The Molecular basis of N-acetylmuramoyl-L-alanine amidase (Rv3915) and Protein Kinase B (PknB) essentiality in Mycobacterium tuberculosis

CURRENT STATUS: POSTED

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DOI:
10.21203/rs.2.13430/v1

SUBJECT AREAS
General Cell Biology & Physiology

KEYWORDS
Mycobacterium tuberculosis, Protein Kinase B, phosphorylation, Rv3915
Abstract

Background: Protein kinase B (PknB) is critical for the survival of Mycobacterium tuberculosis (M. tuberculosis) in vitro and in hosts. It phosphorylates various enzymes involved in biosynthesis of cell wall and a particular autolysin classified as CwIM or Rv3915 has been recently identified as a PknB substrate. However, in-depth knowledge of this protein is still unknown. The aims of this study were to purify and investigate the activity of Rv3915, as well as monitor the phosphorylation of Rv3915 and the influence of phosphorylation on the activity of this protein.

Results: Using the C41 E. coli strain containing a plasmid, with a gene encoding the protein, Rv3915 was either expressed alone or co-expressed with PknB. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was used to observe the amount of Rv3915 purified, anti-poly His and anti-phosphothreonine western blot techniques were used to confirm the presence and phosphorylation of Rv3915 and zymogram assays were run to examine its activity. The results showed that Rv3195 was successfully expressed and was deemed soluble when observed in soluble fraction of E. coli lysate. It was confirmed that Rv3915 is produced as a ~45kDa protein, which does not possess any muralytic activity. However a shorter version of the protein (~25kDA) was active in zymogram, suggesting that Rv3915 is activated by cleavage.

Conclusion: Rv3915 was phosphorylated by PknB and phosphorylation apparently controls stability of the protein.

Background

A common mechanism used by both eukaryotic and prokaryotic cells for the conductance of external signals to bring about changes in gene expression is reversible protein phosphorylation [1]. Bacteria make use of two-component systems, which handle the transfer of phosphate groups from histidine residues on sensor protein kinases to aspartyl residues on response regulator proteins in order to transmit signals [2]. In Gram-positive
and Gram-negative bacteria, serine/threonine protein kinases (STPKs) are widely distributed [3] and the genome of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, contains 11 STPKs and 3 protein phosphatases (Mptp, MstP and SapM) [4-5].

These protein kinases regulate many essential processes in mycobacteria. For example, protein kinase A (PknA) and protein kinase B (PknB) are essential for mycobacterial growth and regulate cell morphology, shape and division [1, 6, 7], while nitrogen metabolism, virulence and the ability of *M. tuberculosis* to adapt inside its host are regulated by protein kinase G (PknG), protein kinase E (PknE) and protein kinase H (PknH) [8, 9, 10, 11]. Protein kinase F (PknF) mediates glucose transport, cell division, growth rate and morphology [12]. The genes pknA and pknB, encoding the proteins PknA and PknB respectively, are found in an operon which contains cistrons for the production of RodA (controls the shape of the cell) and the protein phosphatase PstP and PbpA (plays a role in the synthesis of peptidoglycans).

The PknB kinase belongs to a distinct family of STPKs found only in gram-positive bacteria [13] and the important feature of these kinases is the presence of the PASTA (penicillin-binding protein and serine/threonine kinase associated) domains in the surface-exposed region [14]. In Firmicutes, PASTA domain containing kinases are not essential for growth [15-21] and mycobacteria appear to be a unique bacterial group in which PknB is essential for growth [1, 6, 7]. Over the past years, great progress has been made in the identification of PknB substrates [22-24] and apart from being essential for growth, PknB also regulates an oxygen-mediated replication switch [25] and the regulation of this switch in *M. tuberculosis* is still obscure.

The PknB has many components which includes a conserved catalytic kinase domain, a juxta-membrane part attached to a membrane-spanning region and surface-exposed sensory component, consisting of PASTA designated as PknB_PASTA domain [26-27]. The PknB_PASTA domain from mycobacteria can bind synthetic muropeptides however; it remains unclear whether this binding influences activation of PknB, bacterial growth and resuscitation [28]. The extracellular PASTA domain is believed to recognize peptidoglycan fragments and has been implicated in PknB localization [26, 28], while the juxta-membrane domain recruits FhaA [29] and possibly other proteins that control peptidoglycan biosynthesis. PknB has been shown to phosphorylate multiple substrates, including proteins involved in peptidoglycan biosynthesis and remodelling [30-33]. In addition, PknB interacts with Mur ligases [34] and proteins associated with lipid metabolism [35]. However, the reason for PknB essentiality is currently unknown.

Rv3915, designated as CW1M, is N-acetylmuramoyl-L-alanyl-D-isoglutamine amidase,
which is able to cleave N-acetylmuramoyl-L-alanyl-D-isoglutamine to yield free N-acetyl muramic acid [36]. The Cw1M protein of M. tuberculosis has 21% identity and 31% homology with the Cw1B protein of B. subtilis 168s [37-38]. The gene cw1B codes for an N-acetylmuramoyl-L-alanine amidase, which is one of bacterial peptidoglycan hydrolases implicated in the remodeling of peptidoglycans. The protein Cw1M is predicted to be soluble, to possess a tail, two peptidoglycan-binding domains (one close to the N-terminus of the protein and the other towards the centre of the protein) and a catalytic domain. Three threonines in Cw1M have been shown to be phosphorylated by PknB. The present study was undertaken to purify and investigate the activity of Rv3915, as well as monitor the phosphorylation of Rv3915 and the influence of phosphorylation on the activity of this protein.

Results

Expression and Purification of Rv3915

E. coli lysate were prepared as described in the methods and levels of the Rv3915 expression during E. coli growth were monitored by SDS-PAGE and Western blotting. Initially, the protein was expressed at 37°C using the E. coli strain BL21 (DE3), but the protein produced inclusion bodies and could not be purified. Thus, a different E. coli host (C41) was tested for expression of Rv3915 at 37°C. As shown in figure 1, strong bands were seen at 20kDa for the short version of Rv3915 and 45kDa for the full length version of the protein. No band was observed in the control samples, as expected. Expression after 3hrs showed reasonable amount of protein for the lysate samples, however the highest expression of protein was seen after 20hrs.

Figure 1: SDS PAGE gel for C41 pre and post induction samples.

Next, we attempted to observe solubility/activity of protein, so expression at 37°C was repeated by separating the supernatant and pellet samples and ran on SDS gels, as seen in figure 2.

Figure 2: SDS PAGE gel for C41 pre and post induction supernatant and pellet samples.

In figure 2 above, no particular bands were observed in the supernatant samples, while the pellet samples showed certain amounts of the Rv3915 protein. It could be hypothesized that a very small amount of protein was soluble in the supernatant samples, while a larger amount remained insoluble in the pellet samples. Therefore, we ran anti-poly His western blots for further evaluation, as seen in figure 3.
Figure 3: Anti-Poly His Western Blot for C41 and pre- and post- induction supernatant samples.

In figure 3, certain amounts of Rv3915 was observed in both samples, so the expression was repeated at 37°C for both SH-C and L versions of the protein and purified them using a nickel affinity column. Fractions 3 and 4 had the highest amount of protein and as a result of this, SDS gels for these fractions were ran as seen in figure 4.

Figure 4: SDS PAGE gel for nickel affinity column purified C41 and SH-C supernatant samples.

We observed higher amounts of Rv3915 protein for the SH-C fractions and lower amounts for the L fractions (figure 4). Thus, a zymogram for fraction 4 of both samples were ran for comparison and faint bands of activity were observed at 17kDa for both samples which could be attributed to the degradation of samples due to instability of Pv3915 protein. The SH-C sample of Rv3915 protein was evaluated using mass spectrometry in order to determine sites of cleavage and this resulted in an even smaller fragment.

Phosphorylation of Rv3915

The behavior and activity of a phosphomimetic of Rv3915 and the phosphorylated version of Rv3915 were expressed and monitored. This was done by co-expression of Rv3915 with PknB from a PET-DUET plasmid. It was hypothesized that phosphorylation would improve stability of Rv3915 and possibly affect its activity.

Initially, C41L and a phosphomimetic pET 3915 DDD were compared. Transformed pET 3915 DDD into the C41 E.coli strain carried out expression at 37°C for both it and C41L, purified using the nickel affinity column and ran SDS gels. Although fraction 4 had the highest amount of protein for both samples, the amount of protein expressed was quite low (figure 5). So the expression for both samples was repeated at 20°C instead, and ran on SDS gels, as seen figure 5.

Figure 5: SDS PAGE gel for Nickel affinity column purified C41L and pET 3915DDD supernatant samples.

Higher amounts of Rv3915 protein was observed for samples expressed at 20°C. Both samples showed similar band patterns (figure 5). An anti-poly His western blots were ran on fraction 4 for both samples, as shown in figure 6.

Figure 6: Anti-Poly His Western Blots for Nickel affinity column purified C41L and pET 3915DDD supernatant samples.

A zymogram on fraction 4 of both samples showed a very faint activity for C41L at 17kDa,
but no activity was seen for pET 3915 DDD

The effect of phosphorylation on the stability of Rv3915 was investigated. An experiment using the fraction 4 samples of C41L and pET 3915 DDD was run. A 100µl aliquots for each sample was prepared. One aliquot of each sample had 4x SRB added to them immediately, while another aliquot, for each sample, had glycerol added to it and was left at room temperature for a few days. These aliquots were then run on an SDS gel, which is seen in figure 7. The rest of the aliquots were stored at -80°C for further use.

Figure 7: SDS PAGE gel for Nickel affinity column, preserved and degraded C41L and C41 pET 3915 DDD supernatant samples.

The above experiment was repeated using new aliquots for each sample stored at -80°C. One aliquot of each sample had 4x SRB added to them immediately after removal from storage, while the other aliquot was incubated at 37°C for 1hr before the addition of 4x SRB. These aliquots were then run on an SDS gel, as seen in figure 8.

Figure 8: SDS PAGE gel for Nickel affinity column, purified frozen preserved and degraded C41L and C41 pET 3915 DDD supernatant samples.

The 3915 Duet and PknB 3915 Duet were transformed into C41 E. coli cells, expression performed at 20°C and ran SDS gels on samples. Expression of Rv3915 protein was very low and the expression of both samples was repeated at 37°C. This gave a greater amount of protein and an anti-poly His and anti-phosphothreonine western blot on the pre-induction and 3hr post-induction samples of 3915 Duet and PknB 3915 Duet was ran as seen in figure 9.

Figure 9: Anti-Poly His and Anti-phosphothreonine Western Blots for C41 3915 Duet and PKnB 3915 Duet pre- and post- induction samples

The result showed that the Ami 3 samples, which were quite active, diffused across the gel and interfered with the PknB 3915 Duet samples, which were not so active. This resulted in both samples showing identical bands of activity, thus differentiation could not be achieved and the protein still degraded after nickel affinity purification. So an attempt was made to express protein in inclusion bodies and purify them by centrifugation in different buffers to see if degradation could be prevented. C41WT and Ami 3 at 37°C were expressed in inclusion bodies and ran on SDS gel and zymogram of the samples is as seen in figure 10.

Figure 10: SDS gel for 37°C inclusion bodies Ami3 and C41WT samples with corresponding mycobacterium luteus zymograms

Figure 11: Schematic showing direction of the action of RNA polymerase and the
phosphorylation of RV3915 protein by PKnB

Discussion

Expression and Purification of Rv3915
Not much is known about Rv3915, thus it was attempted to express and purify the protein. First, the full length version (L), short version without the tail and peptidoglycan domains (SH-C) and a control (empty plasmid) were expressed at 37°C. From figure 2, the full length version of the protein was seen at 45kDa, while the short version of the protein was seen at 25kDa. We attempted to observe if the protein would be soluble, as it can only be active when it is soluble. The protein being soluble would mean that it would be seen in the supernatant samples of purified cultures, where all cytoplasmic proteins of a cell are localized. The protein being insoluble, however, would mean that it would be seen in the pellet samples of purified cultures, where membrane proteins of a cell are localized. Since a protein band of interest in the anti-poly His western blot was observed in figure 4, no matter how little the protein amount was, purification of the protein could proceed. Thus, expression at 37°C for the full length version and short version of Rv3915 was repeated, and the samples purified using a nickel affinity column, as seen in figure 8. Usually highest amounts of the protein are found between eluted fractions 3-5. In the gel for C41L, three bands were seen: bands at 40kDa, 25kDa and 17kDa. For C41SH-C, a strong band was seen at ~25kDa and a faint one at 17kDa. It was hypothesized that the protein cleaves itself, possibly at its N-terminus and C-terminus, into two smaller fragments, and that the larger fragment (~25kDa) was the amidase/catalytic domain of the protein. The run of both L and SH-C samples on an M. luteus zymogram (figure 5) produced faint activity at 17kDa for both samples. The principle behind the zymogram assay was that since Rv3915 is a peptidoglycan hydrolase, at the size where it becomes active and starts to break up peptidoglycans on the cell wall of M. luteus, a clear or white band will be seen on the zymogram. Thus, it was also assumed that the protein had to be cleaved to 17kDa in order to become active. Therefore, a sample of SH-C of Rv3915 was evaluated using mass spectrometry, in order to determine the sites at which the protein gets cleaved. An even smaller fragment, Ami 3, was derived. It was believed that this fragment would not degrade or be cleaved further and it would remain active.

Phosphorylation of Rv3915
It was believed that phosphorylation would improve the stability of Rv3915 so that it
would remain at 40kDa. However, the activity of Rv3915 might be compromised as a result, the initial experiment was conducted with C41L and pET 3915 DDD (phosphomimetic) (figure 5). Phosphomimetics are amino-acid substitutions that imitate a phosphorylated protein, thereby activating or deactivating it. In the case of Rv3915, three threonine residues were phosphorylated, each phosphate group carrying three negative charges. In pET 3915 DDD, these three threonine residues were replaced with aspartic acid residues, which carry one negative charge each. However, it should theoretically behave the same way as the phosphorylated Rv3915. From figures 6, both C41L and pET 3915 DDD showed very similar band patterns. However, when samples were run on zymograms, faint activity was seen at 17kDa for C41L, while no activity was noticed for pET 3915 DDD (figure 7), thereby furthering our beliefs. To be more certain, the effect of phosphorylation on the stability of Rv3915 using the phosphomimetic was investigated. This experiment was repeated two times (figures 8-9). From figure 8 in particular, the C41L and pET 3915 DDD samples that had 4x SRB added to them immediately upon removal from storage were preserved and didn’t degrade. However, once the samples were incubated at 37°C for 2hrs before the addition of 4x SRB, C41L degraded completely while a moderate amount of pET 3915 DDD remained, showing that degradation of pET 3915 DDD was slower and it was more stable. Afterwards, the phosphorylation of Rv3915 was monitored. It was hypothesized that phosphorylation would only occur when full length version of Rv3915 was expressed in the presence of PknB.

A vector, pETDuet, which was specifically designed for the co-expression of two target genes, was used for expression of the phosphorylated version of Rv3915. From figure 11, the way the vector system works, RNA polymerase moves from left to right, so PknB is expressed first, followed by Rv3915. As soon as both are present, PknB phosphorylates Rv3915. Therefore, 3915 Duet and PknB 3915 Duet were expressed and an anti-poly His and anti-phosphothreonine western blot was run on the samples. A difficulty with anti-phosphothreonine blots is that since the phosphorylated threonine residues of Rv3915 are small or are not ‘raised’ enough, it proves hard sometimes for the anti-phosphothreonine antibody to bind to the residues. On the anti-poly His blot, both samples are seen as they both possess the His-tag at their C-terminus. However, on the anti-phosphothreonine blot, bands of interest were only observed for PknB 3915 Duet. This was because 3915 Duet was not phosphorylated due to the absence of PknB, so the anti-phosphothreonine antibody did not bind to it.

We went on further to test the hypothesis about Ami 3. It was expressed and a zymogram was run on its 1hr post-induction pellet/cellular extract sample. The difficulty with
zymograms is that it is hard to obtain distinct images of the clear bands most times. So, in order to improve the quality of the zymograms, the zymogram was divided into four pieces and incubated them overnight at 37°C in Tris buffer, 0.1mM ZnSO₄ solution or 40mM potassium phosphate buffer at pH 5.3, 6.7 and 8. As seen from figure 10, the zymogram, which gave the clearest image at pH 5.3, showed that Ami3 is indeed active. For the next experiment, Ami 3 and PknB 3915 Duet was expressed and nickel column purified. Zymograms were run on the fractions that had the highest amount of protein present. The zymograms were then divided into four pieces and incubated at 37°C in 40mM sodium phosphate buffer pH 4.8, and 40mM potassium phosphate buffer at pH 5.3, 6.5 and 7.5. In figure 10, faint bands of activity were seen. However, due to the fact that Ami 3 was much more active than PknB 3915 Duet, the samples interfered with each other on the zymogram, rendering it difficult to differentiate between the two samples. Because of this, for the next experiment, the samples were run on separate zymograms. It was assumed that the reason that such faint activity was seen in figure 10 was because the protein degraded after its nickel affinity column purification. Thus, we tried expressing the protein in inclusion bodies. When E. coli creates these inclusion bodies around the protein, it inactivates it, but the action of SDS on the protein sample should reactivate it again. Thus, Ami 3 and C41WT were expressed and samples were run on an SDS gel (figure 11). Observed a stable amount of protein in both samples, so we ran samples on separate zymograms, divided each zymogram into two pieces and incubated them overnight at 37°C in 40mM citric acid buffer pH6 and 40mM potassium phosphate buffer pH 7. None of the zymograms showed any activity, as seen from samples in figure 11. Therefore, it was concluded that Rv3915 is active in cellular extract samples and nickel affinity column purified samples, but not when expressed in inclusion bodies, although the protein samples avoided degradation in inclusion bodies.

Conclusion

To conclude, it was acknowledged that Rv3915 was produced as a 45kDa protein, which did not possess muralytic activity. The protein was cleaved into smaller fragments and two forms with molecular weights of 25kDa and 17kDa showed activity in zymograms. Rv3915 is apparently activated by cleavage; however the molecular mechanism for this cleavage is still unknown. Only in the presence of PknB can the protein become phosphorylated and phosphorylation apparently has effects on the stability and activity of the protein Rv3915.
Methods

The aims of this study were to purify and investigate the activity of Rv3915, as well as monitor the phosphorylation of Rv3915 and the influence of phosphorylation on the activity of this protein.

Bacterial Strains:

BL21 (DE3): Derived from B834 when transduced to Met\(^+\), an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacI\(^q\). Cells were grown aerobically in standard Luria-Bertani broth supplemented with kanamycin (50 µg/ml). Transformed plasmids containing T7 promoter driven expression are repressed until IPTG induction of T7 RNA polymerase from a lac promoter.

C41 (DE3): Derived from BL21 (DE3), an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacI\(^q\). Cells were grown aerobically in standard Luria-Bertani broth supplemented with kanamycin (50µg/ml). Transformed plasmids containing T7 promoter driven expression are repressed until IPTG induction of T7 RNA polymerase from a lac promoter.

C43 (DE3): Derived from C41 (DE3) through selection for resistance to a contrasting toxin protein, an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacI\(^q\). Cells were grown aerobically in standard Luria-Bertani broth supplemented with kanamycin (50µg/ml). Transformed plasmids containing T7 promoter driven expression are repressed until IPTG induction of T7 RNA polymerase from a lac promoter.

Competent Cells Preparation: 2ml LB bacterial starter culture was inoculated with a single colony of *E. coli* and incubated overnight at 37°C with shaking. 100ml LB bacterial main culture was inoculated with 1ml of starter culture and incubated at 37°C with shaking till OD\(_{580}\) ranged between 0.2 -0.4. Culture was placed on ice for 15mins, than centrifuged at 2500g and 4°C for 15mins. Supernatant was discarded and pellet was re-suspended in 50ml of pre-chilled 100mM CaCl\(_2\). Mixture was placed on ice for 5mins and then centrifuged again, discarded supernatant and gently re-suspended pellet in 50ml of 100mM CaCl\(_2\). Placed on ice for 15mins, and then repeated centrifugation. The supernatant was discarded and gently re-suspended pellet in 2ml of 100mM CaCl\(_2\) and 15% glycerol. A 100µl aliquots were made into pre-chilled eppendorf tubes and stored at -80°C.

*E. coli* Transformation: 100µl aliquot of competent cells was thawed and transferred into a pre-chilled 14ml falcon tube and 1µl of DNA (concentration between 20-200ng/µl) was
added and left on ice for 15 mins. A pre-warmed medium (900μl) was added to cells and incubated at 37°C with shaking for 1 hr. For each transformation, 3 LA plates were used, containing an appropriate antibiotic: plated 100μl of the undiluted culture, 100μl from 10^-1 dilution and 100μl from 10^-2 dilution. The plates were incubated overnight at 37°C. For the negative control, 10μl of competent cells was mixed with 100μl of the recovery medium and incubated at 37°C with shaking for 1 hr.

Preparation of E.coli cultures for protein expression at 20°C: Four 5ml bacterial starter cultures, containing 5μl ampicillin or streptomycin, each inoculated with a single colony of E.coli and incubated at 37°C with shaking overnight. Four 500ml bacterial starter cultures, containing 500μl ampicillin or streptomycin, each inoculated with a 2ml of starter cultures and incubated at 37°C with shaking until A_580 ranged between 0.6-0.85. Left on ice for 1 hr, 5ml of IPTG added and cultures incubated overnight at 20°C with shaking. All main cultures were centrifuged together at 7000G and 4°C for 25 mins. The supernatant was discarded, re-suspended pellet in 5ml of H_2O and centrifuged at 14,000G and 4°C for 10 mins after which the supernatant was discarded and placed pellets at -20°C.

Preparation of E.coli cultures for protein expression at 37°C: Four 5ml bacterial starter cultures, containing 5μl ampicillin (stock: 100μg/ml) or streptomycin (stock: 20μg/ml), each inoculated with a single colony of E.coli and incubated at 37°C with shaking overnight. Four 500ml bacterial starter cultures, containing 500μl ampicillin or streptomycin, each inoculated with a 2ml of starter cultures and incubated at 37°C with shaking until A_580 ranged between 0.6-0.85. A 1 ml Pre- aliquot of cells and media was taken from each main culture and placed on ice in sterile Eppendorf tubes. 5ml IPTG added to remaining cells in main cultures. Main cultures were incubated for 4 hrs at 37°C with shaking. 1 ml Post- aliquot of cells and media was taken from each main culture for each hour and placed on ice in sterile Eppendorf tubes. All main cultures were centrifuged together at 7000G and 4°C for 25 mins. The supernatant was discarded and re-suspended pellet in 5ml of water and centrifuged at 14,000G and 4°C for 10 mins, discarded the supernatant and placed pellets at -20°C.

Purification of Rv3915 from inclusion bodies: 500ml induction pellet were re-suspended in 40ml of lysis buffer (25mM Tris pH 8.0, 150mM NaCl, 0.5% Triton-X100, 1Mm EDTA) and sonicated using a macroprobe; 8-10 pulses, each pulse 30 secs with a 1 min break between each pulse. Incubated at room temperature with shaking for 30 mins after which 5mM MgCl_2 was added and incubated again at room temperature with shaking for 15 mins, centrifuged at 20,000G and 4°C for 20 mins. The supernatant was discarded and pellet re-
suspended in 40ml of 25mM Tris pH 8.0, 0.5M NaCl, 0.5% Triton-X100 and 1mM EDTA, centrifuged, discarded supernatant and the pellet re-suspended in 40ml of 25mM Tris pH 8.0, 0.5M NaCl and 1M urea. The sample was centrifuged, after which the supernatant was discarded and re-suspended pellet in 25mM Tris pH 8.0. 100µl aliquots of sample was made into eppendorf tubes, centrifuged and stored at -80°C.

Affinity Chromatography: After removal of storage solution, the column was washed once with urea, rinsed once with EDTA, washed with MilliQ three times, nickel loading solution added once to column and rinsed column three times with buffer A (5ml of 5M NaCl, 25ml of 1M Tris, made up to 1 litre). 500ml bacterial induction pellet was thawed and re-suspended in 10ml of buffer B (200ml of 5M NaCl, 25ml of 1M Tris, made up to 1 litre), sonicated using a macroprobe; 6 pulses, each pulse 40 seconds with a 40 seconds break between each pulse. The sample was aliquoted into 2ml eppendorf tubes and centrifuged at 15,000g and 4°C for 30mins. The supernatants were pooled and the pellets discarded. The nickel column was rinsed once with buffer B, then loaded pooled supernatant sample. The column was washed twice with 60M imidazole and eluted 1ml fractions with 400mM imidazole.

Gel Filtration: Form analytical gel filtration analysis Hiload 16/600 Superdex 200pg column was used. Equilibrated column with PBS buffer. Protein (1ml), purified on Ni-sepharose at a concentration of 1mg/ml, was filtered using 0.2 micron filter and injected to the column at a flow rate of 1ml/min. The chromatography was carried on protein AKTA system equipped with multi-wavelength UV/Vis detector set at 260 and 280nm, at flow rate of 1ml/min.

Dephosphorylation Assay: Purified culture supernatant on DEAE-sepharose ion exchange column in lysis buffer (50mM Tris HCl pH 8.5, 50mM NaCl, 10mM KCl, 25mM EDTA, 10% Glycerol) and eluted in 25mM Tris pH 8.5, 1M NaCl and 10mM KCl. Purified eluted sample on Ni-sepharose column in 25mM Tris pH 8.5, 1M NaCl and 10mM KCl and eluted in 200mM imidazole. Pooled together and concentrated fractions with the highest amount of protein, incubated at room temperature or 37°C with CIP in CIP buffer (50mM Tris HCl pH 7.9, 100mM NaCl, 10mM MgCl₂, and 1mM DTT) for 0, 0.5, 1 and 2hrs before the addition of 4xSRB.

SDS-PAGE: Samples were loaded and ran gel at 200V for 1hr. The gel was stained with Coomassie blue overnight and washed with water.

Zymogram Assay: 0.06g of Micrococcus luteus was added to 12% resolving gel mixture, in the absence of APS and TEMED. 60µl 10% APS and 20µl TEMED were added to the mixture and pipetted to fill ¾ of gel cassette and left to set for 30mins, with a layer of isopropanol
as a cover. Standard 5% stacking gel was prepared and pre-ran zymogram at 200V for 30mins. Samples were loaded and ran gel at 200V for 1hr. The zymograms were washed for 20mins, three times, in PBS + 0.1% Triton solution, incubated overnight at 37°C in PBS + 0.1% Triton solution and stained with methylene blue for 1hr and rinsed with water.

Western Blotting: Blot was placed in 50ml falcon tube, added 30ml of PBS + 1% milk and washed for 20mins. The PBS solution was discarded and the primary antibody (1µl of anti-poly His/anti-phosphothreonine in 2ml PBS + 1% milk solution) was added to the blot, left blot rolling overnight at -20°C and washed for 10mins, three times, with 30ml of PBS + 0.1% Tween 20 solution. After which 5ml of secondary antibody was added (1µl of Anti-mouse IgG in 10ml PBS + 1% milk solution) and left blot rolling at RT for 2hrs, washed with PBS + 0.1% Tween 20 solution, placed membrane in flat horizontal container and added 2ml of BCIP®/NBT Liquid substrate solution. This was left in dark until the visualization of bands.

Abbreviations

PknB: Protein kinase B, M. tuberculosis: Mycobacterium tuberculosis, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel, STPKs: serine/threonine protein kinases, PknA: protein kinase A, Ami 3: A cleaved/shorter form of the amidase domain of the protein Rv3915, C41: OverExpress C41 competent cells, C43: OverExpress C43 competent cells, E.coli: Escherichia coli, kDa: Kilo Dalton, Duet: Duet plasmid, 3915 Duet: Duet plasmid containing only the Rv3915 gene, pET: pET plasmid, pET Duet: pET Duet plasmid, pET 3915: pET plasmid containing gene for Rv3915 only, pET Duet PknB: pET Duet plasmid containing gene for PknB only, PknB 3915 Duet: pET Duet plasmid containing both genes for Rv3915 and PknB.

Declarations

Acknowledgements
We are grateful to Dr Galina Mukamolova for settling any doubts and answering any questions we had during the course of this research. We thank Maria Luisa Crosatti for the supply of materials needed.

Funding
Nil

Authors' contributions

EIN and OT conceived the experiments. OKC, NLN, OT and EIN conducted the experiments, analysed the results, wrote and reviewed the manuscript.
Competing interests

The authors declare that they have no competing interests.

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Figures
**Left Gel**, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), Pre-pellet of SH-C (Lane 2), Post-pellet of SH-C after 1hr (Lane 3), Post-pellet of SH-C after 3hrs (Lane 4), Pre-pellet of L (Lane 5), Post-pellet of L after 1hr (Lane 6), Post-pellet of L after 3hrs (Lane 7), Pre-pellet of Control (Lane 8), Post-pellet of Control after 1hr (Lane 9) and Post-pellet of Control after 3hrs (Lane 10). **Right Gel**, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 2), Post-pellet of SH-C after 20hrs Duplicate 1 (Lane 3), Post-pellet of SH-C after 20hrs Duplicate 2 (Lane 4), Post-pellet of L after 20hrs Duplicate 1 (Lane 5), Post-pellet of L after 20hrs Duplicate 2 (Lane 6), Post-pellet of Control after 20hrs Duplicate 1 (Lane 7) and Post-pellet of Control after 20hrs Duplicate 2 (Lane 8).

**SH-C**: Short form of protein without tail  
**L**: Long form/Entire protein  
**Control**: Empty Plasmid (PET 15b TEF)

**Figure 1**

SDS PAGE gel for C41 pre and post-induction samples.
**Figure 2**

SDS PAGE gel for C41 pre and post induction supernatant and pellet samples.
Left Blot showing band patterns of the Molecular weight marker (Lane 1), Pre-supernatant of SH-C (Lane 2) and Post-supernatant of SH-C after 3hrs (Lane 3). Right Blot showing band patterns of the Molecular weight marker (Lane 1), Pre-supernatant of L (Lane 2) and Post-supernatant of L after 3hrs (Lane 3).

Figure 3

Anti-Poly His Western Blot for C41 and pre- and post- induction supernatant samples.
Left Gel, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), L Supernatant Eluted Fraction 3 (Lane 2) and L Supernatant Eluted Fraction 4 (Lane 3). Right Gel, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), SH-C Supernatant Eluted Fraction 3 (Lane 2) and SH-C Supernatant Eluted Fraction 4 (Lane 3).

Figure 4

SDS PAGE gel for nickel affinity column purified C41 and SH-C supernatant samples.
Left Gel for samples expressed at 37°C, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), L Supernatant Eluted Fraction 4 (Lane 2) and pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 3). Right Gel for samples expressed at 20°C, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), L Supernatant Eluted Fraction 4 (Lane 2) and pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 3).

Figure 5

SDS PAGE gel for Nickel affinity column purified C41L and pET 3915DDD supernatant samples.
**Left Blot**, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1) and L Supernatant Eluted Fraction 4 (Lane 2). **Right blot**, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1) and pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 2).

**Figure 6**

Anti-Poly His Western Blots for Nickel affinity column purified C41L and pET 3915DDD supernatant samples.
Gel, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), preserved L Supernatant Eluted Fraction 4 (Lane 2), preserved pET 3915 DDD Supernatant Eluted Fraction 3 (Lane 3), preserved pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 4), degraded L Supernatant Eluted Fraction 4 (Lane 5), degraded pET 3915 DDD Supernatant Eluted Fraction 3 (Lane 6) and degraded pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 7).

**Preserved:** Fraction samples that had 4x Sample Buffer added to them immediately they were collected and left on bench  
**Degraded:** Fraction samples which didn’t have any Sample Buffer added after collection. These samples were mixed with 75% Glycerol and left on bench

**Figure 7**

SDS PAGE gel for Nickel affinity column, preserved and degraded C41L and C41  
  
pET 3915 DDD supernatant samples.
Gel, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), preserved L Supernatant Eluted Fraction 4 (Lane 2), preserved pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 3), degraded L Supernatant Eluted Fraction 4 (Lane 4) and degraded pET 3915 DDD Supernatant Eluted Fraction 4 (Lane 5).

**Preserved:** 100μL frozen aliquot of sample that had 4x Sample Buffer added to it as soon as it was taken out from the freezer.

**Degraded:** 100μL frozen aliquot of sample that was incubated for 2hrs at 37°C when it was taken out from the freezer, before 4x Sample Buffer was added to it.

**Figure 8**

SDS PAGE gel for Nickel affinity column, purified frozen preserved and degraded C41L and C41 pET 3915 DDD supernatant samples.
**Left Blot**, which is the anti-poly his western blot, showing band patterns of the Molecular weight marker (Lane 1), Pre-sample of 3915 Duet (Lane 2), Post-sample of 3915 Duet after 3hrs (Lane 3), Pre-sample of PknB 3915 Duet (Lane 4) and Post-sample of PknB 3915 Duet (Lane 5). **Right Blot**, which is the anti-phosphothreonine western blot, showing band patterns of the Molecular weight marker (Lane 1), Pre-sample of PknB 3915 Duet (Lane 2), Post-sample of PknB 3915 Duet after 3hrs (Lane 3), Pre-sample of 3915 Duet (Lane 4) and Post-sample of 3915 Duet (Lane 5).

**Figure 9**

Anti-Poly His and Anti-phosphothreonine Western Blots for C41 3915 Duet and PknB 3915 Duet pre- and post- induction samples
Gel, which was stained with Coomassie blue, showing band patterns of the Molecular weight marker (Lane 1), Ami 3 Inclusion bodies sample (Lane 2) and C41 WT Inclusion bodies sample (Lane 3). **Left Zymogram**, which was incubated at 37ºC in 40mM Citric acid buffer pH 6 and stained with Coomassie blue, showing no band of activity for Ami 3 sample expressed at 37ºC. **Right Zymogram**, which was incubated at 37ºC in 40mM Citric acid buffer pH 6 and stained with Coomassie blue, showing no band of activity for C41 WT sample expressed at 18ºC.

**Figure 10**

SDS gel for 37ºC inclusion bodies Ami3 and C41WT samples with corresponding mycobacterium luteus zymograms
Figure 11

Schematic showing direction of the action of RNA polymerase and the phosphorylation of RV3915 protein by PknB