RESEARCH LETTER

The serine/threonine protein kinase PknI controls the growth of *Mycobacterium tuberculosis* upon infection

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Abstract

The protein kinase PknI is one of 11 functional serine/threonine protein kinases in *Mycobacterium tuberculosis*. Specialized transduction was performed to create a null mutant in the *pknI* gene. The resulting mutant was used to determine the role of PknI in *M. tuberculosis* growth and infectivity. The *pknI* mutant grows better under acidic pH and limited oxygen availability. We observed a modest increased growth of *pknI* mutant within macrophages during an *in vitro* infection and a hypervirulence phenotype in severe combined immunodeficiency mice. The internal signals used to activate PknI are most likely the host-associated signals such as low pH associated with limited oxygen availability. Thus, we have shown that PknI plays a role in sensing the host macrophage's environment and translating it to slow the growth of *M. tuberculosis* within the infected host.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular human pathogen residing in alveolar macrophages and granulomas in the lung. The principal step in mounting a successful infection by *M. tuberculosis* is its ability to circumvent and modify the human host cells' response (Hestvik *et al*., 2005; Bach *et al*., 2008). Following initial infection of the macrophages in the lung, in the absence of an immune response, the bacilli undergoes rapid replication. Once an acquired immune response comes into play, it contains the mycobacterial cells' expansion and disease spread (Honer zu Bentrup & Russell, 2001), followed by the formation of granuloma. In the granuloma, the disease progression is prevented and *M. tuberculosis* survive and persist without getting eliminated (Flynn & Chan, 2001).

In order to adapt its physiology to the changing host environmental conditions, *M. tuberculosis* would need to sense the extracellular milieu to modulate the corresponding adaptive gene expression. The translation of external signals into a cellular response is carried out by a signal transduction mechanism relying on reversible protein phosphorylation carried out by specific protein kinases and phosphatases. In *M. tuberculosis*, signal transduction is principally mediated by serine/threonine protein kinases (STPKs) and two-component systems (Av-Gay & Deretic, 2005). *Mycobacterium tuberculosis* encodes 11 STPKs and three protein phosphatases (Av-Gay & Everett, 2000).

One of these kinases, *pknI*, is flanked by the *dacB2*, *ftsY* and *ffh* genes involved in cell division and protein secretion, indicating a possible role for PknI in one of these processes (Av-Gay & Everett, 2000). PknI has been shown earlier to be a functional kinase autophosphorylated at serine and threonine residues. Sequence comparison with other prokaryotic STPKs revealed its close homology to Stk1 from *Streptococcus agalactiae*, which was shown to play a role in the virulence and cell segregation of the organism (Gopalaswamy *et al*., 2004).

In this study, we gained an insight into the role of PknI in *M. tuberculosis* physiology by constructing a null mutant of *pknI*. We show that PknI is needed for the balanced growth of *M. tuberculosis* under the *in vitro* growth conditions mimicking growth inside the macrophage. As such, the *pknI* mutant is hypervirulent in both human macrophages and severe combined immunodeficiency (SCID) mice.

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Materials and methods

Reagents, bacterial strains and growth conditions

Mycobacterium tuberculosis H37Rv was routinely grown in Middlebrook 7H9-ADS-T medium (7H9 supplemented with 10% ADS (albumin dextrose saline), 0.05% Tween 80) and plated on 7H10 solid agar (supplemented with 10% oleic acid albumin dextrose complex and 0.5% glycerol) and appropriate antibiotics ($50\,\mu g\,mL^{-1}$ of hygromycin and $20\,\mu g\,mL^{-1}$ of kanamycin, wherever applicable). All restriction and modifying enzymes were purchased from New England Biolabs. Liquid cultures and plates were incubated at 37 °C.

Construction of gene knockout

The left (806 bp) and the right (778 bp) arms of *pknI* were PCR amplified using the primers given in Table 1 and cloned into the suicidal delivery vector p0004S to create the gene replacement vector p2914cS (Table 1), which harbors the flanking region of *pknI* and a 5.2-kb Hyg^R-*sacB* cassette in between the arms. Preparation of high-titer phages and specialized transduction was performed as described earlier (Bardarov *et al*., 2002). The transductants were grown, genomic DNA was extracted and Southern hybridization was performed to confirm the presence of disruption. To complement the mutant, the *pknI* coding region along with its 400-bp upstream sequence was cloned into pMV306 (Stover *et al*., 1991) to make pRG3 (Table 1). Integration of this DNA was achieved by electroporation of the Δ*pknI* mutant by pRG3 and selection on hygromycin and kanamycin plates. Reverse transcriptase (RT)-PCR analysis was performed to confirm the expression of *pknI* from the complemented strain. Total RNA extracted from wild-type, Δ*pknI* and complemented strains were DNase I treated, cleaned using the Qiagen RNeasy mini kit and reverse transcribed using Revertaid H minus M-MuLV reverse transcriptase (Fermentas). A 154-bp internal region of *pknI* was then PCR amplified from cDNA using the primers indicated in Table 1.

In vitro growth determinations

To determine the *in vitro* growth characteristics, log-phase cultures grown in 7H9-ADS-T medium were washed thrice and diluted in different growth media to be tested. Wild-type, Δ*pknI* and complemented strains were grown in 7H9-ADS medium, Sauton's medium (Sauton, 1912; Hatfull & Jacobs, 2000) and modified Proskauer Beck (PB) medium (Cowley *et al*., 2004), supplemented with 0.05% Tween 80 at pH 7.0 as well as pH 5.5. All the experiments were performed in duplicate at 3 r.p.m. rolling and under standing culture conditions. Aliquots of 1 mL were taken from the cultures for $OD_{600\,nm}$ readings at specified time points as indicated. Viability was checked at certain time points by measuring CFUs to corelate with the $OD_{600\,nm}$ values.

THP-1 infection studies

The preparation and infection of monolayers of THP-1 cells were performed as described previously (Papavinasasundaram *et al*., 2005). Bacterial inocula were prepared by dilution of log-phase cultures ($OD_{600\,nm}$∼0.6) grown in 7H9-ADS-T and the inoculum CFUs were determined. For individual infection, *M. tuberculosis* strains wild-type, Δ*pknI* and Δ*pknI* complemented strain were infected at a multiplicity of infection (MOI) of 10 : 1 (bacterium : THP-1 cell). For the coinfection studies, the monolayers were coinfected with equal numbers of *M. tuberculosis* wild-type and Δ*pknI*

Table 1. Plasmids and primers used

Plasmids	Description	Source
p0004S	Allelic exchange vector carrying Hyg^R-*sacB* cassette	William R. Jacobs Jr (unpublished data)
p2914cS	Allelic exchange vector carrying Hyg^R-*sacB* cassette, disrupting wild type *pknI* from *M. tuberculosis*	This study
pMV306	Integration-proficient *E. coli*–mycobacteria shuttle vector	Stover *et al*. (1991)
pRG3	pMV306 derivative harbouring *pknI* with its 400-bp upstream sequence	This study
Primers	**Description**	**Sequence**
pknI LL	Forward primer to amplify left flank of *pknI*	TTTTTTTTCCATAAATTGGGGCAGGGCGCTATCCAACC
pknI LR	Reverse primer to amplify left flank of *pknI*	TTTTTTTTCCATTTCTTGGCGGCTACGGCAGTGACGATG
pknI RL	Forward primer to amplify right flank of *pknI*	TTTTTTTTCCATAGATTGGCGGTCCCACTGGACGGCACATA
pknI RR	Reverse primer to amplify right flank of *pknI*	TTTTTTTTCCATCTTTTGGTGCGAATGCCCAGAGTGCGAGTC
pknI 306 Fwd	Forward primer to amplify *pknI* with its 400-bp upstream sequence	CCGGAATTCCGGACATGCGCGACCTGTATGCC
pknI 306 Rev	Reverse primer to amplify *pknI* with its 400-bp upstream sequence	CCCAAGCTTGGGTCGTTGAGTTGCCGCCAAGC
pknI RT-Fwd	Forward primer to amplify 154-bp internal region of *pknI* from cDNA	CGCCTGGACAACTTCTCCAC
pknI RT-Rev	Reverse primer to amplify 154-bp internal region of *pknI* from cDNA	TGGCGGTCGTGTTCGTCTTG

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strains at a MOI of 2 : 1 (bacterium : THP-1). Infection was carried out for 3 h, washed with warm Roswell Park Memorial Institute (RPMI) medium and resuspended in warm RPMI medium (supplemented with 10% fetal bovine serum and 100 $\mu g\,mL^{-1}$ gentamycin), and the plates were incubated at 37 °C. Intracellular bacteria were recovered by lysing the monolayers in cold 0.01% Triton X-100 and then serially diluted and plated to determine their CFUs.

Infection of SCID mice

The methodology for infecting SCID mice has been described in detail previously (Smith *et al.*, 2001). Briefly, mice were injected with 1×10^5 viable mycobacteria in 200 μL of phosphate-buffered saline via a lateral tail vein. At the time of inoculation, the mycobacteria were routinely plated to verify input CFUs. All animals were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. Median survival times were calculated for each group. The log rank test was used to calculate the significance.

Results

Construction of the Δ*pknI* mutant in *M. tuberculosis*

To study the function of PknI, we disrupted *pknI* in *M. tuberculosis* by phage-mediated allelic exchange (Bardarov *et al.*, 2002). The coding region of *pknI* was replaced by the hygromycin resistance gene and the *sacB* cassette in *M. tuberculosis* H37Rv (Fig. 1a). As described in Fig. 1, the successful gene replacement was confirmed by Southern hybridization (Fig. 1b). To complement the disrupted strain, the Δ*pknI* mutant was electroporated with the mycobacterial expression vector pRG3 that contains the parental *pknI* gene under the control of its putative promoter. RT-PCR analysis confirmed the production of *pknI* mRNA from the wild-type and complemented strain, but not the Δ*pknI* mutant (Fig. 1c).

The Δ*pknI* mutant growth is impaired in aerated culture and enhanced in acidic pH

The growth of the Δ*pknI* mutant was monitored in rolling or standing cultures over time in a variety of growth media. The Δ*pknI* mutant was indistinguishable from its parental strain upon growth under a standing culture condition in standard growth media (Fig. 2a). However, when the culture was aerated by rolling at 3 r.p.m., the parental strain grew to a higher density compared with the Δ*pknI* mutant or the complemented strain. Over time, the complemented strain grew to a lesser extent compared with the parental strain or the Δ*pknI* mutant (Fig. 2b). By day 34, the complemented strain was lower than the wild type with a difference in $OD_{600\,nm}$ of 0.8.

Next we compared the growth of the mutant with its parental strain in acidic culture similar in pH (5.5) to the infected phagosome (Fig. 2c and d). Under standing growth, the Δ*pknI* mutant showed better growth compared with the wild type (Fig. 2c). Viable counts measured on day 14 confirmed these results, with the Δ*pknI* mutant CFUs reaching $2.7 \pm 0.14 \times 10^8$ cells mL^{-1} while the parental strain reached $1.77 \pm 0.04 \times 10^8$ cells mL^{-1} and the complemented strain reached $6.5 \pm 0.7 \times 10^7$ cells mL^{-1}. Under oxygen-saturated rolling conditions, the Δ*pknI* mutant grew to a lesser extent compared with its wild-type parental strain (Fig. 2d).

Under both rolling and standing conditions, the complemented strain failed to grow exponentially compared with the wild type and the Δ*pknI* mutant when the growth media were acidic (Fig. 2c and d). These results were verified by viable counts performed on day 14, showing $2.8 \pm 0.29 \times 10^7$ cells mL^{-1}, which are close to 1 log lower than the wild-type counts (at $2 \pm 0.34 \times 10^8$ cells mL^{-1}) and the Δ*pknI* mutant CFUs (at $1.4 \pm 0.11 \times 10^8$ cells mL^{-1}) under rolling conditions.

The Δ*pknI* mutant has increased intracellular growth inside THP-1 cells

The intracellular growth characteristics of the Δ*pknI* strain were studied by infecting differentiated THP-1 cells. Wild-type, Δ*pknI* and complemented strains were infected individually into THP-1 cells and survival of intracellular bacteria was analyzed. The Δ*pknI* mutant grows better than the wild type by day 5 postinfection and reaches 1.7-fold higher CFUs ($P < 0.05$) compared with the wild type and complemented strain (Fig. 3a). The survival of all the strains was similar on day 1, suggesting that there are no differences in bacterial uptake. To confirm this observation, we performed coinfection studies aimed to monitor differences in phagocytosis between the wild type and the Δ*pknI* mutant during infection of THP-1 cells. For this, we mixed and introduced equal numbers of Δ*pknI* mutant and wild-type *M. tuberculosis* into THP-1 cells. By day 1, the Δ*pknI* mutant was 51% of the total population while the wild type was 49%, indicating that the uptake or the entry of both the Δ*pknI* and the wild-type strain were the same. Later on, beginning day 3 and up to day 6 postinfection, the Δ*pknI* mutant yielded higher CFUs compared with the wild-type parental strain (Fig. 3b).

The *M. tuberculosis* Δ*pknI* mutant is hypervirulent in SCID mice

To test the differences in bacterial virulence, the wild-type and Δ*pknI* mutant strains were introduced intravenously into immunodeficient SCID mice. All the mice infected with the Δ*pknI* mutant became moribund and died earlier compared with mice infected with the wild type. Mice infected with the Δ*pknI* mutant had a median survival time

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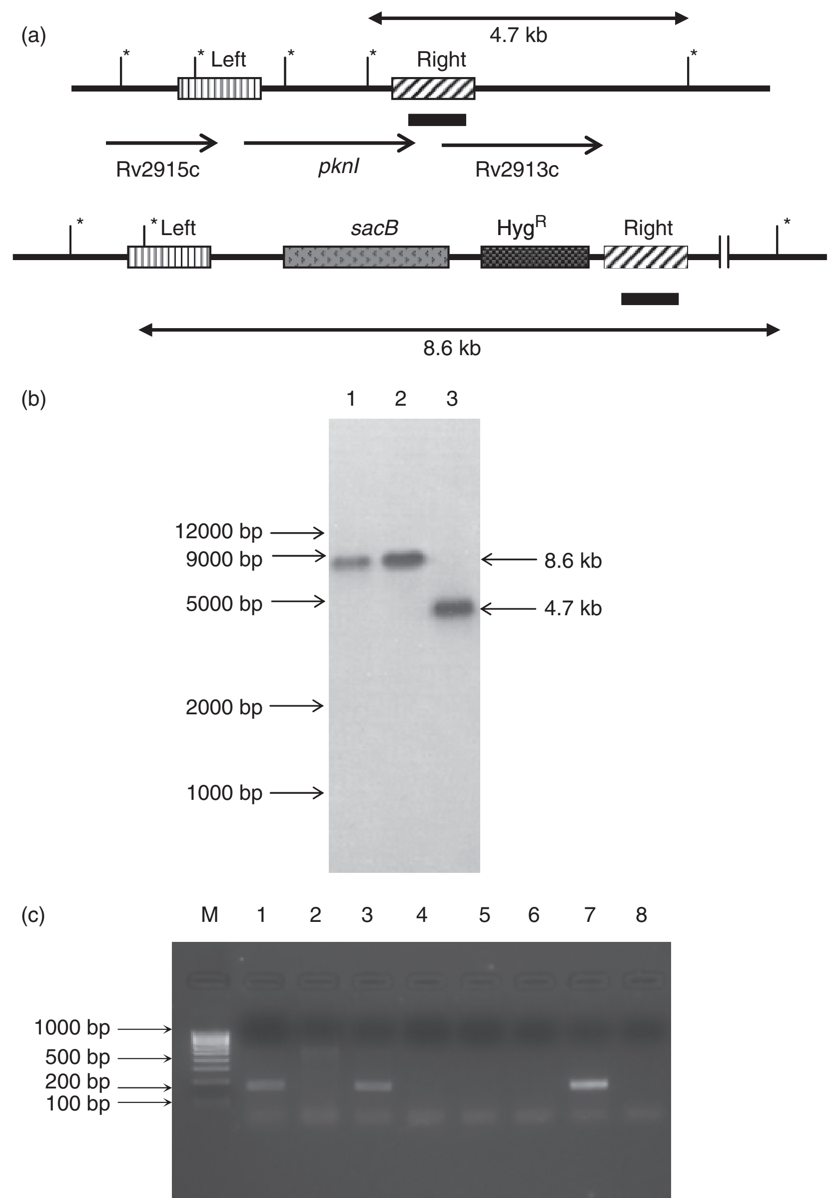

Fig. 1. Genotype and phenotype of *Mycobacterium tuberculosis* knockout of *pknI*. (a) Schematic representation of the *pknI* locus. The left and right arms used for gene replacement are marked as shaded boxes. *pknI* (flanked by Rv2913c encoding a probable D-amino acid hydrolase and Rv2915c encoding a conserved hypothetical protein) was disrupted by Hyg^R-*sacB* following allelic exchange. The solid bar represents the probe used in the Southern hybridization, and '*' indicates the BamHI sites. The expected fragment sizes after hybridization are indicated by double-headed arrows below the probe region. (b) Southern blot analysis of *pknI* disruption. Genomic DNA from Δ*pknI* mutants (lanes 1, 2) and wild type (lane 3) was digested with BamHI, run on 0.8% agarose gel and blotted onto an N^+ membrane. The blot was hybridized with a labeled probe (region indicated earlier) and the sizes were determined using the molecular DNA standards (indicated by arrows) run in parallel. (c) RT-PCR analysis. A 154-bp internal region of *pknI* was amplified from cDNA synthesized from the wild type (lane 1), Δ*pknI* (lane 2) and complemented strain (lane 3) and run on a 2% agarose gel along with the 100-bp ladder. A control reaction without reverse transcriptase was included (lanes 4, 5 and 6, respectively, for wild type, Δ*pknI* and complemented strain). Genomic DNA from wild type (lane 7) and no template (lane 8) were included as PCR controls.

of 18 days while mice infected with the wild type had a median survival time of 28 days (Fig. 4). This difference in the survival ratio was found to be statistically significant ($P < 0.0001$). Mice infected with a Δ*pknI* complemented strain had a median survival time of 21 days. These results demonstrate that Δ*pknI* is hypervirulent in a SCID mice model and demonstrate the role of PknI in controlling *M. tuberculosis* growth *in vivo*.

Discussion

In this study, we have constructed and characterized the Δ*pknI* mutant in *M. tuberculosis*. As shown earlier, our ability to generate a Δ*pknI* mutant indicates that *pknI* is not an essential gene in *M. tuberculosis* (Sassetti *et al*., 2003). We then continued and compared the growth characteristics of the Δ*pknI* mutant with the wild type under different *in vitro* growth conditions. The Δ*pknI* mutant grew slower than the wild type in well-aerated rolling cultures in both standard and acidified media. Interestingly, in standing culture, where there is localized oxygen depletion within the bacterial sediment, the Δ*pknI* mutant showed enhanced growth when grown under acidic conditions. This indicates a clear growth advantage that *M. tuberculosis* has through the loss of *pknI* under conditions of acidic pH and low oxygen availability, which probably mimic the macrophage environment as soon as the bacilli are taken into the host. In studies where mycobacterial transcription has been studied under similar

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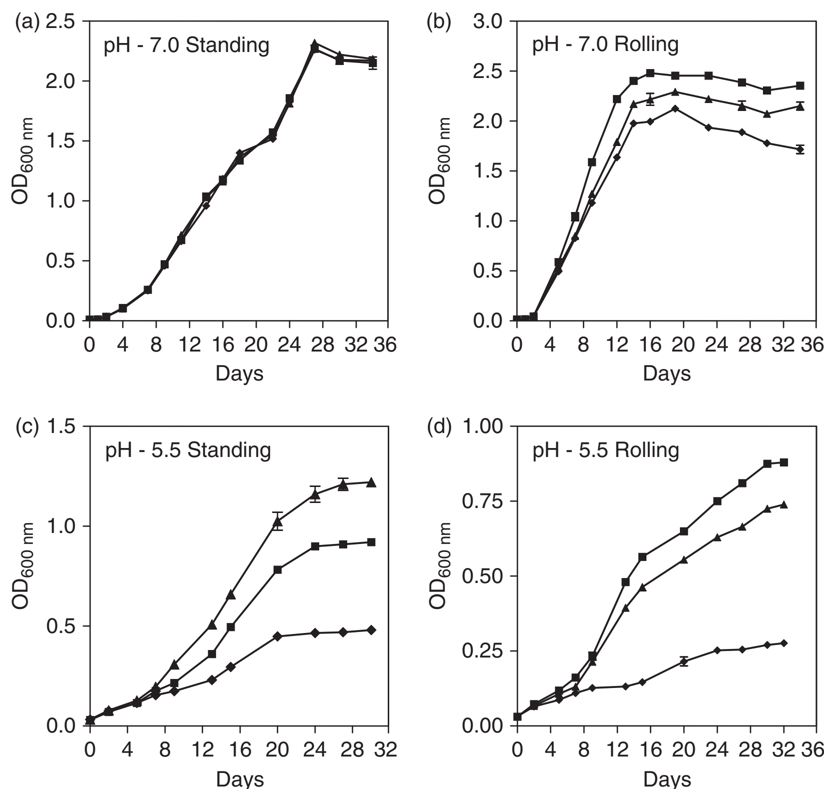

Fig. 2. *In vitro* growth analysis of Δ*pknI*. Wild type (■), Δ*pknI* (▲) and complemented (◆) strains were grown in 7H9-ADS-T medium at pH 7.0 (a, b); pH 5.5 (c, d) under standing (a, c) and rolling (b, d) conditions. The experiment was performed in duplicate and mean values (plotted with SE) represent one of the three independent experiments. Similar results were obtained for rolling and standing cultures using modified PB-T and Sauton's-T media (data not shown).

acidic conditions (Fisher *et al*., 2002; Saviola *et al*., 2003), *pknI* was not listed among the induced genes. This could be due to the low transcript level because signaling molecules are not needed in abundance and the change in the kinase phosphorylation status is responsible for the cellular response rather than its transcription level. Although *pknI* was expressed from its own promoter, complementation was not very effective under certain specific conditions. Our result leads us to speculate that *M. tuberculosis* could induce expression of *pknI* upon entry into a low-pH environment. The overexpression of *pknI* could be attributed to the slow growth of the complementing strain under acidic culture conditions in both rolling and standing type of cultures.

To determine the role of *pknI* in intracellular growth and survival inside the macrophages, we infected the macrophage-like THP-1 cell line. The Δ*pknI* mutant showed enhanced growth in both individual infection and coinfection studies. The role of *pknI* in macrophage uptake or entry was ruled out as we observed limited difference in bacterial uptake. In a previous study, *pknI* expression was shown to be decreased during the course of infection from day 1 to day 3 using THP-1 macrophages. This suggested that *pknI* of *M. tuberculosis* could be a negative regulator that should be removed for active intracellular growth (Singh *et al*., 2005). Interestingly, our studies show that the Δ*pknI* mutant grows better within macrophages when compared with the wild type. One possible explanation for this discrepancy could be that the signaling cascade controlled by PknI is induced at the first 24 h postinfection and then it is winding down. Alternatively, as PknI is a regulatory protein kinase, its expression levels are less significant compared with its phosphorylation status. It is possible that low levels, however, phosphorylated forms of PknI are sufficient for its activity within macrophages. Thus, activated PknI inside the macrophages can be used to contain growth within the host and to bring about sustained infection. Our *in vitro* growth studies strengthen this later hypothesis as they show striking differences between the Δ*pknI* mutant and its parental strain. With the loss of PknI, the mutant possesses enhanced growth under specific conditions mimicking an intracellular environment.

Infection of SCID mice by the Δ*pknI* mutant results in early death of mice compared with the wild type. This indicated a hypervirulent response in these immunosuppressed mice. A similar observation was made with Δ*devR*, a mutant of one of the response regulators (*devR*) of a two-component system in mycobacteria. In this study, disruption of *devR* resulted in hypervirulence observed with both SCID and DBA mice. Three other two-component system mutants, Δ*trcS* (sensor), Δ*tcrXY* and Δ*kdpDE* (both sensor and response regulator), also possess increased virulence, with significantly shorter survival times of infected SCID

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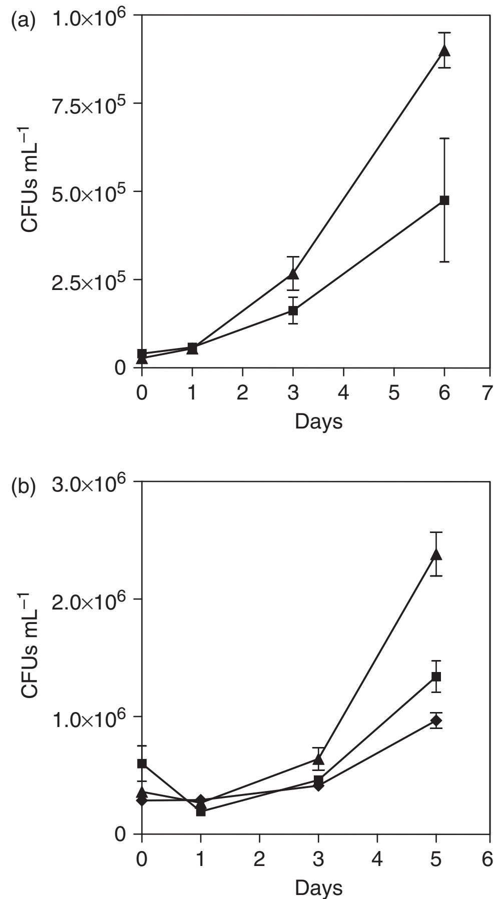

Fig. 3. Intracellular growth of Δ*pknI* within THP-1 cells. (a) Individual infection: THP-1 cells (2.5×10^5) were infected with wild type (■), Δ*pknI* mutant (▲) and complemented strains (◆) at an MOI of 10 : 1 for 3 h. (b) Coinfection: the wild type (■) and Δ*pknI* mutant (▲) were infected for 3 h in equal numbers into 1×10^5 THP-1 cells at an MOI of 2 : 1. Cells were lysed using cold 0.1% Triton X-100 and plated for CFUs at indicated time points. The experiment was performed in triplicate, and the mean CFUs with SE are shown in the graph for each time point.

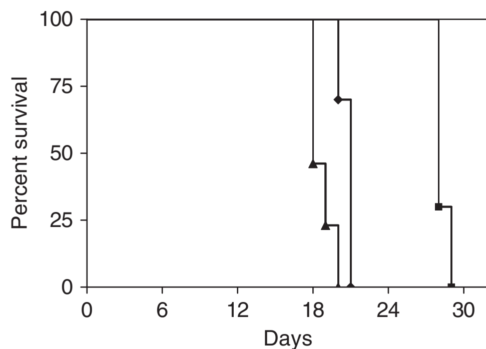

Fig. 4. Virulence of the Δ*pknI* mutant in SCID mice. SCID mice were infected intravenously each with 1×10^5 wild type (■), Δ*pknI* mutant (▲) and complemented (◆) strains. Each group had 10 mice, and the median survival time was plotted.

mice (Parish *et al.*, 2003). We have earlier shown that another kinase, PknH, is important in mediating infection as the Δ*pknH* mutant is hypervirulent in immunocompetent mice (Papavinasasundaram *et al.*, 2005). These results are similar to those observed for mutants in the *mce1* (Shimono *et al.*, 2003), *hspX* (Hu *et al.*, 2006) and *cmaA2* (Rao *et al.*, 2006) genes in mycobacteria. Although we used immunosuppressed mice for studies with the Δ*pknI* mutant, all the above given examples indicate that signaling genes in mycobacteria control the bacterial growth both under innate and adaptive immune responses. In our case, the mutant complementation using the homologous promoter was not fully effective, as SCID mice infected with the complemented strain fail to completely restore the wild-type phenotype. This phenomenon is similar to that observed earlier for PknG (Cowley *et al.*, 2004), and suggests that the tight regulation of kinases activity involves additional physiological conditions such as triggers, activation and phosphorylation status. Nevertheless, the growth advantage for the Δ*pknI* mutant that we observed with growth curves and THP-1 infection clearly supports our SCID mice infection studies. Loss of this kinase-associated control results in hypervirulence that may lead to enhanced immune response and better microbial control. It would be interesting to test the virulence of the Δ*pknI* mutant in immunocompetent mice to further evaluate the increased virulence in this preliminary study.

To conclude, we have shown that *pknI* plays a pivotal role in slowing down the growth of mycobacteria once within the host. Our studies also indicate that internal signals used to activate PknI are most likely the host-associated internal signals of low pH associated with limited oxygen availability.

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