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Role Of Phosphoylation Of The Mycobacterium Tuberculosis Proteasome In Oxidative Stress Response And Regulation Of Homoserine Kinase (thrb) In Corynebacterium Glutamicum

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ROLE OF PHOSPHOYLATION OF THE MYCOBACTERIUM TUBERCULOSIS PROTEASOME IN OXIDATIVE STRESS RESPONSE
-AND-
REGULATION OF HOMOSERINE KINASE (THRB) IN CORYNEBACTERIUM GLUTAMICUM

by

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Part I: Role of Phosphorylation of the *Mycobacterium tuberculosis* Proteasome in Oxidative Stress Response

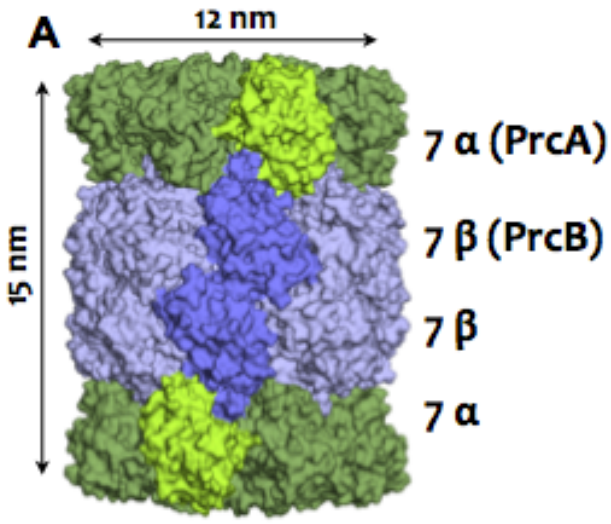
Chapter 1: Introduction

According to the World Health Organization (WHO), tuberculosis is second only to HIV/AIDS as the "greatest killer worldwide due to a single infectious agent." One-third of the world's population is infected with *M. tuberculosis* and most have the latent form of the disease [1, 2]. *M. tuberculosis* must be able to control its cell division and survival to maintain this latent state, even in response to nitrosative and oxidative stress conferred by the host. How *M. tuberculosis* controls these processes is not well known.

One way that *M. tuberculosis* may be able to control dormancy and cell division is through its eleven eukaryotic-like ser/thr protein kinases (STPKs) [3]. Among these STPKs, nine are trans-membrane kinases that may transduce extracellular signals into the cytoplasm [4]. Our lab has been focusing on two of these kinases, PknA and PknB by searching and studying the function of their substrates [5]. Recently, a previous member of the lab, Jae-il Han, has found that PknB phosphorylates the α-subunit of the proteasome in *M. tuberculosis*. It was suggested that this phosphorylation of the α-subunit affects the proteolytic activity of the proteasome. Mr. Han's preliminary data also hinted that PknA expression may affect the assembly of the proteasome complex [6]. My research thus has been focused on how PknA affects assembly of the proteasome complex and what physiological role this phosphorylation may have.

M. tuberculosis and *Mycobacterium smegmatis* contain the *prcBA* operon that encodes the 20S proteasome core complex [3, 7, 8]. The *prcA* gene encodes the α-subunit and the *prcB* gene encodes the β-subunit, which includes an N-terminal pro-peptide that is cleaved before the

half-proteasome assembles into the active holo-proteasome complex (Fig 1A). The 20S core complex is composed of 14 α-subunits and 14 β-subunits: 7α,7β,7β,7α (Fig 1B). While the pro-peptide of PrcB is not required for assembly (when the *M. tuberculosis* proteasome is expressed in *Escherichia coli*), it has been suggested that an inability to cleave the pro-peptide of PrcB causes a defect in assembly of the proteasome core complex [7]. It has previously been shown that *M. tuberculosis* proteasome will arrest at the half-proteasome stage if the pro-peptide is not cleaved, and that this intermediate is very similar to the *Rhodococcus* proteasome intermediate [9]. The proteasome assembly pathway of *Rhodococcus erythropolis*, a related Actinomycete, has been previously determined (Fig. 1B). We use this pathway as a basis for the assembly pathway in *Mycobacterium*. In *Rhodococcus* an α-subunit and a β-subunit with pro-peptide assemble into a dimer. These dimers then come together and form a hexameric ring, which makes a half-proteasome. The β-subunit pro-peptide will be cleaved, presumably by autocatalysis when the half-proteasomes assemble into an active holo-proteasomal complex [10].



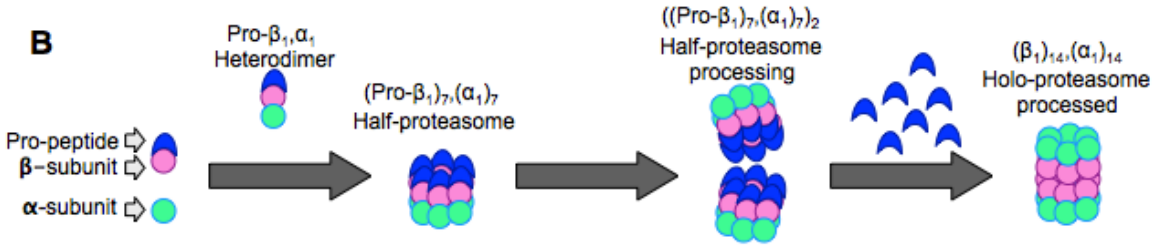


Fig. 1. **Proteasome Structure and Assembly Pathway A.** The proteasome core complex structure **B.** The pathway of proteasome assembly in *Rhodococcus erythropolis NI86/21*.

The 20S proteasome is the core complex, but there are other proteins that associate with the proteasome. It was discovered that the proteasome associates with *Mycobacterium* proteasomal ATPase (Mpa) that is believed to load substrate proteins into the core complex. Another proteasome accessory factor, PafA is believed to recognize a degradation signal on the substrate proteins and modify them with a small protein, prokaryotic ubiquitin-like protein (Pup) for degradation by the proteasome [11]. Mpa forms hexameric rings around the proteasome and is thought to work as a cap similar to a Eukaryotic 26S proteasome [12]. The Eukaryotic proteasome has a 20S core particle and a 19S cap, that includes an ATPase subunit as well as non-ATPase subunits [13]. Mpa in *M. tuberculosis* may functionally be homologous to the eukaryotic 19S cap[14]. Proteins can be targeted for degradation via Pup [15-18]. Pup is first deamidated by Dop (Deamidase of pup) [19], and then conjugated to targeted proteins by PafA [16]. Pup conjugated proteins will be recognized by Mpa [17] and loaded into the proteasome for degradation. The conjugation of Pup to proteins is reversible and Dop is also able to remove Pup [20, 21].

Previous work has shown that mutations in Mpa, PafA, and the proteasome results in lower resistance to reactive nitrogen intermediates (RNI) in *M. tuberculosis* [11, 12, 14, 19]. Interestingly, however, the same mutants in Mpa, PafA, or depletion of proteasome showed a higher resistance to H_2O_2 [22]. In this work we show that PknA phosphorylates the α subunit and

the β subunit of the *M. tuberculosis* proteasome. Moreover we show that this phosphorylation affects the ability of the proteasome to assemble into the core complex, which then improves *M. smegmatis* survival in the presence of H_2O_2.

Chapter 2: Materials and Methods 1

Bacterial strains and growth conditions

M. smegmatis MC^2155 cultures were grown at 37 °C in MiddleBrook 7H9 liquid medium (Difco) supplemented with 10% ADC (ADC = 5% (w/v) BSA fraction V powder, 2% (w/v) glucose and 0.85% (w/v) NaCl), and 0.05% (w/v) Tween-80 (7H9-ADC-Tw), or on Middlebrook 7H9-ADC agar plates supplemented with 0.05% (w/v) glycerol. Kanamycin (50 μg/ml), hygromycin (50 μg/ml) or apramycin (50 μg/ml) was added to culture media as indicated. *Escherichia coli* DH5α (Invitrogen), was used as a cloning host, and *E. coli* BL21 (DE3) was used for expression of recombinant proteins. Cultures were grown at 37 °C in LB broth or solid medium with apramycin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml) or ampicillin (50 μg/ml) where appropriate.

Expression, purification, and gel electrophoresis of protein

E. coli BL21(DE3) strains containing pACYCDuet1-P_{T7}-*prcB*-*prcA*-strep (pCK343) were transformed with pGEX-4-T3-*pknA* (pCK3) or pGEX-4-T3. Cultures were grown in the Overnight Express Autoinduction medium (Novagen) containing chloramphenicol and kanamycin. Cultures were grown for 10 hours at 37 °C and harvested. Cell pellet was stored at -80 °C until cells were lysed in a 1x PBS buffer containing protease inhibitors (Roche) and phosphatase inhibitors (Roche) by using a French Press (Thermo Scientific). PrcA-strep was affinity purified by using the Strep-Tactin resin (GE Healthcare) and the Profinia Protein Purification system (BioRad). Protein was dialyzed overnight with Thermo-Scientific Slide-A-Lyzer 10K MWCO Dialysis Cassettes in dialysis buffer (Tris-base 6 g, NaCl 16 g, KCl 0.4 g 50% glycerol per L, pH 7.4) at 16 °C. Purified protein was prepared for 2-D SDS-PAGE analysis

using Bio-Rad ReadyPrep 2-D Cleanup Kit, and stored at -80 °C. IPG strips pH 4-7 were rehydrated overnight. Isoelectric focusing (IEF) was done in Protean IEF Cell (Bio-Rad). After IEF, the strips were used for SDS-PAGE with 4-12% Criterion XT Pre-Cast gels (Bio-Rad). Gels were stained with GelCode Blue (Pierce Biotechnology Inc.).

15 μg of purified PrcA-strep and co-purified PrcB from *ΔprcBA M. smegmatis* MC^2155 containing pUAB100A-P_{Hsp60}-*prcB*-*prcA*-strep (pCK322, strain KMS108) and either pMH94-P_{acet}-*pknA* (pCK5, strain KMS111) or pMH94-P_{acet}-*pknB* (pCK7, strain KMS110) were prepared for 2-D SDS-PAGE analysis. Analysis was done as previously described.

E. coli cells containing ACYCDuet1-P_{T7}-*prcB*-*prcA*-strep (pCK343) and over-expressing pGEX-4-T3-*pknA* (pCK3) were used for affinity purification of PrcA-strep. Cells were grown in the Overnight Express Autoinduction medium (Novagen) containing chloramphenicol and kanamycin. Cultures were grown for 16 hours at 37 °C and harvested. Cells were stored at -80 °C, and then purified as previously described. 1 μg of purified PrcA-strep and co-purified PrcB was loaded onto an 8% SDS-PAGE followed by Western blot with phospho-T antibody (Cell signaling Technology) or anti-strep-II monoclonal antibody (Novagen). Protein was transferred with Trans-blot SD Semi-Dry Transfer Cell (Bio-Rad) for 15 min at 15V. Protein was visualized from HRP-conjugated secondary antibody and Immobilon Western Chemiluminescent HRP substrate (Millipore). It was developed with SNAP i.d. (Millipore) and visualized with ChemiDoc XRS and Quantity One ® 1-D analysis software.

In-gel kinase assay

E. coli BL21(DE3) strains containing pACYCDuet1-P_{T7}-*prcA*-P_{T7}-*prcB*-his (pCK426) were transformed with pKan5-P_{Ara}-his-GFP. Cultures were grown in the Overnight Express Autoinduction medium (Novagen) containing chloramphenicol and kanamycin. Cultures were

grown for 10 hours at 37 °C and harvested. Cell pellet was stored at -80 °C. PrcB-his was affinity purified using Bio-Scale Mini Profinity IMAC cartridge (Bio-Rad) with the Profinia Protein Purification system.

Kinase reactions were performed using about 250 nM PknA or PknB, with about 200 nM of purified prcBA, and 1 mM ATP. Reactions performed in kinase assay buffer (50 mM HEPES, 50 mM NaCl, 10 mM MgCl, 2 mM MnCl) and incubated for 2 hours at 37 °C. Proteins were separated on Native PAGE and either visualized by staining with Flamingo^{TM} Fluorescent Gel Stain (BioRad) or used for an in-gel proteasome assay. For the in-gel proteasome assay, the activity of proteasome was tested by incubating the gel in proteasome assay buffer (1 M HEPES pH 7.4, 0.5 M EDTA, water) and Suc-LLVY (Boston Biochem) for 30 minutes at 30 °C. Gel was analyzed using ChemiDoc XRS.

As a control in the assay, to test the activity of kinases used, a kinase activity assay was also performed by including Rv1422 (about 500 nM), a known substrate of PknA and PknB [5]. Reaction was incubated for 1 hour at 30 °C. Reaction was stopped with 6x SDS-Dye and analyzed by running a 1-D SDS-PAGE followed by Western blot with a phospho-T antibody.

H_2O_2 treatment of *M. smegmatis*

To test the *M. smegmatis* survival in H_2O_2 with or without the *M. tuberculosis* proteasome the *ΔprcBA M. smegmatis* MC^{2}155 (KMS98) strain was transformed with either pUAB100A (pCK315, strain KMS128) or pUAB100A-P_{Hsp60}-*prcB*-*prcA*-strep (pCK322) via electroporation. The strains were then cultured in a shaking incubator at 37 °C overnight and diluted to an initial OD_{600} = 0.01 in a Middlebrook 7H9 media supplemented with 10% ADC and 0.25% Tween-80 and containing hygromycin and apramycin. Cultures were treated with 4 mM

or 0 mM H_2O_2 and further incubated at 37° C for 45 min. After treatment the cultures were serially diluted into a buffer containing 0.8% NaCl and 0.01% Tween-80 and plated on Middlebrook 7H9 agar supplemented with 10% ADC and 0.02% glycerol. Plates were incubated at 37° C for three days. Colony forming units (CFU) and relative survival rate were determined.

To further test PknA effect on proteasome and *M. smegmatis* MC^2155 survival in H_2O_2, *ΔprcBA M. smegmatis* strain (KMS98) containing pUAB100A-P_{Hsp60}-*prcB-prcA-strep* (pCK322) was transformed with pMH94-*pknA* (pCK5) or pMH94 via electroporation. Seed cultures were grown overnight in Middlebrook 7H9 media supplemented with 10% ADC and 0.25% Tween-80 and containing 25 μg/ml of hygromycin, 25 μg/ml of apramycin, and 10 μg/ml of kanamycin. To induce the expression of kinases the initial seed was re-inoculated to provide an initial OD_{600} = 0.05 and 0.1% acetamide added. Cultures were grown at 37 °C for 5 hours. Treatment with H_2O_2 was repeated as previously described.

Chapter 3: Results

PknA affects the integrity of the *M. tuberculosis* proteasome core complex.

A previous member of our lab Mr. Jae-il Han demonstrated that PrcB was not co-purified with PrcA when he attempted to purify strep-tagged PrcA from *M. smegmatis* MC^2155 over-expressing PknA (Fig 2, middle lane) [6]. Mr. Han also demonstrated that the total levels of the proteasome complex were lower than those of cells with vector control or PknB over-expression because he had to load 5-10 times more purified protein in order to have a similar PrcA band as shown in Fig. 2.

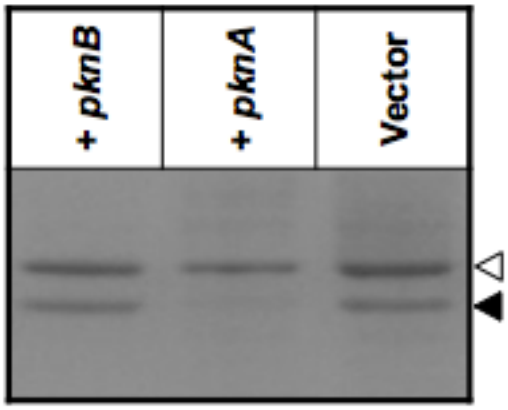


Fig. 2. 1-D SDS-PAGE of purified PrcA-strep from *M. smegmatis*. Mr. Han purified PrcA-strep (white arrow) and analyzed PrcA-step and co-elution of PrcB (black arrow) on a 1-D SDS-PAGE. Left lane, purified PrcA-strep with over-expression of *pknB*; Middle lane, purified PrcA-strep with over-expression of *pknA*; Right lane, purified PrcA-strep with vector alone. Note that he had to load 5-10 times more of elution from PknA-expressing cells than those of vector or PknB-over-expression to achieve the intensities of the protein bands shown in this figure.

In order to determine whether there was any PrcB in the sample or if mutations in the *pknA*-over-expressing construct caused this result, we reexamined the purified protein by 2-D PAGE analysis. This 2-D analysis allowed us to load more protein samples (15 μg) per gel and therefore to examine if there is processed and unprocessed PrcB in the sample. Similar to Mr.

Han we had to load more protein from the *pknA*-over-expression sample in order to have similar PrcA-strep bands. Fig. 3 shows that when PknA is over-expressed there are low levels of both unprocessed and processed PrcB suggesting that *pknA*-over-expression affects either the assembly or the stability of the proteasome complex. This result also suggests that if the assembly is indeed arrested, it could be at the half-proteasome stage. Taken together, PknA expression caused less proteasome to be purified (remember that we had to load 5-10 times more of elutions) and less holo-proteasome complex formation (about half of PrcB is in its unprocessed form when PknA was expressed, as compared to the near complete processing of PrcB when PknB was expressed). This suggested that PknA affects either the assembly or the stability of the proteasome complex.

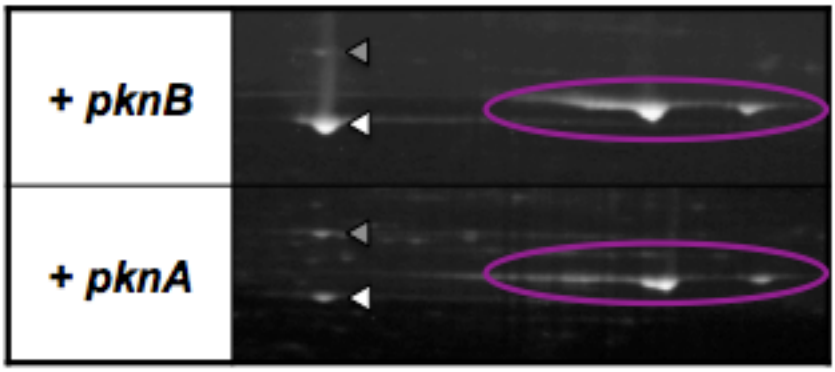


Fig. 3. PknA affects the integrity of the proteasome core complex. 2-D and Flamingo staining of purified PrcA-strep (purple circle) (15 μg) and unprocessed PrcB (gray arrow) and processed PrcB (white arrow) in the presence of PknB (top) or PknA (bottom) in *M. smegmatis* MC^2155.

PknA has a direct effect on the proteasome complex formation

To determine if the effect of PknA on the proteasome complex formation is direct we performed a similar experiment in *E. coli*, which does not have the proteasome system or a PknA kinase homolog. We grew *E. coli* cells containing pACYCDuet-1-P_{T7}-*prcB*-*prcA*-strep (pCK343)

with either pGEX-4-T3 or pGEX-4T-3-P_{T7}-*pknA* (pCK3), and purified PrcA-strep using the Strep-Tactin resin (GE Healthcare) and the Profinia Protein Purification system (BioRad). We then analyzed the purified PrcA-strep and co-eluted PrcB in a 2-D SDS-PAGE gel, stained with GelCode Blue and analyzed with ChemiDoc XRS. As shown in Fig. 4, when PknA is over-expressed the ratio of PrcB to PrcA is 0.32 compared to the ratio of PrcB to PrcA when there is vector alone (0.56), meaning there is less PrcB in relation to PrcA when *pknA* is over-expressed. In Fig. 4, the white arrow points to the unprocessed PrcB, which demonstrates that when *pknA* is over-expressed there is less processing of PrcB. Also, it was necessary to load two-fold more protein when PknA was over-expressed compared to vector alone. Therefore there is a similar effect by PknA on the proteasome complex formation in *E. coli* where there is less proteasome as well as less processed PrcB. Since *E. coli* does not have the proteasome machinery, this result further suggests that PknA directly affects the formation of the proteasome complex, probably by phosphorylation.

	vector	***pknA*-overexpression**
GelCode staining		
Ratio of PrcB/PrcA	0.56	0.32
Total lysate protein used to purify	60 µg	120 µg

Fig. 4. PknA has a direct effect on the proteasome complex formation. 2-D and GelCode Blue staining of purified PrcA-strep (purple circle) and co-purified unprocessed PrcB (gray arrow) and processed PrcB (white arrow) with *pknA*-over-expression or pGEX-4-T3 alone. The ratio of PrcB/PrcA determined by ChemiDoc XRS and Quantity One ® 1-D analysis software.

PknA affects the integrity of the complex through phosphorylation of PrcA and unprocessed PrcB

If the effect of PknA is direct, we predicted that PknA exerts its effect on the proteasome complex formation via phosphorylating the processed PrcB, unprocessed PrcB, or PrcA. To test this prediction, we purified PrcA-strep from *E. coli* over-expressing PknA. We performed Western blot analyses with a phospho-T and anti-Strep antibodies. Fig. 5 shows that when PknA is over-expressed in *E. coli* both PrcA and unprocessed PrcB are phosphorylated (Lane 1). Lane 2 shows the location of PrcA-strep (white arrow). This result suggests that, since the effect of PknA on the proteasome is direct, the direct effect is most likely due to the phosphorylation of the unprocessed PrcB and the PrcA subunits.

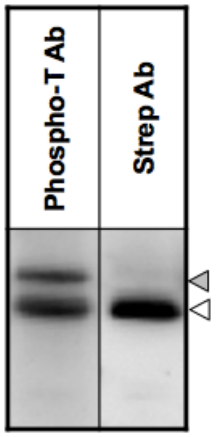


Fig. 5. PknA affects the integrity of the complex through phosphorylation of PrcA and unprocessed PrcB. Western blot analyses of purified PrcA-strep (white arrow) and co-purified prcB (gray arrow) in the presence of *pknA*-over-expression in *E. coli* BL21 (DE3) with phospho-T antibody (lane 1) or strep (lane 2) antibody.

PknA does not affect the stability of the proteasome complex

Less processing of PrcB suggested that the proteasome complex formation may be arrested by PknA-over-expression at the half-proteasome stage, but we also wanted to

understand why there is less total proteasome purified. One possibility is that phosphorylation by PknA causes the proteasome complex to alter its assembly.

To test this, we incubated the purified holo-proteasome complex with PknA and analyzed the possible disassembly of the proteasome complex and its proteolytic activity by using a native PAGE and the in-gel proteasome assay. In the in-gel assay, we used a fluorescent peptidyl substrate: succinyl-leu-leu-val-tyr-AMC (Suc-LLVY-Amc), which will fluoresce when the AMC is cleaved off by the proteasome. Before we conducted the actual assays, we first wanted to test the activity of the purified kinases, PknA and PknB, by incubating with a known substrate Rv1422. After the reaction, we ran an SDS-PAGE and did a Western blot analysis with a phospho-T antibody, and visualized with the ChemiDoc system. Both of the kinases were active since both PknA and PknB autophosphorylate (white arrow), and they phosphorylate Rv1422 (gray arrow) (Fig. 6B).

We then conducted kinase reactions with PknB and the purified proteasome, PknA and proteasome, PknB alone, PknA alone, and proteasome alone. We used half of the kinase reactions for a native PAGE and staining with Flamingo stain (Fig. 6A), in order to test the integrity of the proteasome complex. We used the other half of the kinase reactions for the in-gel assay on a second native gel where we incubated the gel with the fluorescent substrate (Suc-LLVY-AMC) to test the proteolytic activity of the proteasome complex. As shown in Fig. 6, the proteasome complex was still able to degrade Suc-LLVY-AMC, suggesting that PknA did not affect the stability of the proteasome complex *in vitro* (Fig. 6, black arrow bottom). Even in the presence of either kinase the proteasome is still functional, suggesting the holo-proteasome complex does not destabilize or fractionate.

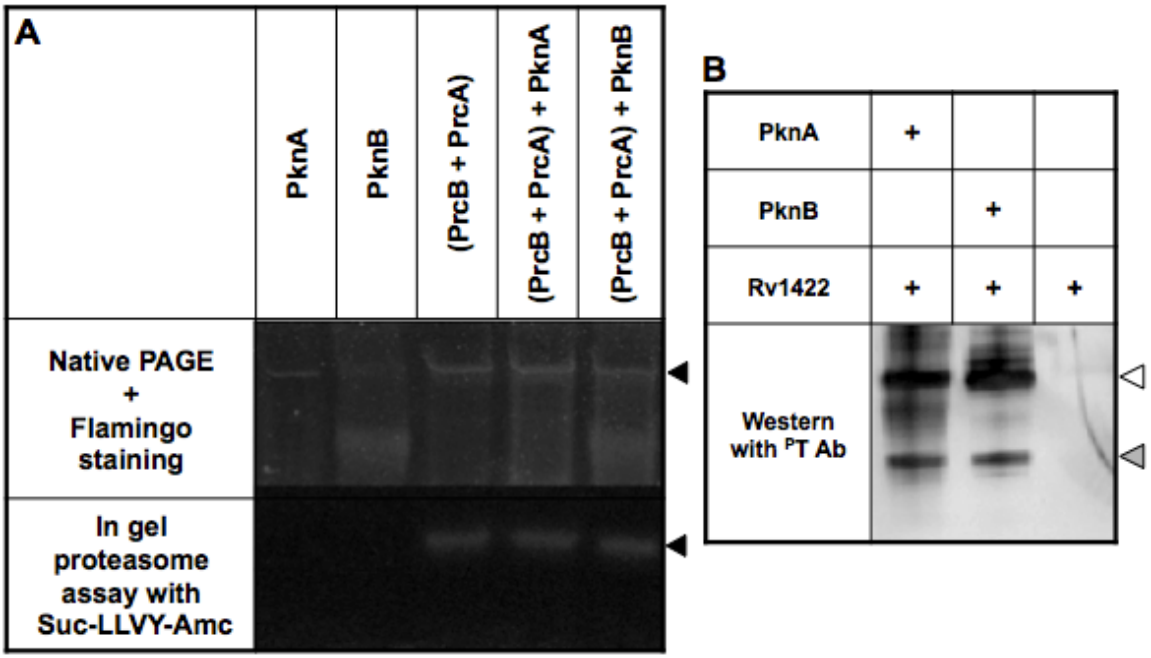


Fig. 6. PknA does not affect the stability of the proteasome complex. **A**. Native PAGE for in-gel proteasome assay or Flamingo staining to determine proteasome (black arrow) activity in the presence of PknA. **B**. SDS-PAGE and Western, with phospho-threonine antibody, of kinase assay showing activity of both kinases. White arrow is PknA or PknB, and gray arrow is Rv1422.

PknA may help *M. smegmatis* survive in the presence of H_2O_2

It has been shown previously in *M. tuberculosis* that having the proteasome complex causes a decrease in its survival in the presence of H_2O_2 [22]. We wanted to test if this effect would be the same in *M. smegmatis*, because if the proteasome also negatively affected the response of *M. smegmatis* to H_2O_2, it would provide us with a system to test the effect of the *M. tuberculosis* PknA on the response to H_2O_2 in *M. smegmatis*. For this we treated Δ*prcBA M. smegmatis* MC^2155 cells containing either pUAB100A or carrying pUAB100A-*prcBA* with 4 mM or 0 mM H_2O_2 for 45 minutes. After treatment we diluted the samples and plated them to measure CFU. We divided the number of colonies on the 0 mM plates by that of the 4 mM plates in order to determine the survival rate. We then set the survival rate of Δ*prcBA M. smegmatis* MC^2155 + pUAB100A-*prcBA* to one and determined the relative survival rate of the two strains.

As shown in Fig. 7., the relative survival rate of Δ*prcBA M. smegmatis* MC^{2}155 with vector only was two times higher than that of Δ*prcBA M. smegmatis* MC^{2}155 carrying pUAB100A-*prcBA*. This experiment was performed in triplicate and the p-value was determined to be 0.007 (one-tailed, paired t-test). This suggests that the proteasome in *M. smegmatis*, similar to *M. tuberculosis*, negatively affects its survival in the presence of H_2O_2.

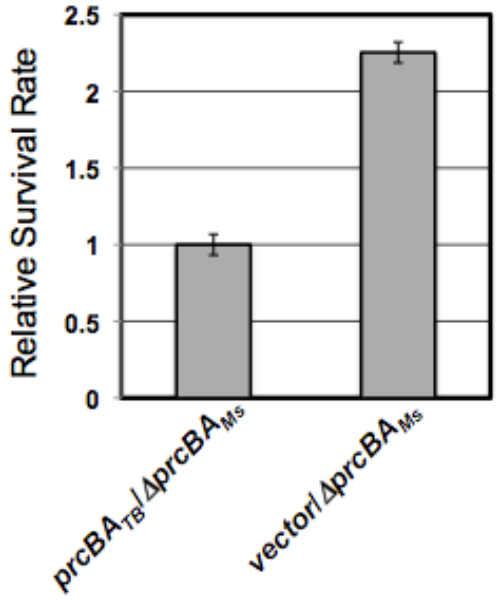


Fig. 7. Lack of proteasome may help *M. smegmatis* survive better in the presence of H_2O_2. Relative survival rate of *ΔprcBA M. smegmatis* MC^{2}155 containing constitutive *prcBA* expression or vector alone. Survival rate was determined by CFU from 4mM H_2O_2 sample divided by CFU from 0 mM H_2O_2 sample after 45 min. Survival of ΔprcBA cells with *prcBA* is set to 1 in order to determine the relative survival rate of the two strains. The p-value of prcBA$_{TB}$/ΔprcBA$_{MS}$ vs. vector/ΔprcBA$_{MS}$ is 0.007. Experiment performed in triplicate.

Since *M. smegmatis* shows the similar effect by the depletion of proteasome as *M. tuberculosis* we were able to use it to test the effect of PknA on the integrity of the proteasome in the presence of H_2O_2. We showed earlier that PknA over-expression affected the assembly of the *M. tuberculosis* proteasome complex in *M. smegmatis*. Since lack of proteasome allows a higher survival rate of *M. smegmatis* in the presence of H_2O_2 we wanted to test if *M. smegmatis* cells

with PknA and proteasome will survive better in the presence of H_2O_2 than *M. smegmatis* cells without PknA but with proteasome. We hypothesized that the PknA will increase the survival of cells containing proteasome by affecting the assembly of the proteasome complex.

To test this hypothesis we used Δ*prcBA*$_{Ms}$ *M. smegmatis–prcBA* carrying pUAB100A-*prcBA* and transformed with pMH94-P_{acet}-*pknA* or pMH94. We performed the H_2O_2 experiment as previously described. We determined the survival rate of the strains by determining CFU and dividing the number of colonies on the 0 mM H_2O_2 plate by the number of colonies on the 4 mM H_2O_2 plate. We then set the pcrBA + pMH94 strain to 1 to determine the relative survival rate (Fig. 8). The strain over-expressing PknA (as shown in Fig. 8) confers a significantly higher relative survival rate (1.39), with a p-value 0.01 (one-tailed, paired t-test). PknA over-expressing cells are able to survive better in the presence of H_2O_2, and PknA over-expression disrupts the proteasome assembly, we therefore determined from this experiment that PknA can potentially increase *M. smegmatis* resistance to H_2O_2 by arresting proteasome formation.

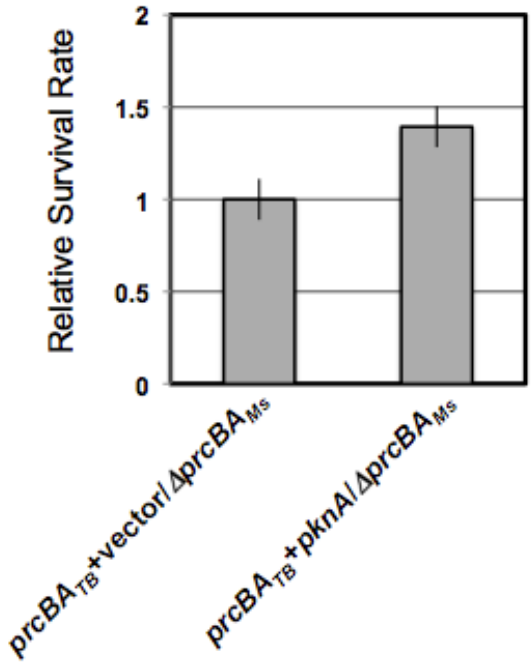


Fig. 8. PknA may help *M. smegmatis* survive in the presence of H_2O_2. Relative survival rate of *ΔprcBA M. smegmatis* MC^2155 containing constitutive *prcBA* expression containing pknA over-expression or vector alone. Survival rate determine by 4mM H_2O_2 CFU after 45 min. divided by 0 mM H_2O_2 CFU after 45 min. prcBA + vector alone in ΔprcBA cells is set to 1 in order to determine the relative survival rate of the two strains. P-value of $prcBA_{TB}$+vector/Δ$prcBA_{MS}$ vs. $prcBA_{TB}$+pknA/Δ$prcBA_{MS}$ = 0.01. Experiment performed in triplicate.

Chapter 4: Conclusions and future directions

M. tuberculosis proteasome is necessary for the resistance of *M. tuberculosis* to nitrosative stress conditions, for persistence in mice, however it hinders *M. tuberculosis* from being able to grow in the presence of H_2O_2 [11, 22]. Understanding how the proteasome is regulated may better explain why it is necessary for nitrosative resistance, but harmful in the presence of H_2O_2. Previous data have shown that eukaryotic 20S proteasomes first assemble into half proteasome complexes before assembling into a holo-proteasome complex [23], and that *M. tuberculosis* proteasome is most similarly related phylogenetically to the eukaryotic 20S proteasome [24]. Because they are phylogenetically related and have a similar assembly process, previous research into eukaryotic 20S proteasome regulation may be potentially applicable to *M. tuberculosis* proteasome regulation. It has also been previously shown in eukaryotic 20S proteasome assembly, ser/thr phosphatase inhibitors caused "decreased processing of assembly intermediates" and that kinases "play a role in the assembly and disassembly of 26S complexes"[25]. Phosphorylation of the eukaryotic proteasome intermediates is one way that phosphorylation affects the regulation of proteasome, and we have shown in this work that PknA directly phosphorylates the α and unprocessed β subunits of the *M. tuberculosis* proteasome. This phosphorylation potentially hinders the processing of the β subunit, and consequently causes a loss of holo-proteasome complex. It will be important to determine which residue is phosphorylated in these subunits. Previous work has shown the pro-peptide on the β subunit can hinder the formation of the holo-proteasome core complex in *M. tuberculosis* [7]. Thus, it can be speculated that the phosphorylation could occur on the pro-peptide of the β subunit in *M. tuberculosis*. Our lab is currently elucidating the phosphorylation sites on the unprocessed PrcB and PknA.

Another kinase of *M. tuberculosis* STPK's, PknK, has been shown to be beneficial for survival in the presence of oxidative stress, at the early stages of infection in mice. Both PknK and PknA are expressed at the same time in *M. tuberculosis* [26]. We show that phosphorylation of the proteasome affects the assembly of the proteasome potentially in order to increase resistance to oxidative stress. While the effect that we demonstrate is not great, we were able to replicate this effect over several experiments.

After further research into the effect of PknA on proteasome, it will be necessary to characterize how PknA is able to arrest proteasome formation. H_2O_2 has been previously shown to directly activate a IκB kinase through serine residues in an activation loop [27]. H_2O_2 also directly activates Protein Kinase C which in turn is able to phosphorylate other proteins in response to this stressor [28], This would suggest that it is possible that PknA may be directly activated by H_2O_2 and further testing of this possibility will be important in understanding how *M. tuberculosis* can persist and survive inside the macrophage.

Part II: Regulation of Homoserine kinase (ThrB) in *Corynebacterium glutamicum*

Chapter 5: Introduction

When the livestock are deficient in an amino acids, they are less able to utilize other amino acids and consequently add more nitrogen compounds into the atmosphere [29]. Adding L-threonine into livestock feed will therefore make healthier animals as well as a cleaner environment. Another importance of L-threonine is that it is used in the pharmaceutical and cosmetic industries. *C. glutamicum* is a grade-A food safe microorganism, which makes it ideal for the industrial production of amino acids compared to other bacterial species, such as *E. coli*, which produces lipopolysaccharides and other endotoxins[30].

L-threonine is one of the aspartate family amino acids. It is synthesized from aspartate through a series of reactions, some of which are feedback inhibited by L-threonine (Fig. 9). Two genes, *hom* and *thrB* make up an operon that encodes homoserine dehydrogenase (Hom) and homoserine kinase (ThrB), respectively. ThrB is feedback inhibited by 25-30 mM threonine [31] (Fig. 9, D), and homoserine dehydrogenase is also feedback inhibited by 5 mM threonine [32] (Fig. 9, C). Aspartate kinase is inhibited by L-threonine [33] (Fig. 9, A). None of the other enzymes in this pathway are inhibited by L-threonine [33, 34] (Fig. 9, B,E). The inhibition of homoserine kinase requires larger amounts of threonine, but when *hom-thrb* is over-expressed intracellular concentration of threonine can increase up to 100 mM, compared to 9 mM threonine concentration in the lysine over-producing strain [35].

Our long-term goal of this project is to construct a strain of *C. glutamicum* that over-produces threonine. To achieve this goal, I focused on the feedback inhibition of homoserine kinase (ThrB). While it was possible to release the feedback inhibition of enzymes in the pathway such as aspartate kinase and homoserine dehydrogenase [36, 37] it has not been possible to do so with homoserine kinase (ThrB) because threonine feedback inhibits ThrB by competing with the substrate L-homoserine for the same active site [38].

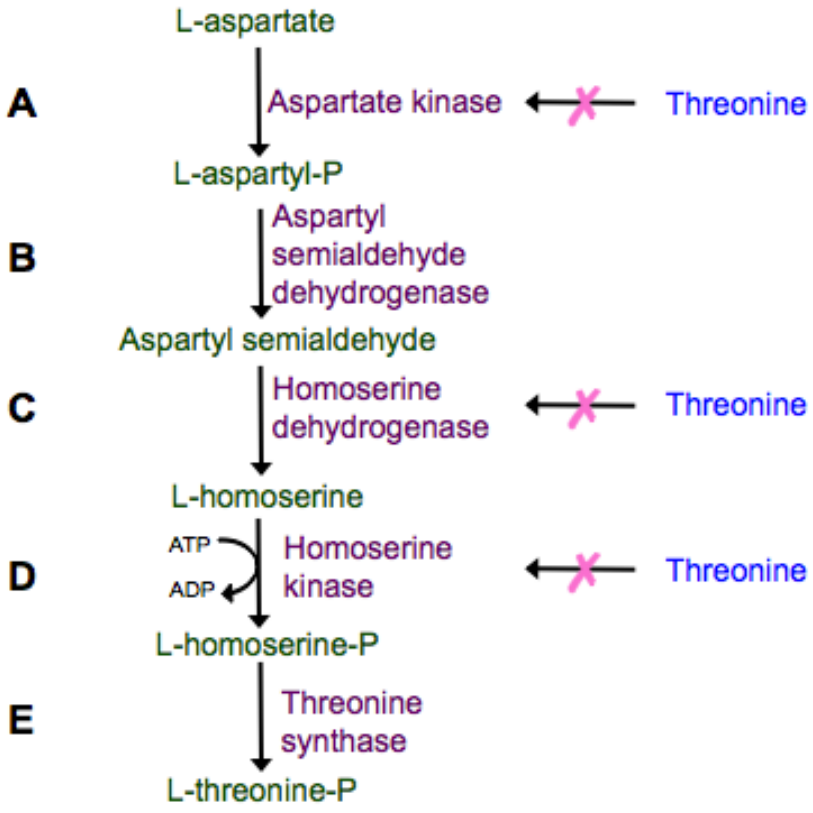


Fig. 9. Pathway for L-threonine biosynthesis. Homoserine kinase protein encoded by *thrB* gene in *C. glutamicum*.

When we analyzed the 3-D structure of ThrB with either homoserine or threonine bound, we found that Ala16 reside is located at the entrance of the active site (Fig. 10). Through the collaboration with Dr. Don Ronning group at the University of Toledo, it was suggested that the

alteration of this Ala16 residue may differentially affect the substrate (homoserine) and inhibitor (threonine) bindings, which then contribute to the release of feedback inhibition of ThrB by threonine. I will address this possibility by constructing various mutations of Ala16 and examining the effect of these mutations on the enzymatic activity of ThrB.

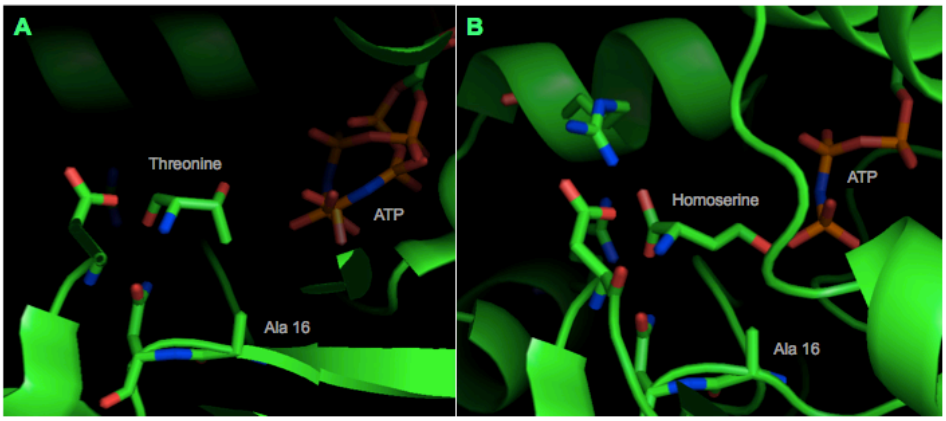


Fig. 10. Active Site of *C. glutamicum* homoserine kinase A. Active site of ThrB when threonine is present. **B.** Active site of ThrB when homoserine is bound.

Chapter 6: Materials and Methods

Bacterial strains and growth conditions

Please refer to Materials and Methods Part 1.

Plasmid construction

The *thrB-his* genes were amplified by PCR using ThrBFor and ThrBRev primers (Table 2s) and the *C. glutamicum* ATCC 13032 genomic DNA as a template. PCR product was cloned into the pACYCDuet-1 plasmid (Novagen) behind a T7 promoter. Ala16 of *thrB* was mutated to glycine, valine, serine, or leucine using Stratagene's QuickChange Site-Directed Mutagenesis kit. The *thrBA16G* allele was PCR amplified using primers c59g and c59g_antisense, and pACYCDuet-1-P_{T7}-*thrB-his* as template. The *thrBA16V* allele was amplified with c59t and c59t_antisense primers, the *thrBA16S* allele with g58t and g58t_antisense, and the *thrBA16L* allele with g58c_c59t and g58c_c59t_antisense primers. All primers are listed in Table 2.

Expression and purification of the ThrB proteins.

All constructs were transformed into *E. coli* BL21 (DE3) and transformants were grown in LB broth with 34 μg/ml chloramphenicol. Gene expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at mid-log phase for 3 hours at 37 °C. Cell pellet was stored at -80 °C until cells were lysed in a 1x PBS buffer containing protease inhibitors (Roche) and phosphatase inhibitors (Roche) by using a French Press (Thermo Scientific). Protein was purified using Bio-Scale™ Mini Profinity™ IMAC Cartridges and the Profinia Protein Purification System (Bio-Rad). Protein was dialyzed overnight as previously described. Purified protein run

on 1-D SDS-PAGE, stained with GelCode Blue (Pierce Biotechnology Inc.), and visualized on ChemiDoc XRS.

In vitro Kinase Assay of ThrB

Kinase activity was determined via the ADP Quest Assay kit (DiscoveRx). Ultra-pure ATP (Sigma-Aldrich) was diluted in 10 mM Tris-Cl, pH 7.0. Enzyme and homoserine were diluted in an assay buffer provided by the manufacturer. Reactions were performed as described in the provided protocol with about 40 nM kinase, 1 mM homoserine, 3 mM ATP, and kinase in a 96 well microtiter plate at 30 °C. Fluorescence from the reactions were monitored by Gemini XPS at λ_{EX} = 530 nm and λ_{EM} = 590 nm.

Chapter 7: Results

Active Site of Homoserine Kinase

In order to better understand the homoserine kinase (ThrB), we aligned the amino acid sequences of ThrB from various microbes. We found that there is a SSAN motif that is conserved among those microbes we examined. As an example, Fig 10 shows the sequence similarity between *E. coli* and *C. glutamicum*. Based on the 3D structure, we hypothesized that the Ala16 may play an important role in binding of homoserine (substrate) and threonine (inhibitor), and the alteration of this residue may differentially affect the binding of these molecules. To test this hypothesis, we mutated the Ala16 residue into glycine, valine, serine and leucine, and test their effects on the activity of ThrB.

```
E.coli      ---------MVKVYAPASSANMSVGFDVLGAAVTPVDGALLGDVVTVEAAETFSLNNLGR  51
C.Glu       MAIELNVGRKVTVTVPGSSANLGPGFDTLGLALSVYDTVEVEIIPSGLEVEVFGEG----  56
                      *.*  *.****:. ***.** *    * . :  : :   .*.*. .
```

Fig. 11. Alignment of *C. glutamicum* homoserine kinase sequence with that of *E. coli*. Conserved motif in *E. coli*, top line, pink. Conserved motif in *C. glutamicum*, bottom line, green.

Homoserine Kinase Purification and Activity

We made these mutations by site-directed mutagenesis of the homoserine kinase gene using Stratagene's QuickChange Site Directed Mutagenesis Kit. We mutated the Ala16 residue to glycine, valine, serine, or leucine. Some microbes we examined have glycine instead of Ala, and thus it may have a unique role in ThrB. Valine and leucine are both bulkier and more hydrophobic than the conserved alanine, which may enhance the binding of homoserine more than that of threonine. (Fig. 9, B and C). We also mutated Ala16 to serine, which has a similar

size with Ala but has a hydroxyl group that may block the access of threonine that is more hydrophilic with a hydroxyl group than homoserine.

PCR was used to amplify the *thrB-his* gene from *C. glutamicum* ATCC 13032 genomic DNA. It was then cloned into the pACYCDuet-1-P_{T7} plasmid, which was sequenced and used as a template for the PCR reactions of the site-directed mutagenesis. Each construct was transformed into *E. coli* BL21(DE3) to express and purify recombinant proteins. Protein expression and purification were confirmed through 1-D SDS-PAGE (Fig. 12).

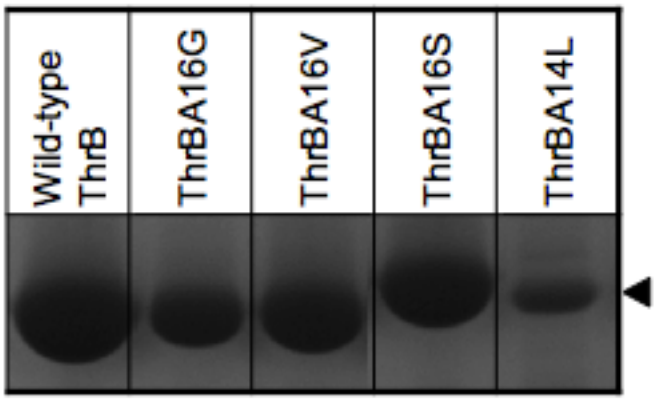


Fig 12. Purification of ThrB and ThrB mutants. Confirmation of the purification of the wild-type ThrB (first lane) and ThrBA16G (second lane), ThrBA16V (third lane), ThrBA16S (fourth lane), ThrBA16L (last lane). Protein run on a 1-D SDS-PAGE and stained with GelCode Blue (Pierce Biotechnology Inc.). Visualized with ChemiDoc XRS.

Purified protein was then subjected to the ADP Quest Assay Kit. ADP Quest uses an enzyme couple reaction where ADP is detected and a detectable resorufin fluor is created (ADP + PEP + cofactors → H_2O_2 +ADHP → Resorufin Fluor). The Fluor can be detected by a microplate reader at λ_{EX} = 530 nm and λ_{EM} = 590 nm. Wild-type ThrB had a slope of about 13.3, with the glycine mutant showing slightly slower activity with a slope of about 8. The leucine mutant showed activity, but at a much slower rate with a slope of about 1.3 (Fig. 13). Neither the

valine, nor the serine mutants had activity, as their slope was the same as wells that did not contain kinase (about 1) (Fig. 13).

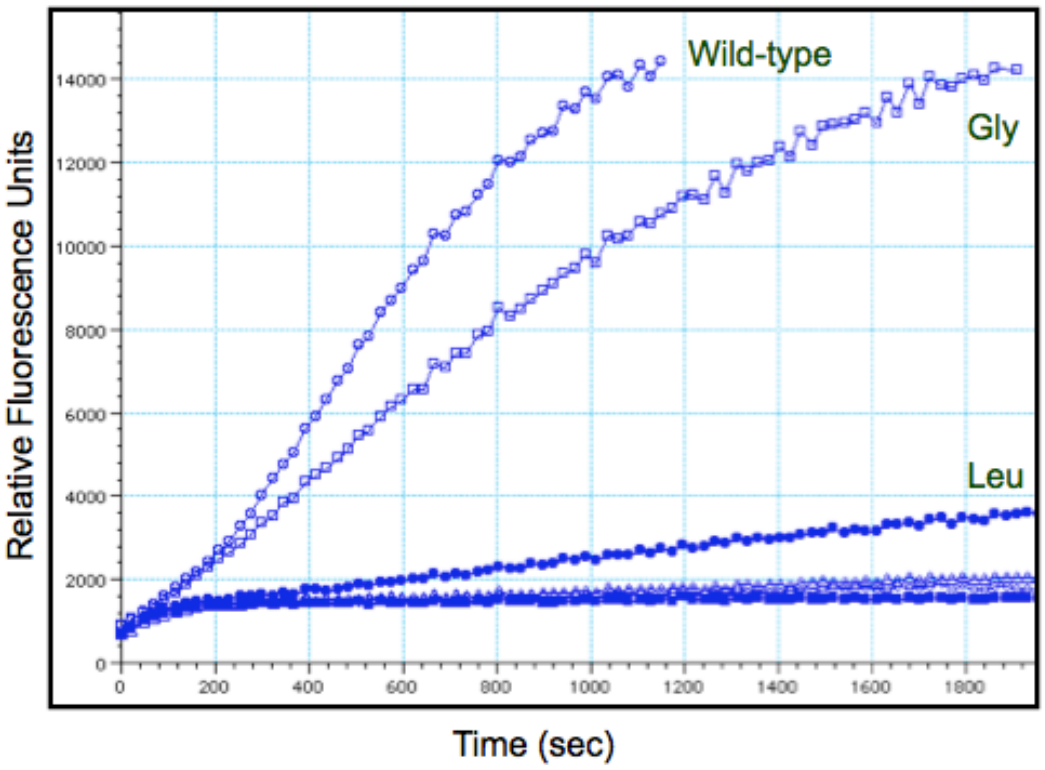


Fig. 13 Kinase activity assay of Homoserine Kinase. Wild-type homoserine kinase, A16G homoserine kinase, and A16L homoserine kinase all show activity. A16S homoserine kinase and A16V homoserine kinase are both fluorescing at the same level as wells without enzyme.

Chapter 8: Conclusions and future directions

We found that two mutant forms of ThrB (A16G and A16L) remained but decreased activity. Thus, the next step is to test if these mutant forms are still feedback inhibited and if so by how much. However, repeating the experiment described above should be done with a more stabilized assay method since we found that the assay results fluctuated. If we find any mutant form of ThrB that has decreased the feedback inhibition more than that of enzymatic activity toward homoserine, then we will introduce the allele into the *Corynebacterium glutamicum* strain to test if it can increase the biosynthesis of threonine inside the cell.

It is possible that since the inhibition of homoserine kinase requires large amounts of threonine, compared to homoserine dehydrogenase, it would be a good idea to mutate homoserine dehydrogenase instead of homoserine kinase. However, *hom* mutations can cause high intracellular concentrations of threonine [38], so it would eventually be beneficial to mutate *thrB*. Our approach to *thrB* mutation was novel because we attempted to genetically separate the catalytic activity and feedback inhibition that act on the same active site. Upon successful completion this approach may then be applied to similar situations in other amino acid production in *C. glutamicum* and other microbes.

It will eventually be necessary to mutate certain excretion mechanisms, as they are limiting factors for intracellular threonine levels [35, 39]. In addition, our lab intends to analyze the metabolomics and phenotypic microarray of a known lysine over-producing strain of *C. glutamicum,* that was engineered through random mutagenesis, in order to find novel mutation sites that may be applicable to our final goal of threonine over-production, since they are both produced through the same aspartate family of amino acids.

Table 1: List of Plasmids and Strains

Plasmids

pMH94	mycobacterial integration vector (Kan^r)
pCK5	pMH94-P_{acet}-*pknA* (Kan^r)
pCK7	pMH94-P_{acet}-*pknB* (Kan^r)
pCK343	pACYCDuet-1-P_{T7}-*prcB-prcA*-strep (Cm^r)
	pGEX-4T-3
pCK3	pGEX-4T-3-P_{T7}-*pknA*
pCK426	pACYCDuet-1-P_{T7}-*prcA*-P_{T7}-*prcB*-his
pCK315	pUAB100A Km^r replaced with $Apra^r$
pCK322	pUAB100A-P_{Hsp60}-*prcB-prcA*-strep
	pKan5-P_{Ara}-*his-GFP*
pCK448	pACYCDuet-1-P_{T7}-*thrB-his*
pCK449	pACYCDuet-1-P_{T7}-*thrBA(16)G-his*
pCK450	pACYCDuet-1-P_{T7}-*thrBA(16)V-his*
pCK454	pACYCDuet-1-P_{T7}-*thrBA(16)S-his*
pCK453	pACYCDuet-1-P_{T7}-*thrBA(16)L-his*

Strains

Top 10	*E. coli* for molecular cloning
BL21 (DE3)	*E. coli* for protein expression
Mc^2155	WT *M. smegmatis*
KMS98	Δ*prcBA*$_{Ms}$ *M. smegmatis – prcBA* replaced with Hyg^r

KMS108	KMS110 carrying pCK322
KMS109	KMS111 carrying pCK322
KMS110	KMS98 carrying pCK7
KMS111	KMS98 carrying pCK5
KMS128	KMS98 carrying pCK315

Table 2: List of Primers

Primers

ThrBFor	5’ cct cat atg gca att gaa ctg aac gtc 3’
ThrBRev	5’ gga ctc gag cta gtg gtg atg atg gtg gtg agg ttg gtt aac ttc aac ctt g 3’
c59g	5’ tac ctg gat ctt ctg gaa acc tcg gac ctg g 3’
c59g_antisense	5’ cca ggt ccg agg ttt cca gaa gat cca ggt a 3’
c59t	5’ gta cct gga tct tct gta aac ctc gga cct ggc 3’
c59t_antisense	5’ gcc agg tcc gag gtt tac aga aga tcc agg tac 3’
g58t	5’ ggt acc tgg atc ttc ttc aaa cct cgg acc tgg 3’
g58t_antisense	5’ cca ggt ccg agg ttt gaa gaa gat cca ggt acc 3’
g58c_c59t	5’ ggt acc tgg atc ttc tct aaa cct cgg acc tgg c 3’
g58c_c59t_antisense	5’ gcc agg tcc gag gtt tag aga aga tcc agg tac c 3’

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ABSTRACT

ROLE OF PHOSPHOYLATION OF THE MYCOBACTERIUM TUBERCULOSIS PROTEASOME IN OXIDATIVE STRESS RESPONSE -AND- REGULATION OF HOMOSERINE KINASE (THRB) IN CORYNEBACTERIUM GLUTAMICUM

by

HEATHER BAUN

May 2013

Advisor: Choong-Min Kang

Major: Biological Sciences

Degree: Master of Science

Part 1:

One-third of the world's population is infected with the latent form of *Mycobacterium tuberculosis*. *M. tuberculosis* must be able to control this latent even in response to nitrosative and oxidative stress conferred by the host. It is not well known how *M. tuberculosis* controls this state.

One possibility for this control is through its eleven eukaryotic-like serine/threonine protein kinases. Our lab has focused research on two eukaryotic-like ser/thr protein kinases, PknA and PknB. It has been found that PknA expression may affect the stability or the assembly of the proteasome complex, which is essential for *M. tuberculosis* to persist in mice. This work shows that PknA phosphorylates the α subunit and the unprocessed β subunit of the *M. tuberculosis* proteasome, and that this direct effect arrests the proteasome assembly at the half-proteasome stage. Furthermore this effect confers resistance in *Mycobacterium smegmatis* to oxidative stress.

The significance of this research is that it potentially elucidates one way that *M. tuberculosis* is able to persist in host cells, because it shows an effect of PknA on a protein complex that is necessary for resistance to nitrosative stress, and whose lack confers resistance to oxidative stress.

Part 2:

L-threonine is the second-most deficient amino acid in livestock, and its addition to livestock feed will create healthier animals and a cleaner environment. *Corynebacterium glutamicum* is a Grade-A food-safe microorganism that is used for the industrial production of amino acids, which makes it ideal for creating an over-producing strain of threonine.

A key enzyme in the pathway for threonine production in *C. glutamicum* is homoserine kinase (*thrB*). Homoserine kinase is competitively inhibited at the active site by threonine, which makes it difficult to relieve feedback inhibition through mutation. This work shows that at the entry of the active site of homoserine kinase there is a conserved alanine residue. We propose that mutation of this residue could relieve feedback inhibition.

The significance of this research is that it provides a novel approach for relief of feedback inhibition, since we attempted to genetically separate the catalytic activity and feedback inhibition in the active site of homoserine kinase. This approach may be applied later to other amino acid production in *C. glutamicum* and other amino acid over-producing organisms.

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