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# Phosphorylation of *Mycobacterium tuberculosis* protein tyrosine kinase A PtkA by Ser/Thr protein kinases

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ABSTRACT

*Mycobacterium tuberculosis (Mtb)*, the causative agent of tuberculosis (TB), has inflicted about one third of mankind and claims millions of deaths worldwide annually. Signalling plays an important role in *Mtb* pathogenesis and persistence, and thus represents attractive resource for drug target candidates. Here, we show that protein tyrosine kinase A (PtkA) can be phosphorylated by *Mtb* endogenous eukaryotic-like Ser/Thr protein kinases (eSTPKs). Kinase assays showed that PknA, PknD, PknF, and PknK can phosphorylate PtkA in dose- and time-dependent manner. Enzyme kinetics suggests that PknA has the highest affinity and enzymatic efficiency towards PtkA. Furthermore, protein–protein interaction assay in surrogate host showed that PtkA interacts with multi-eSTPKs *in vivo*, including PknA. Lastly, we show that PtkA phosphorylation by eSTPKs occurs on threonine residues and may effect tyrosine phosphorylation levels and thus PtkA activity *in vitro*. These results demonstrate that PtkA can serve as a substrate to many eSTPKs and suggests that's its activity can be regulated.

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## 1. Introduction

Tuberculosis (TB) is a major threat of global public health, caused by single etiological agent-*Mycobacterium tuberculosis* (*Mtb*) [1]. A hallmark of TB is that *Mtb* can persist in humans for an extended period due to its ability to adapt to the hostile host environment. Such adaptation is dependent on several factors: immune evasion mechanism [2], distinctive nutrient metabolism [3], and intricate signal transduction systems [4]. *Mtb* uses an array of effector molecules to arrest phagosome maturation [5,6], including: the lipids phosphatidylinositol mannoside (PIM) [7] and lipoarabinomannan (LAM) [8], phosphatidylinositol-3-phosphate (PI(3)P) phosphatase SapM [9], protein-tyrosine phosphatase PtpA [10,11], nucleoside diphosphate kinase (Ndk) [12], and low molecular weight protein antigens EsxH and EsxG [13]. *Mtb* possesses a variety of metabolic capabilities to tackle the hostile microenvironment of the human host, thought to include hypoxic, acidic, nutrient poor conditions, and immune effectors such as nitric oxide (NO) [3]. Signal transduction systems provide *Mtb* with the ability to sense its environment based on external signals and mount an effective adaptive response. Signalling pathways represent an attractive target candidate for novel drug development against tuberculosis [14–17].

*Mtb* possesses multiple families of signal transduction systems, including eleven eukaryotic-like Ser/Thr protein kinases (eSTPKs)-phosphatases [18] (some of them work as dual specificity kinases that also phosphorylate Tyrosine [19]), twelve two-component regulatory systems (TCSs) [20,21], thirteen sigma factor system (especially the 11 extracytoplasmic function sigma factor, ECF-σ) [22,23], one protein-tyrosine kinase (PTK) PtkA [24,25], and two protein-tyrosine phosphatases PtpA and PtpB [26,27].

PtkA is the first protein tyrosine kinase identified in *Mtb*; its physiological function is still unknown. PtpA is the only known substrate identified thus far for PtkA [24,25]. PtpA blocks the fusion between phagosome and lysosome, and inhibits phagosome acidification by binding to the subunit H of $H^+$-ATPase and dephosphorylating human class C Vacuolar Protein Sorting VPS33B in

*Abbreviations:* TB, tuberculosis; Mtb, mycobacterium tuberculosis; PtkA, protein tyrosine kinase A; eSTPK, eukaryotic-like Ser/Thr protein kinases.

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homotypic vacuole fusion and vacuole protein sorting complex (HOPS) [10,11]. PtpA was shown to inhibit innate immune response through dephosphorylation of Jnk and p38 [28]. PtpA also represses host apoptosis by dephosphorylating GSK3α (Glycogen Synthase Kinase 3α) [29] and potentially macrophage's bioenergetics state [30]. Our most recent study showed that PtkA positively regulates PtpA phosphatase activity [31]. Taken together, this set of evidence suggests a potential role for PtkA in the control of PtpA and related pathophysiological events in *Mtb*.

Protein kinase generally phosphorylates multiple target substrates. An earlier report suggested a tyrosine-phosphorylated protein with an approximate size of 55 kDa [32]. PtpA and PtkA have different sizes of 18 kDa and 30 kDa respectively. Thus, PtkA might phosphorylate different substrates depending on different environmental signals. Therefore, we looked for potential additional protein substrates for PtkA. For this, *Mtb* cellular extract was incubated with purified recombinant protein PtkA. To our surprise the phosporylation level of PtkA protein itself was increased when incubated with an *Mtb* cellular extract. This suggests that PtkA can serve as a substrate for endogenous protein kinases in *Mtb*. Indeed, a previous report showed phosphorylated threonine residue in PtkA [33]. As TCSs have specific cognate substrates [22], we hypothesized that PtkA phosphorylation is due to endogenous eSTPKs. We incubated eight of the eleven *Mtb* eSTPKs in the form of truncated proteins expressing only the active kinase domains with PtkA. We found that four eSTPKs (PknA, PknD, PknF, and PknK) can phosphorylate PtkA and the tyr phosphorylation inactive mutant, $PtkA^{Y262A}$, *in vitro*. Enzyme kinetics parameters suggest that PknA shows the highest affinity for PtkA binding, compared to that by PknD, PknF, and PknK. *In vivo* protein–protein interaction assay between multi eSTPKs and PtkA further suggests an interaction between these two independent families of protein kinases. Phosphoamino acid assay showed that PknD and PknK might enhance PtkA auto-phosphorylation activity. Taken together, a signalling network combining eSTPKs and PtkA in *Mtb* may play a role mediating signal transduction and controlling physiological events in Mtb.

## 2. Materials and methods

### 2.1. Protein expression and purification

The cloning and expression of PtkA and $PtkA^{Y262A}$, PknG, and PknH were performed as described before respectively [24,34,35]. The expression of kinase domains of PknA, PknB, PknD, PknE, PknF, PknI, PknJ, PknK, and PknL from *Mtb* were performed as described before [31].

### 2.2. Mtb culture and cellular extracts preparation

*Mtb* H37Rv strain was grown in Middlebrook 7H9 broth (BD Diagnostic Systems) supplemented with 10% (v/v) OADC and 0.05% (v/v) Tween-80 (Sigma-Aldrich) at 37 °C standing. Exponentially growing *Mtb* H37Rv culture was diluted to $OD_{600} \approx 0.05$ in 10 mL medium in 50 mL sealed tubes, and grown at 37 °C rolling. When $OD_{600}$ was around 1, bacteria were harvested by centrifugation, washed twice with extraction buffer (20 mM Tris-HCl, pH7.2, 1 mM DTT and 1 mM PMSF) and resuspended in 1/100 vol. 1/3 volume of 0.1 mm glass beads (BioSpec) was added, and the suspensions were subjected to 3 × 30s pulses in the Fastprep FP120 cell disrupter (Thermo). Cell debris was removed by spinning at 13,000 rpm for 15 min at 4 °C and cell-free extracts were filtered through 0.22 μm filter membrane and stored at −80 °C in 10% glycerol.

### 2.3. In vitro kinase assay

Kinase assays were carried out using γ-[$^{32}$P] ATP(Perkin Elmer) as the phosphate donor in reaction buffer (20 mM Tris-HCl, pH7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT and 2 mM $Na_3VO_4$) according to published protocols [36]. Briefly, the reactions were started by addition of 5 μCi of γ-[$^{32}$P] ATP and the incubation was performed at room temperature (25 °C). At the end of the incubation period, reactions were stopped by the addition of SDS-sample loading buffer and heated at 95 °C for 10 min. Samples were resolved by SDS-PAGE, and the gels were silver stained and dried. The $^{32}$P-radioactively labelled protein bands were detected using a PhosphorImager SI (Molecular Dynamics). Bands corresponding to the phosphorylated proteins were cut out and subjected to scintillation count when necessary (Beckman Coulter LS 6500).

### 2.4. Mycobacterial Split-Trp protein–protein interaction assay

The Split-Trp assay [37] was carried out as described previously [38]. Briefly, genes of interest were cloned into pJC10 and pJC11 vectors to generate translational fusion constructs with the N-trp and C-trp fragments, respectively, of the N-(5′-phosphoribosyl)-anthranilate isomerase under the control of the acetamidase promoter. Co-transformed *Mycobacterium smegmatis (Msm)* $Trp^-$ was spotted (5 μL) onto 7H10, 1% glucose, 60 μg/mL histidine, 50 μg/mL hygromycin and 30 μg/mL apramycin plates, supplemented with either 0.02% acetamide, 120 μg/mL Trp, or neither, and grown for 2–3 weeks at 25 °C. Oligonucleotide sequences and vectors used in this work are listed in Table S1. The concentrations of antibiotic used were 25 μg/mL of kanamycin, 50 μg/mL of hygromycin and 30 μg/mL of apramycin for tryptophane auxotrophic strain *Msm ΔhisA*.

### 2.5. Mycobacterial protein complementation assay

The mycobacterial protein fragment complementation assay was performed as described [31,39]. The genes of interest were PCR-amplified and cloned into pUAB100 (expressing mDHFR fragment F1, 2) or pUAB200 (expressing mDHFR fragment F3). *M. smegmatis (Msm)* was co-transformed with both plasmids; the co-transformants were selected on 7H11 agar plates with 25 μg/mL kanamycin and 50 μg/mL hygromycin and tested for growth over 3–4 days on 7H11 kanamycin/hygromycin plates supplemented with 20 μg/mL trimethoprim (TMP).

### 2.6. In vitro protein interaction assay

ALPHAScreen assay was performed using the Histidine (Nickel Chelate) Detection Kit, according to the manufacturer's protocol (Perkin Elmer). Purified recombinant protein was biotinylated using the EZ-link Biotinylation Kit (Pierce) according to the manufacturer's protocol. PtkA was cloned as a donor, while PknE and GST protein were cloned as acceptor respectively.

### 2.7. Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described [10]. eSTPKs phosphorylated PtkA, and autophosphorylated PtkA were separated by SDS-PAGE and transferred onto 0.45 μm PVDF membranes. The $^{32}$P-labelled protein band corresponding to the migration of PtkA was excised from the membrane and hydrolysed with 6 M HCl at 110 °C for 1 h, concentrated by vacuum drying, and suspended in 20 μL $H_2O$. 2 μL samples were separated on a cellulose TLC plate in one dimension with isobutyric acid/0.5 M $NH_4OH$ (5:3 v/v).

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## 3. Results

### 3.1. PtkA is phosphorylated under in vitro assays conditions

To identify potential substrates for PtkA, we incubated recombinant PtkA with cellular extracts of *Mtb*. As shown in Fig. 1A, we could not determine new phosphorylated proteins; however, we observed that PtkA phosphorylation was increased upon incubation with cellular extracts from *Mtb* compared to PtkA alone. Prisic et al. [33] previously showed that PtkA can undergo phosphorylation on $Thr^{36}$. Taken together, we suggest that PtkA can serve as a substrate for *Mtb* eSTPKs.

### 3.2. Multi-eSTPKs phosphorylate PtkA in vitro

To examine eSTPK phosphorylation activity on PtkA, recombinant kinase domains of all eleven *Mtb* eSTPKs were expressed, purified, and incubated with PtkA in a radioactive kinase assay, and the corresponding phosphorylation profile was analysed by autoradiography. PknI, PknJ, and PknL fail to show kinase activity (Data not shown). The presence of radioactive signals indicated that PtkA can be phosphorylated by multiple kinases, including PknA, PknD, PknF, and PknK (Fig. 1B). No signal was observed in the presence of PknB, PknE, PknG, or PknH. To rule out the autophosphorylation of PtkA, which might be enhanced after phosphorylation by eSTPKs, $PtkA^{Y262A}$, an autokinase-defective mutant [24], was used to repeat this experiment. As shown in Fig. 1C, PknA, PknD, PknF, and PknK display similar phosphorylation on $PtkA^{Y262A}$. These results clearly indicate that PtkA can be phosphorylated by these STPKs *in vitro*.

### 3.3. Multi-eSTPKs phosphorylate $PtkA^{Y262A}$ in dose and time dependent manner

To further characterize eSTPKs' substrate specificity towards phosphorylation, kinetics was examined with recombinant PknA, PknD, PknF, and PknK. The autokinase-defective $PtkA^{Y262A}$ mutant protein was used in order to reduce background signal of PtkA autophosphorylation. As shown in Fig. 2A and B, the above STPKs phosphorylate $PtkA^{Y262A}$ in time- and dose-dependent manners, respectively. The radioisotope levels of $PtkA^{Y262A}$ protein bands in the dose-dependent experiments were determined by scintillation counting, which were used for calculating the enzyme kinetics (Table 1). The $K_m$ values indicate that PknA has the highest affinity for PtkA binding while PknD, PknF, and PknK showed lower interaction affinity (1.074 μM, 2.692 μM, 3.530 μM, and 2.092 μM for PknA, PknD, PknF, and PknK respectively). PknA also exhibited the highest enzymatic efficiency of PtkA phosphorylation, which is followed by PknD, as evident from the $K_{cat}/K_m$ values measured ($1.983 \times 10^3$ $M^{-1}$ $S^{-1}$, $3.388 \times 10^2$ $M^{-1}$ $S^{-1}$, $4.218 \times 10^{-3}$ $M^{-1}$ $S^{-1}$, $5.249 \times 10^{-3}$ $M^{-1}$ $S^{-1}$ for PknA, PknD, PknF, and PknK respectively).

### 3.4. Multi-eSTPKs interact with PtkA in vivo

Protein–protein interaction is a prerequisite for protein phosphorylation. Here, we performed both Split-Trp protein–protein interaction assay and MPF-C protein–protein assay in *Msm* [37,38] to characterize the interaction between eSTPKs and PtkA. In Split-Trp protein–protein interaction assay, protein–protein interaction leads to the reassembly of the N- and C-fragments ($N_{Trp}$ and $C_{Trp}$) of N-(5′-phosphoribosyl)-anthranilate isomerase, an enzyme

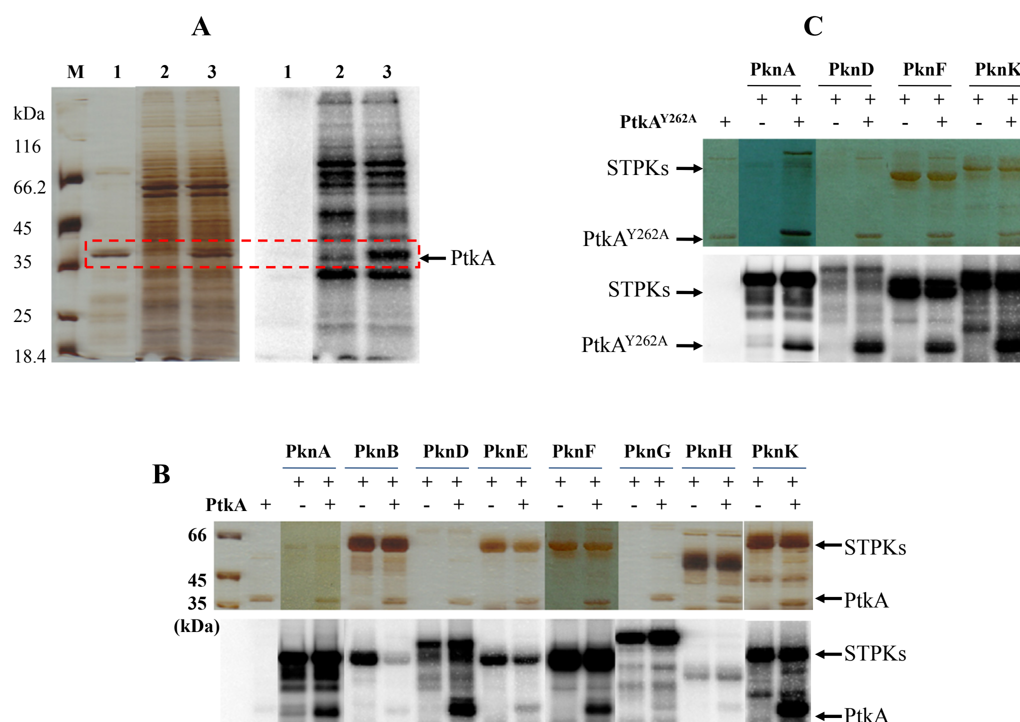


**Fig. 1.** PtkA is phosphorylated by multiple eSTPKs in *Mtb*. A. PtkA is phosphorylated by endogenic protein kinase in *Mtb*. Left: SDS-PAGE (10%); right: autoradiograph. M: unstain protein marker; 1: PtkA protein (1.5 μg); 2: *Mtb* cellular extract (1.5 μg); 3: *Mtb* cellular extract (1.5 μg) and PtkA protein (1.5 μg). B. Phosphorylation of PtkA by eSTPKs. Eight recombinant kinase domains of eSTPKs encoded by the *Mtb* genome were expressed and purified as GST-tag or His-tag fusions and incubated with purified His-tagged PtkA (0.5 μg) and γ-[$^{32}$P] ATP for 30 min. The quantity of the different eSTPKs varied from 0.26 μg to 8.9 μg to obtain the optimal autophosphorylation activity for each kinase. Samples were separated by 8% SDS-PAGE and stained with silver (upper panel) and visualized by autoradiography after overnight exposure to a film (lower panel). The upper bands illustrate the autokinase activity of each eSTPK, and the *lower bands* represent phosphorylated PtkA. C. Phosphorylation of $PtkA^{Y262A}$ by eSTPKs. Upper panel: SDS-PAGE; Lower panel: autoradiograph. Conditions as described above.

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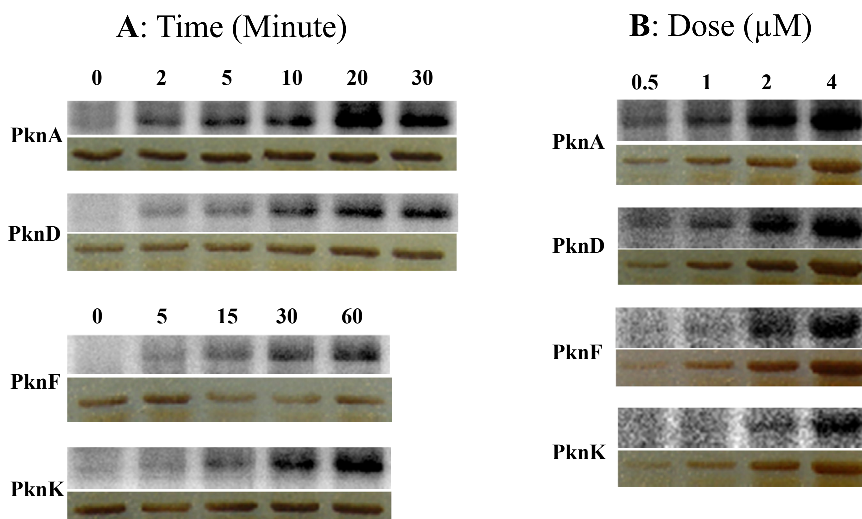


**Fig. 2.** eSTPKs phosphorylate PtkA$^{Y262A}$ in time and dose dependent manner. Up: autoradiograph. Down: SDS-PAGE. A, Time dependent. PknA, PknD, PknF and PknK were allowed to autophosphorylate for 30 min at 25 °C by adding γ-[$^{32}$P] ATP. Then, PtkA$^{Y262A}$ was added, mixed and 20 μL of sample were pipetted out and stopped by SDS-sample loading buffer at different time points. B, Dose dependent. After PknA, PknD, PknF and PknK were autophosphorylated for 30 min at 25 °C, same amount were added into tubes that contain same volume of serially increasing concentration of PtkA$^{Y262A}$. The reactions were continued for another 5 min for PknA, PknD, and 10 min for PknK and PknL respectively. All experiments were repeated 3 times.

**Table 1**
Enzyme kinetics of PknA, PknD, PknF and PknK for PtkA$^{Y262A}$.

| | *V*max (μM Min$^{-1}$ μM$^{-1}$) | *K*m (μM) | *K*cat (S$^{-1}$) | *K*cat/*K*m (M$^{-1}$ S$^{-1}$) |
|---|---|---|---|---|
| PknA | $1.278 \times 10^{-1}$ | 1.074 | $2.13 \times 10^{-3}$ | $1.983 \times 10^{3}$ |
| PknD | $5.472 \times 10^{-2}$ | 2.692 | $9.12 \times 10^{-4}$ | $3.388 \times 10^{2}$ |
| PknF | $8.932 \times 10^{-7}$ | 3.530 | $1.489 \times 10^{-8}$ | $4.218 \times 10^{-3}$ |
| PknK | $6.586 \times 10^{-7}$ | 2.092 | $1.098 \times 10^{-8}$ | $5.249 \times 10^{-3}$ |

required for tryptophan (Trp) biosynthesis. A Trp auxotrophic strain of *Msm* only grows in the absence of Trp if the tested proteins were expressed by acetamide induction and interact with each other. Ntrp-Esat6 and Cfp10-Ctrp were used as a positive control and the negative control consisted of $N_{Trp}$ and $C_{Trp}$ fragments alone. As shown in Fig. 3A, co-expression of PknA, PknE, PknF, PknG, PknH, PknI, and PknL with PtkA fusion proteins restored mycobacterial growth on 7H9 agar plates, confirming their interaction *in vivo*. No interaction between PtkA and PknB, PknD, PknJ, or PknK was detected in this system.

In the M-PFC protein–protein assay, reassembly of the complementary fragments [F1, 2] and [F3] of mDHFR confers resistance to TMP. Here, PtkA–PtpA and Esat6-Cfp10 were used as positive controls. As shown in Fig. 3B, co-expression of PknA, PknB, PknF and PknH with PtkA fusion proteins restored mycobacterial growth on 7H9 agar plates, further confirming their interaction *in vivo*. A selected representative was tested in an *in vitro* ALPHAScreen assay to characterize the kinetics of selected eSTPK binding to PtkA. As shown in Fig. 3C, PknD and PtkA show dissociation constant of Kd 0.0175 μM, indicating an affinity between GST-tagged PknD and PtkA, compared to PknE and GST proteins (which have a dissociation constant of Kd 883.4 μM and 144.3 μM respectively).

### 3.5. Multi-eSTPKs phosphorylated PtkA on Thr residues in vitro

To determine the residues which eSTPKs phosphorylate on PtkA and monitor the effect on its autophosphorylation activity, PtkA was incubated with PknA, PknD, PknF, and PknK respectively, and quantitative phosphoamino acid analysis of the phosphorylated PtkA was performed using TLC. As shown in Fig. 4, PknA, PknD, PknF, and PknK phosphorylated PtkA on threonine residues, and PtkA autophosphorylation was below detection level. We also noticed an increase in the amount of the phospho-tyrosine residues in Thr-phosphorylated PtkA samples. These results further demonstrate that PknA, PknD, PknF, and PknK phosphorylate PtkA on threonine residues, and that PknD and PknK can enhance PtkA autophosphorylation activity *in vitro*.

## 4. Discussion

Here, we report that protein tyrosine kinase PtkA in *Mtb* can undergo threonine phosphorylation by multiple eSTPKs (PknA, PknD, PknF, and PknK) in both dose- and time-dependent manners. Two independent *in vivo* protein–protein interaction assays further confirm the interaction between eSTPKs and PtkA. The Split-Trp assay failed to detect the interaction between PtkA and PknD, or PtkA and PknK, which could phosphorylate PtkA *in vitro*. This might be due to transient interaction or incompatible conformation. Some discrepancy can be observed between the Spit-Trp and the mDHFR interaction assays that can be explained by the different technological design. In the M-PFC system both examined genes are located at the N-terminal of mDHFR fragments, and in the Split-Trp system one is located at N-terminal while the other gene is located at the C-terminal. Nevertheless, the interaction observed between PtkA and PknA and PtkA and PknF in both systems clearly indicated that they can interact *in vivo*. We further characterized a potential binding using the ALPHAScreen *in vitro* protein–protein interaction assay. A representative result examining PknD interact with PtkA suggests binding with a dissociation constant of Kd 0.0175 μM, compared to that of PknE and GST proteins (which shown Kd of 883.4 μM and 144.3 μM respectively).

Protein phsophorylation can effect enzymatic activity. For example, activity of enoyl-ACP reductase InhA is negatively regulated by PknA and PknA mediated phosphorylation in *Mtb* [40]. In this study, we show that PknD and PknK phosphorylate PtkA and enhance its autophosphorylation activity *in vitro*. More interestingly, our recent work has shown that PtkA and PknA enhance PtpA phosphatase activity through phosphorylation of tyrosines and threonine residues on PtpA [31]. However, PknA does not effect

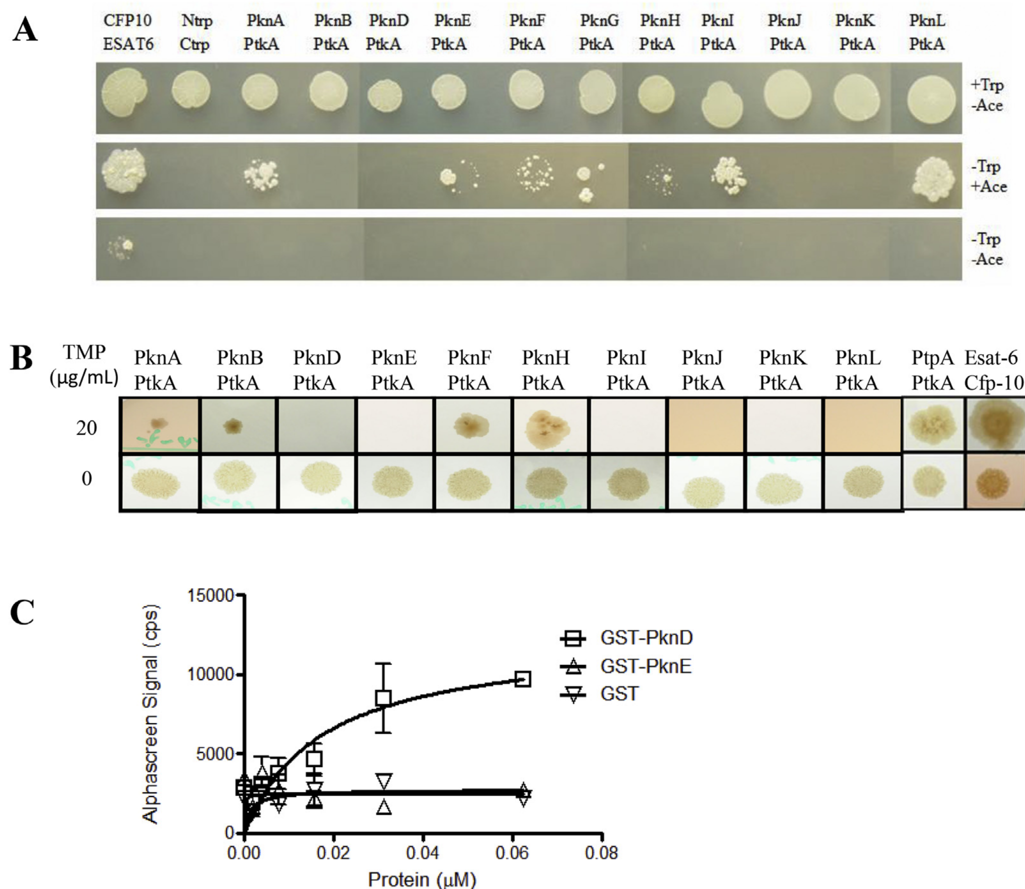


**Fig. 3.** PtkA interacts with Multi-eSTPKs. A. Split-Trp protein–protein interaction assay. eSTPKs and PtkA interacted in the Split-Trp protein fragment complementation assay to facilitate Ntrp and Ctrp reassembly required for Trp biosynthesis, thus enabling growth of the *Msm* Trp$^-$ strain coexpressing $N_{Trp}$-eSTPKs and PtKA-$C_{Trp}$ under acetamide (ACE) induction (Middle). (Top) Transformed strains are capable of growing on Trp supplemented media. (Bottom) Excepting the positive control, no growth is observed in the absence of acetamide induction and exogenous Trp. B. MPF-C protein–protein assay. PknA, PknF and PtkA protein–protein interaction facilitated the reassembly of the [F1, 2] and [F3] domains of mDHFR, enabling growth of *Msm* strains in the presence of 20 μg/mL TMP. Identical spots on control plates without TMP revealed growth of all strains. Positive control, *Saccharomyces cerevisiae* GCN4 dimerization domains fused to [F1, 2] and [F3], respectively. C. PknD interacts with PtkA *in vitro*. GST-tagged PknD was subjected to an ALPHAScreen assay with increasing concentration of PtkA. GST and PknE served as control. Curve fitting yielded Kd = 0.0175 μM, 883.4 μM and 144.3 μM for PknD, PknE and GST protein respectively.

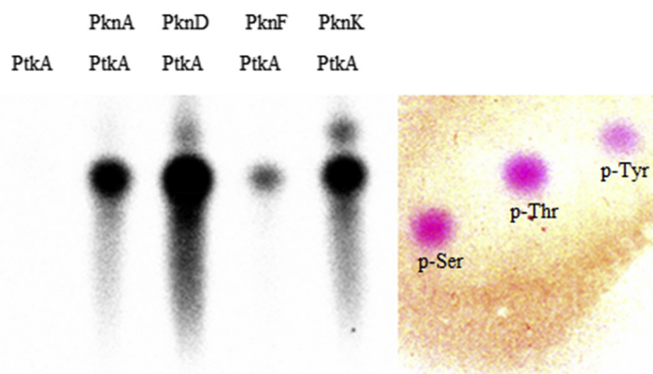


**Fig. 4.** Multi-eSTPKs phosphorylated PtkA on Thr residues *in vitro*. One-dimensional phosphoamino acid analysis of PtkA autophosphorylation when incubate with PknA, PknD, PknF and PknK. Control phospho-Tyr, -Thr, and -Ser were visualized by spraying with ninhydrin and radiolabeled PtkA residues were visualized by phosphorimaging.

PtkA phosphorylation, suggesting that phosphorylation might effect other aspects like substrate specificity or affinity. For example, the DNA binding activity (affinity) of Rv2175c, a function unknown transcriptional regulator, is negatively regulated by PknL [41].

In *Mtb*, most eSTPKs possess a sensor domain for sensing special environmental signals [18]; for example, PknB uses a PASTA (penicillin and Ser/Thr kinase associated domain) sensor domain to sense specific muropeptides [42]. However, tyrosine kinases like PtkA could not sense the environment directly and thus may relay on transmitted signals from other proteins – including membrane proteins such as the eSTPKs. In future, more experiments need to be performed to mechanistically analyse eSTPKs regulation of PtkA *in vivo*, and its subsequent effects on downstream substrates such as PtpA.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.124.

## Declaration of conflict of interest

None declared.

## References

[1] WHO, Global tuberculosis report 2014.
[2] V. Poirier, Y. Av-Gay, Mycobacterium tuberculosis modulators of the macrophage's cellular events, Microbes Infect. 14 (2012) 1211–1219.
[3] S.T. Cole, R. Brosch, J. Parkhill, et al., Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence, Nature 393 (1998) 537–544.
[4] P.K. Chakraborti, N. Matange, V.K. Nandicoori, et al., Signalling mechanisms in Mycobacteria, Tuberc. Edinb. 91 (2011) 432–440.
[5] R.S. Flannagan, G. Cosio, S. Grinstein, Antimicrobial mechanisms of phagocytes and bacterial evasion strategies, Nat. Rev. Microbiol. 7 (2009) 355–366.
[6] Y. He, W. Li, G. Liao, et al., Mycobacterium tuberculosis-specific phagosome proteome and underlying signaling pathways, J. Proteome Res. 11 (2012) 2635–2643.
[7] I. Vergne, R.A. Fratti, P.J. Hill, et al., Mycobacterium tuberculosis phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion, Mol. Biol. Cell. 15 (2004) 751–760.
[8] I. Vergne, J. Chua, V. Deretic, Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade, J. Exp. Med. 198 (2003) 653–659.
[9] I. Vergne, J. Chua, H.H. Lee, et al., Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 4033–4038.
[10] H. Bach, K.G. Papavinasasundaram, D. Wong, et al., Mycobacterium tuberculosis virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B, Cell Host Microbe 3 (2008) 316–322.
[11] D. Wong, H. Bach, J. Sun, et al., Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 19371–19376.
[12] J. Sun, V. Singh, A. Lau, et al., Mycobacterium tuberculosis nucleoside diphosphate kinase inactivates small GTPases leading to evasion of innate immunity, PLoS Pathog. 9 (2013) e1003499.
[13] A. Mehra, A. Zahra, V. Thompson, et al., Mycobacterium tuberculosis type VII secreted effector EsxH targets host ESCRT to impair trafficking, PLoS Pathog. 9 (2013) e1003734.
[14] M. Matsushita, K.D. Janda, Histidine kinases as targets for new antimicrobial agents, Bioorg Med. Chem. 10 (2002) 855–867.
[15] Y. Gotoh, Y. Eguchi, T. Watanabe, et al., Two-component signal transduction as potential drug targets in pathogenic bacteria, Curr. Opin. Microbiol. 13 (2010) 232–239.
[16] T. Alber, Y. Av-Gay, Prospects for TB therapeutics targeting Mycobacterium tuberculosis phosphosignaling networks, in: B. Klebl, M. Hamacher (Eds.), Protein Kinases as drug targets, Wiley, Hoboken, 2011, pp. 349–364.
[17] R. Szekely, F. Waczek, I. Szabadkai, et al., A novel drug discovery concept for tuberculosis: inhibition of bacterial and host cell signalling, Immunol. Lett. 116 (2008) 225–231.
[18] Y. Av-Gay, M. Everett, The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis, Trends Microbiol. 8 (2000) 238–244.
[19] U. Kusebauch, C. Ortega, A. Ollodart, et al., Mycobacterium tuberculosis supports protein tyrosine phosphorylation, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 9265–9270.
[20] C. Demetriadou, T.C. Zahrt, D.J. Bretl, Adaptation to environmental stimuli within the host: two-component signal transduction systems of Mycobacterium tuberculosis, Microbiol. Mol. Biol. Rev. 75 (2011) 566–582.
[21] P. Zhou, Q. Long, Y. Zhou, et al., Mycobacterium tuberculosis two-component systems and implications in novel vaccines and drugs, Crit. Rev. Eukaryot. Gene Expr. 22 (2012) 37–52.
[22] P. Sachdeva, R. Misra, A.K. Tyagi, et al., The sigma factors of Mycobacterium tuberculosis: regulation of the regulators, FEBS J. 277 (2010) 605–626.
[23] A. Staron, H.J. Sofia, S. Dietrich, et al., The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family, Mol. Microbiol. 74 (2009) 557–581.
[24] H. Bach, D. Wong, Y. Av-Gay, Mycobacterium tuberculosis PtkA is a novel protein tyrosine kinase whose substrate is PtpA, Biochem. J. 420 (2009) 155–160.
[25] J.D. Chao, D. Wong, Y. Av-Gay, Microbial protein-tyrosine kinases, J. Biol. Chem. 289 (2014) 9463–9472.
[26] A. Koul, A. Choidas, M. Treder, et al., Cloning and characterization of secretory tyrosine phosphatases of Mycobacterium tuberculosis, J. Bacteriol. 182 (2000) 5425–5432.
[27] S.C. Cowley, R. Babakaiff, Y. Av-Gay, Expression and localization of the Mycobacterium tuberculosis protein tyrosine phosphatase PtpA, Res. Microbiol. 153 (2002) 233–241.
[28] J. Wang, B.X. Li, P.P. Ge, et al., Mycobacterium tuberculosis suppresses innate immunity by coopting the host ubiquitin system, Nat. Immunol. 16 (2015) 237–245.
[29] V. Poirier, H. Bach, Y. Av-Gay, Mycobacterium tuberculosis promotes anti-apoptotic activity of the macrophage by PtpA protein-dependent dephosphorylation of host GSK3alpha, J. Biol. Chem. 289 (2014) 29376–29385.
[30] M. Margenat, A.M. Labandera, M. Gil, et al., New potential eukaryotic substrates of the mycobacterial protein tyrosine phosphatase PtpA: hints of a bacterial modulation of macrophage bioenergetics state, Sci. Rep. 5 (2015) 8819.
[31] P. Zhou, W. Li, D. Wong, et al., Phosphorylation control of protein tyrosine phosphatase A activity in Mycobacterium tuberculosis, FEBS Lett. 589 (2015) 326–331.
[32] K. Chow, D. Ng, R. Stokes, et al., Protein tyrosine phosphorylation in Mycobacterium tuberculosis, FEMS Microbiol. Lett. 124 (1994) 203–207.
[33] S. Prisic, S. Dankwa, D. Schwartz, et al., Extensive phosphorylation with overlapping specificity by Mycobacterium tuberculosis serine/threonine protein kinases, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7521–7526.
[34] S. Cowley, M. Ko, N. Pick, et al., The Mycobacterium tuberculosis protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo, Mol. Microbiol. 52 (2004) 1691–1702.
[35] X. Zheng, K.G. Papavinasasundaram, Y. Av-Gay, Novel substrates of Mycobacterium tuberculosis PknH Ser/Thr kinase, Biochem. Biophys. Res. Commun. 355 (2007) 162–168.
[36] Y. Av-Gay, S. Jamil, S.J. Drews, Expression and characterization of the Mycobacterium tuberculosis serine/threonine protein kinase PknB, Infect. Immun. 67 (1999) 5676–5682.
[37] H. O'Hare, A. Juillerat, P. Dianiskova, et al., A split-protein sensor for studying protein-protein interaction in mycobacteria, J. Microbiol. Methods 73 (2008) 79–84.
[38] J.D. Chao, K.G. Papavinasasundaram, X. Zheng, et al., Convergence of Ser/Thr and two-component signaling to coordinate expression of the dormancy regulon in Mycobacterium tuberculosis, J. Biol. Chem. 285 (2010) 29239–29246.
[39] A. Singh, D. Mai, A. Kumar, et al., Dissecting virulence pathways of Mycobacterium tuberculosis through protein-protein association, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11346–11351.
[40] S. Khan, S.N. Nagarajan, A. Parikh, et al., Phosphorylation of enoyl-ACP reductase InhA impacts mycobacterial growth and survival, J. Biol. Chem. 285 (48) (2010) 37860–37871.
[41] M. Cohen-Gonsaud, P. Barthe, M.J. Canova, et al., The Mycobacterium tuberculosis Ser/Thr kinase substrate Rv2175c is a DNA-binding protein regulated by phosphorylation, J. Biol. Chem. 284 (2009) 19290–19300.
[42] P. Barthe, G.V. Mukamolova, C. Roumestand, et al., The structure of PknB extracellular PASTA domain from mycobacterium tuberculosis suggests a ligand-dependent kinase activation, Structure 18 (2010) 606–615.

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