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A Serine/threonine kinase *PknL*, is involved in the adaptive response of *Mycobacterium tuberculosis*

Ahmed Kabir Refaya [a], Divakar Sharma [b], Virendra Kumar [b], Deepa Bisht [b], Sujatha Narayanan [a,*]

[a] Department of Immunology, National Institute for Research in Tuberculosis, #1, Mayor Sathiyamoorthy road, Chetpet, Chennai, 600 031, India
[b] National JALMA Institute for Leprosy & other Mycobacterial Diseases, Taj Ganj, Agra 282004, India

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ABSTRACT

Mycobacterium tuberculosis adapts itself to various environmental stress conditions to thrive inside the phagosome for establishing a chronic infection. Serine/threonine protein kinases (STPKs) play a major role in the physiology and pathogenesis of *Mycobacterium tuberculosis*. Some of these STPKs are involved in regulating the growth of the mycobacterium under nutrient stress and starvation conditions. In this study, we have investigated the role of *PknL*, a STPK in the adaptive responses of *M. tuberculosis* by conditional inactivation of the gene using antisense technology. The inhibition of *PknL* in the knockdown strain was validated by RT-PCR. The *in vitro* growth kinetics of *M. tuberculosis* strain following inhibition of *PknL* was found to be bacteriostatic. The knock down strain of *PknL* exhibited a better survival in pH 5.5 when compared to its growth in pH 7.0. Similarly, it also exhibited more resistance to both SDS(0.01%) and Lysozyme stress (2.5 mg/ml), indicating that loss of *PknL* enhances the growth of mycobacterium under stress conditions. SEM pictographs also represent an increase in the cell length of the knock down strain compared to Wild type stressing its role in cellular integrity. Lastly, the proteome analysis of differentially expressing *PknL* strains by 2D gel electrophoresis and mass spectrometry identified 19 differentially expressed proteins. Our findings have shown that *PknL* plays an important role in sensing the host environment and adapting itself in slowing down the growth of the pathogen and persisting within the host.

1. Introduction

Mycobacterium tuberculosis, responsible for causing tuberculosis still remains to be one of the deadliest communicable diseases. In 2013,WHO reported an estimated 9.0 million cases of TB and 1.5 million deaths including 3,60,000 people with HIV (World Health Organisation, 2014). Almost 30% of the world's population is affected with latent *M. tuberculosis* infection providing a large reservoir for the disease to reactivate and 2–10% of latently infected individuals are estimated to reactivate the disease with an increase of >20 folds in case of HIV co-infection (Getahun et al., 2010). In order to survive inside the macrophage of the host, Mycobacteria senses and responds to the changes in the immediate environment such as pH differences and other stress conditions that occur in the phagosome (Tan et al., 2013) The rigid cell wall of *M. tuberculosis* and changes in cell wall composition in response to various environmental stimuli are critical for the adaptation of the pathogen during infection. (Daffe and Draper, 1998) Elucidating the environmental cues and the response of *M. tuberculosis* to such signals is very critical in understanding the pathogenesis and persistence of the bacilli inside the host environment. The ability to sense environmental signals and implement adaptive changes is a key feature of a living cell. These signal transduction networks are often regulated by reversible protein phosphorylation. The mechanism of Ser/Thr/Tyr protein phosphorylation-based signaling were extensively studied in eukaryotes, whereas it has only begun in prokayotes (Deutscher and Saier, 2005).

The whole genome sequence of *M. tuberculosis* has revealed the presence of eukaryotic like 11 serine/threonine protein kinases(STPKs *PknA-PknL* except C) (Cole et al., 1998). These STPKs influence a wide range of biological functions through phosphorylation, such as adaptation to various environmental conditions, stress, cell wall synthesis, cell division and pathogenicity (Narayan et al., 2007). These kinase proteins are mainly localized in the cell membrane and cell wall of *M. tuberculosis*, except *PknG* which is predominantly found in cytoplasm (Koul et al., 2001). Functional

* Corresponding author.
E-mail addresses: refayanasar@gmail.com (A.K. Refaya), divakarsharma88@gmail.com (D. Sharma), vksJalma@gmail.com (V. Kumar), abd1109@rediffmail.com (D. Bisht), sujatha.sujatha36@gmail.com (S. Narayanan).

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Table 1
Primers used for sense and anti-sense cloning.

Primers	Description	Sequence
PknL-ASF	Fwd primer for PknL-AS	5′-ATTCCAACATATG-GTGGTCGAAGCTGGCACGAG-3′
PknL-ASR	Rev primer for PknL-AS	5′-AGGCCTAGGATCC-TAGAGCAGGCCGCTCAGGT-3′
PknL-SF	Fwd primer for PknL-S	5′ TCCGGATCCTAGG-GTGGTCGAAGCTGGCACGAG-3′
PknL-SR	Rev primer for PknL-S	5′ TAAGGTTGTATAC-TTAGAGCAGGCCGCTCAGGT-3′

characterization of *PknA, PknB* and *PknF* indicated a role in determining cell shape, morphology and cell division and *PknF* is also responsible for biofilm formation and glucose transport. (Deol et al., 2005; Kang et al., 2005; Dasgupta et al., 2006; Fernandez et al., 2006). *PknI* and *PknK* have a potential role in growth regulation of *M. tuberculosis* (Gopalaswamy et al., 2009; Malhotra et al., 2010). The other kinases *PknD*, *PknE* and *PknG*, have been shown to be required for the survival and persistence of *M. tuberculosis* inside the host. (Parida et al., 2005; Jayakumar et al., 2008; Scherr et al., 2009) *PknH* mutant also induced hypervirulent phenotype in BALB/c mice in terms of bacterial load in mouse organs. (Papavinasasundaram et al., 2005). Four out of the 11 kinases in *M. tuberculosis* (*PknA, PknB, PknG* and *PknL*) which are conserved in *M. leprae* are proved to be essential in *M. tuberculosis* except *PknL* (Sassetti et al., 2003; Greenstein et al., 2005; Fernandez et al., 2006). A study based on a phospoproteome approach on *M. tuberculosis* serine/threonine phosphorylation have identified 301 proteins involved in a broad range of functions. Many of the phosphorylation events were specific to growth conditions encountered during infection such as low pH, nitric oxide exposure and hypoxia (Prisic et al., 2010). These reports supports the fact that serine/threonine phosphorylation is involved in signaling mechanism in response to changing environments. (Prisic et al., 2010) A recent report on *PknB* serving as a major regulator of the oxygen-dependent replication switch, where *PknB* levels were reduced during hypoxia and restored upon reaeration signifying its role in transducing growth and replication signals. (Ortega et al., 2014). In view with the above findings, we wanted to elucidate the role of *PknL* in regulating mycobacterial growth, survival, cell homeostasis and/or pathogenesis.

Previously characterized substrate/kinase pair in *M. tuberculosis*, *PknL/Rv2175c* and the association of *PknL* with the_30-kb *dcw*(division cell wall) gene cluster, encompassing several genes involved in cell wall synthesis and cell division (Narayan et al., 2007; Canova et al., 2008; Molle and Kremer, 2010), raises the possibility that *PknL* might be involved in the cell wall homeostasis and survival inside the host. We have already demonstrated the role of *PknL* in adaptive response during nutrient limitation using site directed mutagenesis in *M. Smegmatis*. (Lakshminarayan et al., 2009). We wanted to extend our study on the role of *PknL* in the obligate human pathogen *M. tuberculosis*, which has to deal with a more restricted set of host environmental variables such as low pH, surfactant and lysozyme stress. Since the attempts to create a gene knockout of *PknL* was unsuccessful, we aimed to generate a knockdown strain of *PknL*. Conditional inactivation of the expression of essential genes by cloning them in an IPTG inducible vector using antisense approach and the down regulation of polyphosphate kinase (*ppk)* by a similar approach resulted in bactericidal activity (Kaur et al., 2009; Jagannathan et al., 2010) which prompted us to use a similar approach to understand the survival kinetics of *M. tuberculosis* following *PknL* knockdown. Moreover, a conditional knockdown strain of *PknL* generated using a tetR expression system reported *PknL* to be a poor target, as the reduction of the naturally low level of expression had no effect (Carroll et al., 2011). Owing to these reports, we decided to use the IPTG inducible antisense strategy for knocking down the expression of *PknL* and to study the changes involved in the adaptive response of *M. tuberculosis* following inhibition of *PknL*.

Table 2
Primers used for RT-PCR.

Primers	Description	Sequence	Dye
PknLF	Fwd primer	CGGTTGCCCGGCTAAATA	FAM and TAMRA
PknLR	Rev primer	GATGAGCTCCATCACCAGAAA	
PknLP	Taqman probe	TCTACGACCAGGGCAAAGACGG	

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

The *Escherichia coli* strain DH5α was used for cloning and was grown in Luria-Bertani (LB) broth or on LB agar (Difco) for plasmid isolation, transformation and isolation of recombinant clones. Mycobacterial strains were grown in 7H9 (Middlebrook) medium supplemented with 10% ADS (Albumin dextrose saline) and 0.05% Tween80. Antibiotics were used in prescribed concentration (Hygromycin (150 mg/ml) for *E. coli* and (50 mg/ml) for *M. tuberculosis*) when required. All plasmids and constructs are listed in Table S1. Gene sequences required for primer designing was retrieved from Tuberculist and primers were procured from Shrimpex biotec services. The primers used for this cloning are listed in Table 1 and primers used for quantifying gene expression by RT-PCR experiments are listed in Table 2.

2.2. Construction of the antisense and sense construct of PknL (Rv2176) in tuberculosis

The sense and antisense oriented *PknL* were cloned using the IPTG inducible mycobacterial shuttle vector pAZI9018b (Kaur et al., 2009) (received from Dr. Santanu Datta, AstraZeneca). The full length *PknL* (Rv2176) gene from *M. tuberculosis* was amplified with the respective primers (Table 1). This amplified product was inserted into the vector at 5'*BamH1* and 3'*Nde1* sites to obtain *PknL* in the sense orientation (PknL-S) and similarly the antisense orientation of *PknL* (PknL-AS) was obtained by inserting the amplified product at the 5'*NdeI* and 3'*BamHI* sites of the vector. The orientation of clones were confirmed by DNA sequencing and Restriction Enzyme Digestion. The inserts released from the plasmid harboring PknL-S and PknL-AS were around 1.2 kb which correlates with the length of *PknL*. The sequence confirmed clones along with the vector were transformed into *M. tuberculosis* by electroporation and equal amount of transformants were plated in 7H10 agar plates containing Hygromycin (50 μg/ml) as a resistant marker. The transformants obtained were grown in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% albumin, dextrose along with 50 μg/ml of Hygromycin. The cultures were stored in −80 °C until further use.

2.3. Regulation of tuberculosis PknL gene expression during different phases of growth

All the *M. tuberculosis* strains Wild type, PknL-S and PknL-AS were grown in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% albumin, dextrose along with 50 μg/ml of Hygromycin. IPTG was used in the concentration of 0 mM,1 mM and 10 mM to induce the expression of sense and