Title: *In Silico* Evidence for Extensive Ser/Thr Phosphorylation of *Mycobacterium tuberculosis* Two Component Signalling Systems

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Abstract/ Summary

Mycobacterium tuberculosis has the innate ability to adapt and survive the intracellular environments during infection. Two component signalling systems and Serine/Threonine protein kinases facilitate metabolic and growth adaptation by directing transcriptomic reprogramming in response to environmental stimuli. Presently, very little is known about the post-translational regulation of TCS proteins through *O*-phosphorylation. Using the NetPhosBac 1.0 *in silico* tool, we screened components of *M. tuberculosis* TCS systems for potential Ser/Thr phosphosites. We report extensive Ser/Thr phosphorylation of sensor kinases and response regulator proteins suggesting it maybe a distinct mechanism enabling co-regulation of pathways impacting adaptive changes in mycobacterial growth and metabolism.

Keywords. *M. tuberculosis*, Serine/Threonine Protein Kinase, two-component systems (TCS) response regulators, cross-talk, post translational modification.

Tuberculosis is a respiratory disease caused by *Mycobacterium tuberculosis*; an intracellular pathogen responsible for about 1.5 million deaths every year[1]. The pathogenic success of *Mycobacterium tuberculosis* lies in its ability to adapt and survive the changing growth environments during infection. Signal transduction systems are central to this adaptability and are known to play a vital role in mycobacterial pathogenesis, virulence, and persistence. There are two major arms of signalling pathways in mycobacteria, namely the traditional Two-component signalling systems (TCS) and the "eukaryotic-like" Serine/Threonine Protein Kinases (STPKs)[2,3] that regulate diverse cellular pathways ranging from cell division, transport, metabolism, persistence, and virulence.

Typically, a two-component system is composed of a *sensory component*-Histidine or Sensory Kinase (SK), and a *response generating component*-Response Regulator (RR)[4]. This paired system facilitates the adaptation and survival of the bacteria under different environmental stress conditions like nutrient starvation, hypoxia, nitrosative and oxidative stress (reviewed in [5]). The environmental stimulus is detected by the N-terminal variable 'input domain' of the SK, which leads to phosphorylation of the histidine residue in the autocatalytic kinase domain. The SK phosphorylates a conserved aspartate residue in the "receiver domain" of RR via a phosphotransfer reaction which in turn modulates the DNA binding ability of the variable 'output domain' of the RR. *M. tuberculosis* has 12 completely paired two-component regulatory systems that includes five orphan regulators and one orphan histidine kinase (compiled in Table 1). Of these, multiple TCS systems are known to be involved in virulence[6] and two have been shown to be essential for mycobacterial survival[7,8].

Post-translational modification via Ser/Thr/Tyr phosphorylation also known as *O*-phosphorylation is a key regulatory mechanism ubiquitous to living organisms. Phosphorylation of proteins inactivates or activates them impacting their cellular function or the downstream regulatory pathways. In comparison to eukaryotic organisms, few prokaryotes

including bacteria also have Ser/Thr protein kinases (STPKs) that stimulate a wide variety of signalling networks[3]. The STPKs integrate many cellular or extracellular signals by reversible phosphorylation and dephosphorylation of proteins. In *M. tuberculosis*, there are 11 STPKs named PknA-B, PknD-L that regulate metabolic homeostasis, transportation, transcription, cell growth and division (compiled in Table 1)[9–14].

Typically, the paired TCS system is very specific since the SKs have a kinetic preference for their cognate RRs. This intrinsic preference helps SKs to differentiate their cognate RRs from all other likely substrates. However, recently it has been shown that the absence of either RR or SK generally does not eliminate the associated response of the TCS, suggesting the possibility of crosstalk amongst TCS proteins *in vivo*[15]. Novel interactions between a SK and a non-cognate RR, and between different DNA-binding RR proteins to form heterodimers have been shown to help coregulate the downstream expression of regulon genes[16].

Post-translational regulation via *O*-phosphorylation of TCS proteins is an emerging paradigm of the signalling mechanisms in mycobacteria. Convergence of PknB and RegX3-SenX3 signalling pathways - integrating two different signals i.e Pi limitation and replication state of *M. tuberculosis*[17] suggests that such cross-interactions ultimately lead to a more efficient gene expression and balanced coordination within the cell. The DevR RR of the DevRS two component system known to play a role in hypoxic adaptation[18] of mycobacteria is regulated by its cognate SK along with PknB[19] and PknH[20] kinases. Recent findings from our laboratory established PknK, a cytosolic STPK, as a nodal point connecting atypical signalling pathways[21,22]. Ser/Thr phosphorylation of two essential RRs, MtrA and PrrA by PknK underscores the significance of such interactions increasing the complexity of underlying regulatory mechanisms.

From an evolutionary point of view, cross interactions between two distinctively different signalling pathways resulting in multiple intricate and often overlapping regulatory circuits

may be responsible for the robust survival fitness of microorganisms. Four main types of interactions have been reported namely, SK-RR (cognate pairs), SK-RR (non-cognate pairs), RR-RR (heterodimers) and STPK-RR interactions[15]. So far, *M. tb* STPKS, PknB and PknH have been shown to phosphorylate multiple RR proteins[19,20] with PknK being a recent addition to the list. However, there is no information on Ser/Thr phosphorylation of TCS sensor kinases (STPK-SK interaction) till date.

Figure 1 shows a compilation of all experimentally validated interactions of TCS and STPKs in *M. tuberculosis.* It is noteworthy that out of the 12 paired TCS, PdtaS-PdtaR, SenX3-RegX3 and TrcS-TrcR TCS systems are seen to be completely specific, and do not interact with any other pair other than their cognate partners[15,16]. This however does not exclude the possibility of these TCS being modified by Ser/Thr phosphorylation, thereby allowing a distinctly separate control of the regulatory pathway.

In this study, we screened 31 TCS components listed in Table 1 for potential Ser/Thr phosphorylation sites using the NetPhosBac 1.0 tool[23]. NetPhosBac is based on an artificial neural networking model that predicts bacteria specific *O*-phosphorylation. The web address (http://www.cbs.dtu.dk/services/NetPhosBac/) hosting the NetPhosBac 1.0 was used for the identification of bacteria-specific putative Ser/Thr phosphorylation sites[23]. It should be noted that the NetPhosBac 1.0 tool takes into account the full-length of the protein irrespective of any specific domain regions such as transmembrane regions in its analysis, and only reports phosphosites with scores > 0.5.

In addition to the phosphorylation sites predicted by the NetPhosBac 1.0 tool, we scored the extent of phosphorylation for any RR or SK protein in terms of the percentage number of Ser or Thr residues that can be potentially phosphorylated. In our analysis, we found that both SK and RR proteins are susceptible to extensive STPK-mediated phosphorylation in addition to their canonical phosphorylation sites on Histidine residues and Aspartate residues,

respectively. The complete data from the NetPhosBac 1.0 server is provided as supplementary data (Table S1).

As shown in Table 2, NetPhosBac predicted Ser/Thr phosphosites for all TCS proteins analysed. While the number of phosphosites varied, the propensity of Serine vs Threonine sites also varied with phosphorylation on former being more prevalent than the latter. Remarkably, KdpD sensor kinase showed the highest number of predicted phosphorylation sites. A large percentage of the serine residues in KdpD (55%) and MtrB (52%) SKs can be potentially phosphorylated by STPKs. The sensor kinase Rv0601c showed the least number of Ser/Thr phosphorylation sites. Amongst the RR proteins, TcrA had the highest percentage of Serine phosphorylation. About 83% of the total number of serine sites in TcrA were potential sites amenable for phosphorylation closely followed by orphan RRs, Rv0195 (~75%) and Rv2884 (~ 60%) (Table 2).

The results obtained with NetPhosBac 1.0 were compared with recently published data[24] wherein the authors reported widespread *O*-phosphorylation in *M. tuberculosis* H37Rv strain. We found a distinct overlap between our observations and the data reported by Frando et al. With the exception of PhoR sensor kinase, there were common phosphosites for all TCS proteins, thus providing validation to the *in silico* predictions (Table S1). Of all the other experimentally validated phosphosites reported so far in RegX3[17], DevR[20] and PrrA[22] RRs, only one phosphosite, Thr151 in RegX3[17] RR having a score of 0.621 was common with our analysis. Incidentally, most of the published Ser/Thr phosphosites have low scores and thus, were not identified by the NetPhosBac tool. Being a prediction tool, these differences are conceivable; however, they cannot undermine the implication of widespread Ser/Thr phosphorylation scored by NetPhosBac across all *M. tuberculosis* TCS systems.

Notably, the MtrB sensor kinase exhibits cross-interactions not only with multiple non-cognate RRs[15,16] but was also susceptible to STPK-mediated Ser/Thr phosphorylation. It is not surprising that MtrAB signalling pathway, known to be essential for mycobacterial growth and cell division[7] is subjected to multiple layers of regulation. The SK1-SK2-TcrA forms a three-component system[25]; however, the function is not yet defined. Our analysis showed that while the SK1 and SK2 showed the least number of Ser/Thr phosphorylation sites, TcrA presented most of its Ser/Thr residues as potential sites for phsophorylation. It is possible that the lack of dual phosphorylation of SK1 and SK2 is compensated by the *O*-phosphorylation of the TcrA RR. Furthermore, it is interesting to note that TcrA RR is also a target of MtrB[16], which itself is subjected to regulation by dual phosphorylation. Since SKs and RRs have specific functional domains such as the histidine kinase and DNA binding motifs, we mapped the predicted phosphosites onto these domains (data not shown). While no specific pattern was observed in the localization of Ser/Thr phosphosites in RR proteins, most of them were mapped in the histidine kinase domain of the sensor kinases. These observations suggest that STPK mediated phosphorylation of TCS proteins may play an important role in signal sensing, integration and transduction.

Although the functional significance of these interactions is unknown as yet and warrants further experimentation, it is clear that post translational modification of both sensor kinases and response regulator proteins must be taken into account in order to fully understand the dynamics of a signalling pathway. This study highlights several questions. For example, under what conditions do the non-canonical Ser/Thr phosphorylation of TCS dominate the traditional His/Asp phosphorylations and if they contribute to the promiscuity observed within the TCS systems? Since 9 out 11 STPKs are membrane receptor kinases, the feasibility of STPK-mediated modification of membrane histidine kinases bring forth the issue about spatial organisation of these proteins. It should be noted that two *M. tb* STPKs are soluble, cytosolic

proteins[13,26,27] with PknK being also associated with the cell wall[13]. It is possible that cytosolic STPKs may be part of a larger scaffold assembly that facilitates protein-protein interactions and post-translational modifications of SKs. Indeed, data suggests that PknK mediates ~86% phosphorylation of RR proteins[24]. Generally, cross interactions of TCS proteins have been believed to be restricted to within the TCS only. However, recently it has been shown that DevS SK of the DevR/DevS two component system can phosphorylate non-TCS proteins[28]. This highlights the possibility of signal transduction through TCS sensor kinases outside the domain of TCS components. In such a scenario, it will be particularly interesting to determine if STPK-mediated regulation of TCS sensor kinases is a contributory factor.

Our findings indicate that signalling pathways are best studied in composite rather than in isolation, and pave way for deeper investigations to reveal novel mechanisms for regulation of mycobacterial gene expression. In all likelihood, these cross interactions may well account for the survival fitness of this remarkable intracellular pathogen. We envision that uncovering these non-traditional regulatory circuits will facilitate the development of newer therapeutic strategies to combat tuberculosis.

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Table 1: *M. tuberculosis* Ser/Thr Protein Kinases and Two Component Systems

STPK	Gene	[a] Known Environmental Activating Signal
PknA	*Rv0015c*	ND
PknB	*Rv0014c*	Oxygen-dependent replication
PknD	*Rv0931c*	Osmotic Stress
PknE	*Rv1743*	Nitric oxide stress
PknF	*Rv1746*	ND
PknG	*Rv0410c*	Nutritional Stress (Glutamine/Glutamate levels)
PknH	*Rv1266c*	Nitric oxide stress
PknI	*Rv2914c*	Low pH associated with low oxygen availability
PknJ	*Rv2088*	*In vivo* concentrations of Ni^{2+} and Co^{2+}
PknK	*Rv3080c*	Stationary phase stress
PknL	*Rv2176*	ND
TCS	**Gene**	**[a] Known Environmental Activating Signal**
PhoP/ PhoR	*Rv0757/Rv0758*	Acid Induction
NarL/NarS	*Rv0844c/Rv0845*	Nitrate
PrrA/PrrB	*Rv0903c/Rv0902c*	Nitrogen limitation, Macrophage infection
MprA/MprB	*Rv0981/Rv0982*	Detergents SDS, Alkaline pH, Triton X-100, Nutrient limitation
KdpE/ KdpD	*Rv1027c/Rv1028c*	K^+ limitation, osmotic stress, turgor pressure
TrcR/ TrcS	*Rv1033c/Rv1032c*	ND
MtrA/ MtrB	*Rv3246c/Rv3245c*	ND
TcrX/ TcrY	*Rv3765c/Rv3764c*	Starvation, Low Iron
PdtaR/ PdtaS	*Rv1626/ Rv3220c*	Nutrient Limitation
RegX3/SenX3	*Rv0491/ Rv0490*	Phosphate Starvation
DevR/DevS, DosT	*Rv3133c/ Rv3132c, Rv2027c*	Hypoxia, Nitrate, Nitric Oxide, Carbon monoxide, Vitamin C
TcrA/ SK1, SK2	*Rv0602c/Rv0600c, Rv0601c*	Antibiotic stress
Orphan SK/RR		
OSK	*Rv2027c*	
ORR	*Rv0195*	
ORR	*Rv0260c*	
ORR	*Rv0818*	
ORR	*Rv2884*	
ORR	*Rv3143*	

[a]Data compiled from[5,13,26,29–37]

ND- No Data

Table 2: Predicted Ser/Thr Phosphorylation in SKs and RRs of TCS systems

SK	Gene	Total no. of Serine (Ser) and Threonine (Thr) residues	Number of potential Ser/Thr phosphorylations	[a] Percentage Ser phosphorylation
PhoR	*Rv0758*	S-35; T-29	S-16; T-1	S ++
NarS	*Rv0845*	S-25; T-23	S-10; T-1	S ++
PrrB	*Rv0902c*	S-30; T-28	S-10; T-2	S ++
MprB	*Rv0982*	S-39; T-24	S-10; T-2	S ++
KdpD	*Rv1028c*	S-40; T-56	S-22; T-3	S +++
TrcS	*Rv1032c*	S-36; T-38	S-14; T-5	S ++
MtrB	*Rv3245c*	S-38; T-34	S-18; T-1	S +++
TcrY	*Rv3764c*	S-33; T-36	S-12; T-2	S ++
PdtaS	*Rv3220c*	S-32; T-25	S-13; T-4	S ++
SenX3	*Rv0490*	S-30; T-20	S-10; T-2	S ++
DevS	*Rv3132c*	S-26; T-33	S-11; T-1	S ++
DosT [b]	*Rv2027c*	S-25; T-30	S-9; T-3	S ++
SK1	*Rv0600c*	S-6; T-18	S-1; T-1	S +
SK2	*Rv0601c*	S-3; T-18	S-1	S ++

RR	Gene	Total no. of Serine (Ser) and Threonine (Thr) residues	Number of potential Ser/Thr phosphorylations	[a] Percentage Ser phosphorylation
PhoP	*Rv0757*	S-9; T-17	S-3; T-3	S ++
NarL	*Rv0844c*	S-12; T-5	S-7; T-1	S +++
PrrA	*Rv0903c*	S-14; T-13	S-7; T-2	S ++
MprA	*Rv0981*	S-12; T-12	S-7; T-2	S +++
KdpE	*Rv1027c*	S- 8; T-15	S-4; T-2	S ++
TrcR	*Rv1033c*	S-17; T-17	S-9	S +++
MtrA	*Rv3246c*	S-5; T-14	S-1; T-1	S +
TcrX	*Rv3765c*	S-15; T-12	S-7	S ++
PdtaR	*Rv1626*	S-7; T-14	S-2	S ++
RegX3	*Rv0491*	S-13; T-12	S-7; T-1	S +++
DevR	*Rv3133c*	S-10; T- 8	S-4	S ++
TcrA	*Rv0602c*	S-6; T-16	S-5; T-2	S ++++
Rv0195 [c]	*Rv0195*	S-12; T-15	S-9; T-2	S +++
Rv0260c [c]	*Rv0260c*	S-26; T-18	S-10; T-3	S ++
Rv0818 [c]	*Rv0818*	S-15; T- 12	S-5; T-3	S ++
Rv2884 [c]	*Rv2884*	S-10; T-17	S-6; T-2	S +++
Rv3143 [c]	*Rv3143*	S-7; T-9	S-4	S +++

[a] *Percentage of Serine Phosphorylation based on the number of potential phosphosites as predicted by NetPhosBac 1.0 server. Percentage of putative Ser phosphorylation was denoted as + (0-25%), ++ (25-50%), +++ (50-75%) and ++++ (75-100%)*

[b] *Orphan Histidine Kinase*

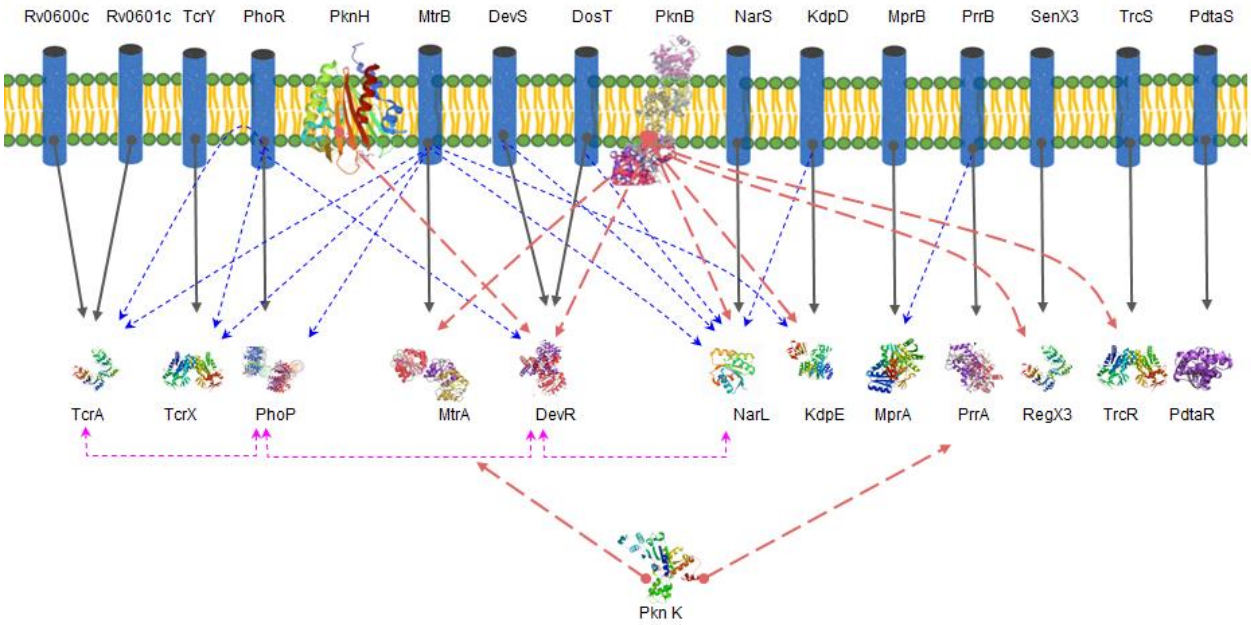
[c] *Orphan Response Regulator*

Figure Legend.

Figure 1. A Signalling Interactome of *M. tuberculosis* TCS and STPK proteins.

A cartoon illustrating previously reported interactions between STPKs and TCS components[16,19,20,31,38–40]. The TCS sensor kinases alongwith STPKs PknB and PknH are shown as membrane receptor kinases while the response regulators are shown as cytosolic proteins. STPK PknK being a soluble kinase is shown as a cytosolic protein. Black solid arrows indicate SK-RR cognate pairs, Blue Dotted arrows indicate SK-RR Non-cognate pairs, Pink Dotted arrow indicate RR-RR and Orange Dotted arrows indicate STPK-RR interactions. The 3D structures of specific proteins were obtained from STRINGS database[41].

Figure 1.


Rv0600c
Rv0601c
TcrY
PhoR
PknH
MtrB
DevS
DosT
PknB
NarS
KdpD
MprB
PrrB
SenX3
TrcS
PdtaS
TcrA
TcrX
PhoP
MtrA
DevR
NarL
KdpE
MprA
PrrA
RegX3
TrcR
PdtaR
Pkn K