were induced at an optical density at 600 nm (OD600) of 0.6 with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown for 3 h at 37°C. The cells were harvested and lysed by sonication in a lysis buffer (50 mM Tris [pH 7.9] at 4°C, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged, and the supernatant was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA)–agarose column preequilibrated with the same buffer. The
protein was eluted using 500 mM imidazole and dialyzed in buffer containing 25 mM Tris (pH 7.9) at 4°C, 125 mM NaCl, 25 mM imidazole, and 5% glycerol.

**In vitro (pp)pGpp synthesis assay.** The (pp)pGpp synthesis activity of the proteins was assayed as described earlier using 10-μl reaction mixtures containing 1 mM ATP, 10 μCi of [γ-32P]ATP ml⁻¹ (3,000 Ci mmol⁻¹; BRIT, India), 1 mM GDP/GTP/CTP, 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM diethiothreitol (DTT), and 1 mM MgCl₂ in the presence of 1 μM protein (16). The reaction mixture was kept at 37°C for 30 min, and the reaction was stopped by adding 1 N formic acid. Then, 2 μl of the reaction mixture was spotted onto a PEI-cellulose sheet (Merck) and resolved by one-dimensional TLC in 1.5 M KH₂PO₄ (pH 3.4). The nucleotides were visualized by phosphorimaging (Molecular Imager; Bio-Rad) and quantified by densitometric analysis using ImageJ software.

Kinetic constants were determined for RelZ with GMP as the substrate. We used 10 mM ATP and 1 μM enzyme in all reactions. The GMP concentration was varied from 10 μM to 5 mM, and the Mg²⁺ concentration was kept at 5 mM. The reaction was analyzed by TLC as mentioned above.

To analyze the substrate preference for Rel₉, and RelZ, the reaction mixtures were equilibrated by adding all nucleotide equimolar concentration (1.5 mM) of GMP, GDP, and GTP, along with 5 mM ATP, 5 mM Mg²⁺, and 1 μM protein and analyzed as described above.

**Alkali hydrolysis.** Alkali hydrolysis of pGpp was carried out as described previously (27, 41). Briefly, 0.5 M NaOH and 10 mM MgCl₂ was added to the pGpp reaction mixture, followed by incubation at 37°C for 2 h. Formic acid was added to a final concentration of 1 N, and the reaction products were analyzed by TLC as described above. The control mixture lacked protein.

**Preparation of (pp)pGpp.** To make pure pGpp, 2 μM RelZ was incubated with 1 mM ATP and 2 mM GMP in the same reaction buffer used to assay pGpp synthesis. Phenol-chloroform extraction was performed to remove the protein. The sample was diluted 1:3 in 25 mM Tris (pH 7.9) and subjected to anion-exchange chromatography (MonoQ 10/100 GL; GE Healthcare). The nucleotides were resolved by using a 0 to 1 M LiCl gradient. The peak fraction was collected and precipitated by the addition of 1 M LiCl and 4 volumes of ethanol. After incubation on ice for 30 min, the fraction was centrifuged at 5,000 × g for 20 min at 4°C. The pellets were washed twice with absolute ethanol, air dried, and stored at –20°C. The quality was controlled by HPLC and electron spray ionization-mass spectrometry (ESI-MS).

32P-labeled pGpp was purified as described above, but the reaction mixture contained radiolabeled ATP.

ppGpp and pppGpp were purchased from Jena Biosciences, Germany.

**pGpp hydrolysis assay.** The reaction mixture contained 1 mM 32P-pGpp, 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, 2 mM MnCl₂, and 1 μM protein. The reaction mixture was kept at 37°C, and aliquots were spotted onto TLC plates at specified time intervals. The reaction products were visualized and quantified as described above. Pure pyrophosphate was cored out from TLC of a Rel₉, pppGpp hydrolysis reaction and extracted in 10 mM Tris-HCl.

**Activity inhibition assays.** R-loops were synthesized as described earlier by annealing the oligonucleotides D1, D2, and R3 mentioned in Table S2 (20). In vitro pGpp synthesis reaction mixture containing RelZ was incubated with either 2 or 4 μM single-stranded DNA (80-nucleotide [nt] D1), single-stranded RNA (20-nt R3), double-stranded DNA (80-bp D1.D2), or R-loop. The reaction mixture was kept at 37°C for 30 min, and then the reaction was stopped, visualized, and quantified as described earlier.

To analyze the effect of (pp)pGpp on RelZ pGpp synthesis, the pGpp synthesis reaction mixture was incubated with either 1 mM pGpp, ppGpp, or pppGpp at 37°C for 30 min and analyzed as described above. For Rel₉, the inhibition was tested by using GTP as a substrate instead of GMP. All assays were performed with multiple replicates.

**In vitro RNase H fluorescence assay.** An RNA/DNA hybrid (AH) was synthesized by annealing the 12-mers 5'-fluorescein-poly(rA) and 3'-dabsyl-poly(dT) as described earlier (16). The hydrolysis reaction mixture consisted of 75 nM RelZ and 500 nM AH in a reaction buffer (50 mM Tris-Cl [pH 7.5], 50 mM KCl, and 5 mM MnCl₂) and was kept at 37°C for 15 min. A control mixture lacking protein was used as the negative control. A concentration of 4 μM pGpp was added to assess its effect on RNase H activity. Fluorescence was recorded using an excitation wavelength of 490 nm. Then, 4 μM pGpp was added to assess its effect on RelZ activity.

**M. smegmatis strain construction.** The Δrel ΔrelZ strain was generated using phage-mediated specialized transduction method as described earlier (20, 42). The allele exchange substrate plasmid (pφAE159-ms, rhl-rsd) used to generate the ΔrelZ strain previously was transduced into the M. smegmatis Δrel strain and screened for apramycin resistance (Table S1). The deletion of the gene was confirmed by sequencing.

**Biofilm formation and quantification.** Stationary-phase cultures grown in MB7/H medium were harvested, washed, and suspended in Sauton’s medium containing 2% glucose. For biofilm formation, 2 ml of Sauton’s medium was poured into a 24-well plate, and each well was inoculated with 2 μl of the culture. Each sample was aged, kept in a humidified incubator at 30°C for 5 days, and imaged.

Biofilm quantification was done using the crystal violet method described by Gupta et al. (15) and O’Toole (43). The stationary-phase cultures were resuspended in Sauton's medium (containing 2% glucose) to a final OD₆₀₀ of 0.05. Subsequently, 200 μl of the culture was poured well of the 96-well plates, sealed, and kept in a humidified incubator at 30°C for 7 days. Subsequently, the cells were removed from the wells, and the wells were washed twice with water. The adherent biofilms attached to the wells were stained with 0.1% crystal violet for 45 min. The residual dye was removed, and the wells were washed again with water and allowed to dry. The bound dye was solubilized in 200 μl of 80% ethanol, and the absorbance at 570 nm was recorded using a microtiter plate reader (SpectraMax
The experiment was repeated three times with seven technical replicates, and the resulting graph was analyzed using GraphPad software.

**Phenotype microarray analysis.** PM analysis was performed using the protocol of Gupta et al. (15). In brief, the transmittance of the cultures was adjusted to 81%, and tetrazolium violet was added to a final concentration of 0.01%. Portions (100 μl) of these cultures were inoculated into the wells of PM plates 11 to 20 coated with antibiotics/chemicals and then kept in a Biolog incubator at 37°C for 60 h. The dye reduction values were converted to areas under the curve (AUCs) by using the parametric software module of the Biolog Omnilog system. The AUCs for the strains were compared and overlaid as a test strain versus reference strain AUC. A cutoff of 4,000 in the AUCs was used to estimate the number of antibiotics in which the growth difference was significant. Dye reduction curve values were plotted using ggplot2 in R statistical software (http://cran.r-project.org).

REMA. MIC values were estimated using REMA adapted from an earlier protocol (44). In brief, the transmittance of the cultures was adjusted to a McFarland turbidity standard of 1 and then diluted 1:10. Next, 100-μl portions of the diluted culture were inoculated into 96-well microtiter plates containing a 2-fold dilution series of the antibiotics rifampin, ofloxacin, and bleomycin. The plates were sealed and incubated in a humidified incubator at 37°C. After 36 h, 30 μl of 0.01% resazurin was added to the wells of the microtiter plates, and the plates were further incubated for 4 h. The color of the resazurin dye changes from blue to pink due to bacterial growth. The MIC is defined as the minimum antibiotic concentration at which the resazurin dye did not change color.

**Analysis of cell surface properties.** M. smegmatis wild-type, knockout, and complementation strains were grown until reaching the stationary phase in MB7H9 medium supplemented with 2% glucose and 0.05% Tween 80. The protocol for the aggregation assay was adapted from that of Deshayes et al. (45). The aggregates were removed by centrifuging the cells at 10 × g for 1 min. The OD600 values of the supernatants were recorded. The OD600 values of the cultures in which the aggregates were broken by vortexing with glass beads were measured. The percent aggregation was calculated by using the OD600 of the supernatant and the OD600 of the vortexed culture.

The motility assay was adapted from Martinez et al. (46). MB7H9 plates were solidified with 0.3% agarose and inoculated in their centers with 5 μl of culture (OD600 normalized to 1). The plates were incubated at 37°C for 4 days, and the motility was evaluated by measuring the diameter of the halo of growth formed by the cells. The Congo red accumulation assay was adapted as follows (30). The cultures were cultivated to stationary phase in MB7H9 broth containing Congo red at 100 μg ml⁻¹ and 0.05% Tween 80. The cells were then washed extensively with water until the supernatants were colorless. The Congo red dye associated with the cells was extracted with 400 μl of acetone for 1 h with gentle shaking. The absorbance of the extract was measured at 488 nm. The Congo red binding index was defined as the A420 of the acetone extracts divided by the OD600 of the culture. All assays were performed in replicates and repeated at least three times.

The statistical analysis was performed using unpaired t tests (two-tailed) and plotted by using GraphPad Prism 5.0 software.

**Measurement of intracellular nucleotides by TLC.** M. smegmatis cultures were grown to mid-log phase in MB7H9 media containing glucose. Secondary cultures were grown in morpholinepropanesulfonic acid medium supplemented with 2% and 0.02% glucose, 0.08 mg/ml Casamino Acids, and 0.05% Tween 80 as described earlier (16). The cultures were labeled at an OD600 of 0.3 by adding 32P-β-glycerophosphoric acid (specific activity, >3,000 mCi/mmol; BRIT, Hyderabad, India) to a final concentration of 100 μCi/ml. The cells were harvested after 36 h of growth, washed, and lysed using formic acid. The nucleotide extracts, normalized to an OD600 of 2, were spotted onto TLC plates and visualized as described above.

Mass spectrometry analysis of nucleotides (p)pGpp was carried out according to an earlier protocol (19). The cultures were lysed by the addition of 50% ethanol and homogenized in a Beadbeater (Biospec), and the dried extracts analyzed by ESI-MS.

**Estimation of RelZ promoter activity.** The upstream sequences (200 bp for p200 and 1,000 bp for p1000) of the relZ gene were cloned in a pSDS8 plasmid upstream of the lacZ gene (21). The M. smegmatis wild-type strain was transformed with promoter-lacZ fusion constructs and grown in MB7H9 medium containing 2% glucose. LacZ expression levels were estimated by performing β-galactosidase activity assays using ONPG (o-nitrophenyl-β-D-galactopranoside) as a substrate. The activity was calculated as described earlier using the following formula: activity (Miller units) = 1,000 × ([A420 − 1.75(OD550)]/(time × culture volume × OD600)).

**CFU analysis for antibiotic sensitivity.** The M. smegmatis strains were grown until mid-log phase, and the OD600 was normalized to 0.6. The cultures were subsequently exposed to 4 μg/ml bleomycin, 20 μg/ml rifampin, 4 μg/ml ofloxacin, and 30 μg/ml erythromycin for 24 h. The bacteria were diluted by 10-fold, and the serial dilutions were plated into MB7H9 agar medium. The bacterial CFU were estimated after 3 to 4 days of growth at 37°C. The survival percentage was calculated as the percentage of CFU remaining after exposure to antibiotics compared to the CFU before antibiotic treatment.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.**
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A.P., S.Y.N., and D.C. contributed to the conception and design of the study. A.P. and S.Y.N. performed the experiments. A.P., S.Y.N., and D.C. participated in data interpretation and manuscript preparation.

REFERENCES


