Functional characterization of the *Mycobacterium tuberculosis* serine/threonine kinase PknJ

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Eukaryotic-like Ser/Thr protein kinases (STPKs) are present in many bacterial species, where they control various physiological and virulence processes by enabling microbial adaptation to specific environmental signals. PknJ is the only member of the 11 STPKs identified in *Mycobacterium tuberculosis* that still awaits characterization. Here we report that PknJ is a functional kinase that forms dimers *in vitro*, and contains a single transmembrane domain. Using a high-density peptide-chip-based technology, multiple potential mycobacterial targets were identified for PknJ. We confirmed PknJ-dependent phosphorylation of four of these targets: PknJ itself, which autophosphorylates at Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³ residues; the transcriptional regulator EmbR; the methyltransferase MmaA4/Hma involved in mycolic acid biosynthesis; and the dipeptidase PepE, whose encoding gene is located next to *pknJ* in the mycobacterial genome. Our results provide a number of candidate phospho-targets for PknJ and possibly other mycobacterial STPKs that could be studied to investigate the role of STPKs in *M. tuberculosis* physiology and virulence.

Received 13 January 2010

Revised 15 February 2010

Accepted 18 February 2010

INTRODUCTION

Protein phosphorylation in response to environmental changes is a common mechanism of adaptation in both eukaryotes and prokaryotes. In prokaryotes, the primary signal transduction mechanism relies on a two-component system consisting of a histidine kinase and a response regulator. The histidine kinase undergoes autophosphorylation in response to an environmental signal, and subsequently phosphorylates an aspartate residue in the response regulator, which binds to specific DNA sequences (Inouye & Nariya, 2008). This two-component system is ubiquitous in prokaryotes and regulates a variety of processes, including nutrient acquisition, energy metabolism and virulence, while eukaryotes mostly use serine/threonine and tyrosine phosphorylation systems to create highly sophisticated regulatory networks (Zhang *et al.*,

1998). The first eukaryotic-like protein serine/threonine kinase (STPK) identified in a prokaryote was discovered in *Myxococcus xanthus* (Munoz-Dorado *et al.*, 1991). Accumulating bacterial genome sequencing data has further shown that many prokaryotes use eukaryotic-like STPKs for translating external signals into cellular responses (Cozzzone, 2005; Deutscher & Saier, 2005). The completion of the *Mycobacterium tuberculosis* genome sequencing project showed that this pathogen contains 11 STPK-encoding genes, namely *pknA*, *pknB* and *pknD–L* (Cole *et al.*, 1998). All the corresponding enzymes except PknJ have been structurally and/or functionally characterized (Av-Gay *et al.*, 1999; Canova *et al.*, 2008; Chaba *et al.*, 2002; Cowley *et al.*, 2004; Dasgupta *et al.*, 2006; Duran *et al.*, 2005; Fernandez *et al.*, 2006; Gay *et al.*, 2006; Good *et al.*, 2004; Gopalaswamy *et al.*, 2004; Greenstein *et al.*, 2007; Grundner *et al.*, 2005; Kang *et al.*, 2005; Koul *et al.*, 2001; Kumar *et al.*, 2009; Lakshminarayan *et al.*, 2008; Mieczkowski *et al.*, 2008; Molle *et al.*, 2003a, b, 2004, 2006b; Ortiz-Lombardia *et al.*, 2003; Parikh *et al.*, 2009;

Abbreviation: STPK, Ser/Thr protein kinase.

Supplementary material is available with the online version of this paper.

Park *et al.*, 2008; Perez *et al.*, 2006; Sharma *et al.*, 2006b; Thakur & Chakraborti, 2006, 2008; Veyron-Churlet *et al.*, 2009; Villarino *et al.*, 2005; Young *et al.*, 2003). They are involved in the control of various processes, including cell growth and morphology (Chaba *et al.*, 2002; Dasgupta *et al.*, 2006; Gopalaswamy *et al.*, 2009; Kang *et al.*, 2005, 2008), molecular transport (Molle *et al.*, 2004), glucose uptake and metabolism (Deol *et al.*, 2005), glutamate metabolism, at least in *M. tuberculosis* (Cowley *et al.*, 2004; O'Hare *et al.*, 2008) but possibly not in *Mycobacterium bovis* BCG (Nguyen *et al.*, 2005), fatty acid synthesis (Molle *et al.*, 2006a; Veyron-Churlet *et al.*, 2009), transcription factor activity and gene transcription (Canova *et al.*, 2008; Cohen-Gonsaud *et al.*, 2009; Greenstein *et al.*, 2007; Kumar *et al.*, 2009; Park *et al.*, 2008; Sharma *et al.*, 2006b), and host–pathogen interactions (Walburger *et al.*, 2004). The sensor domains of these molecules have been barely investigated to date, but it is thought that ligand recognition by the sensor domain induces activation through transmission of conformational changes to the intracellular kinase domain (Good *et al.*, 2004). Some of these STPKs are essential for mycobacterial growth and thus constitute promising antibacterial targets (Fernandez *et al.*, 2006; Szekely *et al.*, 2008).

In contrast to all other *M. tuberculosis* STPKs, PknJ has no detectable orthologue in mycobacterial species outside the *M. tuberculosis* complex (Narayan *et al.*, 2007), and we recently proposed that the PknJ-encoding gene arose in the *M. tuberculosis* ancestor though horizontal gene exchange with environmental species (Becq *et al.*, 2007). This suggests that PknJ may be involved in unique aspects of *M. tuberculosis* physiology. Here we used a combination of heterologous expression, high-density peptide-chip analysis and mass spectrometry to show that PknJ has a functional kinase domain that can phosphorylate a number of substrates.

METHODS

Strains and growth conditions. The bacterial strains used in this study are listed in Supplementary Table S1, available with the online version of this paper. *M. tuberculosis* CDC 1551 and *M. tuberculosis* CDC 1551 *pknJ*::Tn and *pepE*::Tn mutants were obtained from Colorado State University (Lamichhane *et al.*, 2003), and were grown in Middlebrook 7H9 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco) and 0.05% Tween 80. When required (for the mutant strains), kanamycin (20 µg ml⁻¹) was added to the mycobacterial cultures. The *pknJ*/Rv2088 mutant carries a transposon insertion at nt 60; the *pepE*/Rv2089c mutant carries a transposon insertion at nt 704.

Protein topology analysis. Membrane topology predictions for PknJ were examined using TMHMM Server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). We constructed a series of translational fusions of the *pknJ* gene fragments to both the *phoA* gene expressing a truncated alkaline phosphatase (PhoA) and the *gfp* gene expressing green fluorescent protein (GFP). PCR with a forward primer for the 5' end of the *pknJ* gene paired with three different reverse primers (Table S2) was used to amplify appropriate sequences using chromosomal DNA from *M. tuberculosis* strain H37Rv as a

template. The PCR amplicons were inserted into pUC19-*phoA* (pPhoA) and pJFX2, and the resulting plasmids containing different truncated *pknJ*::*phoA* and *pknJ*::*gfp* fusions were used to transform *Escherichia coli* strains CC118 (a strain with the *phoA* gene deleted) and XL1-Blue, respectively. The plasmids used in this study are described in Table S1. The alkaline phosphatase assay was performed as described by LeBlanc & Beatty (1996). GFP expression was observed under a fluorescence microscope (Leica).

Purification of recombinant PknJ, PepE, EmbR and MmaA4/Hma. An 843 bp *pknJ* fragment was amplified from *M. tuberculosis* H37Rv genomic DNA using primers PknJFd and PknJRv (Table S2). The amplified PCR fragment was inserted between the *NdeI* and *HindIII* sites of pET-28b(+) (Novagen). *E. coli* BL21 Star (DE3) cells (Invitrogen) were transformed with the resulting plasmids and the purified recombinant protein was used for the PepChip array kinase assay only. In parallel, an 807 bp PCR product of *pknJ* amplified with primers pMAL*pknJ*Fd and pMAL*pknJ*Rv (Table S2) was ligated into vector pMAL-c2X (New England Biolabs) for overproduction of its gene product. The resulting ligation product, pMAL-c2X-*pknJ*, was transformed into *E. coli* BL21 Star (DE3) (Invitrogen) and the resulting recombinant protein was used for all assays described in this paper, except the PepChip assay. The DNA fragments encoding recombinant PepE and EmbR were generated using various primers containing the additional 4 bp sequence 5'-CACC-3' necessary for directional cloning on the 5' end (Table S2). The amplicons generated were ligated into vector pET100/D-TOPO (Invitrogen). The BL21 Star (DE3) cells were transformed with the plasmids and incubated overnight at 37 °C with shaking in LB medium. The resulting culture was added to 500 ml of the same medium and grown at 37 °C to OD₆₀₀ ~0.4. Enzyme overproduction was induced by adding IPTG (1 mM final concentration, Sigma) and shaking (120 r.p.m.) at 16 °C for 30 h. For protein purification, cells were harvested by centrifugation and lysed by sonication in a buffer containing 20 mM Tris/HCl (pH 7.4), 200 mM NaCl and protease inhibitor cocktail (Roche). Recombinant protein was purified using HisTrap HP columns (GE Healthcare) and affinity columns (amylose resin, New England Biolabs) according to the manufacturer's instructions. This purified protein was used for *in vitro* kinase assays. Recombinant MmaA4/Hma was produced as previously described (Boissier *et al.*, 2006).

In vitro kinase assay. Kinase assays were conducted in 20 µl kinase buffer (25 mM Tris/HCl, 5 mM MgCl₂, 2 mM MnCl₂, pH 7.4). All reactions were started by adding ATP (100 µM) and recombinant PknJ (4.5 µg). The reaction mixture was incubated with 15 µg recombinant substrate protein at 32 °C for 25 min and terminated by addition of Laemmli SDS sample buffer. For protein migration under non-denaturing conditions, DTT was omitted from the loading buffer. The proteins were resolved by SDS-PAGE and phosphorylations were revealed by Pro-Q Diamond Phosphoprotein Gel staining (Invitrogen) according to the manufacturer's instructions (Martin *et al.*, 2003; Sun *et al.*, 2008). The phosphorylation was visualized by Typhoon 9400 PhosphorImager (Molecular Dynamics). In some experiments, proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose membrane and Western blotting detection was performed using a mouse anti-phosphothreonine antibody (Invitrogen).

LC-MS/MS analysis. Appropriate bands were cut out of gels, and excised gel slices were digested with endoprotease Glu-C (Sequencing Grade from *Staphylococcus aureus* V8, Sigma) followed by trypsin (Sequencing Grade Modified Trypsin, V511A, Promega). The tryptic digests were analysed by online LC-MS/MS on an LTQ-FT Orbitrap hybrid mass spectrometer (Thermo Electron). The samples were run on a 75 µm i.d. × 15 cm PepMap C18 column after loading onto a 300 µm i.d. × 5 mm PepMap C18 precolumn (Dionex). The flow rate

was set at 300 nl min⁻¹ (U3000 system, Dionex). Peptides were eluted using a 5–50 % linear gradient of solvent B in 50 min (solvent A was 0.2 % formic acid in 5 % acetonitrile and solvent B was 0.2 % formic acid in 90 % acetonitrile). The LTQ-FT Orbitrap mass spectrometer was operated in the data-dependent mode. In brief, a scan cycle was initiated with a full scan of high mass accuracy (m/z 300–2000) in the Orbitrap, which was followed by five MS/MS scans in the linear ion trap on the five most abundant precursor ions, with dynamic exclusion of previously selected ions. Singly charged ions were excluded from the MS/MS analysis.

MS/MS data analysis. The MASCOT search engine was used for protein identification by searching against *M. tuberculosis* complex in the Sprot-Trembl_20080901 database (24 526 sequences). Specificity of Glu-C and trypsin digestion was set for cleavage after D, E, K or R, and two missed cleavage sites were allowed. Carbamidomethylation of cysteines, oxidation of methionines and phosphorylation of serine and threonine were set as variable modifications. All MS/MS spectra of modified peptides of interest were manually validated.

Site-directed mutagenesis. Mutants of *pknJ* were generated by using a Phusion site-directed mutagenesis kit (Finnzymes). The pMAL-c2X-*pknJ* template (10 pg), sense and antisense primers (0.5 μ M) were added to PCR tubes containing 0.2 mM dNTPs, 0.01 U Phusion DNA polymerase and 1 $\times$ reaction buffer at final concentration. The programme used for the PCR consisted of 30 s of initial denaturation at 98 °C, followed by 25 cycles of 98 °C, 55 °C and 68 °C, each for 20 s. The final extension step of the PCR was 72 °C for 10 min. All fragments were separated on a 1 % (w/v) agarose gel, purified with a QIAquick Gel Extraction kit (Qiagen) and were used to transform XL1-Blue competent cells. The resultant mutations were confirmed by sequencing.

PepChip array kinase assay. The PepChip Microarray kinase assay was conducted by LC Sciences (Houston, TX, USA). The full slide contained 582 sequences matching the xx(LVI)TxTx consensus sequence detected in the *M. tuberculosis* deduced proteome, with two redundancies. Each sequence is 8-mer or longer and each substrate has its own corresponding negative control (i.e. substitute S, T or Y with A). Multiple quality-control probes were included in each chip. For kinase assay, the peptide chip was incubated with blocking buffer (1 $\times$ TBS, 1 % BSA, 0.05 % Tween 20, pH 6.8) at 4 °C overnight with circulation. After incubation, the chip was washed with 1 ml deionized water. For the kinase reaction, kinase reaction buffer containing purified recombinant PknJ (10 μ g ml⁻¹) was added to the PepChip slide and incubated for 16 h at 25 °C. The kinase reaction buffer contains 1 $\times$ TBS, 5 mM MgCl₂, 2 mM MnCl₂, 0.1 mM ATP, pH 7.5. After incubation, the slide was washed twice with washing buffer (1 $\times$ TBS pH 7.5 and 1 $\times$ TBS pH 6.8, respectively). For the detection of phosphorylated peptides, the peptide chip was stained with fivefold-diluted Pro-Q Diamond Phosphoprotein Gel Stain at 25 °C for 20 min according to manufacturer's instructions. Finally, the phosphorylated peptides were scanned and the result was quantified and analysed. Net signals for kinase substrate sequences (except internal controls) were determined by subtracting detectable signals for negative sequences from those for the corresponding kinase substrate sequences. These values are given in Supplementary Table S3. The ratios of the net signals and corresponding kinase substrate signals are presented as 'Net Signal Percentiles'. These percentiles are approximate measures of the contribution of the kinase to the signal of a kinase substrate sequence as percentages. Only sequences meeting at least the following conditions were listed as detectable: signal intensity higher than 3 $\times$ (background standard deviation); spot CV <0.5. CV is calculated as (standard deviation)/(signal intensity) and the signals from at least 50 % of the repeat probes were above the detection threshold. Background was defined as 10 % of the lowest signals from the 3968 spots; background

standard deviation was defined as the standard deviation of the 10 % of the lowest signals (about 397 spots). Only signals higher than 3 $\times$ (background standard deviation) were listed as detectable in the 'simple detectable' sheet. Spot CV was defined as the CV within a spot after its intensity and standard deviation were extracted from an image by ArrayPro software. It measures spot uniformity. Only spots with spot CV <0.5 were used for calculation of detectable signals. There were usually three replicates on an array. If the signals were from at least 50 % of the repeat probes, then it meant that 2 out of the 3 repeats, or 3 out of the 3 repeats, showing signals were considered as positives, and 1 out of 3 repeats showing signals were not considered as positive.

Macrophage and mouse infection. Bone-marrow-derived macrophages from female BALB/c mice were isolated and infected as described previously (Rousseau *et al.*, 2004). For activation, macrophages were pre-stimulated with IFN- γ at a concentration of 1000 U μ l⁻¹ for 4 h prior to infection. The cells were infected for 4 h, then washed six times with pre-warmed culture medium, lysed at various times thereafter and plated onto 7H11-agar medium containing 10 % OADC for c.f.u. scoring. For the virulence and survival analysis, 6- to 8-week-old female BALB/c mice were infected intranasally with 10³ c.f.u. of *M. tuberculosis* strain CDC 1551 or its *pknJ* transposon mutant. At each time point, mice were killed, and the spleen and lungs were harvested and homogenized. Tenfold serial dilutions of organ homogenates were plated onto complete 7H11 medium and c.f.u. counted.

Lipid analysis and cytokine secretion assay. Details of these methods are given in the supplementary material.

RESULTS

pknJ encodes a functional kinase

Genomic organization of the *pknJ* region is shown in Fig. 1(a). We inserted the kinase-domain-encoding genomic fragment of the *pknJ* gene (bp 1–807) into an appropriate expression vector. The recombinant product was soluble and its purity was confirmed by gel migration and staining (not shown). We tested this construct for expression of a functional kinase using recombinant myelin basic protein (MBP) as a substrate. Gel staining with Pro-Q Diamond (Martin *et al.*, 2003; Sun *et al.*, 2008) showed MBP phosphorylation in a time-dependent manner (Fig. 1b). This result was confirmed by Western blotting using an anti-phosphothreonine antibody (Fig. 1c). Autophosphorylation of PknJ was also observed (Fig. 1d) unless ATP was omitted from the reaction mixture. We did not detect a Pro-Q signal in the absence of ATP in the reaction mixture (Fig. 1d). This excludes the possibility of false-positive results.

PknJ autophosphorylates at Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³

Mass spectrometry analysis of PknJ proteolytic digest revealed one phosphorylation site at threonine residue 168 and two phosphorylation sites at residues 171–173 (Fig. 2a). STPKs can phosphorylate both threonine and serine residues, so we investigated which two residues in the

Fig. 1. *M. tuberculosis* PknJ contains a functional kinase domain. (a) Genomic organization of the *pknJ*/Rv2088 region in the *M. tuberculosis* chromosome according to Tuberculist nomenclature. IS, insertion sequences. (b, c) Phosphorylation assay using recombinant *M. tuberculosis* PknJ kinase domain produced in *E. coli*, and myelin basic protein (MBP) as a substrate. (d) Autophosphorylation assay of recombinant PknJ kinase domain in the presence or absence of ATP. Phosphoproteins in (b) and (d) were detected using Pro-Q Diamond; in (c), phosphoproteins were detected by Western blotting using an anti-phosphothreonine antibody.

T¹⁷¹ST¹⁷³ tripeptide were in fact phosphorylated. A triple PknJ mutant where Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³ were replaced by Ala residues showed no sign of phosphorylation, confirming the specificity of Pro-Q staining for phosphoprotein in our experiment (Fig. 2b). We conclude that *in vitro* PknJ autophosphorylates at threonine residues 168, 171 and 173 only; in particular our results exclude the possibility that Ser¹⁷² is a phosphorylated residue. However, they do not rule out the possibility that there may be diverse PknJ isoforms containing variable numbers of phosphothreonine residues in the analysed samples.

PknJ forms dimers and has a single transmembrane domain

STPKs can form dimers, which probably contributes to signal transduction (Gay *et al.*, 2006), and we assessed whether this was also true for PknJ. Analysis of the recombinant protein under non-denaturing conditions revealed two bands with apparent masses of 38 and 76 kDa, suggesting that the protein can form dimers *in vitro* (Fig. 3a). Most *M. tuberculosis* STPKs have a predicted transmembrane fragment (Av-Gay & Everett, 2000; Narayan *et al.*, 2007; Wehenkel *et al.*, 2008) but the topology has been rigorously analysed for only a few of

them. *In silico* analysis of PknJ revealed two potential transmembrane domains, which is unusual for mycobacterial STPKs (Fig. 3b). In order to study the topology of the protein, we constructed PhoA and GFP fusions with PknJ fragments of different lengths (Fig. 3c). PhoA is active only when expressed extracellularly (Manoil & Beckwith, 1986), whereas GFP emits fluorescence only when located in the host cell cytoplasm (Feilmeier *et al.*, 2000). The PhoA/GFP topology analysis has been successfully used to analyse protein topology in *E. coli* (Drew *et al.*, 2002). Fluorescence (Fig. 3d) and phosphatase activity analysis (Fig. 3e) revealed that PknJ contains only one transmembrane fragment, most probably from residue 344 to residue 363.

PknJ phosphorylates EmbR, PepE and MmaA4/Hma *in vitro*, and may have additional substrates

The autophosphorylation residues of several *M. tuberculosis* STPKs, namely PknB, PknD–F and PknL, have been identified (Duran *et al.*, 2005; Lakshminarayan *et al.*, 2008; Molle *et al.*, 2003a, 2004; Perez *et al.*, 2006). We used these results together with our results showing that PknJ Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³ are phosphorylated (Fig. 2), to generate a consensus sequence of phosphorylation sites. We searched the *M. tuberculosis* genome for the consensus sequence xx(LVI)TxTxx (Fig. 4a), and thereby identified 582 potential phosphorylation targets. These peptides were synthesized and included in a high-density peptide chip. The EmbR-derived peptide PLWTQLITAYY was also included in the chip because EmbR has been reported to be phosphorylated by several STPKs, including PknH (Molle *et al.*, 2003b; Sharma *et al.*, 2006a). Peptides in which all possible phosphorylation residues (Thr, Ser) in each target were replaced by Ala were included in the chip as negative controls. The chip was incubated with recombinant PknJ and phosphorylation was revealed by Pro-Q staining (Fig. 4b). A number of peptides were phosphorylated by incubation with PknJ, although the corresponding Ala control peptides remained unphosphorylated. The peptides giving a net signal percentile of 100 % (see Methods) and the corresponding mycobacterial proteins according to Tuberculist nomenclature are given in Table 1. The EmbR-derived peptide gave a particularly strong signal (Fig. 4b, Table 1). Phosphorylation assays confirmed that PknJ can phosphorylate recombinant EmbR *in vitro* (Fig. 4c). Another peptide (kDvTRTYS) similar to the putative dipeptidase PepE and the methyltransferase MmaA4/Hma involved in mycolic acid biosynthesis was also identified, although with a net signal percentile below 100 % (Fig. 4b, Table S3). The PepE-encoding gene maps next to *pknJ* in the *M. tuberculosis* genome (Fig. 1a); many genes encoding the substrate(s) for several mycobacterial STPKs map in the vicinity of the gene encoding the cognate kinase itself (Narayan *et al.*, 2007). Phosphorylation assays showed that recombinant PepE is a substrate for PknJ (Fig. 4d). Several mycobacterial STPKs have been shown to phosphorylate various enzymes involved in mycolic acid synthesis (Molle *et al.*, 2006a;

Fig. 2. The *M. tuberculosis* PknJ kinase domain autophosphorylates on Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³. (a) The MS/MS spectrum shows a specific triphosphorylated peptide sequence in PknJ, which is displayed above the spectrum. The ESI-MS/MS spectrum of the peptide with m/z 1210.01 (doubly charged ion) indicates that the peptide sequence ¹⁶⁴ALGD_pTGLTSTGSLATLAYAAPE₁₈₆ is phosphorylated at Thr¹⁶⁸ and contains two other phosphorylation sites in the underlined amino acid sequence TST. The phosphorylation status of threonine (T) and serine (S) residues is indicated positively by the letter p in the annotated spectrum. (b) Phosphorylation analysis of a PknJ kinase domain mutant form in which Thr¹⁶⁸, Thr¹⁷¹ and Thr¹⁷³ are each replaced by an Ala residue. Phosphoproteins were detected using Pro-Q Diamond staining.

Veyron-Churlet *et al.*, 2009), and we show here that recombinant Hma can be efficiently phosphorylated by PknJ, at least *in vitro* (Fig. 4e). These results show that PknJ has at least four substrates *in vitro*, namely EmbR, PepE, MmaA4/Hma and PknJ itself. The numerous peptide substrates identified in our peptide-chip screening assay suggest additional potential targets for PknJ.

PknJ does not contribute to mycobacterial virulence in the mouse model

A role for some *M. tuberculosis* STPKs in mycobacterial virulence *in vitro* and *in vivo* has been suggested (Gopalaswamy *et al.*, 2009; Papavinasundaram *et al.*,

2005; Walburger *et al.*, 2004). We checked *pknJ* expression by RT-PCR. A *pknJ* transcript was indeed detected, at least in axenic culture conditions (data not shown). *M. tuberculosis* transposon mutants in *pknJ* and *pepE* were generated elsewhere (Lamichhane *et al.*, 2003). Interestingly, both the *pknJ*- and the *pepE*-deficient strains showed a growth defect in minimal Sauton's broth, unless BSA was added to the medium (Fig. S1). To assess whether PknJ plays a part in host-cell infection, we infected bone-marrow-derived macrophages with the *pknJ*::Tn mutant or with the corresponding wild-type strain CDC1551. No difference in intracellular growth of the bacilli was observed (Fig. 5a). Infection of immunocompetent mice again revealed no particular phenotype for the *pknJ* mutant

Fig. 3. The *M. tuberculosis* PknJ kinase domain forms dimers and PknJ has a single transmembrane fragment. (a) Electrophoretic mobility assay of recombinant PknJ kinase domain under non-denaturing conditions. Monomers (38 kDa) and dimers (76 kDa) are detected. (b) PknJ topology prediction (posterior probability of inside/outside/TM helix) using the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM/>). TM, transmembrane domain. (c) Gene fusion constructs used in topological analysis of PknJ. DNA fragments encoding PknJ regions were fused to GFP or to PhoA as indicated, and expressed in *E. coli*. (d) Fluorescence analysis of GFP fusions shown in (c). (e) Phosphatase activity of PhoA fusions shown in (c).

(Fig. 5b). The *pknJ*-deficient mutant was slightly hyper-virulent in immunodeficient SCID mice (Fig. 5c), although we could not restore the phenotype of the wild-type strain

in the complemented mutant strain. Mycobacterial lipids, such as phthiocerol dimycocerosates (DIMs) play a major part in *M. tuberculosis* virulence (Camacho *et al.*, 1999; Cox

Fig. 4. The *M. tuberculosis* PknJ kinase domain phosphorylates multiple substrates. (a) A consensus phosphorylation sequence was deduced from known sequences in PknB, PknD–F, PknL and PknJ. (b) Phospho-chip analysis of peptide phosphorylation by recombinant PknJ kinase domain. Internal controls (phosphorylated and non-phosphorylated peptides), and the peptide substrates identified in EmbR (full match) and PepE (partial match), are shown. (c–e) Confirmation assays using recombinant *M. tuberculosis* EmbR (c), PepE (d) and MmaA4 (e) produced in *E. coli* as PknJ substrates in the presence or absence of ATP. Ovalbumin (OVA) is shown as a control. Phosphoproteins were revealed by Pro-Q Diamond staining.

et al., 1999), and our results showing that the polyketide synthases PpsA and PpsB, involved in DIM synthesis, are potential substrates for PknJ (Table 1) suggested that DIM synthesis may be under the control of PknJ-mediated phosphorylation. Extensive analysis of the lipid content of *M. tuberculosis* CDC1551 and its *pknJ*::Tn mutant revealed

no difference as regards DIM (Fig. S2a), other lipids, such as trehalose derivatives (TMM, TDM or DAT) (Fig. S2b) or phospholipids (PI, PE, CL, PIM₂, PIM₆) (Fig. S2c). Protein macroarray analysis of the host cell response to the mutant and the wild-type strains showed no difference in cytokine and chemokine synthesis (Fig. S3).

Table 1. List of peptides, and corresponding *M. tuberculosis* genes and derived whole protein sequences, found to be phosphorylated by PknJ with no signal detected for the cognate (T/S→A) control

No.†	Probe ID†	Sequence	Signal‡	Gene§	Function§
801	PknJ_0401	TPVTATRH	172.8	Rv3073c	
211	PknJ_0106	PDITATPA	173.5	<i>pgi</i>	Glucose-6-phosphate isomerase
645*	PknJ_0323	AWLTATPV	173.6	Rv1318c/Rv1320c	Adenylate cyclase
963	PknJ_0482	AVETNTFG	173.9	<i>metH</i>	Methionine synthesis
477	PknJ_0239	MQLTLTEL	174.9	<i>ppsB</i>	PDIM synthesis
311	PknJ_0156	TSVTLTMS	176.9	<i>lprA</i>	Lipoprotein
839	PknJ_0420	VQVTSTGV	177.0	Rv0479c	
571	PknJ_0286	DTVGTAS	177.2	<i>tal</i>	Transaldolase
1087	PknJ_0544	DNTVGTDE	177.3	<i>rpoB</i>	RNA polymerase
799	PknJ_0400	VLVTGTVA	177.7	Rv2926c	
781	PknJ_0391	KPITGTHA	178.2	Rv2633c	
397	PknJ_0199	THVTATHY	178.8	<i>nadB</i>	L-Aspartate oxidase
267	PknJ_0134	GTVTLTFT	179.6	<i>icd1</i>	Isocitrate dehydrogenase
279	PknJ_0140	SYLTQTTL	180.2	<i>lytB1</i>	
649*	PknJ_0325	AWLTATPV	180.3	Rv1318c/Rv1320c	Adenylate cyclase
125	PknJ_0063	DSVTITGF	180.5	<i>hupB</i>	Histone-like
831	PknJ_0416	ITLTSTAD	181.4	Rv3740c	Triacylglycerol synthase
1107	PknJ_0554	GVTTQLGDDVA	182.1	<i>lppI</i>	Lipoprotein
399	PknJ_0200	EMVTLTID	182.4	<i>nuoG</i>	NADH dehydrogenase I
1067	PknJ_0534	VQETYTTL	182.6	Rv0635	Fatty acid synthesis
1147**	PknJ_0574	EKLTQLGNTVGT	183.5	<i>pknE</i>	STPK
129	PknJ_0065	VVLATFA	186.9	<i>dipZ</i>	Cytochrome biogenesis
117	PknJ_0059	DLITGTDL	187.9	<i>ctpI</i>	P-type ATPase
1011	PknJ_0506	SIETPTPV	188.4	<i>tatA</i>	Sec-independent protein translocase
847	PknJ_0424	DGITGLV	188.6	<i>mshA</i>	Glycosyltransferase
333	PknJ_0167	VPLTETAY	189.4	<i>mfd</i>	Transcription-repair coupling factor
547***	PknJ_0274	MLVTTTDA	189.9	<i>sodC</i>	Superoxide dismutase
761	PknJ_0381	VDITMTPA	190.3	Rv2560	
487	PknJ_0244	FWVTGTSI	191.2	<i>ppsA</i>	PDIM synthesis
835	PknJ_0418	IGITVTDA	191.6	Rv3884c	
601	PknJ_0301	TLVTDTPG	191.8	Rv0010c	
141	PknJ_0071	LGVTVTVD	192.9	<i>dnaJ2</i>	Chaperone
543***	PknJ_0272	MLVTTTDA	193.5	<i>sodC</i>	Superoxide dismutase
459	PknJ_0230	TGLTSTGSII	194.0	<i>pknJ</i>	STPK
745	PknJ_0373	GDVTETIR	194.5	<i>pitB</i>	Phosphate transport permease
401	PknJ_0201	GALTGTAY	196.3	<i>nuoG</i>	NADH dehydrogenase I
1085	PknJ_0543	NMTVGTVS	197.5	<i>pknF</i>	STPK
859	PknJ_0430	IHLTVTDV	198.5	Rv0887c	
481	PknJ_0241	MLVTQTSS	199.0	<i>pra</i>	Proline-rich antigen
405	PknJ_0203	AILTMTVG	201.1	<i>nuoN</i>	NADH dehydrogenase I
57	PknJ_0029	KLITYTSD	202.3	<i>blaC</i>	Beta-lactamase
225	PknJ_0113	LPVTWTVA	202.4	<i>idsA1</i>	Lipid synthesis
1035	PknJ_0518	MPETPTGD	203.9	Rv1910c	
775	PknJ_0388	CRLTWTMA	205.1	Rv2574	
65	PknJ_0033	TIITATFL	205.6	<i>cdsA</i>	Phosphatidate cytidyltransferase
569	PknJ_0285	SAVTGTDI	207.7	<i>trpS</i>	tRNA synthetase
173	PknJ_0087	LHVTWTGE	208.5	<i>esxE</i>	ESAT6-like protein
773	PknJ_0387	TKVWTWSP	209.1	Rv2574	
755	PknJ_0378	VYLVTVGY	209.6	Rv2331	
807	PknJ_0404	GVLTDITAS	209.9	Rv3400	
261	PknJ_0131	DDVTETLE	210.7	<i>hemC</i>	Porphyrin synthesis
981	PknJ_0491	RAETSTPV	212.9	<i>pyrD</i>	Dihydroorotate dehydrogenase
567	PknJ_0284	SAVTGTDI	213.7	<i>trpS</i>	tRNA synthetase
353	PknJ_0177	VFVTSTVS	214.0	<i>mmpL11</i>	Fatty acid transport
409	PknJ_0205	PALTSTDY	216.4	<i>aceE</i>	Pyruvate dehydrogenase

Table 1. cont.

No.†	Probe ID†	Sequence	Signal‡	Gene§	Function§
181	PknJ_0091	LVITYTGA	219.1	<i>recC</i>	Exonuclease
583	PknJ_0292	DELTTT	219.3	<i>trpD</i>	Tryptophan synthesis
919	PknJ_0460	GNETTKL	220.7	<i>cyp123</i>	Cytochrome P450
189	PknJ_0095	DEITLTMK	221.8	<i>fadD11</i>	Fatty acid-CoA ligase
779	PknJ_0390	YTVTTSD	222.4	Rv2576c	
519	PknJ_0260	VDVTGTSK	226.1	<i>rplC</i>	50S ribosomal protein
929	PknJ_0465	EGETLTIR	228.0	<i>dapB</i>	Dihydrodipicolinate reductase
1059	PknJ_0530	LEETVTYD	229.8	Rv2913c	Amino acid hydrolase
551	PknJ_0276	ADVTATPS	230.9	<i>suhB</i>	Extragenic suppressor protein
985	PknJ_0493	DLETSTLR	246.7	<i>recG</i>	DNA helicase
1083**	PknJ_0542	GNTVGTLY	254.0	<i>pknE</i>	STPK
1143	PknJ_0572	PLWTQLITAYY	267.9	<i>embR</i>	Arabinan synthesis
1063	PknJ_0532	EAETLTAR	270.6	Rv3788	
507	PknJ_0254	DVITDTYS	332.7	<i>recG</i>	DNA helicase
475	PknJ_0238	LRVTATRS	366.9	<i>ppsA</i>	PDIM synthesis

†No. and probe ID are given as in the raw data sheet (Table S3). Asterisks (*) indicate redundant probesets.

‡See Methods.

§Gene and function are given as in Tuberculist (<http://genolist.pasteur.fr/TubercuList/>). Function is putative or documented (see Tuberculist for details).

||Residues identified by MS analysis as being phosphorylated are underlined.

DISCUSSION

We report the first topological and functional characterization of PknJ, the only *M. tuberculosis* STPK that has not previously been investigated. We recently suggested that the PknJ-encoding gene arose in the *M. tuberculosis* complex ancestor by horizontal gene transfer from environmental species (Becq *et al.*, 2007). This may explain why PknJ is found only in species of the *M. tuberculosis* complex and not in other mycobacteria (Narayan *et al.*, 2007). However, proteins annotated as ‘PknJ’ are found in other mycobacterial genomes available through various online databases. Close examination of the amino acid sequences of these homonyms showed similarity with PknJ either only in the N-terminal kinase domain (e.g. in *Mycobacterium marinum* ‘PknJ’) or in the C-terminal extracellular domain (e.g. in *Mycobacterium avium* ‘PknJ’). The *M. avium* ‘PknJ’, for example, lacks a detectable kinase domain (data not shown). This suggests either that (i) *M. tuberculosis* PknJ arose from gene fusion between an *M. avium* ‘PknJ’-related protein and a functional kinase domain already present in an ancestral mycobacterial genome, or that (ii) *M. avium* ‘PknJ’ results from deletion of the kinase domain of an ancestral protein that has been transmitted to *M. tuberculosis*. Nevertheless, we propose that the name PknJ is kept only for Rv2088/PknJ found in *M. tuberculosis* and close relatives of the *M. tuberculosis* complex.

We show here that PknJ is a functional kinase that undergoes autophosphorylation when incubated with ATP, forms dimers *in vitro* and has a single transmembrane

domain. These three features are shared by many other *M. tuberculosis* STPKs (Wehenkel *et al.*, 2008). MS analysis and site-directed mutagenesis showed that the PknJ kinase domain autophosphorylates at threonine residues 168, 171 and 173. We cannot rule out the possibility that there may also be multiple isoforms of the protein containing different numbers of phosphorylated threonine residues. Our peptide-chip analysis shows multiple potential targets for PknJ (~70 proteins). Phosphorylation of the peptides does not imply that all proteins containing these sequences are indeed phosphorylated, and we do not claim that these proteins are all PknJ substrates *in vitro* or *in vivo*, which should be evaluated on a case-by-case basis. STPKs can phosphorylate numerous substrates, and several STPKs can phosphorylate the same substrates (Grundner *et al.*, 2005; Molle *et al.*, 2006a). It is therefore likely that some of these proteins may be substrates for several *M. tuberculosis* STPKs, as well as for PknJ. We have confirmed four of these targets: (1) PknJ itself, (2) EmbR, which is also a substrate for PknH (Molle *et al.*, 2003b) and other mycobacterial STPKs (Sharma *et al.*, 2006a), (3) MmaA4/Hma, a methyltransferase involved in the synthesis of mycolic acids, and (4) PepE, a putative dipeptidase with proline specificity (prolidase), whose gene maps next to *pknJ* in the mycobacterial genome (Fig. 1a). This suggests that PknJ may be involved in nitrogen and amino acid metabolism, possibly through phosphorylation of PepE. Prolidase activation through phosphorylation has already been reported in other species (Surazynski *et al.*, 2001), but it is not known whether this is also the case in *M. tuberculosis*. The finding that both the *pknJ*- and

Fig. 5. PknJ does not contribute to mycobacterial virulence in the mouse model. (a) Inactivated (left) and activated (right) mouse bone-marrow-derived macrophages were infected with wild-type *M. tuberculosis* CDC1551 or with a CDC1551-derived *pknJ* transposon mutant. Cell lysates were plated and c.f.u. scored at various times. (b) Wild-type and mutant strains were used to infect BALB/c mice intranasally (1000 c.f.u. per mouse). After 1, 21 and 42 days of infection, lungs and spleen were homogenized and plated for c.f.u. scoring. (c) Wild-type and mutant strains were used to infect SCID mice intranasally (1000 c.f.u. per mouse), and animal survival was recorded. The figure shows one representative out of two independent experiments.

pepE-deficient mutants grow poorly in minimal medium unless BSA (a possible nitrogen source) is added supports this hypothesis.

The *pknJ* mutant was found to be slightly hypervirulent in immunocompromised SCID mice. Although we could not restore the phenotype of the wild-type strain in the complemented mutant, this phenotype was strikingly similar to that of mycobacterial mutants defective in other STPKs, namely PknH (Papavinasundaram *et al.*, 2005) and PknI (Gopalaswamy *et al.*, 2009). It may be explained by the low virulence of the CDC1551 mother strain as compared to other *M. tuberculosis* strains (Manca *et al.*, 1999), and the role of PknJ in mycobacterial virulence may become more apparent in other experimental conditions (e.g. in other *M. tuberculosis* mother strains or in other animal models); it may also suggest that STPKs such as

PknH–J may play a part in mycobacterial virulence, which may be more apparent in other model systems. The exact function of PknJ (possibly in association with PepE) will thus require further investigation. More generally, our proteome-wide screening provides a list of putative substrates for *M. tuberculosis* PknJ, which may help better understanding of the function of this unique kinase in mycobacterial physiology.

ACKNOWLEDGEMENTS

We thank A. Wehenkell, M. Bellinzoni and P. M. Alzari for providing help at the start of the project. We thank Colorado State University for providing mycobacterial mutants (NIH, NIAID Contract No. HHSN266200400091C, 'Tuberculosis Vaccine Testing and Research Materials', awarded to CSU). B.M. was supported by grants from the Région Midi-Pyrénées, the FRM, and the ANR PFTV (Agence

Nationale de la Recherche PlateFormes Technologiques du Vivant). This work was supported by the CNRS and the Institut Pasteur. J. J. was a fellow of the Fondation pour la Recherche Médicale (FRM). O. N. is a beneficiary of an ATIP grant from the CNRS.

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Edited by: S. V. Gordon