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Minireview

MINIREVIEW

The stringent response and *Mycobacterium tuberculosis* pathogenesis

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One sentence summary: The authors discuss (p)ppGpp metabolism and how this functions as a critical regulator of Mycobacterium tuberculosis pathogenesis.
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ABSTRACT

During infection, the host restrains *Mycobacterium tuberculosis* (*Mtb*) from proliferating by imposing an arsenal of stresses. Despite this onslaught of attacks, *Mtb* is able to persist for the lifetime of the host, indicating that this pathogen has substantial molecular mechanisms to resist host-inflicted damage. The stringent response is a conserved global stress response in bacteria that involves the production of the hyperphosphorylated guanine nucleotides ppGpp and pppGpp (collectively called (p)ppGpp). (p)ppGpp then regulates a number of cellular processes to adjust the physiology of the bacteria to promote survival in different environments. Survival in the presence of host-generated stresses is an essential quality of successful pathogens, and the stringent response is critical for the intracellular survival of a number of pathogenic bacteria. In addition, the stringent response has been linked to virulence gene expression, persistence, latency and drug tolerance. In *Mtb*, (p)ppGpp synthesis is required for survival in low nutrient conditions, long term culture and during chronic infection in animal models, all indicative of a strict requirement for (p)ppGpp during exposure to stresses associated with infection. In this review we discuss (p)ppGpp metabolism and how this functions as a critical regulator of *Mtb* virulence.

Keywords: tuberculosis; stringent response; mycobacteria; starvation; polyphosphate; Rel

(p)ppGpp SYNTHESIS IN BACTERIA

(p)ppGpp (also referred to as magic spot due to initial identification as a spot in chromotagraphy experiments (Cashel 1969; Cashel and Gallant 1969)) is the effector molecule of the stringent response. (p)ppGpp metabolism in bacteria is controlled by Rel/SpoT homolog proteins (RSHs), named for their sequence similarity to RelA and SpoT enzymes in *Escherichia coli* (Atkinson, Tenson and Hauryliuk 2011). RelA is a monofunctional (p)ppGpp synthetase while SpoT is a bifunctional enzyme with (p)ppGpp hydrolysis activity and weak (p)ppGpp synthetase activity. Despite being the basis for the nomenclature of RSH proteins, phylogenetic analyses indicate that the presence of two functionally divergent RSH homologs (RelA and SpoT) is unique to the γ- and β- proteobacteria lineages (Atkinson, Tenson and Hauryliuk 2011). Most other bacteria encode a single bifunctional RSH enzyme that contains both a (p)ppGpp synthetase and hydrolase domain as well as several regulatory domains. The common nomenclature for multi-domain RSH proteins is long RSHs. Orthologs of long RSHs are widely-distributed throughout bacteria and are believed to originate from a common ancestral long RSH (Atkinson, Tenson and Hauryliuk 2011). The *E. coli* homologs RelA and SpoT likely arose from a lineage-specific duplication.

Mtb encodes a single bifunctional long RSH named Rel_{Mtb} (Avarbock *et al.* 1999) (Fig. 1) that is conserved in all *Mycobacterium* species and has been shown to complement an *E. coli relA* mutant for growth on minimal media (Ojha, Mukherjee and Chatterji 2000), confirming its ability to promote the

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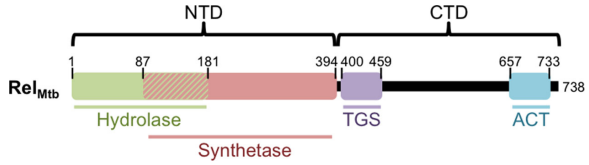

Figure 1. Diagram of the full-length Rel$_{Mtb}$ protein illustrating functional domains. The N-terminal domain (NTD) contains the catalytic domains and the C-terminal domain (CTD) contains the regulatory domains. The CTD contains two structural domains: the TGS domain (named for its initial identification in ThrRS, GTPase and SpoT) and the ACT domain (named for its initial identification in aspartate kinase, chorismite mutase and TyrA). The numbers designate the amino acid positions that form the borders of each domain.

stringent response. The *rel*$_{Mtb}$ locus is syntenous with the *rel* locus in *Bacillus subtilis*, encoding *apt* (adenine phosphoribosyltransferase) upstream and *cypH* (cyclophilin) downstream (Avarbock *et al.* 1999). Deletion of *rel*$_{Mtb}$ (Δ*rel*$_{Mtb}$) results in a (p)ppGpp null mutant ((p)ppGpp^0) (Primm *et al.* 2000), suggesting that Rel$_{Mtb}$ is the only functional (p)ppGpp synthetase in *Mtb*. (p)ppGpp synthesis by Rel$_{Mtb}$ is necessary for chronic *Mtb* infection in both mice and guinea pigs (Dahl *et al.* 2003; Karakousis *et al.* 2004; Klinkenberg *et al.* 2010; Weiss and Stallings 2013), which is when the immune system is activated to impose growth restrictive stresses on the bacteria. In contrast, the Δ*rel*$_{Mtb}$ mutant is not attenuated for growth in THP-1 macrophages in cell culture (Primm *et al.* 2000), indicating that the stringent response is essential for *Mtb* survival during stress conditions specific to the chronic phase of *in vivo* infection.

Streptomyces, Frankia and *Salinispora species* encode a second long RSH homolog named RshA, which is believed to have arisen from an Actinobacteria-specific duplication but has subsequently been lost in *Mycobacterium species* (Sun, Hesketh and Bibb 2001; Atkinson, Tenson and Hauryliuk 2011). In addition to long RSHs, two other classes of RSH proteins have been discovered, the Small Alarmone Synthetases (SASs) and Small Alarmone Hydrolases (SAHs). In contrast to the long RSHs, SASs and SAHs are monofunctional, single-domain enzymes. Actinobacteria SASs belong to their own monophyletic clade (actRel) and are orthologous to the well-studied *B. subtilis* SAS proteins RelP (YjbM) and RelQ (YwaC) (Atkinson, Tenson and Hauryliuk 2011). *Mtb* encodes a single SAS (Rv1366), but the enzyme is believed to be non-functional (Bag *et al.* 2014) and deletion of *rv1366* in *Mtb* has no phenotypes in culture or during infection of mice (Weiss and Stallings 2013). In other bacteria, SAS activity is toxic in the absence of a functional (p)ppGpp hydrolase (Lemos *et al.* 2007; Nanamiya *et al.* 2008; Abranches *et al.* 2009; Das *et al.* 2009; Natori *et al.* 2009), which is not observed in *Mtb*, further supporting that Rv1366 is not a functional (p)ppGpp synthetase (Weiss and Stallings 2013). The only other SAS that has been studied in mycobacteria is MS_RHII-RSD in *Mycobacterium smegmatis* (*Msm*), a non-pathogenic model organism for *Mtb*. Unlike Rv1366, MS_RHII-RSD has been shown to be a functional pppGpp synthetase and encodes an amino-terminal RNase HII domain (Murdeshwar and Chatterji 2012) that can hydrolyze R-loops (Krishnan *et al.* 2016) that may form as a result of collisions between the replication and transcription machinery, indicating that MS_RHII-RSD has evolved functions distinct from Rv1366.

(p)ppGpp METABOLISM BY Rel$_{Mtb}$

Rel$_{Mtb}$ is comprised of two catalytic domains, a (p)ppGpp hydrolysis domain (1-181 amino acids (aa)) and a (p)ppGpp synthetase domain (87-394 aa), that include an overlapping three helix bundle (87-181), as well as a regulatory C-terminal domain (CTD, 395-738 aa) (Fig. 1) (Avarbock *et al.* 2005; Singal *et al.* 2017). The (p)ppGpp synthetase domain transfers the 5′-β,γ-pyrophosphate from ATP to the 3′-OH of GDP or GTP to synthesize ppGpp and pppGpp, respectively (Avarbock *et al.* 1999). Crystal structures of the N-terminus of Rel$_{Mtb}$ (1-394 aa) and the N-terminus of a homolog from *Streptococcus dysgalactiae* subspecies *equisimilis* (Rel$_{Seq}$, 1-385 aa included in the crystal structure) have provided insight to the functional features of Rel catalytic domains (Hogg *et al.* 2004; Singal *et al.* 2017). The (p)ppGpp synthetase domain is comprised of five β-sheets surrounded by five α-helices (Singal *et al.* 2017). The (p)ppGpp synthetase domain was determined to be structurally homologous to DNA polymerase β (pol β) and the D190 and D256 residues in pol β that are essential for coordinating the Mg^{2+} cofactor correspond to D265 and E325 in Rel$_{Mtb}$ (Hogg *et al.* 2004). Both D265 and E325 of Rel$_{Mtb}$ are required for (p)ppGpp synthesis *in vitro* and Rel$_{Mtb}$ rescue of an *E. coli* Δ*relA*Δ*spoT* ((p)ppGpp^0) strain growth on minimal media (Hogg *et al.* 2004; Bag *et al.* 2014). Rel$_{Seq}$ co-crystallized with GDP in a ligand-binding pocket partially formed by 176-185 aa within the synthetase domain. The conservation of the majority of the residues comprising the GDP binding pockets between Rel$_{Seq}$ and Rel$_{Mtb}$ suggests that Rel$_{Mtb}$ and Rel$_{Seq}$ recognize and bind to GDP in a similar fashion. The GDP pocket also includes Rel$_{Seq}$ Y308 (Rel$_{Mtb}$ Y309), which face-to-face stacks with GDP and is required for Rel$_{seq}$ to rescue growth of an *E. coli* (p)ppGpp^0 strain on minimal media (Hogg *et al.* 2004; Bag *et al.* 2014). The Rel$_{Mtb}$ ATP binding pocket likely involves the positively charged residues R242, K244 and K252 coordinating the β and γ phosphates of ATP at a site proximal to the GDP binding pocket and the Mg^{2+} binding site (Hogg *et al.* 2004; Singal *et al.* 2017). The importance of the basic patch created by R242, K244 and K252 is supported by experiments showing that Rel$_{Mtb}$ R242H is unable to rescue growth of an *E. coli* (p)ppGpp^0 strain on minimal media (Bag *et al.* 2014). In addition, Rel$_{Mtb}$ G241 and H344, which were initially identified in *E. coli* RelA as being important for ATP binding (Gropp *et al.* 2001), are required for pppGpp synthesis *in vitro* (Avarbock *et al.* 2005). Additionally, H344 is required for Rel$_{Mtb}$ to rescue growth of an *E. coli* (p)ppGpp^0 strain on minimal media (Bag *et al.* 2014) and mutation of Rel$_{Mtb}$ H344 attenuates *Mtb* in mice (Weiss and Stallings 2013).

In addition to being able to synthesize (p)ppGpp, Rel$_{Mtb}$ also hydrolyzes (p)ppGpp into pyrophosphate (PP_i) and GDP or GTP via an N-terminal catalytic HD superfamily domain (Avarbock *et al.* 2005) (Fig. 1). HD superfamily members are phosphohydrolases with conserved histidine and aspartate residues that are involved in the coordination of divalent cations, which is essential for their activity (Aravind and Koonin 1998). The Rel$_{Mtb}$ hydrolase domain is comprised of 11 α-helices, which includes a (p)ppGpp binding pocket spanning residues 41-53 located in the region connecting the second and third α-helices (α2 and α3). The HD motif comprised of the conserved histidine (H80) and aspartate (D81) residues is located in a β-turn that connects α4 and α5 (Singal *et al.* 2017). Alanine substitution of H80 or D81 in Rel$_{Mtb}$ abolishes hydrolase activity *in vitro* without affecting pppGpp synthesis (Avarbock *et al.* 2005).

The Rel$_{Mtb}$ catalytic domains have strict cation co-factor requirements where (p)ppGpp synthesis requires Mg^{2+} or Mn^{2+} and Rel$_{Mtb}$ (p)ppGpp hydrolysis requires Mn^{2+}. The Mg^{2+} and Mn^{2+} concentrations required for optimal (p)ppGpp synthesis is equal to the combined concentration of the substrates, ATP and GTP. Rel$_{Mtb}$ synthetase activity is inhibited by concentrations of Mg^{2+} (or Mn^{2+}) that exceed the GTP and

ATP substrate concentrations due to an RXKD motif within the synthetase domain that is highly conserved among bifunctional Rel enzymes (Avarbock, Avarbock and Rubin 2000; Sajish et al., 2007, 2009). The model for how the RXKD motif sensitizes the Rel_{Mtb} synthetase catalytic activity to Mg^{2+} concentrations that exceed ATP and GTP substrate concentrations is that the negatively charged phosphates of GTP can bind to either the positive charges of Rel_{Mtb} RXKD motif (R and D) or positively charged Mg^{2+} ions. Mg^{2+} bound GTP is unable to bind the positive charges of RXKD in a way that is required for (p)ppGpp synthesis. Thus, Mg^{2+} ions compete with the Rel_{Mtb} RXKD motif for GTP binding. In contrast, *E. coli* RelA has an EXDD motif instead of the RXKD motif and is not sensitive to high concentrations of Mg^{2+}. Instead, due to the presence of two negatively charged residues (E and D), the *E. coli* EXDD motif requires Mg^{2+} to bind GTP, allowing the *E. coli* RelA synthetase domain to function when Mg^{2+} concentrations are higher than the combined ATP and GTP concentration (Sajish et al., 2007, 2009).

REGULATION OF (p)ppGpp SYNTHESIS BY Rel_{Mtb}

Rel_{Mtb} is constitutively expressed at basal levels, likely via an almost consensus -10 promoter element upstream of the rel_{Mtb} gene that would be recognized by the housekeeping σ-factor σ^A (Jain *et al.* 2005). However, in the absence of stress or stimuli, the Rel_{Mtb} synthetase activity is repressed by the presence of the CTD (Avarbock *et al.* 1999; Jain *et al.* 2006) and only a low level of (p)ppGpp is produced (Stallings *et al.* 2009; Weiss and Stallings 2013). Although the level of (p)ppGpp produced in unstressed conditions is low, this low level production is still required for WT growth rates and a Δrel_{Mtb} mutant is attenuated for growth in unstressed, nutrient-rich media under aerobic conditions (Primm *et al.* 2000; Weiss and Stallings 2013), suggesting that the low levels of (p)ppGpp produced in unstressed conditions still provide a growth advantage to *Mtb*. In addition, (p)ppGpp hydrolysis is essential for preventing toxic (p)ppGpp accumulation in *Mtb* in unstressed culture conditions, as well as during both acute and chronic stages of infection (Weiss and Stallings 2013). These observations demonstrate that Rel_{Mtb} constitutively produces (p)ppGpp and may act to maintain homeostasis in all growth conditions. This is also similar to observations in other bacteria where (p)ppGpp hydrolysis is essential for viability in unstressed conditions due to the presence of a constitutive level of (p)ppGpp synthesis (Lemos *et al.* 2007; Nanamiya *et al.* 2008; Das *et al.* 2009).

The induction of (p)ppGpp synthesis by Rel homologs is best characterized during amino acid starvation where Rel is associated with ribosomes when an uncharged tRNA enters the A site of the ribosome and stimulates Rel-mediated (p)ppGpp production (Haseltine and Block 1973; Avarbock, Avarbock and Rubin 2000). Accordingly, *Mtb* accumulates (p)ppGpp in a Rel_{Mtb}-dependent manner during nutrient starvation in liquid culture (Primm *et al.* 2000; Stallings *et al.* 2009). In agreement with stringent response mutants in other bacteria, deletion of rel_{Mtb} reduces the viability of *Mtb* in nutrient-poor conditions (Primm *et al.* 2000), supporting a role for the *Mtb* stringent response in responding to starvation. In addition to during starvation, (p)ppGpp accumulates in *Mtb* in response to hypoxia and oxidative stress (Primm *et al.* 2000; Stallings *et al.* 2009), and it is not known whether this is because these stresses cause amino acid starvation that triggers Rel_{Mtb} (p)ppGpp synthetase activity or if there are other ways to induce Rel_{Mtb}-mediated (p)ppGpp synthesis.

The induction of (p)ppGpp synthesis during amino acid starvation requires the Rel_{Mtb} CTD and can be recapitulated *in vitro* by adding the Rel activating complex (RAC) consisting of ribosomes, uncharged tRNAs, and mRNA (Avarbock, Avarbock and Rubin 2000; Avarbock *et al.* 2005; Jain *et al.* 2006). The Rel_{Mtb} C-terminal domain (CTD) contains two structural domains: the TGS domain (400-459 aa, named for its initial identification in ThrRS (threonyl tRNA synthetase), GTPase (Obg family of GTPases) and SpoT) and the ACT domain (657-733 aa, named for its initial identification in aspartate kinase, chorismite mutase and TyrA) (Wolf *et al.* 1999; Chipman and Shaanan 2001) (Fig. 1). Although a crystal structure for the Rel_{Mtb} CTD has not yet been solved, multiple cryo-EM structures of *E. coli* RelA in complex with a ribosome with an uncharged tRNA (Phe) occupying the A site have provided a structure-based model for the function of TGS and ACT domains (Arenz *et al.* 2016; Brown *et al.* 2016; Loveland *et al.* 2016). These structures suggest that the TGS domain acts as a sensor to distinguish charged versus uncharged tRNAs that enter the A-site of the ribosome. The sensor function of the TGS domain is accomplished by the ability of the TGS domain to interact with the 3'CCA of the A-site tRNA. If an aminoacylated tRNA occupies the A-site of the ribosome, the amino acid attached to the 3'CCA will sterically occlude the interaction of the 3'CCA with the TGS domain. However, when a deacylated tRNA occupies the A-site of the ribosome, the TGS domain is able to interact with the 3'CCA, resulting in structural changes within the RelA synthetase domain that activates (p)ppGpp synthesis. The ACT domain serves an anchor function, allowing RelA to bind the ribosome regardless of whether the A-site is occupied by a charged or uncharged tRNA. The ACT domain occupies a cavity between the A and P sites of the ribosome and is held in place through several interactions with the A-site finger (helix 38) of the 23S ribosomal RNA (rRNA), the L16 ribosomal protein and the P-site tRNA. The cryo-EM structures also revealed a domain between the TGS and ACT domains of RelA that resembles a zinc-finger domain. This previously uncharacterized domain provides a second binding interface between RelA and the ribosome, and is expected to be conserved in mycobacteria. Loveland *et al.* (2016) termed this new domain in RelA the Ribosomal-InterSubunit (RIS) domain because it bridges the 30S and 50S ribosomal subunits through interactions with the A-site finger and the hydrophobic patch on the 30S S19 ribosomal protein.

Induction of (p)ppGpp synthesis is accompanied by a repression of (p)ppGpp hydrolysis. The Rel_{Seq} crystal structure supports a model where Rel does not simultaneously catalyze (p)ppGpp synthesis and (p)ppGpp hydrolysis (Hogg *et al.* 2004). In this study, two conformations of the Rel_{Seq} monomer (1-385) were observed, a hydrolase-OFF/synthetase-ON conformation and a hydrolase-ON/synthetase-OFF conformation. Transition between the two conformations is dependent on ligand binding to the respective active site and indicates that at any given time only one enzymatic function is active, thus preventing simultaneous synthesis and hydrolysis of (p)ppGpp (Hogg *et al.* 2004). In support of this model, Avarbock *et al.* have shown that Rel_{Mtb} hydrolase activity is inhibited in the presence of synthetase substrates ATP and GTP (Avarbock, Avarbock and Rubin 2000).

In what appears to be a negative feedback loop, pppGpp has been shown to bind to the CTD in the region that separates the TGS and ACT of the Rel protein from *Msm* (Syal *et al.* 2015). Binding of pppGpp to the Rel_{Msm} CTD represses pppGpp synthesis while increasing pppGpp hydrolysis. As such, Rel_{Msm}-mediated

pppGpp synthesis is decreased at saturating pppGpp concentrations compared to synthesis at non-saturating pppGpp concentrations. Rel_{Msm} point mutants with lower affinity for pppGpp (F533A, R615A, F620A and R633A) lose this effect. pppGpp binding to Rel_{Msm} decreases protein compaction and causes Rel_{Msm} to become less structured, suggesting that conformational changes within Rel_{Msm} may lead to repression of the synthetase activity (Jain, Saleem-Batcha and Chatterji 2007).

Studies *in vitro* have suggested that Rel_{Mtb} could also be regulated through oligomerization (Avarbock *et al.* 2005; Jain *et al.* 2006; Singal *et al.* 2017). The full-length Rel_{Mtb} has been shown to form trimers in gel filtration experiments, which is facilitated by the CTD, although this domain is not required for oligomerization (Avarbock *et al.* 2005). The Rel_{Mtb} NTD (amino acids 1-394) alone formed dimers in small angle X-ray scattering analysis and X-ray crystallography experiments (Singal *et al.* 2017). In both cases, oligomerization was shown to inhibit pppGpp synthesis (Avarbock *et al.* 2005; Jain *et al.* 2006). These *in vitro* observations have led to some speculation that this could be another way that Rel_{Mtb} activity is regulated, however, it is still unknown if oligomerization occurs *in vivo*.

REGULATION OF TRANSCRIPTION BY (p)ppGpp

Once (p)ppGpp is synthesized, it has been shown to bind multiple targets in bacteria to modify their function and promote changes in physiology that allow for survival during stress. The most well-characterized outcome of the stringent response in bacteria is a downregulation of rRNA and ribosomal proteins accompanied by an upregulation of amino acid biosynthesis genes to restore amino acid levels during starvation (Eymann *et al.* 2002; Traxler *et al.* 2008). Hallmarks of this canonical stringent response appear to be conserved in *Mtb*. In a microarray experiment comparing expression profiles of a Δrel_{Mtb} strain to WT *Mtb* starved in Tris-buffered saline with Tween-80 (TBS-T), expression of 54 of the 58 ribosomal proteins was downregulated during starvation in WT *Mtb* but not in the Δrel_{Mtb} mutant (Dahl *et al.* 2003). In addition, quantitative reverse transcription-PCR (qRT-PCR) experiments showed that the Δrel_{Mtb} strain was unable to downregulate rRNA levels during starvation (Stallings *et al.* 2009). These data support the conservation of the canonical stringent response involving a Rel-dependent repression of genes encoding the translation machinery. However, WT *Mtb* starved in TBS-T does not induce expression of amino acid biosynthesis genes (Betts *et al.* 2002; Dahl *et al.* 2003). One potential explanation for *Mtb* failing to induce expression of amino acid biosynthesis genes is that the studies with *Mtb* used a general carbon starvation condition by incubating *Mtb* in TBS-T, whereas studies in *E. coli* and *B. subtilis* have used amino acid analogs to directly interfere with tRNA charging. Therefore, it is possible that *Mtb* would upregulate amino acid biosynthesis genes in response to specifically interfering with tRNA charging, but this has yet to be tested. Alternatively, Zhang and Rubin (2013) attribute this observation to the relatively unique amino acid prototrophy of *Mtb* where the bacteria constitutively synthesizes its own amino acids and these pathways may have evolved to become independent of stress signaling.

The mechanisms by which (p)ppGpp alters gene expression during starvation have been most well-characterized in the gram-negative γ-proteobacteria *E. coli* and the gram-positive firmicute *B. subtilis*. Numerous differences in the mechanisms used by (p)ppGpp in these two organisms has established two divergent paradigms of the stringent response: the *E. coli* paradigm, which is utilized by most proteobacteria, and the *B. subtilis* paradigm, which occurs in most other bacteria species (Boutte and Crosson 2013; Hauryliuk *et al.* 2015; Liu *et al.* 2015). One of the primary differences between the two stringent response paradigms is the direct binding target of (p)ppGpp. In *E. coli*, (p)ppGpp directly binds to RNA polymerase (RNAP) and changes gene expression by destabilizing RNAP-promoter open complexes (RP_o) that form during transcription initiation (Barker, Gaal and Gourse 2001; Ross *et al.* 2013; Zuo, Wang and Steitz 2013). Two distinct ppGpp binding sites have been identified on the *E. coli* RNAP. The first ppGpp binding site (Site 1) is a pocket in between the β' and ω subunits of RNAP (Ross *et al.* 2013; Zuo, Wang and Steitz 2013). ppGpp binding to Site 1 is thought to allosterically prevent the RNAP from making stable contacts with the promoter DNA, thus destabilizing RP_o (Ross *et al.* 2013; Zuo, Wang and Steitz 2013). A second ppGpp binding site (Site 2) is introduced by the binding of the DksA protein to RNAP (Ross *et al.* 2016). DksA binds to the β' subunit of RNAP and inserts its coiled-coil domain into the RNAP secondary channel, creating (p)ppGpp binding Site 2, located at the interface between the RNAP β' subunit and DksA. Recent crystal structures revealed that ppGpp binding stabilizes the DksA-RNAP interaction by relieving the mechanical stress in RNAP caused by DksA binding (Molodtsov *et al.* 2018). In addition, ppGpp binding induces a conformational change in DksA, allowing it to insert its coiled-coil domain further into the RNAP secondary channel to potentially interfere with RNAP catalytic activity (Molodtsov *et al.* 2018). Binding of DksA is necessary for the full effects of ppGpp on RP_o stability *in vitro* (Paul *et al.* 2004; Paul, Berkmen and Gourse 2005; Lennon *et al.* 2012; Molodtsov *et al.* 2018). (p)ppGpp and DksA are thought to regulate transcription initiation by destabilizing the transition states between the RNAP-promoter closed complex (RP_c) and RP_o complex, thus decreasing the activation energy of this transition (Paul, Berkmen and Gourse 2005; Rutherford *et al.* 2009). At promoters that form intrinsically unstable RP_o, such as *E. coli* rRNA promoters, this effect of ppGpp and DksA negatively regulates transcription by facilitating RP_o collapse back to RP_c (Paul *et al.* 2004). In contrast, at promoters where transcription is not rate-limited by RP_o stability, such as promoters of the *E. coli* amino acid biosynthesis genes, this effect of ppGpp and DksA positively regulates transcription by allowing the isomerization of RP_c to RP_o to occur more readily (Paul, Berkmen and Gourse 2005). Thus, (p)ppGpp is able to exert differential effects on transcription initiation based on the kinetics of the RNAP complex at a given promoter.

In *B. subtilis*, (p)ppGpp exerts its effects on transcription indirectly through changes in intracellular guanosine nucleobase metabolism (Kriel *et al.* 2012). (p)ppGpp directly binds and inhibits enzymes involved in both *de novo* and salvage pathways of GTP synthesis. Specifically, (p)ppGpp potently inhibits the activity of guanylate kinase (GMK), which phosphorylates GMP to GDP (Kriel *et al.* 2012; Liu *et al.* 2015), and HprT, which converts hypoxanthine to inosine monophosphate (IMP) in the purine salvage pathway (Kriel *et al.* 2012), collectively inhibiting GTP biosynthesis. At high (millimolar) concentrations, (p)ppGpp also inhibits the enzymatic activity of IMP dehydrogenase (IMPDH or GuaB), which converts IMP to xanthosine monophosphate (XMP) and controls a branch point between ATP and GTP synthesis in the purine salvage pathway, in both *B. subtilis* (Kriel *et al.* 2012) and *E. coli* (Pao and Dyess 1981). Inhibition of the GTP biosynthesis pathways upon nutrient starvation alters the metabolite landscape, most notably causing depletion of GTP and concordant accumulation of ATP (Kriel *et al.* 2012; Liu *et al.* 2015). In addition,

pppGpp synthesis depletes free GTP by using it as a substrate for pppGpp synthesis, further decreasing intracellular GTP levels and increasing the intracellular ATP:GTP ratio. ATP and GTP are the most common initiating nucleotide triphosphates (iNTP) used in *B. subtilis* and since binding of the iNTP to the transcription initiation complex stabilizes RP_o (Kuzmine, Gottlieb and Martin 2003), changes in the ATP:GTP ratio impacts gene expression. Consequently, *B. subtilis* genes that initiate with ATP, such as amino acid biosynthesis genes, are up-regulated while genes that initiate with GTP, such as rRNA genes, are down-regulated (Krásný *et al.* 2008; Tojo *et al.* 2010; Kriel *et al.* 2012). Depletion of GTP also activates a starvation response through the transcription factor CodY (Handke, Shivers and Sonenshein 2008; Kriel *et al.* 2012). In nutrient replete conditions, GTP binds CodY and blocks transcription of stress adaptive genes such as those involved in sporulation and branched chain amino acid biosynthesis (Ratnayake-Lecamwasam *et al.* 2001; Belitsky and Sonenshein 2008). When GTP levels decrease during the stringent response, CodY-mediated repression is relieved and transcription of these genes is activated. Together, the ATP:GTP ratio and CodY contribute to the gene expression profile that results from the *B. subtilis* stringent response. However, the role of guanosine metabolites in the stringent response is likely more complex because GTP depletion alone is not sufficient for starvation survival (Liu *et al.* 2015).

Intriguingly, *Mtb* does not appear to strictly follow either stringent response paradigm, and the mechanisms underpinning the *Mtb* stringent response remain mostly unknown. In terms of the *E. coli* paradigm of the stringent response, the sites known to be targeted by ppGpp on the *E. coli* RNAP are largely not conserved in the *Mtb* RNAP. Site 1 where ppGpp binds the *E. coli* RNAP involves numerous residues at the interface of the β' and ω subunits, including β' K615, β' I619, β' D622, β' Y626, β' R632, β' R417, ω A2, ω R3, ω V4 and ω T5 (Ross *et al.* 2013; Zuo, Wang and Steitz 2013). The *Mtb* RNAP shares identity or similarity with only two of these residues, *E. coli* β' D622 (*Mtb* β' D714) and *E. coli* β' R362 (*Mtb* β' K437). Site 2 on the *E. coli* RNAP that is targeted by ppGpp is DksA-dependent (Ross *et al.* 2016) and *Mtb* does not encode DksA. In addition, the Site 2 residues located in the *E. coli* β' subunit are not conserved in the *Mtb* RNAP.

Nonetheless, there is evidence that pppGpp directly binds the *Mtb* RNAP, raising the possibility that although the features of the (p)ppGpp-RNAP interaction in *Mtb* may be different than described in *E. coli*, this interaction could still occur. Addition of pppGpp to *in vitro* transcription reactions inhibited RP_o formation by *Mtb* RNAP at the *Mtb* rRNA *rrn*AP3 promoter (China *et al.* 2012) and transcript formation by *Mtb* RNAP at the *Mtb rrn*AP3 and *gyrB* promoters (Tare, China and Nagaraja 2012; Tare, Mallick and Nagaraja 2013). Addition of Rv3788, a protein that binds near the secondary channel of the *Mtb* RNAP, blocks the effects of pppGpp on RP_o formation at the *Mtb rrn*AP3 promoter (China *et al.* 2012), suggesting that the effects of pppGpp require its interaction with the RNAP. The effect of pppGpp on transcript formation by *Mtb* RNAP at the *Mtb rrn*AP3 promoter was also dependent on a thymine positioned three nucleotides downstream of the -10 element and L232 in σ^A, both of which affected RP_o stability, thus supporting a link between pppGpp binding to the RNAP and RP_o stability (Tare, Mallick and Nagaraja 2013). However, in all of these cases, effects of pppGpp were only observed at concentrations $\geq$100 μM, and often only at $\geq$500 μM, whereas the IC_{50} of ppGpp on the *E. coli* RNAP at the *rrnBP1* promoter is 12 μM (Ross *et al.* 2013). Therefore, differences exist in how (p)ppGpp impacts RNAP activity in *E. coli* and *Mtb* and future studies will be necessary to determine whether the high concentrations of pppGpp used *in vitro* are physiologically relevant *in vivo* in *Mtb*.

Mycobacteria also share some similarities with the *B. subtilis* stringent response paradigm. For example, when *M. smegmatis* was engineered to accumulate higher levels of (p)ppGpp through mutation of the Rel_{Msm} hydrolase domain, ATP levels were increased and GTP levels were decreased (Weiss and Stallings 2013), indicating that (p)ppGpp synthesis affected purine metabolism. Accordingly, rRNA production is downregulated in mycobacteria during the stringent response and the two promoters (AP1 and AP3) upstream of the *Mtb* rRNA locus both initiate with GTP, which mirrors the pattern for transcriptional start sites at rRNA operons that has been reported in *B. subtilis* (Krásný *et al.* 2008). Nonetheless, it has yet to be directly tested whether the identity of the iNTP for *Mtb* promoters is sufficient to determine the direction of gene expression during the stringent response. Unlike *B. subtilis*, pppGpp is not able to inhibit *Mtb* GMK (Rv1389) *in vitro*, even though GMK homologs from other Actinobacteria such as *Streptomyces coelicolor* were inhibited by pppGpp (Liu *et al.* 2015). This is possibly explained by the lack of conservation in multiple residues in the Rv1389 active site that are required for the interaction of GMK with pppGpp in *B. subtilis* and *S. coelicolor* (Fig. 2). These polymorphisms are exclusive to *Mtb*, as even the (p)ppGpp-insensitive proteobacteria GMK homologs are conserved at these residues and their (p)ppGpp insensitivity is due to polymorphisms elsewhere in the protein (Liu *et al.* 2015). Another difference between the *B. subtilis* paradigm and *Mtb* is that mycobacteria do not encode a CodY homolog. The effect of (p)ppGpp on the *Mtb* homologs of other *B. subtilis* (p)ppGpp targets in the purine biosynthesis pathway (HprT and IMP dehydrogenase) has not yet been tested and, therefore, could be (p)ppGpp targets in *Mtb* and explain the decreased GTP levels during (p)ppGpp synthesis. Alternatively, (p)ppGpp synthesis could be sufficient to deplete GTP by using it as a substrate. Together these data indicate that the classic model of (p)ppGpp impacting RP_o to alter gene expression during the stringent response could be conserved in *Mtb*, but the mechanistic features of this response are unique from model organisms.

(p)ppGpp, POLYPHOSPHATE AND PERSISTENCE

In addition to RNAP and enzymes involved in GTP metabolism, polyphosphatase, a key regulator of polyphosphate (polyP) metabolism, has been investigated as a target of (p)ppGpp in *Mtb*. PolyP chains are linear polymers of phosphates that can reach hundreds to thousands of phosphates in length (Rao, Roberts and Torriani 1985). *Mtb* accumulates polyP chains when exposed to nutritional, nitrosative, oxidative, acidic and antibiotic stresses as well as during its transition to stationary phase (Thayil *et al.* 2011; Singh *et al.* 2013; Chuang *et al.* 2015), overlapping with conditions that induce (p)ppGpp accumulation (Primm *et al.* 2000; Stallings *et al.* 2009). PolyP accumulation results in an overhaul of *Mtb* metabolism that arrests growth and facilitates *Mtb* persistence. In *Mtb*, polyP is synthesized primarily by the polyphosphate kinase PPK1 (Rv2984). *Mtb* encodes another polyphosphate kinase PPK2 (Rv1026) that can synthesize polyP, but PPK2 more efficiently carries out the reverse reaction as a polyphosphate dependent nucleoside diphosphate kinase that utilizes polyP to produce ATP and GTP (Sureka *et al.* 2009; Shum *et al.* 2011; Chuang, Belchis and Karakousis 2013). In addition, Δ*ppk1* mutants produce only negligible amounts of polyP

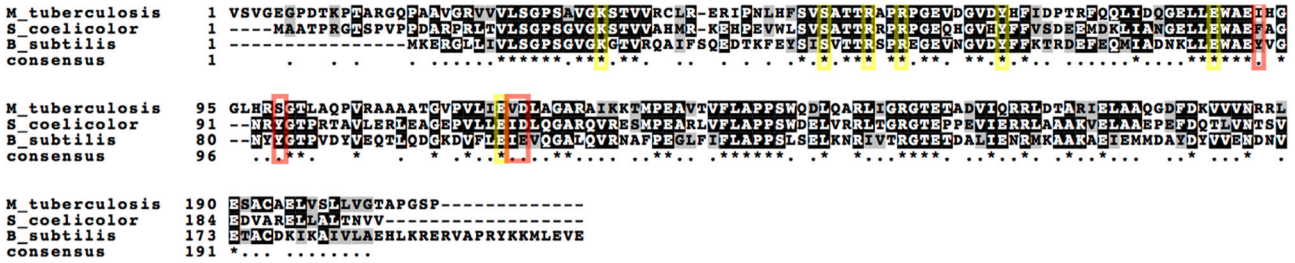

Figure 2. Sequence alignment of GMK homologs from *Mycobacterium tuberculosis* (*Mtb*), *Streptomyces coelicolor* and *Bacillus subtilis*. Yellow boxes indicate residues that interact with pppGpp and are conserved in all three species. Red boxes indicate residues that interact with pppGpp and are polymorphic in *Mtb*.

(Singh *et al.* 2013), supporting that PPK1 serves as the primary polyP synthase in *Mtb*. *Mtb* encodes two polyphosphatases, PPX1 (Rv0496) and PPX2 (Rv1026), that hydrolyze polyP by cleaving phosphates from the termini of polyP chains. PPX1 and PPX2 differ in their substrate preference where PPX1 preferentially catabolizes short polyP chains and PPX2 specializes in breaking down long polyP chains (Choi *et al.* 2012; Chuang *et al.* 2015). (p)ppGpp directly inhibits the hydrolytic activity of *Mtb* PPX1 and PPX2 during *in vitro* exopolyphosphatase assays (Choi *et al.* 2012; Chuang *et al.* 2015). Inhibition of PPX1 or PPX2 would lead to the accumulation of polyP in *Mtb* and this has been proposed to contribute to the role of the stringent response in persistence (Thayil *et al.* 2011; Chuang *et al.* 2015). In support of the physiological relevance of this mechanism, deleting rel_{Mtb} causes mid- and late- log phase cultures of *Mtb* to accumulate less polyP (Singh *et al.* 2013).

How changes in polyP levels affect *Mtb* has been investigated using *Mtb* mutants that produce lower levels of polyP (Δ*ppk1*) or over-accumulate polyP (*ppx1*:Tn, *ppx2* knockdown, and *ppk2*:Tn) (Table 1). The Δ*ppk1* and *ppx1*:Tn strains grow slower in liquid culture and the Δ*ppk1*, *ppx1*:Tn, *ppx2* knockdown and *ppk2*:Tn mutants all reach a lower maximum optical density at stationary phase (Thayil *et al.* 2011; Chuang, Belchis and Karakousis 2013; Singh *et al.* 2013; Chuang *et al.* 2015). Δ*ppk1*, *ppx1*:Tn, and *ppk2*:Tn mutant strains are also attenuated *in vivo* (Thayil *et al.* 2011; Chuang, Belchis and Karakousis 2013; Singh *et al.* 2013). The Δ*ppk1* and *ppx1*:Tn strains both showed decreased lung CFU starting at 14 days post-infection in the guinea pig infection model (Thayil *et al.* 2011; Singh *et al.* 2013). The *ppk2*:Tn strain showed decreased lung CFU at 14 days but grew back to levels comparable to WT *Mtb* in a mouse infection model (Chuang, Belchis and Karakousis 2013). Unlike the Δrel_{Mtb} mutant, the Δ*ppk1*, *ppx1*:Tn, and *ppk2*:Tn strains were impaired for survival in macrophages (Thayil *et al.* 2011; Chuang, Belchis and Karakousis 2013; Singh *et al.* 2013), suggesting that the virulence mechanisms mediated by polyP and the stringent response are not completely overlapping. Unexpectedly, the *ppx2* knockdown strain grew better in macrophages (Chuang *et al.* 2015), suggesting that the role of polyP metabolism in virulence requires further study to reconcile these seemingly contradictory findings. Nonetheless, these data demonstrated that proper regulation of polyP levels is important for *Mtb* virulence and the ability of (p)ppGpp to modulate polyP levels by direct inhibition of polyphosphatases could be one mechanism by which the stringent response impacts pathogenesis.

Metabolomics studies revealed that the *ppx1*:Tn, *ppk2*:Tn and *ppx2* knockdown mutants that accumulate polyP contain significantly less glycerol-3-phosphate (G3P), a major scaffold for phospholipids, accompanied by differences in the expression of enzymes involved in G3P metabolism. The enzymes responsible for G3P synthesis from dihydroxyacetone phosphate and glycerol, Rv0564c and GlpK, respectively, were significantly downregulated in the *ppx1*:Tn mutant and the enzyme responsible for the reverse reaction, Rv1692, which hydrolyses G3P to glycerol, was significantly upregulated in the *ppk2*:Tn strain. In addition, an enzyme involved in the recycling of glycerophospholipids for G3P synthesis, Rv2182c, was significantly downregulated in both the *ppx1*:Tn and *ppk2*:Tn mutants (Chuang et al., 2015, 2016). These data suggest that polyP accumulation signals for changes in gene expression that are linked to a reduction in *de novo* G3P synthesis, a reduction in recycling pathways for G3P synthesis, and increased G3P turnover. The *ppx1*:Tn, *ppk2*:Tn, and *ppx2* knockdown mutants also exhibit decreased levels of metabolites involved in peptidoglycan synthesis and both *ppx1*:Tn and *ppk2*:Tn mutants express lower transcript levels for the peptidoglycan L,D-transpeptidases *ldtA* and *ldtB* (Chuang *et al.*, 2015, 2016). Several metabolites involved in NADH metabolism, the TCA cycle and arginine metabolism also accumulate in the *ppx1*:Tn and *ppk2*:Tn mutants (Chuang *et al.* 2016), while fatty acid biosynthesis and nucleotide biogenesis metabolites are reduced in the *ppx2* knockdown strain (Chuang *et al.* 2015). The accumulation of metabolites involved in the TCA cycle also occurs during exposure to hypoxia, a condition that induces the formation of drug and stress tolerant persisters (Wayne and Hayes 1996; Watanabe *et al.* 2011; Eoh and Rhee 2013). Overall, the decrease in central metabolism and anabolic pathways during polyP accumulation is indicative of a shift towards metabolic quiescence.

PolyP accumulation in *Mtb* also affects antibiotic tolerance. The Δ*ppk1* mutant that does not produce polyP showed increased susceptibility to rifampicin, levofloxacin and isoniazid (Singh *et al.* 2013). In contrast, the polyP accumulating *ppx1*:Tn, *ppk2*:Tn and *ppx2* knockdown mutants displayed decreased sensitivity to isoniazid (Thayil *et al.* 2011; Chuang, Belchis and Karakousis 2013; Chuang *et al.* 2015). However, the *ppx1*:Tn strain displayed increased sensitivity to clofazimine, which is thought to kill *Mtb* through membrane destabilization and altered redox cycling (Cholo *et al.* 2017), while the *ppk2*:Tn strain displayed increased sensitivity to meropenem, which inhibits peptidoglycan L,D-transpeptidases (Chuang *et al.* 2016). Therefore, although polyP production is important for antibiotic tolerance, over-accumulation of polyP results in changes in physiology that compromise antibiotic tolerance, indicating the polyP levels must be tightly regulated. Together, the changes that result from polyP accumulation coordinate a program that favors persistence, stress resistance and antibiotic tolerance, which would be induced when (p)ppGpp inhibits PPX1 and PPX2 and polyP accumulates.

In addition to (p)ppGpp promoting polyP accumulation, polyP also promotes Rel_{Mtb} expression and the production of (p)ppGpp

Table 1. Summary of the phenotypes of polyP accumulating and polyP deficient *Mtb* strains. References are provided within the main text.

Phenotype	Δ*ppk1*	*ppx1*:Tn	*ppx2* knockdown	*ppk2*:Tn
polyP levels	Decreased	Increased	Increased	Increased
Growth in liquid culture	Decreased growth rate in late log and stationary phase	Decreased growth rate	Decreased cell density during stationary phase	Decreased cell density during stationary phase
Growth in macrophages	Decreased survival in macrophages	Decreased survival in macrophages	Increased survival in macrophages	Decreased survival in macrophages
Virulence	Decreased virulence in guinea pigs	Decreased virulence in guinea pigs	Unknown	Decreased virulence in mice
Biofilm formation	Unknown	Decreased biofilm formation	Decreased biofilm formation	Decreased biofilm formation
Antibiotic and Stress tolerance	Increased sensitivity to isoniazid, levofloxacin, rifampicin and nitrosative stress	Decreased sensitivity to isoniazid. Increased sensitivity to clofazimine and hypoxia	Decreased sensitivity to isoniazid, heat, cell surface and acid stress	Decreased sensitivity to isoniazid. Increased sensitivity to naphthoquinone plumbagin and meropenem
Metabolism	Unknown	Decreased levels of peptidoglycan and phospholipid metabolism intermediates. Increased levels of TCA cycle and arginine metabolism intermediates	Decreased levels of glycolysis, fatty acid metabolism, phospholipid metabolism and pentose phosphate pathway intermediates	Decreased levels of peptidoglycan and phospholipid metabolism intermediates. Increased levels of arginine metabolism intermediates

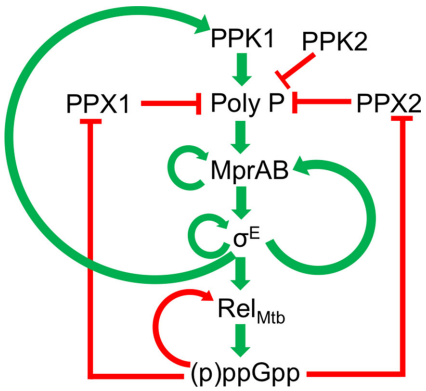

Figure 3. Signaling network involving Rel_{Mtb}, (p)ppGpp and polyP in *Mtb*. The network shows the proteins and molecules comprising the signaling cascades that positively and negatively regulate (p)ppGpp and polyP accumulation. Polyphosphate kinase 1 (PPK1), Polyphosphate kinase 2 (PPK2), Polyphosphatase 1 (PPX1) and Polyphosphatase 2 (PPX2) are shown. Green arrows indicate positive regulation. Red lines indicate negative regulation.

through a signaling cascade involving the two component system MprAB and the alternative σ-factor σ^E (Sureka *et al.* 2007) (Fig. 3). PolyP serves as a phosphodonor to the histidine kinase MprB, which then phosphorylates the response regulator MprA. Phosphorylated MprA activates expression of σ^E, which binds to a promoter sequence upstream of the rel_{Mtb} gene to activate transcription of rel_{Mtb} (He *et al.* 2006; Sureka *et al.* 2007). Accordingly, increasing intracellular polyP levels via mutation of *ppx1* also increased transcript levels of rel_{Mtb} (Thayil *et al.* 2011). However, the Δ*ppk1* mutation does not affect the expression of rel_{Mtb}, suggesting that polyP is not necessary for Rel_{Mtb} expression but can induce higher expression (Singh *et al.* 2013). This signaling cascade is amplified by several layers of positive feedback loops that rest on three molecular fulcrums: σ^E, MprA and (p)ppGpp. MprAB activates its own expression in addition to *sigE* (He and Zahrt 2005; He *et al.* 2006) and σ^E activates expression of *ppk1*, the *mprAB* operon, and itself (Manganelli *et al.* 2001; Sanyal *et al.* 2013), thereby positively regulating this signaling cascade. (p)ppGpp also positively regulates its own production by inhibiting polyphosphatases to promote polyP accumulation. Thus, crosstalk between the polyP metabolism and stringent response activation pathways results in synchronized promotion of polyP and (p)ppGpp production.

PolyP accumulation and the stringent response are both linked to *Mtb* persistence. The ability of *Mtb* to persist in the host is attributed to the formation of persister cells that exhibit decreased replication, altered metabolism, increased antibiotic tolerance and increased stress resistance (Gomez and McKinney 2004; Keren *et al.* 2011). Persister cell formation occurs through bacterial bistability, a strategy used by bacterial populations to maintain phenotypically heterogeneous subpopulations that protect the population from environmental insults (Dubnau and Losick 2006; Smits, Kuipers and Veening 2006). Cells transition between phenotypic states through stochastic changes in gene expression that are reinforced by feedback loops. In *E. coli*, a subpopulation of persister cells is maintained through phenotypic heterogeneity in *relA* expression (Balaban *et al.* 2004). Similarly, the *mprAB-sigE-rel* feedback loops underpin the maintenance of 'bistable' *rel* expression in mycobacteria (Sureka *et al.* 2008; Ghosh *et al.* 2011). When Sureka *et al.* sorted a population of *M. smegmatis* cells based on GFP expression from the rel_{Msm} promoter, they observed a bi-modal distribution of GFP expression with 'high' and 'low' subpopulations (Sureka *et al.* 2008). As the cultures of *M. smegmatis* approach stationary phase, the 'low' rel_{Msm}-expressing subpopulation declines and the 'high'

population increases. When the *mprAB-sigE-rel* feedback loop is disrupted by mutations to the σ^E binding site in the rel_{Msm} promoter, the 'high' subpopulation does not form (Sureka *et al.* 2008), suggesting that this loop is necessary for maintaining high rel_{Msm} expression. Although the existence of bistable rel_{Mtb} expression has not yet been examined in *Mtb*, the *mprAB-sigE-rel* feedback loop presents a model that connects the stringent response, polyP and mycobacterial persistence.

THE STRINGENT RESPONSE AND *Mtb* VIRULENCE GENE EXPRESSION

(p)ppGpp accumulates in *Mtb* during exposure to multiple stresses, including starvation, hypoxia and oxidative stress (Primm *et al.* 2000; Stallings *et al.* 2009), which are all conditions *Mtb* is exposed to during infection (Stallings and Glickman 2010). As a result, stress survival strategies and virulence mechanisms have converged such that activating the stringent response leads to expression of multiple virulence factors (Dahl *et al.* 2003; Dalebroux *et al.* 2010). A microarray study examining the effect of nutrient starvation on gene expression in WT *Mtb* versus the Δrel_{Mtb} mutant identified a Rel_{Mtb} regulon as the set of 159 genes whose expression changed significantly during starvation in WT bacteria but not in the Δrel_{Mtb} mutant (Dahl *et al.* 2003). Genes involved in lipid metabolism comprised a significant proportion of the Rel_{Mtb} regulon indicating that Rel_{Mtb} may coordinate changes in the production of *Mtb* lipids and lipoproteins that can play important protective and immunomodulatory roles during infection (Dahl *et al.* 2003; Cambier *et al.* 2014). With a few exceptions, the trend of gene expression suggests that Rel_{Mtb} may coordinate a shift from lipid anabolism to catabolism during starvation, which could represent the bacteria liberating nutrients from lipids but may also affect lipid modulators of virulence. Indeed, the Rel_{Mtb} regulon also included several polyketide synthases (PKS) and polyketide associated protein (Pap) genes that are required to synthesize complex lipids (Dahl *et al.* 2003; Gokhale *et al.* 2007). Notably, during starvation, Rel_{Mtb} upregulates *pks2* and *papA1*, which are responsible for synthesizing sulfolipid-1 (SL-1) (Dahl *et al.* 2003; Bhatt *et al.* 2007). SL-1 is the most abundant sulfolipid in the *Mtb* cell wall and purified SL-1 has been reported to have immunomodulatory effects (Pabst *et al.* 1988). PKS-synthesized surface glycolipids are also critical for biofilm formation *in vitro* (Pang *et al.* 2012; Cambier *et al.* 2014). Accordingly, loss of Rel_{Mtb}-mediated (p)ppGpp synthesis resulted in delayed formation of biofilms and pellicles in static liquid cultures (Weiss and Stallings 2013). The relevance of biofilm formation for *Mtb* pathogenesis is currently uncertain, but *Mtb* during chronic infection shares many characteristics with *Mtb* within biofilms, including decreased replication rates, and increased stress tolerance (Ojha *et al.* 2008; Richards and Ojha 2014). In addition, multiple groups have also reported the observation of extracellular communities of *Mtb* that resemble biofilms during infection (Lenaerts *et al.* 2007; Wong and Jacobs 2016).

The Δrel_{Mtb} mutant also exhibited lower expression of the *mce1*, *mce3* and *mce4* (mammalian cell entry) operons that have been associated with macrophage entry and are critical for *Mtb* infection (Zhang and Xie 2011) as well as lower expression of the major secreted antigen ESAT-6 (Dahl *et al.* 2003; Ganguly *et al.* 2007; Wang *et al.* 2009; Sreejit *et al.* 2014). Additionally, multiple genes belonging to the PE-PPE (Pro-Glu-rich) family of *Mtb* proteins were up- or downregulated in the Δrel_{Mtb} mutant (Dahl *et al.* 2003). PE-PPE proteins are either secreted or are cell surface proteins that are thought to contribute to *Mtb* virulence (Singh *et al.* 2016). Collectively, this data suggests that the *Mtb* stringent response has crosstalk with numerous virulence pathways. The connection between the stringent response and virulence suggests that *Mtb* may have coopted sensing changes in the host environment to activate virulence mechanisms and changes in metabolism to promote adaptation and persistence.

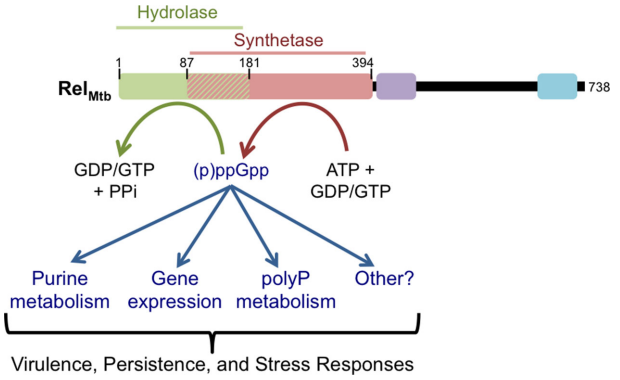

Figure 4. Summary of the effects of (p)ppGpp in *Mtb*. The RelMtb synthetase domain transfers the 5′-β,γ-pyrophosphate from ATP to the 3′-OH of GDP or GTP to synthesize (p)ppGpp, which can then be hydrolyzed by the RelMtb hydrolase domain. (p)ppGpp accumulates in response to a number of stresses, including starvation and oxidative stress, leading to alterations in purine metabolism, gene expression and polyP metabolism. It is also possible that there are other unidentified targets of (p)ppGpp in *Mtb*. These effects of (p)ppGpp accumulation contribute to enhanced virulence, persistence and stress responses.

TARGETING Rel_{Mtb} AS AN ANTI-VIRULENCE STRATEGY TO TREAT TUBERCULOSIS

There has been significant interest in identifying compounds that target (p)ppGpp synthesis as an anti-virulence and anti-persistence strategy in many pathogenic bacteria, including *Mtb* (Zhang, Yew and Barer 2012). Relacin, a 2′-deoxyguanosine-based analogue of ppGpp with the original pyrophosphate moieties at positions 5′ and 3′ replaced by glycyl-glycine dipeptides, was designed based on the Rel_{Seq} crystal structure and shown to inhibit *E. coli* RelA *in vitro* and (p)ppGpp synthesis in *B. subtilis* (Wexselblatt et al., 2010, 2012). However, Relacin is unable to penetrate the mycobacterial cell envelope to access its cytoplasmic target Rel_{Mtb}. More recently, Syal *et al.* designed and synthesized analogs of Relacin that had activity in mycobacteria by functionalization of the amine group at C-2 position of the guanine base in Relacin (Syal *et al.* 2017). These next generation Rel inhibitors were able to inhibit long-term survival and biofilm formation of mycobacteria. However, all compounds that have been designed to target the stringent response so far are analogs of ppGpp, which has limits in terms of improving potency and cell permeability. Therefore, the identification of new chemical matter will likely be important to translate (p)ppGpp synthesis inhibitors to clinical use.

SUMMARY AND OPEN QUESTIONS

A model has emerged where Rel_{Mtb}-synthesized (p)ppGpp alters gene expression profiles, purine metabolism and polyP metabolism to enhance Mtb stress responses, virulence and persistence (Fig. 4 and Table 2). In addition to the RNAP, GTP synthesis enzymes, and polyphosphatases, other molecular targets

Table 2. Molecular targets of (p)ppGpp that have been investigated in *Mycobacterium tuberculosis* (*Mtb*).

Molecular targets	Cellular process	Effects in *Mtb*	References
RNAP	Transcription	Inhibited at high [(p)ppGpp] *in vitro*	China *et al.* (2012), Tare, China and Nagaraja (2012) and Tare, Mallick and Nagaraja (2013)
GMK, Guanlyate Kinase	Purine metabolism	Insensitive to (p)ppGpp	Liu *et al.* (2015)
PPX1 and PPX2, Polyphosphatases	Polyphosphate metabolism	Inhibited by (p)ppGpp *in vitro*	Choi *et al.* (2012) and Chuang *et al.* (2015)

Table 3. Molecular targets of (p)ppGpp that have been identified in other bacteria but not yet explored in mycobacteria.

Cellular process	Molecular targets	References
DNA replication	DnaG	Wang, Sanders and Grossman (2007) and Maciag *et al.* (2010)
Translation	IF2, EF-G, EF-Tu, EF-Ts, Obg, RsgA	Legault, Jeantet and Gros (1972), Rojas *et al.* (1984) and Persky *et al.* (2009)
Nucleotide metabolism	AprT, HprT, GprT, Upp, GuaB, PurA, GdpP	Gallant, Irr and Cashel (1971), Hochstadt-Ozer and Cashel (1972), Fast and Sköld (1977), Pao and Dyess (1981) and Rao *et al.* (2010)
Amino acid metabolism	HisG, LdcI, LdcC, SpeC	Kanjee *et al.* (2011)
Lipid metabolism	PlsB, PgsA, AccA, AccD, FabA, FabZ,	Merlie and Pizer (1973), Polakis, Guchhait and Lane (1973) and Stein and Bloch (1976)
Central metabolism	GdhA, Pcc	Pao and Dyess (1981) and Maurizi and Rasulova (2002)
Glycogen biosynthesis	GlgC	Dietzler and Leckie (1977)

of (p)ppGpp have been identified in non-mycobacterial species, but have yet to be explored in mycobacteria (Table 3) (Kanjee, Ogata and Houry 2012; Hauryliuk *et al.* 2015; Liu, Bittner and Wang 2015). Many of these additional (p)ppGpp targets share a common theme of being GTPases or GTP-binding proteins with binding pockets for other guanine nucleotides that likely provide a natural binding site for (p)ppGpp. In addition to the GTPases or GTP-binding proteins, (p)ppGpp has been shown to target *E. coli* lipid and amino acid metabolism enzymes (Merlie and Pizer 1973; Polakis, Guchhait and Lane 1973; Stein and Bloch 1976; Heath, Jackowski and Rock 1994; Kanjee *et al.* 2011; Kanjee, Ogata and Houry 2012). Whether (p)ppGpp binds and modulates similar enzymes in *Mtb* remains an open question for further study, but it is possible that (p)ppGpp effects on *Mtb* physiology are much more complex than our current understanding. In particular, our current understanding of how the *Mtb* stringent response promotes pathogenesis is based on transcriptional responses that are lost in the Δrel_{mtb} mutant. Importantly, there exists a clear precedent for (p)ppGpp interacting with and affecting the activity of proteins post-translationally, so a purely transcriptome-level approach may miss key mechanistic details of the stringent response. Nonetheless, it is clear that by some mechanism (p)ppGpp metabolism is important for survival during stress, antibiotic treatment and virulence in animal models, highlighting the stringent response as a key mediator of *Mtb* pathogenesis.

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Conflict of interest. None declared.

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