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Involvement of Serine Threonine Protein Kinase, PknL, from Mycobacterium Tuberculosis $H_{37}Rv$ in Starvation Response of Mycobacteria

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Abstract

The adaptation to nutrient depletion in bacteria involves a highly organized series of intracellular events that enable them to adapt to starvation conditions. The regulatory effect of serine threonine protein kinase, PknL, from *Mycobacterium tuberculosis* strain $H_{37}Rv$ was investigated under nutrient deprived conditions that simulate circumstances leading to latency. Recombinant PknL was expressed in *Mycobacterium smegmatis* strain mc^{2}155 in its wild type and mutant forms. *In vitro* growth kinetics experiments revealed that clone expressing active PknL had a significant growth advantage under nutrient limiting conditions. Experiments were conducted to ascertain the *in silico* predictions of the involvement of PknL in regulating glutamine metabolism in mycobacteria. Furthermore, a role for PknL in cell wall biogenesis/cell division was shown by scanning electron microscopy.

Keywords: Serine threonine protein kinase (STPK); PknL; Starvation response; Glutamine metabolism; Nutrient uptake; HPLC; Scanning electron microscopic studies

Abbreviations: $(NH_4)_2SO_4$: Ammonium Sulphate; K_2HPO_4: Potassium Sulphate; BLASTP: Basic Logistic Alignment and Search Tool; E value: Expect value; GlnA: Glutamine synthetase; kb: kilobase; kV: kilovolt; µm: micrometer; PknL: Protein Kinase L; STPK: Serine Threonine Protein Kinase; MOPS: 3-(N-morpholino) propanesulfonic acid; nm: nanometer; OPA: Orthopthalaldehyde reagent; PBS: Phosphate Buffered Saline; PMSF: Phenyl Methyl Sulfonyl Fluoride; UTase/UR Uridylyl Transferase and Uridylyl Removing enzyme; Tim: Translocases of mitochondrial inner membrane; Tom: Translocases of mitochondrial outer membrane.

Introduction

An estimated one-third of the world's population is latently infected with tuberculosis (TB) and most active cases of TB arise from this vast reservoir (Riska et al., 2002). Little is known about the nature of the persistent state *in vivo* or the factors that induce and maintain it. Several latency models have been experimented by Cornell (1998) – antibiotic induced latency, Wayne (1994) – oxygen depletion induced latency and Loebel (1933) – nutrient starvation model for latency. These models have facilitated identification and validation of drugs active against latent TB.

Bacterial cells enter into the stationary phase due to limitations in major nutrients such as carbon, nitrogen and phosphorous or in trace elements. Sensing the famine triggers adaptive responses in bacteria. Although many studies have focused on understanding the regulation of gene expression and the phenotypic consequences, there is limited understanding of how bacteria sense environmental changes and thereby produce signals for the genetic machinery to respond appropriately. Currently, there is a wide interest in the role of signaling molecules in adaptation to starvation (Kjelleberg, 1993).

Phosphorylation is a key component of signal transduction network of both eukaryotic and prokaryotic cells. It is an extremely subtle and sophisticated mechanism by which information is expertly transduced from the environment into the cell (Hunter, 1995). PknL (*Rv*2176) is a transmembrane STPK in *Mycobacterium tuberculosis.* The *pknL* gene is proximally placed with a gene coding for a putative transcriptional regulator (*Rv2175*c) on the *M. tuberculosis* chromosome and has been shown to phosphorylate the latter (Canova et al., 2008). The existence of a gene (ML0897c) sharing 75% identity with *pknL* in *M. leprae,* a bacterium that has undergone massive gene decay (Cole et al., 2001), has led to speculations that this kinase could play a pivotal role in regulating mycobacterial growth, survival and/or pathogenesis.

In our endeavour to functionally characterize PknL, we sought to investigate its role in starvation response of mycobacteria. Serine threonine kinases could serve as valuable drug targets as they mastermind the intracellular lifestyle of this pathogen. We have previously described the biochemical characterization of PknL, (Lakshminarayan et al., 2008) and here we describe our attempts to investigate the role of this kinase in the starvation response of mycobacteria.

Materials and Methods

Pfam analysis to identify functional domains among PknL-$H_{37}Rv$ and its orthologs

The primary amino acid sequence of PknL from *M. tuberculosis* strain $H_{37}Rv$ was downloaded in the FASTA format from the Pasteur Institute website (http://genolist.pasteur.fr/Tuberculist).

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Study on the protein orthologs of PknL among different bacteria was performed using the BLASTP program at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The amino acid sequence of PknL and its related prokaryotic orthologs were searched against the Pfam database using the HMMER program available from (http://pfam.sanger.ac.uk).

In vitro growth kinetics under nutrient limiting conditions

Recombinant PknL was expressed as a full length enzyme in *Mycobacterium smegmatis* strain mc^{2}155 in its wild type (pHL4) and kinase inactivated (pHL8) forms. The clones pHL4, pHL8 and pAL (vector control) were grown in Sautons broth and induced for protein expression with acetamide (0.2% w/v) (Lakshminarayan, 2008). Nitrogenous supplements were provided by including L-glutamine or ammonium sulphate [$(NH_4)_2SO_4$] to provide a final concentration of 4mM and 30mM respectively. The cultures were grown with continuous shaking at 130 rpm at 37 C and were regularly sampled at 3 - 4 hourly intervals to measure the cell density (OD_{600} values) spectrophotometrically. A colony count was also performed by plating suitable dilutions on selective Middlebrooks 7H10 agar plates (Hygromycin 50µg/ml).

Nutrient starvation experiments

To investigate the role of H_{37}*Rv*-PknL in the starvation response of *M. smegmatis* strain mc^{2}155, the clones pHL4, pHL8 and pAL were grown in Hartmans-de-Bont minimal medium (Smeulder et al., 1999) and their growth profiles were monitored following induction. To achieve conditions of nitrogen and carbon starvation, $(NH_4)_2SO_4$ and glycerol were adjusted to final concentrations of 0.15mM and 11mM respectively in this minimal medium. For phosphorous starvation, both K_2HPO_4 and NaH_2PO_4 were reduced 100-fold to 0.16mM, 3-(N-morpholino) propanesulfonic acid (MOPS) was added at 50mM to replace lost buffering capacity, and the pH was adjusted to 7.

High performance liquid chromatography (HPLC) for estimating intracellular pools of glutamine and glutamic acid

For generating the cell lysate used in this estimation, cells were grown to mid logarithmic phase under conditions of starvation as described in the previous section. The cell pellet obtained by spinning down the culture was washed with phosphate buffered saline (PBS) and disrupted by sonication. A protease inhibitor (PMSF) was added to the lysate at a concentration of 1mM and stored at 4 C while processing. Following OPA derivatization with orthopthalaldehyde reagent, the 1100 series HP-HPLC system with UV detection at 338nm was used to perform the amino acid estimations. The protein concentration of the cell lysates was normalized prior to HPLC analysis.

Scanning electron microscopy

Induced *M. smegmatis* clones were spun down and washed twice with PBS. The cell suspension was fixed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 hours. The cells were filtered through a Whatmann No.5 filter paper and post fixed in 1% osmium tetroxide for 2 hours. The filter paper discs were dehydrated through a graded series of acetone and air dried. Following coating with platinum for 3-4min, using JEOL-JFC-1600 Auto Fine Coater, the samples were scanned and micrographs were taken, using JEOL JSM 6360 scanning electron microscope. A working distance of 15mm and an accelerating voltage of 10kV were used to view the specimens.

Statistical analyses

The culture experiments were carried out in triplicate. The significance of the results was determined by analysis of variance. *P* values of < 0.05 were considered significant.

Results

Pfam analysis to identify functional domains among PknL-H_{37}*Rv* and its orthologs

By comparing the amino acid sequence of H_{37}*Rv*-PknL and its orthologs with the Pfam protein domain family database several functional domains could be identified. Motifs belonging to GlnD-Uridylyl transferase (E value = 0.33) and Tim inner mitochondrial membrane family of proteins (E value = 0.71) were

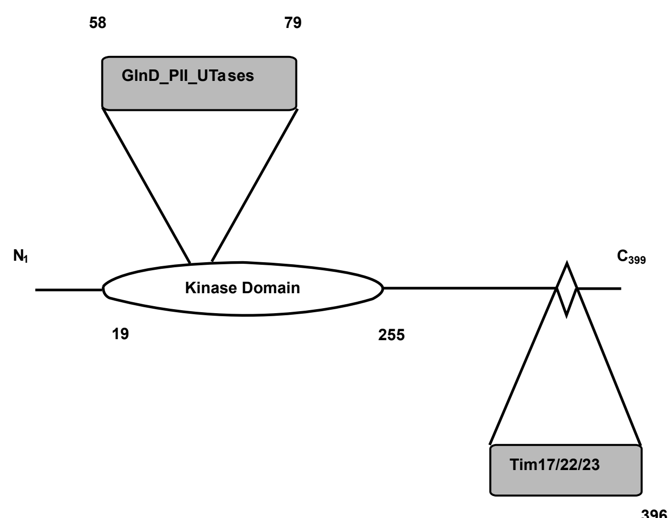


Figure 1: Identifying functional motifs in PknL. The numbers indicate amino acid positions on PknL.

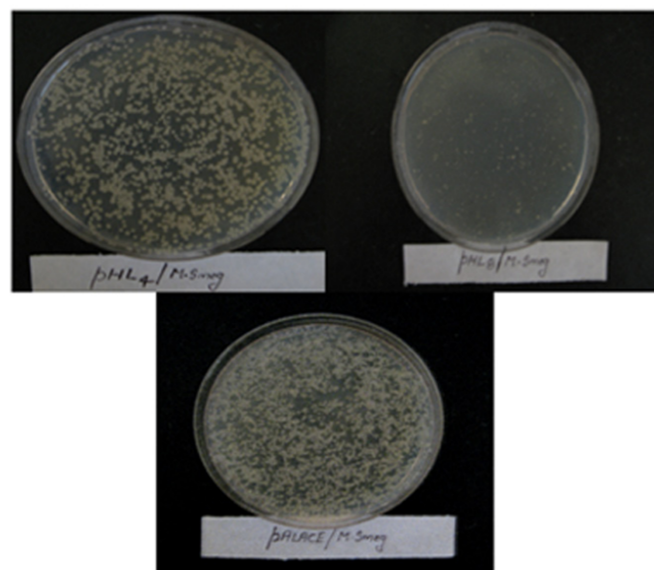


Figure 2: Colony morphology of *M. smegmatis* cells expressing H_{37}*Rv*-PknL. Following induction in Sautons medium with acetamide (0.2% w/v) the cells were plated on 7H10 agar plates.

identified in the C-terminus of PknL from *M. tuberculosis* and *M. bovis* (Figure 1). Though the serine threonine kinases shared a common kinase core (20-30% identity), they differed in their functional domains which contribute to the diversity in their function.

In vitro growth kinetics under nutrient limiting conditions

In the acetamide induced cultures grown in complete Sautons medium, strains expressing kinase inactive PknL (pHL8) showed a considerable retard in growth. When shifted to nutrient rich 7H10 agar both pHL4 and pHL8 revived with equal efficiency.

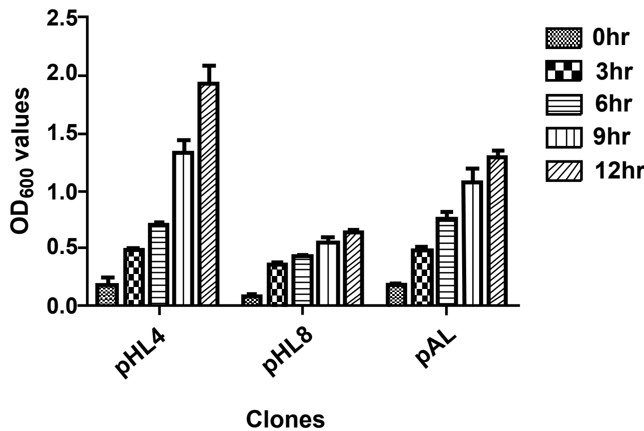


Figure 3: *In vitro* growth kinetics in minimal medium. All strains were induced with acetamide (0.2% w/v) for protein expression. OD readings were taken at 600nm.

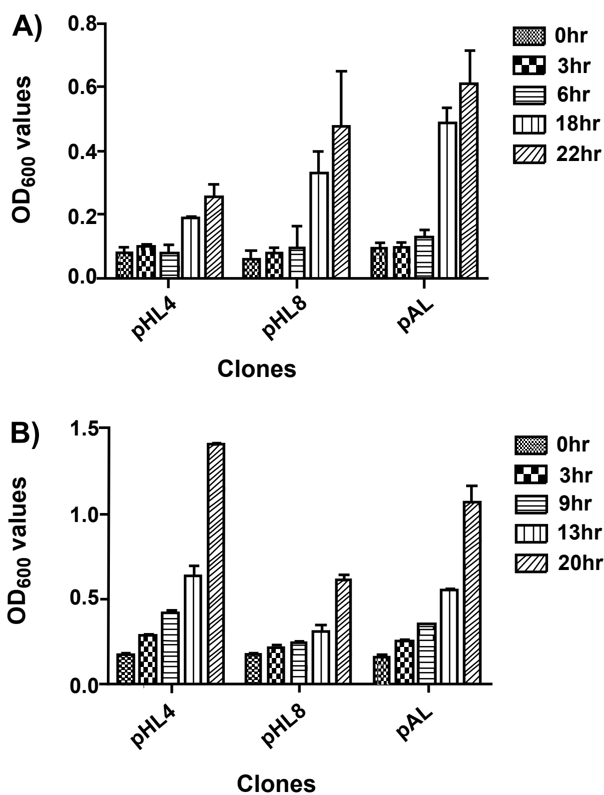
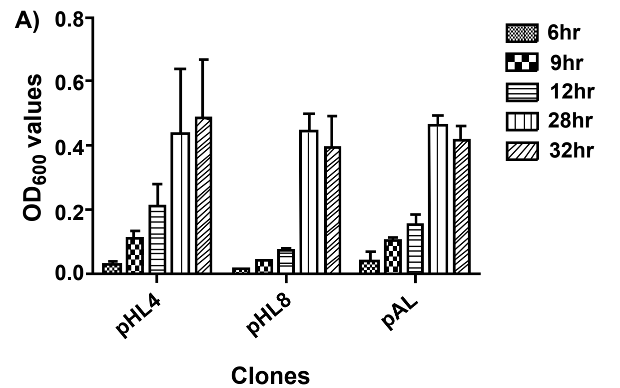
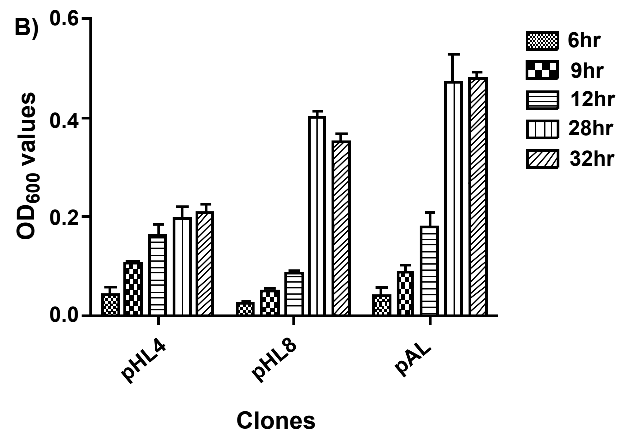


Figure 4: *In vitro* growth kinetics in minimal Sautons medium supplemented with nitrogen sources. The cultures were grown at 37 C and induced for protein expression with acetamide (0.2%w/v). **A)** Ammonium source was provided by addition of ammonium sulfate [$(NH_4)_2SO_4$] to a final concentration of 30mM. **B)** L-glutamine (4mM) was used as the serum free growth supplement and a source of nitrogen.





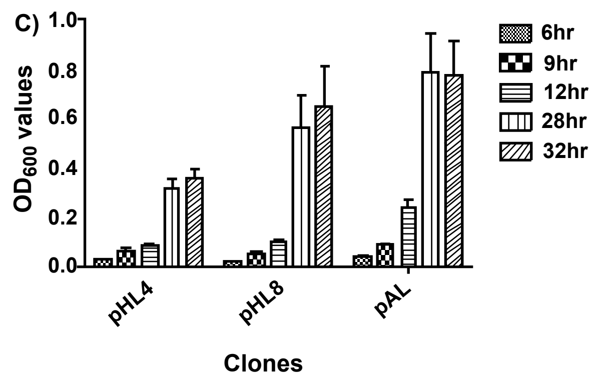


Figure 5: Growth profile of H_{37}*Rv*-PknL over expressing M. smegmatis clones under nutrient limiting conditions. Clones were grown in Hartman's de Bont minimal medium with added supplements and induced for protein expression with acetamide (0.2% W/V). **A)** Nitrogen starvation conditions. Nitrogen sources were provided by adding ammonium sulphate (0.15mM) **B)** Carbon starvation conditions. Carbon source was provided in the form of glycerol (0.08% v/v). Acetamide added to induce gene expression may also function as a carbon source. **C)** Phosphate limiting conditions. PO_4 were provided at 0.16mM and MOPS (50mM) was added to compensate for buffering capacity.

However the colonies of pHL8 clone were much smaller in size than those of pHL4 or pAL (Figure 2).

Under nutrient limiting conditions, growth in minimal Sautons without added supplements, pHL4 clone expressing the wild type enzyme had a significant growth advantage. It grew to higher cell densities before entering the stationary phase (Figure 3). When ammonium salts were present in the culture medium the growth of pHL4 was retarded while the kinase inactive mutant (pHL8) showed a pronounced growth almost equalling that of

the vector control (Figure 4A). However, with glutamine supplementation the pHL4 clone displayed a growth advantage as compared to pHL8 and the vector control (Figure 4B).

Nutrient starvation experiments

Under conditions of nitrogen starvation, the pHL4 clone showed growth advantage (Figure 5A). In the carbon starved cultures (Figure 5B), pHL4 clone grew efficiently in the early phase (up to 12 hrs) with the growth matching that of the vector control (pAL). In the later stages of growth (22-36 hrs), pHL8 and pAL clones attained high cell densities while the pHL4 clone was retarded in growth. Under conditions of phosphate starvation (Figure 5C) pHL4 clone displayed diminished growth.

Estimating intracellular pools of glutamine and glutamic acid by high performance liquid chromatography

The cells were grown under varying conditions of carbon, nitrogen and phosphate starvation and the intracellular pools of glutamine and glutamic acid were determined using HPLC. The concentrations of the respective amino acids are shown in Table 1.

Scanning electron microscopy indicates a role for M. tuberculosis H_{37}Rv-PknL in regulating mycobacterial cell division and cell wall biogenesis

The three clones, pHL4, pHL8 and pAL were grown in minimal Sautons medium with acetamide induction and viewed with a scanning electron microscope. The cell length measurements of a total of 100 cells in each category were determined by manual and automated methods. It was observed that each clone contained a heterogeneous population of cells with respect to cell length. The cell length measurements have been plotted in Table 2A and 2B. It could be appreciated that *M. smegmatis* cells over expressing the active kinase PknL (pHL4) grew to greater cell lengths (>3.5 µm) (Table 2A). Even the cells expressing the point mutant (pHL8) were larger (3-3.4 µm) when compared to the vector control. Table 2B which represents cell length data obtained by automated counting also projects a similar picture. pHL4 clones measured around 2-3 µm, followed by pHL8 and pAL.

Discussion

It is well known that mycobacteria survive within the granuloma in a living yet non replicating state. The process of adaptation to latency involves bringing the metabolic responses to a halt without compromising the viability of the organism. Nutrient limitation induced *in vitro* has been able to mimic the conditions of latency (Betts et al., 2002). This ability of the bacterium to keep the cellular machinery intact without access to an exogenous energy source is considered to reflect an extensive reorganization of the cellular makeup, initiated after the onset of nutrient starvation. Several observations have recently been made providing evidence for the role of cellular signaling molecules in adaptive responses to oxidative stress, a prime factor associated with latency (Rodriguez et al., 2008; Zhart et al., 2003).

Eukaryotic like serine threonine protein kinases and associated phosphatases are important signal transduction systems

Growth condition	Clone name	Total protein content (µg/ml)	Glutamic acid (µmol/ml)	Glutamine (µmol/ml)
Nitrogen starvation	pHL4	440	8.42	3.39
	pHL8		11.66	6.26
	pALACE		8.05	4.54
Carbon starvation	pHL4	337	11.39	6.61
	pHL8		10.54	4.59
	pALACE		11.86	10.79
Phosphate starvation	pHL4	55	16.91	9.26
	pHL8		8.95	5.26
	pALACE		8.95	6.71

Table 1: HPLC report for glutamic acid and glutamine concentration in cell lysates generated under different conditions of nutrient starvation. Following derivatization with orthopthalaldehyde reagent the amino acid estimations were performed with UV detection at 338nm.

A)

Cell Size	pHL4	pHL8	pAL
<2 µ m	0.18	0.12	0.20
2-2.95 µ m	0.40	0.48	0.50
3-3.4 µ m	0.12	0.25	0.09
>3.5 µm	0.27	0.14	0.09

B)

Cell Size	pHL4	pHL8	pAL
< 2 µ m	0.82	0.70	0.65
2-3 µ m	0.74	0.63	0.14
>3 µm	0.006	0.04	0.14

Table 2: Cell length measurements of *M. smegmatis* cells expressing wild type (pHL4) and mutant (pHL8) forms of PknL. A) Cell length determined by manual measurements B) Cell length determined by automated measurements. The numbers indicate cell length ratios (Ratio = Actual No. of cells in each category / Total No. of cells).

which modulate the behaviour of the bacteria in response to external stimuli (Zhang et al., 1996). By phosphorylating transcription factors, STPKs may take part in global regulatory response influencing the activity of several genes. To address the possibility that the regulation of adaptive response is mediated by the signalling molecules produced by *M. tuberculosis*, we studied the growth profile of *M. smegmatis* cells over expressing H_{37}*Rv*-PknL.

Identifying functional domains in PknL

Motifs for UR/UTases, GlnD (E value = 0.33) and Tim family (E value = 0.71) of proteins were identified in the C-terminus of PknL from *M. tuberculosis* and *M. bovis* (Figure 1). GlnD is a pivotal protein in sensing intracellular levels of fixed nitrogen. The family of bifunctional Uridylyl-removing enzymes/ Uridylyl transferase (UR/UTases, GlnD) is responsible for the modification of the regulatory protein P-II, or GlnB (Stadtman et al., 1990). In response to nitrogen limitation, these transferases catalyse the uridylylation of PII protein, which in turn stimulates deadenylylation of glutamine synthetase (GlnA). Deadenylylated glutamine synthetase is the more active form of the enzyme which catalyses the assimilation of ammonia in an ATP dependent reaction (Stadtman et al., 1990). Tim 17 and Tim 23 are thought to form the translocation channel of the inner mitochondrial membrane (Bomer et al., 1996). The findings from *in silico* analysis encouraged us to probe into the role of PknL in nitrogen metabolism of *M. tuberculosis*.

In vitro growth kinetics with PknL over expressing clones implicates a role in nitrogen metabolism

The growth advantage of the active kinase expressing clone (pHL4) grown in minimal Sautons medium without added supplements such as glycerol, glutamine or ammonia, implies that the kinase has a role in sensing nutrient shortage and modulates compensatory metabolic activities to tide over the stress. On the other hand, the clone expressing kinase inactive PknL (pHL8) shows a considerable retard in growth (Figure 3). When the acetamide induced cultures were shifted to 7H10 agar, the colonies of pHL8 clone were much smaller in size than those of pHL4 or pAL (Figure 2). This critical finding reflects a shortcoming in nutrient uptake by the kinase inactive clone (pHL8) which may have a bearing on the recovery from stress, inflicted on the host cell through kinase over expression.

When ammonium was used as the nitrogen source, pHL4 displayed restricted growth as compared to the mutant and vector control (Figure 4A), suggesting of a role for PknL in sensing extra cellular nitrogen levels. The principle means of ammonia assimilation in bacteria grown under nutrient limiting conditions is through the *de novo* synthesis of glutamine in an ATP dependent reaction catalysed by glutamine synthetase (Forchhammer, 2007). Thus, it is hypothesised that sensing abundant extra cellular ammonium sets off a feedback inhibitory circuit where the cell avoids expending all its cellular reserves of ATP on assimilation. This observation is in accordance to the prevailing paradigm for nitrogen control in bacteria mediated by GlnD-PII-GlnE loop (Stadtman, 1990).

The growth advantage conferred by PknL expression on the *M. smegmatis* cells when grown with glutamine supplementation (Figure 4B) is indicative of its involvement in glutamine metabolism. In *M. tuberculosis* genome, the serine threonine protein kinase PknG was postulated to have a role in glutamine metabolism as it is located within the same operon as glnH substrate binding protein (Av-Gay and Everett, 2000). A pknG null mutant in *M. tuberculosis* showed an increased intra cellular concentration of glutamate and glutamine when grown in nutrient depleted medium (Cowley et al., 2004). Though the gene encoding PknL is not chromosomally positioned near the glutamine permease operon, its expression responds to extra cellular nitrogen sources (Fig 4A, 4B). As multiple mycobacterial STPKs have been shown to phosphorylate ABC transporter *Rv*1747 under physiological conditions (Grunder et al., 2005), it is probable that PknL regulates inducible glutamine transport in mycobacteria.

However we needed to distinguish between amino acid uptake and *de novo* glutamine synthesis as a mechanism for providing nitrogen sources to the cell and the involvement of PknL in regulating these processes. Thus the nutrient starvation experiments were initiated.

Nutrient starvation experiments

The growth advantage of the pHL4 clone in nitrogen starved conditions (Figure 5A) may be due to the heightened adaptability of this clone by virtue of PknL expression.

In the carbon starved environment a biphasic pattern of growth was observed. In the early phase (up to 12 hrs) the pHL4 clone expressing active PknL took the lead, subsequently (22-36 hrs) pHL8 and pAL clones could attain high cell densities (Figure 5B). Pronounced growth observed in later stages may be due to the additional nutrients provided by the dead cells, a phenomenon that is correlated with the conditions encountered in a caseating granuloma (Medlar, 1926). Starvation response is to ensure that the bacteria generate minimal levels of energy to ensure that they remain viable. During carbon starvation fatty acids can serve as an alternative energy source as evidenced by the importance of isocitrate lyase (*icl*) in mycobacterial persistence (Flynn et al., 2001).

Owing to its central role in energy metabolism, phosphate is an essential nutrient for cell growth (Kornberg et al., 1999). We compared the *in vitro* growth of *M. smegmatis* clones, under phosphate limitation. The pHL4 clone showed a decrease in growth (Figure 5C).

Estimating intracellular glutamine and glutamate levels by high performance liquid chromatography

The intracellular concentrations of glutamine/glutamic acid were determined by HPLC with UV detection at 338 nm. The chromatogram revealed a heightened intracellular pool of glutamic acid (16.7μmol/ml) in the pHL4 clone grown under phosphate limitation. The increase is almost two fold, with pAL and pHL8 cultures having intracellular glutamic acid concentrations of 8.95 μmol/ml (Table 1).

We targeted glutamic acid because of the hypothesised involvement of PknL in glutamine metabolism as discussed earlier. However there are reports associating glutamate and glutamine synthetase with osmotic stress response (McLaggan et al., 1994; Gralla et al., 2006). Thus, PknL may influence the global transcriptional response to nutritional stress by increasing the intra-

cellular levels of glutamate. Such an adaptive response involving moderations in intra cellular glutamate-glutamine levels has also been observed in Δ pknG mutants in *Mycobacterium tuberculosis* grown in nutrient limiting medium (Cowley et al., 2004).

Scanning electron microscopy indicates a role for *M. tuberculosis* H_{37}*Rv*-PknL in regulating mycobacterial cell division and cell wall biogenesis

The conservation of genes coding for division and cell wall biogenesis (dcw gene cluster) in the 30kb locus encompassing PknL, the phylogenetic relatedness to PknB/PknA and the existence of homologs in *Corynebacterium glutamicum* with carboxy terminal PASTA domains, represent the biological line of evidence that implicates a role for PknL in regulating cell wall biogenesis (Narayan et al., 2007).

It was seen that *M. smegmatis* cells over expressing the active kinase (pHL4) grew to greater cell lengths (>3.5μm). Even the pHL8 clones expressing the point mutant were large (3-3.4μm) when compared to the vector control pAL (2-2.95μm) (Table 2A).

These finding are suggestive of a role for PknL in the cell wall biosynthetic activity in mycobacteria. It is reasoned that PknL by its ability to regulate the synthesis of the cell wall polymer, poly-L-glutamine, or access to nutrients by promoting uptake from the surrounding medium has a positive influence on the health of mycobacteria allowing them to grow to greater lengths.

Many mycobacterial kinases have been shown to play a part in cell division. Mycobacterial strains over expressing PknA and PknB showed a tendency to form chains which is a sign of anomalies in cell septation (Kang et al., 2005). PknA phosphorylates the cell division protein FtsZ and inhibits its GTPase activity, resulting in the observed chain phenotype (Thakur et al., 2006). PknB exerts its action on septal peptidoglycan biosynthesis by regulating the activity of penicillin binding protein PBPA (Datta et al., 2006). Cell division protein Fts Z, cell wall biosynthetic MurD amide ligase and PbpB are conserved near PknL, thus becoming easy targets for the regulatory protein.

Starvation results in a programmed pattern of gene regulation aimed at promoting bacterial survival in the dormant state. Thus, PknL by it ability to sense extra cellular nutrient levels may mediate extensive reorganization of the cellular makeup, initiated after the onset of nutrient starvation. PknL could directly respond to changes in cAMP or pppGpp levels which occur during starvation. The predicted role for PknL in influencing transcription has been confirmed with the demonstration of its ability to phosphorylate a transcription factor *Rv*2175c co-localized with it in the chromosome. This adds strength to our hypothesis that PknL has a regulatory role in interceding adaptive response to nutrient starvation in *Mycobacterium tuberculosis* by influencing gene transcription.

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