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Evidence that a eukaryotic-type serine/threonine protein kinase from *Mycobacterium tuberculosis* regulates morphological changes associated with cell division

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A eukaryotic-type protein serine/threonine kinase, PknA, was cloned from *Mycobacterium tuberculosis* strain H37Ra. Sequencing of the clone indicated 100% identity with the published *pknA* sequence of *M. tuberculosis* strain H37Rv. PknA fused to maltose-binding protein was expressed in *Escherichia coli*; it exhibited a molecular mass of ≈ 97 kDa. The fusion protein was purified from the soluble fraction by affinity chromatography using amylose resin. *In vitro* kinase assays showed that the autophosphorylating ability of PknA is strictly magnesium/manganese-dependent, and sodium orthovanadate can inhibit this activity. Phosphoamino-acid analysis indicated that PknA phosphorylates at serine and threonine residues. PknA was also able to phosphorylate exogenous substrates, such as myelin basic protein and histone. A comparison of the nucleotide-derived amino-acid sequence of PknA with that of functionally characterized prokaryotic serine/threonine kinases indicated its possible involvement in cell division/differentiation. Protein–protein interaction studies revealed that PknA is capable of phosphorylating at least a ≈56-kDa soluble protein from *E. coli*. Scanning electron microscopy showed that constitutive expression of this kinase resulted in elongation of *E. coli* cells, supporting its regulatory role in cell division.

Keywords: autophosphorylation; phosphorylation; PknA; serine/ threonine kinase; signal transduction.

Signal-transduction pathways in both prokaryotes and eukaryotes often utilize protein phosphorylation as a molecular switch in regulating different cellular activities such as adaptation and differentiation. It is well known that protein kinases play a cardinal role in the process. They are grouped into two superfamilies, histidine (His) and serine/ threonine (Ser/Thr) or tyrosine (Tyr) kinases, based on their sequence similarity and enzymatic specificity [1,2]. Signal transduction in prokaryotes usually uses His kinases, which autophosphorylate at histidine residues [2]. In eukaryotes, such signalling pathways are mediated by Ser/Thr or Tyr kinases, which autophosphorylate at serine/threonine or tyrosine residues [1].

Interestingly, analysis of genome sequences revealed the presence of putative genes encoding eukaryotic-type Ser/Thr kinases in many bacterial species. A search of the *Escherichia coli* genome also indicated the presence of sequences exhibiting homology with eukaryotic-type Ser/Thr kinases, but they have not been characterized biochemically or functionally. Involvement of such kinases in regulating growth and development has largely been documented in soil bacteria such as *Myxococcus* [3–6], *Anabaena* [7] and *Streptomyces* [8,9]. In *Yersinia pseudotuberculosis*, YpkA has been identified as the first secretory prokaryotic Ser/Thr kinase involved in pathogenicity [10]. Besides these, eukaryotic-type Ser/Thr kinases have been implicated in virulence in opportunistic pathogens such as *Pseudomonas aeruginosa* [11]. Thus a detailed study of these kinases, especially in pathogenic bacteria, could produce important insights into their contributions to signal transduction. This may help in the design of drug intervention strategies in a situation where the emergence of drug-resistant strains of several pathogenic bacteria has resulted in the rapid resurgence of diseases thought to be near irradication. We focused on tuberculosis, a disease caused by *Mycobacterium tuberculosis*, which is responsible for considerable human morbidity and mortality world wide [12].

In the *M. tuberculosis* genome, 11 putative eukaryotic-type kinases have been reported [13]. Among these Ser/Thr kinases, four (PknB, PknD, PknF, PknG) have been biochemically characterized [14–16], but their biological functions are not known. The *M. tuberculosis* genome sequence further indicated that the gene for a putative Ser/ Thr kinase, *pknA*, is located adjacent to those encoding bacterial morphogenic proteins. Interestingly, the presence of a Ser/Thr kinase at this location in the mycobacterial genome is unique among prokaryotes [17]. We therefore concentrated on PknA. In this paper, we report the cloning and expression of PknA as a fusion with maltose-binding protein (MBP). Characterization of the fusion protein revealed that it is capable of phosphorylating itself as well as basic protein substrates not present in *M. tuberculosis*. Furthermore, we present strong evidence that the constitutive expression of this kinase causes elongation of cells in *E. coli*, supporting a regulatory role for PknA in cell division.

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MATERIALS AND METHODS

Bacterial strains and vectors

M. tuberculosis strain H37Ra [18] used in this study was grown at 37 °C using oleic acid/albumin/dextrose/catalase/Tween-80/glycerol-supplemented Middle brook 7H9 broth or 7H10 agar. *E. coli* strains DH5α and TB1 were cultured on Luria–Bertani agar or broth. Vectors such as pUC19 and pMAL-c2X were obtained from commercial sources. The *Mycobacterium–E. coli* shuttle vector, p19Kpro, was a gift from D. B. Young and M. Blokpoel, Imperial College School of Medicine at St Mary's, London, UK.

PCR amplification, site-directed mutagenesis, and construction of recombinant plasmids

Genomic DNA was isolated from *M. tuberculosis* strain H37Ra as described elsewhere [19] except that the spheroplast lysis step was carried out for 24 h at 37 °C with SDS (4%) and proteinase K (500 $\mu g \cdot mL^{-1}$). DNA thus obtained was used for PCR amplification of *pknA*. The Expand Long Template PCR system (mixture of *Pwo* and *Taq* DNA polymerases; Roche Molecular Biochemicals) was used for this purpose. The forward (CC7: 5′-CATATGAGCCCC CGAGTTGG-3′) and reverse (CC8: 5′-TCATTGCGCTA TCTCGTATCGG-3′) primers were designed on the basis of the published *M. tuberculosis* genome sequence [13] of *pknA* (Rv0015c). Oligonucleotides used in this study were custom-synthesized from IDT, Coralville, IN, USA. PCR was carried out for 30 cycles (denaturation, 95 °C for 30 s per cycle; annealing, 50 °C for 30 s per cycle; elongation, 68 °C for 2 min for first 10 cycles and then for the remaining 20 cycles the elongation step was extended for an additional 20 s in each cycle).

PCR was also used to generate the K42N (replacement of lysine by asparagine at residue 42) point mutant of PknA. Two forward primers, CC58 (5′-CACAGGAATTCCATA TGAGCCCCCGAGTTGG-3′), CC62 (5′-GTGTTGCGG TGAATGTGCTCAAGAGCG-3′) and two reverse primers, CC61 (5′-CTGCCCGGTGGGGGGTGATCAAGA TG-3′), CC63 (5′-CGCTCTTGAGCACATTCACCGCA ACAC-3′), were synthesized. Base mismatches (underlined bases) for the desired mutations were incorporated in primers CC62 and CC63. To generate the mutant, two sets of primary and one set of secondary PCR reactions were carried out as described elsewhere [20] using the gel-purified *pknA* ($\approx$ 1.3 kb) as template. Primary reactions were carried out with primers CC58/CC63 and CC61/CC62, while for secondary reactions, PCR primers CC58 and CC61 were used. Thus, the K42N mutation was contained within the amplified $\approx$ 460-bp fragment of *pknA*, which has a unique *Xho*I site in addition to the *Eco*RI and *Nde*I sites incorporated in the primer CC58.

All manipulations with DNA were performed by standard methods [21]. Restriction/modifying enzymes and other molecular biological reagents used in this study were obtained from New England Biolabs. After PCR amplification, *pknA* was treated with Klenow, and the blunt-ended fragment was cloned at the *Sma*I site of pUC19 (pPknA). Plasmid DNA was prepared after transformation of pPknA in *E. coli* strain DH5α and sequenced in an automated sequencer (ABI; PE Applied Biosystems).

To monitor expression of PknA fused with MBP, *E. coli* vector pMAL-c2X was used. After digestion of pPknA and pMAL-c2X with *Nde*I and *Bam*HI, respectively, they were treated with Klenow to obtain blunt-ended fragments. Both these fragments were further restriction-digested with *Hin*dIII, ligated and transformed in *E. coli* strain TB1 to obtain clones containing the plasmid (pMAL-PknA) bearing in-frame fusion of $\approx$ 1.3 kb *pknA* (confirmed by junction sequencing) at the 3′ end of MBP. To express the K42N mutant as an MBP fusion protein, a $\approx$ 460-bp fragment of mutated *pknA* was digested with *Eco*RI/*Xho*I and substituted for the corresponding wild-type fragment in the pMAL-PknA backbone. The resulting construct, pMAL-K42N, was sequenced to confirm the mutation.

pknA or the K42N mutant was also cloned in the *Mycobacterium–E. coli* shuttle vector p19Kpro [22] to obtain the constitutive expression plasmids (p19Kpro-PknA or p19Kpro-K42N). The strategy adopted was same as for construction of pMAL-PknA. To clone *pknA* in an antisense orientation, pPknA was initially digested with *Nde*I and treated with Klenow to obtain a blunt-ended fragment. After restriction digestion with *Bam*HI, this fragment was subsequently ligated to p19Kpro, which was already digested with *Bam*HI and *Eco*RV. The antisense construct of *pknA* was designated p19Kpro-aPknA. All three constructs, p19Kpro-PknA, p19Kpro-K42N and p19Kpro-aPknA were transformed in *E. coli* strain DH5α. Clones carrying the gene of interest were confirmed at all steps by restriction analysis and Southern-blot hybridization. The probe (PCR-amplified *pknA*) used was radiolabelled by random priming with [α-^{32}P]CTP (BRIT, Hyderabad, India).

Expression of recombinant protein

pMAL-PknA or pMAL-K42N cultures were grown at 37 °C and induced with 0.3 mM isopropyl thio-β-D-galactoside (IPTG) at an A_{600} of 0.5. Cells were harvested after 3 h, lysates were prepared, and expression was monitored by SDS/PAGE (8% gel) and Coomassie Brilliant Blue staining. To find out the solubility of the expressed fusion protein, after induction cells were suspended in lysis buffer and sonicated. Supernatant and pellet fractions obtained after sonication were subjected to SDS/PAGE. Finally, the fusion protein was purified by affinity chromatography on an amylose column according to the manufacturer's instructions (New England Biolabs). In a similar manner, MBP–βgal fusion protein expressed by pMAL-c2X was also purified for its use as a control. To examine the constitutive expression of the protein and its solubility, overnight cultures (at 37 °C) of constructs in p19Kpro were processed in the same way as pMAL-PknA except that IPTG induction was not required.

Kinase assay

The ability of PknA or the K42N mutant, as a purified fusion protein with MBP, to autophosphorylate and phosphorylate exogenous substrates such as histone (from calf thymus, type II-AS; Sigma) or myelin basic protein (from bovine brain; Sigma) was determined in an *in vitro* kinase assay. Aliquots (usually 800 ng to 6 μg in 20 μL reaction volume) of fusion protein (MBP–PknA or

MBP–K42N or MBP–βgal) were mixed with $1 \times$ kinase buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MnCl_2$), and the reaction was initiated by adding 2 µCi [γ-^{32}P]ATP. After incubation at 24 °C for 20 min, the reaction was stopped by adding SDS sample buffer (30 mM Tris/HCl, pH 6.8, 5% glycerol, 2.5% 2-mercaptoethanol, 1% SDS and 0.01% bromophenol blue). Samples were boiled for 5 min and resolved by SDS/PAGE (8–12.5% gels). Gels were stained with Coomassie Brilliant Blue, dried in a gel dryer (Bio-Rad) at 70 °C for 2 h and finally exposed to Kodak X-Omat/AR film. To monitor the effect of bivalent cations, the 10 mM $MnCl_2$ in the $1 \times$ kinase buffer was substituted with 1, 10 or 100 mM $Mn^{2+}/Mg^{2+}/Ca^{2+}$. The autophosphorylating ability of the constitutively expressed PknA was determined using p19Kpro-PknA-transformed *E. coli* extract in a similar manner.

To identify proteins that interacted with PknA, MBP–PknA (100 µg) was immobilized on amylose resin and incubated in the presence of soluble protein extracts (250 µg) prepared from *E. coli* strain DH5α for 10 h at 4 °C. Amylose beads were washed (4500 ***g*** for 5 min) four times to remove unbound proteins. After suspension of washed beads in TEN buffer (20 mM Tris/HCl, pH 7.5, 200 mM NaCl and 1 mM EDTA), aliquots (12 µL) were used for phosphorylation assays.

Western blotting

Phosphoamino-acid analysis was carried out by Western blotting. Purified fusion proteins or cell extracts (800 ng to 3 µg protein per slot) were resolved by SDS/PAGE (8% gel) and transferred at 250 mA for 45 min to nitrocellulose membrane (0.45 µm) in a mini-transblot apparatus (Bio-Rad) using Tris/glycine/SDS buffer (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol, pH ≈ 8.3). Primary antibodies (anti-MBP, anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine) used for different immunoblots were commercially available (New England Biolabs, Santa Cruz Biotech and Sigma). Horseradish peroxidase-conjugated anti-(mouse IgG) Ig or anti-(rabbit IgG) Ig secondary antibody (Roche Molecular Biochemicals) was chosen depending on the primary antibody used, and the blots were processed by the ECL detection system (Amersham Pharmacia Biotech) following the manufacturer's recommended protocol.

Northern blotting

Total RNA was isolated from cultures harbouring p19Kpro or p19Kpro-PknA plasmid by the hot phenol extraction method [23]. For Northern-blot analysis, RNA samples were electrophoresed on 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane. The membrane was UV cross-linked and then hybridized with [α-^{32}P]CTP-labelled *pknA* as a probe following the standard protocol [21].

Scanning electron microscopy

Overnight cultures (*E. coli* strain DH5α transformed with p19Kpro, p19Kpro-PknA, p19Kpro-aPknA or p19Kpro-K42N) were reinoculated such that initial A_{600} was 0.05 and grown for a further 12 h. After harvesting, cells were washed three times with ice-cold $NaCl/P_i$. The cells were then resuspended in $NaCl/P_i$, adhered to coverslips that had been coated with 0.1% poly(L-lysine). Adherent cells were washed with $NaCl/P_i$ and then dehydrated using an ascending series of ethanol incubations (30 min each step). Finally, cells on coverslips were infiltrated with *t*-butyl alcohol and freeze-dried in a lyophilizer [24]. Dried samples were sputter-coated with gold/palladium and then observed under a scanning electron microscope.

Bioinformatic analysis

Nucleotide-derived amino-acid sequences were compared with 'nr database' in the PSI-BLAST program using the mail server at NIH. The multiple sequence alignments of the retrieved sequences were carried out using the CLUSTAL W 1.74 program [25]. The gap opening and extension penalties of 10 and 0.05, respectively, were used during the alignments. The multiple sequence alignments for generating the phylogenetic tree were performed by excluding highly variable N-terminal and C-terminal stretches of the sequences. The tree was constructed after 100 cycles of bootstrapping using PROTDIST, UPGMA and CONSENSE programs, which are available at the PHYLIP site [26], and was drawn with TREEVIEW [27].

RESULTS AND DISCUSSION

Analysis of the *M. tuberculosis* genome sequence revealed the presence of 11 eukaryotic-type Ser/Thr kinases [13]. However, so far the functions of such a large number of regulatory proteins in this intracellular facultative pathogen have not been elucidated. As the focus in the postgenomic era has been characterization of individual genes deduced from the genome for biological understanding of an organism, we concentrated on one such homologue of mycobacterial Ser/Thr kinases, *pknA*. It is located adjacent to genes encoding bacterial morphogenic proteins, which seems to be unique among prokaryotes [17] and therefore demands special attention.

We decided to amplify *pknA* from *M. tuberculosis* strain H37Ra by PCR. The primers were designed from the published *M. tuberculosis* H37Rv genome sequence [13] of *pknA* (Rv0015c). PCR at an annealing temperature of 50 °C with primers CC7 and CC8 and genomic DNA from *M. tuberculosis* H37Ra resulted in amplification of the expected ≈ 1.3-kb fragment. Only reaction mixtures that contained template DNA, primers and enzymes showed the amplification (data not shown). Sequencing of this ≈ 1.3-kb fragment (exactly 1293 bp or 431 amino acids) after cloning in pUC19 indicated 100% identity at the nucleotide level with the published *pknA* sequence of the pathogenic strain, H37Rv, of *M. tuberculosis*. This observation possibly exclude its direct association in pathogenicity/virulence. Southern-blot analysis using *pknA* as a probe revealed the presence of a similar gene in *Mycobacterium bovis* BCG but not in a saprophyte such as *Mycobacterium smegmatis* (data not shown).

PknA fused with MBP was expressed after subcloning in pMAL-c2X. SDS/PAGE analysis of the cell lysate prepared from *E. coli* strain TB1 harbouring plasmid pMAL-PknA indicated expression of at least three different bands (≈ 97, ≈ 70 and ≈ 42 kDa) after IPTG induction (Fig. 1A,
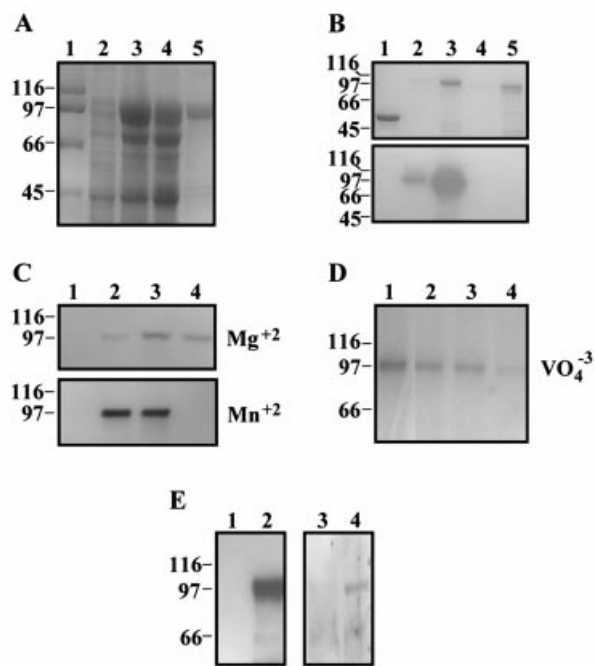
Fig. 1. MBP–PknA fusion protein has autophosphorylating ability.
(A) Expression and purification of MBP–PknA fusion protein. Protein samples at various stages of purification were subjected to SDS/PAGE (8% gel) followed by Coomassie Brilliant Blue staining. Lane 1, molecular mass marker; lane 2, uninduced lysate; lane 3, induced lysate; lane 4, soluble fraction; lane 5, amylose resin-purified fusion protein. (B) *In vitro* kinase assay with the purified fusion protein; 6 µg MBP–βgal control (lane 1), 800 ng (lane 2) and 6 µg (lane 3) MBP–PknA, 800 ng (lane 4) and 6 µg (lane 5) MBP–K42N mutant protein after Coomassie Brilliant Blue staining (upper panel) or γ-^{32}P labelling (lower panel). (C) Effect of bivalent cations on the autophosphorylation of PknA. *In vitro* kinase assays were carried out in the presence of 0 (lane 1), 1 (lane 2), 10 (lane 3) and 100 (lane 4) mM Mg^{2+} (upper panel) or Mn^{2+} (lower panel). (D) Effect of sodium orthovanadate on the enzyme activity. MBP–PknA fusion protein samples were pre-incubated for 15 min at room temperature with 0 (lane 1), 0.5 (lane 2), 1 (lane 3) and 2.5 (lane 4) mM sodium orthovanadate and then assayed for phosphorylation activity. (E) Phosphoamino-acid analysis of PknA. MBP–βgal control (lanes 1 and 3) and MBP–PknA fusion protein (lanes 2 and 4) after Western-blot analysis with antibodies to phosphothreonine (left panel) and phosphoserine (right panel). Numbers denote size of the molecular mass standards.

compare lanes 2 and 3). All these induced proteins were found in the soluble fraction (Fig. 1A, lane 4). Subsequent affinity purification of the soluble proteins revealed binding of only the one of molecular mass 97.1 ± 1.3 kDa (mean ± SD, $n = 4$) on amylose resin (Fig. 1A, lane 5). The expression was further confirmed by Western-blot analysis with the antibody to MBP (data not shown). However, the molecular mass of the purified fusion protein was higher than that of the one predicted from the sequence ($\approx$ 88.7 kDa). This anomalous migration is not unusual as it has already been reported that the autophosphorylating proteins may show slower mobility on SDS/PAGE analysis [28]. In fact a kinase-deficient variant of PknA was found to run at 89.3 ± 6.8 kDa (mean ± SD, $n = 6$) on SDS/PAGE (Fig. 1B, upper panel; compare lanes 3 and 5).

Moreover, migration of a protein on SDS/PAGE has often been correlated with the number of proline residues present. Interestingly, comparison of the nucleotide-derived amino-acid sequence of PknA revealed the proline content to be 10.4% of total molecular mass, which is comparable to that of other proteins that showed such anomalous mobility [28].

The autophosphorylating ability of the fusion protein was monitored by incubating it with $[\gamma$-^{32}P]ATP in the presence of Mn^{2+}, followed by separation of reaction products by SDS/PAGE. Finally, the labelled protein was identified by autoradiography of dried gel. *In vitro* kinase assays revealed that MBP–PknA fusion protein is capable of phosphorylating in a concentration-dependent manner. On the other hand, neither MBP nor MBP–K42N showed any labelling (Fig. 1B). Thus, lysine at residue 42 in subdomain II is essential for catalyzing the phosphorylation reaction. This result is in agreement with those for known Ser/Thr kinases [3]. Autophosphorylation of the $\approx$ 97-kDa band could not be seen when boiled protein was used in the kinase assays (data not shown and also see below Fig. 2A, lanes 3 and 7 or Fig. 2B, lane 5). Incorporation of γ-^{32}P from ATP to the fusion protein occurred by 20 min (data not shown).

To investigate whether bivalent cations have an effect on the autophosphorylation of PknA, *in vitro* kinase assays were carried out in the presence and absence of Mg^{2+} or Mn^{2+}. As shown in Fig. 1C, phosphorylation is only detectable in the presence of either Mg^{2+} or Mn^{2+} (compare lanes 1 and 2). Compared with a concentration of 1 mM, 10 mM Mg^{2+} produced an approximately fivefold increase in autophosphorylation of PknA (Fig. 1C, upper panel). The autophosphorylating ability of PknA was also augmented up to a concentration of 10 mM Mn^{2+} (Fig. 1C, lower panel). However, both Mg^{2+} and Mn^{2+} had an inhibitory effect on enzyme activity at higher concentrations (Fig. 1C). Interestingly, it seems that PknA is distinct from one of its homologues, PknD, for which Mg^{2+} did not influence the enzyme activity [14]. Furthermore, bivalent cations such as Ca^{2+} in the presence of Mn^{2+} did not affect autophosphorylation of PknA (data not shown), which is in contrast with PknD, for which it did have an inhibitory effect on the *in vitro* kinase activity [14].

The literature indicates that vanadate being a phosphate analogue binds to a large number of phosphotransferases and phosphohydrolases and thus specifically inhibits phosphoryl-transfer reactions [29]. The effect of sodium ortho-vanadate on *in vitro* protein phosphorylation was therefore assessed. Preincubation (15 min at room temperature) of vanadate (0.5–2.5 mM) with the fusion protein inhibited its ability to incorporate γ-^{32}P (Fig. 1D). This inhibition by vanadate is specific because another oxyanion, tungstate, did not have any effect on phosphorylation of PknA (data not shown).

The autophosphorylating amino acids in PknA were identified by immunoblot analysis using specific antibodies against phosphoserine and phosphothreonine. Both antibodies recognized PknA, suggesting that the phosphorylated residues are serine and threonine (Fig. 1E, lanes 2 and 4). However, both antisera do not recognize PknA equally, as phosphorylation of threonine was more than that of serine (Fig. 1E, compare lanes 2 and 4). This observation does not seem to be unusual as PknD, another Ser/Thr kinase from *M. tuberculosis*, mainly phosphorylated at
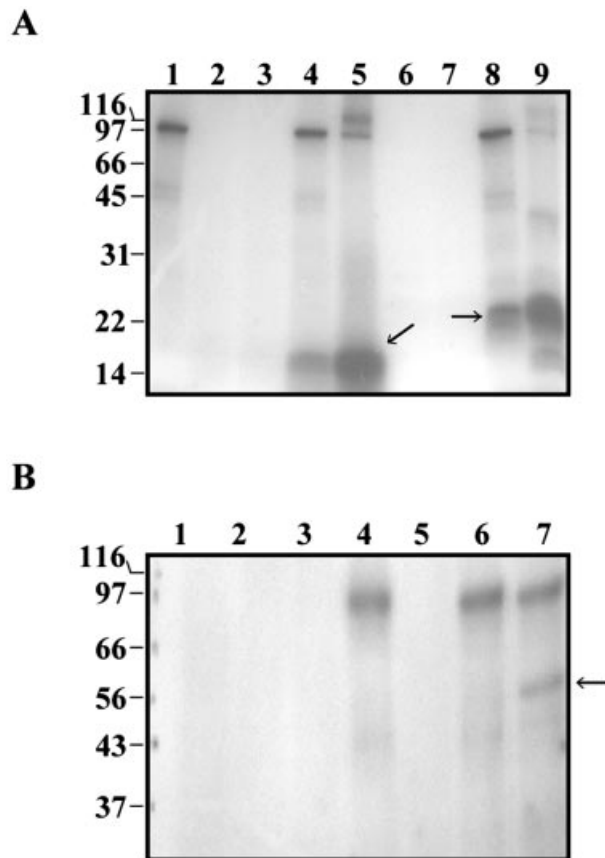
Fig. 2. Substrate phosphorylation by PknA. (A) Phosphorylation of exogenous substrates. *In vitro* kinase assays were carried out as described in Materials and methods. Lane 1, MBP–PknA; lane 2, histone (50 µg); lane 3, histone (50 µg) with boiled MBP–PknA; lane 4, histone (1 µg) with MBP–PknA; lane 5, histone (50 µg) with MBP–PknA; lane 6, myelin basic protein (50 µg); lane 7, myelin basic protein (50 µg) with boiled MBP–PknA; lane 8, myelin basic protein (1 µg) with MBP–PknA; lane 9, myelin basic protein (50 µg) with MBP–PknA. The positions of phosphorylated exogenous substrates are indicated by arrows. (B) Phosphorylation of soluble protein of *E. coli* by PknA. MBP–βgal or MBP–PknA (100 µg) was immobilized on amylose resin and incubated with crude soluble protein extracts of *E. coli* strain DH5α (250 µg) for 10 h at 4 °C. *In vitro* kinase assays were carried out with aliquots (12 µL) of washed amylose beads suspended in buffer as described in Materials and methods. Lane 1, resin only; lane 2, resin incubated with crude soluble protein extracts of *E. coli*; lane 3, resin incubated with MBP–βGal and crude soluble protein extracts of *E. coli*; lane 4, resin incubated with MBP–PknA; lane 5, resin incubated with boiled MBP–PknA and crude soluble protein extracts of *E. coli*; lane 6, resin incubated with MBP–PknA and boiled crude soluble protein extracts of *E. coli*; lane 7, resin incubated with MBP–PknA and crude soluble protein extracts of *E. coli*. The position of the ≈ 56-kDa band is indicated by an arrow. The numbers denote the size of molecular mass markers.
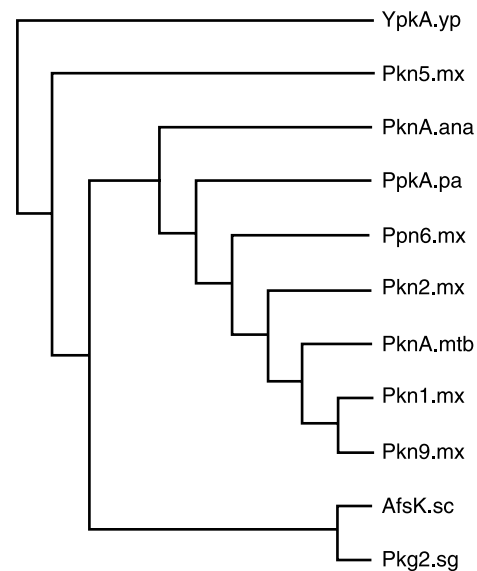

Fig. 3. Dendrogram exhibiting the phylogenetic placement of PknA from *M. tuberculosis* with respect to other bacterial Ser/Thr kinases with known function. Criteria for the selection of these bacterial Ser/Thr kinases and procedure for the generation of the phylogenetic tree are described in the text. Abbreviations used: PknA.mtb, PknA from *M. tuberculosis* [13]; Pkn1.mx, Pkn1 [3], Pkn2.mx, Pkn2 [4], Pkn5.mx, Pkn5 [5], Pkn6.mx, Pkn6 [5] and Pkn9.mx, Pkn9 [6] from *M. xanthus*; AfsK.sc, AfsK from *Streptomyces coelicolor* [8]; Pkg2.sg, Pkg2 from *Streptomyces granaticolor* [9]; PpkA.pa, PpkA from *P. aeruginosa* [31]; PknA.ana, PknA from *Anabaena* [7]; YpkA.yp, YpkA from *Y. pseudotuberculosis* [10].

$[\gamma^{-32}P]ATP$ and either histone or myelin basic protein. The reaction products were subjected to SDS/PAGE (12.5% gel), gels were dried, and labelled proteins were identified by autoradiography. As shown in Fig. 2A, in addition to an autophosphorylating band of MBP–PknA at ≈ 97 kDa, substrate phosphorylation was also observed (lanes 4, 5, 8 and 9). In contrast, exogenous substrates alone showed negligible phosphorylation (Fig. 2A, lanes 2 and 6). Even in the presence of boiled fusion protein, phosphorylation of histone/myelin basic protein could not be seen (Fig. 2A, lanes 3 and 7).

To elucidate the possibility of its interaction with unknown protein(s), the soluble fraction of cell lysates from *E. coli* strain DH5α was incubated for 10 h at 4 °C with MBP–PknA fusion protein that was immobilized on amylose resin. *In vitro* kinase assays with aliquots of the resin after thorough washing indicated the phosphorylation of a 56.36 ± 0.83 kDa (mean ± SD, $n = 3$) protein in addition to the ≈ 97-kDa autophosphorylating MBP–PknA (Fig. 2B, lane 7). The MBP–PknA-immobilized amylose resin when incubated with or without boiled lysate showed the phosphorylation of only the ≈ 97-kDa fusion protein (Fig. 2B, lanes 4 and 6). This ≈ 56-kDa band did not seem to be an experimental artifact, because it was absent from the controls (resin only, resin with either lysate or MBP–βgal and lysate) used in the assay. Furthermore, immobilization of the boiled MBP–PknA on amylose resin followed by incubation with the lysate neither showed autophosphorylation of the fusion protein nor highlighted
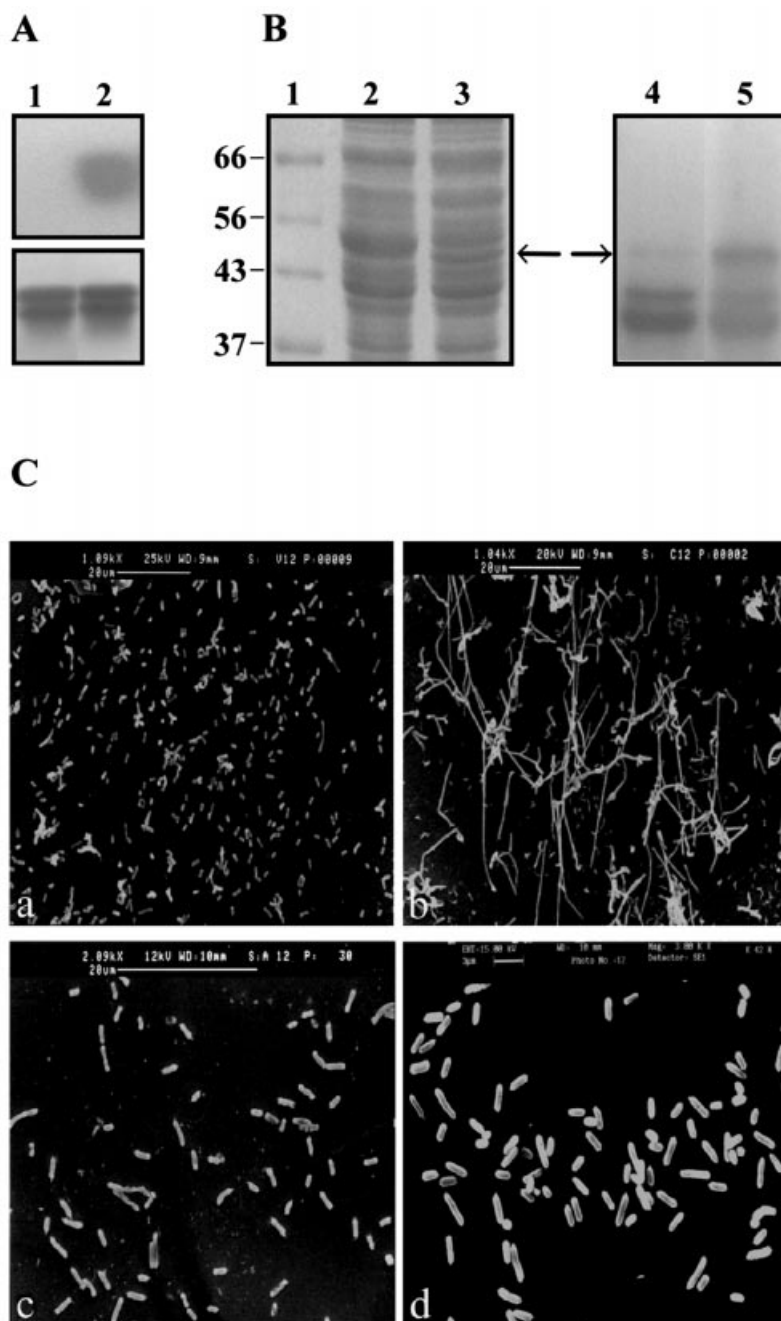
threonine [14]. On the other hand, no specific signal was obtained in Western blots using antibody to phosphotyrosine (data not shown).

The ability of PknA to phosphorylate known exogenous substrates was also examined. Purified MBP–PknA fusion protein was added to reaction mixtures containing

Fig. 4. Effect of constitutive expression of PknA on the morphology of *E. coli* cells. (A) Northern-blot analysis indicating constitutive expression of *pknA* in *E. coli* at the mRNA level. Total RNA was isolated from *E. coli* DH5α cells transformed with either p19Kpro (lane 1) or p19Kpro-PknA (lane 2), electrophoresed on 1.2% agarose gel containing formaldehyde, transferred on to a nylon membrane, and processed as described in the text. Upper panel: the blot after hybridization using [α-^{32}P]CTP-labelled *pknA* as the probe. Lower panel: the same blot after methylene blue staining, serving as a loading control. (B) Expression of the ≈ 45-kDa PknA protein which is able to autophosphorylate. Soluble fractions of crude lysates of *E. coli* DH5α cells transformed with either p19Kpro vector or p19Kpro-PknA were subjected to SDS/PAGE and Coomassie Brilliant Blue staining (left panel). *In vitro* kinase assay was carried out with the same lysate as described in Materials and methods (right panel). Lane 1, Molecular mass marker; lanes 2 and 4, p19Kpro; lanes 3 and 5, p19Kpro-PknA. Numbers denote size of the molecular mass standards, and arrows indicate the position of the constitutively expressed PknA protein with autophosphorylating ability. (C) Phenotypic alteration of *E. coli* strain DH5α after expression of PknA. The morphology of the cells was determined by scanning electron microscopy as described in the text. Panels a–d: *E. coli* DH5α cells transformed with p19Kpro (a), p19Kpro-PknA (b), p19Kpro-aPknA (c), or p19Kpro-K42N (d). The bar in each panel indicates magnification.

phosphorylation of the ≈ 56-kDa band (Fig. 2B, lane 5). Thus our results indicate that at least a ≈ 56-kDa soluble protein of *E. coli* interacts with PknA.

Bacterial Ser/Thr kinases characterized so far have been shown to be involved in different processes, namely regulation of development, stress responses, and pathogenicity

[3–10,30,31]. To relate PknA to other bacterial Ser/Thr kinases for which functions have already been assigned, we carried out sequence database comparisons using BLAST and PSI-BLAST programs. Nine different bacterial Ser/Thr kinase sequences were retrieved through these searches; the homology score varied from 80 to 162 with expected values of between e^{-15} and e^{-39}. In contrast, YpkA, a Ser/Thr kinase from *Y. pseudotuberculosis* known to be associated with virulence [10], showed insignificant homology (score = 39.9, expected value = 0.054). In a phylogenetic tree generated by multiple sequence alignment of different bacterial Ser/Thr kinases excluding highly variable N-termini and C-termini, PknA is found to be very close to Pkn1 and Pkn9 of *Myxococcus xanthus* (Fig. 3). As these kinases, are involved in sporulation or cell division/differentiation, it seems likely that PknA has similar functions.

In the *M. tuberculosis* genome, *pknA* (Rv0015c) is located adjacent to *pbpA* (Rv0016c) and *rodA* (Rv0017c) genes, which encode putative morphogenic proteins belonging to the SEDS (shape, elongation, division and sporulation) family [32]. Members of this family of proteins have been reported to be present in all eubacteria in which a constituent of the cell envelope is peptidoglycan. These proteins are known to be involved in controlling cell shape and peptidoglycan synthesis in bacteria such as *Bacillus subtilis* [32] and *E. coli* [33]. Thus the presence of a kinase at this location in the genome suggests a regulatory role in mycobacterial cell division.

Alteration in cell shape is the initial event in bacterial cell division which involves ordered assembly of proteins [34,35]. These proteins are fairly conserved among different prokaryotes. This is evident from the fact that a ≈ 56-kDa soluble protein of *E. coli* interacted with the mycobacterial PknA (Fig. 2B). In a preliminary study, we observed that pMAL-PknA-transformed cells of *E. coli* (strain TB1) grown for 2–10 h after IPTG induction exhibited an unusual elongation pattern compared with that of the cells harbouring only the pMAL-c2X plasmid. To investigate further the involvement of PknA in this process, we sought to express the protein constitutively in the *E. coli* host strain DH5α using a low-copy vector. However, expression of mycobacterial protein in *E. coli* is known to be difficult, especially under the control of a heterologous promoter [36]. We therefore used a *Mycobacterium–E. coli* shuttle vector p19Kpro, derived from p16R1 [22] containing a mycobacterial 19-kDa antigen promoter. These series of vectors are known to elicit a low level of mycobacterial gene expression in *E. coli* [36]. *pknA* was cloned in p19Kpro, and, after transformation in *E. coli*, its expression was monitored at the mRNA and protein levels. Northern-blot analysis of total RNA extracted from cells transformed with either p19Kpro (vector) or p19Kpro-PknA using *pknA* as a probe confirmed expression of the kinase at the mRNA level (Fig. 4A, upper panel, compare lanes 1 and 2). The constitutive expression of PknA at the protein level was also evident from the expected ≈ 45-kDa band on SDS/PAGE after Coomassie Brilliant Blue staining (Fig. 4B, left panel, compare lanes 2 and 3). The protein was found in the soluble fraction. *In vitro* kinase assay of crude cell extracts indicated autophosphorylating ability of the expressed protein (Fig. 4B, right panel, compare lanes 4 and 5). The effect of constitutive expression of *pknA* on the phenotype of the *E. coli* cells was evaluated by scanning electron microscopy. As shown in Fig. 4C, *E. coli* strain DH5α transformed with p19Kpro (panel 'a') were normal rods of size 1–2 μm. On the other hand, *E. coli* cells transformed with p19Kpro-PknA (panel 'b') showed remarkable elongation (more than 95% of the cells were in the range 60–70 μm). Interestingly, *E. coli* transformed with either the antisense construct, p19Kpro-aPknA (panel 'c') or the kinase-deficient mutant, p19Kpro-K42N (panel 'd') did not show such phenotypic alteration. Furthermore, cell elongation did not seem to result in any toxicity from 'out of context' expression of the mycobacterial gene as experimental and control growth curves were similar (data not shown). There are, in fact, examples of mycobacterial gene expression using *E. coli* as a host [16]. Thus, all these lines of evidence convincingly establish the participation of mycobacterial PknA in regulating morphological changes associated with cell division.

Finally, our study in a heterologous setting has shown the involvement of PknA in cell shape regulation; it is the first report describing the functionality of any eukaryotic-type Ser/Thr kinase from *M. tuberculosis*. Identification of the natural substrate of PknA in mycobacteria would aid progress towards its utilization as a drug target, which is a top priority in this era of bacterial drug resistance.

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