1 **Mycobacterial Serine/Threonine phosphatase PstP is phospho-**
2 **regulated and localized to mediate control of cell wall**
3 **metabolism**

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Abstract

The mycobacterial cell wall is profoundly regulated in response to environmental stresses, and this regulation contributes to antibiotic tolerance. The reversible phosphorylation of different cell wall regulatory proteins is a major mechanism of cell wall regulation. Eleven Serine/Threonine protein kinases (STPKs) phosphorylate many critical cell wall-related proteins in mycobacteria. PstP is the sole serine/ threonine phosphatase, but few proteins have been verified as PstP substrates. PstP is itself phosphorylated but the role of its phosphorylation in regulating its activity has been unclear. In this study we aim to discover novel substrates of PstP in *Mycobacterium tuberculosis* (*Mtb*). We show *in vitro* that PstP dephosphorylates two peptidoglycan regulators of *Mtb*, FhaA and Wag31. We also show that a phospho-mimetic mutation of T137 on PstP negatively regulates its catalytic activity against the cell wall regulatory substrates FhaA, Wag31, CwlM, PknB and PknA, and that the corresponding mutation in *Mycobacterium smegmatis* (*Msmeg*) causes mis-regulation of peptidoglycan *in vivo*. We show that PstP is localized to the septum, which likely restricts its access to certain substrates. These findings on the regulation of PstP provide insight into the control of cell wall metabolism in mycobacteria.

Introduction

Mycobacterium tuberculosis (*Mtb*), which causes Tuberculosis (TB), remains the age-old menace, infecting about 10 million and killing at least a million people worldwide each year (World Health Organization, 2021). TB infections require extended multi-drug antibiotic treatment regimens (Nguyen, 2016). The mycobacterial cell wall contributes to *Mtb*'s inherent antibiotic tolerance (Jarlier and Nikaido, 1994; Sarathy *et al.*, 2013; Batt *et al.*, 2020). The cell wall consists of a covalently linked peptidoglycan-arabinogalactan-mycolic acid layer core surrounding the plasma membrane (Marrakchi *et al.*, 2014). Peptidoglycan is a network of glycan chains cross-linked by small peptides, and is the innermost layer of the wall (Abrahams and Besra, 2021). The wall serves as permeability

barrier (Jarlier and Nikaido, 1990; Hett and Rubin, 2008; Hoffmann *et al.*, 2008; Sarathy *et al.*, 2013), and is strongly regulated in response to environmental stresses (Cunningham and Spreadbury, 1998; Betts *et al.*, 2002; Bhamidi *et al.*, 2012; Sarathy *et al.*, 2013), including infection (Sharma *et al.*, 2006; Doerks *et al.*, 2012). The cell wall changes in response to environmental stresses, and these changes correlate with increased antibiotic tolerance (Xie *et al.*, 2005; Liu *et al.*, 2016; Sarathy *et al.*, 2017). The expression and activity of many cell wall enzymes and regulators are also tightly coordinated over the course of the cell cycle in order to maintain cell wall integrity during elongation and division (Kieser and Rubin, 2014; Dulberger *et al.*, 2020; Bandekar *et al.*, 2020).

A crucial regulatory mechanism for transmission of environmental signals to control cell growth is reversible Serine/Threonine (S/T) phosphorylation of a myriad of proteins (Wang *et al.*, 1998; Juris *et al.*, 2000; Echenique *et al.*, 2004). *Mtb* regulates cell physiology and growth (Kang *et al.*, 2005; Gee *et al.*, 2012; Baer *et al.*, 2014; Boutte *et al.*, 2016) through 11 Serine/Threonine kinases (STPKs) (PknA-B and PknD-L) and one essential S/T protein phosphatase PstP (Cole *et al.*, 1998; Bach *et al.*, 2009). PstP is also essential in *Msmeg* (Sharma *et al.*, 2016; DeJesus *et al.*, 2017).

PstP belongs to the protein phosphatase 2C (PP2C) subfamily of the strictly divalent metal-ion dependent protein serine/threonine phosphatases (Cohen, 1989; Barford, 1996; Chopra *et al.*, 2003), known to regulate prokaryotic and eukaryotic cell growth and division, sporulation, stress response and metabolic processes by responding to various environmental signals (Vijay *et al.*, 2000:2; Irmeler and Forchhammer, 2001; Mougous *et al.*, 2007; Lu and Wang, 2008; Bradshaw and Losick, 2015; Bradshaw *et al.*, 2017). PstP_{*Mtb*} shares structural architecture and conserved catalytic residues with the human PP2Cα (Pullen *et al.*, 2004) (Figure 1), which is considered the prototypical member of the PP2C family. The N-terminal cytoplasmic enzymatic domain of PstP_{*Mtb*} (Figure 1) connects to its C-terminal extracellular domain via a transmembrane helix (Pullen *et al.*, 2004). PstP_{*Mtb*} is itself phosphorylated on T134, T141, T174 and T290

(Figure 1) and its phosphorylation affects its activity against small molecule substrates *in vitro* (Sajid *et al.*, 2011) and cell wall metabolism *in vivo* (Shamma *et al.*, 2021).

Two essential STPKs, PknA and PknB, play important roles in cell wall regulation by phosphorylating enzymes or regulatory proteins involved in cell division and growth (Sasseti and Rubin, 2003; Kang *et al.*, 2005; Fernandez *et al.*, 2006; Kusebauch *et al.*, 2014; Boutte *et al.*, 2016). Phosphorylation of different cell wall substrates has different effects on their activities. For example, phosphorylation activates synthesis of peptidoglycan precursors through regulatory protein CwIM (Boutte *et al.*, 2016), and inhibits synthesis of mycolic acids by inactivating several mycolic acid biosynthesis enzymes in *Mtb* (Molle *et al.*, 2006; Molle and Kremer, 2010; Veyron-Churlet *et al.*, 2010; Khan *et al.*, 2010; Slama *et al.*, 2011; Vilchèze *et al.*, 2014). PstP is suggested to be a global negative regulator of STPK-mediated phosphorylation (Iswahyudi *et al.*, 2019). Being the only S/T protein phosphatase (Cole *et al.*, 1998), it seems likely to dephosphorylate many of the substrates phosphorylated by the 11 STPKs (Prisic and Husson, 2014). However, only a few proteins have been identified to be substrates of PstP. The biochemically-verified cell wall substrates of PstP include the STPKs PknA and PknB (Boitel *et al.*, 2003; Chopra *et al.*, 2003; Durán *et al.*, 2005; Sajid *et al.*, 2011), KasA and KasB, enzymes of the fatty acid synthase –II (FAS-II) system of mycolic acid biosynthesis (Molle *et al.*, 2006), the arabinogalactan layer regulator EmbR (Sharma *et al.*, 2006), and CwIM, which regulates the peptidoglycan layer (Shamma *et al.*, 2021).

CwIM is crucial for peptidoglycan synthesis; it interacts with MurA (Boutte *et al.*, 2016), the first enzyme of peptidoglycan precursor synthesis (Marquardt *et al.*, 1992), and CwIM's phosphorylation is required for MurA's activity (Boutte *et al.*, 2016). So dephosphorylation of CwIM~P by PstP (Shamma *et al.*, 2021) should halt peptidoglycan synthesis. Phosphorylated CwIM~P also interacts with FhaA (Turapov *et al.*, 2018), which is involved in modulating peptidoglycan biosynthesis (Gee *et al.*, 2012; Viswanathan *et al.*, 2015; Viswanathan *et al.*,

2017) and unphosphorylated CwIM interacts with the peptidoglycan precursor lipid-II flippase MurJ (Turapov *et al.*, 2018), suggesting that CwIM's activity and interactions with other peptidoglycan regulators and enzymes are phospho-regulated and that both CwIM and FhaA are part of a phosphorylation-controlled peptidoglycan regulatory hub (Turapov *et al.*, 2018).

FhaA and Wag31 are both regulators of mycobacterial cell wall metabolism. FhaA helps maintain cell envelope integrity, cell length and antibiotic tolerance (Viswanathan *et al.*, 2015; Viswanathan *et al.*, 2017). Available data suggest that FhaA binds and inhibits the MurJ flippase (Gee *et al.*, 2012) and stabilizes PbpA (Viswanathan *et al.*, 2017). FhaA is phosphorylated by PknB (Grundner *et al.*, 2005; Prsic *et al.*, 2010; Roumestand *et al.*, 2011), though the role of its phosphorylation in regulation is not yet clear. Wag31 localizes at the cell poles (Kang *et al.*, 2005), is essential for polar peptidoglycan synthesis (Kang *et al.*, 2008; Jani *et al.*, 2010) and is phosphorylated by the STPK PknA (Kang *et al.*, 2005). Experiments with phospho-mimetic and phospho-ablative mutations of *wag31T73* suggest that its phosphorylation has a role in controlling peptidoglycan metabolism (Jani *et al.*, 2010). It was not known whether FhaA and Wag31 were actively dephosphorylated, or if their phosphorylated forms were removed through protein degradation.

In this study we show that FhaA_{Mtb} and Wag31_{Mtb} are substrates of PstP_{Mtb} *in vitro*, and that PstP dephosphorylates Wag31 much faster than it does FhaA or CwIM. PstP's activity against different substrates could be controlled by its localization, by its own phosphorylation (Sajid *et al.*, 2011) or through interactions with other regulatory proteins. Here, we show that the T137E phospho-mimetic mutation of PstP_{Mtb} inhibits PstP_{Mtb}'s catalytic activity against the substrates CwIM, FhaA, Wag31, PknB and PknA *in vitro*. Furthermore, the corresponding phospho-mimetic mutation in *Msmeg* leads to dis-regulation of PG metabolism. We also find that PstP is enriched at the cell division site, suggesting that its access to substrates at the pole might also control its activity. We suggest a model where PstP's activity against assorted substrates is regulated by substrate

affinity and protein localization, and further toggled by the phosphorylations on PstP.

Methods and Materials

Bacterial strains and culture conditions

All *Mycobacterium smegmatis* mc²155 ATCC 700084 cultures were grown in 7H9 (Becton, Dickinson, Franklin Lakes, NJ) medium containing 0.003 g/liter catalase, 5 g/liter bovine serum albumin (BSA), 2 g/liter dextrose, 0.2% glycerol, 0.85 g/liter NaCl, and 0.05% Tween 80 and incubated at 37°C until log. phase. 1xPBS supplemented with 0.05% Tween 80 was used for starvation assays. Cultures were plated on LB Lennox agar (Fisher BP1427-2).

E. coli Top10, XL1-Blue, and Dh5α strains were used for cloning and *E. coli* BL21 Codon Plus and BL21(DE3) strains were used for expressing proteins. Antibiotic concentrations for *M. smegmatis* were 25μg/ml kanamycin 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.

Cloning and Strain construction

N-terminally Histidine (His) tagged cytosolic domains (1-300 amino acids (Gupta *et al.*, 2009)) of *pstP*_{cT137E_{Mtb} and *pstP*_{cT141E_{Mtb}, and *fhaA*_{WT_{Mtb} codon-optimized for expression in *E. coli* were ordered from IDT, Inc. and cloned in to pET28a.}}}

The *Msmeg* mc²155 strains Δ*pstP*::lox L5::pCT94-p766tetON6-*pstP*_{*Msmeg*}T134E and Δ*pstP*::lox L5::pCT94-p766tetON6-*pstP*_{*Msmeg*}WT were generated by L5 allele swapping as described previously (Pashley and Parish, 2003; Shamma *et al.*, 2021). All vectors and strains are described in supplementary tables 1-3.

A vector that expressed mCherry-2B-PstP_{*Msmeg*} was constructed using isothermal assembly and integrated at the tweety phage integration site in *Msmeg*. In brief,

pstP was amplified from *Msmeg* genomic DNA, and subcloned into a plasmid along with *mCherry2B* and the *uvr15* ribosomal promoter. The resulting vector was transformed into *Msmeg*.

Growth Curve Assay

At least three biological replicates of *pstP*_{*Msmeg*}T134E and WT allelic variants were grown in 7H9 medium up to the mid-log. phase. The growth curve assays were performed in non-treated non-tissue culture plates using a plate reader (BioTek Synergy neo2 multi mode reader) in 200µl of 7H9 medium at 37°C starting at an Optical Density at 600nm (OD₆₀₀) of 0.1.

The doubling times of each strain were calculated in the non-linear (curve fit) method using an exponential growth equation with the least squares (ordinary) fit in GraphPad Prism (version 7.0d). *P* values were calculated using two-tailed unpaired t-tests.

Protein purification

His-MBP-PknA_{*Mtb*} was expressed in *E. coli* BL21(DE3) and all other proteins were expressed using *E. coli* BL21 Codon Plus cells. The *Mtb* genes of all studied factors were cloned into expression vectors. His-MBP-PknB_{*Mtb*}, His-SUMO-CwlM_{*Mtb*}, His-PstPcWT_{*Mtb*} and His-PstPcT174E_{*Mtb*} were expressed and purified as previously described (Kieser *et al.*, 2015; Shamma *et al.*, 2021). His-PstPcT137E_{*Mtb*} and His-PstPcT141E_{*Mtb*} were expressed and purified using the same conditions and buffers used to purify His-PstPcWT_{*Mtb*} and His-PstPcT174E_{*Mtb*} (Shamma *et al.*, 2021).

His-Wag31_{*Mtb*} was induced with 0.5mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6h at 18°C, and purified on Ni-NTA resin (G-Biosciences 786-940) then dialyzed and concentrated. The buffer for His-Wag31_{*Mtb*} was 50mM Tris pH 8, 350mM NaCl, 1 mM dithiothreitol (DTT), and

10% glycerol. Imidazole (10mM and 20mM) was added to the buffers for lysis of the cell pellets and washing the Ni-NTA resin respectively, and 250mM imidazole was added for elution. The lysis buffer was supplemented with 1mM PMSF and 0.2% Triton X100.

His-MBP-PknA_{Mtb} (1-279 amino acids containing the kinase domain (Baer *et al.*, 2014)) was induced with 0.5mM IPTG at 18°C overnight, purified with 5ml Ni-NTA resins (BIO-RAD EconoFit Nuvia IMAC, 12009287), then run over the size exclusion resin (Enrich SEC650, BIO-RAD 780-1650) after dialysis and concentration to get soluble protein. The buffer for purifying His-MBP-PknA_{Mtb} was 50mM Tris pH 7.5, 150mM NaCl, 20% glycerol. PMSF (1mM) was added to the buffer while resuspending the cell pellets. Imidazole was used in buffer for application to (20mM) and for elution from Ni-NTA resin (200mM).

His-FhaA_{Mtb} was induced with 1mM IPTG at 18°C overnight and purified on Ni-NTA resin (G-Biosciences, 786-940 in 5 ml Bio-Scale Mini Cartridges, BIO-RAD 7324661), then dialyzed, concentrated, and run over size exclusion resin (GE Healthcare Sephacryl S-200 in HiPrep 26/70 column). His-FhaA_{Mtb} was purified using the buffers described in (Roumestand *et al.*, 2011).

***In vitro* dephosphorylation assays**

His-SUMO-CwlM_{Mtb} at 0.15 µg/µl was phosphorylated *in vitro* with His-MBP-PknB_{Mtb} at 0.015 µg/µl for 30 minutes at room temperature with 0.15µCi/µl ATP [γ -³²P] (3000Ci/mmol, 10mCi/ml, PerkinElmer BLU002A250UC) and 2mM MnCl₂ in buffer (50mM Tris, pH 7.5, 250mM NaCl, 10% glycerol and 1mM DTT). Right after collecting kinase reaction samples, the leftover mixture was split into equal parts in different microcentrifuge tubes to carry on phosphatase assays by adding replicates of His-PstP_cWT_{Mtb}, His-PstP_cT137E_{Mtb}, His-PstP_cT141E_{Mtb} or His-PstP_cT174E_{Mtb} at 0.012 µg/µl to the kinase reaction mixtures containing 0.12 µg/µl of His-SUMO-CwlM_{Mtb} and incubated in phosphatase buffer (50mM Tris pH 7.5, 10mM MnCl₂, and 1mM DTT) at room temperature up to 40 minutes. His-

FhaA_{Mtb} at 0.15 µg/µl was phosphorylated *in vitro* with His-MBP-PknB_{Mtb} at 0.015 µg/µl for 45 minutes at 37°C with 0.15µCi/µl ATP [γ -³²P] in presence of 5mM MnCl₂ in 25mM Tris pH 7.5 and 1mM DTT. Right after collecting 0 minute kinase reaction sample, the reaction mixture was then split into equal parts and incubated with either PstP_cWT_{Mtb} or different phospho-mimetic variants of it at 0.012 µg/µl up to 80 minutes at room temperature in presence of the phosphatase buffer where the concentration of the substrate was 0.12 µg/µl For CwlM and FhaA, the amount of kinase and phosphatases was 1/10th the amount of substrate, by weight.

His-Wag31_{Mtb} at 0.15 µg/µl was phosphorylated with His-MBP-PknA_{Mtb} at 0.03 µg/µl for 2 hours at room temperature in presence of 0.2µCi/µl ATP [γ -³²P], 10mM MnCl₂ and buffer (50mM Tris pH 7.5, 20% glycerol and 150mM NaCl). The amount of PknA was 1/5th the amount of Wag31 substrate, by weight.

The kinase reaction mixture was equally split in to separate tubes after the 0 second kinase reaction sample was taken. His-PstP_cWT_{Mtb} or its different phospho-mimetic variants at 0.0013 µg/µl were added to the mixture containing 0.13 µg/µl substrate and incubated at room temperature for up to 120 seconds in the phosphatase buffer. The amount of phosphatase was 1/100th the amount of Wag31 substrate in the phosphatase reactions, by weight.

Control samples with no kinase and with no phosphatase were performed in all the above-mentioned substrate dephosphorylation assays. All *in vitro* dephosphorylation assays were carried out separately with two individually purified batches of WT and phospho-mimetic mutant of PstP as biological replicates.

Quantification of γ -³²P signals

Samples from each *in vitro* biochemical reaction were collected before (0 minute or second) and after addition of each of the phosphatases at several different time points. The samples were run on 12% (Mini-Protean TGX, BIO-RAD,

4561046) gels and stained with Coomassie blue solution. After destaining, gels were dried using the GD200 Vacuum Gel Drying system (Hoefer Inc.) and imaged with phosphor screens (Molecular Dynamics) and a scanner (Storm 860, Amersham Biosciences). ImageQuant software (Molecular Dynamics) was used to process the images.

The protein bands on the gel and the phospho-signals on the autoradiogram were then quantified with FIJI. The intensities of the substrate protein bands on the Coomassie-stained gel and their corresponding phospho-signals on the autoradiograms at each time point were normalized against the respective protein band and the phospho-signal at 0 minute (His-SUMO-CwlM_{Mtb} and His-FhaA_{Mtb}) or 0 second (His-Wag31_{Mtb}) before addition of the phosphatase. These relative intensities were used to calculate the intensity of phospho-signal over the amount of substrate for each time point. These values were used to plot the graph. The P32 signal intensities of the kinases on autoradiograms at each time point were normalized against the respective phospho-signal on the kinase at 0 minute (His-MBP-PknB_{Mtb}) or 0 second (His-MBP-PknA_{Mtb}) before addition of the phosphatase. These relative P32 signal intensity values were then used to plot the graph. All values were plotted and p-values were calculated using two-tailed, unpaired Student's t-test in GraphPad Prism (version 7.0d).

Cell staining

At least three biological replicates of *pstP*_{Msmeg} T134E and WT allelic variants were grown in 7H9 medium up to mid log. phase. Cultures were washed and then starved in 1xPBS with 0.05% Tween 80 (PBST) at OD₆₀₀= 0.3 for 15 minutes. 100µl of starved culture was then treated with 3µl of 10mM fluorescent D-amino acid HADA for 10 minutes. Cells were then pelleted and resuspended in PBST and fixed with 10µl of 16% paraformaldehyde for 10 minutes at room temperature. Cells were then pelleted and resuspended in PBST before imaging on agarose pads.

For staining cells in the log. phase, 1µl of 1mM HADA was added to 100µl culture grown up to the mid-log. phase and incubated at 37°C for 15 minutes. Stained cells were then pelleted, washed and fixed as described above.

Static microscopy and image analysis

A Nikon Ti-2 widefield epifluorescence microscope with a Photometrics Prime 95B camera and a Plan Apo 100x, 1.45 NA objective lens was used to image cells. A 350/50nm excitation filter and a 460/50nm emission filter were used to take the blue fluorescence images for HADA staining. All images were captured using the NIS Elements software and analyzed using FIJI and MicrobeJ (Ducret *et al.*, 2016). Appropriate parameters for length, width and area were set in MicrobeJ to detect cells. All detected dividing (V-snapping) cells in the images were split at the septum so that each daughter cell could be considered as a single cell. No overlapping cells in the images were included in the analysis.

Length and mean-intensities of HADA signals of at least 330 cells from each of *pstPT134E_{Msmeg}* and *pstPWT_{Msmeg}* (at least 110 cells from each of three biological replicate strains of each genotype) were quantified using MicrobeJ. The values of the mean intensities of 330 cells of each *pstP* allelic mutant and WT are represented in the graphs generated using GraphPad Prism (version 7.0d).

The medial intensity profiles of HADA signals in cells from different *pstP* allele strains in log. phase and starvation analyzed with MicrobeJ were plotted on the Y-axis over relative positions of cells using the “XStatProfile” plotting feature in MicrobeJ to show the subcellular localization of fluorescent intensities in cells. Demographs of HADA signal intensity across cell lengths in log. phase and starvation were built using the “Demograph” feature of MicrobeJ by plotting the medial intensity profiles of HADA signals.

Time-lapse microscopy

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300 An inverted Nikon Ti-E microscope was used for the time-lapse imaging. An
301 environmental chamber was maintained at 37°C. Cells were cultivated in an B04
302 microfluidics plate from CellAsic; continuously supplied with fresh medium; and
303 imaged every 15 minutes using a 60x 1.4 N.A. phase contrast objective, with an
304 intermediate magnification of 1.5x. mCherry2B was excited with a SpectraX
305 Illuminator at 561-nm. Fluorescence was detected with a bandpass filter of
306 595/40 and detected with an ORCA Flash Digital sCMOS.

307

308 **Results**

309

310 **PstP_{Mtb} dephosphorylates FhaA_{Mtb} and Wag31_{Mtb} *in vitro***

311 FhaA_{Mtb} is phosphorylated by PknB_{Mtb} *in vivo* (Prisic *et al.*, 2010) and *in vitro*
312 (Grundner *et al.*, 2005; Roumestand *et al.*, 2011) on T116 (Roumestand *et al.*,
313 2011). Wag31_{Mtb} is phosphorylated at T73 by PknA_{Mtb} (Kang *et al.*, 2005). To
314 determine if FhaA_{Mtb} and Wag31_{Mtb} are substrates of PstP_{Mtb}, we performed *in*
315 *vitro* dephosphorylation assays with purified full-length His-FhaA_{Mtb}, His-
316 Wag31_{Mtb}, N-terminally His-MBP tagged cytosolic kinase domains of PknA_{Mtb} and
317 PknB_{Mtb}, and the cytosolic region of PstP_{Mtb} containing the catalytic domain (His-
318 PstP_cWT_{Mtb}).

319 We phosphorylated His-FhaA_{Mtb} with His-MBP-PknB_{Mtb} and ATP [γ -³²P]. After
320 addition of 1/10th the amount of His-PstP_cWT_{Mtb}, the phosphorylation on His-
321 FhaA_{Mtb}~P decreases by ~80% in 80 minutes (Figure 2a,b). Our control assay
322 with no phosphatase shows that the phosphorylation on His-FhaA_{Mtb}~P is stable
323 over time in the phosphatase buffer (Figure 2a, b). These data show that the cell
324 wall regulator FhaA_{Mtb} is a substrate of PstPWT_{Mtb} *in vitro*.

325 We performed similar phosphatase assays to determine if PstP_cWT_{Mtb} can
326 dephosphorylate Wag31_{Mtb}. We first phosphorylated His-Wag31_{Mtb} with His-MBP-

PknA_{Mtb} and ATP [γ -³²P], then added His-PstP_cWT_{Mtb}. With 1/100th the amount of PstP compared to Wag31, Wag31_{Mtb} phosphorylation decreased by about ~90% in 2 minutes (Figure 3a,b), only in the presence of His-PstP_cWT_{Mtb}. These data show that Wag31_{Mtb} is a substrate of PstPWT_{Mtb} *in vitro*.

Our previous biochemical data that demonstrated PstP_{Mtb} dephosphorylates CwIM_{Mtb} (Shamma *et al.*, 2021) is reproducible in our *in vitro* assays with ATP [γ -³²P] where we observe a 97% decrease in phosphorylation on His-SUMO-CwIM_{Mtb}~P over a time period of 40 minutes after addition of His-PstP_cWT_{Mtb} (Figure 4a, b). In all the assays, we see no phosphorylation signals on the WT and phospho-mimetic PstPs on the autoradiograms (data not shown) since PstP can quickly dephosphorylate itself (Sajid *et al.*, 2011) and the purified forms are thus unphosphorylated.

Because measuring the rates of dephosphorylation by PstP against different substrates required different PstP concentrations and timing, we calculated the initial linear rate of dephosphorylation per unit concentration of PstP, in order to compare PstP's activity against different substrates. This comparison indicates that His-PstP_cWT_{Mtb} dephosphorylates His-Wag31_{Mtb} more than 500 times faster than His-SUMO-CwIM_{Mtb} and about 2500 times faster than His-FhaA_{Mtb} (Figure 4c).

Phospho-mimetic mutation at PstPT137_{Mtb} inhibits dephosphatase activity

PstP_{Mtb} is phosphorylated on the conserved threonine (T) residues 137, 141 and 174 (Figure 1), and when phosphorylated, shows increased activity against small molecule substrates *in vitro* (Sajid *et al.*, 2011). We hypothesized that phosphorylation of the threonine residues of PstP individually might help regulate PstP's activity through switching its catalytic activity against different substrates or by affecting substrate affinity. To test this, we made phospho-mimetic (T to E) (Cottin *et al.*, 1999) mutants of PstP_{Mtb} at each of the three conserved phosphorylation sites of PstP_{Mtb} in the catalytic domain and purified them (His-

PstP_cT137E_{Mtb}, His-PstP_cT141E_{Mtb} and His-PstP_cT174E_{Mtb}). We previously showed that *in vitro* His-PstP_cWT_{Mtb} and His-PstP_cT174E_{Mtb} dephosphorylate His-SUMO-CwIM_{Mtb} at the same rate (Shamma *et al.*, 2021). We performed separate *in vitro* phosphatase assays with the substrates His-FhaA_{Mtb}, His-Wag31_{Mtb} and His-SUMO-CwIM_{Mtb} with the phospho-mimetic versions of PstP. His-PstP_cT137E_{Mtb} is completely inactive *in vitro* against all three substrates (Figures 2,3,4). This suggests that phosphorylation on T137 of PstP may inhibit its activity against multiple substrates.

His-MBP-PknB_{Mtb} and His-MBP-PknA_{Mtb}, the STPKs used in these assays, are also confirmed substrates of PstP (Sajid *et al.*, 2011). PknA and PknB are activated to phosphorylate their own substrates when they themselves are phosphorylated at their activation loops (Baer *et al.*, 2014). Because we already had these kinases in our phosphatase assays, we were able to measure dephosphorylation of His-MBP-PknB_{Mtb} and His-MBP-PknA_{Mtb} by PstP in the context of a 10-fold and 5-fold excess of His-SUMO-CwIM_{Mtb} or His-Wag31_{Mtb}, respectively. We found that His-PstP_cT137E_{Mtb} dephosphorylates both His-MBP-PknB_{Mtb} (Figure 5a,b) and His-MBP-PknA_{Mtb} (Figure 5c,d) more slowly than His-PstP_cWT_{Mtb} and the other PstP mutants. This shows that His-PstP_cT137E_{Mtb} is inhibited against at least five different substrates.

Phospho-mimetic mutations at PstPT141_{Mtb} and PstPT174_{Mtb} moderately inhibit Wag31's dephosphorylation

His-PstP_cT141E_{Mtb} and His-PstP_cT174E_{Mtb} dephosphorylate His-FhaA_{Mtb} (Figure 2,4c) and His-SUMO-CwIM_{Mtb} (Figure 4) at about the same rate as His-PstP_cWT_{Mtb}, but dephosphorylate His-Wag31_{Mtb} about half as fast as His-PstP_cWT_{Mtb} (Figure 3, 4c). These biochemical data support our hypothesis that phosphorylation on PstP may modulate its activity by affecting its substrate specificity. Phosphorylation at T141 and T174 appear to inhibit binding or

catalysis of Wag31 but not FhaA or CwIM.

Phospho-site T134 of PstP_{Msmeg} regulates peptidoglycan metabolism in starvation

Phosphorylation of CwIM allows it to activate the peptidoglycan precursor enzyme MurA, thereby licensing peptidoglycan synthesis (Boutte *et al.*, 2016). Phosphorylation of CwIM also affects its binding to FhaA and the lipid II flippase MurJ (Turapov *et al.*, 2018). CwIM is quickly dephosphorylated in the transition to stasis (Boutte *et al.*, 2016) likely by PstP (Shamma *et al.*, 2021). The function of phosphorylation on FhaA and Wag31 is not yet clear, but available data (Jani *et al.*, 2010; Gee *et al.*, 2012; Viswanathan *et al.*, 2016) suggest it could also affect peptidoglycan metabolism. Dephosphorylation of these regulators could be important for downregulation of peptidoglycan metabolism in stasis.

As the phospho-mimetic mutation at PstPT137_{Mtb} inhibits PstP's dephosphorylation of three peptidoglycan regulatory proteins *in vitro* (Figure 2,3,4), we sought to determine if the corresponding phosphosite on PstP (T134) might affect peptidoglycan metabolism *in vivo* in *Msmeg*. We made *Msmeg* strains with a phospho-mimetic *pstPT134E_{Msmeg}* allele replacing the wild-type *pstP*. We reported previously that the biological replicates of *pstPT134E_{Msmeg}* mutant strains had inconsistent doubling times (Shamma *et al.*, 2021) probably due to potential suppressor mutations. We confirmed that the slow growing clones could, after passaging, accumulate suppressor mutations that allowed them to grow as fast as the *pstPWT* strain. To characterize the original *pstPT134E_{Msmeg}* strain, we isolated numerous clones from fresh transformations, and measured the doubling times of each before continuing with experiments. We show data only from clones that grew at the slower rate.

We used three biological replicate *pstPT134E_{Msmeg}* strains that had consistently slower growth than the *pstPWT_{Msmeg}* strains (Figure 6a, red bar) in our next experiments. We stained both log.phase and starved *pstP T134E_{Msmeg}* and

WT_{Msmeg} strains using a fluorescent D-amino acid HADA (Kuru *et al.*, 2012) as a reporter of active peptidoglycan metabolism. We saw a slight increase in length (Figure 6b,d and Supplementary Figure S2a,b) and HADA intensity (Figure 6c,d and Supplementary Figure S2a,b) in the *pstP* T134E_{Msmeg} strains in the log. phase compared to the WT_{Msmeg} strains. After starvation, these differences increased (Figure 6b,c,e and Supplementary Figure S2c,d). WT_{Msmeg} strains quickly downregulate peptidoglycan metabolism upon starvation (Boutte *et al.*, 2016), as the decreased HADA staining (Figure 6c,d,e,f,g) shows. The starved *pstPT134E_{Msmeg}* strains show significantly higher HADA signal than the WT_{Msmeg} (Figure 6c, top right panels of d,e and Supplementary Figure S2b,d) indicating a defect in downregulating peptidoglycan metabolism. This suggests that the presence of a constant negative charge on phosphosite T134 of PstP_{Msmeg} impairs downregulation of peptidoglycan synthesis, likely by inhibiting PstP's dephosphorylation of peptidoglycan regulators (Figure 2,3,4).

Our data showing that the *pstPT134E_{Msmeg}* strains have increased length (Figure 6b) and higher HADA intensity (Figure 6c,d,f and Supplementary Figure S2b) in the log. phase support the notion (Jani *et al.*, 2010; Turapov *et al.*, 2018) that peptidoglycan regulation involves a balance in phosphorylated and dephosphorylated forms of its regulatory substrates and PstP's phosphoregulation helps toggle its activity against substrates in log. phase. The misregulation of peptidoglycan metabolism (Figure 6c,d,e,f,g and Supplementary Figure S2b,d) may account for the slower growth of the *pstPT134E_{Msmeg}* strains that we see in our data (Figure 6a), though it is likely that other processes beyond peptidoglycan metabolism are also misregulated, as PstP likely has many substrates involved in diverse processes (Molle *et al.*, 2006; Iswahyudi *et al.*, 2019).

PstP localizes at the septum in exponentially growing *Msmeg* cells

We sought to determine if PstP's activity against its diverse substrates could be regulated through protein localization. We made an *Msmeg* strain carrying an additional copy of *pstP_{Msmeg}* fused to *mCherry2B*. By time-lapse fluorescence microscopy, we observed that mCherry2B-PstP_{Msmeg} appears to be uniformly distributed along the membrane throughout most of the cell cycle, but becomes localized at the septum before cell separation (Figure 7). These data suggest that PstP is also regulated by its access to substrates at different sites in the cell.

Discussion

Previous works have suggested that PstP plays a crucial role as a negative regulator of protein Serine/ Threonine phosphorylation (Boitel *et al.*, 2003; Molle *et al.*, 2006; Sajid *et al.*, 2011; Boutte *et al.*, 2016; Iswahyudi *et al.*, 2019; Dulberger *et al.*, 2020; Shamma *et al.*, 2021). Our findings indicate that PstP is regulated by its own phosphorylation (Figure 2,3,4,5) to regulate cell wall metabolism (Figure 6), and that it may also be regulated by its localization (Figure 7).

We see that PstPWT_{Mtb} dephosphorylates CwIM_{Mtb}, FhaA_{Mtb} and Wag31_{Mtb} at different rates *in vitro* (Figure 4c), with Wag31_{Mtb} being the fastest to be dephosphorylated and FhaA the slowest (Figure 4c). We showed that PstP localizes at the septum in the exponentially growing *Msmeg* cells (Figure 7). Both FhaA and Wag31 localize at the poles and septum (Gee *et al.*, 2012; Hannebelle *et al.*, 2020). Our localization data (Figure 7) suggest that both Wag31 and FhaA (Figure 2, 3, 4c) may be more highly phosphorylated at the poles than at the septum. PstP's rapid dephosphorylation of Wag31 (Figure 3, 4c) suggests that it is likely to be nearly completely unphosphorylated at the septum.

Our data in Figure 2, 3 and 4 show that negative charges at PstPT141_{Mtb} and T174_{Mtb} slow dephosphorylation of Wag31_{Mtb}, but not FhaA_{Mtb} and CwIM_{Mtb}. Phosphorylation site T141 is on the flap subdomain and T174 is on the β -sheet

core ($\beta 8$) on PstP_{Mtb} (Pullen *et al.*, 2004), but both are facing into the same groove between these domains (Figure 1). T137 is the first residue of the flap subdomain (Pullen *et al.*, 2004), and it faces away from the groove with the other phospho-sites (Figure 1). We propose that the T141/ T174 groove may contribute to toggling specificity of PstP against certain substrates, depending on the phosphorylation status.

Our *in vitro* data in Figure 2,3, 4 and 5 show that PstPT137E_{Mtb} inhibits its activity against CwlM_{Mtb}, FhaA_{Mtb} and Wag31_{Mtb}, and also against the STPKs PknA and PknB that phosphorylate them, all of which are involved in regulation of peptidoglycan metabolism (Thakur and Chakraborti, 2006; Kang *et al.*, 2008; Jani *et al.*, 2010; Gee *et al.*, 2012; Prsic and Husson, 2014; Boutte *et al.*, 2016; Viswanathan *et al.*, 2016; Viswanathan *et al.*, 2017; Carette *et al.*, 2018; Turapov *et al.*, 2018). We expected the corresponding phospho-mimetic mutant *pstPT134E_{Msmeg}* allele to cause mis-regulation of peptidoglycan synthesis *in vivo*. Our *in vivo* data show that the *pstPT134E_{Msmeg}* strains grow more slowly, and have an increase in cell length and HADA staining in log. phase and starvation (Figure 6). We see relatively mild defects in peptidoglycan metabolism in these strains in log. phase (Figure 6b,c), which suggests that phospho-site T134 on PstP may not be important for controlling peptidoglycan metabolism in active growth. Alternatively, the function of the negative charge at T134 may be different in the cell: phosphorylation here could help recruit an interacting protein that could alter PstP's activity in a different way. As expected, however, we do see greater defects in peptidoglycan metabolism during starvation (Figure 6c,d,e,f,g), suggesting that dephosphorylation of CwlM, FhaA, Wag31, PknB or PknA, and/or other substrates, is more important in the transition to stasis than in log. phase. The cells still largely downregulate peptidoglycan metabolism, indicating that other regulatory systems contribute to this response, or that *pstPT134E* has different effects *in vivo* than *in vitro*.

Although our findings are limited to PstP's activity on certain substrates only, altogether they prove that the individual phosphosites are important in regulating

PstP. *In vitro*, we see that a negative charge on T137 of *PstP_{Mtb}* inhibits its activity against three PG regulators (Figure 2,3,4), as well as the essential kinases PknB and PknA when they are mixed together (Figure 5). In the cell, the substrates and kinases also co-exist, and *PstP* may exert its effect directly by dephosphorylating the substrates or indirectly by acting on the kinase (Bhaskara *et al.*, 2019), or both simultaneously. There are likely other mechanisms of cell wall regulation involved in the complex *in vivo* scenario like protein localization or *PstP*'s interaction with other regulatory proteins that may contribute to *PstP*'s regulation as well. We do not know the conditions under which *PstP* is phosphorylated, or the combinations of *PstP*'s phosphorylations that exist in the cell. So the *in vitro* data may not mirror the deep complexity of the STPK-substrate-*PstP* network *in vivo*.

In this work we show that *PstP* is regulated by both phosphorylation and localization. This work demonstrates the importance of protein dephosphorylation, not just phosphorylation, in regulating the activity of proteins involved in cell wall metabolism in mycobacteria. Future work that addresses when all of these important phospho-sites are phosphorylated and dephosphorylated will help us to understand how all these protein regulators contribute to coordinating the construction and integrity of the cell wall in changing conditions.

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PstP T137 paper 2022 Figures

Figure 1 Phosphorylation sites are not near the active site in PstP

Schematic of the crystal structure of PstP from *M. tuberculosis* (PstP_{Mtb}WT) (Pullen *et al.*, 2004) (PDB code 1TXO) showing the conserved threonines (T) which are phosphorylated by the kinases PknA and PknB (Sajid *et al.*, 2011). Highlighted on the structure are: red - PstP_{Mtb}T137 (PstPT134 in PstP_{Msmeg}); blue - PstP_{Mtb}T141 (PstPT138 in PstP_{Msmeg}); green - PstP_{Mtb}T174 (PstPT171 in PstP_{Msmeg}); yellow - Mn²⁺ present at the conserved two-metal center typically seen in the PP2C subfamily of protein Serine/Threonine phosphatases, which are part of the active site core; pink - the unique third Mn²⁺ ion bound in the catalytic core of PstP_{Mtb} but not present in the human PP2Cα which sits in a groove created by the flap subdomain next to the active site; orange - Ser60 and Asp118 that act as ligands for the third Mn ion; lime green - residues of the catalytic sites surrounding the two-metal center. Arrows point to position of the OH-groups on T137, T141 and T174 that are phosphorylated.

FIGURE 2 Dephosphorylation of FhaA by PstP and phospho-mimetic isoforms

(a) Autoradiograms and corresponding Coomassie stained SDS-gels of *in vitro* phosphatase assays performed with ATP-[γ-³²P]-phosphorylated His-FhaA_{Mtb} and His-PstP_cWT_{Mtb} (first panel), His-PstP_cT137E_{Mtb} (second panel), His-PstP_cT141E_{Mtb} (third panel), His-PstP_cT174E_{Mtb} (fourth panel) and no phosphatase control reaction (bottom panel). One set of representative images for each assay is shown here from two individual assays performed with individually purified batches of phosphatases.

(b) Quantification of relative intensities of [γ-³²P] on His-FhaA_{Mtb} on autoradiogram over amount of His-FhaA_{Mtb} on the SDS-gel. P values were calculated using two-tailed unpaired t-test.

P-values of T137E at 0 min vs. T137E 80 min = 0.7598, WT vs. T137E at 80 min = 0.0487, WT vs. T141E at 80 min = 0.3712, WT vs. T174E at 80 min = 0.2000. The error bars represent standard error of mean.

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FIGURE 3 Dephosphorylation of Wag31_{Mtb} by PstP and phospho-mimetic isoforms

(a) Autoradiograms and their respective Coomassie stained SDS-gels of *in vitro* phosphatase assays performed with ATP-[γ -³²P]-phosphorylated His-Wag31_{Mtb} and His-PstP_cWT_{Mtb} (first panel), His-PstP_cWT_{Mtb} (second panel), His-PstP_cT141E_{Mtb} (third panel), His-PstP_cT174E_{Mtb} (fourth panel) and no phosphatase control reaction (bottom panel). One set of representative images for each reaction is shown here from two individual assays performed with individually purified batches of phosphatases.

(b) Quantification of relative intensities of [γ -³²P] on His-Wag31_{Mtb} on autoradiogram over amount of His-Wag31_{Mtb} on the SDS-gel. P values were calculated using two-tailed unpaired t-test.

889 P-values of T137E at 0 sec vs. 15 sec = 0.01947 and 0 sec vs. 120sec= 0.7493,
890 WT vs. T137E at 120 sec= 0.0130, WT vs. T141E at 120 sec =0.0106 and WT
891 vs. T174E at 120 sec = 0.170. The error bars represent standard error of mean.

892

FIGURE 4 Dephosphorylation of CwIM_{Mtb} by PstP_{Mtb} its phospho-mimetic isoforms and linear rate of dephosphorylation of CwIM_{Mtb}, FhaA_{Mtb} and Wag31_{Mtb}

(a) Autoradiograms and their respective Coomassie stained SDS-gels of *in vitro* phosphatase assays performed with ATP-[γ -³²P]-phosphorylated His-SUMO-CwIM_{Mtb} and His-PstP_cWT_{Mtb} (first panel), His-PstP_cWT_{Mtb} (second panel), His-PstP_cT141E_{Mtb} (third panel), His-PstP_cT174E_{Mtb} (fourth panel) and no phosphatase control reaction (bottom panel). One set of representative images for each reaction is shown here from two individual assays performed with individually purified batches of phosphatases.

(b) Quantification of relative intensities of [γ -³²P] on His-SUMO-CwIM_{Mtb} on autoradiogram over amount of His-SUMO-CwIM_{Mtb} on the SDS-gel. P values were calculated using two-tailed unpaired t-test. P-values of T137E at 0 min vs. 40 min= 0.1107, WT vs. T137E at 40 min= 0.0127, WT vs. T141E at 40 min=0.3683 and WT vs. T174E at 40 min= 0.5918. The error bars represent standard error of mean.

(c) Quantification of linear rate of change in [γ -³²P] signal on 10 μ g of His-SUMO-CwIM_{Mtb}, His-FhaA_{Mtb} and His-Wag31_{Mtb} in presence of His-PstP_cWT_{Mtb} and its phospho-mimetic mutants per minute. P values were calculated using two-tailed unpaired t-test. **, P value = 0.0026 and *, P value 0.0306. The error bars represent standard error of mean.

FIGURE 5 Dephosphorylation of PknB_{Mtb} and PknA by PstPWT_{Mtb} and its phospho-mimetic variants

(a) Autoradiograms showing [γ -³²P]-phospho-signals on His-MBP-PknB_{Mtb} in the *in vitro* phosphatase assays with His-SUMO-CwlM_{Mtb} and His-PstP_cWT_{Mtb} (first panel), His-PstP_cWT_{Mtb} (second panel), His-PstP_cT141E_{Mtb} (third panel) and His-PstP_cT174E_{Mtb} (fourth panel), where His-MBP-PknB_{Mtb} was used to phosphorylate His-SUMO-CwlM_{Mtb}. One set of representative images for each reaction is shown here from two individual assays performed with individually purified batches of phosphatases.

(b) Quantification of relative intensities of [γ -³²P] on His-MBP-PknB_{Mtb} on autoradiogram. P values were calculated using two-tailed unpaired t-test. P value of T137E at 0 min vs. 40 min= 0.0523. *, P value= 0.0375 (WT vs.T137E at 40 min); *, P value=0.0314 (T141E vs.T137E at 40 min); *, P value= 0.0217 (T174E vs.T137E at 40 min). The error bars represent standard error of mean.

(c) Autoradiograms showing [γ - 32 P]-phospho-signals on His-MBP-PknA_{Mtb} in the *in vitro* phosphatase assays with His-Wag31_{Mtb} and His-PstP_cWT_{Mtb} (first panel), His-PstP_cWT_{Mtb} (second panel), His-PstP_cT141E_{Mtb} (third panel) and His-PstP_cT174E_{Mtb} (fourth panel), where His-Wag31_{Mtb} was first phosphorylated with His-MBP-PknA_{Mtb}. One set of representative images for each reaction is shown here from two individual assays performed with individually purified batches of phosphatases.

(d) Quantification of relative intensities of [γ - 32 P] on His-MBP-PknA_{Mtb} on autoradiogram. P values were calculated using two-tailed unpaired t-test. P value of T137E at 0 sec vs. 120 sec= 0.0643. *, P value= 0.0163 (WT vs. T137E at 120 sec). The error bars represent standard error of mean.

Figure 6 The phosphosite on T134 on PstP_{Msmeg} affects PG metabolism

(a) Doubling times of *Msmeg* strains carrying the *pstP*_{*Msmeg*} WT and the phospho-mimetic T134E alleles. Each bar represents the mean of individual average doubling times of three biological replicates of each genotype from two different experiments on different dates. The error bars represent mean with standard deviation. **, P value= 0.0026.

(b) Quantification of cell lengths of isogenic *pstP*_{*Msmeg*} allele strains (WT and T134E) grown in 7H9 in log. phase and starved in PBS with Tween80. At least 100 cells from each of three biological replicates were quantified. P values were calculated by unpaired t-test. *, P value = 0.0122, ****, P value= 0.000000031.

(c) Quantification of mean intensities of HADA of *pstP*_{*Msmeg*} allele strains (WT and T134E) grown in 7H9 in log. phase and starved in PBS with Tween80. Cells were stained after 15 minutes of starvation and washed and fixed after another 15 minutes (starved for a total of 30 minutes). At least 100 cells from each of three biological replicates were quantified. P values were calculated by unpaired t-test. *, P value = 0.0109, ****, P value= <0.000000000000001.

(d) and (e) Representative HADA and phase images of cells from *pstP*_{*Msmeg*} allele strains (WT and T134E) grown in 7H9 in log. phase (d) and after 30 minutes of starvation in PBS with Tween80 (e).

(f) and (g) Intensity profiles of HADA signals in cells of *pstP*_{*Msmeg*} allele strains (WT and T134E) in log. phase (f) and after starving for 30 minutes in PBS supplemented with Tween80 (g). The shaded regions denote standard deviations and the solid lines represent the mean intensity values. Signal intensities from at least 110 cells from each of three biological replicates of every *pstP*_{*Msmeg*} allelic variant genotype were quantified and used to analyze the signal profiles in MicrobeJ. 0 to 1 on the X-axes represent cells from the old pole to the new pole, respectively.

Figure 7 Localization of PstPWT_{*Msmeg*}

Msmeg cells expressing mCherry2B-PstP were imaged over time in a CellAsic Microfluidic device at 15-minute intervals. Intensity is seen at the midcell (white

arrows) for several frames before cell separation can be visualized by phase contrast microscopy (yellow arrows).

Supplemental Figures:

Supplementary Figure S1: Dephosphorylation of His-Wag31_{Mtb} by His-PstP_c WT_{Mtb}. Autoradiogram and the respective Coomassie stained SDS-gel of *in vitro* phosphatase assays performed with ATP-[γ-³²P]-phosphorylated His-Wag31_{Mtb} and His-PstP_c WT_{Mtb}. Ten times more substrate than the phosphatase was used in the assay.

Supplementary Figure S2: Demographic profiles of HADA signal intensity in *pstP*_{Msmeg} strains in growth and starvation. (a) and (b) Demographs showing intensities of the fluorescent dye HADA signal in individual cells from *pstP*_{Msmeg}WT allele strains (a) and *pstP*_{Msmeg}T134E allele strains (b) in log. phase. (c) and (d) Demographs showing intensities of the fluorescent dye HADA signal in individual cells from *pstP*_{Msmeg}WT allele strains (c) and *pstP*_{Msmeg}T134E allele strains (d) starved in PBS with Tween80 for a total time period of 30 minutes.

Biological triplicates of each *pstP*_{Msmeg} allelic variant were analyzed. Signal intensities from at least 340 cells from each *pstP* allelic variant (at least 100 cells from each biological triplicate of each genotype) in log. phase and starvation were plotted in the demographs.