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Polyphosphate kinase is involved in stress-induced *mprAB-sigE-rel* signalling in mycobacteria

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Summary

Polyphosphate kinase 1 (PPK1) helps bacteria to survive under stress. The *ppk1* gene of *Mycobacterium tuberculosis* was overexpressed in *Escherichia coli* and characterized. Residues R230 and F176, predicted to be present in the head domain of PPK1, were identified as residues critical for polyphosphate (polyP)-synthesizing ability and dimerization of PPK1. A *ppk1* knockout mutant of *Mycobacterium smegmatis* was compromised in its ability to survive under long-term hypoxia. The transcription of the *rel* gene and the synthesis of the stringent response regulator ppGpp were impaired in the mutant and restored after complementation with *ppk1* of *M. tuberculosis*, providing evidence that PPK1 is required for the stringent response. We present evidence that PPK1 is likely required for *mprAB-sigE-rel* signalling. σ^E regulates the transcription of *rel*, and we hypothesize that under conditions of stress polyP acts as a preferred donor for MprB-mediated phosphorylation of MprA facilitating transcription of the *sigE* gene thereby leading finally to the enhancement of the transcription of *rel* in *M. smegmatis* and *M. tuberculosis*. Downregulation of *ppk1* led to impaired survival of *M. tuberculosis* in macrophages. PolyP plays a central role in the stress response of mycobacteria.

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Introduction

Inorganic polyphosphate (polyP) is a polymer of tens or hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. PolyP is ubiquitous (Kornberg *et al*., 1999). The enzyme that is primarily responsible for reversible polyP synthesis in *Escherichia coli* is polyphosphate kinase 1 (PPK1) which catalyses the polymerization of the γ-phosphate of ATP into a polyP chain (Ahn and Kornberg, 1990; Akiyama *et al*., 1992). PPK1 is highly conserved in many bacterial species including some of the major pathogens, but it is absent in eukaryotes (Tzeng and Kornberg, 2000), making it an attractive target for drug development. Studies in *E. coli* and *Pseudomonas aeruginosa* have shown that *ppk1* plays an important role in bacterial survival under conditions of stress such as in the stationary phase, under nutrient limitation and under oxidative stress (Rao and Kornberg, 1996; Kuroda *et al*., 1999). In *P. aeruginosa*, decreased levels of polyP have been linked to defect in biofilm formation and quorum sensing, with associated attenuation of virulence (Rashid *et al*., 2000a,b). The possible physiological roles of polyP have been elegantly reviewed by Brown and Kornberg (2004).

A third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. New drugs are needed to shorten the course of therapy. At the same time, there is an urgent need to develop an effective vaccine. In order to meet these goals it is imperative to understand the biology of the tubercle bacillus, to identify gene products that are essential for mycobacterial growth *in vivo*, and those that when inactivated would lead to an attenuated phenotype. *M. tuberculosis* is an intracellular pathogen which survives and replicates within macrophages. Persistent mycobacteria in lung lesions encounter hypoxia and nutritional deprivation and these mycobacterial cells regain acid-fastness and start growing even 2 years after removal of the starvation-induced stress (Nyka, 1974). Overpowering the persistent bacteria, therefore, poses a challenge. Drug-induced inhibition of translation is associated with the downregulation of *ppk1*, the polyphosphate kinase 1 (PPK1) of *M. tuberculosis* (Boshoff *et al*., 2004), suggesting a role of polyP metabolism in the response to translational inhibition, a situation encountered during starvation. In spite of the widening interest in

polyP metabolism, there has been no detailed investigation on polyP metabolism in the genus *Mycobacterium*. Genes that likely encode counterparts of *ppk1* have been identified both in *M. tuberculosis* and in its fast-growing non-pathogenic counterpart *Mycobacterium smegmatis*, widely used as a model system to study mycobacterial physiology. In this study, the putative *ppk1* genes of *M. tuberculosis* and *M. smegmatis* have been cloned and expressed in *E. coli*, and the corresponding protein products have been characterized. The *ppk1* gene of *M. smegmatis* has been inactivated in order to study the role of *ppk1* in survival of the bacterium under conditions of stress. We report that *ppk1* is involved in survival of the bacterium under conditions of hypoxia, detergent stress and oxidative stress. We have uncovered a novel regulatory signalling cascade in which *ppk1* is required for the transcription of the two-component signal transduction system *mprAB*, which in turn regulates the expression of *sigE*, a stress-regulated σ-factor. We show for the first time that σ^E regulates the transcription of the stringent response regulator *rel*. Delineation of this pathway explains how inactivation of *ppk1* downregulates *rel* and the alarmone ppGpp. Downregulation of *ppk1* of *M. tuberculosis* is associated with a loss in the ability of the bacterium to survive in macrophages supporting the view that *ppk1* plays an important role in the intracellular life of the bacterium.

Results

Expression of ppk1 *of* M. tuberculosis *and* M. smegmatis *and activity assays*

The genes encoding putative counterparts of PPK1 have been identified in *M. tuberculosis* H37Rv (Rv2984) and *M. smegmatis* mc^2155 (MSMEG2390). The putative PPK1 of *M. tuberculosis* and *M. smegmatis* were of 742 and 736 amino acid residues respectively (Fig. S1). *M. tuberculosis* and *M. smegmatis* were 85% similar and 79% identical. PPK1 of *M. tuberculosis* was 74%, 54% and 56% similar to PPK1 of *Streptomyces coelicolor*, *E. coli* and *P. aeruginosa*, respectively; and 59%, 33% and 38% identical to the respective proteins. The gene products were expressed as N-terminal His-tagged proteins in *E. coli* and purified by affinity chromatography on Ni^{2+}-NTA agarose (Fig. S2A).

Antibody against PPK1 of *M. tuberculosis* was raised in rabbits. In *E. coli*, PPK1 is present in the membrane (Kuroda and Kornberg, 1997). The Kyte–Doolittle hydrophobicity plot suggested the presence of several stretches of hydrophobic amino acids in PPK1 of *M. tuberculosis* (Fig. S2B). In harmony with this, immunoblotting of the membrane fraction of *M. tuberculosis* with anti-PPK1 showed a prominent band of 81 kDa corresponding to the position of recombinant PPK1. The antibody also cross-reacted with a band of similar size in membrane fractions of *M. smegmatis* (Fig. S2C).

His-PPK1 of *M. tuberculosis* and *M. smegmatis* were both capable of converting [γ-^{32}P]-ATP to acid-insoluble polyP *in vitro*. The recombinant *M. tuberculosis* and *M. smegmatis* PPK1 catalysed polyP synthesis with specific activities of 4.5 ± 0.16 and 4.20 ± 0.23 μmol min^{-1} per mg of protein respectively. PPK1 was immunoprecipitated from membrane extracts of *M. tuberculosis* in order to assay specifically for PPK1 activity. The immunoprecipitated enzyme showed a specific activity of 0.05 μmol min^{-1} per mg of total membrane protein. The supernatant after immunoprecipitation was subjected to immunoblotting with anti-PPK1 antibody. No cross-reactive band was detected, suggesting that no residual PPK1 was left in the supernatant after immunoprecipitation.

Similar to other PPKs, *M. tuberculosis* PPK1 transferred the γ-phosphate of ATP processively to generate polyP chains of lengths 200–800 residues (data not shown). Addition of unlabelled ATP in the reaction mixture could inhibit (> 90%) incorporation of radiolabel into polyP, whereas unlabelled GTP was less effective (< 15%). *M. smegmatis* PPK1 also showed similar characteristics (data not shown). Both proteins could also synthesize ATP by breaking down polyP isolated from mycobacteria, with activities of 0.67 and 0.7 μmol min^{-1} per mg of protein for the *M. smegmatis* and *M. tuberculosis* proteins respectively. These results validated the functions of the identified proteins as PPKs in mycobacteria.

Analysis of some PPK1 point mutations

Directed mutagenesis of *E. coli* PPK1 (Tzeng and Kornberg, 2000) has helped to identify critical residues required for activity, within the 300-residue carboxy-terminus (which is highly conserved among species). However, little is known about the less conserved region which encompasses the head domain (Zhu *et al.*, 2004). In this study, we therefore focused on this less conserved region (spanning residues 155–376 of *M. tuberculosis* PPK1) for mutational analysis in order to gain insight into the critical residues in this region that are required for PPK1 function. Based on sequence similarity, seven conserved residues within this region were mutated individually to alanines. PPK activity was assayed in all these mutants. Five of the mutants exhibited PPK activities similar to that of the wild type. Two mutants, F176A and R230A, showed significant reduction in the ability to synthesize polyP as well as to regenerate ATP by the breakdown of polyP (Table 1). The mutation R230K did not cause the marked decrease in activity observed with the R230A mutant. The F176Y mutation also showed a far lesser inhibitory effect than the F176A mutation. PPK1 is

Table 1. PolyP-synthesizing activity of PPK1 mutants.

Mutants	Synthesis of polyP from ATP	Generation of ATP by the breakdown of polyP
F176A	8 ± 1	5 ± 1
F176Y	84 ± 7	89 ± 6
P225A	86 ± 6	90 ± 7
R230A	5 ± 0.5	2 ± 0.5
R230K	80 ± 3	86 ± 4
L234A	88 ± 8	92 ± 6
R309A	59 ± 4	65 ± 7
G347A	90 ± 7	94 ± 8
D395A	82 ± 5	90 ± 5
H491A	35 ± 4	40 ± 3
H510A	35 ± 4	41 ± 4
H491A, H510A	7 ± 1	4 ± 1

The data are expressed relative to the activity of wild-type PPK1 as 100%. PolyP of *M. smegmatis* was used as substrate for measuring the breakdown of polyP from ATP. Results are the means ± SD of three experiments.

known to require autophosphorylation for its enzymatic activity (Tzeng and Kornberg, 2000). *M. tuberculosis* PPK1 also underwent autophosphorylation (Fig. 1A). Based on sequence similarity with PPK1 of *E. coli*, H491 and H510 were predicted to be the residues required for autophosphorylation. Mutations of histidines 491 and 510 individually led to partial loss of activity, whereas simultaneous mutation of both these histidines showed almost complete inhibition of PPK1 activity (Table 1). Autophosphorylation was also reduced in the F176A and R230A mutants (Fig. 1A). On the other hand, autophosphorylation was not affected in the mutant L234A, which was also not affected in its polyP-synthesizing activity, suggesting that the observed effects were specific for F176 and R230.

PPK1 is known to form oligomers (Tzeng and Kornberg, 2000). The dimer has been reported to be the active form of the enzyme for the synthesis of polyP. Dimerization of PPK1 was affirmed by non-denaturing gel electrophoresis (Fig. 1B). Autophosphorylation of PPK1 in the presence of [γ-^{32}P]-ATP showed that the label was associated with the dimer (Fig. 1C). Gel filtration analysis by FPLC suggested that under these conditions the ratio of dimer : monomer was 7:1 (data not shown) for the wild-type PPK1. This ratio was 1:10 in the case of the R230A as well as the F176A mutants. These results suggested that dimerization and autophosphorylation were correlated, and that F176 and R230 were involved in dimerization.

Transcription of ppk1 *at different stages of growth*

The transcription of *ppk1* at different stages of growth of *M. tuberculosis* was assessed by reverse transcription polymerase chain reaction (RT-PCR). Expression of *ppk1* was detectable during exponential growth but increased in the stationary phase in *M. tuberculosis* H37Rv (Fig. 1D). A similar observation was also made for the *ppk1* of *M. smegmatis* (Fig. S3A).

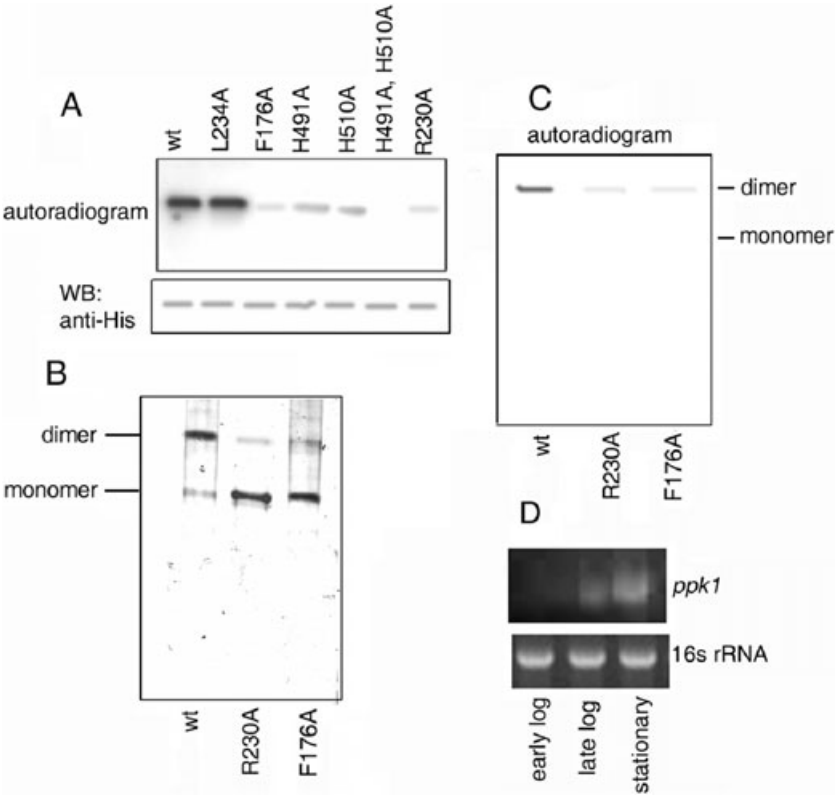

Fig. 1. Autophosphorylation and oligomerization of PPK1, and transcription of its gene at different stages of growth.
A. Forty nanograms of recombinant wild type (wt) or mutated (as indicated in the figure) PPK1 of *M. tuberculosis* was incubated with [γ-^{32}P]-ATP (1 mM) on ice, separated by SDS-PAGE, and incorporation of radioactivity into the protein was monitored by autoradiography. In a separate set of experiments, the same amount of protein was separated by SDS-PAGE followed by immunoblotting with anti-His antibody (bottom) in order to ensure equal loading of proteins. WB, Western blot.
B. PPK1 of *M. tuberculosis* or its variants were run on non-denaturing polyacrylamide gels, followed by staining with Coomassie brilliant blue R-250.
C. Autophosphorylation was performed as described in (A), followed by separation of proteins by PAGE under non-denaturing conditions and autoradiography.
D. RNA isolated from *M. tuberculosis* H37Rv at early, late log and stationary phase was reverse-transcribed by RT-PCR, using primers specific for *ppk1*.

Effect of oxidative and surface stress as well as anaerobiosis on the survival of a ppk1 *knockout mutant of* M. smegmatis *(PPK-KO)*

PPK1 is conserved across several prokaryotic organisms including pathogenic and non-pathogenic mycobacterial species suggesting that its function is likely to be conserved. In view of this, we used the fast-growing non-pathogenic *M. smegmatis* as a model organism to investigate the role of PPK1 in mycobacterial physiology. Knockout of *ppk1* of *M. smegmatis* was confirmed by PCR of genomic DNA which generated products of 1 and 0.5 kb in the case of wild-type mc^2155 and the *ppk1* knockout (PPK-KO) respectively (Fig. S3B). PolyP content of the wild-type *M. smegmatis* was 15.6 ± 1 nmol per mg of protein. This was reduced to 2.5 ± 0.2 nmol per mg of protein in the PPK-KO supporting the view that PPK1 is required to maintain polyP levels in *M. smegmatis*.

In *E. coli*, the inability to accumulate polyP has been associated with impaired long-term survival and the inability to withstand oxidative, osmotic and thermal stress (Rao and Kornberg, 1996; 1998; Shiba *et al*., 1997; Ault-Riche *et al*., 1998). A *Streptomyces lividans*, *ppk* mutant has been reported to be more sensitive towards oxidative stress compared with its wild-type counterpart (Ghorbel *et al*., 2006). Considering that mycobacteria encounter a variety of stresses within a granulomatous lesion, we subjected the wild type and the *ppk1* mutant to various conditions of stress. The *ppk1* mutant was compromised in terms of its ability to withstand oxidative stress (Fig. 2A) imparted by exposing cells to H_2O_2. Protein carbonylation was monitored as a marker of protein oxidation using antibody against derivatized carbonyl groups (Levine *et al*., 1990; Berlett and Stadtman, 1997). Higher levels of carbonylation were observed in the PPK-KO compared with the wild type in cells exposed to oxidative stress (Fig. S4). Similar results were obtained when oxidative stress was induced using 0.25 mM cumene hydroxide (data not shown). The susceptibilities of the wild type and PPK-KO to oxidative stress were also tested by determining the LD_{50} values of H_2O_2 (Wu *et al*., 1997). The LD_{50} for PPK-KO was 0.46 mM compared with 1.28 mM for the wild type. The LD_{50} value rose to 1.20 mM upon complementation with *ppk1* of *M. tuberculosis*.

In harmony with these observations, polyP levels increased fourfold in the oxidatively stressed wild type under stress (compared with unstressed cells). No such increase was observed in the PPK-KO. The PPK-KO was also attenuated in terms of its ability to survive under surface stress induced by SDS (Fig. 2B). PolyP levels increased 2.5-fold in the wild type under surface stress (compared with unstressed cells), whereas no such increase was observed in the PPK-KO.

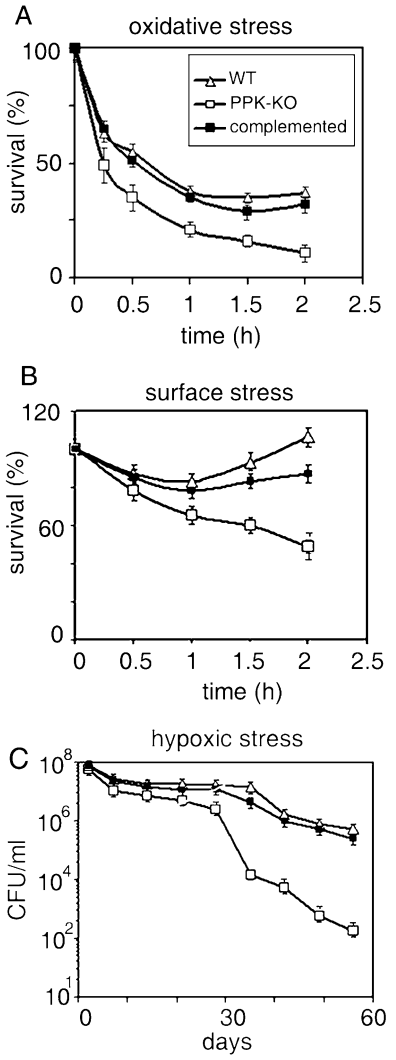

Fig. 2. Role of PPK1 in growth under oxidative or surface stress or anaerobiosis. Cultures were subjected to oxidative (A) or surface (detergent) (B) or hypoxic (C) stress as described under *Experimental procedures*. Growth was monitored over the course of time by determining cfu. –△–, wild type; –□–, PPK-KO; –■–, PPK-KO complemented with *ppk1* of *M. tuberculosis.* Results represent the means ± SD of three separate experiments.

Next we compared the ability of the wild type and PPK-KO to withstand hypoxic stress. Anaerobic survival was assessed as described by Primm *et al*. (2000). PPK-KO was compromised in its ability to survive under hypoxic conditions. After 50 days of incubation, the mutant lost more than 4 logs of viability, while the viability of the wild type declined only slightly (Fig. 2C). Reduced survival of PPK-KO under oxidative and surface stress as well as in hypoxia could be restored after complementation with the wild-type *ppk1*, but not with *ppk1* expressing F176A or R230A or H491A, H510A mutants (Fig. S5) under the conditions described above.

In summary, *ppk1* appeared to be necessary for the bacterium to survive under conditions of anaerobiosis,

oxidative stress and surface stress. The slight differences observed between the survival of the wild type and the complemented strain under surface stress (Fig. 2B) could possibly be due to the fact that the exogenously supplied *ppk1* was not expressed from its endogenous promoter.

Characterization of the mprAB-sigE-rel *signalling cascade in* M. smegmatis

Considering that an *M. tuberculosis rel* mutant is impaired in its ability to withstand hypoxic stress (Primm *et al*., 2000), we investigated whether *rel* expression is altered in the *ppk1* mutant. Ojha *et al*. (2000) have established the role of *rel* under conditions of carbon starvation, and reported the accumulation of the alarmone ppGpp (which is synthesized by the product of the *rel* gene) when *M. smegmatis* is grown in carbon-starved (0.2% glucose) medium. ppGpp could not be detected by Ojha *et al*. in enriched medium. We therefore evaluated *rel* transcription and ppGpp levels in the wild type and PPK-KO under carbon-starved condition only. *rel* transcription was lower in PPK-KO compared with the wild type (Fig. 3A), supporting the view that PPK1 is required for *rel* transcription in mycobacteria. PPK-KO showed reduced levels of ppGpp (Fig. S6A) compared with that of the wild type under similar conditions. *rel* transcription, ppGpp levels and survival under hypoxia were restored after complementation with *ppk1* of *M. tuberculosis*. These results suggested that PPK1 is required both for the ability of mycobacteria to survive under hypoxic conditions and for the stringent response which is central to the persistence of *M. tuberculosis*.

σ-Factors provide the promoter recognition function to bacterial RNA polymerase (RNAP) holoenzymes. *M. tuberculosis* encodes 13 σ-factors belonging to the σ^{70} class of σ-factors (Gomez *et al*., 1997; Rodrigue *et al*., 2006). Of these, 10 are extracytoplasmic σ-factors (Manganelli *et al*., 1999), which transcribe sets of genes that enable the bacterium to counter conditions of stress. The *sigE* gene is conserved across mycobacterial species. Although σ^E is not essential for growth *in vitro* (Ando *et al*., 2003), deletion of *sigE* results in increased susceptibility to oxidative stress as well as surface stress (Fernandes *et al*., 1999; Manganelli *et al*., 1999; Ando *et al*., 2003) and decreased viability in macrophages (Manganelli *et al*., 2001). Considering the above, attempts were made to decipher the role of σ-factors in the transcription of *rel.* We scanned the sequence upstream of the translational start site of the *rel* gene for prediction of the putative *rel* promoter using the neural network promoter prediction program of Reese (2001) (http://www.fruitfly.org/seq_tools/promoter.html). A putative transcription start site was identified 202 bp upstream of the *rel* gene with a probable σ^E consensus motif in the –35 and –10 region (Fig. S6B). This region was found to be similar in *M. tuberculosis* and *M. smegmatis*. In order to further test the dependence of *rel* transcription on σ^E, we performed *in vitro* transcription assays. *M. tuberculosis* RNAP core enzyme was reconstituted after expression and purification of the α-, β-, β′- and σ-subunits as described by Jacques *et al.* (2006). The *rel* DNA template encompassed the region –610 to +930 relative to the first translated nucleotide. Based on the predicted transcriptional start site located 202 bp upstream of the *rel* open reading frame, a product of 615 bp would be expected using the σ^E-containing holoenzyme. A product of approximately this size was obtained (Fig. 3B). Addition of increasing amounts of σ^E showed that the formation of this product

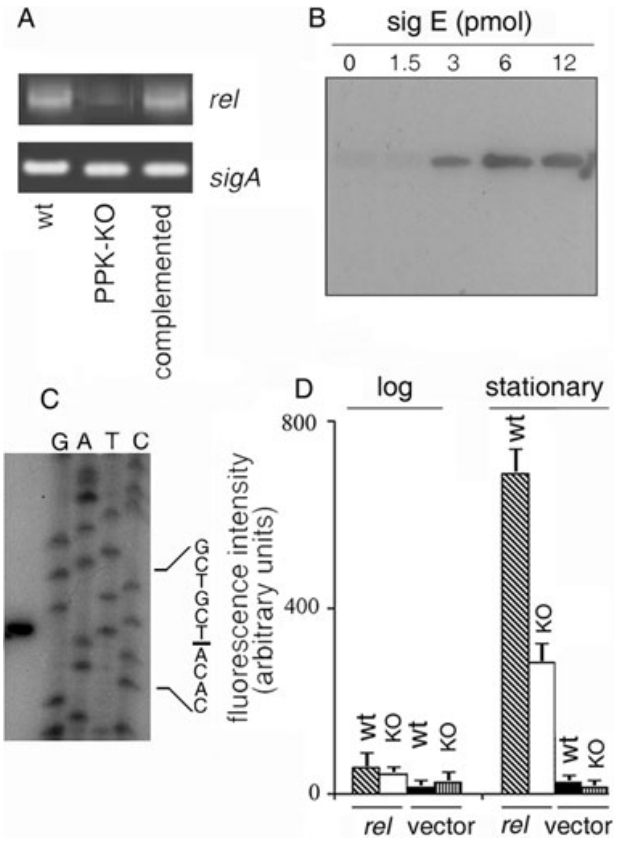

Fig. 3. Regulation of *rel* expression by σ^E.
A. RNA was isolated from *M. smegmatis* cells grown under carbon-starved conditions and transcription of *rel* was followed by RT-PCR.
B. *In vitro* transcription of the *rel* promoter was carried out in the presence of increasing concentrations of σ^E.
C. The transcription start site was determined by primer extension analysis using total RNA isolated from *M. smegmatis* as described under *Experimental procedures*. The reaction product was run in the first lane along with the sequencing reaction represented by G, A, T and C. The identified (+1) transcription start site (T) is underlined on the right.
D. *M. smegmatis* (Wt or PPK-KO) harbouring a reporter plasmid carrying the *rel* (–610 to +1) promoter fused to *gfp* was grown and fluorescence resulting from the expression of GFP was monitored as described under *Experimental procedures*.

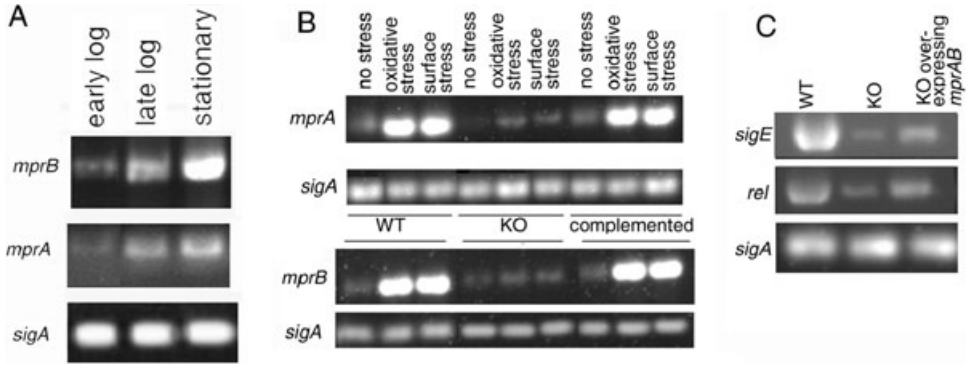

Fig. 4. Effect of inactivation of *ppk1* on the transcription of *mprA*, *mprB*, *sigE* and *rel* in *M. smegmatis*.
A and B. RNA was isolated from cells at different stages of growth, or from exponentially growing cells subjected to different conditions of stress as indicated. The levels of *mprA* and *mprB* were determined by RT-PCR using primers specific for *mprA* or *mprB*.
C. RNA was isolated from wild type (WT) or PPK-KO (KO) or PPK-KO overexpressing *mprAB* (KO overexpressing *mprAB*) and RT-PCR was carried out using primers specific for *sigE* or *rel* of *M. smegmatis*.

was dependent on the concentration of σ^E (Fig. 3B), suggesting the specificity of the reaction. In addition, analysis of the DNA sequence in the region of the transcriptional start site confirmed the σ^E-dependent transcription of rel (Fig. 3C). No product was found using either the σ^B- or σ^F-containing holoenzymes (Fig. S7A). As positive control (to show that the reconstituted RNAP was functional), transcription potential of the σ^F holoenzyme was tested using the *usfX* template (Beaucher *et al*., 2002), and the desired product was obtained (Fig. S7A). The role of σ^E was further confirmed by performing primer extension analysis on the RNA isolated from wild-type *M. smegmatis* and an isogenic *sigE* mutant. A product of expected size was obtained in the case of the wild type, whereas no product was obtained in the case of the *sigE* mutant (a gift from Professor Robert Husson, Children's Hospital, Harvard Medical School, Boston, MA) (Fig. S7B). In order to confirm that *ppk1* was regulating the *rel* promoter, *rel* promoter-driven GFP expression was studied by inserting the putative *rel* promoter (starting 610 bp upstream of the translational start site of the *rel* gene and ending at the start site) upstream of the *gfp* gene in order to drive the expression of GFP. The levels of expression of GFP were compared at different stages of growth. In the stationary phase, GFP expression (although induced) was consistently lower in PPK-KO compared with the wild type (Fig. 3D). This strengthened the view that the expression of *rel* was linked to *ppk1*.

Two-component signal transduction systems consist of a sensor histidine kinase and its cognate cytosolic response regulator. Several such systems regulate the response of mycobacteria to environmental stress. Considering that transcription of the *sigE* gene has recently been reported to be regulated by the two-component system encoded by the genes *mprA* and *mprB* and that *mprAB* is part of the *sigE* regulon (He *et al*., 2006), we analysed the expression of *mprAB* in the wild-type *M. smegmatis*. *mprAB* expression was upregulated in stationary phase in the wild type (Fig. 4A). Expression of *mprA* and *mprB* was also compared in exponentially growing cells which were subjected to oxidative or surface stress. Both *mprA* and *mprB* expression was elevated in the wild type compared with the unstressed cells (Fig. 4B). Our view that *mprAB* was likely regulating the expression of both *sigE* and *rel* was supported by the observation that overexpression of *mprAB* under the control of the *hsp60* promoter in the PPK-KO led to an increase in expression of both *sigE* and *rel* in the stationary phase compared with PPK-KO harbouring the vector alone (Fig. 4C). However, *mprAB* overexpression could not restore the transcription of either *sigE* or *rel* to the level observed in the wild type.

Downregulation of mprAB-sigE-rel *signalling cascade in PPK-KO*

PPK-KO was attenuated in terms of its ability to withstand surface and oxidative stress (Fig. 2). We considered the possibility that the levels of *sigE* expression could be altered in the mutant. Semi-quantitative RT-PCR showed that indeed at stationary phase, *sigE* gene expression was attenuated in PPK-KO compared with the wild type (Fig. 5A). Similarly, whereas *sigE* expression was increased when exponentially growing wild-type cells were subjected to oxidative or surface stress, PPK-KO was compromised in terms of its ability to upregulate *sigE* expression under these stress conditions (Fig. 5B). This ability was restored in the *ppk1*-complemented strain. σ^H is another σ-factor which has been linked to the response of mycobacteria to oxidative stress (Raman *et al*., 2001). No changes in expression of *sigH* were observed in the PPK-KO under the conditions studied (data not shown).

In view of our observations that the expression of both the *rel* and *sigE* genes is compromised in PPK-KO, we explored the possibility that the expression of these genes

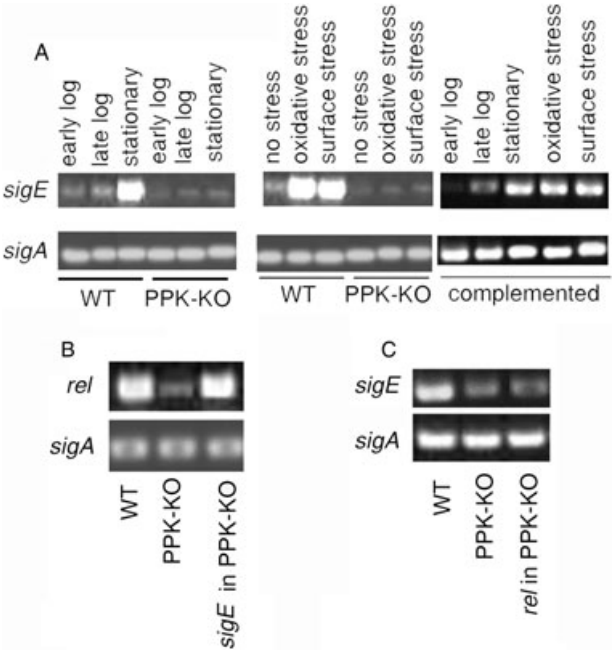

Fig. 5. Expression of *sigE* and its role in *rel* transcription at different stages of growth.
A. RNA was isolated from cells at different stages of growth or subjected to different conditions of stress as indicated. Levels of *sigE* were determined by RT-PCR.
B and C. Quantification of *rel* in *sigE*-overexpressing cells or *sigE* in *rel*-overexpressing cells was carried out using primers specific for *rel* or *sigE* respectively.

could be linked. *sigE* and *rel* were individually overexpressed with 6x His tags in PPK-KO under the control of the *hsp60* promoter. Overexpression of *rel* and σ^E was confirmed by Western blotting with anti-His antibody (Fig. S8A). Overexpression of σ^E led to an increase in the expression of *rel* in PPK-KO (Fig. 5B) at stationary phase, whereas overexpression of *rel* did not affect the expression of *sigE* (Fig. 5C). The increase in *mprAB* expression was not observed when PPK-KO was subjected to oxidative or surface stress (Fig. 4B), whereas the behaviour was rescued by complementation with *ppk1* of *M. tuberculosis.* In addition, we observed downregulation of *mprAB-sigE-rel* cascade in PPK-KO under carbon starvation (Fig. S8B–D) which could be restored after complementation with *ppk1* of *M. tuberculosis*. These results suggested that *ppk1* induces *mprAB-sigE-rel* signalling cascade in *M. tuberculosis* in a manner similar to that observed in *M. smegmatis.*

In vitro *phosphorylation of the sensor kinase MprA by polyphosphates*

In order to explore how PPK1 is linked to *mprAB*, we tested whether MprB, the histidine kinase, could utilize polyP as a donor for phosphorylation of the cognate regulator MprA. Both MprA and MprB were expressed in *E. coli* as His-tagged and S-tagged proteins respectively. Purified MprB was capable of undergoing autophosphorylation, as well as phosphorylating MprA in the presence of polyP as phosphate donor (Fig. 6A). Non-radioactive polyP from *M. smegmatis* could compete with [^{32}P]-polyP (with reference to MprA and MprB phosphorylation) in a dose-dependent manner (Fig. 6B) supporting the specificity of the reaction. Similar competition was observed when polyP was synthesized *in vitro* using recombinant PPK1 as described earlier (Chouayekh and Virole, 2002) (Fig. S9A). Positive controls were run using ATP as phosphate donor (data not shown). Control reactions were carried out where the autophosphorylation of PknB was studied in the presence of radiolabelled ATP or polyP. PknB could not utilize labelled polyP as substrate (Fig. S9B). This experiment also ruled out the possibility of the presence of any contaminating ATP in the polyP preparation used in this study. These data supported the view that appropriate polyP levels are likely required for the phosphorylation of MprA under certain conditions such as ATP depletion. Considering that phosphorylated MprA regulates the transcription of the *mprAB* operon, as well as the transcription of *sigE*, the above data provide a link between PPK1 (which regulates the synthesis of polyP) and the levels of expression of *mprAB* and *sigE*.

Downregulation of ppk1 *attenuates the* mprAB-sigE-rel *cascade and the survival of* M. tuberculosis *in macrophages*

In order to test the implications of our above findings in the context of the pathogen *M. tuberculosis*, we developed a

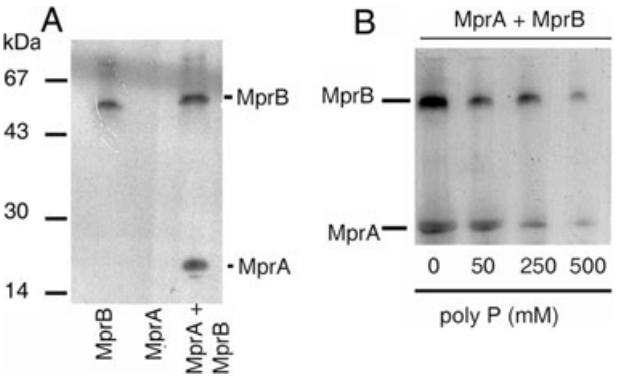

Fig. 6. Role of polyP in MprB-dependent phosphorylation of MprA.
A. Recombinant MprB or MprA or MprA + MprB were incubated with [^{32}P]-polyP as described under *Experimental procedures* and phosphorylated proteins were detected by SDS-PAGE and autoradiography.
B. Phosphorylation reactions were carried out with MprA and MprB as described in (A) in the presence of increasing concentrations of non-radioactive polyP. Phosphorylated proteins were detected as described under panel A.

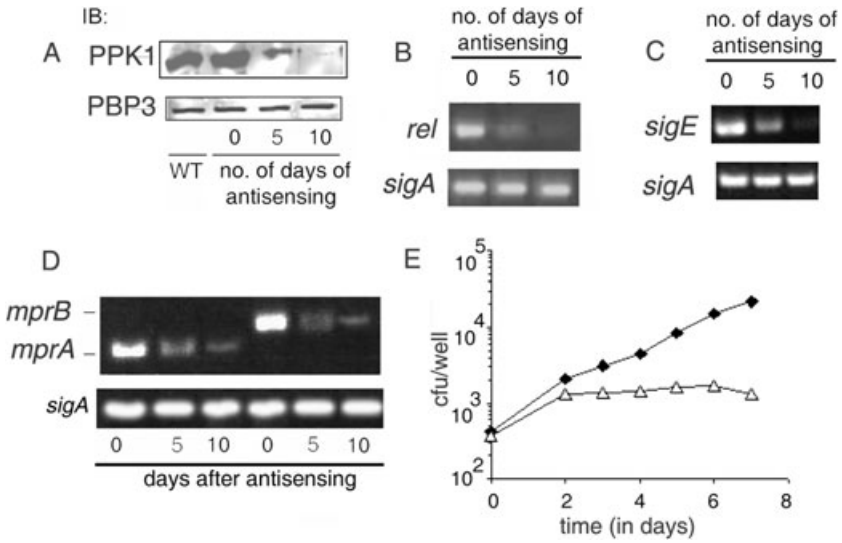

Fig. 7. Effect of inactivation of *ppk1* on the expression of *rel*, *sigE* and *mprA-mprB* and *in vivo* survival of *M. tuberculosis*. *M. tuberculosis* was transformed with pMIND carrying *ppk1* in antisense orientation. Transformed cells were induced with tetracycline for different periods of time as indicated in order to downregulate *ppk1*.
A. Western blotting was carried out with cell lysates from the above cells, using anti-PPK1 antibody. The blot was reprobed with anti-PBP3 antibody in order to ensure equal loading in all wells.
B–D. RNA was isolated from cells before and after antisensing of *ppk1* and reverse-transcribed using primers specific for *rel*(B) or *sigE* (C) or *mprA* and *mprB* (D).
E. THP-1 cells were infected with *M. tuberculosis* harbouring vector alone or *M. tuberculosis* expressing an antisense construct of *ppk1* under the control of the *hsp60* promoter at an moi of 2 for 90 min and washed with fresh medium. Cells were grown for different periods of time and internalized mycobacteria were released by lysing the macrophages followed by cfu determination as described under *Experimental procedures*. –◆–, no antisensing; –△–, after antisensing of *ppk1*.

system for antisensing of *ppk1* of *M. tuberculosis*. The entire *ppk1* gene was cloned in antisense orientation under the control of the *hsp60* promoter or under the control of the inducible tetracycline promoter (Blokpoel *et al*., 2005). Downregulation of *ppk1* was confirmed by Western blotting using anti-PPK1 antibody (Fig. 7A). Maximum antisensing was observed after growing the cells for 10 days in the presence of tetracycline. No antisensing was observed when cells were grown in the absence of tetracycline. PolyP content of *M. tuberculosis* decreased from 14.75 ng per mg of protein to 4 ng per mg of protein after antisensing. Under the conditions where *ppk1* downregulation had occurred, the expression levels of *mprAB*, *sigE* and *rel* were analysed. As would be expected, these genes were all downregulated (Fig. 7B–D).

Inactivation of σ^E of *M. tuberculosis* results in significant attenuation of growth of the bacterium in THP-1 cells (Manganelli *et al*., 2001). Considering that downregulation of *ppk1* leads to decreased expression of *sigE*, we tested the ability of *M. tuberculosis* to survive in macrophages when *ppk1* was downregulated. There was a severe defect in the ability of the bacterium to survive in macrophages over a 7-day period under conditions of *ppk1* antisensing (Fig. 6E). This behaviour was similar to that of the σ^E knockout strain (Manganelli *et al*., 2001).

Discussion

The tubercle bacillus is extremely successful in persisting within the human host. Most people infected with the bacillus show no signs of disease and only develop active disease when their immune system is perturbed. During latency, it is difficult to treat dormant bacteria with antibiotics which are normally effective against replicating bacteria making it necessary to understand the detailed mechanisms of survival of the bacterium in dormancy. Previous studies have demonstrated the role of polyP in survival under conditions of stress of Gram-negative and Gram-positive bacteria (Rao and Kornberg, 1996; Kornberg *et al*., 1999). In this study we have characterized the polyP-synthesizing enzyme PPK1 of *M. tuberculosis* and explored its role in the survival of mycobacteria under conditions of stress.

The cloned and overexpressed PPK1 of *M. tuberculosis* as well as *M. smegmatis* showed comparable polyP-synthesizing activity in harmony with the amino acid sequence similarity between the two enzymes. However, the activity was lower than that of *E. coli* PPK1. PPK1 of *E. coli* catalyses synthesis of polyP of average length 700–800 residues (Zhang *et al*., 2002). *M. tuberculosis* PPK1 catalysed synthesis of polyP with a broader distribution of chain length ranging from 200 to

800 (data not shown). PPK1 could also catalyse the reverse reaction, i.e. generation of ATP from polyP (isolated from mycobacteria as well from *E. coli*) like the *E. coli* enzyme (Kumble *et al*., 1996). By analogy with *E. coli* PPK1, the phosphate group appeared likely to have been transferred on to the conserved histidine residues at position 491 and 510 of *M. tuberculosis* PPK1. The importance of these residues was supported by observations that the H491A, H510A mutation caused complete inhibition of autophosphorylation as well as polyP-synthesizing ability (Table 1). The recently solved crystal structure of *E. coli* PPK1 suggests that PPK1 forms an interlocked dimer and that its active site is located in a tunnel which contains a unique ATP-binding pocket (Zhu *et al*., 2004). The dimerization of PPK1 of *E. coli* is required for its polyP-synthesizing activity. Dimerization and autophosphorylation were correlated in the *M. tuberculosis* PPK1 Homology modelling using the *E. coli* PPK1 as template suggested that F176 and R230 are located in the head domain which likely contributes to one dimer-forming interface (Fig. S10). Our results suggest that a positively charged residue at position 230 and a hydrophobic phenyl ring at position 176 are likely to be involved in dimerization activity.

In order to explore the role of *ppk1* in the stress response of mycobacteria, *M. smegmatis* was chosen as the model system. *M. tuberculosis* encounters different conditions of stress both when it grows within macrophages and when the bacterium resides within a caseous granuloma. The bacterium must have the ability to adapt to both these conditions. *In vitro*, *M. tuberculosis* can persist for years in the absence of detectable replication after nutrient deprivation, whereas replenishing nutrients restores active metabolism and growth (Loebel *et al*., 1933). PPK-KO showed significant reduction in polyP content suggesting the role of PPK1 in the synthesis of polyP in *M. smegmatis.* We tested the ability of PPK-KO to withstand different conditions of stress. PPK-KO was compromised in its ability to survive under conditions of hypoxia. Considering that complementation of *ppk1* of *M. tuberculosis* restored the growth defect, it appeared unlikely that the observed phenotypic changes in PPK-KO were due to polar effects attributable to gene(s) downstream of *ppk1*. Mutants of *ppk1* could not reverse the growth defect (Fig. S6) in harmony with the results of *in vitro* enzymatic assays (Table 1) suggesting the crucial role of the identified amino acid residues in PPK1 function. When microorganisms encounter a nutrient-limited environment they slow their growth rate dramatically and reduce levels of RNA and protein synthesis. This overall alteration in metabolism is known as the stringent response. Using the mouse model, several persistence-defective mutants deficient in persisting in the face of the immunological onslaught of the host have been characterized (Hingley-Wilson *et al*., 2003). In *M. tuberculosis*, the stringent response is reported to be mediated by (p)ppGpp (hyperphosphorylated guanine nucleotides) which are synthesized by the Rel_{MTB} enzyme (Avarbock *et al*., 2000). Microarray analysis has revealed that H37Rv Δrel_{MTB} suffers from a generalized alteration of the transcriptional apparatus as well as specific changes in expression of virulence factors, secreted antigens, heat shock protein and cell wall-synthesizing enzymes (Dahl *et al*., 2003). The fact that *rel* has been identified as a gene that is required for long-term survival under hypoxic conditions (Primm *et al*., 2000) prompted us to explore whether this role was linked to, or independent of the stringent response. The level of the alarmone, ppGpp, was compared between the wild type and PPK-KO. Under conditions of carbon starvation, *rel* expression was markedly impaired in the mutant compared with the wild type, implicating *ppk1* in the regulation of *rel* transcription. This finding assumes particular significance considering the documented role of *rel* in the adaptation to stationary phase and in long-term persistence of *M. tuberculosis* in mice. This is the first report linking polyP metabolism to the stringent response in mycobacteria. The situation in mycobacteria is obviously different from an earlier report where a *ppk1*-inactivated mutant of *S. lividans* did not show any alteration in *rel* expression or ppGpp biosynthesis (Chouayekh and Virole, 2002).

Pathogenic mycobacteria encounter oxidative stress in their intracellular milieu. The PPK-KO was more susceptible to oxidative stress as well as surface stress. Considering that a large number of stress-regulated σ-factors have been linked to the stress response of this bacterium, we focused our attention on the expression of some of these extracytoplasmic σ-factors in the absence of PPK1. σ^E and σ^B have been reported to be upregulated under conditions of oxidative stress, surface stress and low aeration (Wu *et al*., 1997; Manganelli *et al*., 1999). In harmony with these observations, σ^E was downregulated in PPK-KO. We also observed reduced expression of σ^B in PPK-KO, although not to the extent of σ^E (unpublished observations). Considering that the downregulation of σ^E was more pronounced than the downregulation of σ^B, we focused our attention on exploring the link between *sigE* and *rel*. In this report we demonstrate that the transcription of the *rel* gene is regulated by σ^E, establishing an important link between the σ-factor-dependent stress response and the stringent response. We observed an increase in the *rel* promoter-driven GFP expression in the stationary phase in both the wild type and PPK-KO. However, the increase observed was consistently lower in the mutant compared with the wild type (Fig. 3D) suggesting that the presence of *ppk1* potentiates the efficiency of *rel* transcription in mycobacteria. We also observed that overexpression of σ^E from an *hsp60* promoter could over-

come the *rel* expression deficiency associated with inactivation of *ppk1*, suggesting that σ^E is an important regulator of *rel* expression. We hypothesized that optimal polyP levels (regulated by PPK1) are probably required for phosphorylation reactions under certain conditions such as ATP depletion, and therefore searched for the possible regulation of σ^E by phosphorylation-dependent binding of transcription factors. A recent report has demonstrated that both σ^B and σ^E are regulated by the two-component system comprising the histidine kinase MprB and its cognate regulator MprA which shows enhanced binding to its target sequences after MprB-dependent phosphorylation. Interestingly, *mprAB* expression was compromised in the PPK-KO strain. Overexpression of *mprAB* in the PPK-KO was associated with increased levels of *sigE* and *rel* expression in the stationary phase. It therefore appeared likely that decreased levels of *rel* and *sigE* in the PPK-KO were linked to attenuation of *mprAB* expression. Considering that MprA regulates transcription of the *mprAB* operon as well as *sigE* and *sigB* transcription, it appeared possible that MprB was capable of phosphorylating MprA using polyP as the phosphate donor. *In vitro* kinase reactions using recombinant MprA and MprB affirmed our view that MprB could use polyP as a phosphate donor and phosphorylate MprA. In comparison with the wild type, we observed low but detectable levels of transcription of the genes of the *mprAB-sigE-rel* cascade in PPK-KO, by semi-quantitative RT-PCR (Figs 4 and 5). This could be due to the availability of other physiological substrates like ATP or aceyl phosphate (Zahrt *et al*., 2003) which possibly serve as donors for phosphorylation with a low efficiency in PPK-KO. The contribution of other, as yet uncharacterized, polyP-synthesizing enzyme(s) also cannot be ruled out. These possibilities would explain the observation that *mprAB* overexpression in the PPK-KO led to an increase in *rel* and *sigE* expression compared with PPK-KO itself. We speculate that polyP is a preferred substrate under stress and that PPK1 assumes a critical role under such conditions providing a polyP pool for efficient utilization by MprAB. It remains to be tested whether other histidine kinases of two-component systems also utilize polyP as phosphate donor. The mechanism of phosphate donation from polyP is open to further investigation.

PolyP metabolism remains unexplored in the context of mycobacterial physiology and infection. Our studies have shed light on the polyP-synthesizing enzyme PPK1 of mycobacteria. We have uncovered a novel regulatory network linking polyP to the optimal response of a two-component system which controls expression of the stress-regulated σ-factor σ^E, which in turn controls the expression of the stringent response regulator *rel*.

The observation that *ppk1* attenuation compromises the ability of *M. tuberculosis* to survive in macrophages, increases the importance of this gene in the context of the intracellular life of the bacterium. However, data obtained by infecting macrophages with any particular mutant of *M. tuberculosis* do not necessarily correlate to survival characteristics of the mutant in its host. In fact, the impaired survival of the *M. tuberculosis sigE* mutant within macrophages reported by Manganelli *et al.* (2001) does not appear to extend to a survival defect in lung and spleen tissues in C3H/HeJ mice (Ando *et al*., 2003). Nevertheless it would be of considerable interest to test the role of *ppk1* in persistence, considering that the *mprAB* signal transduction system has been reported to be required for persistent infection in the lung (Zahrt and Deretic, 2001).

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study were *E. coli* DH5α, *E. coli* BL21(DE3) and *E. coli* Top 10. *E. coli* strains were grown in Luria–Bertani (LB) Miller media. *M. smegmatis* mc^2155 was grown in Middle Brook (MB) 7H9 broth supplemented with 0.05% Tween 80 or Lemco medium supplemented with 0.05% Tween 80. Early log, late log and stationary phases refer to 16, 28 and 44 h of growth respectively (Dasgupta *et al*., 2006).

Electroporation in mycobacteria

This was carried out using a Bio-Rad Gene Pulser according to the protocol of Snapper *et al.* (1990).

Construction of expression plasmids for PPK1

ppk1 (Rv2984) of *M. tuberculosis* H37Rv and *M. smegmatis* mc^2155 (MSMEG239) were amplified from cosmid MTCY349 (gift from Professor Stewart Cole, Institut Pasteur, Paris) and from the genomic DNA of *M. smegmatis* mc^2155, respectively, followed by cloning between the NdeI and EcoRI sites of the expression vector pET 28a (Novagen), or between the KpnI and HindIII sites of pBAD-His B (Invitrogen). The primers used are given in Table S1. Mutants of *ppk1* were generated by overlap extension PCR. The primers used are given in Table S2 with restriction sites in bold. The integrity of all constructs was checked by sequencing.

Expression and purification of PPKs

Recombinant plasmids derived from pET 28a were transformed in *E. coli* BL21(DE3). Cells were grown to an OD_{600} of 0.6. IPTG was added to a final concentration of 0.1 mM and growth was continued at 37°C with shaking for 2 h. Recombinant plasmids derived from pBAD-His B were transformed into *E. coli* TOP10 and induced with 0.02% arabinose at 37°C for 4 h. Cells were harvested, re-suspended in 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM PMSF, 20 μg ml^{-1} leupep-

tin, 10 μg ml^{-1} pepstatin and 10 μg ml^{-1} aprotinin and disrupted by sonication. Recombinant His-tagged proteins were purified from lysates by chromatography on Ni^{2+}-NTA agarose.

Isolation of polyP

Polyphosphate was extracted by the method of Ault-Riche *et al.* (1998) with minor modifications. *E. coli* or *M. smegmatis* cell pellet was dissolved in 5 M guanidine isothiocyanate (GITC) and cells were lysed by heat, SDS and sonication. After addition of ethanol, DNA-binding resin was added and the resin was washed with 5 mM Tris-HCl (pH 7. 5), 50 mM NaCl, 5 mM EDTA, 50% ethanol. After nuclease treatment, polyP was eluted with water of pH 8 at 95°C. Sizes of polyP were determined after separation by 20% PAGE containing 7 M urea followed by autoradiography (Tzeng and Kornberg, 2000). For isolation of ^{32}P-labelled polyP, *M. smegmatis* was grown in low phosphate (0.15 mM)-containing Hartmans-de-Bont medium (Hartmans and De Bont, 1992) to an OD_{600} of 0.2 followed by the addition of [γ-^{32}P]-H_3PO_4 (BRIT, Hyderabad, India) to a final concentration of 100 μCi ml^{-1}. Cells were grown and [^{32}P]-polyP was isolated as described above.

Autophosphorylation and assay for enzyme activities

Polyphosphate kinase autophosphorylation was assayed by incubating purified enzyme in 50 mM HEPES-KOH (pH 7), 40 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$ and 5 μM [γ-^{32}P]-ATP at 37°C for 5 min (Tzeng and Kornberg, 2000). The reaction was terminated by adding 40 mM EDTA and samples were electrophoresed on a 10% gel followed by autoradiography. The PPK1-catalysed production of acid-insoluble [^{32}P]-polyP from ATP was quantified as described by Ahn and Kornberg (1990). PolyP breakdown catalysed by PPK was quantified using the non-radioactive technique of Ault-Riche *et al.* (1998). The membrane fraction of *M. tuberculosis* H37Rv was kindly provided by Dr John Belisle, Colorado State University, Fort Collins, CO through NIAID, National Institutes of Health Contract NO1-AI-40091. PPK1 of *M. tuberculosis* was immunoprecipitated from the solubilized membranes with rabbit polyclonal antibody raised against recombinant PPK1 of *M. tuberculosis* (at Imgenex, Bhubaneswar, India), followed by measurement of the enzymatic assay with the immunoprecipitate.

Non-denaturing PAGE

This was performed using a discontinuous buffer system (Davis, 1964). SDS and β-mercaptoethanol were omitted from samples and buffers. Acrylamide concentrations ranging from 5% to 12% were used for Ferguson plots (Ferguson, 1964).

Construction of suicidal delivery vector

The unmarked deletion mutant of *ppk1* of *M. smegmatis* mc^{2}155 was constructed using the allelic replacement method of Parish and Stoker (2000). Briefly, the *ppk1* gene of *M. smegmatis* along with its upstream sequence was PCR amplified using the primer pairs indicated in Table S1 and genomic DNA of wild-type mc^{2}155 as template. The amplicon was cloned between the KpnI and Hind III sites of the vector pUC19 to generate pPPK101. A 450 bp was deleted from the *ppk1* gene by digesting pPPK101 with BsaI and religating the vector to give rise to pPPK102. The 2.1 kb insert carrying the disrupted *ppk1* gene was excised with KpnI and PacI and cloned between the same sites of p2NIL to generate pPPK103. The final delivery vector pPPK104 was generated by cloning the PacI cassette (hyg, P_{ag85}-lac Z, P_{hsp60}-sac B) excised from the vector pGOAL 19 at the PacI site of pPPK103.

Generation of a ppk1 *mutant of* M. smegmatis

pPPK104 was electroporated into electrocompetent cells of *M. smegmatis* mc^{2}155 and plated on Lemco agar plates supplemented with hygromycin B (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}) and X-gal (50 μg ml^{-1}). Blue colonies were replated onto Lemco agar; a loopful of cells was re-suspended in Lemco broth, serially diluted and plated onto Lemco agar supplemented with 2% sucrose and X-gal (50 μg ml^{-1}). White kan^S, hyg^S and suc^R colonies were identified and isolated.

Genotype analysis

Genomic DNA was prepared from the wild type and the kan^s, hyg^s and suc^R colonies according to standard procedures. The genotype was checked by PCR using mc^{2}155 genomic DNA and the genomic DNA isolated from kan^s, hyg^s and suc^R colonies as templates and the primer pair 5′-TGT GGG CCC CGC CAC GGC ACA GGC CAT-3′ and 5′-ATG TCG TCG GAA ACT TC-3′ which flanked the deletion site. The PCR products were sequenced.

Complementation of ppk1 *of* M. tuberculosis *in the* M. smegmatis *strain inactivated in the* ppk1 *gene using an integrating vector*

Complementation of *ppk1* was achieved by constructing an integrating vector containing a hygromycin-resistance cassette along with a positive-selection L5 integrase cassette. *ppk1* of *M. tuberculosis* was first cloned in pOLYG (O'Gaora *et al.*, 1997), under the control of the *hsp60* promoter and *hsp60–ppk1* was excised and cloned between the same sites in pUC19 to generate pPPK105. A 3.7 kb Hyg-integrase cassette from pUC-HY-INT (Mahenthiralingam *et al.*, 1998), was cloned at the single HindIII site of pPPK105 to generate the integrating vector pPPK106. pPPK106 was electroporated into electrocompetent cells of the PPK-KO strain in order to complement the mutant strain with a single copy of the wild-type *ppk1* of *M. tuberculosis.* The presence of the *ppk1* gene was confirmed by PCR with *ppk1*-specific primers.

Isolation and detection of ppGpp

Mycobacterium smegmatis was grown in low phosphate (0.15 mM)-containing medium (Hartmans and De Bont, 1992) to an OD_{600} of 0.2 followed by the addition of [γ-^{32}P]-

H_3PO_4 (BRIT, Hyderabad, India) to a final concentration of 100 μCi ml^{-1}. The labelled cells grown up to an OD_{600} of 0.8 were harvested, washed, treated with 1 mg of lysozyme per ml (in 10 mM Tris, pH 8.0 on ice for 20 min), and lysed with 1% SDS. ppGpp was extracted with an equal volume of 2 M formic acid. After centrifugation at 4°C, 5 μl of the supernatant was loaded on a polyethyleneimine (PEI)-coated thin-layer chromatography (TLC) plate (Sigma) (Ojha *et al*., 2000). The plate was developed in 1.5 M KH_2PO_4 (pH 3.5) in one dimension, air-dried and exposed to X-ray film. Labelled ppGpp isolated from *E. coli* strain CF3120, overexpressing the ppGpp synthase gene, *rel* (gift from Professor Dipankar Chatterji, Indian Institute of Science, Bangalore), was used as standard on TLC plates.

RNA preparation and RT-PCR

Cells were lysed by agitation with glass beads (0.1 mm) in a Mini bead beater apparatus (BioSpec Products, Bartlesville, OK) (Stahl *et al*., 2001). RNA was isolated from cell lysates using the RNeasy kit according to the manufacturer's protocol (Qiagen GmbH, Germany). Purified RNA was eluted with RNase-free water and treated with RNase-free DNase I for 30 min at 37°C. RNA (100 ng) was reverse-transcribed using the Titanium one-step RT-PCR kit (Clontech). The primer pairs used for RT-PCR are given in Table S3. Relative quantities of cDNA were determined from the products generated by the amplification of genomic DNA after serial dilutions, using the appropriate primers, and were normalized for amounts of *sigA* or 16s rRNA as their expression remains unchanged during stress or at different growth phases. The DNA-free RNA preparation was checked by DNA-PCR (–RT) of the housekeeping gene. RT was performed only on RNA preparations confirmed to be free of contaminating DNA according to the manufacturer's instructions using annealing temperatures of 55°C for *rel*, *sigA*, 16s rRNA, *mprA* and *mprB*; 60°C for *sigE*; and 50°C for *ppk1*. All samples were subjected to identical reactions lacking reverse transcriptase in order to confirm the absence of any genomic DNA.

PPK1 structural modelling

PPK1 (SWISSPROT Accession No. P65768) from *M. tuberculosis* was modelled by using the First Approach Mode at the Swiss-Model protein structure homology modelling server (http://ca.expasy.org) using the structural template of *E. coli* PPK1. Amino acids 48–742 of *M. tuberculosis* PPK1 were included in the model. The graphics were produced by using Swiss Pdb viewer (Peitsch, 1996; Guex and Peitsch, 1997).

Anaerobic survival assays

These assays were performed as described by Primm *et al.* (2000). Briefly, wild-type *M. smegmatis* and the *ppk1* mutant were grown to an OD_{600} of approximately 0.2–0.3 in 7H9 medium supplemented with 2% glucose and 0.05% Tween 80 (7H9-glucose-Tween 80). For anaerobic survival assays, bacteria were diluted to an OD of 0.05 in fresh 7H9-glucose-Tween 80 and were aliquoted into 1.5 ml screw-cap tubes with rubber septa with no headspace and incubated stationary at 37°C. Control tubes with methylene blue indicator dye demonstrated that all oxygen was consumed after 4–5 days. For all experiments, the number of colony-forming units (cfu) per millilitre was determined by plating in triplicate onto 7H11 plates.

Survival assays under surface or oxidative stress

For determination of survival following detergent or hydrogen peroxide exposure, cultures of wild-type *M. smegmatis* or PPK-KO were grown overnight in 7H9-glucose-Tween 80 at 30°C to an OD_{600} of 0.3–0.4. Cultures were then diluted in fresh medium to an OD_{600} of 0.2, and exposed to 0.01% SDS (detergent stress). Cultures were incubated with shaking at 30°C and aliquots were removed from each culture and plated at the indicated time points. Colonies were counted after 3–4 days with duplicate or triplicate plates counted at each of two dilutions. Experiments were performed at least thrice for each stress condition. For oxidative stress, cells were exposed to 50 μM hydrogen peroxide for 1 h, followed by addition of hydrogen peroxide to 5 mM, followed by plating of dilutions as described above.

Detection of oxidized proteins

Oxidized proteins containing carbonyl groups were detected using the Oxyblot kit (Chemicon) as described (Lee *et al*., 2005).

Analysis of the activity of the rel *promoter*

The *rel* promoter (–610 to +1 relative to the translational start site) was amplified from the genomic DNA of *M. smegmatis* using primers depicted in Table S4 and cloned into the promoter-less replicative *gfp* vector pFPV27 (Valdivia *et al*., 1996) using asymmetric KpnI and BamHI sites. The resulting plasmid was electroporated into wild-type *M. smegmatis* mc^2155, the isogenic PPK-KO and the *ppk1*-complemented strain. Cells were grown in 7H9 broth containing kanamycin (25 μg ml^{-1}) at 37°C. The cells were harvested by centrifugation, washed once in PBS/0.05% Tween 80 and resuspended in the same buffer to an OD_{600} of approximately 1. Fluorescence was measured with a spectrofluorimeter using excitation and emission wavelengths of 480 and 510 nm respectively (Blokpoel *et al*., 2003).

Overexpression of mprAB, sigE *and* rel *in PPK-KO*

The *mprAB*, *sigE* and *rel* genes were amplified from the genomic DNA of *M. smegmatis* using primers shown in Table S4, and the genes were cloned between the NdeI (or EcoRI) and HindIII sites of a shuttle vector under the control of the *hsp60* promoter (Choudhuri *et al*., 2002).

In vitro *transcription assays and analysis of the transcriptional start site*

Recombinant RNAP subunits were purified and reconstituted as described by Jacques *et al.* (2006). Recombinant σ-factors were purified as described by Jacques *et al*.

The *rel* promoter template for *in vitro* transcription assays was a 1540 bp PCR product amplified with the primers 5′-TAAGGTACCGAAGTTGTCGAAATGACGT-3′ (sense) and 5′-TTAAAGCTTTTGACGATCATCTTCTGGTAG-3′ (antisense). The other template used was a 269 bp NheI–HindIII fragment from pYZ99 for the usfXP1 promoter (Jacques *et al*., 2006). The *in vitro* transcription protocol used in this study was adapted from that used by Jacques *et al*. (2006). Briefly, reactions were carried out in a final volume of 40 μl containing 45 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 75 mM KCl, 1 mM dithiothreitol, 250 mg ml^{-1} bovine serine albumin (BSA) and 13% glycerol. Core RNAP (22.5 pmol) and σ-factor (3 pmol) were mixed and incubated for 30 min at 37°C in the transcription reaction buffer. Transcription reactions were initiated by adding 2 μl of NTPs. The final ATP, UTP, GTP concentration were 0.15 mM and the final CTP concentration was 0.4 mM. Immediately after transcription, 2 μl of a solution containing 6 μg of heparin, along with 0.15 mM of CTP, was added. Reactions were left for another 10 min at 37°C. Transcription reactions were stopped by adding 2 μl of 7.5 M ammonium acetate, 1 μl of 5 mg ml^{-1} bacterial tRNA and 100 μl of ice-cold ethanol. RNAs were then precipitated at −80°C and washed with 70% ethanol. The pellets were re-suspended in 10 μl of water. Template-specific oligonucleotides were radiolabelled using [γ-^{32}P]-ATP and T4 polynucleotide kinase and used as probes for detection by primer extension using the Rel- and UsfX-specific oligonucleotides 5′-AGCAGCGCGGCCACCAGCGTGGTG GTGT-3′, 5′-TCTGGTCTTCGAGCTGGTCGGTCATGGTC-3′, respectively, according to standard procedures. Primer extension assays were performed using template-specific oligonucleotides radiolabelled with [γ-^{32}P]-ATP and T4 polynucleotide kinase as described by Beaucher *et al*. (2002). Sequencing reactions were performed using the same primer as that used for primer extension, and run in adjacent lanes of 6% acrylamide-urea sequencing gel to determine the start site of the transcripts.

Construction of a vector for antisensing of ppk1

The *M. tuberculosis ppk1* coding region was amplified by PCR using the oligonucleotide primer pair 5′-TA**GGA TCC**TCAGGGGCTGCGGTGC-3′ and 5′-TAT**CATATG**AT GAGCAATGATCGC-3′, followed by cloning of the PCR product in reverse orientation in the vector pMIND (Blokpoel *et al*., 2005) between the BamHI and NdeI sites (in bold) to generate the plasmid pPPK103. pPPK103 was electroporated in *M. tuberculosis* in order to obtain a strain expressing the antisense mRNA of *ppk1*. Transformants were grown up to mid log phase and induced with tetracycline (20 ng ml^{-1}) for different periods of time up to 15 days. The expression level of PPK1 was determined by immunoblotting. In experiments where the survival of *M. tuberculosis* was assessed in macrophages, *ppk1* was downmodulated by expression of the gene in antisense orientation under the control of the *hsp60* promoter (Choudhuri *et al*., 2002).

Expression and purification of MprA and MprB

The *mprA* and *mprB* genes of *M. smegmatis* were amplified using primers depicted in Table S1 and genomic DNA from *M. smegmatis*, and cloned in pET28a and pET29a respectively. Recombinant plasmids were transformed in *E. coli* BL21(DE3) pLysS. After induction with 0.1 mM IPTG, His-MprA and S tag-MprB were purified from the cell lysates using Ni^{2+}-NTA agarose and S-protein agarose, respectively, according to the manufacturers' protocol.

Phosphorylation of MprA and MprB

The soluble fractions of the cell lysate from *E. coli* cells expressing S-tagged MprB were adsorbed on S-protein agarose pre-equilibriated with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Triton X-100. The bound protein was pelleted, first washed with the same buffer and then with kinase buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM KCl and 50 mM $MgCl_2$, and re-suspended in the same buffer. The reaction was started with the addition of the substrate MprA and 50 mM [^{32}P]-polyP and continued for 30 min at 37°C. Control phosphorylation reactions were carried out in the presence of [γ-^{32}P]-ATP. Reactions were stopped by addition of Laemmli SDS-PAGE denaturation buffer and samples were electrophoresed on a 12.5% gel followed by autoradiography. Phosphorylation in the presence of [^{32}P]-polyP was chased by increasing concentrations of non-radioactive polyP isolated either from *M. smegmatis* or polyP synthesized *in vitro.* As a control, PknB of *M. tuberculosis* was purified (Dasgupta *et al*., 2006) and phosphorylation was carried out with [γ-^{32}P]-ATP.

Infection of THP-1 with M. tuberculosis

THP-1 cells (obtained from the National Centre for Cell Science, Pune, India) were maintained in RPMI1640 supplemented with 10% fetal bovine serum. Cells were treated with phorbol 12-myristate 13-acetate (20 nM) to induce maturation to macrophages (Bhattacharyya *et al*., 2002). Cells were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (moi) of 2 for 1.5 h at 37°C. Wells were washed to remove extracellular bacteria and incubated in the presence of fresh medium at 37°C for the indicated periods of time. Intracellular bacteria were released by lysing the macrophages with 500 μl of 0.05% SDS, diluted in 7H9 broth and plated to determine the viable counts. The media removed from the wells were also plated to determine the number of extracellular bacteria.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Alignment of the deduced amino acid sequences of the PPK1 orthologues of *M. tuberculosis* (M.tb), and *M. smegmatis* (M.smeg) using CLUSTAL W.

Fig. S2. Overexpression, purification and immunological detection of PPK1 of mycobacteria and hydropathy plot.

Fig. S3. Expression of *ppk1* at different stages of growth and PCR analysis of the *ppk1* knockout mutant.

A. RNA isolated from *M. smegmatis* at early log, late log and stationary phase was reverse-transcribed by RT-PCR.

B. Chromosomal DNA from wild-type *M. smegmatis* mc^2155 (wt) or PPK-KO was used as template for PCR analysis. The

sizes of the PCR products were analysed by agarose gel electrophoresis. The first lane is the 1 kb ladder.

Fig. S4. Role of PPK1 in growth under oxidative or surface stress. Cultures growing logarithmically were subjected to oxidative stress. The oxidized (carbonylated) proteins in wild type (WT), PPK-KO and the *ppk1*-complemented PPK-KO (complemented) strain were detected using the OxyBlot kit. Samples prepared from equal numbers of cells were analysed for carbonylated proteins by Western blotting after SDS-PAGE. The results shown are representative of three different experiments.

Fig. S5. Role of PPK1 mutants in growth under oxidative or surface stress or anaerobiosis. Logarithmically growing PPK-KO transformed with full-length wild-type (wt) *M. tuberculosis ppk1* or its mutants (as indicated) were subjected to oxidative (top) or surface (detergent) (middle) or hypoxic (bottom) stress. Growth was monitored over the course of time by determining cfu. Results represent the means ± SD of three separate experiments.

Fig. S6. Synthesis of ppGpp and the sequence of the putative *rel* promoter.

A. Cells were grown in the presence of [γ-^{32}P]-H_3PO_4 and ppGpp present in the formic acid extract was separated by TLC followed by autoradiography.

B. The sequence of the putative *rel* promoter region of *M. smegmatis.* The arrow indicates the start of the gene.

C. Alignment of *rel* promoter region from *M. smegmatis* and *M. tuberculosis*.

Fig. S7. *In vitro* transcription assays and primer extension analysis of *rel*.

A. *In vitro* transcription of the *usfX* promoter was carried out in the presence of σ^F (first lane, positive control) and that of the *rel* promoter was carried out in the presence of σ^B, or σ^F or σ^E as indicated (second to fourth lanes).

B. Primer extension analysis for the *rel* promoter was carried out with RNA isolated from *M. smegmatis* (lane 2) or from the σ^E-inactivated mutant (lane 3) and the size of the product was compared with the product obtained by *in vitro* transcription of the *rel* promoter as described under Fig. 3B (lane 1).

Fig. S8. Western blot analysis of σ^E or *rel* expression and RT-PCR analysis for σ^E, *mprA* and *mprB*.

A. Western blots was performed using anti-His antibody on lysates of the PPK-KO strain harbouring (a) vector alone or vector expressing either (b) His-Rel or (c) His-σ^E.

B. RNA was isolated from stationary-phase cells of the wild type (WT) or PPK-KO (KO), or PPK-KO overexpressing *ppk1*(com).

Quantification of *sigE* (B) or *mprA* (C) or *mprB* (D) was carried out by RT-PCR.

Fig. S9. Phosphorylation of *mprA* and *mprB* by polyP.

A. Role of polyP in MprB and MprA phosphorylation. Recombinant MprA and MprB were incubated with [γ-^{32}P]-polyP (synthesized *in vitro* using PPK1 and [γ-^{32}P]-ATP), and phosphorylated proteins were detected by SDS-PAGE and autoradiography.

B. Phosphorylation reactions were carried out with recombinant PknB of *M. tuberculosis* using [γ-^{32}P]-ATP alone (top, first lane) or in the presence of either increasing concentrations of non-radioactive polyP (top) or non-radioactive ATP (bottom, second to fifth lanes). Phosphorylated proteins were detected as described above. The first lane of the bottom panel shows the result of a phosphorylation reaction carried out with PknB and [γ-^{32}P]-labelled polyP (first lane of the bottom panel).

Fig. S10. Structural model of *M. tuberculosis* PPK1. PPK1 of *M. tuberculosis* was modelled using the structural template of *E. coli* PPK1. The head domain is shown in purple whereas the rest of the protein is depicted in blue. The residues F176 (PHE176) and R230 (ARG230) which are involved in polyP-synthesizing activity are indicated.

Table S1. Primers for cloning the *ppk1* genes of *M. tuberculosis* and *M. smegmatis*, for generating the suicide vector (pPPK101), for inactivation of *ppk1* of *M. smegmatis*, and for cloning the *mprA* and *mprB* genes of *M. smegmatis.*

Table S2. Primers used for mutation of *ppk1.*

Table S3. Primers used for RT-PCR.

Table S4. Primers used for cloning constructs of *rel* and *sigE*.

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