Deficiency of the Novel Exopolyphosphatase Rv1026/PPX2 Leads to Metabolic Downshift and Altered Cell Wall Permeability in *Mycobacterium tuberculosis*

Yu-Min Chuang, a Nirmalya Bandyopadhyay, b Dalin Rifat, a Harvey Rubin, c Joel S. Bader, b Petros C. Karakousis a,d

Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; Department of Biomedical Engineering, High-Throughput Biology Center, and Institute of Computational Medicine, Johns Hopkins University, Baltimore, Maryland, USA; Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA; Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

ABSTRACT *Mycobacterium tuberculosis* can persist for decades in the human host. Stringent response pathways involving inorganic polyphosphate [poly(P)], which is synthesized and hydrolyzed by polyphosphate kinase (PPK) and exopolyphosphatase (PPX), respectively, are believed to play a key regulatory role in bacterial persistence. We show here that *M. tuberculosis* poly(P) accumulation is temporally linked to bacillary growth restriction. We also identify *M. tuberculosis* Rv1026 as a novel exopolyphosphatase with hydrolytic activity against long-chain poly(P). Using a tetracycline-inducible expression system to knock down expression of *Rv1026* (*ppx2*), we found that *M. tuberculosis* poly(P) accumulation leads to slowed growth and reduced susceptibility to isoniazid, increased resistance to heat and acid pH, and enhanced intracellular survival during macrophage infection. By transmission electron microscopy, the *ppx2* knockdown strain exhibited increased cell wall thickness, which was associated with reduced cell wall permeability to hydrophilic drugs rather than induction of drug efflux pumps or altered biofilm formation relative to the empty vector control. Transcriptomic and metabolomic analysis revealed a metabolic downshift of the *ppx2* knockdown characterized by reduced transcription and translation and a downshift of glycerol-3-phosphate levels. In summary, poly(P) plays an important role in *M. tuberculosis* growth restriction and metabolic downshift and contributes to antibiotic tolerance through altered cell wall permeability.

IMPORTANCE The stringent response, involving the regulatory molecules inorganic polyphosphate [poly(P)] and (p)ppGpp, is believed to mediate *Mycobacterium tuberculosis* persistence. In this study, we identified a novel enzyme (Rv1026, PPX2) responsible for hydrolyzing long-chain poly(P). A genetically engineered *M. tuberculosis* strain deficient in the *ppx2* gene showed increased poly(P) levels, which were associated with early bacterial growth arrest and reduced susceptibility to the first-line drug isoniazid, as well as increased bacterial survival during exposure to stress conditions and within macrophages. Relative to the control strain, the mutant showed increased thickness of the cell wall and reduced drug permeability. Global gene expression and metabolite analysis revealed reduced expression of the transcriptional and translational machinery and a shift in carbon source utilization. In summary, regulation of the poly(P) balance is critical for persister formation in *M. tuberculosis*.

Received 1 December 2014 Accepted 5 February 2015 Published 17 March 2015

Citation Chuang Y, Bandyopadhyay N, Rifat D, Rubin H, Bader JS, Karakousis PC. 2015. Deficiency of the novel exopolyphosphatase Rv1026/PPX2 leads to metabolic downshift and altered cell wall permeability in *Mycobacterium tuberculosis*. mBio 6(2):e02428-14. doi:10.1128/mBio.02428-14.

Invited Editor Christina L. Stallings, Washington University in St Louis School of Medicine

Editor L. David Sibley, Washington University School of Medicine

Copyright © 2015 Chuang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported license, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Petros C. Karakousis, petros@jhmi.edu.

Tuberculosis (TB) remains a major global health problem (1, 2). The prolonged duration of therapy required to eradicate TB infection is due to the ability of *Mycobacterium tuberculosis* to persist in host tissues despite antibiotic treatment (3). The stringent response mediates bacterial adaptation to stress conditions (3, 4). Inorganic polyphosphate [poly(P)], a linear polymer of many tens or hundreds of inorganic phosphate residues linked by high-energy phosphoanhydride bonds, has been implicated in the transition to bacterial persistence (5, 6). Intracellular poly(P) content increases when bacteria encounter growth-limiting conditions, such as phosphate depletion, amino acid starvation, or osmotic stress (6, 7). Poly(P) accumulation has been shown to control various bacterial processes, including protein synthesis, nucleotide balance, lipid metabolism, energy utility, and susceptibility to antibiotics (5, 6). Regulation of bacterial poly(P) content has been linked to the stringent response alarmone (p)ppGpp, whose stochastic expression may contribute to bacterial persistence (8).

*M. tuberculosis*, like other bacteria, possesses two groups of enzymes, which control inorganic polyphosphate homeostasis: polyphosphate kinase (PPK1/Rv2984) (9) and exopolyphosphatase (PPX1/Rv0496) (10, 11). *ppk1* plays a central role in the regulatory network controlling expression of *relA* (9), which encodes a dual-function enzyme responsible for synthesis and hydrolysis of (p)ppGpp (12, 13). Despite its name, *M. tuberculosis* Rv3232c/PPK2 catalyzes poly(P)-dependent phosphorylation of ADP to ATP at a rate >800-fold higher than that of (p)ppGpp synthesis (14, 15), and a *ppk2*-deficient mutant inappropriately accumulates
poly(P) (11). PPX1, which hydrolyzes short-chain poly(P) (10, 16), is inhibited by pppGpp (16), suggesting the presence of a positive-feedback regulatory loop in the M. tuberculosis stringent response.

Poly(P) content has been implicated in M. tuberculosis antibiotic tolerance. Thus, poly(P)-accumulating strains deficient in ppx1 (10) or ppx2 (11) showed reduced susceptibility to the bactericidal drugisoniazid, which targets the mycolic acid synthesis pathway (17). Conversely, an M. tuberculosis ppx1 deletion mutant was found to have enhanced susceptibility to isoniazid and fluoroquinolones (18). Furthermore, maintenance of intracellular poly(P) balance is critical for M. tuberculosis survival during host infection. The ppx1 (9), ppx2 (11, 14), and ppx1 (10) genes are each required for optimal M. tuberculosis growth and survival during macrophage infection. A mutant deficient in ppx2 showed impaired growth during acute infection in the lungs of mice (11). In addition, a poly(P)-deficient strain lacking ppx1 (18) and a poly(P)-accumulating strain lacking ppx1 (10) were found to have reduced long-term survival in guinea pig lungs. These findings suggest that M. tuberculosis poly(P) levels must be tightly regulated during different stages of animal infection.

Bioinformatic predictions have identified M. tuberculosis Rv1026 as a putative PPX belonging to the single-domain PpxGppA family (19). However, Rv1026 was shown to lack PPX activity against short-chain poly(P) (16). Rv1026, which appears to be an essential gene (20, 21), was found to be significantly upregulated during chronic TB infection in two different mouse strains (21). Overexpression of Rv1026 in Mycobacterium smegmatis leads to altered sliding motility and biofilm formation (22), and the gene is present in the Mycobacterium leprae minimal genome required for slow growth in human tissues (23). In the current study, we tested the hypothesis that M. tuberculosis Rv1026 encodes a PPX capable of hydrolyzing long-chain poly(P). We studied the intracellular poly(P) content of wild-type M. tuberculosis during exposure to various stress conditions to determine whether poly(P) behaves as a transient molecular signal during bacillary growth restriction. Next, we generated recombinant strains conditionally deficient in Rv1026 to determine whether poly(P) accumulation is sufficient for inducing M. tuberculosis growth restriction and antibiotic tolerance and to test the role of poly(P) accumulation during macrophage infection. Using RNA sequencing and ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS), we characterized the regulatory and metabolic pathways controlled by poly(P)-mediated signaling. Finally, we used transmission electron microscopy (TEM) to characterize the bacillary morphology and cell wall thickness in the context of Rv1026 deficiency and poly(P) accumulation.

RESULTS

Rv1026 exhibits exopolypophatase activity against long-chain poly(P). Rv1026 has been predicted to have PPX activity, but this has not been confirmed experimentally (16, 19, 22). We cloned, expressed, and purified six-histidine-tagged Rv1026 (6×His-Rv1026) from Arctic Escherichia coli, and protein lysate from E. coli transformed with the empty vector was used as the baseline control. Recombinant protein was confirmed by Western blotting using anti-His antibody (Fig. 1A). When incubated with poly(P) substrates of different lengths for 8 h, 6×His-Rv1026 showed the greatest hydrolysis activity against 700-mer poly(P) relative to calf intestinal alkaline phosphatase (CIP) (41%, 11.1%, and 6.7% for 700-mer, 45-mer, and hexametaphosphate, respectively [P < 0.05]; Fig. 1B). The hydrolytic activity of long-chain poly(P) was time and concentration dependent (Fig. 1C) and was accelerated when recombinant protein was incubated with AMP/ADP and GMP/GDP but did not favor any specific substrate (see Fig. S1 in the supplemental material). The addition of ppGpp (8 μM) inhibited the PPX activity of Rv1026 after 6-h incubation (Fig. 1D). On the basis of these findings, we concluded that recombinant Rv1026, hereafter designated PPX2, has PPX activity against long-chain poly(P), which can be inhibited by ppGpp, as in other bacteria (24).

Growth restriction is associated with transient elevation of intracellular polyphosphate content. In order to gain insight into the role of poly(P) in M. tuberculosis growth arrest, we used a 4,6-diamidino-2-phenylindole (DAPI)-based method to evaluate the intracellular poly(P) content during different growth-limiting conditions (10). During axenic growth in nutrient-rich broth, intrabacillary poly(P) levels increased during late log phase and returned to the baseline level after entry into stationary phase (Fig. 2A). Upon nutrient starvation, intrabacillary poly(P) levels peaked at 4 h and remained elevated for 24 h (Fig. 2B), coinciding with bacillary growth restriction, before decreasing to the baseline level. M. tuberculosis subjected to progressive hypoxia showed a transient elevation of poly(P) 5 to 6 days before bacterial entry into nonreplicating persistence stage 2 (Fig. 2C), which is characterized by shutdown of replication and metabolism (25). Similarly, peak M. tuberculosis poly(P) levels were observed 3 to 4 days after phosphate depletion (Fig. 2D), when bacillary growth is curbed due to exhaustion of intracellular phosphate stores (26). Under each condition, transient poly(P) accumulation closely preceded reduced M. tuberculosis growth, suggesting that poly(P) may serve as a molecular signal for M. tuberculosis growth arrest.

Rv1026 deficiency leads to poly(P) accumulation and slowed M. tuberculosis growth. To determine whether poly(P) accumulation is sufficient for M. tuberculosis growth arrest, we used a Tet-on expression system (27) to conditionally knock down the expression of the exopolypophatase gene Rv1026/pxx2 in wild-type M. tuberculosis CDC1551 (see Fig. S2A in the supplemental material). The identity of the ppx2 knockout strains was confirmed by PCR and sequencing (Fig. S2B). As shown in Fig. S2C, the growth rate of the ppx2 knockout strain in supplemented Middlebrook 7H9 broth was inversely proportional to the concentration of the inducing agent, anhydrotetracycline (aTc). Since aTc at 250 ng/ml maximally inhibits growth of the ppx2 knockout strain, while aTc at 1 μg/ml also inhibits growth of wild-type M. tuberculosis (28), the former concentration of inducing agent was used in all subsequent studies. The ppx2 knockout strain displayed reduced expression of PXX2 by immunoblotting (Fig. 3A). By densitometric analysis, the ppx2 knockout strain had 66% PXX2 expression compared to the empty vector control strain after normalization to the expression level of DnaK. The ppx2 knockout strain showed increased intracellular poly(P) content relative to the empty vector control during logarithmic growth in nutrient-rich broth (P < 0.05; Fig. 3B), corroborating the PPX activity of Rv1026. The ppx2 knockout strain reached a lower bacterial density during stationary phase relative to the empty vector control (Fig. 3C); this phenomenon could be attributable to reduced replication or accelerated bacterial death. To address this question, we used a “replication clock” plasmid,
ppx2 deficiency contributes to antibiotic tolerance. Next, we studied the activity of isoniazid, which targets actively multiplying bacilli (31, 32), against the poly(P)-accumulating ppx2 knockdown strain. Relative to the empty vector control strain, the isoniazid MIC increased 8-fold against the ppx2 knockdown strain (Table 1). In contrast, the MIC of rifampin was shifted only 2-fold against this recombinant strain. The ppx2 knockdown strain showed improved survival following exposure to high concentrations of isoniazid (10 μg/ml) compared to the empty vector strain (P < 0.05; Fig. 4A), confirming the importance of ppx2 in bacterial survival during exposure to cell wall synthesis inhibitors. Furthermore, the absolute numbers of isoniazid-resistant colonies were equivalent in the ppx2 knockdown and empty vector strains (Fig. 4A), accounting for 49% ± 11% and 2.5% ± 1.2% of the surviving bacilli, respectively. Together, these data are consistent with increased survival of drug-tolerant bacteria during poly(P) accumulation rather than selection of drug-resistant mutants. **ppx2 deficiency leads to increased M. tuberculosis resistance to various in vitro stresses.** Poly(P) regulates expression of the stringent response alarmone (p)ppGpp, and accumulation of poly(P) is believed to contribute to bacterial stress resistance (3, 8). We exposed the ppx2 knockdown strain to various growth-limiting conditions to study its phenotype in relation to the empty vector control strain. The ppx2 knockdown strain showed increased survival following exposure to 40°C for 24 h (Fig. 4B) and to 0.05% sodium dodecyl sulfate (SDS) for 4 h and 6 h (Fig. 4C) compared to the empty vector control. The recombinant strain also showed improved survival following acid shock compared to the empty vector strain (Fig. 4D).

**ppx2 deficiency leads to increased M. tuberculosis growth in murine macrophages.** To determine whether ppx2 deficiency alters *M. tuberculosis* survival in the host, we infected J774 macrophages with the ppx2 knockdown and empty vector control strains. The ppx2 knockdown strain showed increased intracellu-
lar growth during infection of naive macrophages (Fig. 5A). The supernatant of naive macrophages infected with the ppx2 knockdown strain showed higher levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) \((P/M_H11005/0.02)\), interleukin 5 (IL-5) \((P/M_H11005/0.003)\), IL-12(p40) \((P/M_H11005/0.001)\), and IL-12(p70) \((P/M_H11005/0.002)\) compared to those infected with the empty vector control (Fig. 5B). Other cytokines, including the Th1-type cytokines gamma interferon (IFN-\(\gamma\)) \((P/M_H9253)\), tumor necrosis factor alpha (TNF-\(\alpha\)) \((P/M_H9251)\), and IL-2, were noted to be significantly different in the two strains.

These data suggest that the increased growth of the ppx2 knockdown was not due to reduced macrophage production of proinflammatory cytokines.

Inorganic polyphosphate regulates the transcription of multiple \(M.\) \(tuberculosis\) genes. In order to gain insight into the regulatory and metabolic changes occurring in \(M.\) \(tuberculosis\) during poly(P)-mediated growth restriction we used transcriptome sequencing (RNA-seq) to study the global gene expression of the ppx2 knockdown strain relative to the empty vector control strain during mid-log phase, when the difference in poly(P) content between the two strains is greatest and statistically significant (Fig. 3B). A total of 972 genes were significantly differentially regulated in the ppx2 knockdown strain, including 482 downregulated genes and 490 upregulated genes (see Table S1 in the supplemental material). The poly(P)-dependent stringent response signaling pathway, including mprAB and sigE (9, 33), was significantly upregulated in the ppx2 knockdown strain. In addition, the operon comprising espA \((Rv3616c)\), espC \((Rv3615c)\), and espD \((Rv3614c)\) was upregulated in the ppx2 knockdown strain. This operon has been shown to play a role in \(M.\) \(tuberculosis\) virulence (34–36) and during bacillary growth when long-chain fatty acids are the carbon source (37). The cydA-cydB-cydC-cydD operon, which has been implicated in \(M.\) \(tuberculosis\) drug resistance (38), was also upregulated in the ppx2 knockdown strain. Similarly, the isoniazid-induced iniB gene, mutations of which have been implicated in \(M.\) \(tuberculosis\) resistance to isoniazid and ethambutol (39, 40), was among the most significantly upregulated genes in the ppx2 knockdown strain relative to the empty vector strain.

Based on gene ontology analysis, the ppx2 knockdown strain showed differential expression of genes involved in cell wall, growth, and protein synthesis (see Table S2 in the supplemental material). Among growth-related pathways, 46 of 460 genes were upregulated, and 138 of 460 genes were downregulated. Among cell wall-related pathways, 78 of 504 genes were upregulated, and 113 of 504 genes were downregulated. The ppx2 knockdown strain displayed significant downregulation of genes involved in translation and transcription, including rpoA, rpsB, rpsD, rpsG, rpsJ, rpsK, rpsL, rplK, rplM, rplN, rplU, and rplV, consistent with the hypothesis that ppx2 deficiency-induced poly(P) accumulation is associated with a reduction in the rate of protein synthesis.

**ppx2 deficiency affects global \(M.\) \(tuberculosis\) metabolism.** Metabolomics analysis using UHPLC/MS/MS revealed lower levels of the glycolytic intermediates glucose 6-phosphate \((P/M_H11005/0.11)\) and isobaric metabolites (fructose 1,6-diphosphate, glucose 1,6-
diphosphate, myo-inositol 1,4- or 1,3-diphosphate) in the ppx2 knockdown strain compared to the empty vector control strain (P/H11005 0.02; Fig. 6). Significant decreases were also observed in malonyl-coenzyme A (malonyl-CoA) levels (P/H11005 0.017), a metabolite required for the fatty acid synthesis and pentose phosphate pathway intermediates 6-phosphogluconate (P/H11005 0.002) and ribose-5-phosphate (P/H11005 0.01), which could impact nucleotide biogenesis. The major phospholipid scaffold, glycerol 3-phosphate (G3P), was also lower in the ppx2 knockdown strain (P/H11005 0.04). Statistically insignificantly lower levels of metabolites in the peptidoglycan synthesis pathway, including 1-deoxyxylose-5-phosphate (P/H11005 0.067) and diaminopimelate (P/H11005 0.13), were detected in the ppx2 knockdown strain. The mutant showed reduced levels of precursors in pathways responsible for glucose metabolism, fatty acid synthesis, and nucleotide biogenesis (see Table S3 in the supplemental material).

Previous work has highlighted the importance of alterations in lipid metabolism during M. tuberculosis dormancy (37). To gain further insight into the global metabolic changes associated with M. tuberculosis poly(P) accumulation, we calculated z scores for metabolites and gene expression values and mapped these to the Palsson’s network based on reactions (41). Significant correlations in enzyme-encoding gene expression and corresponding metabolites were observed in pathways related to fatty acid metabolism, membrane metabolism, pentose phosphate, pyrimidine metabolism, and sugar metabolism (see Table S4 in the supplemental material), indicating that transcriptional changes were associated with changes at the metabolite level. For example, upregulation of the Rv1347c (mbtK) and Rv2524c (fas) genes could directly contribute to lower levels of malonyl-CoA. Conversely, expression of the fabH, inhA, and pks15 genes and the Rv2931-Rv2932-Rv2933 (ppxA-ppxB-ppxC) operon was negatively

### TABLE 1 MICs of two antibiotics against the empty vector control and ppx2 knockdown strains

| Strain            | Isoniazid (µg/ml) | Rifampin (µg/ml) |
|-------------------|-------------------|------------------|
| Empty vector control | 0.12              | 0.25             |
| ppx2 knockdown    | 0.96              | 0.5              |
correlated with malonyl-CoA levels, suggesting the former may play a negative-feedback role in this pathway.

According to our transcriptomic and metabolomics analyses, ppx2 deficiency was associated with altered global metabolism. *M. tuberculosis* persistence and antibiotic tolerance have been associated with altered reduction potential (42). In order to characterize the redox potential of the ppx2 knockdown strain, we used the alamarBlue assay (Invitrogen), which measures the reduction potential of viable metabolically active cells to chemically reduce resazurin to the fluorescent molecule resorufin, and can be used as a surrogate of cellular metabolic activity (43, 44). At mid-log phase, the normalized fluorescence signal (based on CFU) in the ppx2 knockdown strain was 68.4% of that of the empty vector strain, consistent with reduced redox potential in the mutant strain.

ppx2 deficiency is associated with altered cell wall permeability and increased cell wall thickness but reduced biofilm formation. Our global transcriptional analysis of the poly(P)-accumulating ppx2 knockdown strain revealed induction of several efflux genes, including iniA, iniB, mmpL10, and Rv2459 (45–47), which could contribute to the isoniazid tolerance phenotype of this strain. To further understand the potential role of altered antibiotic influx and efflux during ppx2 deficiency, we evaluated the accumulation rate of the surrogate compound, ethidium bromide (EtBr) (48), in the ppx2 knockdown and control strains by fluorescence. EtBr showed significantly reduced accumulation in the recombinant strain compared to the empty vector control strain (Fig. 7A). However, we found no difference in the fluorescence decay ratio following preincubation of each strain with EtBr, and treatment with the efflux pump inhibitors verapamil, chlorpromazine, reserpine, and carbonyl cyanide m-chlorophenyl hydrazone led to a similar increase in EtBr accumulation in each strain (data not shown). Interestingly, the ppx2 knockdown strain showed higher uptake of the Nile red dye relative to the empty vector strain (Fig. 7B), consistent with cell wall modifications in the former strain permitting greater diffusion of lipophilic molecules rather than polar molecules such as isoniazid. Transmission electron microscopy revealed no significant difference in the gross morphology of these two strains. However, the mean cell wall thickness of the ppx2 knockdown strain (20.1 nm ± 2.49 nm) was significantly greater than that of the empty vector strain (17.75 ± 2.8 nm; *P* < 0.001). On the other hand, the thickness of the cell capsule (49) of each strain was similar (17.25 ± 2.67 nm in the mutant versus 18.11 ± 2.76 nm in the control; Fig. 8). These data are the first to link induction of the bacterial

FIG 4 Polyphosphate accumulation contributes to *M. tuberculosis* phenotypic tolerance to isoniazid and stress resistance. (A) Logarithmically growing ppx2 knockdown and empty vector control strains were incubated with isoniazid (10 μg/ml) for 7 days. The bacteria were plated on Middlebrook 7H10 agar with or without isoniazid (INH) (1 μg/ml) to determine the number of drug-sensitive and drug-resistant CFU. Data are the means of three independent samples, and the numbers are the numbers of CFU of isoniazid-resistant (gray bar) or isoniazid-sensitive bacteria (white bar) (*, *P* < 0.05; *n* = 3). (B to D) Logarithmically growing cultures of empty vector control and ppx2 knockdown strains were incubated under various stress conditions. The stress conditions were as follows: 40°C for 24 h (B), 0.05% SDS for 4 and 6 h (C), and acidified Middlebrook 7H9 broth (pH 4.5) (D). In panels B and C, the survival ratio is the number of surviving bacteria after challenge divided by the number of bacteria prior to incubation. Values are means ± SD. Values that are significantly different (*P* < 0.05) compared to the value for the empty vector control strain are indicated by an asterisk.

Corresponding author: Yu-Liang Chuang, The Salk Institute for Biological Studies, 10016Torrey Pines Road, La Jolla, CA 92037.
stringent response pathway with changes in cell wall thickness, potentially contributing to antibiotic tolerance.

Finally, we investigated the possibility that ppx2 deficiency and poly(P) accumulation alter formation of biofilms, which have been implicated in mycobacterial antibiotic tolerance (50). The ppx2 knockdown and empty vector control strains were grown for 5 weeks in Sauton’s medium without detergent. We found that the pellicle thickness of the ppx2 knockdown strain was reduced relative to that of the empty vector control strain. Consistent with these data, the ppx2 knockdown strain showed 40% reduced signal by crystal violet staining compared to the empty vector strain (Fig. 7C) (51). On the basis of these findings, we conclude that ppx2 deficiency-induced poly(P) accumulation was not associated with induction of drug efflux pumps or enhanced biofilm formation but was associated with increased cell wall thickness and reduced cell wall permeability to polar compounds, which may contribute to isoniazid tolerance.

**DISCUSSION**

The stringent response contributes to bacterial adaptation during growth-limiting conditions through the induction of specific transcriptional programs (3, 4). Poly(P) serves as a phosphate donor to activate downstream regulatory genes, including mprAB in *M. tuberculosis* (9, 33). Our data are consistent with the hypothesis that poly(P) serves as a transient signal to trigger the bacterial stringent response, which is required for growth arrest under nutrient-limited conditions (6). In this study, we identify a novel exopolyphosphatase (Rv1026, PPX2), and a deficiency of this enzyme leads to poly(P) accumulation, premature growth restriction, and antibiotic tolerance, likely as a result of changes in the cell wall leading to reduced permeability of polar compounds, such as isoniazid. In addition, we show for the first time that poly(P) deficiency in the context of ppx2 deficiency leads to global changes in bacterial transcriptional responses and metabolism, including a downshift in transcription and translation and a shift from utilization of carbohydrate sources of energy, as well as the induction of several important virulence factors.

Despite bioinformatic predictions (19), prior work has challenged the hypothesis that Rv1026 encodes an exopolyphosphatase in *M. tuberculosis* (16). Consistent with these studies, we found that recombinant Rv1026 had very weak PPX activity against short-chain poly(P) (45-mer). However, 6×His-Rv1026 was able to hydrolyze long-chain poly(P) (700-mer) in a concentration- and time-dependent manner. In addition to long-chain poly(P) as a substrate, our studies included the use of ADP, AMP, GDP, and GMP to promote hydrolysis activity, which may account for the discrepant findings between our results and those of Choi et al. (16). As in other bacteria (24), the PPX activity of recombinant Rv1026 was inhibited by the stringent response alarmone ppGpp. Further corroborating the function of Rv1026 as a PPX, we found that the ppx2 knockdown strain had higher intracellular poly(P) content than that of the empty vector control strain. *M. tuberculosis* PPX1 (Rv0496) has been shown to hydrolyze short-chain poly(P) (10, 16). Therefore, *M. tuberculosis* may employ these two enzymes to tightly regulate the number and length of intracellular poly(P) molecules, thereby quenching the transient regulatory signal during growth arrest.

Corroborating the hypothesis that poly(P) accumulation triggers *M. tuberculosis* growth restriction, the ppx2-deficient strain showed premature entry into stationary phase in nutrient-rich broth and reduced susceptibility to the cell wall-active agent isoniazid, which targets actively multiplying bacilli, while retaining susceptibility to the sterilizing drug rifampin which better targets nonreplicating “persisters” (52). The enhanced survival of the ppx2 knockdown strain following exposure to high-dose isoniazid was not due to the selection of isoniazid-resistant mutants, but to the presence of drug-tolerant persisters. Our findings are consistent with previous reports showing increased susceptibility of poly(P)-deficient *M. tuberculosis* to antibiotics (18) and reduced isoniazid susceptibility among *M. tuberculosis* poly(P)-accumulating strains (10, 11). We also found that poly(P) accumulation contributes to *M. tuberculosis* adaptation and survival during various stress challenges, including acid, heat, and detergent stresses. Although the ppx2 knockdown strain induced greater release of IL-5, IL-12, and GM-CSF, these cytokines were ineffective in controlling *M. tuberculosis* replication, as these strains showed enhanced intracellular survival within naive macrophages relative to the empty vector. Taken together, these findings suggest that poly(P) accumulation contributes to *M. tuberculosis* growth restriction, antibiotic tolerance, and survival during growth-limiting conditions.

Based on our transcriptomics analysis, a similar number of genes were found to be downregulated as were upregulated in the ppx2 knockdown strain, indicating that poly(P) accumulation...
does not simply lead to global transcriptional shutdown. According to previous studies, poly(P) appears to play a role in the stringent response signaling pathway, mprAB-sigE-relA (9, 33). mprAB and sigE were both upregulated in the ppx2 knockdown strain. Based on gene ontology analysis, ppx2 deficiency affected regulatory pathways related to growth, cell wall metabolism, transcription, and translation. Among the pathways related to transcription and translation, the majority of genes were downregulated, which is consistent with the putative role of poly(P) as a stringent response regulatory molecule.

FIG 6 Metabolomics analysis of the ppx2 knockdown strain compared to the empty vector control strain during exponential growth. (A) Hierarchical clustering pathway analysis of ppx2 knockdown and empty vector strains. (B and C) Metabolites altered in the ppx2 knockdown strain compared to the empty vector strain include phosphate compounds (B) and components of the pentose phosphate and glucose utilization pathways (C). Values that are significantly different are indicated as follows: *, $P < 0.05$; †, $0.05 < P < 0.1$; ‡, $P < 0.005$. TCA, tricarboxylic acid.
Prior work has highlighted the importance of tricarboxylic acid cycle remodeling, including increased synthesis of succinate and decreased levels of \( \alpha \)-ketoglutarate, in the metabolic adaptation of \( M. \) tuberculosis to hypoxia (53). In addition, accumulation of pyruvate and succinate and depletion of \( \alpha \)-ketoglutarate were noted following \( M. \) tuberculosis exposure to antibiotics (54). Our metabolomics analysis revealed significantly reduced levels of glycerol 3-phosphate (G3P) and glycerol 2-phosphate (G2P) during poly(P) accumulation. G3P serves as an important scaffold for phospholipid biosynthesis (55), and reduced G3P levels are believed to be important in persister formation (56, 57). Significantly reduced intracellular G3P and G2P content has been reported in \( M. \) tuberculosis when cholesterol is the sole carbon source (58). The \( ppx2 \) knockdown strain also showed significant downregulation of the G3P dehydrogenase gene, \( glpD2 \), which has been shown to be downregulated in \( M. \) tuberculosis persisters (59).

In addition, glucose phosphorylation was significantly altered in the \( ppx2 \) knockdown strain, and this process has been shown to have an important role during \( M. \) tuberculosis chronic infection in mice (60). Although several metabolites in lipid biosynthetic pathways were reduced during \( ppx2 \) deficiency, transcriptomic analysis revealed significantly increased expression of the triacyl-

**FIG 7** Polyphosphate accumulation results in decreased ethidium bromide accumulation, increased Nile red staining, and reduced biofilm formation. (A) Mid-log-phase cultures of empty vector control and \( ppx2 \) knockdown strains were incubated in PBST with 2 \( \mu \)g/ml ethidium bromide. The values at each time point are normalized to the time zero reading value (mean ± SD; *, \( P < 0.05; n = 3 \)). RFI, relative fluorescence intensity. (B) Mid-log-phase cultures of each strain were incubated in PBS containing 20 \( \mu \)M Nile red stain (mean ± SD; *, \( P < 0.05; n = 3 \)). (C) Each strain was incubated in Sauton’s medium lacking detergent for 5 weeks, and biofilms were assessed by crystal violet staining (*, \( P < 0.05; n = 3 \)).

**FIG 8** Polyphosphate accumulation is associated with increased cell wall thickness. (A to D) The empty vector control strain (A and C) and \( ppx2 \) knockdown strain (B and D) were evaluated by transmitted electronic microscopy during mid-log-phase growth. The black arrow indicates the cell wall layer. Bars, 100 nm. (E) Dot plot graph of the cell wall thickness in \( ppx2 \) knockdown and empty vector strains. The cell wall thickness in the \( ppx2 \) knockdown strain and empty vector strain were significantly different (\( P < 0.001 \)).
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the loading control. The data represent the averages of three independent experiments. 

**Materials and Methods**

**Bacteria and growth conditions.** Wild-type M. tuberculosis CDC1551 was grown in Middlebrook 7H9 broth (Difco, Sparks, MD) supplemented with 10% oleic acid–albumin–dextrose–catechol (OADC) (Difco), 0.1% glycerol, and 0.05% Tween 80 at 37°C on a roller. Nutrient starvation and phosphate depletion conditions were established as previously described (26, 77). A progressive hypoxia model (Wayne model) was used to study bacterial adaptation during hypoxia, and the change in color of the methylene blue dye was used as an indicator of nonreplicating persistence stage 2 (NRP-2) (10, 25).

**Expression of His-tagged Rv1026 protein.** The full-length sequence of Rv1026 was amplified from M. tuberculosis CDC1551 and cloned into plasmid pET15b with an N-terminal six-His tag (6×-His tag: Novagen) using the restriction enzymes Xhol and BamHI. The resulting plasmid was used to transform E. coli Arctic Express (DE3) RP competent cells (Stratagene). Original plasmid pET15b was transformed as a negative-control strain. The transformed bacteria were selected by ampicillin (100 μg/ml), and cloning was confirmed by DNA sequencing. Confirmed clones were grown in LB broth with gentamicin (20 μg/ml) and ampicillin (100 μg/ml), and the culture was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in 12°C for 24 h to overexpress recombinant Rv1026. The negative-control strain was induced by using the same protocol. Protein was harvested from cell pellets and stored at −20°C.

**Purification of His-tagged Rv1026 protein.** Protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) slurry under native conditions using standard protocols (Qiagen). The dialyzed active protein fractions were quantified using Qubit protein assay (Invitrogen) and analyzed by SDS-PAGE. Protein specificity was confirmed by Western blotting using Penta-His antibody (Qiagen). The recombinant protein was frozen and stored at −80°C until use.

**Assay of polyphosphatase activity of recombinant Rv1026.** Dialyzed recombinant Rv1026 in phosphate-buffered saline (PBS) solution was used to determine PPX activity (10). Briefly, the elution fraction of six-histidine-tagged Rv1026 (6×His-Rv1026) was prepared as previously described, and protein from the original plasmid (pET15b) served as a negative control. 6×His-Rv1026 (5 μg/ml) or eluted protein harvested from negative control was incubated with 10 μg/ml sodium hexametaphosphate (P6; Sigma), 10 μg/ml 45-mer polyphosphate [poly(P)] (P45; Sigma), or 50 μM (phosphate monomer) 700-mer poly(P) (P700; KeraFast, Inc., Boston, MA) in 50 mM Tris-HCl reaction buffer (pH 8.0), which contained 10 mM MgCl2, 1.5 mM KCl, and 50 μg/ml of ADP, AMP, GDP, and GMP (Sigma) for 8 h. Calf intestinal alkaline phosphatase (CIP) (New England Biolabs [NEB]) (40 U/ml) was used as a positive control, and buffer alone was used to determine total poly(P) content. Poly(P) levels were measured in three or four samples at each time point using a 4′,6-diamidino-2-phenylindole (DAPI)-based method (10, 11, 78). Each experiment had triplicate independent reactions and was repeated at least once. The data represent the averages of these triplicate reactions. To determine the effect of ppGpp on the hydrolytic activity of Rv1026, 8 μM ppGpp (TriLink BioTechnologies, San Diego, CA) was incubated with 6×His-Rv1026 (5 μg/ml) and 50 μM (phosphate monomer) P700, and total poly(P) content was measured after 6-h incubation.

**Determination of intrabacillary inorganic polyphosphate.** A DAPI-based method was used to determine intracellular polyphosphate content (11, 78, 79). Briefly, the bacteria were lysed by bead beating in 50 mM Tris-HCl (pH 7.0) buffer supplemented with 5 M guanidinium thiocyanate (GITC) (Sigma), and the total protein levels of the lysates were determined by colorimetric protein assay (Bio-Rad). Poly(P) was harvested by glass milk from GeneClean III kit (MP Biomedicals LLC) and then treated with DNase (Ambion) and RNase (NEB) before elution with 95°C distilled water (pH 8.0). The poly(P) concentration was determined by fluorescence of the DAPI-poly(P) complex following excitation at 415 nm and emission at 525 nm on a FLUOstar OPTIMA microplate reader (BMG Labtech). Increasing concentrations of poly(P) (type 65; Sigma-Aldrich) were used to generate a standard curve to measure the poly(P) contents. All data were from three biological replicate experiments.

**Conditional Rv1026 knockout strains.** A conditional expression plasmid, pUV15terORm, was obtained from Addgene (Addgene plasmid 17975) (27). For generation of the Rv1026 (ppx2) knockout strain, the sequence containing the Rv1026 gene, including 123 bp upstream and
963 bp downstream of the transcription start and stop sites, respectively, was digested using AclI and PacI and cloned in the reverse orientation into this plasmid. The segment containing attB and Int from pMH94 was cloned into puv15tetORm using the MfeI and AclI sites to generate a hygromycin-containing Middlebrook 7H10 plates. Plasmid insertion was confirmed by PCR. For all experiments, the empty vector and Rv1026 knockdown and empty vector strains were diluted to an optical density (OD$_{600}$) of 0.001 and allowed to grow to mid-log phase in the presence of kanamycin containing Middlebrook 7H10 plates. Plasmid insertion was confirmed by ImageJ. For all experiments, the empty vector and Rv1026 knockdown and empty vector strains were diluted to an optical density (OD$_{600}$) of 0.001 and allowed to grow to mid-log phase in the presence of the inducer anhydrotetracycline (aTc) at 250 ng/mL.

**Mouse immunization and generation of polyclonal antisera.** Female BALB/c mice (4 to 5 weeks old) were purchased from Charles River Laboratories. Recombinant Rv1026 protein was harvested as described above and purified from an SDS-polyacrylamide gel. At day 0, five mice were immunized by subcutaneous injection with a mixed emulsion of 6× His-Rv1026 and complete Freund’s adjuvant (Sigma). At weeks 2 and 4, mice received booster immunizations with an emulsion of incomplete Freund’s adjuvant (Sigma) and 6×His-Rv1026. At weeks 6 and 8, polyclonal sera were collected from the tail veins, and immunoblotting was performed to confirm the activity of antisera. Antisera for Rv1026 were used at a 1:100 titer as the primary antibody. DnaK expression was used as a loading control in Western blots using an anti-DnaK antibody (BEI Resources). The densitometric results of Rv1026 and DnaK in the same experiment were analyzed with ImageJ. All experiments were repeated at least once, and similar results were obtained.

**Determination of the strain replication rate by “replication clock” plasmid.** The Rv1026 knockdown and empty vector strains were transformed with plasmid pBP10 (kind gift of David Sherman) (29), and transformants were selected on Middlebrook 7H10 plates containing kanamycin (50 μg/mL). Individual colonies were grown to mid-log phase in kanamycin-containing Middlebrook 7H9 broth, and the bacteria were pelleted and resuspended in enriched Middlebrook 7H9 broth without kanamycin at day 0. At days 0, 3, 7 and 14, bacteria were plated on Middlebrook 7H10 plates with or without kanamycin to determine the proportion of kanamycin-resistant CFU among total bacterial CFU. The ratio of kanamycin-resistant bacteria to total bacteria is considered to be inversely related to the bacterial division rate (29).

**Transmission electron microscopy.** Live mid-log-phase-phase bacteria in Middlebrook 7H9 broth were fixed with equal volumes of 2× fixative and gently rocked for 10 min. Samples were then centrifuged at 8,000 rpm, supernatant was removed, and 1× fixative was added to the pellet, which was rocked overnight in the cold room. The final fixative concentrations were 2.5% glutaraldehyde, 20 mM sodium cacodylate, and 1 mM MgCl$_2$, pH 7.2. After the samples were rinsed three times with 20 mM sodium cacodylate and 1 mM MgCl$_2$ buffer for 15 min each time, samples were postfixed in 1% osmium tetroxide in 20 mM sodium cacodylate containing 1 mM MgCl$_2$ for 1.5 h on ice. After a brief water rinse, samples were dehydrated through a graded series of ethanol to 100%, then transferred to propylene oxide, and gradually infiltrated with a 1:1 resin mixture of Spurr’s resin and Eponate 12 (Polysciences) with the following propylene oxide parts (30%, 50%, and 75% [rocked overnight]). After 3 changes in pure resin (Spurr’s resin—Eponate 12, 1:1), pellets were cured in a 60°C oven for 2 days. Sections were cut on a Reichert Ultracut E ultramicrotome with a Diatome diamond knife. Eighty-nanometer-thick sections were picked up on Formvar-coated 1- by 2-mm copper slot grids and stained first with 1% tannic acid (filtered aqueous), followed by 2% uranyl acetate (filtered aqueous), and then lead citrate. Grids were viewed on a Phillips CM 120 transmission electron microscope (TEM) operating at 80 kV, and digital images were captured with an AMT 8,000 by 8,000 charge-coupled-device (CCD) camera. For cell size measurement, a fixed phosphotungstate negative stain was used (26). Cell size and wall thickness were measured by ImageJ.

**MIC determination.** The MIC of anti-TB drugs was determined as previously described (11). For antibiotic tolerance studies, mid-log-phase cultures were incubated with isoniazid (10 μg/mL). At days 0, 7, and 14, surviving bacteria were plated on Middlebrook 7H10 agar with and without isoniazid (1 μg/mL) to determine the number of surviving bacteria and proportion of drug-resistant mutants.

**Heat shock, SDS, and acid challenge.** Heat shock and SDS challenge were performed as previously described (82), except that samples were incubated in a water bath at 40°C for 24 h. For acid challenge studies, the M. tuberculosis strains were grown in acidified Middlebrook 7H9 broth (83), and serial dilutions were plated onto Middlebrook 7H10 agar at days 1, 3, and 7 after incubation.

**Macrophage infections and cytokine assays.** The mouse macrophage-like cell line J774.1 was used for these studies, as previously described. Naive macrophages were divided 1 day before infection without any treatment. At day 0, 1× macrophages were infected with an equal number of logarithmically growing bacilli of empty vector control and ppx2 knockdown strains in RPMI 1640 medium containing 250 ng/mL aTc. Intracellular M. tuberculosis was recovered and plated on days 0, 1, 3, 5, and 7. At days 1 and 3, the supernatant of each culture was collected and frozen at −80°C until analysis. Macrophage-secreted cytokines were analyzed by immunobead cytokine assays (mouse cytokine 23-plex assay; Bio-Rad) (11).

**RNA-Seq.** Following exposure of logarithmically growing cultures (OD = 0.5) to the inducer aTc for at least 1 week, RNA was harvested using Trizol-based methods (10). RNA samples were treated with DNase, and the quality of the RNA samples was assessed by using an Agilent bioanalyzer (Agilent Technologies). The samples were sent to the Next Generation Sequencing Center at the Sidney Kimmel Comprehensive Cancer Center of the Johns Hopkins University School of Medicine for library construction and sequencing using Illumina HiSeq 2000 (Illumina). The sequence quality of the data sets was checked by fastQ software. The transcriptome sequencing (RNA-seq) data were aligned with the M. tuberculosis CDC1551 genome obtained from Ensembl (bacteria, http://bacteria.ensembl.org) using bowtie2. HTSeq-count script written in python was used to obtain the read counts of each CDC1551 gene from the significance analysis of microarray (SAM) alignment file. The GTF annotation file from the bacterial Ensembl website was used during read counting for gene annotation. Since the library was strand specific, high-throughput sequencing (HTSeq) was employed with appropriate configurations (default). Normalization and analysis of differential expression were performed using DESeq2 package of bioconductor. DESeq2 generates statistics for each of the genes, including the Wald test P value and Benjamini Hochberg (BH) corrected P values. Standard cutoffs of corrected P values (≤0.05) were used to denote differentially expressed genes under specific conditions. Finally, the Bioconductor package UniProt.w5 was used for a detailed annotation of the genes, including mapping between MT and Rv identifiers.

**Gene ontology analysis.** The gene identifier to gene ontology (GO) identifier was obtained using UniProt.ws software. Gene ontology analysis was conducted using the goseq package, which is useful, because it considers the effect of variations of gene length, which is a relevant normalization criterion for RNA-Seq data. The Bioconductor package GO.db was used to obtain a detailed annotation of the GO identifiers. A BH-adjusted P value cutoff of 0.05 was used to identify differentially regulated GO terms.

**Metabolomics analysis.** Sample preparation and analysis were performed as previously described (84) with minor modifications. After at least 1 week of incubation with aTc, logarithmically growing cultures (OD = 0.5) of the ppx2 knockdown and empty vector control strains were...
pelleted, and the samples were extracted in 1 ml of extraction buffer (chloroform-methanol, 2:1) and then concentrated by centrifugal evaporation. Samples were processed and analyzed by Metabolon, Inc. (Durham, NC). The nontargeted metabolic profiling instrumentation employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography-mass spectrometry (GC-MS) (85, 86). Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standards (87). For statistical analyses and data display purposes, any missing values were assumed to be below the limits of detection, and these features in the experimental samples were not considered.

**Resazurin assay for intracellular NAD/NADH ratio.** Serial dilutions of mid-log-growth-phase M. tuberculosis cultures were incubated with resazurin (Invitrogen) for 16 to 18 h, and the fluorescence intensity was measured using a BMG Optima microplate reader at 544-nm excitation and 590-nm emission wavelengths. The signal was corrected with the signal from negative controls (medium alone), and results yielding a linear relationship were used as representative data.

**Ethidium bromide accumulation/efflux assay and Nile red uptake assays.** The ethidium bromide accumulation and efflux assays were measured by fluorescence intensity (48, 89) with minor modifications. Briefly, mid-log-phase cultures were washed with PBS containing 0.05% Tween 80 (PBST) and then stained with 2 μg/ml ethidium bromide (Sigma). Ethidium bromide (1 μg/ml) was used for accumulation assays with efflux inhibitors, including chlorpromazine (10 μg/ml; Sigma), verapamil (100 μg/ml; Sigma), reserpine (6 μg/ml; Sigma), or carbonyl cyanide mchlorophenyl hydrace (1 μg/ml; Sigma). For the ethidium bromide efflux assay, bacteria were washed with PBST and then incubated with 2 μg/ml ethidium and 100 μg/ml verapamil for 60 min. After the bacteria were washed twice with PBST, efflux activity was measured as the decay ratio of fluorescence intensity. For Nile red uptake, mid-log-phase cultures were washed with PBS and then stained with 20 μM Nile red (Sigma) (90). In all assays, the cells were incubated in 96-well plates, and analysis was performed at the indicated time points by excitation at 544 nm and emission at 590 nm on a FLUOstar OPTIMA microplate reader (BMG Labtech). All data were normalized to the time zero reading of each well. All experiments were repeated at least three times and similar results were obtained. Representative results are shown in Fig. 7A and B.

**Biofilm formation assay and crystal violet staining.** Crystal violet staining was performed (51) with minor modifications. Briefly, mid-log-phase cultures (5 ml; density of 10^6/ml) were grown in 50-ml standing phase cultures (5 ml; density of 10^6/ml) were grown in 50-ml standing cultures containing Sauton’s medium (Himedia, India) without deuterium for 5 weeks. The extracellular matrix of biofilm was measured by using crystal violet stain and a FLUOstar OPTIMA microplate reader (BMG Labtech).

**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 t test, and a P value of <0.05 was considered significant.

**Microarray data accession number.** The RNA-seq data were deposited in the GEO database under accession no. GSE57868.

## SUPPLEMENTAL MATERIAL

**SUPPLEMENTAL MATERIAL for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02428-14/-/DCS supplemental.**

**Figure S1, TIF file, 0.2 MB.**

**Figure S2, TIF file, 0.7 MB.**

**Figure S3, TIF file, 0.5 MB.**

**Table S1, XLSX file, 0.1 MB.**

**Table S2, XLSX file, 0.4 MB.**

**Table S3, XLSX file, 0.02 MB.**

**Table S4, XLSX file, 0.03 MB.**

**Table S5, DOCX file, 0.03 MB.**

## ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health by grants R01 HL106786 to P.C.K. and J.S.B. and grant R01 AI083125 to P.K.C., respectively.

The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

## REFERENCES

1. Fauci AS, NIAID Tuberculosis Working Group. 2008. Multidrug-resistant and extensively drug-resistant tuberculosis: the National Institute of Allergy and Infectious Diseases Research agenda and recommendations for priority research. J Infect Dis 197:1493–1498. 
http://dx.doi.org/10.1086/587890.

2. Lawn SD, Zumla AI. 2011. Tuberculosis. Lancet 378:57–72. 
http://dx.doi.org/10.1016/S0140-6736(10)62173-3.

3. Cohen NR, Lobritz MA, Collins HJ. 2013. Microbial persistence and the road to drug resistance. Cell Host Microbe 13:632–642. 
http://dx.doi.org/10.1016/j.chom.2013.05.009.

4. Boutte CC, Crosson S. 2013. Bacterial lifestyle shapes stringent response activation. Trends Microbiol 21:174–180. 
http://dx.doi.org/10.1016/j.tim.2013.01.002.

5. Kulaev I, Kulakovskaya T. 2000. Polyphosphate and phosphate pump. Annu Rev Microbiol 54:709–734. 
http://dx.doi.org/10.1146/annurev.micro.54.1.709.

6. Rao NN, Gómez-García MR, Kornberg A. 2009. Inorganic polyphosphate: essential for growth and survival. Annu Rev Biochem 78:605–647. 
http://dx.doi.org/10.1146/annurev.biochem.77.030709.093039.

7. Kornberg A, Rao NN, Ault-Riché D. 1999. Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68:89–125. 
http://dx.doi.org/10.1146/annurev.biochem.68.1.89.

8. Maisonneuve E, Castro-Camargo M, Gerdes K. 2013. (p)pGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. Cell 154:140–1150. 
http://dx.doi.org/10.1016/j.cell.2013.07.048.

9. Sureka K, Dey S, Datta P, Singh AK, Dagsupta A, Rodrigue S, Basu J, Kundu M. 2007. Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. Mol Microbiol 65:261–276. 
http://dx.doi.org/10.1111/j.1365-2958.2007.05814.x.

10. Thayil SM, Morrison N, Schechter N, Rubin H, Karakousis PC. 2011. The role of the novel exopolyphosphatase MT0516 in Mycobacterium tuberculosis drug tolerance and persistence. PLoS One 6:e28076. 
http://dx.doi.org/10.1371/journal.pone.0028076.

11. Chuang YM, Belchis DA, Karakousis PC. 2013. The polyphosphate kinase gene ppk2 is required for Mycobacterium tuberculosis inorganic polyphosphate regulation and virulence. mBio 4(3):e00039-13. 
http://dx.doi.org/10.1128/mBio.00039-13.

12. Avarbock A, Avarbock D, Teh JS, Buckstein M, Wang ZM, Rubin H. 2005. Functional regulation of the opposing (p)pGpp synthetase/hydrolase activities of RelMt from Mycobacterium tuberculosis. Biochemistry 44:9913–9923. 
http://dx.doi.org/10.1021/bi0505316.

13. Avarbock D, Salem J, Li LS, Wang ZM, Rubin H. 1999. Cloning and characterization of a bifunctional RelA SpoT homologue from Mycobacterium tuberculosis. Gene 233:261–269. 
http://dx.doi.org/10.1016/S0378-1119(99)00114-6.

14. Sureka K, Sanyal S, Basu J, Kundu M. 2009. Polyphosphate kinase 2: a modulator of nucleoside diphosphate kinase activity in mycobacteria. Mol
Polyphosphate deficiency in Mycobacterium tuberculosis is associated with enhanced drug susceptibility and impaired growth in guinea pigs. J. Bacteriol. 195:2839–2851. http://dx.doi.org/10.1128/JB.00038-13.

Lindner SN, Knebel S, Wesseling H, Schobeth SM, Wendisch VF. 2009. Exopolyphosphatases PppX and PppZ from Corynebacterium glutamicum. Appl. Environ Microbiol. 75:3161–3170. http://dx.doi.org/10.1128/AEM.02703-08.

Colt SE, Eigmiller JK, Parkhill J, Thomson NR, Wheeler PR, Gill WP, Harik NS, Whiddon MR, Liao RP, Mittler JE, Sherman DR. 2008. Ehrt S, Gooi XY, Hickey CM, Ryoo M, Monteleone M, Riley LW, Schnappinger D. 2005. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res 33:4256–4266. http://dx.doi.org/10.1093/nar/gki763.

Wayne LG, Hayes LG. 1996. An in vitro model for sequential study of shutoff of Mycobacterium tuberculosis through two stages of nonreplicating persistence. Infect Immun. 64:2062–2069.

Rifat D, Bishai WR, Karakousis PC. 2009. Phosphate depletia: a novel trigger for Mycobacterium tuberculosis persistence. Nat. Rev. Microbiol. 7:116–1135. http://dx.doi.org/10.1038/nrmicro2057.

Guo XY, Monteleone M, Klotzsche M, Kamionka A, Hillen W, Braunstein M, Ehrt S, Schnappinger D. 2007. Silencing Mycobacterium sgenmatis by using tetracycline repressors. J Bacteriol 189:4614–4623. http://dx.doi.org/10.1128/JB.00216-07.

Ehrt S, Gooi XY, Hickey CM, Ryoo M, Monteleone M, Riley LW, Schnappinger D. 2005. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res 33:4256–4266. http://dx.doi.org/10.1093/nar/gki763.

Gilill WP, Harih NS, Whiddon MR, Liao RP, Mittler JE, Sherman DR. 2007. A replication clock for Mycobacterium tuberculosis. Antimicrob Agents Chemother 2:29–35. http://dx.doi.org/10.1128/AAC.2.1.29.

Winder FG, Collins PB. 1970. Inhibition by isoniazid of synthesis of mycolic acids in Mycobacterium tuberculosis. J Gen Microbiol 63:41–48. http://dx.doi.org/10.1128/mBio.01125-13.

Sanjay 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 MprA/MprS and SenX3-RegX3 and the extracytoplasmic function sigma factor, sigma E. Microbiology 159:2074–2086. http://dx.doi.org/10.1099/mic.0.068452-0.

Champion PA, Champion MM, Manianizado P, Cox JS. 2009. ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA AT-Pases in pathogenic mycobacteria. Mol Microbiol 73:950–962. http://dx.doi.org/10.1111/j.1365-2958.2009.06831.x.

Garcès A, Atmakuri K, Chase MR, Woodward JS, Krastins B, Rothchild AC, Ramsdell TL, Lopez MF, Behar SM, Sarracino DA, Fortune SM. 2010. EspA acts as a critical mediator of ESX1-dependent virulence in Mycobacterium tuberculosis by affecting bacterial cell wall integrity. PLoS Pathog 6:e1000957. http://dx.doi.org/10.1371/journal.ppat.1000957.

Millington KA, Fortune SM, Low J, Garcés A, Hingley-Wilson SM, Wickremasinghe M, Kon OM, Lalvani A. 2011. Rif661c is a highly immunodominant RD1 (region of difference 1)-dependent secreted antigen specific for Mycobacterium tuberculosis infection. Proc Natl Acad Sci U S A 108:5730–5735. http://dx.doi.org/10.1073/pnas.1015153108.

Rodríguez JG, Hernández AC, Helguera-Reteputo C, Aguilar Ayala D, Guadarrama-Medina R, Anzólía JM, Bustos JR, Zambrano MM, González YMJ, García MJ, Del Portillo P. 2014. Global adaptation to a lipid environment triggers the dormancy-related phenotype of Mycobacterium tuberculosis. mbio.5(3):e01125-14. http://dx.doi.org/10.1128/mBio.01125-14.

Padiapdu P, Vashisht R, Chandra N. 2010. Protein-protein interaction networks suggest different targets have different propensities for triggering drug resistance. Syst Biol 4:311–322. http://dx.doi.org/10.1007/s11693-011-0976-5.

Ramswamy SV, Joy AG, Gökşen S, Stager CE, Dou SJ, El Sahly H, Moghazeh SL, Kewalramani B, Musser JM. 2000. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother 44:326–336. http://dx.doi.org/10.1128/AAC.44.2.326-336.2000.

Ramswamy SV, Reich R, Doshi P, Pan Y, Wanger A, Quüituga T, Graviss EA. 2003. Single nucleotide polymorphisms in genes associated with isoniazid resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 47:1241–1250. http://dx.doi.org/10.1128/AAC.47.4.1241-1250.2003.

Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simmons S, Liao RP, Mittler JE, Sherman DR. 2002. Evolutionary consideration. Curr Top Microbiol Immunol 273:41–69. http://dx.doi.org/10.1007/3-540-46643-8.

Jamshidi N, Palsson BØ. 2007. Investigating the metabolic capabilities of Mycobacterium tuberculosis H37Rv using the in silico strain iNJ661 and proposing alternative drug targets. BMC Syst Biol 1:26. http://dx.doi.org/10.1186/1752-0509-1-26.

Llasat MD, Belyk PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobotz MA, Briff D, Schwarz EG, Ye JD, Pati M, Verucysse M, Ralifo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ. 2014. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci U S A 111:E2100–E2109. http://dx.doi.org/10.1073/pnas.1408761111.

Gloeckner H, Jonuleit H, Lemke HD. 2001. Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dAlamar Blue. J Immunol Methods 252:131–138. http://dx.doi.org/10.1016/S0022-1759(01)00347-7.

Nakayama GR, Caton MC, Nova MP, Parandoosh Z. 1997. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J Immunol Methods 204:205–208. http://dx.doi.org/10.1016/S0022-1759(97)00043-4.

Louw GE, Warren RM, Gey van Pittius NC, McEvooy CR, Van Helden PD, Victor TC. 2009. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother 53:3181–3189. http://dx.doi.org/10.1128/AAC.01577-08.

Szymowski JD, Adams KN, Edelstein PH, Ramakrishnan L. 2013. Antimicrobial efflux pumps and Mycobacterium tuberculosis drug tolerance: Evolutionary considerations. Curr Top Microbiol Immunol 374:81–108. http://dx.doi.org/10.1007/83_2012_300.

Salathé JP, Dartois V, Lee EJ. 2012. The role of transport mechanisms in Mycobacterium tuberculosis drug resistance and tolerance. Pharmacol (Basel) 5:1210–1235. http://dx.doi.org/10.3390/ph511210.

Rodrigues I, Machado D, Couto I, Amaral L, Viveiros M. 2012. Contribution of efflux activity to isoniazid resistance in the Mycobacterium
tuberculosis complex. Infect Genet Evol 12:695–700. http://dx.doi.org/10.1016/j.meegid.2011.08.009.

49. Sani M, Houben EN, Geurtsen J, Pierson J, de Punter K, van Zon M, WEVER B, Piersma SR, Jiménez CR, Daffé M, Appelmelk BJ, Bitter W, van der Wel N, Peters PJ. 2010. Direct visualization by cryo-EM of the mycobacterial cell wall: a labile structure containing ESX-1-secreted proteins. PLoS Pathog 6:e1000794. http://dx.doi.org/10.1371/journal.ppat.1000794.

50. Sambandam D, Dao DN, Weinrick BC, Vilchèze C, Gurcha SS, Ojha A, Keren I, Minami S, Rubin E, Lewis K. 2012. Upregulation of the phthiocillin dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant Mycobacterium tuberculosis. J Bacteriol 194:715–721. http://dx.doi.org/10.1128/JB.00304-11.

51. Butt NA, Karakousis PC. 2014. Latent tuberculosis infection: myths, models, and molecular mechanisms. Microbiol Mol Biol Rev 78:343–371. http://dx.doi.org/10.1128/MMBR.00010-14.

52. Eoh H, Rhee KY. 2013. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 110:6554–6559. http://dx.doi.org/10.1073/pnas.1219375110.

53. Nakadukamur M, Nathan C, Rhee KY. 2014. Isocitrate lyase mediates broad antibiotic tolerance in Mycobacterium tuberculosis. Nat Commun 5:3034. http://dx.doi.org/10.1038/ncomms5396.

54. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawasaki K, Iwabuchi M, Dimon MiH, Youngner SJ, Nakai K, Kanehisa M. 2014. KEGG for linking genomes to life and the environment. Nucleic Acids Res 43:D274–D284. http://dx.doi.org/10.1093/nar/gkt102.

55. Atamé SM, Fazeh CH, Henry TC, Mok WW, Orman MA, Sandvik EL, Colomina JG, Benitez CO, Ojha A, Jensen AE, Rubin EJ. 2013. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 110:10026–10031. http://dx.doi.org/10.1073/pnas.1302100110.

56. Kianime K, Mutsaas K, Tshifuba S, Mbithi K, Tshidzoro B, Zhunzi K, Thayil SM, Mbbemba JM, Matshewa P, Kishore S, Lyamuya E, Ann P, Ray D, Meboldt J, Mwamba J, Gwira M, Norton S, Schreuder R, Marasini C, Butterworth Rose C, Kucharik I. 2014. Longitudinal study of Mycobacterium tuberculosis in Zambian adults and children. Am J Respir Crit Care Med 189:A1165. http://dx.doi.org/10.1164/rccm.201308-1494MH.

57. Oh J, Son JE, Kang H, Jeong SY, Lee JY, Han S, Cha D, Lee S, Park Y, Park H, Jang S, Yang JH, Lee SH, Baek MS, Ayanna S, Park CS, Lee HK. 2014. Reduced drug uptake in phenotypically resistant nutrient-starved nonreplicating Mycobacterium tuberculosis. Antimicrob Agents Chemother 58:5701–5711. http://dx.doi.org/10.1128/AAC.00507-14.

58. Pratap D, Perusse S, Halim M, Cotta J, Nester C, Patwardhan N, Yao D, Seitz D, Michalowicz E, Sambasiva H, Goward J, Kilgore D, Nutman TB, Mecsas J, Baker S, Rabinovitch P, Seder R, Berendt AR. 2014. BCG protects against Mycobacterium tuberculosis by promoting interferon-γ and CD8 T cell responses. Proc Natl Acad Sci U S A 111:7061–7066. http://dx.doi.org/10.1073/pnas.1316138111.

59. Raikhel SR, Duarte J, Brown PD, Kropinski AM, Godoy V, Antunes RP, de Paula Neto G, de Lima J, de Amorim S, Markwell AR, de Los Rios S, de Oliveira CB, Paiva GM, Laureano FS, dos Santos LL, de Allegro D, de Almeida G, de Carvalho P, de Lima B, de Oliveira F, de Siqueira RM, de Andrade RB, de Brito PM. 2014. Mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. PLoS Pathog 10:e1004339. http://dx.doi.org/10.1371/journal.ppat.1004339.

60. Rhee KY. 2013. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 110:6554–6559. http://dx.doi.org/10.1073/pnas.1219375110.

61. Pooley SH, Baek SH, Li AH, Sassetti CM. 2013. Mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. PLoS Pathog 9:e1003222. http://dx.doi.org/10.1371/journal.ppat.1003222.

62. Pratap D, Duarte J, Kropinski AM, Godoy V, Antunes RP, de Paula Neto G, de Lima J, de Amorim S, Markwell AR, de Los Rios S, de Oliveira CB, Paiva GM, Laureano FS, dos Santos LL, de Allegro D, de Almeida G, de Carvalho P, de Lima B, de Oliveira F, de Siqueira RM, de Andrade RB, de Brito PM. 2014. Mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. PLoS Pathog 10:e1004339. http://dx.doi.org/10.1371/journal.ppat.1004339.
ST, Raman S, Kaufmann SH, Mohney RP, Chelsky D, Moody DB, Sherman DR, Schoolnik GK. 2013. The Mycobacterium tuberculosis regulatory network and hypoxia. Nature 499:178–183. http://dx.doi.org/10.1038/nature12337.

85. Boudonck KJ, Mitchell MW, Német L, Keresztes L, Nyska A, Shinar D, Rosenstock M. 2009. Discovery of metabolomics biomarkers for early detection of nephrotoxicity. Toxicol Pathol 37:280–292. http://dx.doi.org/10.1177/0192623309332992.

86. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. 2009. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem 81:e6656–6667. http://dx.doi.org/10.1021/ac901536b.

87. Dehaven CD, Evans AM, Dai H, Lawton KA. 2010. Organization of GC/MS and LC/MS metabolomics data into chemical libraries. J Cheminform 2:9. http://dx.doi.org/10.1186/1758-2946-2-9.

88. Storey JD, Tibshirani R. 2003. Statistical significance for genomewide studies. Proc Natl Acad Sci USA 100:9440–9445. http://dx.doi.org/10.1073/pnas.1530509100.

89. Rodrigues L, Ramos J, Couto I, Amaral L, Viveiros M. 2011. Ethidium bromide transport across Mycobacterium smegmatis cell-wall: correlation with antibiotic resistance. BMC Microbiol 11:35. http://dx.doi.org/10.1186/1471-2180-11-35.

90. Xu WX, Zhang L, Mai JT, Peng RC, Yang EZ, Peng C, Wang HH. 2014. The Wag31 protein interacts with AccA3 and coordinates cell wall lipid permeability and lipophilic drug resistance in Mycobacterium smegmatis. Biochem Biophys Res Commun 448:255–260. http://dx.doi.org/10.1016/j.bbrc.2014.04.116.