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

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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

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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

antisense *PknL*. The cultures were harvested at regular intervals on 0,2,5,7,10,14&21 days. Trizol reagent (Invitrogen) was added to the cell pellet and disrupted with 0.1 mm zirconia beads in a mini-bead beater. Total RNA was purified using an RNeasy purification kit(Qiagen). Contaminated DNA in the RNA sample was digested with RNase-free DNase I(Qiagen). The purity of the RNA was determined by measuring the absorbance at 260 and 280 nm. The first-strand cDNA was synthesized from 1 μg total RNA using High capacity cDNA reverse transcriptase kit (Applied biosystems). Real-time quantitative RT-PCR (qRT-PCR) was performed in an ABI 7500 system (Applied Biosystems) using TaqMan assays. Each reaction was done in triplicate with independent RNA samples. Negative controls consisting of no reverse transcriptase and no template mixtures were run with all reactions. The threshold cycle (ct) of *PknL* and 16srRNA was monitored in Wild type, PknL-S and PknL-AS in all concentration of IPTG for all the time points. 16srRNA was used for normalization. After baseline correction and determination of threshold settings, relative expression (R) of PknL in the strains with 1 mM and 10 mM of IPTG was compared with strains containing 0 mM IPTG and calculated using the $22\Delta\Delta Ct$ method of Livak and Schmittgen (2001). Results are represented as log10(R), which denotes the up regulation or down regulation of *PknL* in different strains at different phases of growth.

2.4. In-vitro *growth determinations*

To determine the *in vitro* growth kinetics, logarithmic phase cultures of the Wild type H37Rv, PknL-S and PknL-AS were washed thrice and diluted to 0.1 OD_{600} in enriched 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 different concentration of 0 mM,1 mM and 10 mM as inducer. Diluted cultures were then grown in the shaker incubator at 200 rpm and at 37 °C. Aliquots of the cultures were withdrawn at regular intervals on day 0, 2,5, 7, 10, 14 and 21 and the growth was monitored by measuring the culture OD at 600 nm using SpectraMax 250 microplate reader (Molecular Devices). The viability of all the strains were checked by plating serially diluted cultures on the 7H10-ADS plates at every time point. CFU measurements were made after incubation of the plates at 37 °C for 4–5 weeks.

2.5. In vitro *survival kinetics of* PknL *under stress conditions (pH 5.5, Lysozyme and SDS Sensitivity Assay)*

In order to decipher the adaptive response of *PknL* under different stress conditions, all the log phase bacterial cultures of PknL-S, PknL-AS and Wild type strains, washed and adjusted to equal density by measuring the OD at 600 nm using SpectraMax 250 microplate reader (Molecular Devices) were subjected to acidic stress (pH 5.5), surfactant stress (SDS) and cell wall damaging agent (Lysozyme). For pH sensitivity, the growth of all the strains were monitored at pH 5.5 and pH 7.0 in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% albumin, dextrose along with 50 μg/ml of Hygromycin. 10 mM IPTG was used as inducer. Aliquots of the cultures were withdrawn at regular intervals of 0,2,5,7,10,14 and 21 days and OD_{600} nm was monitored using spectrophotometer. Viability was checked at all the mentioned time point by measuring CFUs to correlate with the OD_{600} nm values.

The Lysozyme stress was assessed by incubating the log phase (after day 7) cultures of Wild type, PknL-S and PknL-AS with 2.5 mg/ml of lysozyme at 37 °C. The cultures at 0 h, 24 h and 48 h were spotted in 7H10 agar plates by 10 fold dilution. The cultures were scored for growth after 3 weeks.

As for the surfactant stress, serial dilutions of the logarithmic phase cultures of Wild type, PknL-S and PknL-AS were plated on 7H10-ADS plates supplemented with SDS at different concentrations of 0.1%, 0.01% and 0.001%. The growth was recorded after 3 weeks of incubation.

2.6. *Scanning electron microscopy(SEM)*

5-ml culture aliquots of *M. tuberculosis* strains PknL-AS and Wild type grown until day 21 in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% albumin, dextrose along with 50 μg/ml of Hygromycin and 10 mM of IPTG under normal conditions were concentrated by centrifugation (5000 g) before suspending in fresh 7H9 medium. Aliquots of concentrated cells were spread on glass slides and allowed to dry before fixing it with O.C.T compound(Sakura Finetek). Samples were saturated at 4 °C overnight before removal from a BSL3 containment facility. Samples were then treated with 2% OsO_4 in 0.1 M cacodylate buffer for 2 h at room temperature. A series of sequential ethanol dehydrations were performed for 10 min each (50%, 70%, 95%, and 100%) before drying samples under CO_2 using a critical point drier apparatus (Samdri-PVT-3B; Tousimis Research Co.). Samples were gold sputter coated, and imaged with S-3000N Hitachi scanning electron microscope. Cell lengths measurements were measured automatically by the in-build software.

2.7. *Two-dimensional gel electrophoresis*

The strains 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 and induced with 10 mM IPTG under normal conditions until day 21. The cell extracts of the Wild type, PknL-S and PknL-AS were prepared as reported earlier (Singhal et al., 2012). The cytoplasmic lysates were precipitated using SDS and Trichloro acetic acid (TCA)–acetone precipitation procedure as reported earlier (Bisht et al., 2007). The pellets were air dried and suspended in appropriate volume of 2D-rehydration buffer (BIO-RAD, USA). The protein concentration was estimated using Bradford assay as reported earlier (Singhal et al., 2012).

Isoelectric focusing (IEF) was carried out using "in-gel rehydration" method. 7 cm immobilized pH gradient (IPG) strips of pH 4–7 (BIO-RAD, USA) were rehydrated overnight with 140 μg protein at 20 °C. The strips were then focused on an IEF unit PROTEAN IEF Cell (BIO-RAD, USA) at 18 °C using the following five-step program: (a) 0–250 V for 1.5 h in linear mode; (b) 250 V constant for 1.5 h in rapid mode; (c) 250–3000 V for 4 h in linear mode; (d) 3000 V constant until 15,000 Vh was reached and e) 500 V constant at slow mode until the IPG strips were taken out form IEF cell. After IEF, strips were equilibrated at RT in equilibration buffer I and II (BIO-RAD, USA) for 5 min each and subjected to SDS-PAGE.

Gel images were acquired and analyzed by Chemidoc using Quantity One software (BIO-RAD). Differentially expressed proteins in Wild type, PknL-S and PknL-AS were shortlisted by Student t-test using PDQuest software (Bio-Rad, Hercules, CA, USA).

2.8. *Mass spectrometry analysis*

Protein spots of interest were excised from the Coomassie brilliant blue R250 stained 2D gels. In gel digestion was performed for the excised protein spots. The extracted peptides were purified using C-18 ziptip pipette tips (Millipore) and then spotted with 1:1 α-cyano-4-hydroxycinnamic acid matrix onto an Opti-TOF 96-well MALDI plates. Peptides was analyzed on a Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) MS/MS, Daltonics Ultraflex III mass spectrometer (Bruker). The resulting

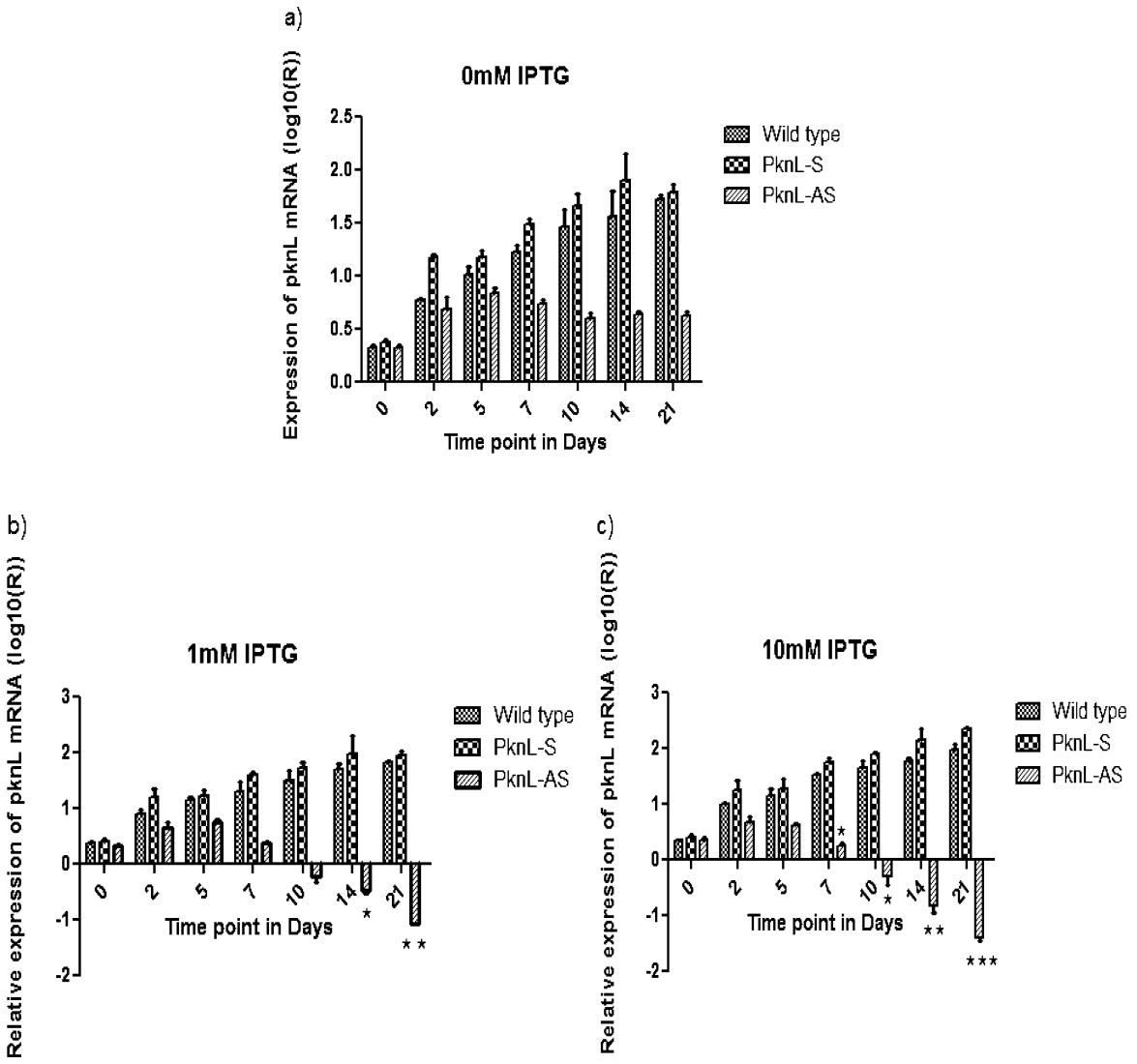

Fig. 1. Differential Expression of *PknL*.
The expression of *PknL* in the knockdown strain (PknL-AS) along with Wild type (H_{37}RV) and over expressed strain (PknL-S) were quantified on days 0,2,5,7,10,14 & 21 by RTPCR with different concentration of IPTG. 16srRNA was used for normalization. The data is representative of three independent experiments. Error bars represent the standard error of the means. The graph represents the quantification ($\log_{10}$(R)) of *PknL* at a) 0 mM IPTG and relative quantification of *PknL* in b) 1 mM IPTG and c) 10 mM IPTG. There was a significant decrease in the expression of *PknL* (*$p < 0.05$ on day14) and **$p < 0.01$ on day 21) in PknL-AS at 1 mM IPTG induction. Similarly the expression of *PknL* further decreased with 10 mM IPTG induction showing a significant reduction (*$p < 0.05$ on day 7 and 10), (**$p < 0.01$ on day 14) and ***$p < 0.001$ on day 21) in PknL-AS compared with PknL-S and Wild type.

spectra were analyzed using Mascot server (www.matrixscience.com) against NCBI database.

2.9. Statistical analysis

Two-way ANOVA with repeated measures using Bonferroni post-test was done for all the *in vitro* growth kinetics, CFU and RTPCR experiments. Significance of differences between the mutant and Wild-type cells in electron microscopy studies was assessed by Student's *t*-test. GraphPad Prism 5.0 Software was used for all the tests. p-value <0.05 were considered significant.

3. Results

3.1. Inhibition of the expression of M. tuberculosis PknL using antisense strategy

Quantification of *PknL* by RT-PCR was done to determine the time period and concentration of the inducer required for inhibiting the expression of *PknL* with respect to the growth of the bacilli. For this total RNA was purified separately from cultures of Wild type (H37Rv), Knockdown strain (PknL-AS) and over expressed strain (PknL-S) at serial time point (0,2,5,7,10,14 and 21 Days) with different concentrations of IPTG (0,1&10 mM) during growth, and quantitative RT–PCR analysis was performed to measure gene expression. The mRNA levels of 16S RNA, was also measured in the same RNA samples. Although the transcription of *PknL* in PknL-AS was less compared to PknL-S and Wild type irrespective of the presence/absence of inducer, but a significant reduction in the expression of *PknL* was observed in PknL-AS on Day 10 in the presence of 1 mM IPTG and the expression of PknL further decreased on Day 7 in the presence of 10 mM IPTG. The expression of *PknL* was almost 2.3 log in PknL-S, 1.9 log in Wild type and −1.3 log in case of PknL-AS in the presence of 10 mM IPTG (Fig. 1). The results observed were statistically significant ($p < 0.05$ to $p < 0.001$) suggesting the successful inhibition of *PknL* by antisense strategy.

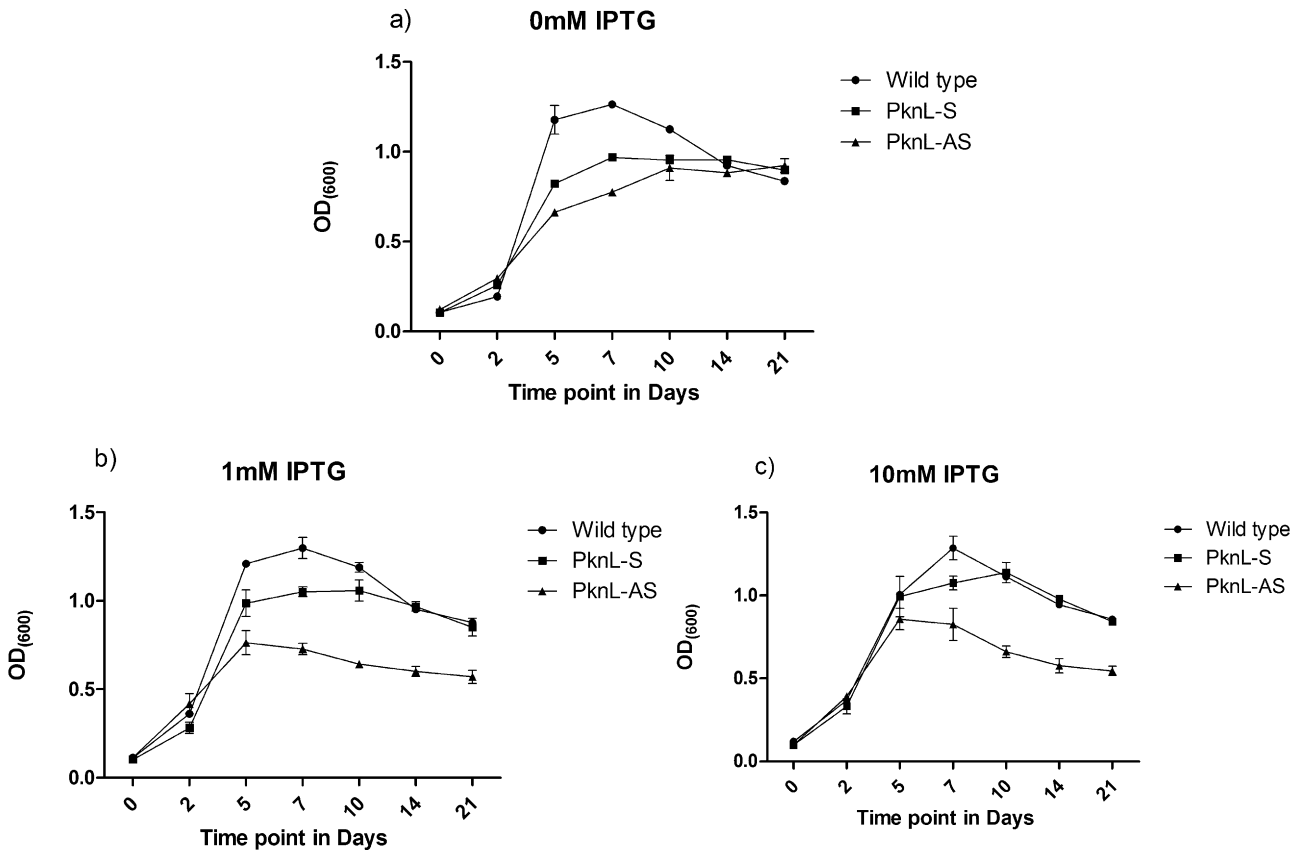

Fig. 2. *In vitro* growth kinetics.
Growth of the strains PknL-AS, PknL-S and Wild type were grown in 7H9 media with differnet concentrations of IPTG and the OD_{600} was monitored on 0,2,5,7,10,14 &21 days. The data is representative of three independent experiments. Error bars represent the standard error of the means. The graph represents the OD_{600} plotted against the respective time point at a) 0 mM IPTG b) 1 mM IPTG and c) 10 mM IPTG.

3.2. Growth and Survival kinetics

To determine whether the antisense inhibition of *PknL* has any effect on the rate of growth and the viability, we compared the growth profile of Wild type, PknL-S and PknL- AS by monitoring the OD_{600} and the viability of the strains were assessed by comparing the Colony Forming units (CFU) at 0,2,5,7,10,14 and 21 days. The growth of all the strains were the same in the absence of the inducer (0 mM IPTG) but the growth of PknL-AS decreased in the presence of 1 mM and 10 mM IPTG (Fig. 2). Similarly, the viability of PknL-AS decreased comparatively after day 10 and day 7 in the presence of 1 mM and 10 mM IPTG respectively (Fig. 3). The OD_{600} of PknL-AS did not go beyond 7.5 and almost 1.2 log difference was observed in the viability of PknL-AS compared to Wild type and PknL-S, indicating a growth defect induced by the inhibition of *PknL*. The decrease in the viability of PknL-AS was much more significant ($p < 0.05$) in the presence of 10 mM of IPTG indicates a dose dependent response (Fig. 3).

3.3. Sensitivity to pH 5.5, SDS and Lysozyme

We studied the growth and survival capacity of Wild type, PknL-S and PknL-AS, in the presence of lysozyme, SDS and during moderate acidic stress that are encountered within the phagosome environment(Geiman et al., 2006; Rohde et al., 2007). The growth and survival kinetics of Wild type remain unaltered in both pH 7.0 and pH 5.5, whereas PknL-AS survived better in pH 5.5 and PknL-S showed a reduced growth in pH 5.5 when compared to their growth in pH 7.0. Similarly, the survival capacity of PknL-S also decreased after encountering the acidic stress of pH 5.5 (Fig. 4) indicating that inhibition of *PknL* has a growth advantage in acidic pH. There was no growth observed in the presence of 0.1% SDS invariably for all the strains and all the strains were resistant to 0.001% of SDS (data not shown). Interestingly, PknL-AS was more resistant to SDS at 0.01% compared to PknL-S and Wild type. There was no visible difference in the colony morphology of all the strains subjected to SDS stress (Fig. 5). Resistance or sensitivity to lysozyme was assessed by comparing the colony count obtained following the incubation of cultures with lysozyme to the log phase cultures of Wild type, PknL-S and PknL-AS at 0 h, 24 h and 48 h. The viability of PknL-AS was comparatively higher and exhibited a significant resistance to lysozyme than PknL-S and Wild type (Fig. 6). No distinct changes in the colony morphology was observed in all the strains.

3.4. Scanning electron microscopy indicates a role for PknL in cell wall biogenesis

To investigate the morphological changes associated with the inhibition of *PknL*, cells of PknL-AS and Wild type strains were visualized using scanning electron microscopy. SEM analysis revealed a significant increase in cell length of PknL-AS (3.622 ± 0.04215 μm, $N = 100$; $p < 0.001$) compared to the Wild-type (2.888 ± 0.04103 μm), N refers to the number of independent measurements. The average difference in cell length between PknL-AS and Wild type cells was ~0.8 μm (Fig. 7).

3.5. Identification of differentially regulated protein by 2-D gel electrophoresis

To determine the effect of *PknL* on the expression of other proteins of *M. tuberculosis* genome, cytoplasmic lysates of Wild type, PknL-S and PknL-AS were separated using 2-D gel elec-

Table 3
Mass spectrometric identifications of proteins.

Spot no	Gene name	Accession No.	Mascot score	No. of matched peptides	Theoretical pl/kDa	Observed kDa	Expression	Functional Category[a]
1	Ppeptidyl-tRNA hydrolase	Rv1014c	53	14	10.1/20.45	20.45	Expressed only in PknL-S	1
2	Hypothetical protein Rv2623	Rv2623	362	62	5.5/31.65	31.747	Expressed only in PknL-S	2
4	Alpha-crystallin protein, partial	Rv2031c	327	48	4.7/16.2	9.808	Expressed only in PknL-S	2
5	Heat shock protein hspX	Rv2031c	325	49	4.7/16.2	11.785	Expressed only in PknL-S	2
6	Membrane protein	N/A	115	18	N/A	9.331	Highly expressed in PknL-AS	N/A
7	Thiosulfate sulfurtransferase, partial	RV3283	80	11	4.8/33.28	5.281	Highly expressed in PknL-AS	1
8	Hypothetical protein[immunogenic protein MPB64]	Rv1980c	573	65	4.5/24.82	24.768	Highly expressed in PknL-AS	3
9	Immunogenic protein MPB64	Rv1980c	572	65	4.5/24.82	25.038	Highly expressed in PknL-AS	3
10	ATP-dependent Clp protease proteolytic subunit	Rv2460	125	17	4.7/23.5	19.518	Highly expressed in PknL-AS	3
11	Heat shock protein 70	Rv0350	562	85	4.5/66.83	66.776	Highly expressed in PknL-S	2
12	Molecular chaperone GroEL	Rv0440	324	47	4.5/56.72	56.722	Highly expressed in PknL-S	2
13	GlnA	Rv2220	242	38	4.8/53.53	53.693	Highly expressed in PknL-S	1
14	Universal stress protein	Rv1636	243	49	5.6/15.31	13.618	Not expressed in PknL-S	2
15	Phosphatidyl-ethanolamine Binding Protein	Rv2140	633	81	5.5/18.63	18.75	Highly expressed in PknL-AS	1
16	Enoyl-CoA hydratase	Rv0632c	169	33	5.6/24.35	24.424	Highly expressed in PknL-AS	4
17	Single-stranded DNA-binding protein	Rv0054	147	37	4.8/17.32	15.905	Highly expressed in PknL-AS	5
18	Serine protease	Rv0125	98	12	4.9/34.92	29.186	Highly expressed in PknL-AS	1
19	Secreted antigen Ag85A	Rv3804c	329	41	6.51/35.68	35.847	Not expressed in PknL-S	4

[a] 1-Intermediary metabolism & respiration; 2-virulence, detoxification & adaptation; 3-cell wall & cell processes; 4-lipid metabolism; 5-information pathways.

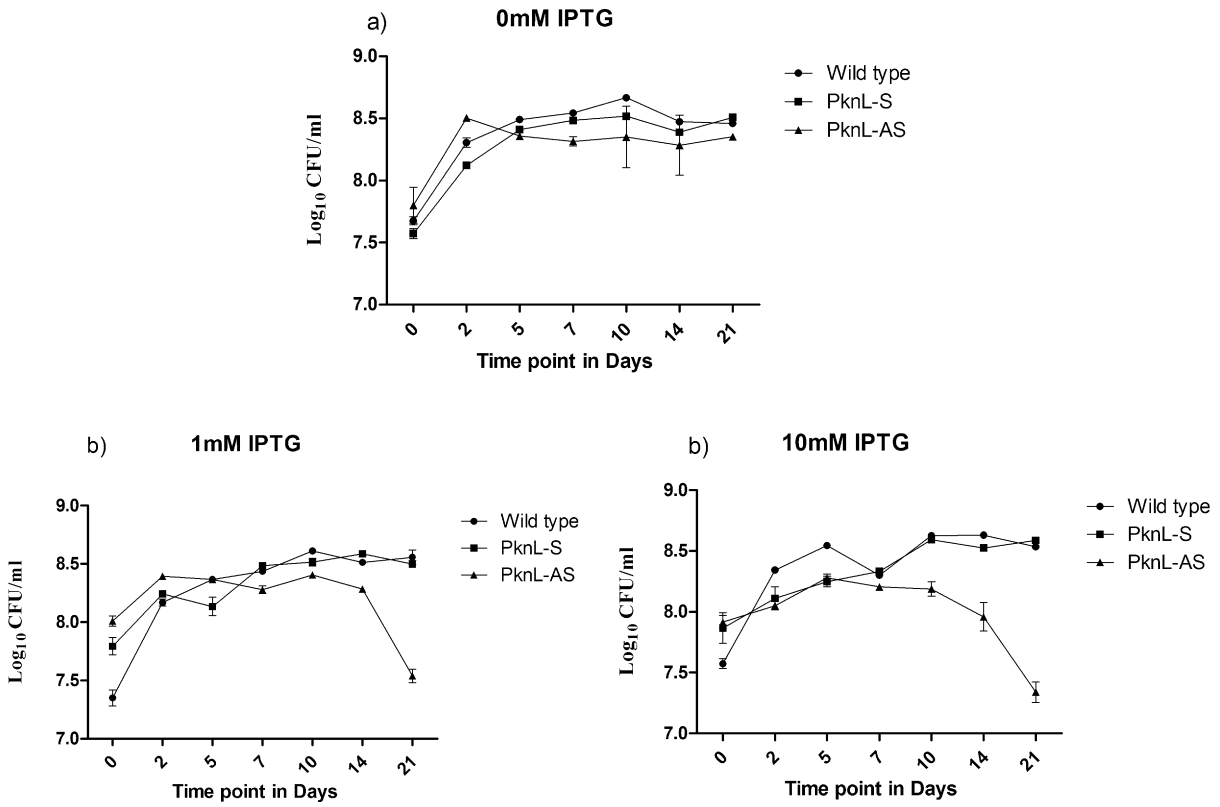

Fig. 3. Survival kinetics.
Viability of the strains PknL-AS, PknL-S and Wild type were monitored by measuring the CFU obtained from serial dilutions on 7H10 agar plates at different concentrations of IPTG. The graph represents the $\log_{10}$(CFU/ml) of the strains at (a) 0 mM IPTG (b) 1 mM IPTG and (c) 10 mM IPTG on 0,2,5,7,10,14 &21 days. The data is representative of three independent experiments. Error bars represent the standard error of the means. There was a significant decrease ($p < 0.001$) in the viability of PknL-AS on day 21 at 1 mM IPTG concentration. Similarly a significant decrease ($p < 0.05$ to $p < 0.001$) in the viability of PknL-AS from day 10 to day 21 compared to Wild type and PknL-S.

trophoresis. Comparison of the gels by PDQuest software revealed nineteen protein spots with increased intensities in PknL-S and PknL-AS compared to Wild type (Fig. 8). Only 18 protein spots were identified using MALDI TOF MS/MS analysis and were categorized into five functional categories based on the information from Tuberculist (Table. 3). The identified proteins were Rv1014c (peptidyl-tRNA hydrolase), Rv2623c (hypothetical protein), Rv2031c (alpha-crystallin protein, Heat shock protein HsPX), Rv3283 (thiosulfate sulfurtransferase), Rv1980c (immunogenic protein MPB64), Rv2460 (ATP-dependent Clp protease proteolytic subunit), Rv0350 (heat shock protein 70), Rv0440 (molecular chaperone GroEL),Rv2220 (Glutamine synthetase glnA1), Rv1636 (universal stress protein), Rv2140 (Phosphatidyl-ethanolamine Binding Protein), Rv0632 (enoyl-CoA hydratase), Rv0054 (single-stranded DNA-binding protein), Rv0125 (serine protease) and Rv3804c (secreted antigen Ag85A).

Among these Rv1014c and Rv2220, involved in intermediary metabolism and stress response were expressed only in PknL-S. Similarly, Rv2031c, Rv0350 and Rv0440 responsible for virulence, detoxification and adaptation showed a higher intensity of expression in PknL-S. PknL-AS strains showed a higher expression of proteins such as Rv1980c and Rv2460, which are involved in cell wall and cell processes. The proteins involved in lipid metabolism and information pathways like Rv0632, Rv3804c and Rv0054 were also highly expressed in PknL-AS (Table 3).

4. Discussion

In this study, we report the adaptive role of a serine/threonine protein kinase *PknL* in response to simulating conditions that are likely to be encountered inside the host cell. Additionally, we have also reported the morphological changes associated with the inhibition of *PknL* by antisense approach. We have also described the proteomic profile of the over expressed and the knockdown construct along with the Wild type.

It is a well known fact that *M. tuberculosis* survives inside the host by evolving effective strategies to overcome most of the macrophage killing mechanisms. *M. tuberculosis* is subjected to multiple stresses within the phagosome, which may act as important environmental cues for the pathogen (Rohde et al., 2007). Understanding the cues that the pathogen recognizes during infection is critical to completely elucidate the impact of the environment on *M. tuberculosis* pathogenesis and persistence and its interaction with fundamental host cell processes. Kinases belong to the signal transduction pathways involved in phosphorylation/dephosphorylation activity in response to environmental alterations.

Although *PknL*, is a non-essential gene in *M. tuberculosis* (Lamichhane et al., 2003; Sassetti et al., 2003; Griffin et al., 2011), we chose to create a knockdown strain of *PknL* to elucidate its role in pathogenesis and stress response, as we were unable to generate a knockout strain even after repeated measures. Moreover, the essentiality of *PknL* is still intriguing since the other three genes *PknA, PknB and PknG* belonging to the cluster of genes share a 75% identity with *M. leprae* and have been proved to be essential (Sassetti et al., 2003; Greenstein et al., 2005; Fernandez et al., 2006). The use of the vector pAZ19018b has been previously validated in generating knockdown strain by antisense technology (Kaur et al., 2009). The cloning of the entire length of *PknL* gene in the antisense and sense orientation by replacing lacZ in this *E.coli – M. tuberculosis* shuttle vector has generated a knockdown and overexpressed strains of *PknL* respectively in *M. tuberculosis*.

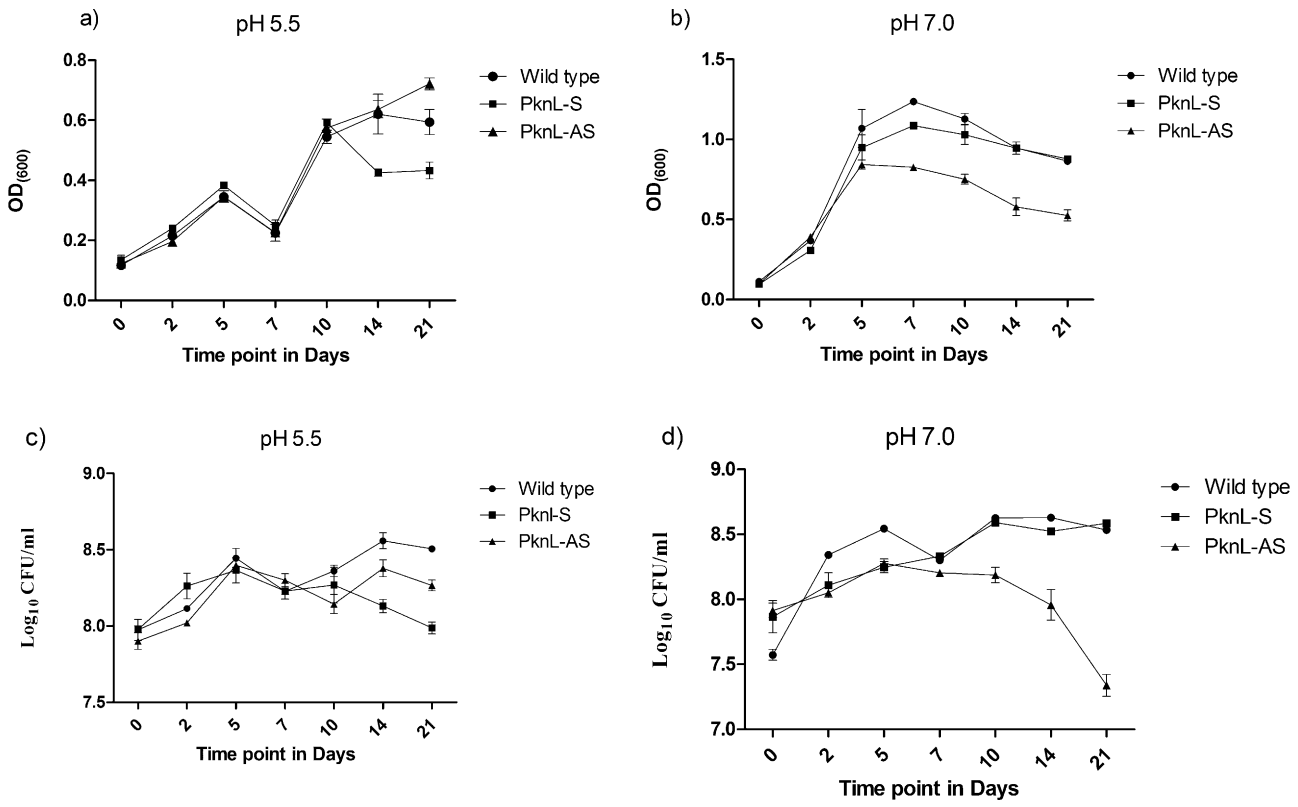

Fig. 4. *In vitro* growth and survival kinetics at variable pH.
Growth and viability of the strains PknL-AS, PknL-S and Wild type were monitored by measuring the OD_{600} and CFU obtained from serial dilutions on 7H10 agar plates at different pH with 10 mM of IPTG on 0,2,5,7,10,14 &21 days. The data is representative of three independent experiments. Error bars represent the standard error of the means. The graph represents the OD_{600} plotted against the respective time point at (a) pH 5.5 (b) pH 7.0. and the $\log_{10}$(CFU/ml) of the strains grown at (c) pH 5.5 (d) pH 7.0. There was a significant decrease ($p < 0.01$ to $p < 0.001$)in the viability of PknL-S compared with PknL-AS and Wild type after day 10.

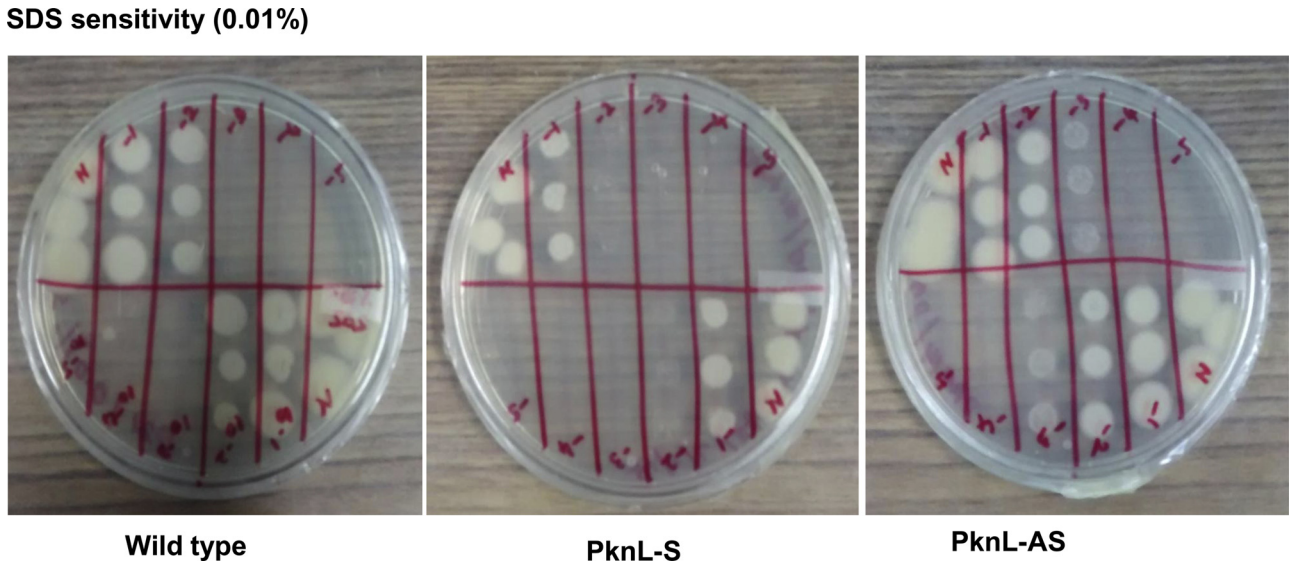

Fig. 5. Sensitivity to SDS.
The log phase cultures of Wild type, PknL-AS and PknL-S were serially diluted and plated on 7H10 agar plates containing SDS at (0.1%, 0.01% and 0.001%) and incubated for 3 weeks. The data represents the sensitivity of the strains in 0.01% SDS. The resistance exhibited by PknL-AS was more compared to PknL-S and Wild type.

We have confirmed the increase and the decrease of the gene expression of *PknL* in PknL-S and PknL-AS respectively in the presence of 1 mM and 10 mM IPTG induction by RTPCR. Though no change in growth was observed in the absence of inducer in the case of PknL-AS, the expression profile showed a reduced expression of *PknL* which may be the reason for the significant drop in the growth and survival rate of PknL-AS after day 7. The *in vitro* growth and survival kinetics pattern of PknL-AS based on the Generic survival kinetics on delineating bactericidal and bacteriostatic targets (Kaur et al., 2009) *PknL* can be classified as 'barely bacteriostatic' (approx 1.2 log CFU reduction). Though quantifying the level of knockdown of gene expression following antisense cloning is difficult, the dose

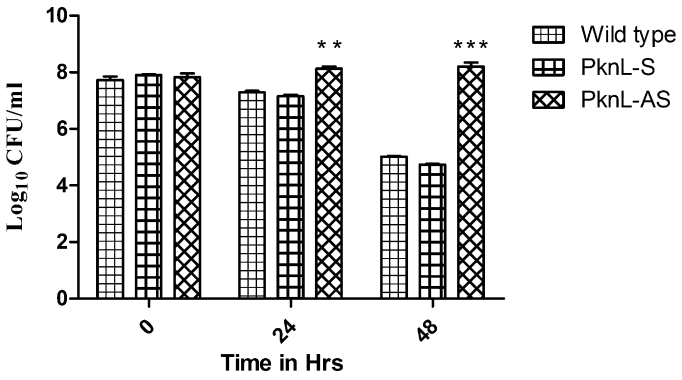

Fig. 6. Sensitivity to Lysozyme.
The log phase cultures of Wild type, PknL-AS and PknL-S were incubated with 2.5 mg/ml of Lysozyme and the sensitivity was monitored by plating serial dilutions on 7H10 agar plates at 0, 24 and 48 h. The data is representative of three independent experiment. Error bars represent the standard error of the means.There was a significant increase ($^{**}p < 0.01$ to $^{***}p < 0.001$)in the growth of PknL-AS compared with PknL-S and Wild type.

dependent reduction in CFU observed was in proportion with the IPTG concentration. The significant reduction in the expression of *PknL* in PknL-AS in the presence of 1 mM and 10 mM IPTG concentration after day 7 indicates that the down regulation of *PknL* reduced the growth of the bacilli in liquid as well as solid enriched media. We observed maximum down regulation of *PknL* in the presence of 10 mM IPTG, so further experiments were performed with only 10 mM IPTG as inducer. The morphological changes associated with down regulation of *PknL* as analyzed by scanning electron microscopy witnessed an elongated cell structure compared to Wild type. Interestingly pHL8, expressing a kinase inactive mutant of *PknL* in *M. smegmatis* (Lakshminarayan et al., 2009) also revealed the same elongated morphology suggesting that phosphorylation triggered by *PknL* have led to the changes in the cell morphology.

In the process of adapting and to survive inside the host, *M. tuberculosis* has to sense the environmental cues of the host and act upon it. One such cue that has received particular attention is pH. It has been reported that the *M. tuberculosis* phagosome acidifies to an intermediate pH of 6.4 (Sturgill-Koszycki et al., 1994; Mwandumba et al., 2004; Pethe et al., 2004) and even in medium, the bacterium exhibits a profound transcriptional response to acidic pH. (Walters et al., 2006; Golby et al., 2007; Rohde et al., 2007). Here we identified that *PknL* is responsible for slowing down the growth of *M. tuberculosis* under acidic conditions since PknL-AS showed a better survival in acidic pH, which was also exhibited by mutant strains of *PknE* and *PknI*. (Jayakumar et al., 2008; Gopalaswamy et al., 2009) . Interrupting or facilitating the binding of *M.tuberculosis* to surfactant proteins leads to either tubercle bacilli invasion or bacterial clearance inside the host cell (Hall-Stoodley et al., 2006). Though all the strains Wild type, PknL-S and PknL-AS were resistant to the surfactant stress, SDS (0.01%) PknL-AS exhibited more resistance suggesting that loss of *PknL* has facilitated the binding aiding the clearance of the mycobacterium inside the host. Another important stress encountered by the bacilli is the lytic stress where the bacilli is encountered with enzymes such as lysozyme which hydrolyze the polysaccharide portion of the peptidoglycan layer causing a strong bactericidal effect. (LH, 2001) Here again, all the strains were able to grow and reactivate after lysozyme stress, but PknL-AS survived better compared to PknL-S and Wild type suggesting that *PknL* plays a role in cell wall homeostasis thereby slowing down the growth of *M. tuberculosis*.

2-Dimensional gel electrophoresis revealed that two of the stress related proteins Rv2031c and Rv2623 which form a part of the dormancy regulon (*dosR*) was expressed only in PknL-S. Although most of the genes in this regulon have not been characterized, it has been indentified that *dosR* plays a significant role in adaptation of *M. tuberculosis* to its host environment (Leistikow et al., 2010). Expression of these stress related proteins in PknL-S signifies the role of *PknL* in stress and adaptive response of *M. tuberculosis*. Recent study on comparison of proteomic profiling of aminoglycosides resistant and susceptible strains of *M. tuberculosis*, the expression of Rv2031 was linked with the resistant strains of *M. tuberculosis* (Sharma et al., 2015). Some proteins that were expressed in all the strains but were highly expressed in

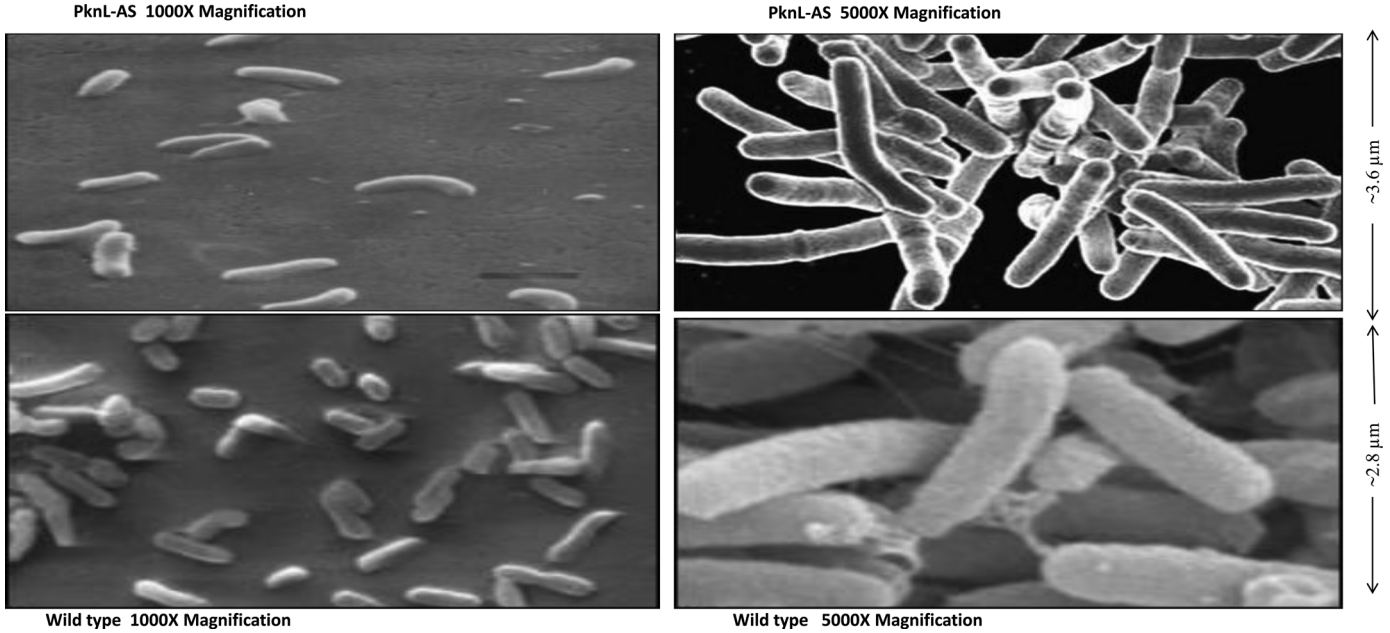

Fig. 7. SEM pictographs.
The cells of the log phase cultures of Wild type and PknL-AS were fixed and scanned. The picture represents the cells of Wild type and PknL-AS in low (1000×) magnification and high (5000×) magnification. A significant difference ($p < 0.001$) was observed in the cell length measurement with an increase of 0.8 µM in case of PknL-AS compared to Wild type.

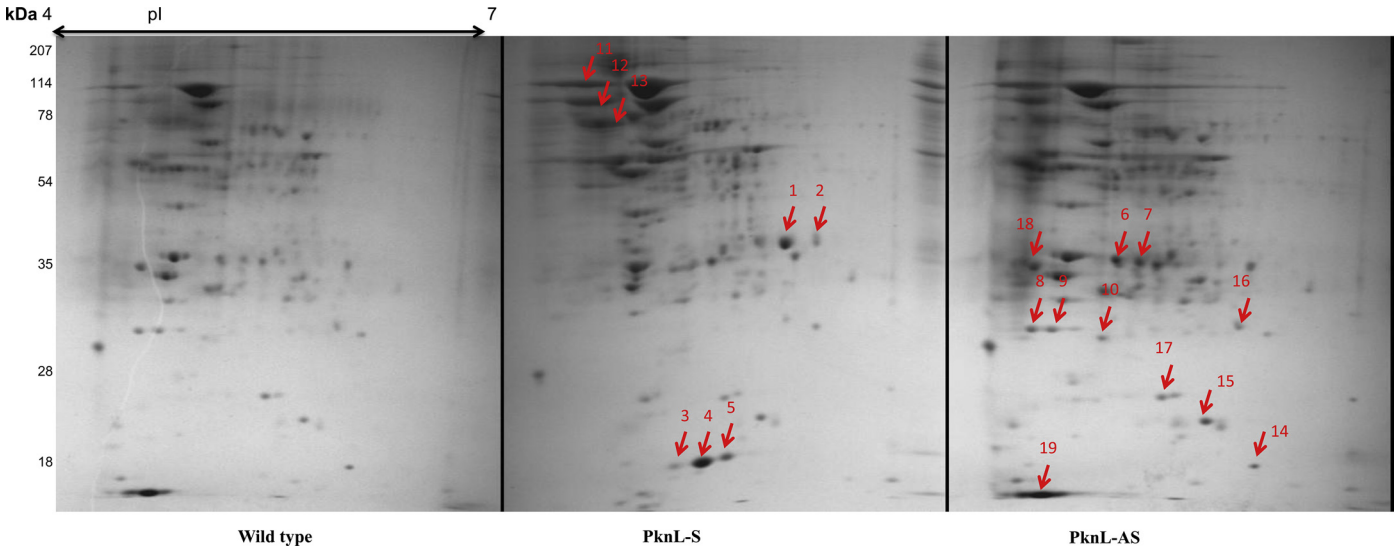

Fig. 8. 2-D gel electrophoresis.
The whole cell extracts of Wild type, PknL-S and PknL-AS were seperated by 2D gel electrophoresis. The arrows represents the difference in spots detected by PDQuest software. These spots were excised from the gel and analyzed by mass spectrometry.

the knockdown strain (PknL-AS) are Rv3283, Rv1980c, Rv2460, Rv2140, Rv0632c, Rv0054 and Rv0125. Among these, two proteins Rv2460 and Rv0054 form a part of the *relA* regulon, responsible for initiating stringent response in *M. tuberculosis* (Primm et al., 2000). The mutant strain of relA failed to survive in nutrient deprived conditions and was also unable to persist in the host contrast to Wild type (Dahl et al., 2003). The expression of these proteins indicates that *PknL* may also play a role in the nutrient starvation response of *M. tuberculosis* as previously reported. Other proteins that were highly expressed in PknL-AS are invovled in various activities like formation of thiosulfate (Rv3283), cell wall process (Rv1980c), lipid metabolism (Rv0632), etc. Rv2220 (*GlnA1*) is an essential gene belonging to the cluster of Glutamine synthetases, involved in regulating the glutamine/nitrogen metabolism and *GlnA1* is known to catalyse the synthesis of L-Glutamine in *M. tuberculosis* (Harth et al., 2005). Rv2220 was expressed in all the strains with a higher intensity in PknL-S compared to Wild type and PknL-AS suggesting the involvement of *PknL* in the glutamine metabolism. Moreover, the presence of GlnD-Uridylyl transferase motif present in the kinase domain of *PknL* may further stress the link between *PknL* and glutamine metabolism (Lakshminarayan et al., 2009). Since *PknG*, a serine/threonine protein kinase is linked to cellular Glutamine/glutamate levels (Cowley et al., 2004), the involvement of *PknL* in glutamine metabolism needs to be identified which will help us to understand its role in metabolic adaptation.

In this study, we have shown that inhibition of *PknL* has a growth advantage under conditions of acidic pH, SDS and Lysozyme stress, which probably mimic the macrophage environment as soon as the bacilli are taken into the host. Our studies also indicate that internal signals used to activate *PknL* are most likely the host-associated internal signals and the ability of *PknL* to respond to such stresses is relevant to their survival *in vivo*. Scanning Electron Microscopy indicates that *PknL* is also responsible for cell wall homeostasis. Proteome profiling studies have also shown the involvement of *PknL* in stress and starvation response of *M. tuberculosis*. In conclusion, it is evident that *PknL* is able to sense the environmental cues and act accordingly thereby helping the bacteria in adaptation to stress conditions inside the host by slowing down the growth of the bacteria leading to dormant state and thereby persisting within the host. The starvation response of *PknL* under different nutrient depletion medium and the study relating *PknL* to glutamine metabolism is currently being investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2016.02.005.

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