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## Phosphorylation on PstP controls cell wall metabolism and antibiotic tolerance in *Mycobacterium smegmatis* [preprint]

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1 **Phosphorylation on PstP controls cell wall metabolism and antibiotic tolerance in**  
2 ***Mycobacterium smegmatis***

3

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6

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11

12 **Abstract**

13

14 The mycobacterial cell wall is a dynamic structure that protects *Mycobacterium*  
15 *tuberculosis* and its relatives from environmental stresses. Modulation of cell wall  
16 metabolism under stress is thought to be responsible for decreased cell wall permeability  
17 and increased tolerance to antibiotics. The signaling pathways that control cell wall  
18 metabolism under stress, however, are poorly understood. Here, we examine the  
19 signaling capacity of a cell wall master regulator, the Serine Threonine Phosphatase  
20 PstP, in the model organism *Mycobacterium smegmatis*. We studied how interference  
21 with a regulatory phosphorylation site on PstP affects growth, cell wall metabolism and  
22 antibiotic tolerance. We find that a phospho-mimetic mutation, *pstP* T171E, slows  
23 growth, misregulates both mycolic acid and peptidoglycan metabolism in different  
24 conditions, and interferes with antibiotic tolerance. These data suggest that  
25 phosphorylation on PstP controls its substrate specificity and is important in the  
26 transition between growth and stasis.

27

28 **Introduction**

29

30 Tuberculosis (TB), an infectious disease caused by the bacterium *Mycobacterium*  
31 *tuberculosis* (*Mtb*), is currently the ninth leading cause of death worldwide (World Health  
32 Organization 2017). The fact that TB treatment requires at least a six month regimen  
33 with four antibiotics is partly due to the intrinsic phenotypic antibiotic tolerance of *Mtb*  
34 (Nguyen 2016; Jarlier and Nikaido 1994). In the host, *Mtb* cells can achieve a dormant,

35 non-replicating state exhibiting antibiotic tolerance, reduced metabolism and altered cell-  
36 wall staining (Boshoff and Barry 2005; Seiler et al. 2003). *In vitro* stresses induce cell  
37 wall thickening and altered staining as well (Cunningham and Spreadbury 1998). The  
38 changes in the cell wall and reduced permeability to antibiotics in stressed cells (Sarathy  
39 et al. 2013) suggest that the regulation of the cell wall is a major contributor to antibiotic  
40 tolerance.

41

42 The accepted cell wall core architecture of *Mtb* consists of a single mycolyl-  
43 arabinogalactan-peptidoglycan molecule which is composed of three covalently linked  
44 layers (Minnikin 1991). A peptidoglycan (PG) layer surrounding the plasma membrane is  
45 covalently bound to an arabinogalactan layer. A lipid layer composed of mycolic acids  
46 surrounds the arabinogalactan layer, and the inner leaflet of this layer is covalently  
47 linked to the arabinogalactan (Kieser and Rubin 2014). The outer leaflet of the mycolic  
48 acid layer contains free mycolic acids, trehalose mycolates and other lipids, glycolipids,  
49 glycans and proteins (Marrakchi, Lan  elle, and Daff   2014). The mycolic acid layer is  
50 the major contributor to impermeability of the cell wall (Hett and Rubin 2008).

51

52 While much is known about the cell wall structure of *Mtb*, little is known about the  
53 regulation of the cell wall during stress or how they contribute to antibiotic tolerance. The  
54 signaling pathways regulating the *Mtb* cell wall likely help it survive in the host.

55 Reversible protein phosphorylation is a key regulatory mechanism used by bacteria for  
56 environmental signal transduction to regulate cell growth (Echenique et al. 2004; Juris et  
57 al. 2000; Galyov et al. 1993; J. Wang et al. 1998). In *Mtb*, Serine/Threonine (S/T)  
58 phosphorylation is important in cell growth regulation (Kang 2005; Gee et al. 2012;  
59 Boutte et al. 2016; Baer et al. 2014). *Mtb* has 11 Serine/Threonine Protein Kinases  
60 (STPKs) (PknA, PknB and PknD-L) and only one S/T protein phosphatase (PstP) (Cole  
61 et al. 1998; Bach, Wong, and Av-Gay 2009). Among the STPKs, PknA and PknB are  
62 essential for *Mtb* growth and phosphorylate substrates involved in cell growth and  
63 division (Sasseti, Boyd, and Rubin 2003; Kang 2005; Fernandez et al. 2006; Kusebauch  
64 et al. 2014; Boutte et al. 2016) Some of these substrates are enzymes whose activity is  
65 directly altered by phosphorylation. The enoyl-ACP reductase activity of InhA, a key  
66 enzyme involved in mycolic acid biosynthesis in *Mtb*, is inhibited when phosphorylated  
67 by multiple STPKs (Molle and Kremer 2010; Khan et al. 2010). All the enzymes in the  
68 FAS-II system of mycolic acid biosynthesis are regulated by threonine phosphorylation

69 (Molle et al. 2006; Vilchèze et al. 2014; Slama et al. 2011; Khan et al. 2010; Veyron-  
70 Churlet and Zanella-Cléon 2010).

71

72 There are also cell wall regulators that are not enzymes but whose phosphorylation by  
73 STPKs affect cell shape and growth. For example, the regulator CwIM activates MurA,  
74 the first enzyme in PG precursor biosynthesis (Boutte et al. 2016; Typas et al. 2011),  
75 when it is phosphorylated by PknB. In the transition to starvation, CwIM is rapidly  
76 dephosphorylated in *Msmeg* (Boutte et al. 2016). Misregulation of MurA activity  
77 increases sensitivity to antibiotics in early starvation (Boutte et al. 2016), implying that  
78 phospho-regulation of CwIM promotes antibiotic tolerance. CwIM may also regulate  
79 other steps of peptidoglycan synthesis (Turapov et al. 2018). A recent  
80 phosphoproteomic study showed that PstP is likely to be the phosphatase of CwIM  
81 (Iswahyudi et al. 2019)

82

83 PstP, the only S/T phosphatase must have to dephosphorylate substrates  
84 phosphorylated by most or all of the 11 STPKs in *Mtb* (Cole et al. 1998). PstP is  
85 essential in *Mtb* and *Msmeg* (DeJesus et al. 2017; Sharma et al. 2016). It is a member of  
86 the Protein phosphatase 2C (PP2C) subfamily of PPM (metal-dependent protein  
87 phosphatase) ser/thr phosphatases (Chopra et al. 2003) which strictly require divalent  
88 metal ions for activity (Shi 2009). In addition to the two characteristic metal binding sites  
89 of PP2C phosphatases, the PstP<sub>Mtb</sub> catalytic domain has a third Mn<sub>2+</sub> binding site close  
90 to the flap subdomain adjacent to the active site (Pullen et al. 2004) (Figure 1A). PstP<sub>Mtb</sub>  
91 shares structural folds and conserved residues with the human PP2C $\alpha$ , which serves as  
92 the representative of the PP2C family (Chopra et al. 2003). PstP<sub>Mtb</sub> has an N-terminal  
93 cytoplasmic enzymatic domain of 237 residues which is joined by a 63 amino acids long  
94 segment to a transmembrane pass of 18 residues connecting the C-terminal  
95 extracellular domain of 191 residues (Chopra et al. 2003).

96

97 PP2C phosphatases are involved in responding to environmental signals, regulating  
98 metabolic processes, sporulation, cell growth, division and stress response in a diverse  
99 range of prokaryotes and eukaryotes (Mougous et al. 2007; Irmeler and Forchhammer  
100 2001; S. Vijay, Mukkayyan, and Ajitkumar 2014; Bradshaw et al. 2017; Lu and Wang  
101 2008; K. Vijay et al. 2000).

102

103 In *Mtb*, PstP can dephosphorylate PknA, PknB, other STPKs (Sajid et al. 2011; Durán et  
104 al. 2005; Boitel et al. 2003), KasA, KasB (Sajid et al. 2011; Molle et al. 2006; Durán et al.  
105 2005) and PBPA (Dasgupta et al. 2006). Dephosphorylation of PknB significantly  
106 reduces protein kinase activity (Boitel et al. 2003), which likely results in downregulation  
107 of growth (Betts et al. 2002; Ortega et al. 2014). Many of the other proteins  
108 dephosphorylated by PstP are involved in cell wall metabolism; however, the effects of  
109 this activity differ. Dephosphorylation of CwIM (Iswahyudi et al. 2019) should decrease  
110 peptidoglycan metabolism (Boutte et al. 2016). But dephosphorylation of KasA (Molle et  
111 al. 2006) and the other FAS-II enzymes (Khan et al. 2010; Slama et al. 2011; Veyron-  
112 Churlet and Zanella-Cléon 2010; Molle and Kremer 2010) should upregulate lipid  
113 metabolism. These phospho-signaling events are likely involved in the transitions  
114 between growth and stasis during infection. However, peptidoglycan and lipid  
115 metabolism should be largely correlated (Dulberger, Rubin, and Boutte 2019), so PstP  
116 must be able to minutely toggle its substrate specificity between growth and stasis. For  
117 example, we expect that PstP should dephosphorylate KasA but not CwIM during  
118 growth, and should switch this specificity in stasis. How does PstP control its substrate  
119 specificity?

120

121 PstP<sub>Mtb</sub> is itself phosphorylated in the catalytic domain on Threonine (Thr) residues 137,  
122 141, 174 and 290 (Sajid et al. 2011). The corresponding phosphothreonine residues in  
123 PstP<sub>smeg</sub> are Thr 134, 138 and 171. We hypothesize that phosphorylation of the  
124 threonine residues of PstP might help determine substrate specificity. Addition of a  
125 phosphate group to a protein will change surface charge, which could affect protein  
126 confirmation, activity (Bibb and Nestler 2005) or substrate binding (Ardito et al. 2017).  
127 Mutating T138 to alanine on the PP2C serine threonine phosphatase PphA of  
128 *Thermosynechococcus elongatus* changes its substrate specificity (Su and  
129 Forchhammer 2012). Thus, changing the surface polarity in this class of enzymes can  
130 change substrate specificity. Interestingly, T138 in *T. elongatus* corresponds to T137 In  
131 PstP<sub>Mtb</sub>, which is phosphorylated (Sajid et al. 2011) . Our model is that the phospho-  
132 threonine sites of PstP are involved in toggling substrate specificity to help regulate  
133 growth and cell wall metabolism in changing conditions.

134

135 We report here that phospho-ablative and phospho-mimetic mutations at the phospho-  
136 site T171 of PstP<sub>Msmeg</sub> alter growth rate, cell length, cell wall metabolism and antibiotic

137 tolerance. Strains of *Msmeg* with *pstP* T171E alleles grow slowly, but are unable to  
138 properly downregulate peptidoglycan metabolism and upregulate antibiotic tolerance in  
139 the transition to starvation. We observed that the same mutation has nearly opposite  
140 effects on mycolic acid layer metabolism and antibiotic tolerance.

141

## 142 **Materials and Methods**

143

### 144 **Bacterial strains and culture conditions**

145 All *Mycobacterium smegmatis* mc<sub>2</sub>155 ATCC 700084 cultures were started in 7H9  
146 (Becton, Dickinson, Franklin Lakes, NJ) medium containing 5 g/liter bovine serum  
147 albumin (BSA), 2 g/liter dextrose, 0.003 g/liter catalase, 0.85 g/liter NaCl, 0.2% glycerol,  
148 and 0.05% Tween 80 and incubated at 37°C up to log phase. Hartmans-de Bont (HdB)  
149 minimal medium made as described previously (Hartmans and De Bont 1992) without  
150 glycerol was used for starvation assays. Serial dilutions of all CFU counts were plated on  
151 on LB Lennox agar (Fisher BP1427-2).

152 *E. coli* Top10, XL1Blue and Dh5 $\alpha$  were used for cloning and *E. coli* BL21 Codon Plus  
153 strains were used for protein expression. Antibiotic concentrations for *M. smegmatis*  
154 were 25  $\mu$ g/ml kanamycin, 50  $\mu$ g/ml hygromycin and 20  $\mu$ g/ml zeocin. Antibiotic  
155 concentrations for *E. coli* were 50  $\mu$ g/ml kanamycin, 25  $\mu$ g/ml zeocin, 20  $\mu$ g/ml  
156 chloramphenicol and 140  $\mu$ g/ml ampicillin.

### 157 **Strain construction**

158 Since *pstP* is an essential gene in mycobacteria, a PstP-knockdown strain was created  
159 in multiple steps; first by creating a merodiploid strain using the *M. tuberculosis pstP*  
160 gene, and then by deleting the native *M. smegmatis pstP* gene from its native  
161 chromosomal location. The merodiploid strain was generated by introducing at the L5  
162 attB integration site, a constitutively expressing *M. tuberculosis pstP* gene cloned on a  
163 StrR plasmid. The *M. smegmatis pstP* gene (MSMEG\_0033) at the native locus was  
164 then deleted by RecET-mediated double stranded recombineering approach using a  
165 1.53 kb loxP-hyg-loxP fragment carrying a 125 bp DNA flanking the *M. smegmatis pstP*  
166 gene (Murphy, Papavinasasundaram, and Sasseti 2015). The recombineering substrate  
167 was generated by two sequential overlapping PCR of the loxP-hyg-loxP substrate  
168 present in the plasmid pKM342. The downstream flanking primer used in the first PCR  
169 also carried an optimized mycobacterial ribosome binding site in front of the start codon  
170 of MSMEG\_0032 to facilitate the expression of the genes present downstream of *pstP* in

171 the *M. smegmatis* *pstP-pknB* operon.

172 Deletion of the *M. smegmatis* *pstP* gene was confirmed by PCR amplification and  
173 sequencing of the 5' and 3' recombinant junctions, and the absence of an internal *M.*  
174 *smegmatis* wild-type *pstP* PCR product. The *M. tuberculosis* *pstP* allele present at the  
175 L5 site was then swapped with a tet-regulatable *M. tuberculosis* *pstP* allele (RevTetR-  
176 P750-*Mtb* *pstP*-DAS tag-L5-Zeo plasmid) (Schnappinger, O'Brien, and Ehrt 2015). The  
177 loxP-flanked *hyg* marker present in the chromosomal locus was then removed by  
178 expressing Cre from pCre-sacB-Kan, and the Cre plasmid was subsequently cured from  
179 this strain by plating on sucrose.

180

181 Different alleles of *pstP* were attained by swapping the zeocin resistance-marked vector  
182 at L5 site with wild-type, phosphoablative (T134A, T138A, T171A) or phosphomimetic  
183 (T134E, T138E, T171E) mutant *pstP* alleles under a p766tetON6 promoter for a  
184 kanamycin resistance-marked vector, as described (Pashley and Parish 2003). The final  
185 genotypes of the strains are thus mc<sub>2</sub>155  $\Delta$ *pstP*::lox L5::pCT94-p766tetON6-*pstP*<sup>*Msmeg*</sup>  
186 wild-type, T134A, T138A, T171A, T134E, T138E or T171E.

187

### 188 **Growth Curve assay**

189 Biological triplicates of each strain were grown in 7H9 media up to log phase. Growth  
190 curve experiment was performed in non-treated 96 well plate using plate reader (BioTek  
191 Synergy neo2 multi mode reader) in 200ul 7H9 media starting at OD<sub>600</sub>=0.1 for upto 16  
192 hours at 37°C. Data was analyzed using the software Prism (version 7.0d).

193

### 194 **Cell staining**

195 For staining cells in their log phase, 100  $\mu$ l culture in 7H9 was incubated at 37°C with 1ul  
196 of 10mM DMN-Tre for 30 minutes and 1 $\mu$ l of 10mM HADA for 15 minutes. Cells were  
197 then pelleted and resuspended in phosphate buffered saline (PBS) supplemented with  
198 Tween 80 and fixed with 10 $\mu$ l of 16% paraformaldehyde (PFA) for 10 minutes at room  
199 temperature. After spinning down, cells were finally resuspended in PBS plus Tween 80.  
200 For starvation microscopy, 500 $\mu$ l of culture of each strain was used after incubating for 4  
201 hours in HdB media without glycerol at 37°C. After pelleting down 500 $\mu$ l culture, 400 $\mu$ l of  
202 the supernatant was discarded and the pellets were resuspended in the remaining 100 $\mu$ l  
203 media. Cells were then incubated at 37°C with 1 $\mu$ l of 10mM DMN-Tre for a total of 1  
204 hour and 3 $\mu$ l of 10mM HADA for 30 minutes. Cells were then pelleted and resuspended

205 in phosphate buffered saline (PBS) with Tween 80 and fixed as mentioned above. The  
206 total time of starvation before fixing them was about five and a half hours.

207

### 208 **Microscopy and Image Analysis**

209 Log-phase and starved cells fixed with PFA were immobilized on agarose pads. Cells  
210 were then imaged using a Nikon Ti-2 widefield epifluorescence microscope having a  
211 Photometrics Prime 95B camera and an objective lens having Plan Apo 100x1.45  
212 numerical aperture (NA). The green fluorescence images for DMN-Tre staining were  
213 taken with a 470/40nm excitation filter and a 525/50nm emission filter. Blue fluorescence  
214 images were taken using 350/50nm excitation filter and 460/50nm emission filter. All  
215 images were captured using NIS Elements software and analyzed using FIJI and  
216 MicrobeJ (Ducret, Quardokus, and Brun 2016)

217

### 218 **Western Blots**

219 For obtaining protein lysates from log phase cultures, cultures were grown in 7H9 upto  
220 log phase ( $OD_{600}=0.8$ ) in 10ml 7H9 media, then centrifuged at 5000 rpm for 10 minutes  
221 at 4°C. Pellets were resuspended in 500 $\mu$ L PBS with 1mM PMSF and lysed  
222 (MiniBeadBeater-16, Model 607, Biospec). Supernatant from the cell lysate was  
223 collected by centrifugation at 14000 rpm for 10 minutes at 4°C. Protein samples were  
224 run on 12% resolving Tris-Glycine gels. Rabbit Strep-tag antibody (1:1000, Abcam,  
225 ab76949) in TBST buffer with 0.5% milk and goat anti-rabbit IgG (H+L) HRP conjugated  
226 secondary antibody (1:1000, ThermoFisher Scientific 31460) in TBST were used to  
227 detect PstP-strep from individual strains on Western blot. For obtaining cell lysates from  
228 starved cultures, cultures were first grown upto log phase, then starved in 50 ml HdB no  
229 glycerol starvation media starting at  $OD=0.5$  for one and a half hour. Cell lysates were  
230 obtained as described above.

231

### 232 **Antibiotic assays**

233 For antibiotic assays with log phase culture, cells were grown up to the log phase and  
234 new 7H9 media with Tween was inoculated at  $OD_{600}= 0.05$ . For starvation assays, cells  
235 were grown upto the log phase, spun down at 5000 rpm for 10 minutes, washed in HdB  
236 starvation (with no glycerol and 0.05% Tween) media. After spinning down at 5000 rpm  
237 for 10 minutes at 4°C, pellets were resuspended in the same media and OD at 600nm  
238 was taken. HdB starvation (with no glycerol and 0.05% Tween) media was inoculated at



239 OD<sub>600</sub>=0.3 and incubated at 37°C for a total of five a half hours. OD<sub>600</sub> was measured  
240 and 5ml of new starvation media was inoculated at OD<sub>600</sub>=0.05. 8 µg/ml and 45 µg/ml  
241 Meropenem was used for log-phase and starved cultures respectively. 10 µg/ml and  
242 90ug/ml Isoniazid was added to log-phase and starved culture respectively. Samples  
243 from the culture were serially diluted and plated on LB agar before meropenem or  
244 isoniazid was added and then at several time points after.

245 **Protein Purification:** (to be rewritten/edited later again)

246 N-terminally his-MBP tagged Pkn<sub>B</sub><sup>Mtb</sup> was expressed using *E. coli* BL21 Codon Plus  
247 cells at 18°C for 17 hours with 1mM IPTG. Cell pellets were resuspended in 50mM Tris  
248 pH 7.5, 150mM NaCl, 20mM Imidazole, 1mM DTT and 10% glycerol) and sonicated to  
249 lyse in presence of lysozyme. Supernatant was run over Ni-column (BioRad Nuvia IMAC  
250 5ml). Proteins were eluted in 50mM Tris pH 7.5, 150mM NaCl, 250mM Imidazole, 1mM  
251 DTT and 10% glycerol and dialyzed. Dialyzed sample was run over Ssize exclusion  
252 resins (GE Biosciences Sephacryl S200 in HiPrep 26/70 column) to obtain soluble  
253 proteins in 50mM Tris pH 7.5, 150mM NaCl and 10% glycerol.

254 His-SUMO-CwIM<sub>Mtb</sub> was expressed in *E. coli* BL21 Codon Plus cells at 25°C for 6 hours  
255 with 1.3 mM IPTG. His-PstP<sub>C<sub>Mtb</sub></sub> was expressed in *E. coli* BL21 Codon Plus at 25°C for 6  
256 hours with 1mM IPTG.

257

258

259

## 260 **Results**

### 261 **Phosphosite T171 on PstP<sub>Msmeg</sub> has an impact on growth**

262 PstP is necessary for cell growth, division and cell wall synthesis in *M. smegmatis* and it  
263 has been shown that phosphorylation regulates the activity of PstP<sub>Mtb</sub> *in vitro* (Sharma et  
264 al. 2016; Iswahyudi et al. 2019; Sajid et al. 2011). We wanted to see if the  
265 phosphorylations on PstP have a role in regulating cell growth. Threonines (T) T134,  
266 T138, and T171 in PstP<sub>Msmeg</sub> correspond to the phospho-sites on PstP<sub>Mtb</sub> (Sajid et al.  
267 2011) (Figure 1A). We constructed an *M. smegmatis* strain with one copy of *pstP* at the  
268 L5 phage integrase site using recombineering (van Kessel and Hatfull 2008). We then  
269 exchanged the wild-type allele for either phospho-ablative (T->A) or phospho-mimetic  
270 (T>E) alleles at each of the three conserved phosphorylation sites (Pashley and Parish  
271 2003; Cottin, Van Linden, and Riches 1999).

272

273 We performed growth curves with several clones of each mutant allele. We found that  
274 the biological replicates of the T134A, T134E, T138A and T138E mutant strains had bi-  
275 modal distributions of doubling times. T134 and T138 map to the flap subdomain of  
276 PstP<sub>Mtb</sub> (Figure 1A). This subdomain varies greatly in sequence and structure across  
277 different PP2C family members and has been shown to be important in regulating  
278 substrate binding, specificity and catalytic activity (Pullen et al. 2004; Su and  
279 Forchhammer 2012; Greenstein et al. 2006; Schlicker et al. 2008). Particularly, T138A  
280 and T138E variants of the serine threonine phosphatase tPphA from  
281 *Thermosynechococcus elongatus* showed differences in substrate reactivity (Su and  
282 Forchhammer 2012). This suggests that phosphorylations at T134 and T138 could be  
283 very important in regulating the normal activity of PstP<sub>Msmeg</sub> in the cell. We suspect that  
284 these mutations impaired growth so severely that suppressor mutations formed in  
285 several of the biological replicates, giving rise to the inconsistent growth rates.

286

287 The *Msmeg* strains with *pstP* T171A and T171E mutations showed consistent and  
288 reproducible growth rates (Figure 1B). The T171A mutants grew normally, but the T171E  
289 grew more slowly than the wild-type (Figure 1C). Since T171E mimics constitutive  
290 phosphorylation, this result suggests that the continuous presence of a phosphate on  
291 T171 may inhibit cell growth.

292

### 293 **Phosphosite T171 of PstP<sub>Msmeg</sub> regulates cell length**

294 To assess how phosphorylation on T171 affects cell morphology, we observed the  
295 *Msmeg pstP* T171 mutant and isogenic wild-type cells in log phase using phase  
296 microscopy and quantified mean lengths (Figure 2A,B). *pstP* T171A cells were about 0.5  
297  $\mu\text{m}$  shorter than the wild-type cells, on average. Because this strain grew at the same  
298 rate as wild-type (Figure 1C), we assume that the rate of cell elongation is the same, but  
299 that septation may be cued at shorter cell lengths. The *pstP* T171E strain has cell  
300 lengths similar to the wild-type (Figure 2A) despite the slower growth (Figure 1C). This  
301 suggests that phosphorylation on T171 may downregulate elongation and division  
302 equally.

303

304 PstP could promote the transition to growth stasis by downregulating the activity of  
305 PknA, PknB and CwIM (Iswahyudi et al. 2019; Boutte et al. 2016; Sajid et al. 2011; Boitel  
306 et al. 2003; Chopra et al. 2003). To test if the phosphosite T171 of PstP<sub>Msmeg</sub> affects cell

307 length in the transition to stasis, we transferred the T171 phosphomutants and wild-type  
308 strains from log phase to minimal HdB media with Tween80 as the only source of  
309 carbon. We aerated the cultures for 5.5 hours before imaging (Figure 2C,D), which leads  
310 *Msmeg* cells to reductively divide (Wu, Gengenbacher, and Dick 2016). The effects of  
311 phosphomutations of PstP<sub>*Msmeg*</sub> on starved cells were the inverse of what we saw in the  
312 log phase. *pstP<sub>Msmeg</sub>* T171E cells in starvation were longer than the wild-type and  
313 T171A. These data imply that phosphorylation on PstP<sub>*Msmeg*</sub> T171 either slows reductive  
314 division or inhibits the downregulation of cell elongation in the transitions to stasis. These  
315 data suggest that phosphorylation on T171 of PstP<sub>*Msmeg*</sub> may reverse the protein's  
316 activity or substrate specificity towards cell growth substrates.

317

318

### 319 **Phosphosite T171 on PstP<sub>*Msmeg*</sub> is important in regulating cell wall metabolism**

320 Since *pstP<sub>Msmeg</sub>* T171 seem to play a role in regulating cell length in growth and stasis,  
321 we hypothesized that it affects cell wall metabolism in different phases. To test this, we  
322 used fluorescent dyes that are incorporated into either the peptidoglycan or mycolic acid  
323 cell wall layers and which preferentially stain metabolically active cell wall (Kuru et al.  
324 2012; Baranowski, Rego, and Rubin 2019; Kamariza et al. 2018). We stained  
325 phosphomutant and wild-type cells from log. phase and after 5.5 hours of carbon  
326 starvation with both the fluorescent D-amino acid HADA (Kuru et al. 2012; Baranowski,  
327 Rego, and Rubin 2019) and the fluorescent trehalose DMN-Tre (Kamariza et al. 2018)  
328 (Figure 3).

329

330 The peptidoglycan staining was consistent between the strains in log. phase (Figure 3A),  
331 but in starvation, the *pstP<sub>Msmeg</sub>* T171E mutant stained much more brightly than the other  
332 strains. This suggests that phosphorylation on PstP<sub>*Msmeg*</sub> T171 likely inhibits the  
333 downregulation of PG synthesis in the transition to stasis, but that this phospho-site is  
334 not important in peptidoglycan metabolism during rapid growth. Phosphorylated CwIM is  
335 a major activator of peptidoglycan synthesis in log. phase growth and is  
336 dephosphorylated upon starvation (Boutte et al. 2016). One possible mechanism to  
337 explain these data is that PstP is dephosphorylated at T171 upon starvation, and this  
338 activates PstP to dephosphorylate CwIM~P, thereby downregulating peptidoglycan  
339 precursor synthesis.

340

341 Staining with DMN-Tre, which correlates with assembly of the mycolic acid cell wall  
342 layer (Kamariza et al. 2018), shows the inverse pattern. The strains stain similarly in  
343 starvation (Figure 3E). In log. phase; however, both mutants show a significant decrease  
344 in DMN-Tre signal compared to the wild-type (Figure 3B), though the *pstP<sub>Msmeg</sub>* T171E  
345 mutant has weaker staining than *pstP<sub>Msmeg</sub>* T171A. These data imply that mycolic acid  
346 synthesis is regulated by phosphorylation of PstP T171 in log. phase, but not in  
347 starvation. Since all the FAS-II enzymes, which make mycolic acids, are inhibited by  
348 threonine phosphorylation (Slama et al. 2011; Veyron-Churlet and Zanella-Cléon 2010;  
349 Khan et al. 2010; Molle and Kremer 2010; Molle et al. 2006), one explanation of these  
350 data is that PstP is partially phosphorylated at T171 in log. phase, and the balance of  
351 PstP in different phospho-states helps maintain a balanced population of active and  
352 inactive FAS-II enzymes to properly modulate the flow of lipid intermediates through the  
353 FAS-II pathway. When PstP is misregulated in either direction by the phospho-  
354 mutations, lipid synthesis is likely not coordinated properly through the FAS-II pathway.  
355 Thus, we hypothesize that the impaired trehalose staining is an indication more of  
356 misregulation rather than downregulation of the FAS-II enzymes.

357

358 These data directly show that the misregulation of phosphorylation on T171 of PstP<sub>Msmeg</sub>  
359 affects cell wall metabolism in *M. smegmatis*. Furthermore, they suggest that this  
360 phospho-site has a role in determining substrate specificity in order to regulate multiple  
361 cell wall metabolism factors in the transition between growth and stasis.

362

### 363 **Phosphosite T171 of PstP<sub>Msmeg</sub> affects antibiotic tolerance**

364 Drug tolerance is a feature of dormant, non-replicating mycobacterial cells in stress  
365 conditions like oxygen depletion and starvation in PBS (Deb et al. 2009; Betts et al.  
366 2002; Zhang 2003; Wayne and Hayes 1996; Sarathy et al. 2013). We hypothesized that  
367 if *Msmeg* fails to downregulate peptidoglycan synthesis in starvation, (Figure 3C), then it  
368 should be more susceptible to a peptidoglycan targeting drug. We meropenem-treated  
369 PstP<sub>Msmeg</sub> wild-type, T171A and T171E strains in log phase and after 5.5 hours of  
370 starvation, and quantified survival using a CFU assay. We saw that the *pstP<sub>Msmeg</sub>* T171E  
371 strain was more tolerant to meropenem in log. phase, but more susceptible in starvation,  
372 compared to *pstP<sub>Msmeg</sub>* T171A and wild-type strains (Figure 4B, left panel). The slower  
373 growth of the *pstP<sub>Msmeg</sub>* T171E strain in log. phase may account for the greater tolerance

374 in that condition. The apparent failure of the *pstP<sub>Msmeg</sub>* T171E strain to downregulate PG  
375 synthesis (Fig. 3) likely makes it more sensitive to peptidoglycan inhibitors in starvation.  
376

377 Next, we treated our wild-type and *pstP<sub>Msmeg</sub>* T171 mutant strains with isoniazid, which  
378 targets InhA in the FAS-II pathway of mycolic acid synthesis (Marrakchi, Lan  elle, and  
379 Qu  mard 2000). In log phase, we see that the *pstP<sub>Msmeg</sub>* T171E strain is more  
380 susceptible to isoniazid than the *pstP<sub>Msmeg</sub>* T171A and the wild-type strains (Figure 4A,  
381 right panel). Phosphorylation inhibits the activity of InhA (Molle and Kremer 2010; Khan  
382 et al. 2010). Our model is that, PstP might be the phosphatase of InhA and the T171E  
383 phospho-form may not be able to activate InhA~P by dephosphorylation in log phase.  
384 Thus the pool of active InhA is decreased in this strain and the cells are sensitized to  
385 further InhA inhibition by Isoniazid. Another possibility is that PstP<sub>Msmeg</sub> T171E likely  
386 cannot properly regulate its activity against the FAS-II enzymes, and the misregulation of  
387 the pathway increases sensitivity to pathway inhibitors. We don't see significant  
388 differences in isoniazid sensitivity between the strains in starvation (Fig. 4B), which  
389 corroborates the observation that there is no difference in DMN-Tre staining either in  
390 starvation (Fig. 3). Thus, it seems that phosphorylation on T171 affects PstP's activity  
391 against mycolic acid enzymes in log phase, but not starvation.

392

393 These results suggest that the phosphosite T171 of PstP<sub>Msmeg</sub> is important in controlling  
394 antibiotic susceptibility in *Msmeg*. A myriad of regulatory proteins and enzymes involved  
395 in this complex network of cell wall biosynthesis regulation are threonine phosphorylated  
396 and thus likely substrates of PstP. It is unknown exactly which of these substrates are  
397 being misregulated by the phospho-mutants of *pstP*.

398

399

## 400 Discussion

401

402 In dormant, non-replicating *Mtb* cells, cell wall synthesis is downregulated (Galagan et  
403 al. 2013) and remodeled (Dulberger, Rubin, and Boutte 2019). This regulation of the wall  
404 protects *Mtb* from both the immune system and antibiotics during infection. Our results  
405 suggest that PstP may be important for this regulation.

406

407 PstP is essential in *Mtb* and *Msmeg* (DeJesus et al. 2017; Sharma et al. 2016) and has  
408 been shown to regulate cell morphology, division and global S/T phosphorylation in the  
409 cell (Chopra et al. 2003; Sajid et al. 2011; Sharma et al. 2016; Iswahyudi et al. 2019).  
410 PstP dephosphorylates the essential S/T kinases PknA and PknB as well as assorted  
411 cell wall regulatory proteins and enzymes (Sajid et al. 2011; Molle et al. 2006; Molle and  
412 Kremer 2010; Irmiler and Forchhammer 2001). PstP and Pkn A and B seem to have a  
413 mutual feedback regulatory loop (Iswahyudi et al. 2019). Previous work has shown that  
414 dephosphorylation of PknA and PknB downregulates their activity (Sajid et al. 2011;  
415 Boitel et al. 2003) which would be expected to broadly downregulate cell growth (Betts et  
416 al. 2002; Ortega et al. 2014; Dulberger, Rubin, and Boutte 2019) The phosphorylation of  
417 PstP has been shown to stimulate its activity against small molecule substrates (Sajid et  
418 al. 2011). However, dephosphorylation by PstP is known to both upregulate cell wall  
419 synthesis through KasA (Molle et al. 2006) and to downregulate cell wall synthesis  
420 through CwIM (Boutte et al. 2016) (Fig. 5). Because synthesis of the various cell wall  
421 layers must be largely correlated to maintain cell wall integrity, it stands to reason that  
422 PstP's regulation must include switches of substrate specificity between growth and  
423 stasis.

424

425 Our data suggest that phosphorylation on T171 of PstP<sub>*Msmeg*</sub> may be involved in  
426 switching substrate specificity between growth and stasis. One model to explain our  
427 results is that in log. phase PstP dephosphorylates FAS-II enzymes and has little effect  
428 on peptidoglycan factors, while in stasis it dephosphorylates the peptidoglycan factor  
429 CwIM and is no longer active against FAS-II enzymes. The phosphate on the T171 site  
430 could alter substrate specificity by changing the charge on a surface of the phosphatase  
431 domain that binds substrates, or it might change the geometry of the active site region in  
432 order to discriminate against certain substrates (see Fig. 1) (Pullen et al. 2004).

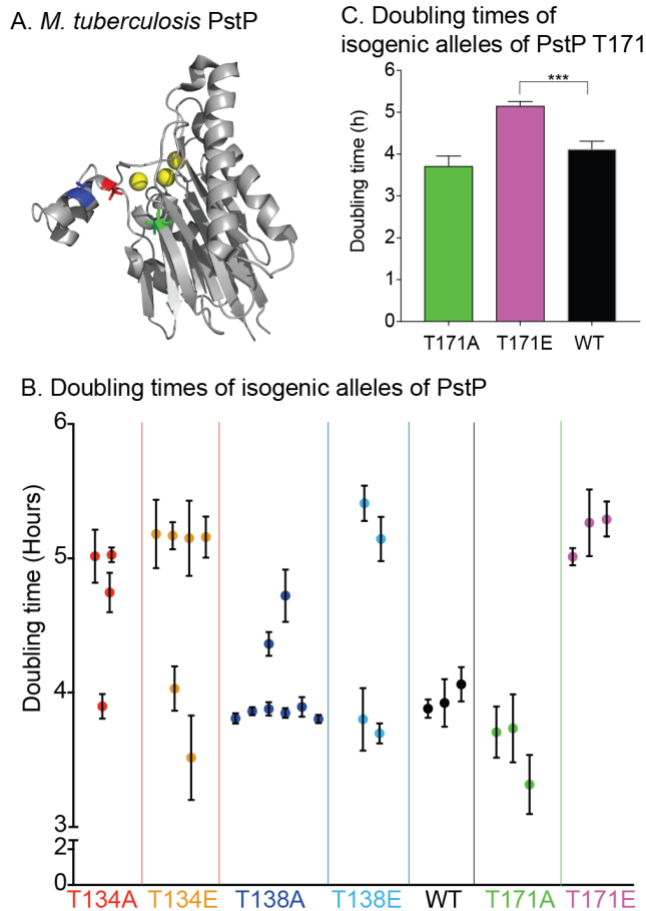
433

434 The antibiotic sensitivity experiments that we performed in *Msmeg* suggest that  
435 misregulation of PstP could sensitize mycobacteria to various antibiotics in both growth  
436 and stasis. We find it very appealing to consider PstP to be an Achilles' heel of *Mtb*. It is  
437 an essential enzyme, so inhibiting it should kill *Mtb* directly. But, it is also a master  
438 regulator of antibiotic tolerance, so inhibiting should misregulate the cell wall and  
439 increase permeability to other antibiotics.

440

441 **Figures**

442



443

444 **Figure 1: Phosphosite T171 on PstP affects growth.**

445

446 A) Crystal structure of PstP from *M. tuberculosis* (PstP<sub>Mtb</sub>) (Pullen et al. 2004). The  
447 threonine (T) sites on PstP<sub>Mtb</sub> phosphorylated by the kinases PknA and PknB (Sajid et al.  
448 2011) are highlighted on the structure: red- PstP<sub>Mtb</sub> T137 (the corresponding threonine in  
449 PstP<sub>Msmeg</sub> is T134), blue- PstP<sub>Mtb</sub> T141 (the corresponding threonine in PstP<sub>Msmeg</sub> is T138)  
450 and green- PstP<sub>Mtb</sub> T174 (the corresponding threonine in PstP<sub>Msmeg</sub> is T171).

451

452 B) Doubling times of biological replicates of WT (L5::pCT94-p766tetON6-*pstP*<sub>Msmeg</sub> WT),  
453 phosphoablative mutant strains (L5::pCT94-p766tetON6-*pstP*<sub>Msmeg</sub> T134A, T138A and  
454 T171A) and phosphomimetic mutant strains (L5::pCT94-p766tetON6-*pstP*<sub>Msmeg</sub> T134E,  
455 T138E and T171E). Each dot is the mean of doubling times from two to three different  
456 experiments on different dates. The error bars represent the standard deviation.

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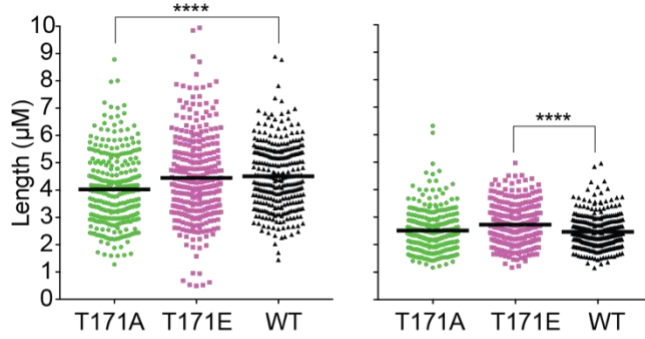
458 C) Mean doubling times of biological replicates of PstP<sub>Msmeg</sub> WT, T171A and T171E strains  
459 (GraphPad Prism 7.0d). The error bars represent the standard deviation. The p-value was  
460 0.0009 by the Student's t-test.

461

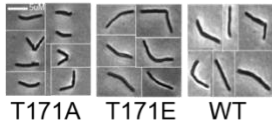
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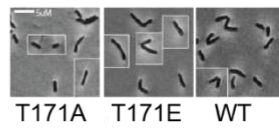
A. Length of cells in log phase C. Length of cells in starvation



B. Micrographs of cells in log phase



D. Micrographs of cells in starvation



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465

**Figure 2: Phosphosite T171 on PstP<sub>Msmeg</sub> is important in regulating the cell length.**

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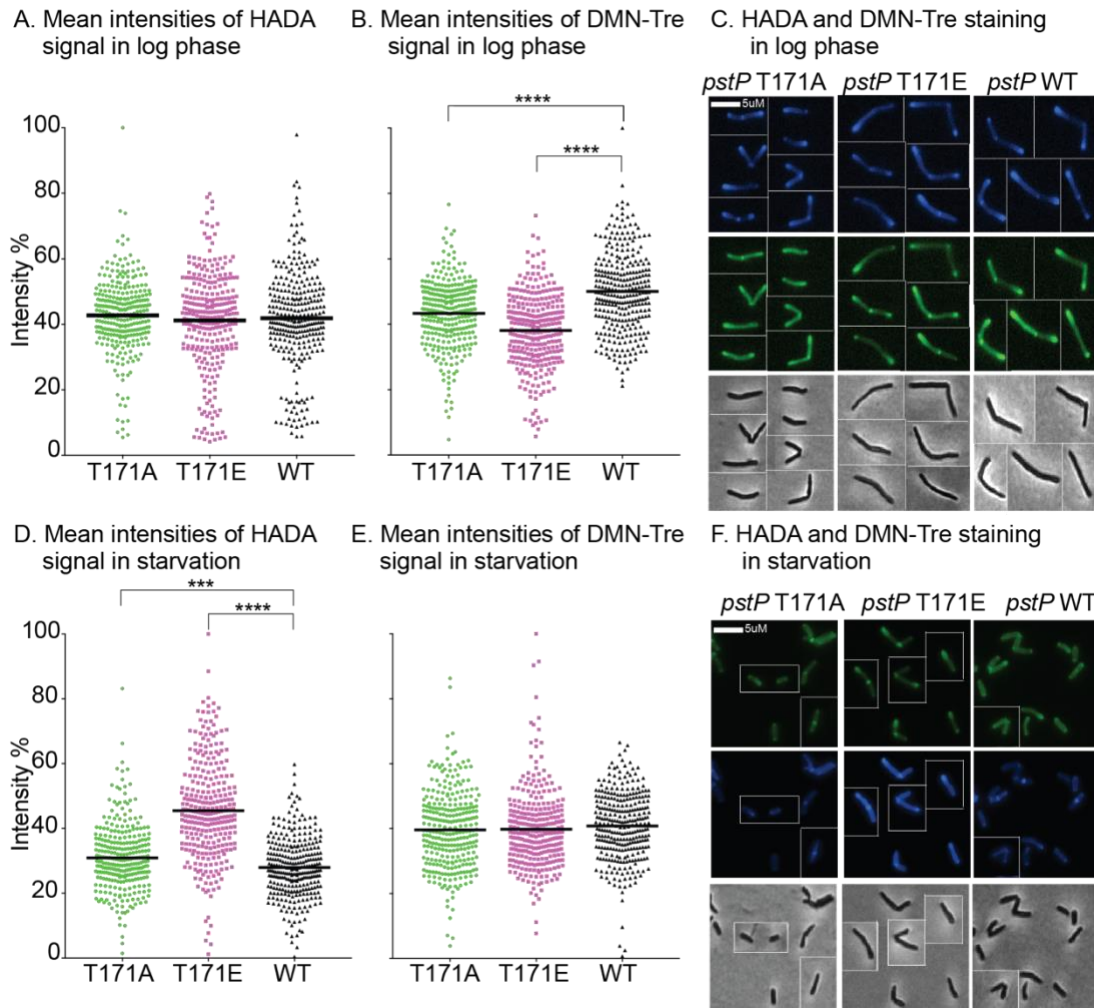
A) Quantification of cell lengths of isogenic *pstP* allele strains (WT, T17A and T171E) grown in 7H9 in log phase. 100 cells from each of three biological replicates were measured. P values were calculated by unpaired t-test. P value <0.0001.

B) Representative phase images of cells from (A).

C) Quantification of cell lengths of isogenic *pstP* allele strains (WT, T17A and T171E) after starvation in HdB (no glycerol, 0.05% Tween) for five and a half hours. 100 cells from each of three biological replicates were measured. P values were calculated by unpaired t-test. P value <0.0001.

D) Representative phase images of cells from (C).





488

489

**Figure 3: Phosphosite T171 of PstP contributes to regulating cell wall metabolism.**

490

491 A) and B) Quantification of mean intensities of HADA and DMN-Tre signals of isogenic  
 492 *pstP* allele strains (WT, T17A and T171E) in log-phase cells. Signals from 100 cells from  
 493 each of three biological replicates were measured using MicrobeJ. P values were  
 494 calculated by unpaired t-test. P value <0.0001.

495

496 C) Representative micrographs of log-phase cells from (A) and (B) stained with the  
 497 fluorescent dye HADA and DMN-Tre respectively. Corresponding phase images are  
 498 shown on the bottom panel. The scale bar applies to all images.

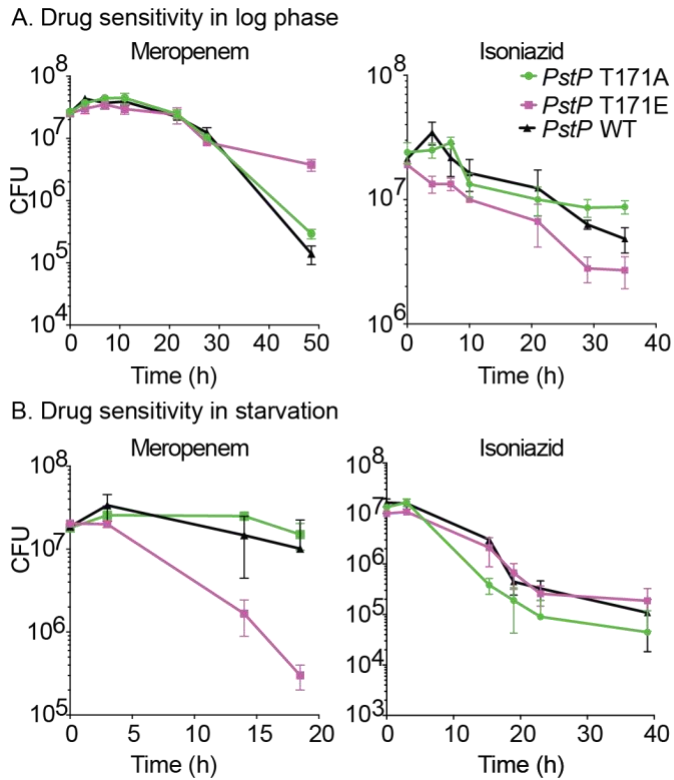
499

500 D) and E) Quantification of mean intensities of HADA and DMN-Tre signals of starved  
 501 isogenic *pstP* allele strains (WT, T17A and T171E) in HdB (no glycerol, 0.05% Tween).  
 502 Signals from 100 cells from each of three biological replicates were measured using  
 503 MicrobeJ. P values were calculated by unpaired t-test. P value <0.0001.

504

505 F) Representative micrographs of starved cell from (D) stained with the fluorescent dye  
 506 HADA and (E) stained with the fluorescent dye DMN-Tre. Corresponding phase images  
 507 are shown on the bottom panel. The scale bar applies to all images.

508



509

510 **Figure 4: Phosphosite T171 of *PstP* plays a role in antibiotic sensitivity.**

511

512 A) Survival curve of isogenic *pstP* allele strains (WT, T17A and T171E) grown in 7H9  
513 treated with 8ug/ml of Meropenem and 10ug/ml of Isoniazid respectively.

514

515 B) Survival curve of isogenic *pstP* allele strains starved in HdB (no Glycerol, 0.05% Tween)  
516 for five and a half hours and then treated with 45ug/ml of Meropenem and 90ug/ml of  
517 Isoniazid respectively.

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