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Novel substrates of *Mycobacterium tuberculosis* PknH Ser/Thr kinase

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Abstract

PknH Ser/Thr protein kinase of *Mycobacterium tuberculosis* controls the expression of a variety of cell wall related enzymes and regulates the *in vivo* growth in mice. Therefore, we predicted that the PknH kinase could phosphorylate several substrates controlling different metabolic and physiological pathways. Using a bioinformatic approach, we identified 40 potential substrates. Two substrates were shown to be phosphorylated by recombinant PknH kinase *in vitro*. Point mutation studies verified that substrates are phosphorylated at the in silico-predicted sites. Kinetic studies revealed a similar relative-phosphorylation rate (V_{max}) of PknH towards two new substrates and the only previously known substrate, EmbR. Unlike the EmbR protein, the Rv0681 and DacB1 proteins do not contain an FHA domain and are possible participants of new signaling pathways mediated by the PknH kinase in *M. tuberculosis*.

Keywords: Ser/Thr kinase; *Mycobacterium tuberculosis*; TB; Bioinformatics; Activation loop

Reversible protein phosphorylation mediated by signaling kinases and phosphatases are important mechanisms by which cells respond to environmental stimuli. The genome of the human pathogen *Mycobacterium tuberculosis* (*Mtb*) encodes 11 Ser/Thr protein kinases (STPKs) which were extensively studied in the last decade [1]. Based on their genomic context, functions of many of these STPKs were suggested to be involved in regulation of diverse physiological functions [2]. Experimental data suggest that at least two of the kinases, PknG and PknH, play a role in *Mtb* virulence [3,4]. Nevertheless, the network of metabolic pathways and biological functions controlled by the STPKs remain largely to be elucidated.

In vitro kinase analysis of recombinant *Mtb* proteins have been demonstrated for 8 of the 11 STPKs [3,5–12]. Endogenous substrates were identified for PknA, B, D, F, H, and G [9,13–18] and all of the identified substrates, except MmpL7 and Wag31, were shown to contain a Forkhead-associated domain (FHA) implicated in mediating phospho-dependent protein–protein interactions [9,10,13–15,18]. All cloned *Mtb* STPKs were shown to undergo autophosphorylation in Ser and Thr residues. Several of the phosphorylated amino acids were localized to a segment termed the activation loop [19–21] in a similar manner to eukaryotic STPKs [22]. The exact role of the activation loop has not yet been clarified, nevertheless, its phosphorylation increases its kinase activity while dephosphorylation decreases it [20,23].

Recently, we have studied the physiological and virulence properties of the *Mtb* PknH kinase [4]. Surprisingly, we found that the PknH mutant is hypervirulent when used to infect BALB/c mice [4]. Moreover, our study had also indicated that the *pknH* knockout strain showed greater resistance to acidified-nitrite treatment suggesting that PknH could control the *in vivo* growth of *Mtb* in response to nitric oxide. PknH phosphorylates EmbR, a putative transcriptional regulator of *embAB* genes encoding arabinosyl transferases which are involved in the biosynthesis of arabinogalactans, a key component of the mycobacterial cell wall [9,24]. In ethambutol-treated wild-type *Mtb*, PknH induces the expression of a set of genes within the *emb*CAB and *ini*ABC operons [4]. Ethambutol treatment failed to elicit similar effects in the *pknH* knockout strain indicating that PknH phosphorylation

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regulates signaling cascades mediating expression of the genes encoding the cell wall associated proteins. Although until now PknH has been shown to phosphorylate only EmbR [9], our studies and recent publications [17,25] suggest that PknH could phosphorylate multiple substrates.

Phosphorylation of EmbR by PknH is achieved via direct binding of the FHA domain on EmbR to the autophosphorylated PknH kinase [9]. Since autophosphorylation of PknH is a prerequisite to its kinase activity, and both its autophosphorylation and its kinase activities utilize the same active site, we predicted that the cognate substrates of PknH kinase may contain either the same or a homologous amino acid motif located around the phosphorylation site of the PknH kinase. Following this rationale, using a bioinformatic approach, we have identified that a number of proteins could serve as substrates of PknH kinase. Using *in vitro* kinase assays, we have demonstrated that Rv0681, a TetR-class transcription factor, and DacB1, a penicillin-binding protein, are phosphorylated by the PknH kinase. Neither of these targets was predicted to contain an FHA domain. The identification of these non-FHA substrates revealed new signaling pathways mediated by PknH kinase in *Mtb*.

Materials and methods

Construction of expression plasmids. The primers used for cloning and site-directed mutagenesis and the plasmids constructed in this study are listed in Table 1. *Mtb* H37Rv genomic DNA was used as a template in the PCR using *Pfu* polymerase (MBI Fermentas). The PCR products were digested with appropriate restriction enzymes and ligated to His-tag vectors to construct the expression plasmids. Site-directed mutagenesis was carried out by following the Stratagene QuickChange protocol. The nucleotide sequences of the cloned and mutated genes were verified by DNA sequencing (UBC NAPS facility, Vancouver).

Expression and purification of recombinant His-tagged proteins. Over-expression of the cloned genes was induced following incubation of BL21 strain carrying the expression plasmids with 1 mM IPTG for 4 h at 37 °C. The recombinant proteins were produced in the soluble form in *Escherichia coli* BL21 and purified on Ni-NTA columns (Qiagen) as recommended by the supplier. The final elutions were checked by SDS–PAGE for purity before dialyzing with 20 mM Tris–HCl at pH 7.2 containing 1 mM DTT and 5% glycerol.

In vitro kinase assay. The *in vitro* kinase reactions contained 25 mM Tris–HCl, pH 7.2, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 10–300 ng of recombinant PknH kinase, and 0–500 nM of substrates. The reactions were started by addition of 10 μCi of γ-[^{32}P]ATP (Perkin-Elmer) followed by 5–30 min incubation at room temperature (21 °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 5 min. Samples were resolved by

Table 1
Strains, plasmids, and primers

	Characteristics
Strains	
E. coli DH5α	
E. coli BL21(DE3)	
Plasmids	
pET15b	Produces N-term His_6-tagged proteins
pET22b	Produces C-term His_6-tagged proteins
pWAB105	pET22b with a *Xho*I/*Nde*I insert encoding the cytoplasmic domain of $PknH_{1–401}$
pWAB106	pET15b with a *Nde*I/*Bam*HI insert encoding the entire EmbR protein
pWAB107	pET15b with a *Nde*I/*Bam*HI insert encoding EmbR T209A
pWAB110	pET22b with *Nde*I/*Hin*dIII insert encoding the entire Rv0681 protein
pWAB111	pET22b with *Nde*I/*Hin*dIII insert encoding Rv0681 T35A
pWAB112	pET22b with *Nde*I/*Hin*dIII insert encoding $DacB1_{133–350}$
pWAB113	pET22b with *Nde*I/*Hin*dIII insert encoding $DacB1_{133–350}$ T336A
Cloning primers	
S15-pknH-For	CTTCTTC*CATATG*AGCGACGCACAGGACTCG
S16-pknH-Rev	TTCT*CTCGAG*CGGGTTGGTTTTGCGCGGGGTCTG
S20-embR-For	AGGACCC*CATATG*GCTGGTAGCGCGACAGTGGAG
S21-embR-Rev	TTGT*GGATCC*CATCGGTGTTAAGGGCTTGTGTCC
S141-Rv0681For	CATCACT*CATATG*GCTCGCCCGGCCAAACTGAGC
S142-Rv0681Rev	CTTC*AAGCTT*CGTTGACGCGGTACCACCGTGTGT
X1-$dacB1_{133–350}$ For	CTACTGT*CATATG*AACAAGTCGGTCGCGGTCGCCGGAAC
X2-$dacB1_{133–350}$ Rev	CATA*AAGCTT*ATTGCGGTCGGTGGACATCAG
Mutagenesis primers	
S111-Rv0681-T35A-s	ATGCGCTGGCGGCCCAGCTCGGG
S112-Rv0681-T35A-as	CCCGACCTGGGCCGCCAGCGCAT
S113-DacB1-T336A-s	CACCCCGGCAGGCGCCCAGATCGGGAC
S114-DacB1-T336A-as	GTCCCGATCTGGGCGCCTGCCGGGGTG
S115-EmbR-T35A-s	GGAGCCGCTGTGGGCACAGCTGATCAC
S116-EmbR-T35A-as	GTGATCAGCTGTGCCCACAGCGGCTCC

Plasmid vectors pET15b and pET22b were obtained from Novagen. All other plasmids listed above were constructed in this study.

SDS–PAGE on 8% polyacrylamide gels and the gels were silver stained, and dried. The ^{32}P-radioactively labeled protein bands were detected using a PhosphorImager SI (Molecular Dynamics). Bands corresponding to the phosphorylated proteins were cut out and subjected to scintillation count (Beckman Coulter LS 6500).

Results

In silico identifcation of PknH potential substrates

PknH was shown to possess autokinase activity on nine or 10 Ser and Thr residues [9,25]. One of the phosphorylation sites, Thr170, is located within the PknH activation loop and is required for PknH phosphorylation [9]. The peptide sequencing flanking the Thr170 in PknH is DEKLTQLGT. Rationalizing that both autophosphorylation and kinase activity target similar amino acid motifs, a search for other cognate substrates was carried out against *Mtb* proteome (http://www.pasteur.fr/Bio/TubercuList/). Short peptide sequences of 5 amino acids long; each containing N-terminal (DEKL**T**), C-terminal (**T**QLGT), or both sides of the Thr170 residue (KL**T**QL) were used for the search.

EmbR, the only known *in vitro* phosphorylation substrate for PknH [9], was found when the C-terminal motif TQLGT with one mismatch was used in the search. The predicted phosphorylation site on EmbR is TQLIT. Besides PknH, only one other protein, designated Rv0681, had an exact match to the query sequence TQLGT. Therefore, we narrowed the search using the sequence TQLGT with mismatches permitted in the last 3

Table 2
Potential PknH substrates

Functional class	Gene/ORF	Position	Sequence
Lipid metabolism	acrA1: Acyl-CoA reductase	287	RNMAA**TQLGI**PAEIF
Information Pathways	gpsI: bifunctional protein polyribonucleotide nucleotidyltransferase (PNPase)	150	AASAS**TQLGG**LPFSG
	ligB: ATP-dependent DNA ligase	37	RAAPD**TQLVT**IIVSW
	Rv3263: probably DNA methylase	116	TWLHK**TQLGS**WDGV
Cell wall and cell processes	dacB1: probable penicillin-binding protein	336	NTPAG**TQIGT**LIEPD
	EsxI, L, N, O, V: ESAT-6 like proteins (5 proteins)	55	FI**TQLGR**NFQ
	fecB: probably Fe(III)-dicitrate-binding periplasmic lipoprotein	216	FAEHA**TQVGT**KHDAT
	lppI: probable lipoprotein lppI	79	RDGVT**TQLGD**DVAFS
	mmpL8: integral membrane transport protein	337	LGMVF**TQLGI**LKTVG
	Rv1333: hydrolase	300	ALSPE**TQLVT**AVGAA
	Rv1517: conserved hypothetical transmembrane protein	186	GAEIG**TQLGA**FVVFT
	Rv1824: conserved hypothetical membrane protein	93	QLGVG**TQLST**AIIVV
	Rv2091c: probable membrane protein	83	FGAQP**TQLGV**PGQYG
	Rv2333c: probable conserved integral membrane transport protein	4	MNR**TQLLT**LIATG
PE/PPE	PPE33: PPE family protein	463	VALPD**TQLGS**H
Intermediary metabolism and respiration	argF: probable Ornithine carbamoyltransferase	76	VDSGS**TQLGR**DETLQ
	phoH2: PhoH-like protein	77	PIPVG**TQGGT**LHVEL
	Rv0083: probable oxidoreductase	142	WYAVM**TQLGF**IAILV
	Rv1006: hypothetical protein	281	SAFPH**TQLVT**SPANP
	Rv1178: probable aminotransferase	140	RADAL**TQLGP**QSPAL
	Rv1264: adenylyl cyclase	227	DLVGF**TQLGE**VVSAE
	Rv1318c: adenylyl cyclase	371	DIVGS**TQLVT**SRPPA
	Rv1320c: adenylyl cyclase	371	DIVGS**TQLVT**SRPAA
	Rv1817: possible flavoprotein	464	GYASG**TQLGE**GSFFG
	Rv2251: possible flavoprotein	248	ALRTI**TQTGT**GPTVV
Regulatory proteins	embR: probable transcriptional regulatory protein	209	REPLW**TQLIT**AYYLS
	pknD: transmembrane Serine/Threonine protein kinase D	169	SDPGL**TQTGT**AVGTY
	pknE: transmembrane Serine/Threonine protein kinase E	170	TDEKL**TQLGN**TVGTL
	pknH: transmembrane Serine/Threonine protein kinase H	170	TDEKL**TQLGT**AVGTW
	Rv0681: probably transcriptional regulatory protein (possible TetR-family)	35	INALA**TQLGT**KGPSL
Conserved hypotheticals	Rv0500A: conserved hypothetical protein	22	GQQAK**TQLLT**VAEVA
	Rv1006: hypothetical protein	281	SAFPH**TQLVT**SPANP
	Rv1593c: conserved hypothetical protein	195	RRRVI**TQTGT**IAQSG
	Rv1830: conserved hypothetical protein	2	V**TQLVT**RARSA
	Rv2133c: conserved hypothetical protein	65	AYLVS**TQLGW**NLVPH
	Rv2616: conserved hypothetical protein	35	HLDVS**TQIGT**GRQRF

residues (LGT) to screen the *Mtb* database. Forty *Mtb* proteins were identified matching the above criteria. The search results are summarized in Table 2. This result suggests that the PknH kinase has the potential to phosphorylate a wide range of mycobacterial proteins. Among the members of the list, we have identified two other STPKs, PknE and PknD which were shown to undergo autophosphorylation and act as kinases [8,17,21].

PknH phosphorylates Rv0681 and DacB1

From among the list of putative substrates identified by bioinformatics analysis, we chose Rv0681 and DacB1 to test experimentally whether they could be phosphorylated *in vitro* by the PknH kinase. The Rv0681 protein is the only substrate identified to have the perfect motif match to that of the TQLGT motif in the activation loop of PknH. Since Rv0681 was predicted to be a transcriptional regulator, we considered that it is likely to be targeted by the PknH kinase. Even though our search revealed many proteins belonging to different functional groups with one mismatch in the motif, we chose DacB1 for experimental analysis. DacB1 is a cell division-related protein hypothesized to be involved in peptidoglycan synthesis. Even though we do not have direct evidence relating a role for DacB1 during *in vivo* replication, the fact that the ΔpknH mutant had displayed increased growth and bacillary load in infected mouse organs suggested that the PknH kinase may regulate expression or activities of proteins associated with *in vivo* cell growth. Therefore, we wanted to investigate whether a cell-wall associated protein containing the homologous activation loop motif such as DacB1 could serve as a substrate for PknH.

First, we tested whether our recombinant truncated PknH possesses autophosphorylation and kinase activities. As expected, the His-tagged $PknH_{1–401}$ was able to phosphorylate itself and the substrate EmbR (Fig. 1). *In vitro* kinase assays using the recombinant Rv0681 and DacB1 proteins in the kinase reactions revealed that the recombinant PknH kinase phosphorylated both these substrates (Fig. 1). Phosphorylation was not detected when the PknH kinase was omitted from the reaction in both cases (Fig. 1), indicating that neither Rv0681 nor DacB1 is capable of autophosphorylation. Rv0681 and DacB1 require the trans-phosphorylating activity of the PknH kinase and thus are genuine substrates of PknH. Neither DacB1 nor Rv0681 was found to serve as substrates for the other *Mtb* STPKs PknG or PknB (data not shown).

Table 3 summarizes the kinetic characteristics of PknH for EmbR, DacB1, and Rv0681. The maximum enzymatic velocities of PknH towards all three substrates are at the same scale. However, we obtained a higher affinity of PknH to EmbR compared to Rv0681 and DacB1. The K_m value for Rv0681 is about 33-fold higher than the K_m for EmbR and 12-fold higher than that of DacB1.

Phosphorylation of Rv0681 and DacB1 are at the TQLGT motifs

To verify that the first Thr residue in the TQLGT motif is phosphorylated by PknH, we tested whether we can completely abolish phosphorylation of target proteins by site directed mutagenesis of this residue. Therefore, the PknH-T_{170} homologs of the three targets (Rv0681, DacB1, and EmbR) were mutated to Ala and *in vitro* kinase assays were used to test whether the mutated proteins can serve as

Table 3
Enzyme kinetics of PknH for EmbR, DacB1, and Rv0681

	EmbR	DacB1	Rv0681
K_m (μM)	0.597	1.59	19.82
V_{max} (μM/min)	3.72	11.49	8.62

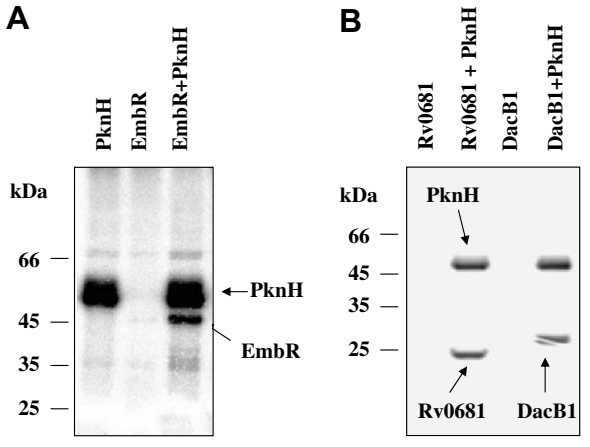

Fig. 1. Phosphorylation of PknH and substrates. (A) *In vitro* autophosphorylation of PknH kinase and phosphorylation of EmbR. (B) *In vitro* phosphorylation of Rv0681 (23 kDa) and truncated DacB1 protein (27 kDa) substrates by PknH kinase.

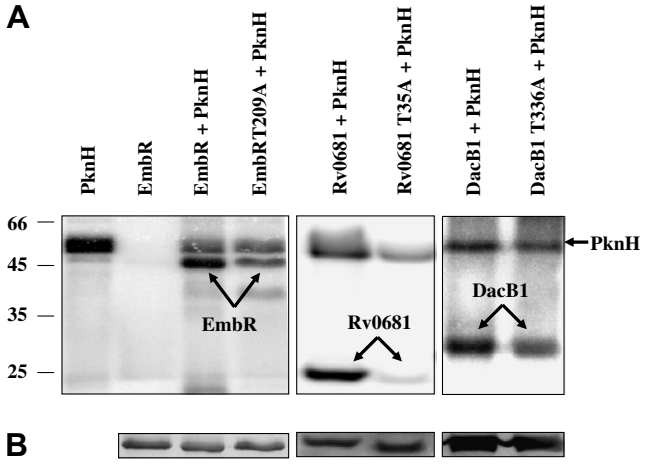

Fig. 2. *In vitro* kinase assay comparing wild-type and T170 mutant homologs of EmbR, Rv0681, and DacB1. (A) Phosphorylation levels of substrates and corresponding point mutants. (B) Silver stained gel showing equal loadings of protein substrates.

substrates for PknH. As shown in Fig. 2, the Thr to Ala point mutation almost completely abolished Rv0681 phosphorylation by PknH. However, in the case EmbR and DacB1, the mutation did not abolish phosphorylation; the protein bands corresponding to the mutated EmbR and DacB1 still possessed ≈40% phosphorylation compared to the wild type protein band indicating that in both these proteins, in addition to the Thr170 homolog, other Ser or Thr residues are phosphorylated by the PknH kinase.

Discussion

Earlier we have shown that PknH controls the expression of a variety of cell wall associated enzymes and regulates the *in vivo* growth of *Mtb* in mice [4]. These results indicate that PknH acts by translating environmental signals to adaptive gene expression. Using point mutants of PknH, Molle et al. [9] have confirmed the essential residue of the enzymes such as the Lys45 residue present in the Hanks subdomain II of the PknH kinase and demonstrated that the catalytic role of Thr170 in the activation loop is required for its phosphorylation activity. However, so far, only the transcriptional regulator EmbR has been identified as a cognate substrate of the PknH kinase. Due to the diverse phenotypes of the ΔpknH mutant, we propose that the PknH kinase acts through diverse signaling pathways involving multiple key substrates. In this study, we hypothesized that both autophosphorylation and kinase activities of PknH utilize similar amino acid motifs as target substrates and by applying this principle, we initiated studies to predict novel substrates of the PknH kinase. We examined this hypothesis using combined bioinformatics and biochemical approach, and have shown that proteins that share similar amino acid sequences with the autophosphorylation site at the PknH activation loop are indeed able to undergo *in vitro* phosphorylation by the PknH kinase.

Using the bioinformatics approach we have identified about 40 potential substrates for PknH (Table 2). In another non-pathogenic actinomycete, *Corynebacterium glutamicum*, phosphoproteome studies determined experimentally 120 unique Ser/Thr phosphorylated proteins that are phosphorylated by four STPKs [26]. We suggest that many of the 40 proteins predicted using the bioinformatics approach are potential substrates for PknH. It is possible that some of the substrates may also be phosphorylated by other kinases as described for PknB, D, E, and F [17]. Interestingly, two other kinases, PknD and E are among the five regulatory proteins identified as potential substrates, which is clearly not possible. A possible explanation is that the substrate binding cleft is similar in these kinases. Indeed similar sequences were identified at the activation loops of these two kinases and PknH. Therefore, it is likely that PknD, E, and H share some substrates between them. The substrates predicted in this study belong to diverse functional classes (Table 2) and suggest that the PknH kinase can regulate multiple cellular processes by phosphorylating diverse key substrates.

Of the predicted target proteins, we chose a transcriptional regulatory protein Rv0681 and a cell-wall associated protein DacB1 to test whether these can serve as targets for phosphorylation by PknH in *in vitro* kinase assays. We used the EmbR as a positive control in our kinase assays. EmbR, Rv0681, and DacB1 were all phosphorylated by the recombinant PknH kinase in our study; none of them by themselves possessed any autophosphorylating activities. The Rv0681 protein is a 21.2 kDa protein predicted to be a transcription factor that was reported to be non-essential for survival by transposon mutagenesis studies [27]. Analysis of the primary protein structure of Rv0681 with MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) revealed a TetR-type Helix-turn-helix (HTH) DNA binding motif from amino acids 6-66. The predicted phosphorylation site (T_{35}QLGT) is in the middle of the HTH-motif. It is possible PknH-mediated phosphorylation of the Rv0681 protein will affect expressions of genes/operons that are under the transcriptional regulatory control of Rv0681.

DacB1 is membrane-bound protein containing 405 amino acids and is predicted to be a probable penicillin-binding protein/carboxy peptidase with a predicted mass of 42 kDa. It is homologous to the *Bacillus subtilis* D-alanyl D-alanine carboxy peptidase with 31% identity in a 249 amino acid overlapping region. The *B. subtilis* DacB protein is a sporulation-specific protein involved in cell envelope biosynthesis [28]. Phosphorylation of the DacB1-T_{336}QIGT motif, which is located just outside the peptidase domain (80–320 aa), might play a regulatory role by affecting DacB1 function. DacB1 is localized in the mycobacterial membrane, thus creates a local high concentration suggesting the actual phosphorylation efficiency of DacB1 by PknH could be much higher inside *Mtb*. The *dacB1* gene itself is not a part of an operon and the expression of *dacB1* is not considered essential for *in vitro* growth [27]; however, our study demonstrates that DacB1 is a substrate for PknH phosphorylation. The phosphorylation of the T336A mutant form of DacB1 by PknH kinase indicates that, besides the motif identified in this study, DacB1 is phosphorylated at other residues as well. This is not surprising since the PknH kinase can phosphorylate both at Thr and Ser residues, and in the mutagenesis study, only the Thr336 was targeted. Further studies with the phosphorylated form of the wild type DacB1 protein by LC–MS/MS analysis will help to identify all the phosphorylated residues in DacB1 protein, and help define other motifs that are targeted by PknH kinase.

The *Mtb* EmbR is a putative transcriptional regulatory protein characterized by the presence of a putative DNA binding domain located in its N-terminal region followed by a bacterial transcriptional activation domain and a Forkhead-Associated Domain (FHA) in its C-terminal region [9,29]. Molle et al. [9] also suggested that the EmbR protein is phosphorylated at additional multiple Thr sites. Indeed in a recent study, it was reported that EmbR possess up to five

PknH-dependent phosphorylation states [29] in agreement with our study which demonstrated that point mutation of Thr170 homolog did not abolish EmbR phosphorylation.

The FHA-domains are protein–protein interaction domains found in protein kinases and transcription factors, and mediate binding to mainly phosphothreonine motifs in proteins in a sequence-specific manner [30]. Six proteins of *Mtb* including EmbR contain FHA domains [1]. In the case of *Mtb* EmbR, truncation of the FHA domain or point mutations in three specific residues in the FHA domain of EmbR abolished PknH-mediated phosphorylation of EmbR indicating the critical role of FHA-mediated interaction between the substrate and PknH kinase [9]. However as demonstrated for PknB and PknD [14,18], phosphorylation of other endogenous substrates need not always involve FHA domains.

We have demonstrated experimentally that both Rv0681 and DacB1 are phosphorylated by PknH even though both these proteins do not contain any FHA-domains. It is possible that the phosphorylation of endogenous substrates by PknH could involve two mechanisms. In the case of EmbR, an FHA-dependent phosphorylation modulates activity through substrate recruitment. In contrast, in the case of Rv0681 and DacB1, a non-FHA dependent mechanism, which could involve free movement of the substrate in and out of the active site, could potentially modulate the phosphate transfer step.

In conclusion, we have identified two new substrates of PknH kinase in *Mtb*; Rv0681 a transcriptional regulatory protein and DacB1, a penicillin binding-protein. Despite the fact that both these substrates do not contain an FHA domain, PknH possess a slightly higher V_{max} towards Rv0681 and DacB1 when compared to the FHA-domain containing protein EmbR. Since PknH kinase plays a regulatory role in the pathogen virulence, further characterization of the various components of the signaling cascades will add to our understanding of the biology of *Mtb* and its pathogenicity.

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