The stringent response factors PPX1 and PPK2 play an important role in Mycobacterium tuberculosis metabolism, biofilm formation, and sensitivity to isoniazid in vivo.

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Abstract

*Mycobacterium tuberculosis* (Mtb) remains a global health threat largely due to the lengthy duration of curative antibiotic treatment, contributing to medical nonadherence and the emergence of drug resistance. This prolonged therapy is likely due to the presence of Mtb persisters, which exhibit antibiotic tolerance. Inorganic polyphosphate (poly(P)) is a key regulatory molecule in the Mtb stringent response mediating antibiotic tolerance. The polyphosphate kinase PPK1 is responsible for poly(P) synthesis in Mtb, while the exopolyphosphatases PPX1 and PPX2, and the GTP synthase PPK2 are responsible for poly(P) hydrolysis. In the present study, we show by LC-MS/MS that poly(P)-accumulating Mtb mutant strains deficient in *ppx1* or *ppk2* had significantly lower intracellular levels of glycerol-3-phosphate (G3P) and 1-deoxy-xyulose-5-phosphate. RT-PCR revealed decreased expression of genes in the G3P synthesis pathway in each mutant. The *ppx1*-deficient mutant also showed significant accumulation of metabolites in the tricarboxylic acid cycle, as well as altered arginine and NADH metabolism. Each poly(P)-accumulating strain showed defective biofilm formation, while deficiency of *ppk2* was associated with increased sensitivity to plumbagin and meropenem, and deficiency of *ppx1* led to enhanced susceptibility to clofazimine. A DNA vaccine expressing *ppx1* and *ppk2*, together with two other members of the Mtb stringent response, *rel*Mtb and *sigE*, did not show protective activity against aerosol challenge with Mtb, but vaccine-induced immunity enhanced the killing activity of isoniazid in a murine model of chronic TB. In summary, poly(P)-regulating factors of the Mtb stringent response plays an important role in Mtb metabolism, biofilm formation, and antibiotic sensitivity *in vivo*.

Key words: inorganic polyphosphate, polyphosphate kinase, exopolyphosphatase, therapeutic vaccine, isoniazid, persistence, antibiotic tolerance, biofilm, metabolomics, host-directed therapy
**Introduction**

*Mycobacterium tuberculosis* (Mtb) remains a major threat to global public health (1). The primary obstacles to eradicating infection are the need for combination antibiotic treatment and the prolonged duration of treatment, which is believed to be due to the presence of replication-deficient, antibiotic-tolerant persistent bacteria or “persisters” (2). An improved understanding of the regulatory pathways underlying persister formation is paramount to the development of novel strategies to more effectively target these persisters, thereby shortening the duration of TB treatment.

The stringent response regulatory molecules, inorganic polyphosphate (poly(P)) and hyperphosphorylated guanosine ((p)ppGpp), have been shown to mediate persister formation in bacteria (3-5). Although the mechanism remains unclear, (p)ppGpp induces toxin-antitoxin systems, leading to increased numbers of persisters in *E. coli* (5, 6). Mtb expresses two polyphosphate kinases (PPK1, PPK2) and two exopolyphosphatases (PPX1, PPX2) to regulate intracellular poly(P) homeostasis (7-10). PPK1 synthesizes poly(P) through hydrolysis of ATP (4). Although PPK2 enzymes can synthesize poly(P), Mtb PPK2 has nucleoside diphosphate kinase A-like activity (11), catalyzing poly(P) hydrolysis and ATP synthesis 800-fold faster than poly(P) synthesis (12). The mycobacterial stringent response appears to be a positive feedback loop, as poly(P) phosphorylates and activates the two-component system MprAB, which induces expression of *sigE* and *relMtb* (13), leading to increased synthesis of (p)ppGpp, which inhibits the hydrolysis of poly(P) by PPX2 (10). Poly(P) accumulation is associated with antibiotic tolerance (9-11), and poly(P) deficiency renders Mtb more sensitive to antibiotics (14). In addition, poly(P) homeostasis is required for Mtb survival during acute infection in murine lungs (15) and during chronic infection in guinea pig lungs (11, 14). Previously, we found that an Mtb *ppx2* (*Rv1026*)
knock-down strain showed global transcriptional changes, including reduced expression of genes encoding enzymes involved in glycerol-3-phosphate synthesis (10). This recombinant strain also displayed altered cell wall thickness and permeability, and tolerance to the cell wall-active agent, isoniazid. However, it is unknown whether these profound metabolic and phenotypic changes were a specific consequence of ppx2 deficiency or more generally related to poly(P) accumulation.

In the current study, we studied the metabolome and gene expression of two poly(P)-accumulating strains containing transposon insertions in the ppx1 (MT0516) gene or the ppk2 gene (ppx1::Tn and ppk2::Tn, respectively) (9, 15). In addition, we determined the susceptibility of each strain to various antibiotics and other stresses, as well as their ability to develop biofilms, a potentially important property of persistent Mtb infection (16, 17). Finally, in order to investigate the role of the Mtb stringent response pathway in bacillary persistence during antibiotic treatment of chronic murine TB infection, we evaluated the therapeutic efficacy of an adjuvant DNA vaccine expressing ppx1, ppk2, as well as sigE and relMtb, two other members of the positive-feedback cascade regulating Mtb intracellular poly(P) homeostasis. Our results suggest that enhanced immunity targeting the Mtb stringent response pathway promotes host control of mycobacteria during antibiotic treatment in mice.

Material and Methods

Bacteria and growth conditions

Wild-type M. tuberculosis (Mtb) CDC1551 strains deficient in ppx1/MT0516 (ppx1::Tn) and ppk2/Rv3232c (ppk2::Tn) were generated by transposon mutagenesis, and the respective
complement strains, *ppx1::Tn Comp* and *ppk2::Tn Comp*, were generated and genetically and phenotypically confirmed as previously described (9, 15). All strains, including wild-type *M. tuberculosis* (Mtb) CDC1551, were grown in Middlebrook 7H9 broth (Difco, Sparks, MD) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC, Difco), 0.1% glycerol and 0.05% Tween-80 at 37°C on a roller.

**Metabolomics analysis**

Sample preparation and analysis were performed as previously described (10, 18). Briefly, mid-logarithmically growing cultures (optical density (O.D.) = 0.5) of the *ppx1::Tn*, *ppk2::Tn*, and wild-type strains were pelleted and the samples were extracted in 1 ml of extraction buffer (chloroform: methanol, 2:1) and then concentrated by centrifugal evaporation. The samples were processed and analyzed by ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) and gas chromatography/mass spectrometry (GC/MS) by Metabolon, Inc (Durham, NC) (19, 20). Statistical analysis of log-transformed data was performed (10) and Welch’s t-tests were performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values (21).

**Sensitivity to plumbagin, meropenem and clofazimine by disc diffusion**

Each Mtb strain was grown in enriched 7H9 broth until mid-log growth phase and 10^7 bacteria were plated on Middlebrook 7H10 plates. Plumbagin (10µl of 20mM or 100mM, dissolved in ethanol), meropenem (10µg/ml or 20µg/ml, dissolved in water), or clofazimine
(10µg/ml, dissolved in DMSO) was added to separate sterile disks, which were placed on plates individually. The zone of growth inhibition was measured after 10 days of incubation and each drug concentration was tested for each strain in triplicate (22).

**Biofilm formation assay and crystal violet stain**

Crystal violet staining was performed (23) with minor modifications. Briefly, mid-log phase cultures (0.5 ml, density= 10⁶/ml) were grown in 24-well conical-bottom plates with in Sauton's medium without detergent and shaking for 5 weeks. The extracellular matrix of biofilm was measured by crystal violet stain using FLUOstar OPTIMA (BMG LABTECH).

**RT-PCR**

Realtime PCR was performed as previous described (15). Gene-specific primers are listed in Table S2.

**Ethidium bromide accumulation/efflux assay and Nile red uptake assays**

The ethidium bromide accumulation and efflux assays were measured by florescence intensity (10). For Nile red uptake staining, mid-log phase cultures were washed with PBS and then stained with 20 µM Nile red (Sigma) (24). In all assays, the cells were incubated in 96-well plates and analysis was performed at the indicated time point by excitation at 544 nm and
emission at 590 nm by FLUOstar OPTIMA (BMG LABTECH). All data were normalized to the time zero reading of each well.

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**Antigen preparation**

The open reading frames of the genes *ppx1*, *ppk2* and *sigE* were amplified from *M. tuberculosis* CDC1551 and cloned individually into plasmid pET28a using the restriction enzymes Ndel and BamHI (Table S3). The previously generated *relMtb* expression plasmid, pET15b[relMtb](25), was used for expression of RelMtb protein. The resulting plasmids were used to transform *E. coli BL21* (DE3) RP competent cells (Stratagene). The transformed bacteria were selected by ampicillin (100 µg/ml), and cloning was confirmed by DNA sequencing. The expression and purification of protein were performed using standard protocols (Qiagen).

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**DNA vaccination of mice**

The plasmid pSectag2B was used to express individual genes, *ppx1*, *ppk2*, *relMtb* and *sigE*. Briefly, each gene was cloned individually into pSectag2B using the restriction enzymes BamHI and HindIII (Table S3). The insertion was confirmed by sequencing and the expression of target genes was confirmed by transfection of 293T cells. Each DNA vaccine was delivered as previously described (26, 27) and all procedures were performed according to protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Briefly, each plasmid was delivered by intramuscular injection into the quadriceps femoris muscle of mice, followed by local electroporation using the ECM830 Square Wave...
Electroporation System (BTX Harvard Apparatus company, Holliston, MA, USA). Each of the two-needle array electrodes delivered fifteen pulses of 72 V (20-ms pulse duration at 200 ms intervals). 10µg of each plasmid was injected into each hind leg once weekly for three weeks. One week after the last vaccination, the mice were scarified and the blood and splenocytes were collected (27).

**Enzyme-linked immunosorbent assay**

Antigen-specific antibody responses were measured by ELISA as described previously (28), with minor modifications in coating and sera incubation. The 96-well microplate was coated with 1µg/ml of purified RelMtb or SigE protein overnight. For PPK2 or PPX1, the coating concentration was 5µg/ml. After blocking, sera from vaccinated mice were diluted 1:100 with PBS, added to wells and incubated at room temperature for 2 hours.

**Intracellular cytokine stain and flow cytometry analysis**

To detect antigen-specific CD4+ T-cell responses by IFN-γ intracellular staining, splenocytes were stimulated individually with the purified recombinant proteins, PPK2, PPX1, RelMtb or SigE (10µg/ml) for 24 hours at 37°C before addition of GolgiPlug (BD Pharmingen, San Diego, CA) overnight. After incubation, the splenocytes were washed once with FACScan buffer and then stained with PE-conjugated monoclonal rat anti-mouse CD4. Cells were permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA). Intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ and APC-conjugated rat anti-
mouse TNFα (BD Pharmingen, San Diego, CA). Flow cytometry was performed by FACSCalibur and analyzed with FlowJo software.

**Aerosol infection of mice with M. tuberculosis and DNA vaccine study endpoints**

The stringent response (SR) vaccine, containing 5µg each of plasmids expressing *ppx1, ppk2, relMtb* or *sigE* or the empty vector, pSectag2B, was delivered as described above once weekly for four weeks. Three weeks after the last vaccine dose, mice were aerosol-infected with ~100 bacilli of wild-type Mtb CDC1551. Mice were sacrificed on Days 14 and 56 after aerosol challenge and lungs were homogenized and plated for CFU (15) to evaluate the protective efficacy of the SR vaccine. Separate groups of naïve, vaccinated, and sham-vaccinated mice were treated with isoniazid (10mg/kg) by esophageal gavage once daily (5 days/week) for 4 weeks beginning on Day 14 after aerosol challenge. The lungs were homogenized and plated after 56 days of isoniazid treatment to determine CFU. A partial sample of each infected lung was fixed with 10% buffered formaldehyde, processed, and paraffin-embedded for histological staining with hematoxylin and eosin staining. Morphometric analysis of histology was performed as previously described (29).

**Statistical analysis**

Data from at least three biological replicates were used to calculate means and standard deviation (SD) for graphing purposes. Statistical analysis employed the unpaired Student’s *t* test, and a *p*-value of < 0.05 was considered statistically significant.
Results

Polyphosphate accumulation alters the metabolic profile of Mtb

Previously, we have shown that Mtb mutant strains deficient in *ppk2* or *ppx1* accumulate poly(P) relative to the isogenic wild-type strain (9, 15). The *ppx1* mutant showed higher gene expression of *relMtb*, *sigE* and *mprB* by RT-PCR during mid-log phase growth (9), while the *ppk2*-deficient mutant showed higher expression of *sigE* (-change in Ct ($\Delta$Ct) = 1.26; p=0.035) but lower expression of *mprB* (-$\Delta$Ct = -0.75; p=0.017 ) and unchanged expression of *relMtb* (-$\Delta$Ct = -0.25; p=0.4 ) compared to the isogenic wild-type strain during late-log phase growth after normalization with *sigA* expression. We used metabolomics to further characterize the physiological changes in these two poly(P)-accumulating mutants (Table S1). In addition to the accumulation of poly(P) and pyrophosphate, both strains had significantly lower levels of glycerol 3-phosphate (G3P) and glycerol 2-phosphate (G2P) (Fig 1A). The peptidoglycan biosynthetic metabolite, 1-deoxy-xyulose-5-phosphate (DXP), which is required for the synthesis of isoprenoid (30), was also less abundant in the mutant strains (Fig1B). These changes suggest that peptidoglycan synthesis may be affected by poly(P) accumulation. NAD is a cofactor utilized in several redox reactions that are vital to metabolism. In comparison to the wild type, the levels of the oxidized form of the metabolite, NAD$^+$, were increased in *ppx1::Tn* relative to wild-type bacilli (Fig 1C). The level of nicotinate ribonucleoside, an intermediate in the salvage pathway of NAD, was significantly reduced in both *ppk2::Tn* and *ppx1::Tn*. The level of nicotinic acid mononucleotide (NaMN), an intermediate in the *de novo* synthesis pathway of NAD, was relatively lower in both strains. These findings suggest that the intracellular NAD pool may be regenerated by salvage pathways in these mutant bacteria.
Lipid oxidation and citrate cycle activity increase during polyphosphate accumulation in the *ppk2* and *ppx1* mutant strains

*ppx1::Tn* showed increased abundance of several metabolites indicative of increased lipid oxidation, including acetyl-CoA, propionyl-CoA, and 2-methylcitrate (Table S1). Acetyl-CoA can be formed from the oxidation of even-chain fatty acids and propionyl-CoA is often generated from the oxidation of odd-chain fatty acids. Both acetyl-CoA and propionyl-CoA can enter the TCA cycle to produce precursors for amino acids, lipids, and energy. When propionyl-CoA enters the TCA cycle, the metabolite 2-methylcitrate is generated. In addition to acetyl-CoA and 2-methylcitrate, several other TCA cycle intermediates were elevated in *ppx1::Tn*, including malate and succinate (Fig 1D). An increase in TCA cycle intermediates was also observed in *ppk2::Tn*, possibly reflecting a need to generate greater amounts of metabolites involved in energy production, such as NADH.

Several amino acid metabolites were more abundant in the poly(P)-accumulating strains, especially in *ppx1::Tn* (Table S1). The arginine deiminase pathway provides a mechanism by which ATP can be generated from the metabolism of arginine. In this pathway, arginine is converted to citrulline, which is then converted to ornithine and carbamoyl phosphate. ATP can be generated when carbamoyl phosphate is dephosphorylated by carbamate kinase (31, 32). This pathway is known to be particularly active in response to environmental stress (33, 34) and is of special interest since poly(P) accumulation is associated with bacterial stress resistance. The levels of arginine, citrulline, and ornithine were higher in *ppk2::Tn* and *ppx1::Tn* relative to wild type (Fig 1E). The increase in the intermediates of this pathway may reflect an increased
demand for energy and could serve as a potential mechanism by which these strains resist different stresses.

*Mtb polyphosphate accumulation is associated with altered expression of G3P-related genes*

Based on our previous report (10) and the current study, Mtb poly(P) accumulation is associated with significantly decreased levels of G3P. This may be due to either decreased *de novo* synthesis of G3P or increased turnover. Several Mtb genes encode enzymes that regulate G3P homeostasis (35). Using RT-PCR, we analyzed the expression levels of these genes in the *ppk2* and *ppx1* mutant strains (Fig 2). The genes encoding proteins responsible for G3P synthesis from dihydroxyacetone phosphate (*Rv0564c*) or hydrolysis (*glpD1, glpD2*) were both significantly down-regulated in *ppx1*::Tn compared to wild type. Among the genes involved in recycling the glycerophospholipid polar head, *Rv2182c* was significantly down-regulated in both mutant strains. The gene encoding the enzyme responsible for synthesis of G3P from glycerol (*glpK*) was down-regulated in *ppx1*::Tn, while the gene encoding the enzyme responsible for G3P hydrolysis was upregulated in *ppk2*::Tn. Thus, poly(P) accumulation associated with deficiency of *ppk2* or *ppx1* led to altered expression of genes involved in G3P homeostasis, contributing to reduced intracellular G3P content.

*Poly(P) homeostasis is required for Mtb biofilm formation*

We found that biofilm formation was impaired in a poly(P)-accumulating strain deficient in the exopolyphosphatase *ppx2* gene (10). However, it is unclear if poly(P) homeostasis is
generally required for biofilm formation, or if this phenomenon was related specifically to PPX2
deficiency. The mutants ppx1::Tn and ppx2::Tn and their respective complemented strains were
grown in Sauton’s medium for 5-6 weeks. As shown in Figure 3, neither of the two mutant
strains formed pellicle biofilms at the air-fluid interface. The pellicle formed by the ppx2::Tn
complemented strain was less dense than that formed by the wild type and the density of the
pellicle formed by the ppx1::Tn complement was similar to that of the wild type. Crystal violet
staining corroborated the finding that Mtb poly(P) accumulation is associated with defective
biofilm formation. It is unlikely that these results can be explained by defective growth in the
mutants, since these differences persisted even after prolonged observation of cultures till 8th
week (data not shown).

**Deficiency of ppx2 leads to increased sensitivity to plumbagin and meropenem, while ppx1
deficiency increases sensitivity to clofazimine**

Previous studies have shown that meropenem inhibits both the D,D-carboxypeptidase and
L,D-transpeptidase of Mtb (36). L,D-transpeptidase plays an important role in cell wall
remodeling during mycobacterial entry into stationary phase (37). We studied the sensitivity of
ppx2::Tn and ppx1::Tn to meropenem by the disc diffusion method (Table 1). ppx2::Tn showed
increased sensitivity to meropenem 10µg/ml and 20µg/ml, and the ppx2::Tn complemented
strain partially restored the wild-type sensitivity pattern. Similarly, ppx1::Tn showed similar
trends in increased susceptibility to meropenem, although this did not reach statistical
significance. Next, we tested whether poly(P) accumulation altered mycobacterial resistance to
oxidative stress (38). ppx2::Tn was more sensitive to the naphthoquinone plumbagin and the
complemented strain restored wild-type resistance. Similarly, *ppx1::Tn* showed mildly increased sensitivity to plumbagin compared to wild type (*p* = 0.1). We then tested the effect of poly(P) accumulation on sensitivity to clofazimine, which destabilizes the bacterial membrane and alters redox cycling (39). Although *ppk2* deficiency did not alter clofazimine susceptibility, *ppx1::Tn* was more sensitive to clofazimine (*p* = 0.006) and the complemented strain restored the wild-type levels of susceptibility.

Deficiency of *ppx1* or *ppk2* alters expression of peptidoglycan biosynthesis genes but does not alter cell wall permeability

*ppk2::Tn* and *ppx1::Tn* showed increased susceptibility to meropenem, suggesting potential changes in the mycobacterial cell wall structure associated with poly(P) accumulation. Our metabolomics analysis revealed decreased abundance of 1-Deoxy-D-xylulose-5-phosphate in both mutants. To investigate the possibility that poly(P) accumulation leads to altered peptidoglycan biosynthesis, we used RT-PCR to evaluate the expression of relevant genes (40). The genes encoding L,D-transpeptidase, *ldtA* and *ldtB*, were down-regulated in both mutant strains (41). The *ppk2* mutant showed higher expression of *phpA*, while the *ppx1* mutant showed lower expression relative to wild type (Fig 3C).

During macrophage infection, Mtb can acquire phenotypic tolerance to antibiotics through induction of efflux pumps (42). Previously, we found that a poly(P)-accumulating strain (*ppx2* knock-down) altered cell wall permeability based on ethidium bromide uptake assay (10). In the current study, we did not observe any significant difference in ethidium bromide accumulation or efflux pump activity between each mutant and the respective complement and
wild-type strains (data not shown). For further evaluation of cell wall permeability, we used Nile red staining to determine whether deficiency of *ppx1* or *ppk2* was associated with altered diffusion rates of lipophilic molecules across the Mtb cell wall (24). Nile red staining was equivalent between *ppx1::Tn* and wild type, but *ppk2::Tn* mutant showed reduced Nile red accumulation relative to the wild type (Fig 4). The *ppk2* complemented strain showed similar uptake of Nile red as compared to the wild type.

**DNA vaccination with poly(P)-regulatory genes of the Mtb stringent response pathway**

*generates antigen-specific CD4+ T-cell responses and immunoglobulin*

Although previous reports have highlighted the importance of poly(P)-regulatory genes in antibiotic tolerance and persistence in animal lungs (9, 15), it remains to be determined whether the stringent response pathway can be therapeutically targeted during chronic Mtb infection. To test this possibility, we first sought to determine whether specific immunity could be engendered to specific poly(P)-regulatory factors by DNA vaccination. We cloned four poly(P)-regulatory genes, *relMtb*, *sigE*, *ppk2*, and *ppx*, individually into the eukaryotic expression plasmid, pSectag2b. Separate groups of C57BL/6J mice were vaccinated intramuscularly with each individual plasmid or empty vector once weekly for three weeks and electroporation was performed immediately following each injection (Fig 5A). Significant IgG responses to each antigen were detected by ELISA in the serum of vaccinated mice one week after the last DNA vaccine dose (Fig 5B). Next, splenocytes from vaccinated mice were collected and stimulated with each recombinant protein (10 µg/ml) for 24 hours. DNA vaccination with each poly(P)-regulatory gene generated significant antigen-specific IFNγ-producing CD4+ T cells compared...
with the vector control (Fig 5C). Only DNA vaccination with \( \text{rel}\text{Mtb} \) generated significant antigen-specific TNF-producing CD4\(^+\) T cells compared with the vector control.

**Immunity engendered by DNA vaccination with poly(P)-regulatory genes enhances the bactericidal activity of isoniazid in mice**

To address the potential preventive and therapeutic efficacy of immunity induced by \( \text{rel}\text{Mtb}, \text{sigE}, \text{ppk}2, \) and \( \text{ppx}1 \), we combined the four individual DNA plasmids expressing each of these poly(P)-regulatory genes, yielding the “stringent-response” DNA vaccine (SR vaccine). C57BL/6J mice were vaccinated either with SR vaccine (5\( \mu \)g of each plasmid) or empty vector control (20 \( \mu \)g) once weekly for four weeks (Fig 6A). These mice were aerosol-challenged with the virulent Mtb strain CDC1551 three weeks after the last vaccine dose. The lung bacillary burden was not significantly different between vaccinated mice and naïve mice 14 days and 42 days after aerosol challenge (Fig 6B). A separate group of mice received human-equivalent doses of isoniazid once daily (5 days/week) by esophageal gavage beginning on Day 14 after aerosol challenge (43). Following 28 days of treatment with isoniazid, we observed a significant (\( p=0.013 \)) reduction in lung CFU in the SR-vaccinated group relative to the sham vaccine group (Fig 6C). Analysis of lung histology revealed pulmonary inflammation, which was commensurate with the lung bacillary burden (Fig S2). As far as we are aware, this is the first study to show that immunity to poly(P)-regulatory factors of the stringent response pathway enhances the bactericidal activity of the first-line drug isoniazid.

**Discussion**
The bacterial stringent response appears to play a key role in antibiotic tolerance (2, 44). Poly(P) may serve as a phosphate donor, activating downstream stringent response genes, including *mprAB, sigE,* and *relMtb,* thereby leading to antibiotic tolerance in *Mtb* (7, 9, 10, 15, 45). Previously, we found that the *ppk2*-deficient mutant showed attenuated growth during the acute phase of infection in the murine model but did not show reduced persistence beyond Day 56 after infection (15). The *ppx1*-deficient mutant showed a persistence defect in the guinea pig model (9). Similarly, when the *ppk2* gene was disrupted by a mycobacteriophage, the resulting *ppk2*-deficient *Mtb* strain showed reduced persistence in guinea pigs (11). Our metabolomics analysis revealed that poly(P) accumulation alters the intracellular G3P content in *Mtb* (Fig 1). Transcriptional analysis of both mutant strains showed reduced expression of genes involved in G3P synthesis and recycling (Fig 2). Importantly, G3P can be used as a scaffold for phospholipid biosynthesis (46). Overexpression of glycerol-3-phosphate dehydrogenase in *E. coli* leads to reduced intracellular G3P levels and increased formation of persister cells following exposure to antibiotics (47). G3P and G2P levels are reduced in *Mtb* when cholesterol is the sole carbon source (48). During chronic TB infection of mice, the use of cholesterol is important for bacillary survival (49). Cholesterol accumulation alters *Mtb* cell wall permeability to rifampin and masks cell wall antigen from binding antibody (50). We have shown previously that a poly(P)-accumulating strain (*ppx2* knock-down) exhibited significant down-regulation of the G3P dehydrogenase gene, *glpD2* (10), which is also down-regulated in *Mtb* persisters (10, 51). Taken together, these findings suggest that poly(P) serves an important role in down-regulating G3P synthesis, which may promote persister formation and antibiotic tolerance (52).

The role of the arginine deiminase pathway during chronic bacterial infections remains controversial. Previous studies have reported the importance of this pathway during
microaerobic survival of *Pseudomonas aeruginosa* in the airways of patients with cystic fibrosis (53). L-arginine deiminase degradation may serve as an alternative carbohydrate utilization pathway during chronic infection in *P. aeruginosa* (54). Infection with *M. bovis* BCG leads to enhanced arginine uptake by IFN-γ-stimulated macrophages (55). However, Mtb deficient in the arginine deiminase gene (*arcA*) did not show defective survival during chronic murine infection (31). Our data raise the possibility that poly(P) accumulation leads to upregulation of the arginine deiminase pathway, perhaps reflecting a switch to alternative energy sources.

Remodeling of the TCA cycle, including increased production of succinate and decreased production of α-ketoglutarate, has been observed during Mtb adaptation to hypoxia and following bacillary exposure to antibiotics (56, 57). In *E. coli*, citrate and succinate were found to accumulate during exposure to bactericidal antibiotics (58). In the current study, we found that deficiency of *ppx1* was associated with significantly increased abundance of succinate and malate. These trends also were observed during *ppk2* deficiency, although they did not achieve statistical significance. Our data suggest that poly(P) accumulation leads to remodeling of the TCA cycle, although the mechanisms by which these metabolic changes are regulated and how they contribute to antibiotic tolerance remain to be elucidated.

Prior work has focused on the role of poly(P) as a molecular chaperone for stabilizing proteins (59). In the current study, we found accumulation of several amino acid metabolites in the two poly(P)-accumulating strains, particularly in *ppx1::Tn*. These findings differ from those of a previously reported poly(P)-accumulating strain deficient in *ppx2* (10). This discrepancy may be explained potentially by differences in the function of the two Mtb exopolyphosphatases, PPX1 and PPX2. Specifically, PPX1 hydrolyzes short-chain poly(P) (60), while PPX2 hydrolyzes predominantly long-chain poly(P) (10). The length of individual molecules may...
dictate the function of poly(P) (59). In addition, these mutant strains were generated using distinct genetic techniques. The previously reported ppx2-deficient mutant was generated using a Tet-inducible system to overexpress the antisense version of the ppx2 gene, thereby knocking down its expression, whereas the ppx1-deficient mutant used in the current study contains a transposon insertion at bp 732 (total gene length, 1035bp), likely disrupting ppx1 function. Further studies are needed to elucidate the role of short-chain and long-chain poly(P) on regulating individual Mtb metabolic processes.

Poly(P) homeostasis appears to be required for the formation of biofilms. Thus, poly(P) accumulation in Campylobacter jejuni (61) and P. aeruginosa (62) was associated with defective biofilm formation. Furthermore, deficiency of a polyphosphate kinase responsible for poly(P) synthesis in P. aeruginosa also resulted in defective biofilms (63). Null mutants of PPK and PPX in Bacillus cereus showed similar defects in biofilm formation (64). Consistent with these findings in other bacteria, we have shown previously (10) and in the current study that maintenance of intracellular poly(P) balance is required for biofilm formation in Mtb. Interestingly, a recent study found that ppk2 deficiency did not alter biofilm formation in Mtb (11). Notably, the parental strain used to generate the mutant in our study is CDC1551. This strain has been shown previously to form less biofilm relative to H37Rv (23), which was used by Singh et al. Furthermore, the latter study used homologous recombination with temperature-sensitive mycobacteriophages to delete the ppk2 open reading frame, while our study used transposon mutagenesis to disrupt ppk2. Relatively little is known about the mechanism by which poly(P) levels modulate biofilm formation. In E. coli, poly(P) hydrolysis during stationary phase appears to trigger the formation of biofilms via the LuxS quorum sensing
Intracellular accumulation of poly(P) has been linked to Mtb growth restriction and tolerance to the bactericidal drug, isoniazid (9, 15). Our metabolomics analysis revealed alterations in cell wall lipid composition and DXP during poly(P) accumulation. By RT-PCR, both mutants showed reduced expression of the L,D-transpeptidase genes, ldtA and ldtB, which play an important role in peptidoglycan formation of non-replicating mycobacteria (41). Unlike the ppx1-deficient mutant, the ppk2-deficient mutant showed increased sensitivity to the L,D-transpeptidase inhibitor, meropenem (36), as well as increased expression of pbpA. Further studies are required to understand the role of poly(P) accumulation in peptidoglycan synthesis.

The respiration and electron transport pathways are required for the transition of Mtb into persistence and antibiotic tolerance (66, 67). We used the oxidative agent, plumbagin, to determine if poly(P) accumulation alters sensitivity to inhibitors of respiratory electron transport (38, 68). ppk2::Tn was more sensitive to plumbagin, suggesting that ppk2 deficiency shifts bacteria to a greater dependence on the TCA cycle and the electron transport chain (69). Furthermore, the ppx1-deficient mutant showed increased susceptibility to clofazimine, which destabilizes the bacterial membrane and targets the redox cycling pathway by enzymatic reduction of the drug by NDH-2, the primary respiratory chain NADH:quinone oxidoreductase of mycobacteria, and nonenzymatic oxidation of reduced clofazimine by O2, yielding reactive oxygen species (39, 70). Future studies will address whether the discrepancies in antibiotic sensitivity between these two poly(P)-accumulating strains result from differences in the enzymatic function of PPX1 and PPK2.
Recently, there is significant interest in host-directed therapy to treat TB (71). Although BCG vaccination does not enhance the bactericidal activity of chemotherapy in the murine model (72), a DNA vaccine expressing heat shock protein 65 has been shown to synergize with conventional antitubercular drugs, further reducing the bacterial burden in the lungs of Mtb-infected mice or non-human primates (72, 73). A fragment whole cell lysate therapeutic vaccine, RUTI, has shown efficacy in generating protective immunity in pre-clinical studies (74, 75). However, the primary factors responsible for TB immunity remain unknown. In the current study, we have shown that a DNA vaccine targeting key poly(P)-regulatory factors of the Mtb stringent response generates IgG and antigen-specific CD4+ T cells, but these immune responses did not offer protection against Mtb challenge in the murine model. However, we observed a significant reduction in lung bacillary burden when immunity to these Mtb stringent response factors was combined with isoniazid. One potential explanation for this phenomenon is that the population of persistent bacilli in untreated, chronically infected mice is relatively small (76), but exposure to isoniazid further induces the formation of persisters (77, 78). In favor of this hypothesis, isoniazid exposure induces Mtb expression of \textit{relMtb}, which are required for persister formation (2, 44, 79). Further studies are required to determine the utility of the SR vaccine as a therapeutic vaccine in shortening the duration of treatment in combination with the standard antitubercular regimen or for treating latent TB infection.

In summary, poly(P) accumulation appears to induce changes in Mtb metabolism, resulting in altered susceptibility to oxidative stresses and antibiotic exposure, and defective biofilm formation. Poly(P) chain length may play an important role in these processes, since we observed several differences between the \textit{ppk2}-deficient and \textit{ppx1}-deficient mutants. Immunity targeting key poly(P)-regulatory factors of the Mtb stringent response augments the
tuberculocidal activity of isoniazid in mouse lungs. Future studies will focus on the utility of the
stringent response as a potential new target in host-directed therapy for TB.
Funding

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Transparency declarations

None to declare.

Disclaimer

The funding sources had no role in the study design, data collection, data analysis, data interpretation or writing of the report.
Table 1. Sensitivity of each strain to plumbagin, meropenem, and clofazimine by the disc-diffusion method.

<table>
<thead>
<tr>
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<th>Wild type</th>
<th>ppk2::Tn</th>
<th>ppk2::Tn Comp</th>
<th>ppx1::Tn</th>
<th>ppx1::Tn Comp</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>Disc zone (mm)</td>
<td>Disc zone (mm)</td>
<td>Disc zone (mm)</td>
<td>Disc zone (mm)</td>
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<tr>
<td>Plumbagin</td>
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<td>0.042</td>
<td>24.3±0.6</td>
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<tr>
<td>100mM</td>
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<td>24±2</td>
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<td>20.6±1.2</td>
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<tr>
<td>Meropenem</td>
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<td>29.3±1.2</td>
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<td>31.3±3.1</td>
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<td>10µg/ml</td>
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<td>0.820</td>
<td>17.3±1.2</td>
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<tr>
<td>20µg/ml</td>
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<td>0.820</td>
<td>17.3±1.2</td>
<td>0.251</td>
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Figure Legends

Figure 1. Polyphosphate accumulation alters Mtb metabolism. Metabolites altered in the Mtb ppk2-deficient and ppx1-deficient mutants relative to the wild-type strain include those belonging to the following categories: A. Phosphate compounds; B. Cell wall synthesis pathways; C. NADH metabolism; D. TCA cycle; E. Arginine metabolism. ppk2=ppk2::Tn, ppx1=ppx1::Tn. n=5, *p< 0.05 compared to wild type.

Figure 2. RT-PCR of Mtb genes related to glycerol 3-phosphate (G3P) metabolism during poly(P) accumulation. The cycle threshold (Ct) of each gene was normalized to that of the housekeeping gene sigA in each strain, and this value was subtracted from the similarly normalized Ct of each gene in the wild-type strain to yield the change in Ct (ΔCt). Open bars represent ppk2::Tn and gray bars represent ppx1::Tn. Error bars represent S.D. from at least three replicates. NS= no significant difference.

Figure 3. Polyphosphate accumulation alters expression of peptidoglycan biosynthesis genes and reduces biofilm formation. ppx1::Tn (A) and ppk2::Tn (B) strains were incubated in Sauton's medium lacking detergent for 5 weeks and biofilms were assessed by Crystal violet staining (*p< 0.05 relative to wild type; n=3). C. RT-PCR of Mtb genes related to peptidoglycan biosynthesis during mid-log phase of growth. The cycle threshold (Ct) of each gene was normalized to that of the housekeeping gene sigA in each strain, and this value was subtracted from the similarly normalized Ct of each gene in the wild-type strain to yield the change in Ct (ΔCt). Open bars represent ppk2::Tn and gray bars represent ppx1::Tn. Error bars represent S.D.

Figure 4. Decreased Nile red staining in the ppk2-deficient mutant. Each strain was grown to mid-log phase and stained with 20 μM Nile red. The fluorescence intensity was normalized to
Figure 5. Immunogenicity of DNA vaccines targeting poly(P)-regulatory genes. A. 6-8 week-old female C57BL/6J mice (n=3 or 4) were vaccinated with DNA plasmid (pSectag2B) encoding relMtb, sigE, ppx1, ppp2 or empty vector as illustrated in the scheme. The vaccination and electroporation were performed once weekly for three weeks. One week after the last vaccination, sera and splenocytes from each group were collected. B. ELISA detection of antigen-specific IgG responses from the different mouse vaccination groups. C. Summary of antigen-specific CD4+ T-cell responses (IFNγ+ and TNFα+) intracellular staining following DNA vaccination. Splenocytes from individual vaccinated groups were stimulated with 10µg/ml of each recombinant protein for 24 hours at 37°C, then GolgiPlug (1 μl/ml) was added overnight. The cells were then stained with anti-mouse CD4, followed by intracellular IFNγ+ and TNFα+ staining. The data were acquired with FACSCalibur and analyzed with FlowJo. *p<0.05 compared to empty vector control.

Figure 6. Immunity to key poly(P) regulatory factors of the Mtb stringent response augments the bactericidal activity of isoniazid in mice. A. Experimental scheme. C57BL/6J mice were vaccinated intramuscularly once weekly with the Stringent Response (SR) vaccine comprising four different DNA plasmids, each encoding relMtb, sigE, ppx1 and ppp2, or with sham vaccine (EV). A separate group of mice received no vaccination (naïve). Three weeks later, all mice were aerosol-infected with wild type Mtb. Beginning on day 14 after aerosol infection, subgroups of mice in each group were treated orally with human-equivalent doses of isoniazid for 4 weeks. INH= isoniazid 10 mg/kg by esophageal gavage once daily (5 days/week). B. Lung bacillary burden (log10 CFU) on Day 14 (D14) and Day 42 (D42) following Mtb aerosol
challenge of naïve mice and those receiving SR vaccine or sham vaccine (EV). C. Lung bacillary burden (log_{10} CFU) CFU at day 42 of isoniazid treatment. n=4 animals per data point.


Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-544.


45. Sanyal S, Banerjee SK, Banerjee R, Mukhopadhyay J, Kundu M. 2013. Polyphosphate kinase 1, a central node in the stress response network of Mycobacterium tuberculosis, connects the two-component systems MprAB and SenX3-RegX3 and the extracytoplasmic function sigma factor, sigma E. Microbiology 159:2074-2086.


A  **Phosphate Compounds**

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<th>ppx1</th>
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B  **Cell Wall Synthesis**

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C  **NADH Metabolism**

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D  **TCA cycle**

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<tr>
<td>2-methylcitrate</td>
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<tr>
<td>acetyl CoA</td>
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E  **Arginine Metabolism**

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<tr>
<td>citrulline</td>
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<td>ornithine</td>
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Glycerol 3-phosphate

Dihydroxyacetone phosphate

Glycerol

Glycerophosphoryl diester phosphodiesterase
Rv0317c, Rv3842c

Lipid polar head

Acytransferases
Rv1551, Rv2182c, Rv2482c

NADH
Rv2982c (NS), Rv0564c

GlpK (Rv3696c)

Rv1692

FAD

glpD1, glpD2

ACI compared to WT

-3
-2.5
-2
-1.5
-1
-0.5
0
1
1.5

ACI compared to WT

-3
-2.5
-2
-1.5
-1
-0.5
0
1
1.5

Rv0564c, Rv1551, Rv2182c, Rv2482c

Rv3842c, Rv0317c

* * *
**A**

Vaccination with individual plasmid → Collection of serum and splenocytes

Day 0 7 14 21

**B**

- **Empty vector**
- **Vaccinated**

<table>
<thead>
<tr>
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**C**

Number of Ag-specific CD4+ T cells/5x10^5 splenocytes

<table>
<thead>
<tr>
<th></th>
<th>IFNγ-TNFα+</th>
<th>IFNγ+TNFα+</th>
<th>IFNγ+TNFα-</th>
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<tbody>
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<td>200±50</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>SigE</td>
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<td>450±50</td>
<td>400±50</td>
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* indicates statistical significance.