

The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth *in vivo*

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Summary

The function of the *Mycobacterium tuberculosis* eukaryotic-like protein serine/threonine kinase PknG was investigated by gene knock-out and by expression and biochemical analysis. The *pknG* gene (Rv0410c), when cloned and expressed in *Escherichia coli*, encodes a functional kinase. An *in vitro* kinase assay of the recombinant protein demonstrated that PknG can autophosphorylate its kinase domain as well as its 30 kDa C-terminal portion, which contains a tetratricopeptide (TPR) structural signalling motif. Western analysis revealed that PknG is located in the cytosol as well as in mycobacterial membrane. The *pknG* gene was inactivated by allelic exchange in *M. tuberculosis*. The resulting mutant strain causes delayed mortality in SCID mice and displays decreased viability both *in vitro* and upon infection of BALB/c mice. The reduced growth of the mutant was more pronounced in the stationary phase of the mycobacterial growth cycle and when grown in nutrient-depleted media. The PknG-deficient mutant accumulates glutamate and glutamine. The cellular levels of these two amino acids reached approximately

threefold of their parental strain levels. Higher cellular levels of the amine sugar-containing molecules, GlcN-Ins and mycothiol, which are derived from glutamate, were detected in the $\Delta pknG$ mutant. *De novo* glutamine synthesis was shown to be reduced by 50%. This is consistent with current knowledge suggesting that glutamine synthesis is regulated by glutamate and glutamine levels. These data support our hypothesis that PknG mediates the transfer of signals sensing nutritional stress in *M. tuberculosis* and translates them into metabolic adaptation.

Introduction

Protein phosphorylation is a principal mechanism by which extracellular signals are translated into cellular responses. Protein phosphorylation is carried out by specific protein kinases and is coupled to dephosphorylation reactions carried out by protein-phosphatases (PPases). In bacteria, the molecular system that is responsible for stimulus response coupling involves the so-called 'two-component system' consisting of histidine kinase sensors and their associated response regulators (Stock *et al.*, 1989). In contrast, protein phosphorylation in eukaryotes occurs mainly on phosphoester (serine, threonine or tyrosine) residues. The eukaryotic protein kinases and PPases are the backbone of signal transduction pathways. Phosphoester protein kinases and their coupled phosphatases were previously thought to be unique to eukaryotes.

In prokaryotes, protein serine/threonine kinases (PSTKs) have been shown to be primarily involved in two different processes, namely development and pathogenicity. Bacteria capable of differentiation into a new developmental state, including *Streptomyces* (Petrickova and Petricek, 2003), *Anabaena* (Zhang, 1996; Zhang *et al.*, 1998; Zhang and Libs, 1998) and *Myxococcus xanthus* (Zhang *et al.*, 1996; Hanlon *et al.*, 1997), contain a large number of PSTK genes in their genomes. In these bacteria, kinases are involved in the control of the late stages of development, sporulation or secondary metabolite production. Alternatively, PSTKs have been shown to be involved in the survival of human pathogens within the host, as exemplified by the *Yersinia pseudotuberculosis* protein kinase YopO (Hakansson *et al.*, 1996) or the

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Pseudomonas aeruginosa PSTK (Wang *et al.*, 1998). Interestingly, both these kinases have been shown to be required for the full virulence of these pathogens in mouse models.

Previously, we have shown that *M. tuberculosis* encodes at least eight eukaryotic-like protein kinases (Av-Gay and Davies, 1997). Furthermore, we have demonstrated that six proteins are phosphorylated *in vitro* (Av-Gay and Davies, 1997), suggesting the presence of functional kinases in *M. tuberculosis*. The completion of the *M. tuberculosis* genome sequencing project has provided a complete list of these eukaryotic-like protein kinases and phosphatases forming the *M. tuberculosis* PSTK family (Cole *et al.*, 1998). Currently, this family is composed of at least 11 protein kinases and four protein phosphatases. Of the 11 predicted serine/threonine kinases, all but two contain putative transmembrane domains that suggest they may be localized to the plasma membrane.

To date, the *M. tuberculosis* PSTKs *pknD*, *pknB*, *pknI* and *pknG* have been cloned, analysed and shown to encode functional serine/threonine kinases (Peirs *et al.*, 1997; Av-Gay *et al.*, 1999; Koul *et al.*, 2001). The focus of the present study is on PknG, which is of particular interest for three reasons: first, it is one of only two potential soluble PSTKs encoded by the *M. tuberculosis* genome; secondly, it is the only PSTK to contain an N-terminal domain that precedes the predicted kinase domain, thus resembling the *Yersinia* PSTK YopO; and thirdly, it contains another eukaryotic signalling motif termed the tet-

atricopeptide (TPR) domain. TPR motifs are structural domains shown to regulate the Hsp70/Hsp90 chaperone system in eukaryotic systems (Brychzy *et al.*, 2003) and mediate protein-protein interactions within a signal transduction pathway of *Bacillus subtilis* (Core and Perego, 2003).

In this paper, we show that recombinant PknG is a functional kinase that is able to phosphorylate its kinase domain as well as its own TPR domain. We also show that targeted knock-out of the *pknG* gene leads to decreased survival of *M. tuberculosis* *in vitro* and *in vivo*. We also demonstrate a direct correlation between PknG and glutamate and glutamine levels in *M. tuberculosis*. This is the first report in which specific physiological events have been linked to a member of the *M. tuberculosis* serine/threonine kinases family.

Results

Expression and characterization of recombinant PknG

The *M. tuberculosis* *pknG* gene was cloned and expressed as a His-tagged protein under the control of a T7 promoter in the *Escherichia coli* expression vector pET-22b as described previously (Av-Gay *et al.*, 1999). As shown by SDS-PAGE and Coomassie blue staining, we observed induction of a protein of ≈ 86 kDa (Fig. 1A). We purified this protein further by cobalt affinity column chromatography (Fig. 1B). To verify that the recombinant pro-

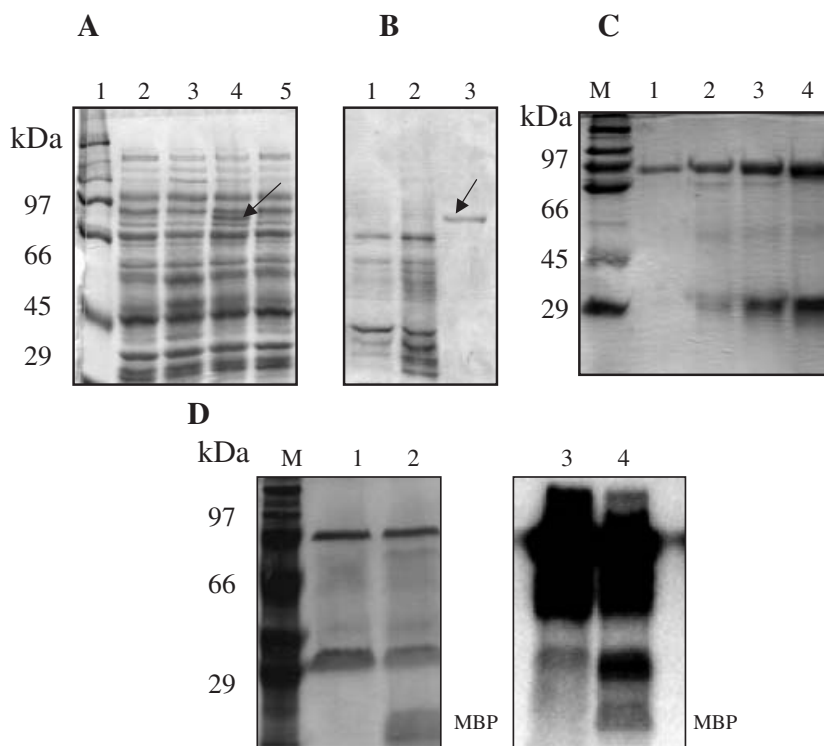


Fig. 1. Expression and analysis of recombinant PknG.

A. SDS-PAGE gel of expression. 1, molecular weight markers; 2, cell-free extract of negative control before IPTG induction; 3, cell-free extract of pET-22b in *E. coli* BL-21 after IPTG induction; 4, cell-free extract of pYA410E expressing PknG after IPTG induction; 5, cell-free extract of pYA410E before IPTG induction.
B. Purification of PknG on Talon metal affinity resins. 1, column flowthrough; 2, column wash fraction; 3, purified PknG eluted with 300 mM imidazole.
C. PknG undergoes self-cleavage to generate a 30 kDa C-terminal fragment. M, molecular weight markers; 1, denatured purified PknG; 2, renatured PknG immediately after purification; 3, renatured PknG after 1 h incubation at room temperature followed by purification; 4, same as lane 3 but after 4 h incubation at room temperature.
D. Phosphorylation of PknG, MBP and the 30 kDa cleavage product as measured by *in vitro* kinase assay. M, molecular weight markers; 1, silver staining of PknG; 2, the same as in lane 1 with the addition of 1 μ g of MBP; 3, *in vitro* kinase assay of PknG; 4, *in vitro* kinase assay of PknG and MBP.

tein was identical to the predicted protein encoded by the *M. tuberculosis* *pknG* gene, we performed N-terminal amino acid sequencing on the IPTG-inducible protein. The first 10 amino acids of this IPTG-induced band were shown to be identical to the amino acid sequence of the predicted *pknG* gene product.

Recombinant PknG possesses self-proteolytic activity and is capable of phosphorylating its TPR motif region

As shown in Fig. 1C, SDS-PAGE analysis of His-tagged recombinant PknG purified without the addition of protease inhibitors revealed the presence of two bands eluted from the column; one was the expected size of the full-length protein at 86 kDa, and the other was only 30 kDa in size. The identity of this 30 kDa band was determined by both matrix-assisted laser desorption ionization (MALDI) and liquid chromatography tandem mass spectrometry (LC/MS/MS) to be the C-terminal portion of PknG containing the aforementioned TPR signalling motif. Thus, we reasoned that the 30 kDa fragment is the result of proteolytic cleavage of the full PknG protein during purification from *E. coli*. This would result in purification of only the His-tagged portions of PknG, namely the uncleaved 86 kDa full-size protein as well as the 30 kDa C-terminal cleavage product, but not the 56 kDa N-terminal cleavage product that would lack a His-tag. We took advantage of this phenomenon and performed *in vitro* kinase assay of the purified PknG samples. As shown in Fig. 1D, PknG is able to phosphorylate itself and the 30 kDa C-terminal cleavage product. It seems that the ability of PknG to phosphorylate its 30 kDa fragment is enhanced upon addition of the artificial kinase substrate myelin basic protein (MBP) as external substrate. As shown previously, MBP is phosphorylated by PknG (Koul *et al.*, 2001).

Subcellular localization of PknG in *M. tuberculosis*

Earlier reports demonstrated that PknG is present in the cytosolic fraction of *M. tuberculosis* (Koul *et al.*, 2001). Given its genomic location in a putative operon together with a glutamine pump gene (*glnH*) and its potential role in mediating signal transduction events, we speculated that PknG might be translocated from the cytosol and, as such, also be present in the membrane compartments of the mycobacterial cell. To address this issue, we produced a rabbit polyclonal antibody against the recombinant PknG for use in cellular localization experiments on subcellular fractions of *M. tuberculosis*. As seen in Fig. 2, Western immunoblots using polyclonal antisera generated against PknG exhibited a reacting band in both cytosolic and membrane fractions. Protein bands reacting with antisera against PknG were also observed in the growth

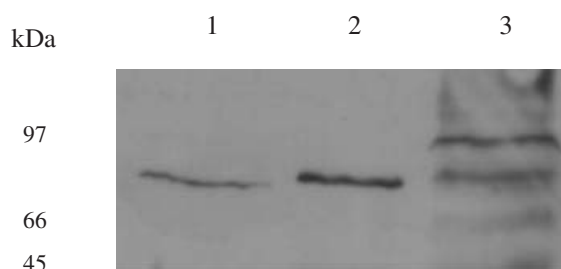


Fig. 2. PknG localization in *M. tuberculosis* cell fractions. Fractions were analysed by PknG Western blotting. Lanes: 1, 45 µg of TB soluble fraction protein; 2, 45 µg of TB membrane protein; 3, 100 µg of *M. tuberculosis* culture filtrate protein. This is a representative blot of three separate experiments using two independent TB preparations.

media of *M. tuberculosis* and were not detected in the $\Delta pknG$ mutant of *M. tuberculosis* (data not shown).

Construction of a $\Delta pknG::hyg$ mutant of *M. tuberculosis*

Allelic exchange of the *pknG* gene in *M. tuberculosis* strain H37Rv with mutant allele $\Delta pknG::hyg$, in which *pknG* was inactivated by deletion of most of the coding sequence and insertion of a hygromycin resistance gene, was carried out using suicide plasmid, pKNG2.1. Two-step mutagenesis using *lacZ* selection (Parish *et al.*, 1999) followed by *sacB* counterselection (Pelicic *et al.*, 1996) was carried out as described previously (Parish and Stoker, 2000). Sucrose-resistant clones corresponding to putative double cross-over recombinants were analysed genotypically by Southern blotting (Fig. 3B). This analysis revealed that the 1202 bp *BglII*-*Bam*HI fragment observed in the wild type (Fig. 3B, lane 1) and single cross-over recombinant (Fig. 3B, lane 2) is lost in the double cross-overs (Fig. 3B, lanes 3 and 4) and replaced instead by a 5685 bp fragment resulting from the deletion of the *Bam*HI and *Bgl*III sites present in the wild-type *pknG* allele. Western analysis using anti-PknG antibodies confirmed the absence of an 85 kDa reacting protein in the allelic exchange mutant strain, which was present in its parental wild type (Fig. 3C). An apramycin-resistant epichromosomal vector capable of transcribing *pknG* via the acetamidase promoter was constructed and transformed into the mutant strains to generate complemented mutants.

In vitro growth of *M. tuberculosis* $\Delta pknG::hyg$ mutant

The *in vitro* growth of an isolate of the mutant strain on Middlebrook 7H10 solid medium and on slants was very poor compared with that of the parental strain. Growth of this mutant in standard 7H9 media was examined and compared with the wild-type *M. tuberculosis* by monitoring

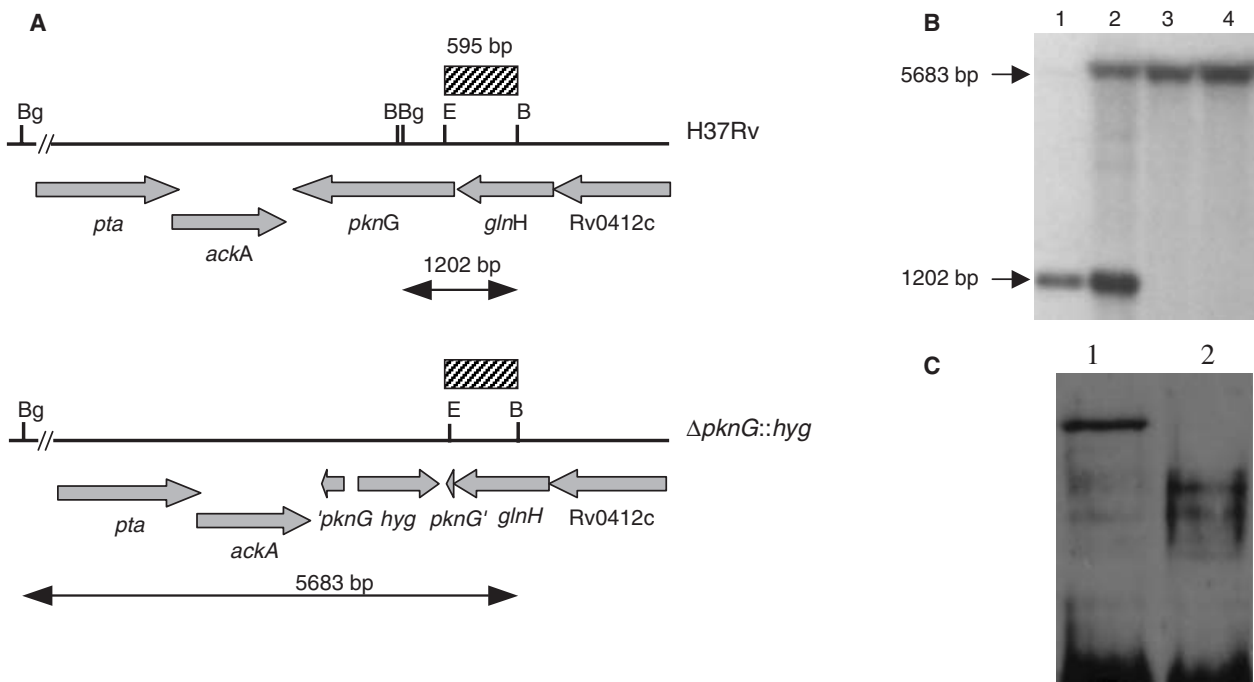


Fig. 3. Genotypic analysis of the $\Delta pknG::hyg$ mutant of *M. tuberculosis*.

A. Schematic representation of the *pknG* locus of H37Rv (Cole *et al.*, 1998) and the corresponding $\Delta pknG::hyg$ mutant allele. Restriction sites have been abbreviated as B for *Bam*HI; Bg for *Bgl*II and E for *Eco*RV.

B. Southern blot analysis of *Bam*HI–*Bgl*II-digested genomic DNA probed with the *Eco*RV–*Bam*HI 595 bp fragment containing the *pknG'* and *glnH* region derived from pKNG2.1. The *Eco*RV site of the wt *pknG* was lost in pKNG2.1, and a new *Eco*RV site generated during the construction of the pKNG2 vector. Lane 1, *M. tuberculosis* H37Rv; lane 2, the parental single cross-over recombinant; lanes 3 and 4, $\Delta pknG::hyg$ mutants.

C. Western analysis of PknG. Lane 1, *M. tuberculosis* H37Rv; lane 2 $\Delta pknG::hyg$ mutant.

the optical densities of the broth cultures over time. As seen in Fig. 4A, the mutant grew significantly slower and achieved a maximum cell density no greater than 50% of its parental wild type. In contrast, under these conditions, the growth rate and maximum cell density achieved by the complemented strain were closer to wild-type levels. Viable count assessments revealed 1 log difference between the mutant, the parental and the complemented strains reaching $1 \times 10^7 \pm 2.2$, $6 \times 10^7 \pm 0.65$ and $1 \times 10^8 \pm 0.05$, respectively, on day 17 after inoculation. When the growth medium was changed to PB-T, a defined medium that lacks oleic acid and bovine serum albumin (BSA), the mutant showed an even more pronounced growth defect, as deduced from optical density (Fig. 4B), whereas the complemented strain appeared to grow to an even higher cell density than the parental wild type.

To test whether the lack of growth results from limitation in nutrient supply, the bacteria were grown in spent (and hence nutrient-depleted) media, which had been used to grow *M. tuberculosis* H37Rv to early stationary phase. The growth rate of the mutant strain was slower than that of the wild type (Fig. 4C), and its growth reached a plateau at an optimal density of 0.5 in spent media compared with 0.85 in 7H9. In this case, the growth characteristics of the

complemented strain were almost identical to those of the parental wild type.

Growth and survival of the M. tuberculosis $\Delta pknG::hyg$ mutant in vivo

To test for bacterial virulence in the absence of specific immunity, SCID mice lacking both T and B cells and previously shown to be highly susceptible to *M. tuberculosis* infection were inoculated with H37Rv or $\Delta pknG$ mutant (Fig. 5). SCID mice were infected with 10^5 bacteria using intravenous (i.v.) inoculation. All mice infected with the parental strain H37Rv became moribund and died with a median survival time (MST) of 31 days. In contrast, mice infected with the $\Delta pknG$ mutant survived significantly longer with an MST of 62 days ($P < 0.001$), suggesting that the $\Delta pknG$ mutant was attenuated.

The survival and growth of the $\Delta pknG$ mutant strain were also assessed in immunocompetent BALB/c and CD-1 mice. BALB/c mice were infected i.v. with either the parental *M. tuberculosis* H37Rv strain or one of two independent $\Delta pknG::hyg$ isolates. Two experiments were performed, one with a low-dose infection ($\approx 10^4$ bacteria per animal) and one with a relatively high dose ($\approx 10^6$ bacteria

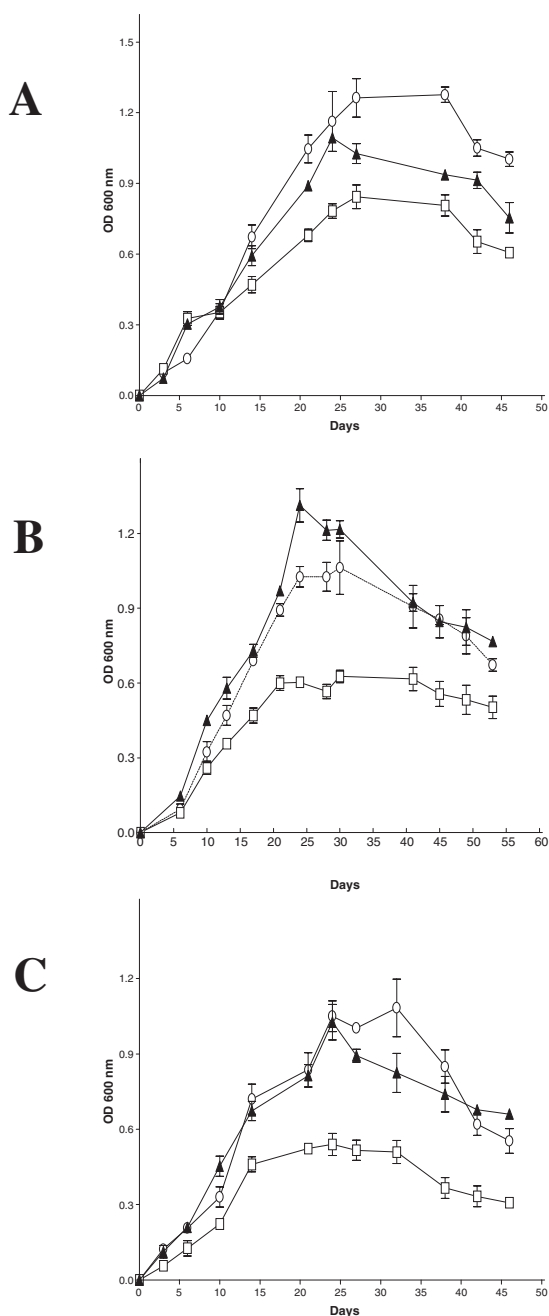


Fig. 4. *In vitro* growth analysis of $\Delta pknG::hyg$ mutant versus the parental and complemented strains. (open circles) denotes H37Rv, (open squares) $\Delta pknG::hyg$ mutant strain or (filled triangles) complemented mutants that were grown in (A) 7H9 media, (B) PB and T media and (C) depleted media.

per animal). Growth of the three strains was measured between day 1 (deposition) and day 28 (peak of the acute phase of growth) by determining bacillary loads in the lungs, spleens and livers of the mice. When compared with the parental strain, growth of the mutants was attenuated in all organs over the 28 day period (Fig. 6A). In a separate experiment, we infected CD-1 mice using a low-

dose aerosol infection and measured colony-forming units (cfus) at days 1, 14, 28 and 56 after infection (Fig. 6B). The deposition or initial survival of the $\Delta pknG$ mutant in the lungs was about 50% that of the parental strain H37Rv and the complemented mutant harbouring an extra chromosomal copy of *pknG*; 94 ± 25 versus 236 ± 16 and 275 ± 47 respectively. This difference was not the result of variation in the inocula used in the aerosol inhalation exposure equipment, which were 4×10^7 for H37Rv and the complemented mutant and 6.5×10^7 for the $\Delta pknG$ mutant. Nevertheless, by day 14, the surviving mutants were able to grow better than their complemented strain. By day 28, cfus of all three strains were comparable and remained so until day 56.

As seen in Fig. 6C, dissemination to the spleens after aerosol infection varied. By day 14, a significant amount of the parental strain and the complemented mutant were present in the spleens of two and three out of four animals, respectively, whereas no bacteria were detected in the spleens of mice infected with the $\Delta pknG$ mutant, demonstrating that the complemented strain was able to restore the parental strain phenotype. However, by day 28, the mutant was able to gain dissemination to the spleen and, by day 56 after infection, all three strains reached the same presence in the spleen.

Inactivation of pknG renders an increase in intracellular levels of glutamine and glutamate followed by a decrease in de novo glutamine synthesis capacity

The *pknG* gene is the terminal gene in the operon containing a gene encoding a possible conserved membrane protein (Rv0412c) and the putative glutamine transport gene *glnH*, suggesting a functional association between these proteins. To examine whether inactivation of PknG has an effect on the function of GlnH, we determined the cellular levels of the amino acids glutamate and glutamine

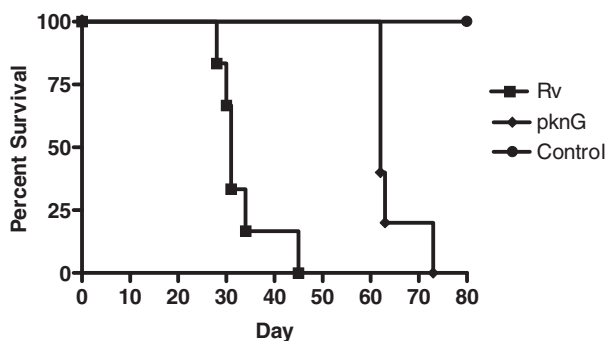
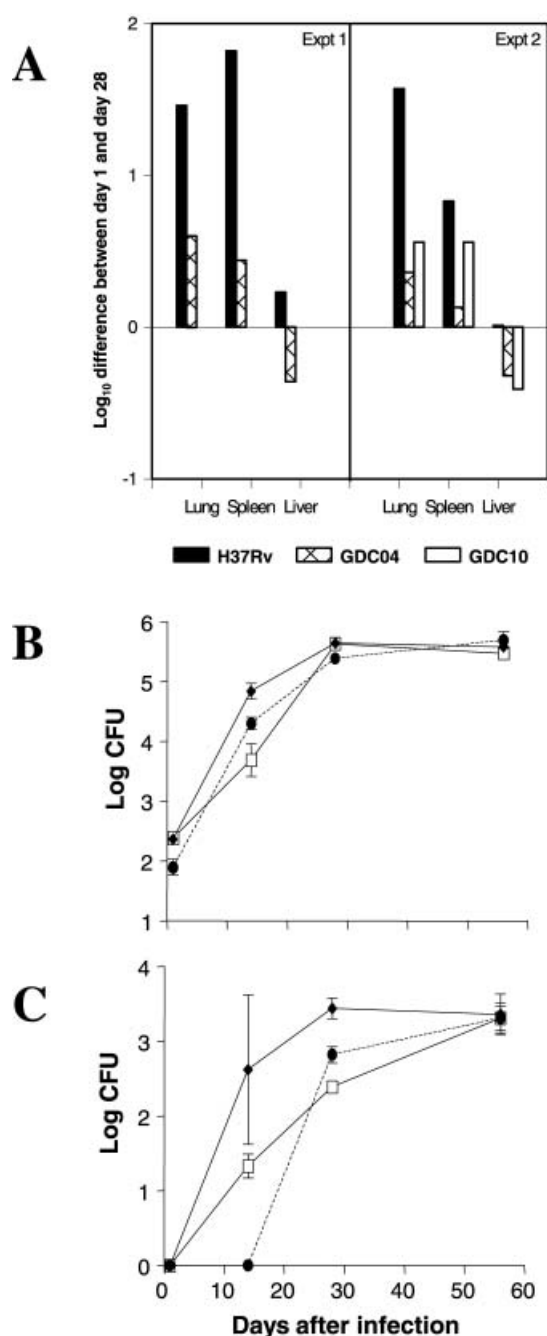


Fig. 5. Virulence of *M. tuberculosis* strains in SCID mice. Mice were infected intravenously with 1×10^5 H37Rv (squares), $\Delta pknG::hyg$ mutant strain (diamonds) or PBS control (circles), and survival was monitored over 80 days. Each group contained six mice, and the results are representative of two separate experiments.



in the wild-type, mutant and complemented strains. As seen in Table 1, the $\Delta pknG$ mutant strain accumulates these two amino acids intracellularly while their levels in the growth media remain constant. High levels of glutamate and glutamine have been shown to correlate with feedback inhibition of glutamine synthetase (GS) expression. *M. tuberculosis* GS accumulates in the growth media of pathogenic mycobacteria (Harth *et al.*, 1994). We therefore compared the levels of glutamine synthesis of the various strains both intracellularly and extracellularly. It is

Fig. 6. Growth of H37Rv and the *pknG::hyg* mutants in immunocompetent mice.

A. The growth of H37Rv and 2 *pknG::hyg* mutants (GDC04 and GDC10) was measured in the lung, spleen and liver of infected female BALB/c mice over 28 days. The cfu at days 1 and 28 were enumerated in four mice, and the log₁₀ difference is shown for each strain in two separate experiments. In experiment 1, $\approx 10^4$ and, in experiment 2, $\approx 10^5$ H37Rv were injected. Approximately 10^5 *pknG::hyg* mutants were injected in both experiments.

B and C. cfu counts in female CD-1 mice after deposition of ≈ 200 bacteria into the lungs via aerosol inhalation exposure. Growth in the lungs (B) and subsequent dissemination and growth in the spleen (C) was followed over 56 days. Symbols: parental strain H37Rv (filled diamonds), $\Delta pknG::hyg$ mutant (filled circles), complemented mutant (open squares). Each point is the mean and SEM from four mice except for the counts on day 14 in the spleens, which are the mean and SEM cfu from two (H37Rv) or three (complemented mutant) positive organs. No bacteria were detected in the spleens of mice infected with the $\Delta pknG::hyg$ mutant on day 14.

expected that accumulation of glutamine will result in reduced expression of glutamine synthetase in both the bacilli and extracellularly. As seen in Table 2, the amount of the enzyme glutamine synthetase did not differ significantly between the samples, but the *de novo* synthesis of glutamine was reduced by >50% in the growth media of the mutant strain compared with the wild-type H37Rv and complemented strains. Furthermore, we could not detect any glutamine synthesis in cell-free extracts of PknG mutant while levels of between 0.05 and 0.13 $\mu\text{mol min}^{-1}$ were observed for the parental and the PknG complemented strains (Table 2). The increase in the cellular levels of glutamate and glutamine resulted in higher levels of metabolites that are synthesized from these two amino acids. As seen in Table 3, higher cellular levels of three amine sugar-containing molecules, 1D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (GlcN-Ins), mycothiol (MSH) and an unidentified amine, U16, were detected by high-performance liquid chromatography (HPLC) analysis of $\Delta pknG$ mutant and compared with the complemented and the parental strains.

Discussion

In this study, we have characterized the *M. tuberculosis* eukaryotic-like serine/threonine kinase PknG and a PknG-deficient mutant strain. To our knowledge, this is the first report of a gene knock-out in this newly described kinase

Table 1. Glutamine and glutamate levels of the various strains in cells and growth media.

Strain (n = 4)	Cell free extract $\mu\text{mol } 10^{-9}$ cells	Growth media $\mu\text{mol } 10^{-9}$ cells
H37Rv parental strain	58.01 \pm 24.565	57.42 \pm 8.392
$\Delta pknG$ mutant	153.20 \pm 17.076	66.66 \pm 18.147
Complemented mutant	56.49 \pm 5.354	48.60 \pm 14.719

Table 2. Glutamine production and glutamine synthetase (GS) units in cells and growth media.

Strain	Glutamine production in media $\mu\text{mol ml}^{-1} \text{min}^{-1}$	Glutamine production in cells $\mu\text{mol ml}^{-1} \text{min}^{-1}$	GS units in media $\mu\text{mol ml}^{-1}$	GS units in cells $\mu\text{mol ml}^{-1}$
H37Rv	1.04 ± 0.14	0.06 ± 0.019	0.20 ± 0.006	0.22 ± 0.053
$\Delta pknG$ mutant	0.40 ± 0.20	ND	0.22 ± 0.005	0.30 ± 0.036
Complement	0.99 ± 0.403	0.13 ± 0.053	0.35 ± 0.009	0.30 ± 0.007

family in *M. tuberculosis*. A recent report (Sasseti *et al.*, 2003) suggested that *pknG* together with *pknA* and *pknB* are likely to be required for growth of *M. tuberculosis* *in vitro*. However, the authors of this paper do state that some of the genes identified by their study may be 'false positive'. In the case of *pknG*, it is likely that the slow growth rate of mutants lacking *pknG* led Sasseti *et al.* (2003) to conclude that *pknG* is essential for *in vitro* growth.

Mycobacterium tuberculosis encodes in its genome 11 putative serine/threonine kinases. Growth and survival of *M. tuberculosis* inside host macrophages together with its long quiescent dormant state represent unique characteristics of this pathogen that may explain the need for a large number of these unique regulatory proteins. An important objective is to identify the roles of each of these protein kinases in the metabolic processes unique to *M. tuberculosis*. The findings of the present study show that PknG is essential for the optimal growth of *M. tuberculosis* *in vitro* and in the target organs of immunocompetent and immunocompromised animal models of tuberculosis.

It appears that the complemented mutant strain is able to restore the $\Delta pknG$ phenotype to that seen in the parental strain only at early stages of infection; up to 24 h after infection in the lungs and up to 14 days in the spleens of infected mice. In these experiments, we used an apramycin-resistant shuttle plasmid to express *pknG*. Without selective pressure to maintain the complementing plasmid in bacteria within infected animals, it is possible that it was lost during long-term growth in mice. Alternatively, it is possible that PknG is only needed at early stages of infection in mice, while the bacteria face initial change in environment-related stress, and has little contribution during long-term infection. To support this hypothesis, we have observed that, in both lungs and spleens of infected mice, the same amount of bacteria was present 56 days after infection. This phenomenon of differential deposition and 'catching up' at a later stage was observed before for several of the Snm proteins of *M. tuberculosis* (Stanley *et al.*, 2003).

The survival and growth of the mutant compared with the wild-type parent differed in the two models of immunocompetent mice used (i.v. infection of BALB/c mice versus aerosol infection of CD-1 mice). After i.v. infection, growth of the mutants over 28 days was significantly retarded in all target organs compared with that in mice

infected with H37Rv. In contrast, after low-dose aerosol infection, growth of H37Rv and the mutant strain in the lungs was comparable over 56 days. However, dissemination to the spleen and subsequent growth was retarded in the $\Delta pknG$ mutant, only reaching levels equal to that seen in H37Rv by day 56. One possible explanation for these observed differences is that the two mouse strains vary in their susceptibility to *M. tuberculosis* (Medina and North, 1998). However, resistance and susceptibility to *M. tuberculosis* is not usually expressed until about day 100 at the earliest (Medina and North, 1998), and we did not take our experiments past day 56. In addition, one of us has shown that the growth of *M. tuberculosis* after aerosol infection of BALB/c and CD-1 mice is identical over 84 days (R. W. Stokes, unpublished results). Furthermore, growth of the $\Delta pknG$ mutant was compromised in the spleen regardless of the route of infection. A more likely explanation for the difference is the route of infection used, i.e. i.v. inoculation versus aerosol inhalation. *M. tuberculosis* is more virulent when given via the respiratory than via the i.v. route (North, 1995). In addition, the pathogenesis of the bacteria following the two routes of infection is very different; after i.v. inoculation, the bacteria are predominantly ingested by phagocytes in the liver and spleen, while about 1% of the inoculum is deposited in the capillary beds within the lung. In contrast, after aerosol infection, the bacteria are deposited in the alveoli, from where they disseminate to the draining lymph nodes and eventually to the spleen (McMurray, 2003). It is quite possible that the mutant cannot cope with the conditions it is exposed to after i.v. inoculation. Our results demonstrate the importance of considering the route of infection when testing the virulence of mycobacterial strains. The observation that the $\Delta pknG$ mutant growth differs in the spleen and lung after aerosol infection is not without precedent.

Table 3. Mycothiol and amine analysis of *M. tuberculosis* strains.

Bacterial strain	MSH	GlcN-Ins $\text{nmol } 10^{-9} \text{ cells}$	Amine U16
H37Rv parental strain	3.69 ± 0.83	0.39 ± 0.04	34 ± 2
$\Delta pknG$ mutant	6.11 ± 1.68	0.53 ± 0.05	79 ± 14
Complemented mutant	3.20 ± 0.47	0.31 ± 0.01	33 ± 5

Mycothiol levels were determined on stationary phase growth (2-month-old standing culture, $n = 3$), and GlcN-Ins and U16 were determined for exponentially grown strains in roller bottles.

Other mutants have been found to fare better in some organs than they do in others. FadD28, FadD33, Pps and MmpL7 mutants all showed tissue-specific growth rates (Cox *et al.*, 1999; Rindi *et al.*, 2002). This may be a result of different physiological conditions or of divergent immune responses in the distinct organs.

PknG is the third serine/threonine kinase described to be involved in microbial pathogenicity. Its homologues in *Yersinia* and *P. aeruginosa* were shown to be essential virulence determinants (Hakansson *et al.*, 1996; Wang *et al.*, 1998; Juris *et al.*, 2002). YpkA (YopO) of *Yersinia* is translocated to the host's plasma membrane and is able to possess kinase activity only in the presence of actin (Juris *et al.*, 2000). We have not yet identified the mechanism by which PknG contributes to enhance the survival of the bacteria inside the host. Nevertheless, it is interesting to note that kinome analysis of mycobacterial infection identified induced phosphorylation of adducin (Hestvik *et al.*, 2003), an enzyme that acts on actin filaments.

The *pknG* gene is located in an operon with *glnH*, which encodes an extracellular glutamine-binding protein with a lipid attachment site to anchor it to the cell membrane. In other bacteria, GlnH is part of a high-affinity glutamine uptake system that is induced under nitrogen-limiting conditions. It is interesting to note that another protein involved in glutamine metabolism, glutamine synthetase, is proposed to have a role in bacterial survival both *in vitro* and in the macrophage (Harth and Horwitz, 1999; Harth *et al.*, 2000). As both *glnH* and *pknG* exist in the same operon, it is likely that *pknG* expression is regulated under similar conditions to the genes involved in glutamine metabolism. Given the role of glutamine synthetase in mycobacterial survival *in vitro* and in the macrophage, it is interesting to speculate that PknG may also have a function related to glutamine metabolism, which contributes to intracellular survival. In relation to the above, we found that PknG function is associated with glutamine–glutamate and molecules derived from these two amino acids in mycobacteria. In particular, we note that the levels of the intracellular antioxidant thiol, mycothiol, are increased. Mycothiol was recently identified as essential for *M. tuberculosis* (Buchmeier *et al.*, 2003; Sareen *et al.*, 2003) and has been shown to protect mycobacteria from oxidative damage (Rawat *et al.*, 2002). Thus, an increase in glutamate and glutamine levels may provide a pool of amines for synthesis of macromolecules required to deal with stress. In *E. coli*, increased levels of glutamate were shown to be the result of the cellular response to osmotic stress (McLaggan *et al.*, 1994), whereas prevention of glutamine synthesis and high-affinity transport attenuates *Salmonella typhimurium* virulence (Klose and Mekalanos, 1997).

In our studies, we have measured glutamine and glutamate levels simultaneously, and glutamine levels were often below our detection level. Thus, it is possible

that *pknG* is involved directly only in controlling glutamate levels. We also note that the transferase assay, which we have used to determine GS units (Table 2), does not distinguish between active and inactive forms of GS as these are controlled by GS adenylation.

Two major roles are fulfilled by glutamate and glutamine in mycobacteria. First, glutamate and glutamine via their synthetases and potential dehydrogenases are the sole means of ammonia assimilation into amino acids in this organism. Secondly, poly L-glutamate/glutamine is an integral part of the cell wall structure of *M. tuberculosis*. *M. tuberculosis* contains four different glutamine synthetases (Cole *et al.*, 1998), whereas most bacteria, including *E. coli*, have a single form of glutamine synthetase. Harth and Horwitz have shown that pathogenic mycobacteria, in contrast to non-pathogenic mycobacteria, release one of the enzymes, GlnA1, into the growth media (Harth *et al.*, 1994). Using specific inhibitors (Harth and Horwitz, 1999) and antisense oligonucleotides (Harth *et al.*, 2000), they have demonstrated inhibition of mycobacterial growth and reduction of the cell wall structure poly L-glutamate/glutamine levels. More recently, gene knock-out experiments revealed that *glnA1* is essential for the growth of *M. tuberculosis* in human macrophages and in guinea pigs (Tullius *et al.*, 2003).

The co-localization of *pknG* and *glnH* suggests that these two genes are co-transcribed and that PknG may act on GlnH by means of controlling its activity. If this were true, then lack of phosphorylation (caused by *pknG* inactivation) would lose control over the GlnH pump and result in over-accumulation of glutamate and glutamine inside the mycobacterial cell. Studies are currently under way to investigate this possibility. However, as GlnH is present in *E. coli* and does not appear to require phosphorylation for its activity, we favour the hypothesis that PknG participates instead in the metabolism of poly L-glutamate/glutamine. PknG, like poly L-glutamine/glutamate, is unique to mycobacteria and can also be detected in the mycobacterial culture filtrate, supporting the notion that it may participate in the synthesis or assimilation of poly L-glutamine/glutamate into the mycobacterial cell wall. Poly L-glutamine/glutamate metabolism is poorly understood, and the enzymes involved in its biosynthesis still need to be identified.

With respect to a possible role for PknG in intracellular survival, it may be relevant to note that PknG is also phosphorylated in its tetratricopeptide (TPR) motif. TPR motifs consist of a degenerate 34-amino-acid sequence that is generally present in tandem arrays of 3–16 motifs. These motifs occur in a wide variety of proteins present in bacteria, eukarya and archaea, and have been shown to be involved in protein–protein as well as protein–lipid interactions. As one example, TPR motifs present within the p67^{phox} portion of the phagocyte NADPH oxidase interact with the small GTPase Rac to form an active oxidase

complex (Koga *et al.*, 1999). Rac and other small GTPases that are members of the Rho family are involved in several important eukaryotic cellular functions, including cytoskeletal reorganization and gene expression. Thus, it is possible that PknG may also interact with other proteins to influence cell wall protein activity or even cell signalling through interaction with a protein complex. In our studies, we have detected natural PknG in *M. tuberculosis* membrane and growth media, thus supporting our hypothesis that PknG may be translocated under specific conditions that have yet to be identified. The localization of PknG within the cell membrane, in spite of its lack of predicted transmembrane domains, is particularly intriguing. This may indicate a strong association with an *M. tuberculosis* membrane protein such as GlnH or a porin, possibly through the TPR motif. It is interesting to speculate that this association may in fact be a prelude to translocation upon exposure to specific environmental conditions such as those found in the host cell.

Experimental procedures

Bacterial strains, vectors and culture conditions

Mycobacterium tuberculosis H37Rv was obtained from the National Collection of Type Cultures, London, UK. Mycobacterial strains were cultured in Middlebrook 7H9 broth or Proskaur–Beck medium with 0.5% Tween 80 (PB-T). Growth on solid media was performed using 7H10 agar (Difco) supplemented with 10% OADC (Difco). All growth experiments were performed in triplicate.

Expression and purification of PknG in E. coli

Competent cells of *E. coli* BL21(DE3) transformed with pYA410E were used for *pknG* expression after induction with 0.4 mM IPTG according to previously established protocols (Av-Gay *et al.*, 1999). In order to obtain pure His-tagged soluble protein, PknG inclusion bodies were resuspended into denaturation extraction buffer (50 mM sodium phosphate, 300 mM NaCl, 8 M urea and 1 mM β -mercaptoethanol, pH 7.0) and slowly added to Talon metal affinity resins (Clontech). Further purification was performed according to the manufacturer's protocol. Purity of PknG was tested by SDS-PAGE. N-terminal amino acid sequence was verified after electrophoresis of samples in SDS-PAGE and electroblotting to polyvinylidene difluoride (PVDF) membrane of samples. SDS-PAGE gels were prepared according to the method of Laemmli (1970). Edman degradation was performed, and the sequence of the first 10 amino acids from the N-terminus was determined at the UBC Protein Sequencing Laboratory. MALDI and LC/MS/MS analysis of proteins excised from SDS-PAGE gels was performed as described previously (Betts *et al.*, 2002).

Production of rabbit anti-PknG antibodies

Approximately 10 mg of purified His-tagged recombinant pro-

tein was separated by 12% SDS-PAGE, and gel slices were excised and stored at 4°C. Protein samples in gel slices were sent to Genemed Synthesis where PknG was electroeluted from gel slices and injected into rabbits. Polyclonal rabbit anti-PknG antibodies were semi-purified using preblotting against a blot of PknG mutant protein extract and were tested against purified PknG.

Western blotting

Western analysis was performed using standard conditions. Samples were electrophoresed on SDS-PAGE and transferred to nitrocellulose by semi-dry electroblotting (Ancos). Blots were blocked with 5% skimmed milk (Difco) in 1 × PBS (pH 7.5) overnight at 4°C on a shaker. Blots were washed with 1 × PBS, 0.25% Tween 20 and incubated for 2 h in 1 × PBS-Tween (pH 7.5) with 1:5000 concentrations of the polyclonal antibodies against the recombinant PknG. Blots were washed for 45 min in 1 × PBS/T and incubated with 1:5000 goat anti-rabbit IgG (H+I) horseradish peroxidase (HRP)-conjugated antibody (Bio-Rad). Blots were incubated in Pierce super signal reagent™ and exposed using Kodak X-OMAT/AR film. Fractions of membrane, cytosol and cell media supernatant from late log phase *M. tuberculosis* H37Rv cultures were obtained from Colorado State University. As each of the fractions contained components other than proteins, the masses of fractions separated by SDS-PAGE were corrected for the amount of protein in each fraction in a ratio of 1:0.55:4.86 for culture supernatant–membrane–cytosol as described earlier (Cowley *et al.*, 2002). Western blotting was done using 1:20 000 anti-PknG primary antibody and 1:5000 goat anti-rabbit IgG (H+I) HRP conjugate (Bio-Rad). Bands were visualized using SuperSignal™ West Pico chemiluminescent substrate (Pierce) and Diagnostic imaging film (Sterling Diagnostic Imaging).

In vitro kinase assay

Total protein samples (20 µg) from the mutant and its parental strain were added to 15 µl of kinase buffer (Koul *et al.*, 2001) with or without 1 µg of MBP (Sigma), and the reaction was initiated by the addition of 10 µCi [α -³²P]-ATP (Perkin-Elmer). Incubation was performed at 37°C for 30 min, and 3 × Laemmli sample buffer was added to stop the reaction. This was boiled for 5 min and resolved by SDS-PAGE. The gels were exposed to Biomax® film (Eastman Kodak), and signals were quantified with a PhosphorImager SF (Molecular Dynamics).

Construction of a Δ pknG::hyg mutant of M. tuberculosis

Synthetic oligonucleotide primers complementary to regions that flank the *pknG* gene (Table 4) were designed to amplify fragments of \approx 1 kb in length on either side of *pknG* from genomic DNA. The resulting polymerase chain reaction (PCR) products were cloned directly into the PCR cloning vector pCR2.1. The Δ pknG::hyg allele was formed by insertion of a hygromycin resistance cassette (*hyg*) between these fragments. This plasmid construct is referred to as pKNG2. The *lacZ-sacB* cassette from pGOAL17 (Parish and Stoker,

Table 4. Strains, plasmids and oligonucleotides used in this study.

	Characteristics ^a	Source or reference
Strains		
<i>M. tuberculosis</i>	H37Rv NCTC 7416	Laboratory collection
<i>M. tuberculosis</i>	H37Rv ATCC 25618	Laboratory collection
<i>M. tuberculosis</i>	$\Delta pknG::hyg$ knock-out mutant; Hyg ^r	This work
<i>M. smegmatis</i> mc ² 155	Host strain for expression and transcription studies	W. R. Jacobs
<i>E. coli</i> BL21	F ^{-ompT hsdS_B(r_B⁻m_B⁻) gal dcm}	Novagen
<i>E. coli</i> DH5 α	F ^{-recA1 hsdR17 thi-1 gyrA96 supE44 endA1 relA1 recA1 deoR Δ (lacZYA-argF) U169 (ϕ80 lacZ ΔM15)}	Laboratory collection
Plasmids		
pET22b	T7 expression vector, Amp ^r	Novagen
pCR2.1	TA cloning vector, Amp ^r	Invitrogen
PYA410E	<i>NdeI</i> – <i>HindIII</i> <i>pknG</i> in pET22b	This study
pALACE	<i>Mycobacterium</i> – <i>E. coli</i> shuttle vector. Expression of proteins under acetamidase promoter	De Smet <i>et al.</i> (1999)
pYA0410pAL	<i>NdeI</i> – <i>AflI</i> <i>pknG</i> in pALACE	This study
pPE207	<i>Mycobacterium</i> vector, Ap ^r	Paget and Davies (1996)
pMK4	pPE207 containing the Ace promoter from <i>palace</i> and <i>pknG</i> . Used for complementation of mutants.	This study
Oligonucleotides		
MK1	5'-ATACATATGGCCAAAGCGTCAGAGACCG-3'	This study
OL1	5'-GTTAAGCTTGAACGTGCTGGTGGGC-3'	This study
OL3	5'-TTTCATATGGAACGTGCTGGTGGGC-3'	This study
OL4	5'-TTACTTAAGATGGCCAAAGCGTCAG-3'	This study

a. Amp^r, ampicillin resistance; Hyg^r, hygromycin resistance; Ap^r, apramycin resistance.

2000) was excised as an *XbaI*–*SmaI* fragment and cloned into the *KpnI* site of pKNG2 to produce pKNG2.1. *M. tuberculosis* H37Rv (ATCC 25618), was electroporated with 5 μ g of UV-pretreated pKNG2.1 (Hinds *et al.*, 1999) and plated onto 7H10 agar containing hygromycin (Hyg), kanamycin (Km) and Xgal. Double cross-over recombinants were obtained by two-step selection, as described by Parish and Stoker (2000). Chromosomal DNA extraction and Southern blotting were carried out using ³²P-labelled probes, as described previously (Gordhan *et al.*, 1996).

Construction of *pknG* complementing vector

The gene encoding *pknG* was amplified by PCR with OL3 and OL4 primers (Table 4). The PCR products were ligated into the vector pCR2.1 of the TA cloning kit (Invitrogen). The *pknG* gene was cloned in the mycobacterial *E. coli* pACE derivative shuttle vector pALACE (De Smet *et al.*, 1999) to form pYA0410E (Table 4). pYA0410E was cut with an *NdeI* site followed by Klenow fill in and digestion with *XbaI*, which generated a fragment of *pknG* downstream of an acetamidase promoter. This fragment was ligated to a *KpnI*-digested pEP207 vector (Paget and Davies, 1996) that was filled in with Klenow and digested with *XbaI* to form pMK4. Thus, pMK4 is an apramycin-resistant vector capable of expressing *pknG* via an acetamidase promoter. An aliquot of 5 μ g of pMK4 was electroporated to the $\Delta pknG::hyg$ mutant of *M. tuberculosis* and plated onto 7H10 agar containing Hyg and apramycin.

Analysis of growth in BALB/c mice

Growth of *M. tuberculosis*, H37Rv and two independent $\Delta pknG::Hyg^r$ mutants was tested in BALB/c mice. An aliquot

of bacteria was thawed, pelleted and resuspended in PBSG (138 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.6 mM CaCl₂, 1 mM MgCl₂ and 5.5 mM D-glucose). To disperse clumps of bacteria, the suspension was sonicated for 45 s in a Sonics and Materials VC50T 50 W sonicator + microcup horn, which had been tuned to the manufacturer's recommendations. Each strain was injected, via a tail vein, into eight female BALB/c mice of 6–10 weeks of age. On days 1 and 28 after infection, four mice in each group were killed, and the spleen, liver and lung were removed and homogenized in Tween saline. Tenfold dilutions of the homogenates were plated on 7H10 agar plates (H37Rv) or 7H10 + 50 μ g ml⁻¹ Hyg ($\Delta pknG::Hyg^r$) and incubated in sealed bags at 37°C for 3–4 weeks, whereupon the resultant cfus were counted after 3–4 weeks. The log₁₀ difference in cfus between day 1 and day 28 was calculated for each strain. The experiment was conducted twice, once with a low dose of $\approx 10^4$ bacteria per mouse and once with a high dose of $\approx 10^6$ bacteria per mouse.

Analysis of growth in SCID mice

CB-17/lcr SCID mice were originally obtained from Dr C. M. Hetherington (National Institute for Medical Research, Mill Hill, London, UK) and bred under barrier conditions at the London School of Hygiene and Tropical Medicine (LSHTM). Female mice between 8 and 10 weeks of age were used in all experiments. Experimental animals were maintained in microisolator cages (Laboratory Care Precision) until use, when they were transferred to negative pressure isolators after infection with *M. tuberculosis* in a class I/III biohazard safety cabinet. All studies were approved by the LSHTM animal ethics committee. Mice were infected with 1×10^5 viable mycobacteria in 200 μ l of pyrogen-free saline via a

lateral tail vein. Where appropriate, infected mice were killed by cervical dislocation in accordance with humane end-point protocols under the Animals Scientific Procedures Act, 1986 (UK). Median survival times were calculated for each group, and statistical analysis was performed using the log rank tests of survival.

Determination of glutamine, glutamate and amino sugar levels

A glutamine/glutamate GLN-1 determination kit (Sigma) was used to determine the levels of these two amino acids in cell extract and growth media of *M. tuberculosis*. Mycobacterial culture (20 ml) grown in 7H9 was centrifuged and separated to supernatant and cells. The supernatant was filtered through a 0.22 µm filter, and the cells were resuspended in 1 ml of PBS and disrupted by beat beating followed by 0.22 µm filtration. Samples were assayed using a spectrophotometric method (Lund, 1986). Glutamate/glutamine levels were measured after 1 h when control standards reached saturation, and results were normalized to 10⁹ cfus. *De novo* glutamine formation were measured by incubating a 250 µl sample with 10 mM adenosine-5'-triphosphate (ATP) and 5 mM glutamate for 180 min. Adenosine 5'-diphosphate (ADP) formation measured at 340 nm was used to calculate the amount of glutamine formation against a glutamine standard curve. Glutamine synthetase units were determined according to the method of Rowe *et al.* (1970). Mycothiol levels and amine-containing molecule analysis were performed as described earlier (Rawat *et al.*, 2002).

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