Inorganic polyphosphate accumulation suppresses the dormancy response and virulence in Mycobacterium tuberculosis

The article has been withdrawn by the authors. Issues related to WT panels were identified. The image from the WT biofilm formation panel published in Agarwal, S., et al. (2018) J. Infect. Dis. 217, 1809–1820 was inadvertently reused in Fig. 3C of the article. The images from 8-week WT-infected guinea pigs published in Arora, G., et al. (2018) Front. Cell. Infect. Microbiol. 8, 385 and Singh, M., et al. (2016) Sci. Rep. 6, 26900 were inadvertently reused in Fig. 5, C and D, of the article. The error happened as experimental data shown in each of the articles were derived from the same experiment in which the growth patterns of multiple mutant strains were compared with the parental WT strain in vitro and in vivo. The authors apologize to the readers and scientific community for these errors and intend to republish these results with necessary corrections and additional data. The authors reaffirm the experimental data and state that these inadvertent mistakes do not affect the results or conclusions of the work.
PolyP homeostasis is essential for \textit{M. tuberculosis} virulence

The levels of intracellular PolyP have been associated with antibiotic tolerance in \textit{M. tuberculosis}. PolyP-deficient or -accumulating \textit{M. tuberculosis} strains display enhanced or reduced susceptibility, respectively, upon exposure to isoniazid, a cell wall inhibitor (18, 19, 23–25). Moreover, maintenance of intracellular PolyP levels is critical for \textit{M. tuberculosis} survival in host tissues.

\textit{M. tuberculosis} strains deficient in either \textit{ppk1} or \textit{ppk2} displayed attenuation in guinea pigs compared with the parental strain (19, 24). Whereas PPX2 was reported to be essential for \textit{M. tuberculosis} growth in vitro, PPX1 is important for \textit{M. tuberculosis} virulence (25–28).

In the present study, we have characterized mycobacterial exopolyphosphatases biochemically and functionally by generating a \textit{ppx2} deletion strain and also the double mutant strain (\textit{dkppx}) in \textit{M. tuberculosis}. RNA-Seq analysis revealed that PolyP accumulation in \textit{M. tuberculosis} reduces the expression of DosR-mediated dormancy and virulence genes. Our findings conclusively show that dysregulation in PolyP levels is associated with a defect in biofilm formation, stress adaptation, and virulence. These findings establish that enzymes involved in PolyP homeostasis are attractive targets to develop novel interventions to combat tuberculosis.

\section*{Results}

\textit{PPX1} and \textit{PPX2} catalyze the release of $P_i$ from PolyP

Using \textit{E. coli} PPX as a reference sequence, multiple-sequence alignment was performed using ClustalW software (Fig. 1A). The genome of \textit{M. tuberculosis} encodes for PPX1 and PPX2 that share an identity of 23 and 27\% with \textit{E. coli} homolog, respectively (Fig. 1A). The residues important for catalysis, Mg$^{2\+}$ binding, and P-loop motif (DXXGGGSX) are conserved in both PPX1 and PPX2 (29–31) (Fig. 1A).

We first determined the substrate specificity of \textit{M. tuberculosis} PPX homologs using PolyP3, PolyP17, GTP, or ATP. For biochemical characterization, both PPX1 and PPX2 were expressed and purified as MBP fusion proteins in \textit{E. coli}. We observed that both PPX1 and PPX2 possessed exopolyphosphatase, ATPase, and GTPase activity (Fig. 1B) (29–31). The maximal polyphosphatase activity of both PPX1 and PPX2 was attained within the initial 10 min of enzymatic reactions.

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{A multiple sequence alignment of protein sequences among species. Multiple-sequence alignment of protein sequences was performed using ClustalW software. The conserved residues have been highlighted in red boxes. The genome of \textit{M. tuberculosis} encodes for PPX1 and PPX2 that share an identity of 23 and 27\% with \textit{E. coli} homolog, respectively (Fig. 1A). The residues important for catalysis, Mg$^{2\+}$ binding, and P-loop motif (DXXGGGSX) are conserved in both PPX1 and PPX2 (29–31) (Fig. 1A).}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure1b.png}
\caption{B, biochemical analysis of \textit{M. tuberculosis} PPX1 and PPX2. The purified MBP, MBP-PPX1, and MBP-PPX2 were incubated with a 100\% concentration of either PolyP3 or PolyP17 or ATP or GTP at 37 °C for 10 min. The amount of $P_i$ released in enzymatic reaction was determined using the Quantichrom phosphate assay kit. The data shown in this panel are the mean ± S.E. (error bars) obtained from three independent experiments.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure1c.png}
\caption{C, Michaelis–Menten plots for PPX1 and PPX2 enzymes. The formation of $P_i$ in enzymatic assays was quantified by measuring absorbance at 630 nm using the Quantichrom phosphate assay kit. The data shown are the mean ± S.E. of initial velocities of $P_i$ released/min obtained from three independent experiments.}
\end{figure}

The presence of two or more exopolyphosphatases within a species has also been reported in other microorganisms, such as \textit{E. coli}, \textit{Vibrio cholerae}, and \textit{Corynebacterium glutamicum}. The residues important for catalysis, Mg$^{2\+}$ binding, and P-loop motif (DXXGGGSX) are conserved in both PPX1 and PPX2 (29–31) (Fig. 1A). We first determined the substrate specificity of \textit{M. tuberculosis} PPX homologs using PolyP$_3$, PolyP$_{17}$, GTP, or ATP. For biochemical characterization, both PPX1 and PPX2 were expressed and purified as MBP fusion proteins in \textit{E. coli}. We observed that both PPX1 and PPX2 possessed exopolyphosphatase activity, and PPX2 activity was higher compared with PPX1 activity (Fig. 1B). Interestingly, both PPX1 and PPX2 also displayed GTPase and ATPase activity in vitro (Fig. 1B). The maximal polyphosphatase activity of both PPX1 and PPX2 was attained within the initial 10 min of in vitro enzymatic reactions (data not shown). As shown in Fig. 1C, both exopolyphosphatase reactions
followed Michaelis–Menten kinetics with a $K_m$ of 36 μM in the case of PPX1 and 23 μM in the case of PPX2 and a $V_{\text{max}}$ of 5.6 μM min⁻¹ for PPX1 and 11.06 μM min⁻¹ for PPX2. The catalytic efficient constant ($k_{\text{cat}}/K_m$) for recombinant PPX1 and PPX2 was 0.077 μM⁻¹ min⁻¹ and 0.216 min⁻¹ μM⁻¹, respectively.

**Stress induces expression of ppx1 and ppx2 in M. tuberculosis**

TB infection in the host is an outcome from adaptation of *M. tuberculosis* to unfavorable environmental conditions within lung granulomas, such as hypoxia, nutrient limitation, nitrogen intermediates, and low pH (32, 33). Recent reports have described transient elevation of PolyP content at later stages of *M. tuberculosis* growth and in *M. tuberculosis* exposed to different growth conditions (18, 19). Therefore, we next examined the transcript levels of *ppx1* and *ppx2* are altered in these stress-related growth conditions (Fig. 2). We observed that transcript levels increased by ~4.0 and 3.0-fold exposed to nitrosative stress conditions (Fig. 2A). The transcript levels increased in most of the conditions tested (Fig. 2B). The transcript levels of *ppx1* and *ppx2* transcript levels increased by 4.0-, 3.0-, 25.0-, and 3.0-fold, respectively (Fig. 2B; *p < 0.05; **, p < 0.01; ***, p < 0.001). This suggested that Polyp homeostasis is required for biofilm formation and motility of various microorganisms including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *V. cholerae*, *Salmonella typhimurium*, and *Bacillus cereus* (11–13, 34–37). Therefore, we next examined the expression of *ppx1* and *ppx2* and *ppk1* and *ppk2* under different conditions. The strains were grown in Sauton’s medium without Tween 80 to induce biofilm formation (Fig. 3). The strains were grown in Sauton’s medium without Tween 80 to induce biofilm formation. Here, we observed that simultaneous deletion of both PKP1 and PKP2 in *M. tuberculosis* resulted in reduced biofilm formation compared with the parental strain (Fig. 3C). We also demonstrated that supplementation with either *ppx1* or *ppx2* was unable to restore the biofilm defect associated with *dkppx* strain (Fig. 3C). The levels of biofilm formation were similar in parental, *Δppx1 Dppx2* and *dkppx* strains of *M. tuberculosis* to form biofilms (Fig. 3C). The strains were grown in Sauton’s medium without Tween 80 to induce biofilm formation. Here, we observed that simultaneous deletion of both *ppx1* and *ppx2* in *dkppx* strain resulted in reduced biofilm formation compared with the parental strain (Fig. 3C). We also demonstrated that supplementation with either *ppx1* or *ppx2* was unable to restore the biofilm defect associated with *dkppx* strain (Fig. 3C). The levels of biofilm formation were similar in parental, *Δppx1* and *Δppx2* strains at 4 weeks postincubation (Fig. 3C). Previous studies have shown that altered lipid composition or metabolic growth rates are responsible for alterations in biofilm formation in *M. tuberculosis* (18, 38–41). Next, we compared the growth rates between WT and *dkppx* strain when grown planktonically in Sauton’s medium containing Tween 80. We observed that the growth kinetics of *dkppx* strain was significantly reduced compared with the parental strain (Fig. 3D). These findings confirmed that simultaneous deletion of both *ppx1* and *ppx2* in *M. tuberculosis* is responsible for altered biofilm formation in the case of *dkppx* strain.

PolyP homeostasis is critical and essential for stress adaptation and virulence of various bacterial pathogens (11–13, 35, 36, 39).
Earlier studies have related metal tolerance to PolyP levels in bacteria, and it has been reported that metals stimulate PolyP degradation and that metal–phosphate complexes are exported from the cell (42–44). Because PPX enzymes are involved in PolyP metabolism, we hypothesized that these enzymes would also contribute to M. tuberculosis adaptation to different stress conditions and persistence in the host. To test this hypothesis, we compared the ability of the parental strain, the ppx1 mutant strain, and the dppx mutant strain to survive in different stress conditions in sealed tubes (Fig. 4A; *, p < 0.05). We noticed that upon 14 days of exposure to nutrient-limiting conditions, relative to WT strain, the dppx mutant strain resulted in increased tolerance to isoniazid, a cell wall inhibitor (Fig. 4B; *, p < 0.05). In contrast, the bacterial loads in dppx-infected macrophages increased marginally by 2.25-fold at day 6 post-infection (Fig. 4C; *, p < 0.05; **, p < 0.01). The Δppx1 strain displayed a growth defect of ~2.5-fold at both days 4 and 6 post-infection (Fig. 4C; *, p < 0.05), but Δppx2 strain did not show any difference at any time point post-infection (Fig. S3C). Furthermore, in concordance with previously published reports, we also observed that PolyP accumulation in dppx strain resulted in increased tolerance to isoniazid, a cell wall inhibitor (Fig. 4D; *, p < 0.05) (18, 19, 23). However, both WT and dppx mutant strains were susceptible to comparable levels upon exposure to levofloxacin (Fig. 4D). This tolerance to isoniazid was also a

**PolyP homeostasis is essential for M. tuberculosis virulence**

Figure 3. A, growth kinetics of WT and dppx strains in MB7H9 medium. The effect of deletion of ppx and dppx on growth was determined by measuring A600 nm until stationary phase using a visible spectrophotometer. The data shown are representative of three independent experiments. B, quantification of PolyP levels in parental, Δppx1, Δppx2, and dppx mutant strains. PolyP levels were determined at mid-log phase (A600 nm ~ 1.0) or late log phase (A600 nm ~ 2.5), and intracellular PolyP levels were determined by measuring A600 nm until stationary phase using a visible spectrophotometer. The data shown are representative of three independent experiments. Significant differences were observed for the indicated groups (paired, two-tailed, t test; *, p < 0.05; **, p < 0.01). C, effect of ppx deletion on biofilm formation of M. tuberculosis. The plates were incubated at 37 °C without shaking for 4 weeks. The images shown are representative of three independent experiments. D, growth kinetics of WT and dppx strain in Sauton’s medium. The effect of deletion of ppx and dppx on growth was determined by measuring A600 nm until stationary phase using a visible spectrophotometer. The data shown are representative of three independent experiments. Significant differences were observed for the indicated groups (paired, two-tailed, t test; *, p < 0.05; **, p < 0.01).
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Figure 4. A and B, susceptibility of WT and dkppx mutant strains to different stress conditions. Early logarithmic phase cultures of various strains were washed, harvested, and exposed to TBS-Tween 80 for either 7 or 14 days. B, parental or mutant strains (Fig. S3D). These findings implicate that PPX-mediated degradation of PolyP is essential for biofilm formation, survival in macrophages, and INH tolerance.

Exopolysphatases are required for M. tuberculosis to establish infection in guinea pigs

M. tuberculosis infection in guinea pig occurs with an initial phase of growth termed as acute infection. However, upon onset of adaptive immunity, bacterial growth slows down, but M. tuberculosis is able to persist in the host. We had previously demonstrated that PolyP deficiency in M. tuberculosis is associated with impaired chronic infection and reduced gross pathology in lung tissues of guinea pigs (19). Moreover, PolyP-accumulating strains with deletions in either PXP1 or PPK2 are also impaired for growth in guinea pigs in both acute and chronic stages of infection (24, 25). To determine the involvement of ppx2 in in vivo infection, guinea pigs were aerosol-infected with either WT or Δppx2 mutant or Δppx2 complemented strains. We observed that Δppx2 strain was attenuated for growth and displayed a 40.0-fold reduction in lungs and splenic bacillary counts compared with WT-infected guinea pigs at 4 weeks post-infection (Fig. 5A; **, p < 0.01; ***, p < 0.001). The Δppx2 mutant strain also exhibited a persistence defect in the chronic stage of infection. The bacterial loads were reduced in spleens and lungs by 70.0- and 20.0-fold, respectively, compared with WT-infected guinea pigs at 8 weeks post-infection (Fig. 5A; **, p < 0.01; ***, p < 0.001). This growth defect associated with Δppx2 strain was restored in the complemented strain, indicating that the observed attenuation in vivo was due to loss of ppx2 function (Fig. 5A; **, p < 0.01).

To further unravel the collective biological function of exopolysphatases, ppx1 and ppx2, in M. tuberculosis persistence, in vivo infection of parental and dkppx mutant strain of M. tuberculosis was performed in guinea pigs. Relative to parental strain, the lung bacillary loads were reduced by 300.0-fold in guinea pigs infected with dkppx mutant strain at 8 weeks post-infection (Fig. 5B; ***, p < 0.001). As shown in Fig. 5B, dkppx mutant strain was cleared more rapidly from spleens of infected guinea pigs at 8 weeks post-infection (***, p < 0.001). In both these experiments, aerosol infection resulted in implantation of ~100 bacilli in lungs of guinea pigs infected with either parental or mutant strains (Fig. 5, A and B). This attenuated phenotype associated with dkppx strain was also evident in histological analysis of the lung sections. Pathology of lung tissues from guinea pigs infected with WT strain at 8 weeks post-infection displayed typical features of M. tuberculosis infection with multiple necrotizing granulomas, whereas the animals infected with dkppx mutant strain showed normal lung parenchyma with minimum inflammation or granuloma formation.
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In agreement with bacterial loads, the total granuloma score in lung sections of guinea pigs infected with the dkppx mutant strain at 8 weeks post-infection was reduced by ~8.0-fold compared with lung sections from WT-infected guinea pigs (data not shown).

Reduced expression of DosR-regulated dormancy genes in dkppx mutant strain of M. tuberculosis

To gain insights into physiological roles of intracellular PolyP, transcriptional profiling was performed on RNA isolated from mid-log phase cultures of WT and dkppx mutant strains of M. tuberculosis. The detailed analysis of the transcriptional profiles using a p value of 0.05 and 2.0-fold cutoff revealed that, relative to WT strain, approximately 202 genes are differentially expressed in dkppx mutant strain (Table S1). Of these, 25 transcripts were up-regulated, whereas 177 transcripts were down-regulated. We further characterized these differentially regulated genes as per their annotations in Tuberculist (www.tuberculist.epfl.ch).

Interestingly, many transcripts down-regulated in the dkppx mutant strain encoded for conserved hypothetical proteins or proteins that are involved in cell wall processes or intermediary metabolism (Fig. 6A). In the case of up-regulated genes, the majority of these transcripts encoded for conserved hypothetical proteins (Fig. 6A).

The DosR (DevR) response regulator of M. tuberculosis forms part of a two-component system with DosS (DevS) and DosT. Several studies have shown that exposure to low-oxygen conditions results in drastic changes of gene expression (46–49). This gene regulation is mediated by the DosSR (DevSR) two-component system and is important for survival of M. tuberculosis under hypoxic conditions and for it to enter the nonreplicating dormant state. Interestingly, DosR-regulated dormancy genes were also significantly down-regulated in dkppx mutant strain (49) (Fig. 6B). Included in these genes were dormancy-regulated genes, α-crystallin or aeg, required for M. tuberculosis survival in infected macrophages and mice (50), and fdeA, a ferredoxin, known to be up-regulated in hypoxia, in acidic conditions, and in macrophages (49, 51). Similarly, the transcripts of narX and narK2, proteins involved in nitrate reduction and transport, were also reduced in dkppx mutant strain (49). Moreover, the transcript levels of universal stress proteins (USPs; Rv1996, Rv2005c, Rv2623, and Rv3134c) were

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Figure 5. A and B, exopolyphosphatases are essential for M. tuberculosis to establish infection in guinea pigs. Guinea pigs were infected with either WT or Δppx2 or Δppx2-CT or dkppx mutant strain via the aerosol route. Bacterial loads were determined in spleens and lungs of infected animals at 4 and 8 weeks post-infection as described under “Experimental procedures.” The data shown in this panel are the mean ± S.E. (error bars) of log_{10} CFU obtained from 7 or 8 guinea pigs/strain. Significant differences were observed for the indicated groups (paired, two-tailed, t test; **, p < 0.01; ***, p < 0.001). C and D, gross pathological and histological analysis of sections of guinea pigs infected with WT or dkppx strain at 8 weeks post-infection. A representative high-resolution image for each group is shown in this panel. Scale bar, 1.0 mm. D, representative photomicrographs (40 magnification) of hematoxylin and eosin–stained lung sections of guinea pigs infected with WT or dkppx strain at 8 weeks post-infection. The lung sections from dkppx-infected guinea pigs displayed less pathological damage compared with sections from WT-infected guinea pigs (Fig. 5, C and D). In agreement with bacterial loads, the total granuloma score in lung sections of guinea pigs infected with dkppx mutant strain at 8 weeks post-infection was reduced by ~8.0-fold compared with lung sections from WT-infected guinea pigs (data not shown).
also reduced in dkppx mutant strain (52). These proteins enable M. tuberculosis to adapt to conditions such as nitric oxide, reactive oxygen, and acidic pH (52). Another DosR-regulated gene, tgs-1, implicated in utilization of host triacyl glycerol for lipid synthesis by M. tuberculosis, was also down-regulated in dkppx mutant strain (53). We confirmed down-regulation of several members of the DosR regulon in mid-log phase cultures of dkppx mutant strain by qPCR using gene-specific primers (Fig. 6C; **, p < 0.01; ***. p < 0.001). Among these down-regulated genes, 17 have been predicted to be essential for in vitro growth of M. tuberculosis (26, 28). However, we did not observe any differences in the transcript levels of these genes in early-log phase cultures of dkppx mutant strain (Fig. 6D).

These observations suggested that PolyP accumulation might result in inhibition of DosS- or DosT-mediated regulation of dormancy-associated genes in mid-log phase cultures (54, 55). To test this hypothesis, we studied the effect of PolyP on autophosphorylation of DosT and DosS in vitro. In concordance with our RNA-Seq data, we observed that PolyP inhibited DosT and DosS autophosphorylation activity in a dose-dependent manner (Fig. 7A). Interestingly, PolyP did not inhibit the autophosphorylation activities of other sensor kinases, such as PdtaS, KdpD, MtrB, and PrrB (Fig. S4). Using microscale thermophoresis assays, in the presence of increasing concentrations of PolyP, $K_{d}$ values of 1.52 ± 0.13 and 1.64 ± 0.11 mM were determined for DosT and DosS binding, respectively (Fig. 7B). Several studies have shown that the M. tuberculosis dormancy regulon is up-regulated in vitro upon exposure to either nitric oxide or carbon monoxide or ascorbic acid or low oxygen (56). Next, we investigated the effect of PolyP accumulation on the expression of DosR-regulated genes in parental and dkppx strains upon exposure to 10 mM ascorbic acid. As shown in Fig. 7C, the transcription of various DosR-regulated genes was increased in parental strain compared with the dkppx strain when exposed to 10 mM ascorbic acid. Taken together, we demonstrate that PolyP accumulation in M. tuberculosis results in suppression of the expression of dormancy-associated genes, which might be responsible for the observed attenuation of the dkppx strain in guinea pigs. However, this PolyP-dependent suppression of the DosR regulon was not responsible for the enhanced isoniazid tolerance of the dkppx strain. We observed that deletion of dosR in the M. tuberculosis genome did not affect persister cell formation upon exposure to either levofloxacin or isoniazid for 14 days (data not shown). These obser-
PolyP homeostasis is essential for M. tuberculosis virulence

In this study, we have enhanced our present understanding of the contribution of PolyP homeostasis in mycobacterial physiology. We biochemically characterized PPX homologs from M. tuberculosis and show that both PPX1 and PPX2 utilize polyphosphates of different chain lengths as substrates. Similar to PPX enzymes from other microorganisms, both homologs displayed a pH optimum of 7.0–8.0 and required Mg²⁺ ion for maximal enzymatic activity in vitro. In addition to exopolyphosphatase activity, these enzymes also possessed ATPase and GTPase activity. Previously, PPX2 has been reported to be an essential gene for M. tuberculosis growth in vitro and up-regulated during the chronic stage of infection (27, 28). However, we were able to generate Δppx2 single and dkppx double mutant strains by homologous recombination in M. tuberculosis. We observed functional redundancy between these two homologs, and only the dkppx mutant strain of M. tuberculosis was slightly impaired for growth in phosphate-limiting growth conditions. This growth defect is most likely to be associated with the inability of mutant strain to degrade PolyP into Pi.

In M. tuberculosis, previous studies have shown that deletion of either PPK2 or PPX2 results in PolyP accumulation along with a defect in biofilm formation, probably as a result of a metabolic adjustment of the TCA cycle and lipid oxidation by an unknown mechanism (18, 23). Additionally, the overexpression of PPK2 in M. smegmatis has been linked to low PolyP levels that affect its sliding motility and biofilm formation (57). Our results showed that dkppx mutant strain was impaired in its ability to form biofilms compared with single PPX mutants and the parental strain of M. tuberculosis in detergent-free Sauton’s medium. We speculate that the biofilm formation defect of dkppx is due to metabolic shift induced by PolyP accumulation rather than due to the absence of certain cell wall components. Contrary to previous reports, biofilm defect was not observed in single ppx mutant strains of M. tuberculosis. This might be attributable to different parental strains used in these studies. The parental strain used in our study was H37Rv, whereas CDC1551 was the parental strain used by Chuang et al. (18). These differences could be due to inherent differences in the intracellular PolyP levels achieved in single-mutant and transposon mutant strains generated in our study and that of Chuang et al. (18), respectively. Further, the differences could be due to other signaling differences, which are known to vary between CDC1551 and H37Rv. In concordance, exopolyphosphatase-mediated PolyP degradation into Pi is essential for biofilm formation in other microorganisms, such as E. coli, B. cereus, Campylobacter jejuni, and P. aeruginosa (57–59).

In addition to biofilm formation, we also show that PPX-mediated PolyP degradation is required for M. tuberculosis survival in nutrient-limiting and low-oxygen growth conditions. Consistent with previous studies, we show that the dkppx mutant strain has reduced susceptibility to the cell wall-targeting drug isoniazid, whereas similar levels of susceptibility...
against levofloxacin, a replication inhibitor, were observed (18, 19, 23, 24). This observed drug tolerance was not due to the emergence of drug-resistant mutants but because of the presence of drug-tolerant persister. Contrary to previous reports, we did not observe increased tolerance to isoniazid in the case of \( \text{H9004ppx2} \) strain (18). The comparison of transcription profiles between RNA isolated from mid-log phase cultures of WT and \( \text{dkppx} \) mutant strain indicates that expression of DosR-regulated dormancy-associated genes was significantly reduced in the mutant strain. We also show that PolyP inhibits the autophosphorylation activity of both DosT and DosS sensor kinases in a dose-dependent manner. These findings suggest that PolyP degradation by exopolyphosphatases is essential for dormancy-associated adaptive response (Fig. 8). These findings implicate that in addition to low oxygen, nitric oxide, carbon monoxide, and vitamin C, DosR-mediated gene expression is also regulated by PolyP levels in the bacteria (55, 60, 61). We also show that PolyP degradation into \( \text{P_i} \) is important for \( M. \text{tuberculosis} \) to establish infection in guinea pigs. As expected relative to WT strain, the growth defect was more pronounced in \( \text{dkppx} \) mutant–infected guinea pigs compared with \( \text{Δppx2} \)-infected guinea pigs. These observations suggest that reduction in expression levels of these DosR-mediated pathways is likely to be associated with the attenuated fitness of \( \text{dkppx} \) mutant strain in vivo.

In conclusion, we expand the present understanding of the multifactorial contribution of PolyP homeostasis in \( M. \text{tuberculosis} \) physiology and pathogenesis. We establish that \( M. \text{tuberculosis} \) PolyP levels are tightly regulated during different stages of growth and propose that modulation of PolyP levels by inhibiting either PolyP synthesis or its degradation might be an attractive target to combat tuberculosis. These modulators of PolyP homeostasis might also be effective against other bacteria that require this balance to maintain their intracellular survival.

**Experimental procedures**

**Chemicals and reagents**

Unless otherwise indicated, all chemicals used in the study were procured from either Merck or Thermo Fisher Scientific.
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Table 1
List of strains and plasmids used in the present study

| Strains and plasmids | Description | Source/Reference |
|----------------------|-------------|-----------------|
| **Bacterial strains** | E. coli XL-1 blue | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIq ZAM15 Trn10 (Tetr)] | Stratagene |
| E. coli HB101 | F’, thi-1, hsdS20 (rK- mK- sU54, mB7 P8 rK+ sM11), supE44, recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 | Promega, UK |
| E. coli TB-1 | F’ araD1 (lac-proAB)/d80lacDX (lacZM15)/rpsL1 (Str”) thi hsdR | New England Biolabs |
| M. tuberculosis H37Rv | M. tuberculosis parental strain | ATCC |
| Δppx1 | Rv0496 mutant strain of M. tuberculosis | This study |
| Δppx2 | Rv1026 mutant strain of M. tuberculosis | This study |
| Δppx2-CT | Rv1026 mutant strain of M. tuberculosis complemented with Rv1026 | This study |
| dkppx | Rv0496 and Rv1026 double mutant strain of M. tuberculosis | This study |
| dkppx-ppx1 | dkppx mutant strain of M. tuberculosis complemented with Rv0496 | This study |
| dkppx-ppx2 | dkppx mutant strain of M. tuberculosis complemented with Rv1026 | This study |

**Plasmids**

| Plasmids | Description | Source/Reference |
|-----------|-------------|-----------------|
| pGEM-T easy | T/A cloning vector | Promega, UK |
| pMal-c2x | Prokaryotic expression vector | New England Biolabs |
| pMal-ppx1 | pMal-c2x harboring Rv0496 | This study |
| pMal-ppx2 | pMal-c2x harboring Rv1026 | This study |
| pYUB854 | Cloning vector | Ref. 63 |
| pYUB854Δppx1:hyg′ | pYUB854 with Rv0496 upstream and downstream region flanking the hygromycin resistance gene | This study |
| pYUB854Δppx2:hyg′ | pYUB854 with Rv1026 upstream and downstream region flanking the hygromycin resistance gene | This study |
| phAE87 | Temperature-sensitive mycobacteriophages | Ref. 63 |
| phAE87Δppx1:hyg′ | phAE87 derivative to replace Rv0496 with hygromycin resistance gene | This study |
| phAE87Δppx2:hyg′ | phAE87 derivative to replace Rv1026 with hygromycin resistance gene | This study |
| phAE87Δppx2:kn′ | phAE87 derivative to replace Rv1026 with kanamycin resistance gene | This study |
| pEB402 | E. coli–mycobacterium shuttle | A kind gift from Dr. Amit Kumar Pandey |
| pEB402-ppx2 | pEB402 harboring Rv0496 | A kind gift from Dr. William R. Jacobs |
| pMV306-apramycin | E. coli–mycobacterium shuttle | This study |
| pMV306-ppx1-apramycin | pMV306 harboring Rv1026 | This study |
| pMV306-ppx2-apramycin | pMV306 harboring Rv0496 | This study |

* The plasmids used for sensor kinase autophosphorylation assays were described previously (69).

Multiple-sequence alignment

The protein sequences were compared in the NCBI database with a search item of polyphosphatase from M. tuberculosis. Multiple-sequence alignment was performed using ClustalW software, and the alignment outcome was formatted using Escrip version 3.0.

Expression, purification, and biochemical assays

The ppx genes were PCR-amplified from M. tuberculosis H37Rv using Platinum TaqDNA polymerase and gene-specific oligonucleotides. The sequences of oligonucleotides used in the present study are shown in Table S2. PPX amplicons were sequenced and cloned into pMALc2x. PPX enzymes were purified as MBP fusion proteins, and purified fractions were pooled and stored in buffer containing 20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol. Biochemical assays were performed in assay buffer (50 mM Tris-Cl, pH 7.4, 20 mM MgCl₂, and 25 mM KCl) containing varying concentrations of ATP, GTP, PolyP₃, and PolyP₅, and 2 µM PPX1 or PPX2. At designated time points, P₅ released in an enzymatic reaction was quantified using the QuantiChrom phosphate assay kit (BioAssay Systems) by measuring absorbance at 630 nm using a Synergy HT Plate reader (BioTek) (62). PolyP, ATP, and GTP for biochemical assays was procured from Merck.

Microorganisms and culturing conditions

E. coli strains XL-1 blue and HB-101 were used for cloning and TB-1 for protein expression and purification. Various M. tuberculosis strains are derived from the H37Rv ATCC27294, respectively (Table 1). Various mycobacterial strains were cultured in either MB7H9 or MB7H11 medium, as described previously (19). When appropriate, antibiotics were added at the following concentrations: kanamycin (25 µg/ml for both E. coli and mycobacteria), hygromycin (150 µg/ml for E. coli and 50 µg/ml for mycobacteria), and apramycin (50 µg/ml for mycobacteria).

Mutant and complemented strain construction

Individual M. tuberculosis mutant strains were generated in a manner such that Rv0496 (ppx1) and Rv1026 (ppx2) were replaced with hygromycin resistance, respectively, using temperature-sensitive mycobacteriophages. Briefly, for the construction of Δppx1 and Δppx2 mutant strains of M. tuberculosis, the first and last 10 codons of the ORF was replaced with a hygromycin resistance cassette by homologous recombination (63). The dkppx double mutant strain of M. tuberculosis was generated by replacing ppx2 with the kanamycin resistance gene in the genome of Δppx1 using temperature-sensitive mycobacteriophages (63). For construction of the Δppx2-CT strain of M. tuberculosis, ppx2 was PCR-amplified and cloned...
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For complementation of the M. tuberculosis *dpkpx* strain with *ppx2*, Rv1026 was cloned under the control of *hsp60* promoter in pMV306-apramycin. For complementation of the *dpkpx* strain with *ppx1*, Rv0496 was PCR-amplified along with its native promoter and cloned into pMV306-apramycin. The deletion of these exopolyphosphatases in their respective mutant strains was confirmed by PCR or qPCR or Southern blotting hybridization.

**PolyP quantification assay**

At designated time points, PolyP was extracted and quantified using a DAPI-based assay as described previously (64–66). The PolyP concentration in bacterial samples was determined by measuring fluorescence of the DAPI–PolyP complex (excitation 415 nm and emission 525 nm) using a Synergy HT plate reader (BioTek). Increasing concentrations of commercially available PolyP were used to generate a standard curve to determine PolyP levels in bacterial samples.

**qPCR analysis**

For qPCR studies, total mRNA was extracted from *M. tuberculosis* exposed to different growth conditions using TRIzol reagent as described previously (19). For qPCR experiments, RNA was isolated from mid-log phase cultures (*A*$_{600 \text{ nm}}$ ~ 1.0) or from early log phase cultures exposed to the following conditions: 5 mM H$_2$O$_2$ in MB7H9 medium for 6 h; 5 mM NaNO$_2$ in MB7H9 medium, pH 5.2, for 6 h; TBS, 0.05% Tween 80 for 7 days, low-oxygen conditions for 30 days, and phosphate-free MB7H9 medium, pH 5.2, for 6 h; 5 mM H$_2$O$_2$ in MB7H9 medium for 7 days. 1 μg of mRNA was treated, and cDNA was prepared using Superscript III reverse transcriptase. The *M. tuberculosis* qPCR using gene-specific primers and SYBR Green mix (Applied Biosystems). The data obtained were normalized to *qPCR* using gene-specific primers and SYBR Green mix reverse transcriptase. The synthesized cDNA was subjected to treatment, and cDNA was prepared using Superscript III enzyme.

**In vitro M. tuberculosis stress, drug tolerance, and biofilm experiments**

For stress experiments, various *M. tuberculosis* strains were grown until late log phase and diluted in fresh medium until *A*$_{600 \text{ nm}}$ reached 0.2–0.3. To measure susceptibility upon exposure to oxidative stress, cultures were diluted and incubated with 5 mM H$_2$O$_2$ for 24 h at 37 °C. For nitrosative stress, cultures were harvested and incubated at pH 5.2 with 5 mM NaNO$_2$ for 3 days. To understand the role of PPX enzymes in the adaptation of *M. tuberculosis* to nutritional stress, early-log phase cultures were washed with 1× TBS-Tween 80 (1× TBST) and resuspended in 1× TBST for either 7 or 14 days. The survival of parental and *dpkpx* strain was also compared after incubation with either 0.25% SDS for 3 days, 2.5 mg/ml lysozyme for 3 days, or 500 μM CuSO$_4$ for 7 days. For drug tolerance experiments, mid-log phase cultures were exposed to either 10 μM/ml levofloxacin or 10 μg/ml isoniazid for 14 days. For bacterial enumeration, 10.0-fold serial dilutions were prepared, and 100 μl was plated on MB7H11 plates at 37 °C for 3–4 weeks. For biofilm formation in *M. tuberculosis*, various strains were grown until mid-log phase, diluted in detergent-free Sauton’s medium in 6-well plates, Parafilm-sealed, and incubated at 37 °C for 4 weeks without shaking (67).

**Macrophage experiments**

For macrophage experiments, THP-1 monocytes were differentiated using 25 ng/ml PMA and seeded at a cell density of 2 × 10^5/well in 24-well plates. The next day, THP-1 macrophages were infected with single cell suspensions of log-phase cultures of *M. tuberculosis* at a multiplicity of infection of 1:1. After 4 h of infection, macrophages were washed with antibiotic-free RPMI medium and overlaid with medium containing 200 μg/ml amikacin for 2 h. At 6 h, 2 days, 4 days, and 6 days post-infection, infected macrophages were lysed with 1× PBS containing 0.1% Triton X-100 (1× PBST). For bacterial enumeration, 10.0-fold serial dilutions were prepared, and 100 μl was plated on MB7H11 plates at 37 °C for 3–4 weeks, as described previously (68).

**Animal experiments**

The animal experiments were performed as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) guidelines. The protocols were reviewed and approved by the institutional animal ethics committee of the International Centre for Genetic Engineering and Biotechnology (ICGEB) and Translational Health Science and Technology Institute (THSTI). For infection studies, female guinea pigs (weight 300 g) were exposed to 10^7 cfu of *M. tuberculosis* through various strains via the aerosol route. For bacterial enumeration, organs were harvested at 4 and 8 weeks post-infection, bacterial loads were enumerated in organs of infected animals at 4 and 8 weeks post-infection, organs were homogenized, and 2 ml of MB7H9 medium, 10.0-fold serial dilutions were prepared, and 100 μl was plated on MB7H11 plates at 37 °C for 3–4 weeks. For histopathology analysis, the upper left lobe from guinea pig lungs was formalin-fixed and stained with hematoxylin and eosin. The number and nature of granulomas and total granuloma score were determined by a histopathologist as described previously (24).

**RNA-Seq experiments**

For RNA-Seq experiments, total RNA was isolated and DNase I–treated, and RNA quality was assessed using an Agilent Bioanalyzer. The RNA samples were sent to AgriGenome Labs Pvt Ltd. (India) for library preparation and sequencing using Illumina HiSeq2000. The preprocessed high-quality reads were aligned with the *M. tuberculosis* H37Rv genome obtained from Ensemble (https://mycobrowser.epfl.ch/) using the Tophat program. Using these aligned reads, differential expression of transcripts was performed using the Cufflinks program (version 2.2.1). Standard cutoffs of 2.0-fold change and corrected *p* values (*p* < 0.05) were used to identify differentially expressed genes.

**Sensor kinase autophosphorylation assays**

10 μM purified recombinant mycobacterial sensor kinases (as indicated) were incubated as reported previously and were preincubated for 10 min at 30 °C with varying concentration of PolyP$_{17}$ in autophosphorylation buffer (50 mM Tris-HCl, pH
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8.0, 50 mM KCl, 10 mM MgCl₂) (69). The autophosphorylation reactions were initiated by the addition of 50 μM ATP and 2 μCi of γ-32P-labeled ATP for 2 h. The reactions were terminated by the addition of 1× SDS-PAGE sample buffer. The samples were resolved on 15% SDS-PAGE, and the gel was washed and exposed to a phosphor screen for 4 h followed by imaging with a Typhoon 9210 phosphor imager (GE Healthcare).

Microscale thermophoresis studies

The fluorescently tagged or labeled proteins were incubated with increasing concentrations of PolyP, and binding was analyzed using a Monolith NT-115 instrument (NanoTemper Technologies, GmbH) (70). The samples were excited for 30 s using the red laser (microscale thermophoresis power = 60%, LED power 100%). The data obtained were analyzed using MO Control software (NanoTemper Technologies, GmbH), and K_D for PolyP binding to these proteins was determined.

Statistical analysis

Prism 5 software (version 5.01; GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis and the generation of graphs. For normally distributed data, comparisons were performed by a paired (two-tailed) t test. Differences between groups were considered significant at p < 0.05.

Author contributions—R. S. conceived the idea and supervised the M. tuberculosis experiments. P. T., T. P. G., M. S., G. A., and S. K. performed M. tuberculosis microbiology experiments. M. S., P. T., and G. A. conducted guinea pig microbiology experiments. S. C. performed in vitro autophosphorylation assays. G. S. performed binding studies. D. K. S. supervised radioactivity assays. R. S., P. T., S. C., and P. T. G. conducted guinea pig experiments. G. S. and S. A. performed guinea pig experiments. D. K. S. wrote the manuscript with input from the other authors.

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