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Marco Bellinzoni, Anne Marie Wehenkel, Rosario Durán, Pedro Alzari. Novel mechanistic insights into physiological signaling pathways mediated by mycobacterial Ser/Thr protein kinases. *Microbes and Infection*, 2019, 21 (5-6), pp.222-229. 10.1016/j.micinf.2019.06.015 . pasteur-02749197

**HAL Id: pasteur-02749197**

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Submitted on 15 Jun 2020

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# Novel mechanistic insights into physiological signaling pathways mediated by mycobacterial Ser/Thr protein kinases

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Running title: **Mechanistic insights into mycobacterial kinases**

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19   **Abstract**

20

21   Protein phosphorylation is known to be one of the keystones of signal sensing and  
22   transduction in all living organisms. Once thought to be essentially confined to the  
23   eukaryotic kingdoms, reversible phosphorylation on serine, threonine and tyrosine  
24   residues, has now been shown to play a major role in many prokaryotes, where the number  
25   of Ser/Thr protein kinases (STPKs) equals or even exceeds that of two component systems.  
26   *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, is one of the most  
27   studied organisms for the role of STPK-mediated signaling in bacteria. Driven by the  
28   interest and tractability of these enzymes as potential therapeutic targets, extensive studies  
29   revealed the remarkable conservation of protein kinases and their cognate phosphatases  
30   across evolution, and their involvement in bacterial physiology and virulence. Here, we  
31   present an overview of the current knowledge of mycobacterial STPKs structures and  
32   kinase activation mechanisms, and we then focus on PknB and PknG, two well-  
33   characterized STPKs that are essential for the intracellular survival of the bacillus. We  
34   summarize the mechanistic evidence that links PknB to the regulation of peptidoglycan  
35   synthesis in cell division and morphogenesis, and the major findings that establishes PknG  
36   as a master regulator of central carbon and nitrogen metabolism. Two decades after the  
37   discovery of STPKs in *M. tuberculosis*, the emerging landscape of *O*-phosphosignaling is  
38   starting to unveil how eukaryotic-like kinases can be engaged in unique, non-eukaryotic-  
39   like, signaling mechanisms in mycobacteria.

40

41

## 42    **Introduction**

43    Reversible protein phosphorylation provides a major mechanism for environmental sensing  
44    in all living systems <sup>1</sup>. In eukaryotes, stable protein phosphorylation (reversed by cognate  
45    phosphatases) essentially occurs on serine, threonine and tyrosine residues (*O*-  
46    phosphorylation) and is mediated by members of a large class of enzymes, the Hanks-type  
47    Ser/Thr/Tyr protein kinases, that share a common catalytic core <sup>2</sup> and represent one of the  
48    largest known superfamilies of homologous proteins <sup>3</sup>. In prokaryotes the situation is  
49    different, because the so-called two-component systems (TCS), which shuttle chemically  
50    labile phosphoryl groups between histidine and aspartate residues, have emerged as the  
51    bacterial signal transduction paradigm since their discovery in the late 1980's <sup>4,5</sup>. Although  
52    the first "eukaryotic-like" Ser/Thr protein kinases (STPKs) and phosphatases were  
53    discovered in bacteria at about the same time <sup>6</sup>, their close association with pathogenic  
54    organisms and their frequent occurrence on mobile genetic elements such as plasmids were  
55    consistent with acquisition from the Eukarya by horizontal gene transfer. Since then,  
56    however, the accumulation of extensive genome sequencing and meta-sequencing data  
57    revealed that the number of known STPKs probably exceeds that of two-component  
58    systems in bacteria <sup>7,8</sup>, and provided strong evidence for a common evolutionary origin of  
59    Hanks-type kinases deeply rooted in the tree of life, before the Eukarya and Prokarya went  
60    separate evolutionary ways <sup>9,10</sup>.

61  
62    Hanks-type STPKs are involved in the regulation of many aspects of bacterial physiology  
63    including virulence, cell division, central and secondary metabolisms (for recent reviews  
64    see <sup>11-16</sup>). However, mechanistic details on how STPKs organize into signaling pathways  
65    (including crosstalk with two-component systems) to perform these different functions are  
66    still largely unknown. Bacterial systems have evolved their own distinct mechanisms to

transfer, recognize and remove phosphate groups from proteins<sup>17</sup> and, while the individual Hanks-type kinases and phosphatases exhibit “eukaryotic-like” features, the actual organization of bacterial signaling cascades and networks most likely differs from that observed in eukaryotes<sup>18</sup>. In this review, we focus on *O*-phosphorylation systems in *Mycobacterium tuberculosis* (*Mtb*), a human pathogen that claims 1.2 million lives every year. Inspection of the *Mtb* genome for the presence of signaling proteins predicted that more than 200 genes could be involved in cellular communication and information processing<sup>19</sup>. Several of these genes encode for proteins involved in O-phosphorylation including 11 Hanks-type STPKs, one PP2C-like Ser/Thr protein phosphatase, two Tyr phosphatases and six proteins containing Forkhead-Associated (FHA) domains that specifically recognize phosphothreonine-containing proteins<sup>20</sup>. The interest of mycobacteria as a model for bacterial *O*-phosphosignaling has been driven in part by the potential of the relevant enzymes as targets for drug development<sup>21,22</sup>. We will not attempt to present an exhaustive survey of the subject, for which the reader is referred to many excellent reviews<sup>15,21,23-27</sup>. Instead, we will focus here on recent work in *Mtb* STPKs with a main emphasis on mechanistic insights concerning the regulatory networks around two of these kinases, PknB and PknG, which are among the best characterized to date and illustrate some specificities of *O*-phosphorylation in bacterial systems.

85

#### 86 **STPK structures and activation mechanisms.**

87

88 The *Mtb* genome contains eleven STPKs named PknA to PknL, two of which are soluble  
89 proteins (PknG and PknK) and the rest are transmembrane kinases. The first crystal  
90 structure of a bacterial kinase domain, showing phosphorylated *Mtb* PknB bound to  
91 different ATP analogs<sup>28,29</sup>, revealed the remarkable conservation of the Hanks-type kinase

92 fold across evolution. The kinase domain exhibits most structural features characteristic of  
93 their eukaryotic homologs (Figure 1). It consists of two lobes, an N-terminal subdomain  
94 made up of the regulatory helix  $\alpha$ -C and a curled  $\beta$ -sheet and a C-terminal lobe essentially  
95 composed of  $\alpha$ -helices. The nucleotide binds in a deep cleft between the two lobes,  
96 engaging the glycine-rich P-loop, a signature  $\beta$ -hairpin with main-chain amides that  
97 coordinate the triphosphate moiety (Figure 1). Other important signatures required for  
98 catalysis are also conserved, including a conserved ion pair in the N-lobe (Lys40 and  
99 Glu59 in PknB), which bridges the nucleotide with the  $\alpha$ -C helix to stabilize the closed  
100 conformation of active STPKs<sup>30</sup>, and the catalytic loop in the C-lobe with the consensus  
101 motif DxKPxN (positions 138-143 in PknB), which includes the catalytic base (Asp138)  
102 for the phosphoryl transfer reaction. Indeed, all the kinase signatures defined for  
103 eukaryotic Hanks-type kinases<sup>2</sup> are strictly conserved in *Mtb* kinase domains, with the  
104 only exception of PknI, which has asparagine instead of lysine in the catalytic loop<sup>22,31</sup>  
105 and whose crystal structure revealed an inactive, pseudokinase-like conformation<sup>32,33</sup>.

106

107 A common mechanism for eukaryotic kinase activation involves phosphorylation of one or  
108 more sites in the activation loop, a centrally located segment between the conserved DFG  
109 and APE sequence motifs (Figure 1). Phosphorylation is usually achieved through protein-  
110 protein interactions (such as receptor homodimerization), and promotes a conformational  
111 transition into an active state that permits ATP access and brings together dispersed  
112 elements of the catalytic machinery. The activation loops of several *Mtb* transmembrane  
113 kinases were indeed found to be phosphorylated in one or more residues<sup>34-37</sup>, and in some  
114 cases this was linked to a higher kinase activity<sup>34,38-40</sup>. The phosphorylated kinase domains  
115 of PknB and PknE, although monomeric in solution, were found to crystallize as  
116 structurally similar homodimers<sup>28,29,41</sup>. The dimerization interface involves the backside of

117 the N-lobe that is highly conserved in PknB-like bacterial STPKs <sup>42</sup>. Elegant work by Tom  
118 Alber and coworkers on PknD and PknB established that ‘back-to-back’ homodimerization  
119 stabilizes an active enzyme conformation and promotes allosteric kinase activation <sup>39,43</sup> *via*  
120 a similar mechanism to that proposed for the human RNA-dependent kinase PKR <sup>44</sup>. It  
121 should be noted, however, that mutations in the dimer interface of PknB or PknD, once  
122 phosphorylated, do not hamper its ability to phosphorylate its substrates, suggesting that  
123 this regulatory mechanism would only be required to trigger the initial activation of non-  
124 phosphorylated forms of the kinase domain.

125

126 An intriguing difference between active bacterial kinases and their eukaryotic counterparts  
127 is the unexpected finding that the phosphorylated activation loop of the former was largely  
128 disordered in many available structures, including those of *Mtb* PknB in different crystal  
129 forms <sup>28,29,42,43</sup>, PknA <sup>45</sup>, PknE <sup>41</sup> and even *S. aureus* PknB <sup>46</sup>. These observations suggest  
130 that, in these cases, phosphorylation of the activation loop may not stabilize a unique  
131 active kinase conformation. The observed activity enhancement upon phosphorylation  
132 might therefore arise from the activation loop acting either as an auto-inhibitor in its  
133 unphosphorylated form, or as a docking site for specific substrates in its phosphorylated  
134 form. In this respect, it is interesting to note that some of the initially reported STPK  
135 substrates were FHA domain-containing proteins capable of binding phosphothreonine  
136 residues, such as the PknH substrate EmrR <sup>34</sup>, the PknF substrate Rv1747 <sup>47</sup> or the PknB  
137 substrate GarA <sup>48</sup>. Actually, a complex network of STPKs was found to phosphorylate  
138 multiple *Mtb* FHA-domain proteins *in vitro* <sup>49</sup>. The recent crystal structure of  
139 phosphorylated *Mtb* PknB in complex with GarA <sup>50</sup> did show that the phosphorylated  
140 activation loop of PknB serves as a docking platform to recruit GarA through canonical  
141 FHA-phosphothreonine interactions that sequesters the loop away from the competent

142 kinase active site. Upon formation of the complex, the PknB regulatory helix  $\alpha$ C moves  
143 away from the conformation observed in the back-to-back PknB homodimer, significantly  
144 weakening the conserved intramolecular salt bridge between Lys40 and Glu59 (Figure 1).  
145 As a consequence, the overall conformation of the kinase N-lobe in complex with substrate  
146 is incompatible with that observed in the PknB homodimer, strongly suggesting that an  
147 important conformational plasticity is associated to the active kinase state.

148

149 The functional versatility of transmembrane *Mtb* STPKs is reflected by the variety of  
150 extracellular domains that accompany their common catalytic core. Structural information  
151 is currently available for four of these domains (Figure 2). The crystal structure of the  
152 PknD extracellular domain was shown to form a highly symmetric six-bladed  $\beta$ -propeller  
153 <sup>51</sup>, that of PknH is an  $\alpha/\beta$  structure made up of a central  $\beta$ -sheet flanked by  $\alpha$ -helices <sup>52</sup>,  
154 and that of PknI is a ten-stranded curved  $\beta$ -sheet that undergo conformational changes  
155 upon dimerization <sup>33</sup>. For PknB, the extracellular domain consists of four repeats  
156 homologous to penicillin-binding protein and serine/ threonine kinase associated (PASTA)  
157 domains <sup>53</sup>. These PASTA domains, consisting of an  $\alpha$ -helix and 3  $\beta$ -strands, are linearly  
158 arranged in an extended conformation <sup>54,55</sup>. The function of the different extracellular  
159 STPK domains remains unclear. By analogy with eukaryotic receptor kinases, it has been  
160 proposed that they might act as extracellular signal sensor domains for kinase activation  
161 control <sup>31</sup>, and/or in STPK targeting to specific cell sites. The putative ligand-binding  
162 specificity is currently unknown for all STPK extracellular regions except for the PknB  
163 PASTA domains. These were shown to bind specific PG fragments <sup>56-58</sup> or LipidII <sup>59,60</sup>, the  
164 latter being synthesized in the cytosol and transferred to the extracellular space for PG  
165 incorporation via the LipidII flippase (MurJ). Recent data on pneumococcal and  
166 mycobacterial PknB homologues <sup>60,61</sup> strongly support a major function of the PASTA



167 domains in ensuring proper PknB localization, targeting the kinase to the sites of PG  
168 production and turnover <sup>57</sup>, but challenge the current model of ligand-induced kinase  
169 activation <sup>54,62</sup>. Indeed, the cell-wall binding capability of the pneumococcal PASTA  
170 domains was found to be dispensable for StkP activation <sup>61</sup>, and abrogation of the  
171 mucopeptide-binding capability of mycobacterial PASTA repeats led to PknB  
172 hyperphosphorylation, rather than inactivation <sup>60</sup>.

173

174 The biological roles of the STPKs depend crucially on their substrate specificity. Initial  
175 efforts in a number of laboratories identified a wide range of *Mtb* STPK substrates, most of  
176 them from kinase assays *in vitro*, that were involved in a large variety of processes,  
177 including cell metabolism, transcriptional regulation, translation, cell-wall biosynthesis,  
178 and cell division <sup>25</sup>. In the last decade, proteome-wide analyses of protein phosphorylation  
179 in mycobacteria have enabled the detection of a vast number of highly confident  
180 phosphosites <sup>37,63-65</sup>. Remarkably, there is limited overlap between the phosphorylation  
181 sites reported in these studies, which may reflect biological differences, but also the high  
182 detection sensitivity and the stochastic nature of data acquisition in high-throughput  
183 proteomic experiments. The meta-analysis of *Mtb* phosphoproteomics data has  
184 nevertheless allowed the identification of a core of systematically identified  
185 phosphoproteins, which represent a reliable starting point for the selection of kinase  
186 substrate candidates <sup>66</sup>. These studies also revealed that *Mtb* supports protein tyrosine  
187 phosphorylation, possibly due to some STPKs functioning as dual-specificity kinases <sup>67</sup>.  
188 Despite this large amount of information, the contribution of phosphoproteomics towards a  
189 better understanding of the biological functions of STPKs has so far been limited, and the  
190 biological relevance of most of these phosphorylation events is still to be demonstrated.

191

## 192    **PknB regulation of peptidoglycan synthesis**

193

194    In *Mtb*, the genes coding for the receptor-like transmembrane kinases PknA and PknB are  
195    the last genes of an operon near the origin of replication, which is highly conserved in  
196    *Actinobacteria*. This operon includes other genes coding for proteins involved in STPK-  
197    mediated signaling networks (including the phosphoserine/threonine protein phosphatase  
198    PstP and the FHA domain-containing proteins FhaA and FhaB/FipA) as well as the *rodA*  
199    and *pbpA* genes involved in peptidoglycan (PG) synthesis. Early work by Kang and  
200    Husson <sup>68</sup> showed that both PknA and PknB are required for normal *Mtb* cell  
201    morphogenesis, as their respective overexpression or inactivation led to broad and irregular  
202    cell shapes with distinct cellular phenotypes for each kinase <sup>68,69</sup>. The *pknB* gene is  
203    transcribed during exponential phase (and upon macrophage infection <sup>70</sup>) but decreases 10-  
204    fold upon entry into stationary phase <sup>68</sup>, in agreement with a regulatory role during the  
205    active phase of mycobacterial growth. Since this initial work, several labs have contributed  
206    towards understanding some of the underlying pathways and dissecting possible roles for  
207    phosphoregulation therein, and significant progress has been made in understanding the  
208    role of PknB, and to a lesser extent that of PknA. We focus below on the experimental  
209    evidence converging to a PknB-mediated signaling network consisting of multiple  
210    phospho-regulatory nodes that control PG synthesis during cell growth (Figure 3). For the  
211    sake of clarity, we have purposely omitted to discuss a large body of data describing purely  
212    *in vitro* kinase substrates, or systemic studies that point to much broader roles of  
213    PknA/PknB in mycobacterial physiology (see for instance <sup>65</sup>), but for which mechanistic  
214    insights and biological validation are missing.

215

216 Significant advances have been made over the last few years towards identifying *bona fide*  
217 substrates of mycobacterial PknB. One major substrate turns out to be CwlM, a PG  
218 hydrolase that has probably undergone evolutionary repurposing to function in the  
219 cytoplasm <sup>71</sup>. The presence of CwlM, but not its presumed hydrolase activity, was shown  
220 to be essential both in *M. smegmatis* <sup>71</sup> and *Mtb* <sup>72</sup>. PknB phosphorylates CwlM at the C-  
221 terminal tail. One phosphorylation site (Thr374 in *M. smegmatis* CwlM, equivalent to *Mtb*  
222 CwlM Thr382) is important for cell growth, because phosphorylated CwlM interacts with  
223 MurA, a cytoplasmic enzyme that catalyzes the first dedicated step in PG precursor  
224 biosynthesis, leading to a 30-fold activation of the latter <sup>71</sup>. More recently Turapov and  
225 coworkers confirmed the importance of CwlM C-terminal tail phosphorylation in *Mtb*, and  
226 proposed CwlM as the major physiological substrate of PknB, as the phosphoablative  
227 (T382A) CwlM mutant strain phenocopies PknB-depleted mycobacteria <sup>72</sup>. Interestingly,  
228 CwlM is differentially localized depending on its phosphorylation status. Non-  
229 phosphorylated CwlM was found in the membrane fractions of *Mtb* cell extracts, most  
230 likely through direct interaction with MviN and/or CwsA, a mycobacterial-specific  
231 membrane protein involved in cell division and elongation <sup>73</sup>. In contrast, the  
232 phosphorylated CwlM was cytosolic and shown to directly interact with the FHA domain-  
233 containing protein FhaA <sup>72</sup>, in addition to the previously identified partner MurA <sup>71</sup> (Figure  
234 3). Like PknB, FhaA also localizes to the septum and poles of mycobacterial cells where  
235 PG is produced <sup>57,74</sup>, and was shown to interact with phosphothreonine residues outside the  
236 kinase domain of both PknB and PknG <sup>75,76</sup>. While its precise role is still unclear, two  
237 independent lines of work place FhaA at the center of the phospho-regulatory node in cell  
238 wall PG biosynthesis <sup>72,74</sup>. The membrane protein MvIN is required for the addition of PG  
239 precursors into the growing cell wall <sup>74</sup>. MviN contains a 14-transmembrane N-terminal  
240 LipidII flippase (MurJ) domain, followed by an intracellular pseudokinase domain and a

241 C-terminal extracellular carbohydrate binding domain. PknB phosphorylates MvIN on the  
242 juxtamembrane region of the pseudokinase domain, and this phosphosite is specifically  
243 recognized by FhaA (Figure 3). Conditional MvIN depletion leads to accumulation of PG  
244 precursors in the cytoplasm whereas FhaA depletion leads to higher incorporation of PG at  
245 poles and septa, and aberrant cell shape, confirming the participation of FhaA in cell  
246 morphogenesis <sup>74</sup>. It was suggested that FhaA negatively regulates the MvIN activity, but  
247 how this regulation occurs and through which mechanism remains to be elucidated.  
248 Phosphorylated CwlM also interacts with FhaA, but how this affects MurA activation  
249 remains to be demonstrated. Indeed, it is not clear how to reconcile the PknB-mediated  
250 phosphorylation of MvIN and the ensuing inhibition of its flippase activity upon FhaA  
251 binding <sup>74</sup>, with the phosphorylation of CwlM and the consequent activation of MurA <sup>71</sup>  
252 leading to increased PG precursor biosynthesis in the cytoplasm.

253

254 In addition to the protein network discussed above, other enzymes involved in PG  
255 synthesis were also shown to be phosphorylated by PknB *in vitro*, such as GlmU <sup>77</sup> or the  
256 bifunctional transglycosylase/transpeptidase PonA1 <sup>78</sup>, suggesting an extended regulatory  
257 network that would control mycobacterial cell growth and morphology at different key  
258 steps (precursors, export and polymerization) of the PG biosynthetic pathway. It is worth  
259 mentioning that PASTA-associated PknB homologues are also involved in cell division,  
260 morphogenesis and developmental processes in other Actinobacteria and Firmicutes <sup>15</sup>,  
261 albeit possibly with species-specific mechanisms, suggesting that STPK-mediated control  
262 of cell division and growth is an ancient process in bacterial evolution.

263

264 Further proteins involved in cell division and elongation were found to be phosphorylated  
265 by PknA and PknB, most notably FtsZ and Wag31. Phosphorylation of the mycobacterial

266 tubulin homolog FtsZ, which was also observed in related bacteria such as *C. glutamicum*  
 267 <sup>79</sup>, was shown to impair its GTPase activity *in vitro* <sup>80</sup>. The tropomyosin-like protein  
 268 Wag31 (DivIVA), one of the first PknA/PknB substrates reported to be phosphorylated in  
 269 mycobacterial cell extracts <sup>68</sup>, is essential for mycobacterial viability and localizes  
 270 asymmetrically to the poles <sup>81</sup>. Upon depletion severe morphological changes such as cell  
 271 rounding and bulging can be observed <sup>81</sup> and the abnormal morphologies observed have  
 272 been linked to defects in cell wall biosynthesis. In *Mtb* Thr73 was identified as the unique  
 273 phosphorylation site and a phosphomimetic mutant (T73E) was shown to have higher  
 274 growth rates, increased polar localization and PG biosynthesis when compared to  
 275 phosphoablative or wild-type cells <sup>81,82</sup>. How phosphorylation leads to increased  
 276 localization remains unclear. It may promote higher Wag31 self-association <sup>82</sup> or serve as a  
 277 docking site for other polar interacting partners such as the histidine kinase MtrB <sup>83</sup>.  
 278 Further biochemical and cellular studies will be required to evaluate these possibilities. It  
 279 should be noted that the Wag31 homologue DivIVA was reported to be phosphorylated in  
 280 other Gram-positive bacteria, such as *Streptomyces coelicolor* <sup>84</sup> where DivIVA  
 281 phosphorylation regulates polarisome localization, or *Streptococcus pneumoniae*, where  
 282 DivIVA phosphorylation by the PknB homologue StkP is required for normal cell division  
 283 and PG synthesis <sup>85,86</sup>. In both cases, however, the phosphorylation sites are located in the  
 284 C-terminus of the protein, as opposed to the N-terminal position in Wag31, suggesting  
 285 distinct modes of action in different species <sup>86,87</sup>.

286

#### 287 **PknG-mediated control of glutamate metabolism**

288

289 PknG is one of the two soluble STPKs in *Mtb*. The protein adopts a unique quaternary  
 290 structure (Figure 4) containing a disordered N-terminal segment, a metal-binding

291 rubredoxin-like domain closely associated to the central kinase domain, and a C-terminal  
292 tetratricopeptide repeat (TPR) domain <sup>88,89</sup>. The first clue about the possible physiological  
293 function of mycobacterial PknG was reported in 2004, when Yossi Av-Gay and coworkers  
294 inactivated the *pknG* gene by allelic exchange in *Mtb* <sup>90</sup>. They showed that the resulting  
295 mutant strain accumulated glutamine and glutamate, and displayed decreased viability both  
296 in nutrient-depleted media as well as upon mice infection. These first observations  
297 confirmed the importance of PknG for *Mtb* growth and linked the kinase to central  
298 metabolism. That same year Jean Pieters and coworkers reported that *Mtb* PknG inhibits  
299 phagosome-lysosome fusion in infected macrophages, possibly by interfering with host-  
300 signaling pathways <sup>91</sup> upon protein secretion <sup>92</sup>. At present, a few aspects of the PknG role  
301 as a secreted virulence factor still remains controversial and it cannot be excluded that  
302 some of the observed effects in infected macrophages might be influenced by PknG-  
303 dependent physiological changes within mycobacteria <sup>25,93</sup>. In contrast, several lines of  
304 evidence have now substantially validated the role of PknG in the regulation of glutamate  
305 metabolism in both mycobacteria and corynebacteria.

306

307 In 2006, Michael Bott and coworkers discovered that the FHA domain protein OdhI  
308 inhibits the 2-oxoglutarate dehydrogenase complex (ODHC) in *Corynebacterium*  
309 *glutamicum*, a non-pathogenic soil actinobacterium. This inhibition was abolished upon  
310 PknG-mediated phosphorylation of OdhI, resulting in regulation of the TCA cycle and  
311 nitrogen accumulation <sup>94</sup>. OdhI is a small protein consisting of an N-terminal segment that  
312 contains the phosphorylation site and a C-terminal FHA domain conserved in, and  
313 restricted to, actinobacteria. Its *Mtb* orthologue is GarA, which had been previously  
314 identified as a substrate of various STPKs <sup>48</sup>. Non-phosphorylated GarA acts as an  
315 allosteric regulator of ODHC, and was also shown to interact with two other metabolic

enzymes, glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT, for glutamate-oxoglutarate amidotransferase)<sup>95,96</sup>. These enzymes share with ODHC the substrate 2-oxoglutarate, situated at a crucial branch point between the TCA cycle and the glutamate/glutamine pool. As determined by enzyme assays *in vitro*, the effect of non-phosphorylated GarA binding is to inhibit ODHC and GDH, and to activate GOGAT<sup>95,96</sup>, thus deviating the metabolic flux towards the synthesis of glutamate (Figure 5). This model was confirmed by phenotypic analysis of a conditional *garA* knock-down mutant in *Mtb*<sup>97</sup>, as well as by a deletion mutant in the same organism, which shows asparagine auxotrophy and a severely attenuated phenotype both *in vitro* (macrophages) and *in vivo* (mouse model)<sup>98</sup>.

PknG phosphorylates GarA at the first threonine residue of the conserved N-terminal motif ETTS (Thr21)<sup>95</sup>, while PknB (and other kinases) phosphorylate the second threonine in the same motif (Thr22)<sup>48</sup>. The kinase autophosphorylation sites (in the activation loop for PknB, and outside the kinase domain for PknG) recruit the substrate FHA domain and stimulate phosphorylation. Once phosphorylated, GarA switches to a closed ‘OFF’ state, due to the intramolecular recognition of the N-terminal phosphothreonine by the FHA domain<sup>99</sup>, as confirmed by NMR studies on both the *Mtb* and *C. glutamicum* proteins<sup>96,100</sup>. Under these conditions, the metabolic flux is reoriented back in the catabolic direction, leading to glutamate consumption. Further work showed that a *pknG*-null *Mtb* mutant was unable to grow on glutamate as the sole carbon source, presumably due to the accumulation of non-phosphorylated GarA, and the same phenotype was observed for a *garA* deletion mutant complemented with an N-terminal truncated form of GarA lacking the phosphorylation sites<sup>98</sup>. It was also observed that the phosphorylation of GarA promptly followed changes in the medium composition (enrichment), while

341 dephosphorylation was a much slower process occurring under starvation conditions <sup>98</sup>.  
 342 GarA therefore acts as a molecular switch, triggered by PknG *in vivo*, to tune the carbon  
 343 and nitrogen metabolic fluxes at the 2-oxoglutarate crossroad according to the  
 344 environmental conditions (nutrient availability).  
 345  
 346 While the GarA FHA domain is a functional phosphothreonine-binding module, it  
 347 mediates phosphoindependent downstream interactions with the different metabolic  
 348 enzymes <sup>96</sup>. If little is currently known about how mycobacterial GarA interacts with GDH  
 349 and GOGAT, the mechanism of ODHC inhibition has been elucidated. GarA inhibits the  
 350 first component (E1o) of ODHC, a thiamine diphosphate-dependent enzyme encoded by  
 351 the Rv1248c gene in *Mtb* that has also been reported to catalyze 2-oxoglutarate  
 352 decarboxylase and 2-hydroxy-3-oxoadipate synthase reactions <sup>101,102</sup>. The structure of the  
 353 GarA-E1o complex <sup>50</sup> reveals that the FHA domain binds far away from the enzyme active  
 354 site, and acts as a dynamic allosteric inhibitor that hampers a conformational transition  
 355 required for E1o catalysis <sup>103,104</sup>. This model is fully consistent with a thorough steady-  
 356 state analysis of the homologous *Mtb* enzymes, showing that GarA acts as a mixed V and  
 357 K-type inhibitor, reducing the  $k_{cat}$  and increasing the  $K_m$  for the substrate 2-oxoglutarate  
 358 <sup>105</sup>. Noteworthy, an exposed aspartate residue (Asp795) from E1o was found to be crucial  
 359 for GarA binding and inhibition, and the structure of the complex showed that the Asp795  
 360 carboxylate occupied the FHA domain phosphate-binding pocket <sup>50</sup>. This clear example of  
 361 functional phosphomimicry illustrates how the GarA FHA domain can mediate both  
 362 ‘canonical’ phosphodependent interactions (kinase recruitment) as well as phospho-  
 363 independent interactions (metabolic regulation), extending the functions of prokaryotic  
 364 FHA domains beyond that of simple phosphorylation ‘reader’ domains in bacterial cell  
 365 signaling.



366

367 Although nutrient availability appears to be the main stimulus leading to GarA  
368 phosphorylation in mycobacteria, the molecular bases for PknG kinase activation are still  
369 poorly understood. PknG is a non-RD kinase that lacks autophosphorylation residues in the  
370 activation loop. All autophosphorylation sites occur in the unstructured N-terminal region  
371 of the protein <sup>95</sup> and do not play any role in activating the kinase domain. The rubredoxin-  
372 like domain, which was shown to downregulate catalysis through specific intramolecular  
373 interactions that modulate access to a deep substrate-binding cleft in the kinase domain  
374 (Figure 4) <sup>89</sup>, has been proposed by independent groups to function as a redox sensor <sup>106,107</sup>.  
375 This putative sensor domain is nevertheless missing in PknG from some suborders of the  
376 *Actinomycetales*, like *Corynebacterium*. A recent study has shed light on how PknG might  
377 be regulated by nutrient levels <sup>108</sup>. The *pknG* gene is located on an operon that bears the  
378 genes coding for GlnH, a periplasmic solute-binding protein, and GlnX, predicted to be an  
379 integral membrane protein. A *M. smegmatis* mutant lacking *glnX* phenocopies the *pknG*  
380 mutant, showing inability to grow on glutamate as the sole nitrogen source, and the  
381 phenotype was rescued by complementing with a functional copy of the gene <sup>108</sup>.  
382 Furthermore, the ligand-binding specificity of GlnH matched the amino acids, notably  
383 glutamate and aspartate, that stimulate GarA phosphorylation. According to the proposed  
384 model, the transmembrane protein GlnX would act as a relay transmitting the signal sensed  
385 in the periplasmic space by GlnH (amino acids availability) to the intracellular kinase  
386 PknG, leading to its activation. However, how this sensor - signal relay system would work  
387 and interact with PknG, ultimately triggering kinase activation and GarA phosphorylation  
388 remains, to be determined.

389

390 **Concluding remarks**

391

392 Current knowledge about *O*-phosphorylation in prokaryotes is based on concepts learned  
393 from eukaryotic systems, not the least because bacterial STPKs and phosphatases exhibit  
394 ‘eukaryotic-like’ structures and biochemical properties, including similar activation  
395 mechanisms <sup>24,109,110</sup>. However, as illustrated by the special bacterial phosphorylation  
396 systems such as BY-kinases <sup>17,111,112</sup> or some features of the PknB and PknG regulatory  
397 networks discussed above, it appears that mycobacteria have evolved their proper *O*-  
398 phosphorylation mechanisms. After identifying the major *Mtb* signaling proteins, the  
399 challenges are now essentially the same as in eukaryotic phosphosignaling <sup>18</sup>, namely  
400 identifying the environmental stimuli that activate the sensor kinases, understanding the  
401 regulation mechanisms of STPK activity, assigning the physiologically relevant  
402 phosphosites to the corresponding STPKs, or yet establishing the existence of crosstalk  
403 between different STPKs (kinase cascades) or between STPKs and two-component  
404 systems. Twenty years after the first report of the presence of STPKs in *Mtb* <sup>19</sup>, a long way  
405 still lies ahead to clarify the extent to which the *O*-phosphosignaling network, and the  
406 complex interplay among its numerous signaling elements, tunes *Mtb* physiology and  
407 regulates key processes in pathogenesis.

408

409 **Acknowledgements:** We thank all colleagues from our laboratories and the former  
410 MM4TB Consortium for insightful discussions and advice.

411

412 **Funding:** This work was partially supported by grants from the Institut Pasteur (Paris), the  
413 Institut Pasteur de Montevideo and the ANR [research contracts ANR-13-JSV8-0003-01,  
414 ANR-18-CE11-0017-01 and ANR-18-CE92-0003-01].

415

416   **Conflict of Interests:** The authors declare that they have no conflict of interests.

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420 **References**

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766

767 **Figure legends**

768

769 **Figure 1.** Structure of the kinase domain of PknB from *M. tuberculosis* in complex with  
770 nucleotide (PDB code 1o6y). The catalytic loop and the partially disordered activation loop  
771 are shown in yellow and red, respectively. The regulatory  $\alpha$ -C helix is shown in green and  
772 the glycine-rich P-loop in blue. The zoom shows the conserved salt bridge between Lys40  
773 and Glu59 that is important to achieve a catalytically competent closed state.

774

775 **Figure 2.** Cartoon representations of the available STPK extracellular domain structures.  
776 Panels A-D correspond to PknB (pdb: 1o6y), PknD (pdb: 1rwl), PknH (pdb: 4esq) and  
777 PknI (pdb: 5xll), respectively.

778

779 **Figure 3.** Schematic interpretation of core interactions in the PknB regulatory network.  
780 Other identified PknB substrates (including PknA) mentioned in the text that might  
781 participate in cell division and morphogenesis control are omitted for the sake of clarity.

782

783 **Figure 4.** Quaternary organization of *Mtb* PknG (unpublished) showing the kinase domain  
784 (blue) in complex with nucleotide, the rubredoxin-like domain (green) and the TPR  
785 domain (yellow).

786

787 **Figure 5.** The PknG pathway that regulates glutamate metabolism in mycobacteria. OG, 2-  
788 oxoglutarate; Asp, aspartate; Glu, glutamate; Gln, glutamine; SucCoA, succinyl-Coenzyme  
789 A. Dephosphorylation of GarA by PstP, the conserved transmembrane protein serine-  
790 threonine phosphatase in actinobacteria, is assumed based on experimental data from *C.*  
791 *glutamicum*<sup>79</sup>, but is yet to be demonstrated in mycobacteria.

Figure 1

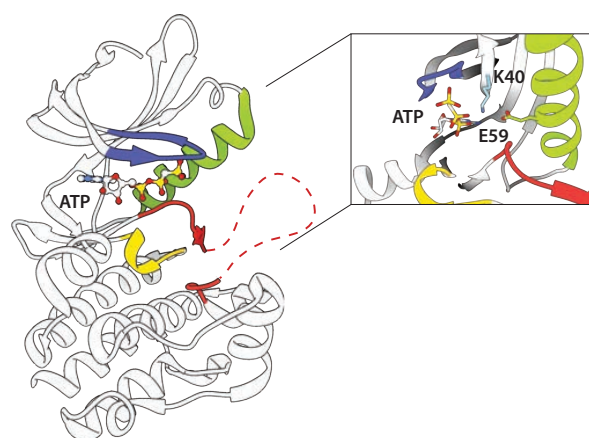


Figure 2

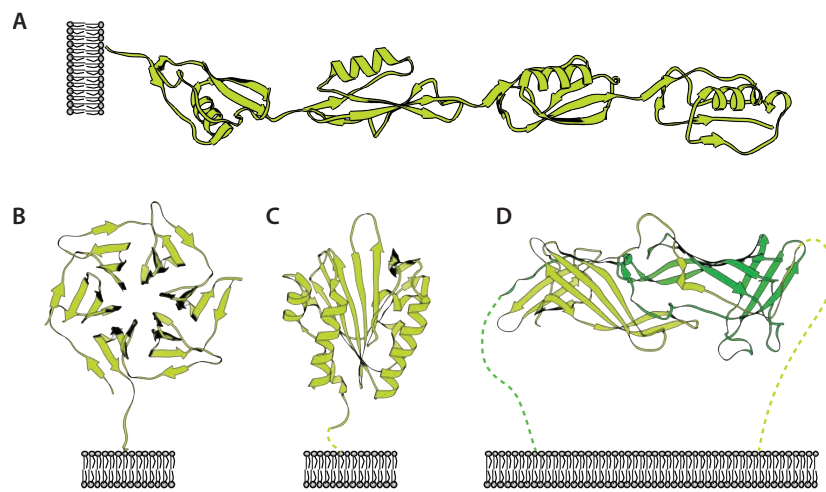


Figure 3

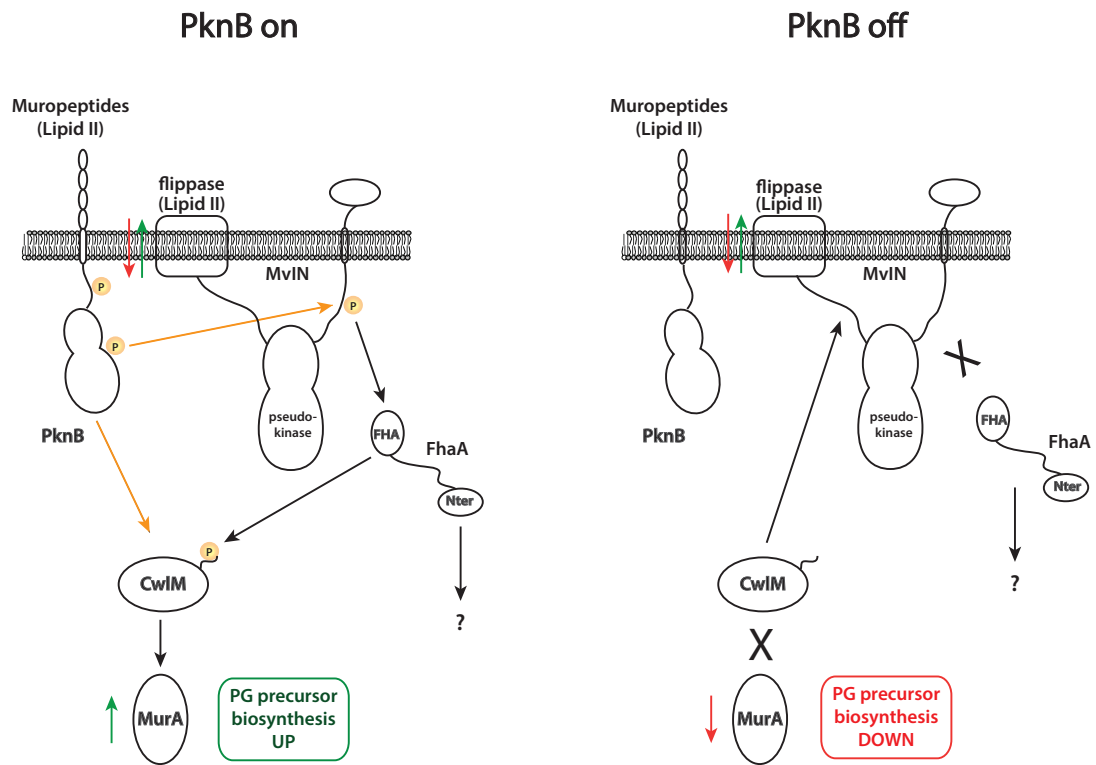




Figure 4

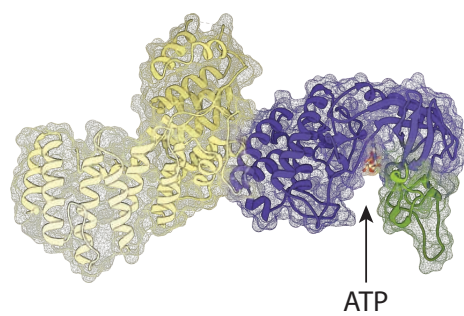


Figure 5

