**Mycobacterium tuberculosis** protein kinase K enables growth adaptation through translation control

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*Mycobacterium tuberculosis* serine/threonine protein kinases (STPKs) are responsible for orchestrating critical metabolic and physiological changes that dictate mycobacterial growth adaptation. Previously, we established that PknK participates in regulatory pathways that slow the growth of *M. tuberculosis* in a variety of *in vitro* stress environments and during persistent infection in mice. In the present study, we have elaborated on the mechanism of PknK-mediated regulation. Through transcription profiling of wild-type H37Rv and a ΔpknK mutant strain during logarithmic and stationary growth phases, we determined that PknK regulates the expression of a large subset of tRNA genes so that regulation is synchronized with growth phase and cellular energy status. Elevated levels of wild-type *M. tuberculosis* PknK (PknK<sub>WT</sub>) but not phosphorylation-defective PknK<sub>MBI</sub> in *Mycobacterium smegmatis* cause significant retardation of the growth rate and altered colony morphology. We investigated a role for PknK in translational control and established that PknK directs the inhibition of *in vitro* transcription and translation processes in a phosphorylation-dependent manner. Increasing concentrations of ATP or PknK exert cooperative effects and enhance the inhibitory function of PknK. Furthermore, truncation and mutational analyses of PknK revealed that PknK is autoregulated via intramolecular interactions with its C-terminal region. Significantly, the invariant lysine 55 residue was only essential for activity in the full-length PknK protein, and the truncated mutant proteins were active. A model for PknK autoregulation is proposed and discussed.

Tuberculosis (TB) continues to be one of the world's leading infectious killers. With only a partially effective vaccine available and consistent annual increases in the percentage of drug-resistant strains causing disease, the global health impact is immense. One key aspect of *Mycobacterium tuberculosis* pathogenesis is its innate ability to alter its growth rate to enable survival in stress environments encountered during *M. tuberculosis* infection in macrophages (2). While the ability of bacterial cells to slow down growth under unfavorable conditions is not uncommon, it has enormous implications for *M. tuberculosis*. Through mechanisms not fully understood, *M. tuberculosis* is able to transition into a state of dormancy or persistence that forms the basis of the global risk of latent TB today (16). According to World Health Organization estimates, one-third of the world’s population is infected with *M. tuberculosis*, 2 million of whom die annually (47) leaving a large reservoir of people who are asymptomatically infected with *M. tuberculosis*. Understanding the regulatory mechanisms that lead to mycobacterial persistence/latency is a current focus of TB research.

The central element in this adaptability is the ability to recognize and interpret external signals from specific environments and the conversion of these signals into transcriptional activation or repression. The *M. tuberculosis* signalome (signaling proteins) is enriched with “eukaryotic-like” Ser/Thr protein kinases (STPKs), a tyrosine kinase, paired two-component systems, and several orphan kinases, regulators, and phosphatases that orchestrate finely tuned and well-coordinated adaptive responses. The discovery of eukaryotic-like STPKs in *M. tuberculosis* (6) has reaffirmed the belief that bacterial pathogens are equipped with exquisite signal transduction mechanisms that not only impact host-pathogen interactions, but also enable metabolic and physiological adaptations within the bacteria.

The genome of *M. tuberculosis* encodes 11 STPK proteins named PknA through PknL (1, 6), 9 of which are transmembrane receptor kinases while 2 are cytoplasmic proteins (PknG and PknK) that are associated with the cell wall/membrane and are secreted during culture (only PknG) and infection (7, 27, 30, 46). *M. tuberculosis* STPKs are key regulators of mycobacterial growth. Two of the 11 STPKs, PknA and PknB, are essential for mycobacterial growth (13, 25), and at least 4 of them, PknH, PknI, PknJ, and PknK, have been implicated in the modulation of growth in culture and during infection (17, 24, 30, 37). Furthermore, convergence of PknH signaling pathways with the DevR-DevS (also known as DosR-DosS) two-component regulatory system (3) that defines the transcriptional adaptation of mycobacteria during hypoxia indicates that *M. tuberculosis* has sophisticated and highly coordinated networks that enable stress-induced growth and survival. Several studies have shown that PknA, PknB, PknD, PknH, PknK, and PknL phosphorlylate proteins involved in cell division and transcription (reviewed in reference 38); however, there is very little information on the mechanisms of STPK-mediated regulation of mycobacterial growth.

The complex regulation of mycobacterial growth requires tight control of STPKs and regulatory proteins. Crystallization studies have indicated that ligand-induced N-lobe dimerization is a mechanism of mycobacterial STPK activation (15, 18, 29, 36, 48); however, at present, we do not understand how these structural changes regulate kinase activity or their downstream effects on mycobacterial growth and physiology. Typically, protein kinases
investigated the phosphorylation-dependent effects of PknK, suggesting an involvement of PknK in translation control. We characterized an autoregulatory role of the PknK C terminus for all molecular manipulations. E. coli cultures were grown in Luria Bertani (LB) broth or LB agar plates at 37°C. For pknK overexpression, M. smegmatis was transformed with acetamide-inducible integrative plasmids pYA1662 and pYA1671 to yield the LIX79 and LIX80 strains overexpressing wild-type and phosphorylation-defective pknK, respectively, and these strains were grown in LB broth supplemented with 0.05% Tween 80 with aeration or on LB agar plates at 37°C. Acetamide was added at a final concentration of 0.2% during exponential growth in liquid medium and in agar plates to induce pknK overexpression. Broth-grown M. tuberculosis H37Rv and a pknK deletion strain, LIX11 (30), were used for comparative expression profiling using microarrays. M. tuberculosis cultures were cultivated in Middlebrook 7H9 medium (Difco Laboratories) supplemented with 10% ADS (0.5% albumin, 0.2% dextrose, 0.085% saline) enrichment as described previously (30). When required, media were supplemented with 25 μg/ml kanamycin (Kan) and 50 μg/ml hygromycin (Hyg) for mycobacteria. All chemicals were obtained from Sigma-Aldrich, St. Louis, MO, unless otherwise indicated.

RNA isolation and integrity. Aliquots for RNA isolation were taken from M. tuberculosis H37Rv and LIX11 cultures at day 3 (log phase) and day 30 (stationary phase) time points during growth, and total RNA was isolated using TRI reagent (Ambion Inc., TX), as described previously (31). Total RNA integrity was assessed using an RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Santa Clara, CA) according to the manufacturer’s protocol. Total RNA purity was assessed with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc., DE). RNA was considered to be good quality when the 23S/16S rRNA ratios were greater than or equal to 1.0. RNA samples from two independent experiments were used for microarray analysis.

Labeling and microarray hybridization. Poly(A) tails were added to the 3’ ends of RNA using the A-plus Poly(A) polymerase tailing kit (Epicentre Biotechnologies, Madison, WI). The samples were labeled using the Quick-Amp labeling Kit (Agilent). Five hundred nanograms of each sample was incubated with reverse transcription mixture at 42°C and converted to double-stranded cDNA (dsDNA) primed by oligo(dT) with a T7 polymerase promoter. The dsDNA was used as the template for cRNA generation by in vitro transcription and incorporation of the dyes Cy3-CTP and Cy5-CTP (Agilent). The cDNA synthesis and in vitro tran-

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<tr>
<td>M. tuberculosis H37Rv</td>
<td>Virulent laboratory strain</td>
<td>ATCC 25618</td>
</tr>
<tr>
<td>M. smegmatis mc²155</td>
<td>ept-1</td>
<td>W. R. Jacobs, Jr., Albert Einstein College of Medicine, NY</td>
</tr>
</tbody>
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**Materials and Methods**

Strains, plasmids, and growth. The strains and plasmids used in this study are listed in Table 1. Escherichia coli JM109 was used as the host strain for all molecular manipulations. E. coli cultures were grown in Luria Bertani (LB) broth or LB agar plates at 37°C. For pknK overexpression, M. smegmatis was transformed with acetamide-inducible integrative plasmids pYA1662 and pYA1671 to yield the LIX79 and LIX80 strains overexpressing wild-type and phosphorylation-defective pknK, respectively, and these strains were grown in LB broth supplemented with 0.05% Tween 80 with aeration or on LB agar plates at 37°C. Acetamide was added at a final concentration of 0.2% during exponential growth in liquid medium and in agar plates to induce pknK overexpression. Broth-grown M. tuberculosis H37Rv and a pknK deletion strain, LIX11 (30), were used for comparative expression profiling using microarrays. M. tuberculosis cultures were cultivated in Middlebrook 7H9 medium (Difco Laboratories) supplemented with 10% ADS (0.5% albumin, 0.2% dextrose, 0.085% saline) enrichment as described previously (30). When required, media were supplemented with 25 μg/ml kanamycin (Kan) and 50 μg/ml hygromycin (Hyg) for mycobacteria. All chemicals were obtained from Sigma-Aldrich, St. Louis, MO, unless otherwise indicated.

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cription steps were carried out at 40°C. The quality of labeled cRNA was assessed for yields and specific activity, followed by hybridization on a custom M. tuberculosis H37Rv 8x15k array designed by Genotypic Technology Pvt. Ltd. (Agilent microarray design identifiers [AMADID] 023057 and 026323; Agilent). Cy3- and Cy5-labeled samples (300 ng each) were hybridized with the Gene Expression Hybridization kit (Agilent) in SureHyb chambers (Agilent) at 65°C for 16 h. The hybridized slides were washed using Gene Expression wash buffers (Agilent) and scanned using the Microarray Scanner G2505C at 5-μm resolution. Data extraction from images was done using Feature Extraction software v10.5.1.1 (Agilent), followed by analysis using GeneSpring GX version 10.2 software (Agilent). Normalization of the data was done in GeneSpring GX using Lowess normalization. In two-color microarray experiments, where two fluorescent dyes (red and green) have been used, intensity-dependent variation in dye bias may introduce spurious variations in the collected data. Lowess normalization is a technique that merges two-color data, applying a smoothing adjustment that removes such variation. Samples were grouped based on the replicates and genes showing up- or down-regulation of ≥2×-fold among the samples. The results are reported as the geometric means of log, expression ratios ± standard deviations between the replicates. Quantitative reverse transcription-PCR (qRT-PCR) was performed to validate the microarray results as described previously (31), using sg4 mRNA as an internal control for normalization.

Cloning and cell-free expression of M. tuberculosis PknK wild-type and mutant proteins. The full-length pknK gene (3,333 bp) and the N-terminal regions encompassing 915 bp and 2,607 bp of pknK were amplified using M. tuberculosis H37Rv genomic DNA as the template and cloned into a pET SUMO expression vector (Invitrogen, Carlsbad, CA) to create plasmids pYA1556 (30), pYA1657, and pYA1658, respectively. The substitution mutation to replace lysine at position 55 with methionine (K55M) was generated in the plasmids pYA1556, pYA1657, and pYA1658 using PknK-K55M F and K55M R primers (27) and the QuickChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer’s recommendations to produce plasmids pYA1659 to pYA1661. The plasmids pYA1659, pYA1660, and pYA1661 served as the template DNA for in vitro synthesis of PknK1-1110-, PknK1-305-, and PknK660-855 mutant proteins, respectively. All clones and mutations were verified by DNA sequencing. For studies with purified PknK protein, PknK wild-type/mutant protein or 5 μg of the purified PknK KD-GST was exogenously added to a fresh IVTT reaction mixture (23 μl), along with 1 μg template DNA (pYA1635 or the Renilla reniformis luciferase gene [Rluc] control DNA). Reactions were supplemented with 1 mM ATP or ADP as needed and incubated at 37°C with shaking for 1 h. Control reactions without any exogenous addition of PknK or ATP were always performed in parallel. Synthesis of His-tagged PhoP was detected by immunoblotting with anti-His antibody, and the extent of inhibition was determined by densitometric analyses using AlphaEase imaging software (Alpha Innotech/Cell Biosciences, Santa Clara, CA).

Luciferase assay. The production of Renilla luciferase from the Rluc control DNA in the S30 T7-coupled IVTT system was monitored by measuring the production of light after addition of the Renilla luciferase assay reagent (Promega). For experiments involving IVTT of firefly luciferase in rabbit reticulocyte lysates (RRL) (Promega) from either luciferase control DNA or presynthesized luciferase mRNA templates (Promega), the samples were diluted 1:20 in nuclease-free water, and luciferase synthesis was assessed by measuring production of light with the luciferase assay reagent (Promega), according to the manufacturer’s recommendations. The reactions were analyzed in a Glomax 96 Microplate luminometer (Promega).

DLS. Dynamic light scattering (DLS) measurements were performed using a 173³ back-scattering Malvern Nano-ZS instrument, as described previously (45). Briefly, PknK KD-GST was subjected to in vitro kinase assay with 1 mM ATP or AMP-PNP (β,γ-imidoadenosine 5′-triphosphate, a nonhydrolyzable ATP analog) and kinase buffer as described above and diluted to 200 μl to give a final concentration of 1.25 μg/μl. The samples were filtered through a 0.45-μm Millipore Millex-HN filter to remove dust particles before DLS measurements. Repeated measurements were taken to ensure reproducibility.

Statistical analysis. Statistical analyses were performed with a Student t test or one-way analysis of variance (ANOVA) using Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA). A P value of <0.05 was considered statistically significant.

Microarray data accession number. The microarray data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE37823 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37823).

RESULTS

PknK regulates M. tuberculosis tRNA synthesis in a growth-phase-dependent manner. We previously demonstrated that PknK levels are elevated in stationary phase and that deletion of the pknK gene in H37Rv results in increased survival of the mutant under stationary-phase stress (30). To extend our investigations on the regulatory role of PknK, we used microarrays to compare the transcription profiles of the LIX11 mutant versus wild-type H37Rv under conditions of logarithmic-phase (3 days) and stationary-phase (30 days) growth in broth cultures. The rationale for using these time points was to maintain correlation with data...
reported earlier (30). Consistent with the idea that PknK plays a distinct role in stationary phase, twice as many genes (Fig. 1A, red dots) \( n = 70, 32 \) up- and 38 downregulated, where \( n \) is the number of differentially expressed genes) were differentially expressed in the mutant during stationary phase than during log phase (Fig. 1A, blue dots) \( n = 37, 7 \) up- and 30 downregulated). Several genes encoding conserved hypothetical proteins and those involved in lipid metabolism and cell wall processes were differentially regulated in the LIX11 mutant; however, the most significant change was observed in the category of tRNA genes (see Fig. S1 and Data sets S1 and S2 in the supplemental material). In the present study, we focused on the expression of tRNA genes in the LIX11 mutant. The tRNA genes, along with their changes in expression in the mutant relative to H37Rv in log and stationary phases, are listed in Table 2. Remarkably, of the 30 genes downregulated in LIX11 during log phase, 80% were tRNA genes (Fig. 1A, circled blue spots). To our surprise, we observed that tRNA expression in the LIX11 mutant was growth phase dependent. Among the 32 upregulated genes in stationary phase, 72% belonged to the tRNA gene category (Fig. 1A, circled red spots). We analyzed the expression of 5 randomly selected tRNA genes—glyU, lysT, gluT, glyV, and argW—in LIX11 and H37Rv by qRT-PCR. The differential expression of two tRNA genes, glyU and lysT, during the log and stationary phases and the upregulation of gluT, glyV, and argW during stationary phase in the LIX11 mutant validated the microarray results (Fig. 1B). Given that PknK levels are induced during stationary phase (30), these results suggest the involvement of PknK in downregulating tRNA expression in H37Rv during stationary phase and, more importantly, are reflective of the energy demand, rate of protein synthesis, and growth of the bacteria. Since all of these are low in stationary phase compared to log phase, these results further substantiate a PknK function in regulatory mechanisms that slow mycobacterial growth in stationary phase. Next, we determined the direct effects of PknK-mediated signaling and its implications in mycobacterial growth regulation.

Overexpression of wild-type, but not phosphorylation-defective, pknKMtb in M. smegmatis inhibits growth rate and colony development. To elucidate the underlying mechanisms of PknK-mediated regulation, we employed the approach of elevating intracellular PknK levels. We reasoned that increased cellular concentrations of PknK could substitute for activating signals and enable us to discern the direct downstream effects of PknK. Despite several attempts to transform M. tuberculosis H37Rv with plasmid pYA1662, which overexpresses the full-length pknKMtb gene from an acetyl-CoA-inducible promoter, we could not recover the transformants. As an alternative, M. smegmatis, which routinely serves as a surrogate host to express M. tuberculosis genes, was used in these experiments. We transformed M. smegmatis with empty vector and pYA1662 to construct the LIX70 and LIX79 (overexpressing pknKMtb) strains, respectively (Table 1). As shown in Fig. 2A, immunoblot analysis of induced (Ind) and uninduced (UI) cultures postinduction were always plated on solid medium containing PknK

![Graphical representation of the trend in tRNA expression in the ΔpknK mutant during logarithmic and stationary-phase growth.](http://jb.asm.org/)

**FIG 1** Graphical representation of the trend in tRNA expression in the ΔpknK mutant during logarithmic and stationary-phase growth. Differences between the transcriptional profiles of an M. tuberculosis ΔpknK strain (LIX11) and the wild-type H37Rv were determined as a function of growth using microarrays. (A) Scatter plot showing the distribution of genes (up- or downregulated) in the LIX11 mutant relative to the wild-type during logarithmic-phase (blue dots) and stationary-phase (red dots) growth based on their respective changes listed in Data sets S1 and S2 in the supplemental material. Note that each spot indicates a unique gene that is differentially expressed in the LIX11 mutant during log and stationary phases. The circled points highlight the biphasic expression of the tRNA genes in the mutant. A large subset of tRNA genes was downregulated in the mutant during logarithmic phase but upregulated during stationary phase. (B) qRT-PCR analyses of 5 randomly selected tRNA genes, glyU, lysT, gluT, glyV, and argW, in LIX11 and H37Rv during log and stationary phases. Expression was normalized using sigA mRNA as an internal control. The change in tRNA expression in the LIX11 mutant relative to H37Rv grown under specific growth conditions is presented as the mean and standard deviation (SD) of data from two independent experiments. The baseline expression of tRNA genes in H37Rv was set to 1.0 (dashed line). **, \( P < 0.01 \).
induced LIX80 cell lysates by immunoblotting (see Fig. S2A in the supplemental material) and used it as a control in these experiments. The growth defect observed with LIX79 was absent in LIX80 (Fig. 2C, bottom), suggesting that PknK\textsubscript{Mtb} mediated effects on the growth and colony morphology of \textit{M. smegmatis} are dependent on its ability to phosphorylate cellular proteins. Continued observation of the individual induced colonies over time (1 day in liquid medium followed by 4, 8, and 12 days of incubation on solid medium containing the inducer) revealed that the LIX79 induced cells were increasing in size (Fig. 2D); however, the rate of growth was significantly lower, and moreover, the colony exhibited vertical growth as opposed to the lateral growth observed with the LIX80 induced cells. Notably, this phenotype was reversed when induced LIX79 cells were grown on noninducing solid medium, confirming that the inhibitory effects of PknK were due to increased PknK\textsubscript{Mtb} synthesis and phosphorylation activity in LIX79 (see Fig. S2B in the supplemental material). These observations corroborate our previous results with a \( \Delta \text{pknK} \) mutant (30) and suggest that increased intracellular levels of PknK have a global effect on the macromolecular synthetic apparatus of the mycobacterium, resulting in an overall growth defect. However, the key question is whether PknK mediates these effects directly or indirectly through phosphorylation of other transcriptional regulators. Considering that microarray results implicated PknK in a role for PknK in the regulation of protein synthesis, we focused our investigation on a role for PknK in the regulation of protein synthesis.

**Cell-free synthesis and functional activity of \textit{M. tuberculosis} PknK wild-type and mutant proteins.** To determine whether PknK directly regulates protein synthesis, we used cell-free IVTT systems. The \textit{E. coli} S30 T7 IVTT system that enables coupled transcription and translation of a gene under the control of a T7 promoter was used to synthesize PknK proteins. Plasmid pYA1556, carrying the \textit{pknK} gene, whose expression is controlled by a T7 promoter, was used as a DNA template to produce His-tagged PknK\textsubscript{Mtb} protein. Phosphorylation-defective PknK\textsubscript{K55M} was synthesized from plasmid pYA1659 (also under the control of a T7 promoter) to serve as a negative control in our investigations. Figure 3A shows the successful production of the PknK\textsubscript{K55M} (lane 1) and PknK\textsubscript{K55M} -IVTT (lane 2) proteins on a Western blot analyzed with anti-PknK antibody. To confirm that the proteins synthesized through the cell-free expression system were functionally active, we analyzed the kinase activities of IVTT-synthesized wild-type and mutant PknK proteins in an \textit{in vitro} kinase assay using MBP as a substrate and \([\gamma-32P]ATP\). As shown in Fig. 3B, wild-type PknK\textsubscript{1-1110} -IVTT protein catalyzed phosphorylation of MBP, whereas the phosphorylation-defective PknK\textsubscript{K55M} was void of any ATP binding or kinase activity (Fig. 3B, lane 3). Thus, we concluded that the \textit{E. coli} S30 T7 expression system could be successfully used for the production of catalytically active mycobacterial kinases that are stable and functional for subsequent use in biochemical enzymatic assays.

PknK inhibits \textit{in vitro} transcription and translation in a phosphorylation-dependent manner. The effect of exogenous addition of IVTT-synthesized PknK\textsubscript{1-1110} on protein synthesis of bacterial cells was analyzed using the \textit{E. coli} S30 T7 system. The choice of template plasmid DNA used in this assay, plasmid

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**Table 2**: Differential expression of tRNA genes in \textit{M. tuberculosis} \( \Delta \text{pknK} \) mutant (LIX11) relative to H37Rv during logarithmic- and stationary-phase growth

<table>
<thead>
<tr>
<th>Gene</th>
<th>Logarithmic Phase (Day3)</th>
<th>Stationary Phase (Day30)</th>
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<tbody>
<tr>
<td>( alaT )</td>
<td>-1.652</td>
<td>0.112</td>
</tr>
<tr>
<td>( glyU )</td>
<td>-2.213</td>
<td>0.228</td>
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<tr>
<td>( tyrT )</td>
<td>-1.833</td>
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<td>( metT )</td>
<td>-1.466</td>
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<td>( thrV )</td>
<td>-1.894</td>
<td>0.320</td>
</tr>
<tr>
<td>( lysT )</td>
<td>-1.921</td>
<td>0.238</td>
</tr>
<tr>
<td>( gluT )</td>
<td>-1.939</td>
<td>0.129</td>
</tr>
<tr>
<td>( aspT )</td>
<td>-0.958</td>
<td>0.039</td>
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<td>( argT )</td>
<td>-1.386</td>
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<td>( gluT )</td>
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<td>( argT )</td>
<td>-1.509</td>
<td>0.012</td>
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<tr>
<td>( leuW )</td>
<td>-1.020</td>
<td>0.126</td>
</tr>
<tr>
<td>( proT )</td>
<td>-1.548</td>
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<td>( asnT )</td>
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<td>( glyW )</td>
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<td>( argW )</td>
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<td>0.159</td>
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<tr>
<td>( hisT )</td>
<td>-1.879</td>
<td>0.239</td>
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<td>( lnuU )</td>
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<td>( valU )</td>
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<td>( gluU )</td>
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<td>( gltU )</td>
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<td>( alaU )</td>
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</tr>
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<td>( metU )</td>
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<tr>
<td>( thrU )</td>
<td>-1.889</td>
<td>0.285</td>
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- \( ^a \) Geometric mean of log\(_2\) ratio from two biological replicates.
- \( ^b \) SD, standard deviation between the replicates. The red and green boxes indicate greater (more than 0.6) or lesser (less than –0.6) fold changes in LIX11 relative to H37Rv, respectively.
- \( ^c \) The 16 tRNA genes common to the logarithmic (downregulated) and stationary (upregulated) phases are shown in boldface.
- \( ^d \) Significant difference between LIX11 and H37Rv (\( P < 0.05 \)) (Student \( t \) test).
- \( ^e \) Significant difference between LIX11 and H37Rv (\( P < 0.01 \)) (Student \( t \) test).
pYA1635 carrying the *M. tuberculosis* phoP gene (Rv0757) expressed from a T7 promoter, was serendipitous. As shown in Fig. 4A, immunoblot analysis with anti-His antibody revealed robust production of His-tagged PhoP upon addition of the plasmid pYA1635 to the IVTT reaction (Fig. 4A, lane 1). Addition of PknK1-1110 to the reaction did not affect the synthesis of PhoP (Fig. 4A, lane 2). In contrast, exogenous addition of PknK 1-1110, together with 1 mM ATP, resulted in a significant decrease in PhoP detection (Fig. 4A, lane 3). No inhibition was observed in the presence of 1 mM ADP (Fig. 4A, lane 4), indicating a requirement for ATP for the inhibitory effect of PknK. It should be noted that all IVTT reactions have background levels of ATP as the energy source for the transcription and translation reaction; however, competition with endogenous ATP was ruled out, as inhibition occurred only in the presence of exogenously added PknK and 1 mM ATP. Addition of ATP alone to the active IVTT reaction did not have any inhibitory effect (see Fig. S3 in the supplemental material), reinforcing the idea that inhibition is specific to PknK and ATP. Densitometric analysis of the immunoblots from three independent experiments revealed significant inhibition (57%) of the phoP-IVTT reaction as a result of direct addition of ATP.
We also tested the phosphorylation-defective PknK1-1110K55M for its ability to inhibit PhoP protein synthesis. In contrast to the wild-type PknK, exogenous addition of PknK1-1110K55M and ATP did not have any inhibitory effect on PhoP synthesis (Fig. 4A, lane 5). These results suggest that PknK-mediated effects on IVTT are phosphorylation dependent and further explain the requirement for ATP for the inhibitory function of PknK.

To demonstrate that the action of PknK on protein synthesis is not gene specific, we determined the effect of PknK on the in vitro synthesis of the Renilla luciferase protein. The control DNA carrying the R. reniformis luciferase gene (Rluc) downstream from a T7 promoter was used as the template for the IVTT reaction with and without exogenous addition of PknK1-1110 and ATP. The Rluc protein levels were monitored by measuring production of light as a readout of luciferase synthesis in a luciferase assay system. We observed a 70% decrease in the production of light and therefore production of the luciferase protein when PknK1-1110 and ATP were added (Fig. 4B, lane 3). Thus, the effect of PknK1-1110 on IVTT is dependent not on the gene undergoing IVTT, but rather, on the mechanism of transcription and translation. Furthermore, inhibitory effects were also observed with exogenously added purified PknK KD-GST protein and ATP (Fig. 4B, lane 4). These results validate our observations with IVTT-synthesized PknK and also indicate that the kinase domain of PknK is sufficient for directing inhibition of in vitro protein synthesis.

In view of the above observations, we analyzed PknK1-1110-dependent phosphorylation of the IVTT components using an in vitro kinase assay followed by immunoblotting with anti-phosphoserine/phosphothreonine antibody. Although some back-
ground signal was observed in the control (IVTT minus PknK) and in PknK<sub>1-1110</sub> K55M reactions, there was a region of distinct kinase activity observed with the wild-type PknK<sub>1-1110</sub> (Fig. 4C, lane 2, black arrows) that was absent with the K55M mutant (Fig. 4C, lane 3). We observed signal corresponding to autophosphorylated wild-type PknK<sub>1-1110</sub> (Fig. 4C, lane 2, white arrow) that was absent in the K55M mutant protein reaction. Thus, we concluded that PknK inhibits in vitro protein synthesis through interactions and phosphorylation of specific components of the IVTT machinery.

PknK-mediated effects on in vitro transcription and translation are synergistic. Bacterial cells have coupled transcription and translation mechanisms; thus, to fully understand the mechanism of PknK-mediated inhibition, it was imperative to analyze the effects of PknK on transcription and translation independently. For coupled transcription and translation, luciferase DNA was used as the template in the rabbit reticulocyte lysate (RRL) system that allows both transcription and translation, whereas presynthesized luciferase mRNA was used as a template for uncoupled RRL systems that only drive translation. Five micrograms of purified PknK KD-GST and 1 mM ATP were exogenously added to both assays and analyzed for any effects on luciferase production. PknK KD-GST drastically inhibited synthesis of luciferase from the DNA template (Fig. 4D, white bars). On the other hand, inhibition with luciferase mRNA as the template was moderate (~35%) (Fig. 4D, black bars), indicating that the effects of PknK on transcription alone contribute to, but do not solely account for, the significant inhibition of luciferase production, as observed in the coupled transcription and translation system. From these results, it is apparent that PknK inhibits both transcription and translation mechanisms so that the effects are synergistic and lead to an overall decrease in the efficiency of protein synthesis.

PknK and ATP concentrations are positive effectors of PknK-mediated inhibition of IVTT. Kinase reactions are typically bimolecular reactions with ATP as one of the substrates. Since PknK undergoes autophosphorylation, PknK acts as a substrate, as well. We rationalized that the effective enzyme (here, PknK) and substrate (ATP or PknK) concentrations can positively or negatively affect the interactions with other substrate proteins in the IVTT mixture; thus, we investigated whether the inhibitory effect of PknK on IVTT was dose dependent. We studied the effects of PknK and ATP concentrations independently on the efficiency of IVTT using plasmid pYA1635 (phoP) as the DNA template. In agreement with the results shown in Fig. 4A, exogenous addition of PknK<sub>1-1110</sub> and 1 mM ATP resulted in a decrease in PhoP synthesis (~48%) compared to the minus-ATP control (Fig. 5A, left blot). Upon increasing the concentration of exogenous ATP to 2 mM, PknK<sub>1-1110</sub> completely inhibited the synthesis of PhoP (Fig. 5A, left blot). On the other hand, there were no observable effects on PhoP synthesis by PknK<sub>1-1110</sub> K55M mutant protein even in the presence of 2 mM ATP (Fig. 5A, right blot) or by 2 mM ATP alone (see Fig. S3 in the supplemental material). These controls highlight the essential role of phosphorylation in PknK-mediated IVTT inhibition and remove any doubts about nonspecific inhibition. To eliminate any variations due to different preparations of PknK-IVTT proteins, we also tested a purified PknK KD-GST. Increasing concentrations of ATP in the presence of a constant amount of PknK KD-GST (5 μg) also led to a decrease in PhoP production (Fig. 5B, left blot). In fact, as with PknK<sub>1-1110</sub>-IVTT protein, there was no detectable protein synthesis in the presence of 2 mM ATP and PknK KD-GST (Fig. 5B, left blot).

To assess the effect of the PknK concentration, we varied the amounts of purified PknK KD-GST (1 to 10 μg) while keeping the amount of ATP constant at 1 mM. We chose 1 mM ATP for consistency with previous experiments. Increasing concentrations of PknK KD-GST led to a concomitant increase in the inhibition of PhoP synthesis (Fig. 5B, right blot). Together, these results indicate that both PknK and ATP levels exert cooperative effects that may govern intra- and/or intermolecular interactions between PknK, ATP, and the IVTT components that further dictate the inhibition of in vitro transcription and translation.

The PknK kinase domain forms oligomeric complexes: role of ATP. In view of the dose-dependent effects of PknK on IVTT (Fig. 5), we wanted to determine if PknK existed as a monomer or a dimer in solution. Western blot analyses of the IVTT-synthesized full-length and kinase domains of wild-type PknK and K55M mutant proteins with an anti-PknK antibody revealed distinct changes in the molecular mass on simultaneously resolved native and denaturing gradient polyacrylamide gels. The linear masses of the His-tagged PknK<sub>1-305</sub> (~130 kDa) and PknK<sub>1-305</sub> (~45 kDa) proteins on an immunoblot of the denaturing gel were as expected (Fig. 6A, left blot). On a Western blot of the native gel, no signal was detected with the full-length proteins, probably due to resolution constraints of the gel; however, a signal corresponding to more than twice the size of the linear kinase domain was observed for the wild-type and mutant PknK<sub>1-305</sub> proteins, suggesting association between PknK KD proteins (Fig. 6A, right blot). To visualize these associations, we analyzed purified PknK KD-GST protein on a Coomassie-stained native gel and compared the sizes of PknK oligomers with respect to simultaneously resolved native protein standard markers. In contrast to a single band corresponding to the PknK KD-GST protein (~60 kDa) obtained on a Coomassie-stained denaturing gel (Fig. 6B, left), we observed two bands of various larger sizes (between 150 kDa and...
480 kDa) on the Coomassie-stained native gel, confirming that PknK KD forms oligomeric complexes (Fig. 6B, right). In accordance with our observations with the His-tagged PknK-KD 1-305 (Fig. 6A, right blot), we observed a lower band between the 146-kDa and 242-kDa protein standards, suggesting the formation of a PknK-KD trimer (Fig. 6B, right). In addition, we noted the presence of a distinct higher band around the region corresponding to 400 to 480 kDa, indicating formation of higher-order oligomeric assemblies (Fig. 6B, right).

We noticed that self-association of PknK occurred in the absence of ATP, and moreover, the K55M mutation that abolishes ATP binding and hydrolysis (Fig. 3B) did not hamper the associations of either the full-length or kinase domain K55M mutant protein (Fig. 6A). Similarly, with the purified PknK KD-GST protein, there was no change in the pattern with or without 1 mM ATP or AMP-PNP (a nonhydrolyzable ATP analog) and compared with a PknK KD-GST (minus ATP) control. The data are presented as the change in diameter of the particle in solution. In comparison to the control, the particle size increased by ~56% in the presence of ATP. The change in the diameter of the particle from 7.5 nm (control) to 11.7 nm (with ATP) is indicated by a shift on the x axis. In contrast, the change in particle size with AMP-PNP was minimal.

FIG 6 PknK forms oligomeric complexes in solution. (A) Western blot (WB) analysis with anti-PknK antibody of IVTT-synthesized PknK1-1110 and PknK1-305 wild type and their K55M counterparts simultaneously resolved on native and denaturing 4 to 15% gradient polyacrylamide gels. Denaturing gel WB analysis showed the linear size of the proteins, whereas WB analysis of the native gel revealed the formation of an oligomer with the wild-type and mutant PknK1-305 proteins. (B) PknK KD-GST forms oligomers of various sizes in solution. A Coomassie-stained gel (C) shows the effect of ATP on the oligomerization pattern of PknK KD-GST (5 µg) after kinase assay with 1 mM ATP, followed by resolution on a native and denaturing 4 to 15% gradient polyacrylamide gel. The presence of ATP did not reveal any changes in the oligomerization pattern of PknK KD-GST. Lane M, native molecular mass protein marker. (C) Effects of ATP binding and hydrolysis on the PknK KD-GST particle size. Dynamic light scattering measurements were made on PknK KD-GST after in vitro kinase assay with 1 mM ATP or AMP-PNP (a nonhydrolyzable ATP analog) and compared with a PknK KD-GST (minus ATP) control. The data are presented as the change in diameter of the particle in solution. In comparison to the control, the particle size increased by ~56% in the presence of ATP. The change in the diameter of the particle from 7.5 nm (control) to 11.7 nm (with ATP) is indicated by a shift on the x axis. In contrast, the change in particle size with AMP-PNP was minimal.

The PknK C terminus exerts intrasteric control that autoregulates PknK activity. The PknK C terminus is a multidomain regulatory region that contains an AAA motif encompassing an ATP/GTP binding site (P loop), a PDZ domain (a common structural domain of 80 to 90 amino acids found in the signaling proteins of bacteria, yeast, plants, and animals [27]), and a region consisting of superhelical (SUPR)- or tetratricopeptide (TPR)-like repeats (1, 28) (Fig. 7A); however, their roles in PknK function have not been elucidated. To investigate any regulatory role of the C terminus, we created and assayed wild-type and K55M mutant proteins of various lengths for their effects on the efficiency of the...
In Fig. 4A, we demonstrated that the wild-type PknK1-1110 inhibits IVTT in an ATP-dependent manner. However, compared to its wild-type counterpart, the full-length K55M mutant protein was completely inefficient in inhibiting the IVTT reaction (Fig. 7B, top, and Fig. 4A). We attributed the lack of any inhibitory effect of PknK1-1110K55M to its inability to undergo ATP-induced activation. We concluded that, as a result of the substitution mutation, the K55M mutant is unable to bind and respond to ATP as an inducer of the conformational changes and autophosphorylation of PknK that are essential for its activity. In contrast, the PknK1-869K55M and PknK1-305K55M proteins inhibited the IVTT reaction, similar to their wild-type counterparts (Fig. 7B, middle and bottom). In light of these unanticipated results, we also investigated the in vitro kinase activities of the truncated K55M proteins. Both PknK1-869K55M and PknK1-305K55M showed consistent but faint autophosphorylation signals (see Fig. S4 in the supplemental material). These results highlight two novel aspects of PknK structure and activation that are illustrated in Fig. 7C:

1. The lysine 55 residue is essential for PknK activity only in the full-length protein, and second, loss of the C-terminal region removes any steric hindrance that may have resulted from interactions between the C terminus and the catalytic site of the full-length protein, thereby leading to ATP binding and inhibitory activity despite the K55M mutation.

DISCUSSION

Our studies have focused on the functional characterization of PknK, a transcriptional regulatory STPK of M. tuberculosis. In this study, we elaborated on several structural and functional aspects of PknK activation and autoregulation: role of the C-terminal regulatory region. (A) Schematic linear representation of the various proposed domains in the full-length and truncated PknK proteins: a kinase domain (amino acids 26 to 283), an AAA motif (white box) encompassing the ATP/GTP-binding site (amino acids 368 to 375), a PDZ domain (amino acids 465 to 533), a helical region containing SUPR repeats (amino acids 777 to 1108), and a putative TPR-like motif (amino acids 904 to 929) (1, 27, 28; http://www.ebi.ac.uk/tools/pfa/iprscan and http://myhits.isb-sib.ch/cgi-bin/motif_scan). Truncated PknK proteins were constructed so that the PknK1-305 protein lacked the entire C-terminal regulatory region while the PknK1-869 lacked only the region containing the TPR-like helical motif (Δ241 amino acids). The K55M mutations in the proteins are marked with asterisks. (B) The PknK wild-type (WT) and mutant (K55M) proteins of various lengths were assayed for their inhibitory effects on IVTT of the target protein (PhoP) in the presence of ATP. CT, positive control for PhoP synthesis in the presence of pYA1635 (phoP) DNA alone. (C) Proposed model for PknK activation and autoregulation. For the sake of clarity, PknK is shown as a monomer; however, we have demonstrated that it can exist as oligomers of different sizes in solution. (1) Based on the results presented in this study, a hypothetical model for PknK activation (in terms of its ability to inhibit the IVTT reaction) is presented wherein, in the absence of any inducer, the interactions between the PknK C-terminal region and the N-terminal active site result in an inactive or autoinhibited kinase. Addition of a positive activator, such as ATP, results in conformational changes that relieve the autoinhibition and direct the autophosphorylation and activation of PknK. Similar structural changes resulting in PknK activation do not occur in the presence of ADP (arrow with an X). (2) Autophosphorylation of threonine residues in the PknK activation loop and some yet-unknown residues in the C-terminal region has been reported (27) and is depicted arbitrarily. The K55M mutation in the full-length protein excludes any possibility of ATP binding and hydrolysis and thus does not respond to structural activation by ATP, resulting in an inactive enzyme. Deletion of the C-terminal region in the truncated PknK1-869 and PknK1-305K55M proteins removes the autoregulatory control and allows ATP binding, resulting in an active protein, a.a, amino acids.
of PknK-mediated growth regulation in vitro and in vivo. We determined that PknK mediates transcriptional reprogramming of M. tuberculosis by regulating tRNA expression during the log- 
mic and stationary growth phases as a means to facilitate adaptation 
to changing growth environments. Furthermore, increases in 
PknK<sub>Mtb</sub> levels and the resulting signaling events in M. smegmatis 
severely reduce the rate of growth and colony development. In 
accordance with its regulatory role in mycobacteria, we estab-
lished that PknK directly inhibits in vitro transcription and 
translation through a phosphorylation-dependent mechanism and, in 
addition, provided valuable insights into the factors that enhance 
PknK-mediated inhibition of protein synthesis and contribute to 
PknK activation and regulation.

Although several STPKs, including PknK, have been impli-
cated in the regulation of mycobacterial growth (13, 17, 24, 25, 30, 
37), the molecular switch that governs the transition of M. tuber-
culus from high to low growth rates is unknown. Given the in-
duced levels of PknK and the increased survival of a PknK mut-
ant during stationary phase (30), we investigated its regulatory 
role by identifying genes that are regulated by PknK during the 
progression of M. tuberculosis from an “active” logarithmic phase 
to a “slow” stationary phase. Predictably, microarray results re-
vealed a distinct regulatory role of PknK during stationary-phase 
growth. The differential expression of genes involved in cell wall 
processes and lipid metabolism in the LIX11 mutant validate our 
previous findings (30), which showed changes in the cell wall 
composition as a result of pknK deletion. A major highlight of 
these studies is the temporal expression of tRNA genes in the 
ΔpknK mutant. It is striking that of the 45 tRNA genes in M. 
tuberculosis (6), a significant number (>50%) were downregu-
lated in the LIX11 mutant during log phase but upregulated in the 
stationary phase. Since M. tuberculosis is a G+C-rich organism, it 
is not surprising that the tRNA genes differentially regulated in 
LIX11 exhibited a codon bias for a G or C base in the third posi-
tion. More importantly, a subset of 16 tRNA genes (up- or down-
regulated) was observed to be common between the logarithmic 
and stationary growth phases (Table 2). Although the precise 
mechanisms are presently unclear, this trend of tRNA expression 
underlines an important feature of PknK-mediated growth regu-
lation. As with most bacteria, availability of nutrients and favor-
able growth conditions dictate the exponential growth of myco-
bacteria. Typically, such growth conditions are marked by high 
metabolic activity, leading to biosynthesis of macromolecules es-
sential for growth and survival. It would then follow that during 
log phase, bacteria would exhibit an increased rate of protein syn-
thesis and therefore require increased tRNA synthesis. Cells enter-
ing stationary phase are exposed to nutrient depletion, acidic pH, 
hypoxia, and oxidative stress, as well as other stress factors. Con-
sequently, growth in stationary phase is slowed, primarily due to 
intracellular metabolic controls that would repress endogenous 
metabolism as a stress response mechanism. Repression of tRNA 
synthesis during stationary phase in the wild-type strain, but not 
in a pknK deletion strain, establishes PknK as a key component of 
such metabolic controls.

In principle, a “loss-of-function” phenotype resulting from a 
gene deletion can be complicated due to the activation of redund-
ant, overlapping pathways, and in the case of well-coordinated 
regulatory effects of multiple transcriptional regulators and 
STPKs on mycobacterial growth, that is a distinct possibility. 
Moreover, the environmental signal for PknK activity is currently 
unknown. In view of these considerations and to elucidate the 
direct regulatory role of PknK, we evaluated the effects of pknK 
overexpression on mycobacterial growth and physiology. In-
creased intracellular PknK<sub>Mtb</sub> levels and phosphorylation activity 
in a fast-growing, nonpathogenic M. smegmatis strain inhibited 
growth significantly and caused “dwarfed” colony morphology. 
Because PknK regulates the expression of genes involved in cell 
wall synthesis and lipid metabolism (see Fig. S1 in the supplemen-
tal material) (27), the defects in colony development are not sur-
prising. It is noteworthy that pknK<sub>Mtb</sub> overexpression did not af-
cct cell viability significantly; rather, it severely reduced the rates 
of growth of the individual colonies. In addition, reversion of the 
inhibition phenotype when LIX79 induced cells were plated on 
noninducing medium suggests that intracellular PknK levels and 
activity are closely regulated. From the data presented, it is clearly 
evident that the cellular abundance of PknK<sub>Mtb</sub> activity slows my-
cobacterial growth and, in addition, provides an explanation for 
our inability to obtain overexpression of pknK in the slow-grow-
ing M. tuberculosis. Together, the role of PknK<sub>Mtb</sub>-dependent 
phosphorylation in the modulation of the growth rate and the 
regulation of tRNA synthesis during M. tuberculosis growth implica-
tes PknK in mechanisms that control translation.

It is well known that phosphorylation of translation initiation 
and elongation factors is subjected to alteration during cell differ-
entiation or bacteriophage infections (14, 21, 34, 41). In strepto-
mymes, ribosome-associated STPKs phosphorylate 11 ribosomal 
proteins and the elongation factor Tu (EF-Tu), resulting in 30% 
loss of ribosomal activity (33). With respect to M. tuberculosis, 
evidence on the role of STPKs in translation control is limited.

With the exception of EF-Tu (42), phosphorylation of RNA poly-
merase subunits, transcription terminators, and ribosomal pro-
teins has been speculated but has yet to be demonstrated (8, 40).

The results presented in Fig. 4, 5, and 7 collectively provide evi-
dence for a direct role of PknK in inhibition of in vitro protein 
synthesis. The cell-free expression system used in our studies pro-
vided an environment that eliminated interference from endoge-
nous signals and transcriptional regulators and allowed direct as-
essment of PknK function. Additionally, IVTT enabled the 
synthesis of large proteins, such as PknK, that were stable, prop-
erly folded, and catalytically functional. This is the first instance of 
a successful demonstration of the synthesis of mycobacterial ki-
nases using cell-free expression systems and their applicability to 
in vitro kinase assays.

Because transcription and translation occur simultaneously in 
bacteria, changes affecting one process automatically affect the 
other. Our data suggest that via phosphorylation of the IVTT 
components, PknK exerts inhibitory effects on transcription and 
translation processes, resulting in an overall decrease in the ef-
ciency of protein synthesis. Although we have not identified the 
specific IVTT components that interact with PknK, our studies 
provide valuable insights into the factors that contribute to PknK 
function. PknK caused inhibition of in vitro transcription and 
translation only in the presence of exogenously supplied ATP. The 
role of ATP was 2-fold: first, it mediated PknK activation by di-
recting conformational changes and autophosphorylation that 
were required for PknK’s inhibitory function, and second, it acted 
as a positive effector of PknK action on the IVTT reaction. The 
increase in PknK activity or IVTT inhibition in the presence of 
increasing substrate (ATP or PknK) concentrations is consistent 
with the effects of excess substrate on enzyme kinetics. However,
considering that the effective ATP and PknK concentrations or turnover are in a flux within the cells, it will be particularly relevant to investigate how these factors contribute to the regulation of PknK activity in changing growth environments. At least with PknK levels, it is sufficiently clear that an abundance of PknK in vitro and in mycobacteria, whether as a result of M. tuberculosis growth during stationary phase (30) or overexpression in M. smegmatis, inhibits protein synthesis, tRNA expression, and the growth rate. While the cell-free IVTT assay afforded a perturbation-free environment to illustrate PknK function, differential regulation of PknK activity due to environmental signals and transcriptional regulators must be considered to understand the mechanism of PknK-mediated growth regulation. The role of one such cellular signal, guanosine tetraphosphate (ppGpp), in the regulation of protein synthesis under stress conditions is well established (4, 44). Repression of tRNA and rRNA by ppGpp (23, 44) and its implications for the long-term persistence of M. tuberculosis (26, 39) necessitate further investigation of possible links between ppGpp signaling and PknK function.

Kinases are molecular switches that exist in either an “off” (inactive) state or an “on” (active) state. Ligand-induced N-lobe dimerization is emerging as the universal mechanism of STPK activation in M. tuberculosis (15, 18, 29, 36, 48). However, the soluble STPKs, PknG and PknK, exhibit structural uniqueness that may require other mechanisms of activation. As opposed to a monomeric PknB, PknG exists as a dimer in solution, and moreover, dimerization does not occur through the kinase domain but rather is mediated by regions at the C-terminal tail (43). In addition, the ability of PknK-KD to form oligomers complex further highlights the structural differences between the M. tuberculosis membrane-bound receptor STPKs and soluble STPKs. PknK belongs to the MalT group of transcriptional activators that share motifs that classify them in a separate class of proteins called the signal transduction ATPases with numerous domains (STAND) proteins (28). STAND proteins are atypical AAA+ (12) proteins involved in signal transduction that integrate signals to assemble them into large scaffolds (11, 28, 32). Unlike E. coli MalT, where oligomerization is regulated by ATP and maltotriose (9, 10), intermolecular interactions of PknK did not appear to depend on ATP, indicating divergence from the MalT activation mechanism. However, the PknK C terminus contains regions that are homologous to MalT domains responsible for ATP binding and hydrolysis and maltotriose binding (9). Thus, it is possible that, in addition to the catalytic site, ATP binds to distinct sites in the C-terminal regulatory region and that a second ligand may be required in conjunction to regulate PknK activation.

The PknK C terminus has SUPR- or TPR-like domains that are typical protein interaction modules with unique regulatory functions. Currently, there is no structural information about PknK available that could aid in understanding the contributions of the PknK C-terminal regulatory region and its phosphorylation (27) towards PknK function. We demonstrated that the PknK C-terminal region autoregulates PknK activity, and we propose a model for PknK activation (Fig. 7C). We define PknK activation as the ability of PknK to inhibit the IVTT reaction, so an inactive PknK would have no effect on IVTT. We hypothesize that in the absence of an inducer, PknK exists in an inactive conformation with no inhibitory effects on IVTT. Addition of a positive activator, such as ATP, but not ADP, leads to autophosphorylation of PknK and induces conformational changes that facilitate PknK activation and, hence, inhibition of protein synthesis. Typically, the invariable lysine residue plays a role in positioning the ATP molecule and the substrate protein for catalysis (19). Previous results have shown a key role of lysine 55 in PknK activity (27). Our results confirm these observations and also show that lysine 55 is essential for the activity of the full-length PknK protein only. The loss of the C-terminal region, specifically the last 241 amino acids, resulted in truncated proteins that were active despite the mutation. It is possible that in the inactive mode the C terminus interacts with residues around the active site, including lysine 55, so its deletion renders the truncated K55M proteins active. We hypothesize that intramolecular interactions with the C terminus may regulate PknK activity by ensuring activation only in the presence of an activator ligand, like ATP. Such a mechanism of autoinhibition is favorable from a regulatory viewpoint, as it allows fine-tuning of an activation mechanism that would otherwise lead to wasteful dissipation of energy. Clearly, in light of PknK’s role in slowing growth, it would not be beneficial to the cell for PknK to be constitutively active.

The autoinhibition mechanism for kinase regulation has not been described for any mycobacterial STPK but is highly conserved in eukaryotic kinases, such as the cyclic AMP (cAMP)-dependent protein kinase and the human twitchin kinase (20). Typically, part of the kinase contains an autoregulatory sequence that folds into the active site and blocks activity. The kinase is switched on by the binding of an allosteric regulator that removes the autoregulatory sequence from the active site. Protein kinase C was found to contain a pseudosubstrate near its N terminus, and truncation mutagenesis of the pseudosubstrate sequence rendered the kinase constitutively active (20). The autoregulatory sequence of PknK resides in the last 241 amino acids at the C terminus, thus implicating the TPR-like motif in PknK autoregulation. In summary, our findings open exciting avenues of research to address questions on PknK structure and function that will shed light on the mechanisms of PknK oligomerization and autoregulation and their implication in the regulation of M. tuberculosis growth and persistence.

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