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

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Abstract

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

Introduction

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

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

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

While much is known about the cell wall structure of *Mtb*, little is known about the regulation of the cell wall during stress or how they contribute to antibiotic tolerance. The signaling pathways regulating the *Mtb* cell wall likely help it survive in the host. Reversible protein phosphorylation is a key regulatory mechanism used by bacteria for environmental signal transduction to regulate cell growth (Echenique et al. 2004; Juris et al. 2000; Galyov et al. 1993; J. Wang et al. 1998). In *Mtb*, Serine/Threonine (S/T) phosphorylation is important in in cell growth regulation (Kang 2005; Gee et al. 2012; Boutte et al. 2016; Baer et al. 2014). *Mtb* has 11 Serine/Threonine Protein Kinases (STPKs) (PknA, PknB and PknD-L) and only one S/T protein phosphatase (PstP) (Cole et al. 1998; Bach, Wong, and Av-Gay 2009). Among the STPKs, PknA and PknB are essential for *Mtb* growth and phosphorylate substrates involved in cell growth and division (Sassetti, Boyd, and Rubin 2003; Kang 2005; Fernandez et al. 2006; Kusebauch et al. 2014; Boutte et al. 2016) Some of these substrates are enzymes whose activity is directly altered by phosphorylation. The enoyl-ACP reductase activity of InhA, a key enzyme involved in mycolic acid biosynthesis in *Mtb,* is inhibited when phosphorylated by multiple STPKs (Molle and Kremer 2010; Khan et al. 2010). All the enzymes in the FAS-II system of mycolic acid biosynthesis are regulated by threonine phosphorylation

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

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

PstP, the only S/T phosphatase must have to dephosphorylate substrates phosphorylated by most or all of the 11 STPKs in *Mtb* (Cole et al. 1998). PstP is essential in *Mtb* and *Msmeg* (DeJesus et al. 2017; Sharma et al. 2016). It is a member of the Protein phosphatase 2C (PP2C) subfamily of PPM (metal-dependent protein phosphatase) ser/thr phosphatases (Chopra et al. 2003) which strictly require divalent metal ions for activity (Shi 2009). In addition to the two characteristic metal binding sites of PP2C phosphatases, the $PstP_{Mtb}$ catalytic domain has a third Mn^{2+} binding site close to the flap subdomain adjacent to the active site (Pullen et al. 2004) (Figure 1A). $PstP_{Mtb}$ shares structural folds and conserved residues with the human PP2Cα, which serves as the representative of the PP2C family (Chopra et al. 2003). $PstP_{Mtb}$ has an N-terminal cytoplasmic enzymatic domain of 237 residues which is joined by a 63 amino acids long segment to a transmembrane pass of 18 residues connecting the C-terminal extracellular domain of 191 residues (Chopra et al. 2003).

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

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

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

We report here that phospho-ablative and phospho-mimetic mutations at the phsopho-site T171 of PstP$_{Msmeg}$ alter growth rate, cell length, cell wall metabolism and antibiotic

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

Materials and Methods

Bacterial strains and culture conditions

All *Mycobacterium smegmatis* mc_2155 ATCC 700084 cultures were started in 7H9 (Becton, Dickinson, Franklin Lakes, NJ) medium containing 5 g/liter bovine serum albumin (BSA), 2 g/liter dextrose, 0.003 g/liter catalase, 0.85 g/liter NaCl, 0.2% glycerol, and 0.05% Tween 80 and incubated at 37°C up to log phase. Hartmans-de Bont (HdB) minimal medium made as described previously (Hartmans and De Bont 1992) without glycerol was used for starvation assays. Serial dilutions of all CFU counts were plated on on LB Lennox agar (Fisher BP1427-2).

E. coli Top10, XL1Blue and Dh5α were used for cloning and *E. coli* BL21 Codon Plus strains were used for protein expression. Antibiotic concentrations for *M. smegmatis* were 25 µg/ml kanamycin. 50 µg/ml hygromycin and 20 µg/ml zeocin. Antibiotic concentrations for *E. coli* were 50 µg/ml kanamycin, 25 µg/ml zeocin, 20 µg/ml chloramphenicol and 140 µg/ml ampicillin.

Strain construction

Since *pstP* is an essential gene in mycobacteria, a PstP-knockdown strain was created in multiple steps; first by creating a merodiploid strain using the *M. tuberculosis pstP* gene, and then by deleting the native *M. smegmatis pstP* gene from its native chromosomal location. The merodiploid strain was generated by introducing at the L5 attB integration site, a constitutively expressing *M. tuberculosis pstP* gene cloned on a StrR plasmid. The *M. smegmatis pstP* gene (MSMEG_0033) at the native locus was then deleted by RecET-mediated double stranded recombineering approach using a 1.53 kb loxP-hyg-loxP fragment carrying a 125 bp DNA flanking the *M. smegmatis pstP* gene (Murphy, Papavinasasundaram, and Sassetti 2015). The recombineering substrate was generated by two sequential overlapping PCR of the loxP-hyg-loxP substrate present in the plasmid pKM342. The downstream flanking primer used in the first PCR also carried an optimized mycobacterial ribosome binding site in front of the start codon of MSMEG_0032 to facilitate the expression of the genes present downstream of *pstP in*

the *M. smegmatis pstP-pknB* operon.

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

Different alleles of *pstP* were attained by swapping the zeocin resistance-marked vector at L5 site with wild-type, phosphoablative (T134A, T138A, T171A) or phosphomimetic (T134E, T138E, T171E) mutant *pstP* alleles under a p766tetON6 promoter for a kanamycin resistance-marked vector, as described (Pashley and Parish 2003). The final genotypes of the strains are thus mc_2155 Δ*pstP*::lox L5::pCT94-p766tetON6-$pstP_{Msmeg}$ wild-type, T134A, T138A, T171A, T134E, T138E or T171E.

Growth Curve assay

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

Cell staining

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

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

Microscopy and Image Analysis

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

Western Blots

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

Antibiotic assays

For antibiotic assays with log phase culture, cells were grown up to the log phase and new 7H9 media with Tween was inoculated at OD600= 0.05. For starvation assays, cells were grown upto the log phase, spun down at 5000 rpm for 10 minutes, washed in HdB starvation (with no glycerol and 0.05% Tween) media. After spinning down at 5000 rpm for 10 minutes at 4°C, pellets were resuspended in the same media and OD at 600nm was taken. HdB starvation (with no glycerol and 0.05% Tween) media was inoculated at

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

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

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

His-SUMO-$CwlM_{Mtb}$ was expressed in *E. coli* BL21 Codon Plus cells at 25°C for 6 hours with 1.3 mM IPTG. His-$PstPc_{Mtb}$ was expressed in *E. coli* BL21 Codon Plus at 25°C for 6 hours with 1mM IPTG.

Results

Phosphosite T171 on $PstP_{Msmeg}$ has an impact on growth

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

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

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

Phosphosite T171 of $PstP_{Msmeg}$ regulates cell length

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

PstP could promote the transition to growth stasis by downregulating the activity of PknA, PknB and CwlM (Iswahyudi et al. 2019; Boutte et al. 2016; Sajid et al. 2011; Boitel et al. 2003; Chopra et al. 2003). To test if the phosphosite T171 of $PstP_{Msmeg}$ affects cell

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

Phosphosite T171 on PstP$_{Msmeg}$ is important in regulating cell wall metabolism

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

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

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

These data directly show that the misregulation of phosphorylation on T171 of PstP$_{Msmeg}$ affects cell wall metabolism in *M. smegmatis*. Furthermore, they suggest that this phospho-site has a role in determining substrate specificity in order to regulate multiple cell wall metabolism factors in the transition between growth and stasis.

Phosphosite T171 of PstP$_{Msmeg}$ affects antibiotic tolerance

Drug tolerance is a feature of dormant, non-replicating mycobacterial cells in stress conditions like oxygen depletion and starvation in PBS (Deb et al. 2009; Betts et al. 2002; Zhang 2003; Wayne and Hayes 1996; Sarathy et al. 2013). We hypothesized that if *Msmeg* fails to downregulate peptidoglycan synthesis in starvation, (Figure 3C), then it should be more susceptible to a peptidoglycan targeting drug. We meropenem-treated PstP$_{Msmeg}$ wild-type, T171A and T171E strains in log phase and after 5.5 hours of starvation, and quantified survival using a CFU assay. We saw that the *pstP*$_{Msmeg}$ 171E strain was more tolerant to meropenem in log. phase, but more susceptible in starvation, compared to *pstP*$_{Msmeg}$ T171A and wild-type strains (Figure 4B, left panel). The slower growth of the *pstP*$_{Msmeg}$ T171E strain in log. phase may account for the greater tolerance

in that condition. The apparent failure of the *pstP*$_{Msmeg}$ T171E strain to downregulate PG synthesis (Fig. 3) likely makes it more sensitive to peptidoglycan inhibitors in starvation.

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

These results suggest that the phosphosite T171 of PstP$_{Msmeg}$ is important in controlling antibiotic susceptibility in *Msmeg.* A myriad of regulatory proteins and enzymes involved in this complex network of cell wall biosynthesis regulation are threonine phosphorylated and thus likely substrates of PstP. It is unknown exactly which of these substrates are being misregulated by the phospho-mutants of *pstP*.

Discussion

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

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

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

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

Figures

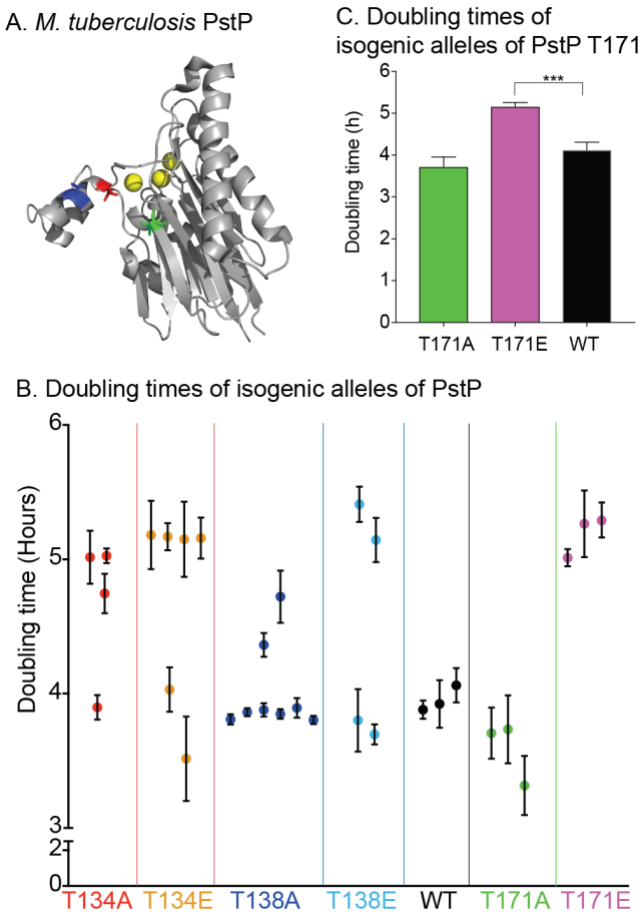

Figure 1: Phosphosite T171 on PstP affects growth.

A) Crystal structure of PstP from *M. tuberculosis (*PstP$_{Mtb}$) (Pullen et al. 2004). The threonine (T) sites on PstP$_{Mtb}$ phosphorylated by the kinases PknA and PknB (Sajid et al. 2011) are highlighted on the structure: red- PstP$_{Mtb}$ T137 (the corresponding threonine in PstP$_{Msmeg}$ is T134), blue- PstP$_{Mtb}$ T141 (the corresponding threonine in PstP$_{Msmeg}$ is T138) and green- PstP$_{Mtb}$ T174 (the corresponding threonine in PstP$_{Msmeg}$ is T171).

B) Doubling times of biological replicates of WT (L5::pCT94-p766tetON6-*pstP*$_{Msmeg}$ WT), phosphoablative mutant strains (L5::pCT94-p766tetON6- *pstP*$_{Msmeg}$ T134A, T138A and T171A) and phosphomimetic mutant strains (L5::pCT94-p766tetON6-*pstP*$_{Msmeg}$ T134E, T138E and T171E). Each dot is the mean of doubling times from two to three different experiments on different dates. The error bars represent the standard deviation.

C) Mean doubling times of biological replicates of PstP$_{Msmeg}$ WT, T171A and T171E strains (GraphPad Prism 7.0d). The error bars represent the standard deviation. The p-value was 0.0009 by the Student's t-test.

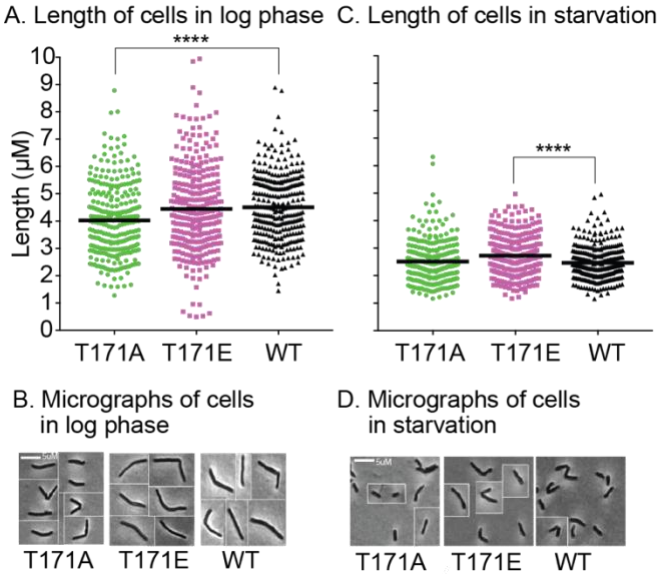

Figure 2: Phosphosite T171 on PstP$_{Msmeg}$ is important in regulating the cell length.

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).

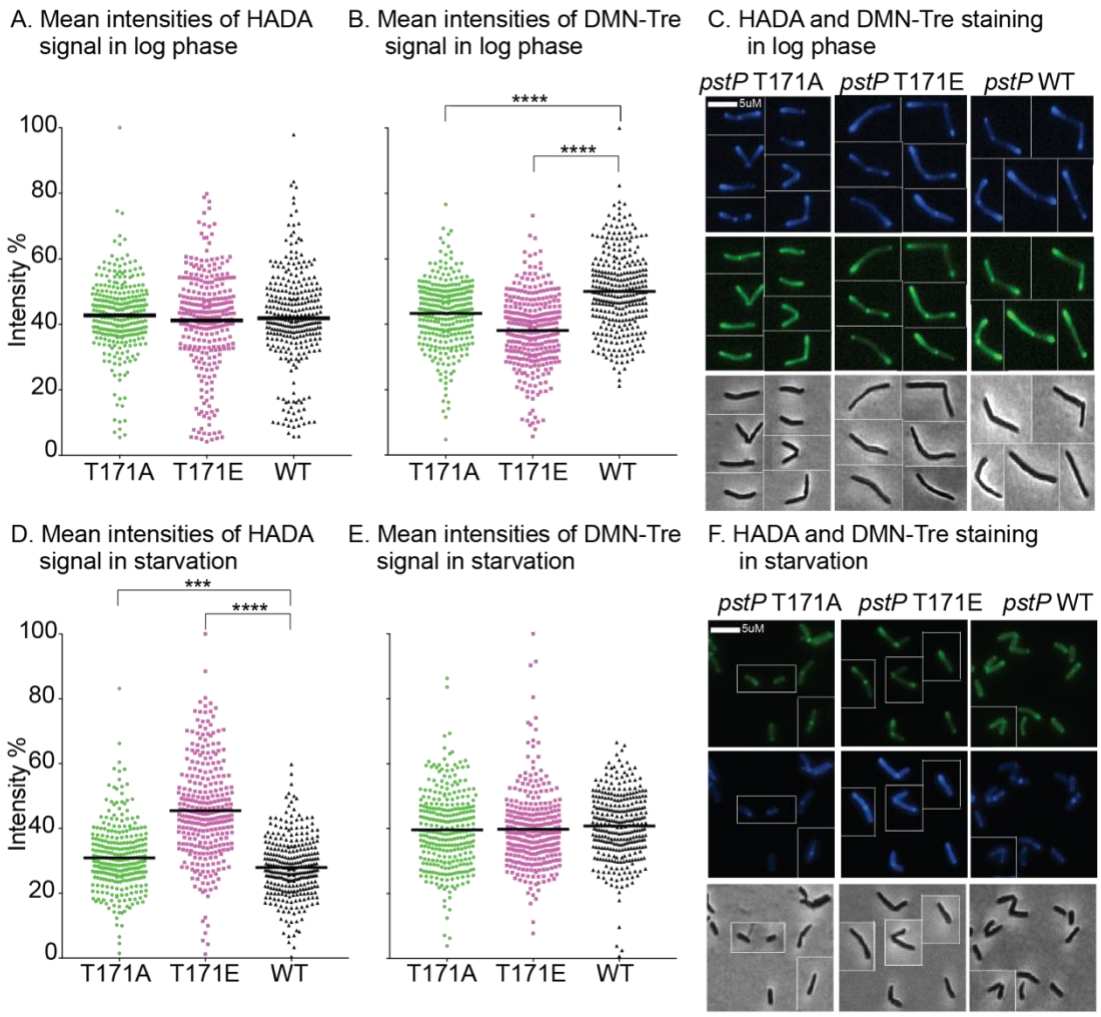

Figure 3: Phosphosite T171 of PstP contributes to regulating cell wall metabolism.

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

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

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

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

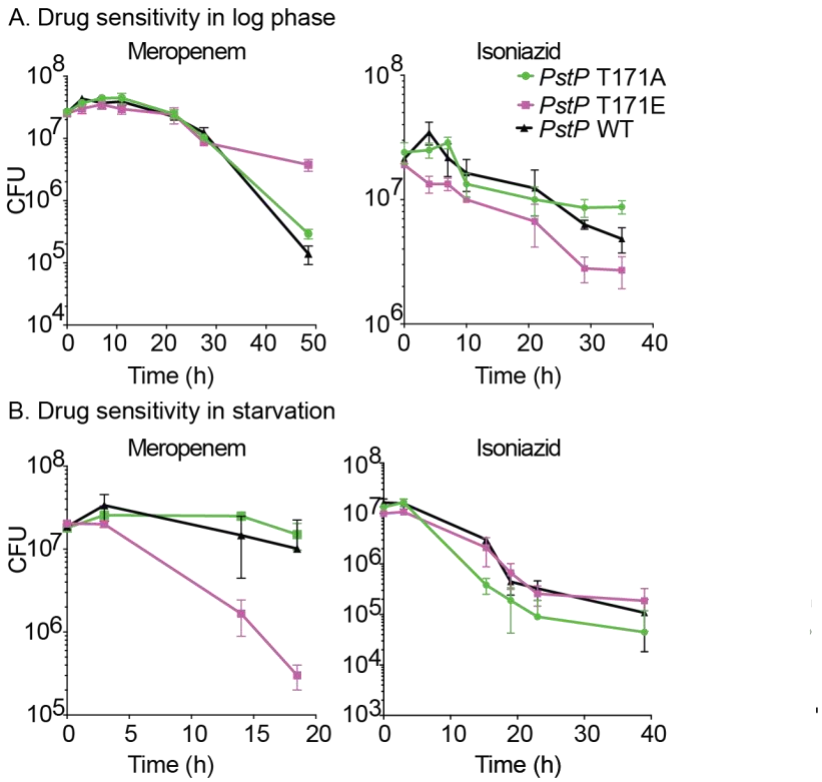

Figure 4: Phosphosite T171 of PstP plays a role in antibiotic sensitivity.

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

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

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