

Expression and Characterization of the *Mycobacterium tuberculosis* Serine/Threonine Protein Kinase PknB

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PknB is a member of the newly discovered eukaryotic-like protein serine/threonine kinase (PSTK) family of proteins. The *pknB* gene was cloned and expressed in *Escherichia coli*. The active recombinant protein was purified and shown to be reactive with antiphosphoserine antibodies, as well as with antibodies to the phosphorylated eukaryotic Ser/Thr kinases mitogen-activated protein kinase kinase 3 and 6, P38, and Creb. In vitro kinase assays demonstrated that PknB is a functional kinase that is autophosphorylated on serine/threonine residues and is also able to phosphorylate the peptide substrate myelin basic protein. Analysis of *pknB* expression in *Mycobacterium tuberculosis* indicates the presence of *pknB* mRNA in (i) organisms grown in vitro in bacteriological media, (ii) a murine macrophage in vitro infection model, and (iii) in vivo alveolar macrophages from a patient with tuberculosis.

Tuberculosis is the primary cause of mortality due to an infectious disease in the world today. The causative agent of tuberculosis is the intracellular pathogen *Mycobacterium tuberculosis*. The ability of *M. tuberculosis* to enter macrophages and to avoid intracellular killing is believed to be pivotal to its virulence. The host immune response to tuberculosis is complex and involves T cells, mononuclear phagocytes, and cytokines (21). Mycobacteria have been suggested to have the ability to subvert normal host immune response mechanisms in order to enhance their intracellular survival. For example, there is evidence to indicate that mycobacteria prevent macrophage acidification (29, 34), inhibit antigen processing (23), and attenuate gamma interferon action (31) and protein kinase C activation in the macrophage (5). These macrophage deactivation mechanisms are considered to be survival strategies of mycobacteria within the host. Induction of these evasive mechanisms in *M. tuberculosis* most probably involves the ability of the organism to adapt its responses to external signals.

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. 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 (33). In contrast, protein phosphorylation in eukaryotes occurs mainly on phosphoester (serine, threonine, or tyrosine) residues. The eukaryotic protein kinases and protein phosphatases are the backbone of signal transduction pathways. Phosphoester protein kinases and their coupled phosphatases were previously thought to be unique to eukaryotes. Recently, evidence arising from the accumulation of bacterial genome sequencing data and the use of antiphosphoprotein antibodies has revealed that some prokaryotes also contain phosphoester kinases and phosphatases (7, 28, 40).

In prokaryotes, protein serine/threonine kinases (PSTKs) have been shown to be involved in two different processes, development and pathogenicity. Bacteria capable of differentiation into a new developmental state, including *Streptomyces* (19, 25, 37) and *Anabaena* (40–42) spp. and *Myxococcus xanthus* (13, 35, 36, 43), 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* plasmid-encoded protein kinase *yopO* (12) or the *Pseudomonas aeruginosa* PSTK (39). Interestingly, both of 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 (3). Furthermore, we have demonstrated that six proteins are phosphorylated in vitro (3), suggesting the presence of functional kinases in *M. tuberculosis*. The completion of the *M. tuberculosis* genome sequencing project provided a complete list of these eukaryotic-like protein kinases and phosphatases that now form the *M. tuberculosis* PSTK family (6). As of today, this family is composed of at least 11 protein kinases and four protein phosphatases. The *M. tuberculosis* PSTK *pknD* gene has previously been cloned and analyzed and shown to encode a functional serine/threonine kinase (27). This paper describes the cloning, expression, and molecular characterization of PknB, a putative *M. tuberculosis* protein serine/threonine kinase encoded by an open reading frame that resides near the origin of replication. The data shows that *pknB* encodes a functional kinase that is constitutively transcribed in *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture conditions. *M. tuberculosis* H37Rv NCTC 7416 was obtained from the National Collection of Type Cultures, London, United Kingdom. *Escherichia coli* DH5 α (Clontech Laboratories, Inc., Palo Alto, Calif.) and *E. coli* BL21(DE3) (Novagen R & D) were used for maintenance of plasmids and expression of foreign proteins, respectively. The plasmid pET-22b (Novagen) was used as an expression vector in *E. coli* BL21(DE3). *E. coli* strains were cultured on Luria-Bertani (LB) agar or broth with or without selective antibiotics. Mycobacterial strains were cultured in Middlebrook 7H9 broth or 7H10 agar (Difco) supplemented with OADC (Difco), Tween 80, and glycerol.

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Amplification and cloning of *pknB*. Genomic DNA of *M. tuberculosis* H37Rv was prepared as described previously (3). The open reading frame Rv 0014c which codes for PknB was amplified from this DNA with the following primers: 1, 5'-AAATACATATGACCAACCCCTTCCCA-3'; and 2, 5'-TTAAGCTTCTA CTGGCCGAACCTCA-3'. Primers 1 and 2 contained *NdeI* and *HindIII* restriction sites, respectively. PCR was performed with *Taq* polymerase obtained from Gibco BRL by using 2 mM MgCl₂ and 5% dimethyl sulfoxide. Annealing temperatures were 58 and 63°C. The PCR products were separated on a 1% agarose gel. The appropriate PCR product was ligated into the vector pCR2.1 of the TA cloning kit (Invitrogen) and transformed into *E. coli* DH5 α or INVF α by standard chemical transformation procedure. Clones containing the vector were selected on LB-plus-ampicillin (100 μ g/ml) plates, and plasmid DNA was digested with restriction endonucleases *NdeI* and *HindIII* (Fermentas). Restriction enzyme-digested plasmids were isolated with a QIAquick gel extraction kit (Qiagen Ltd.). A corresponding digestion was also applied to plasmid pET-22b, and the two products were ligated together with T4 DNA ligase to obtain the plasmid pYA102 (Fig. 1).

Expression and purification of PknB. Competent cells of *E. coli* BL21(DE3) were prepared according to the CaCl₂ method (30) and were transformed by the heat shock method for 2 min at 42°C with 100 ng of pYA102. The transformed *E. coli* cells were then plated onto LB agar supplemented with ampicillin (100 μ g/ml). Single colonies were inoculated into 5 ml of LB broth also containing ampicillin (100 μ g/ml). After overnight incubation at 37°C with shaking, the individual cultures were diluted 1:100 in the same medium and incubation was continued at 37°C with shaking. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM when the optical density at 600 nm reached 0.6. Cultures were centrifuged at 5,000 \times g for 15 min at room temperature, and pellets were lysed in B-Per (Pierce) bacterial protein extraction reagent. Proteins were separated by centrifugation (15,000 \times g, 4°C, 15 min) into soluble and insoluble fractions. PknB inclusion bodies contained in the insoluble fractions were purified from *E. coli* membrane proteins by washing in a solution of 10% B-Per reagent and centrifugation (45,000 \times g, 90 min, 4°C). PknB was separated by sodium dodecyl sulfate-7.5% polyacrylamide electrophoresis (SDS-PAGE) and stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The N-terminal amino acid sequence was verified after electrophoresis of samples in SDS-PAGE gels and electroblotting onto PVDF membranes. Edman degradation was performed, and the sequence of the first 10 amino acids from the NH₂ terminus was determined at the University of British Columbia Protein Sequencing Laboratory. In order to obtain soluble protein, PknB inclusion bodies were resuspended in 1 \times phosphate-buffered saline (PBS) (pH 7.4) and slowly added drop-wise to a solution of 16 M urea and 2 M dithiothreitol (DTT) to make a final concentration of 8 M urea and 1 M DTT. Soluble PknB was then dialyzed via a Spectra/Por 8000 cellulose membrane (VWR Scientific) against 200 volumes of 1 \times Tris-buffered saline (pH 7.4) at 4°C for 16 to 24 h. The sample was then centrifuged for 15 min at 4°C and 15,000 \times g (Baxter), and approximately 20 mg of protein was loaded onto a 50-ml Macro-Prep SE agarose size-exclusion column (Bio-Rad), which was used as a desalting column. Proteins were eluted over time at 4°C with a size-exclusion buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 0.01 mM EDTA (18). The purity of PknB was tested by subjecting samples to SDS-PAGE followed by Coomassie blue staining. SDS-PAGE gels were prepared by the method of Laemmli (17). The gels were stained with Coomassie blue R-250 or silver stain. Protein concentrations were determined by the Bradford protein assay reagent (Bio-Rad).

Production of polyclonal antiserum to PknB. The gel electrophoresis band corresponding to PknB was excised from an SDS-7.5% PAGE gel and homogenized in PBS. Homogenized gels were mixed with Titremax adjuvant (1:1 [vol/vol]) and were injected subcutaneously into 8-week-old BALB/c mice. Two weeks after the immunization, the animals were bled and sera were prepared. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibodies and enhanced chemiluminescence reagents were used to detect antibodies bound in Western blots.

In vitro kinase assay. PknB autophosphorylation and myelin basic protein (MBP) phosphorylation were determined by an in vitro kinase assay. Twenty-microgram samples of PknB protein were added to 15 μ l of kinase buffer {20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 7.2], 10 mM MgCl₂, 10 mM MnCl₂} with or without 50 μ g of MBP (Sigma), and the reaction was started by addition of 1 μ Ci of [γ -³²P]ATP (Mandel Scientific). Incubation was performed at room temperature. One-third of the incubation mixture was then loaded onto P81 phosphocellulose filter paper (Baxter) for incorporation measurements, and to the remainder 3 \times Laemmli sample buffer was added to stop the reaction. The latter mixture was boiled for 5 min and resolved by SDS-PAGE. The gels were electroblotted onto nitrocellulose or PVDF membranes (Bio-Rad) and then exposed to Kodak X-Omat/AR film. Radioisotope levels in filter paper assays were determined by scintillation counting (Beckman LS 1800).

Phosphoamino acid analysis, Western blotting, and cross-reactivity with anti-eukaryotic PSTKs. Autophosphorylated PknB was excised from PVDF membranes and subjected to acid hydrolysis as described by Kamps and Sefton (15). Samples were spotted onto a cellulose thin-layer chromatography plate (Eastman, Rochester, N.Y.) and subjected to two-dimensional liquid thin-layer chromatography. Control phosphotyrosine, phosphothreonine, and phosphoserine amino acids were stained with ninhydrin, and radiolabelled amino acids were

visualized via autoradiography. Phosphoamino acid analysis was also performed by loading PknB on SDS-PAGE gels followed by Western blot analysis with antiphosphoserine and antiphosphothreonine monoclonal antibodies (Sigma) as described in the manufacturer's instructions. Soluble PknB was electrophoresed on an SDS-7.5% PAGE gel and transferred to nitrocellulose by semidry electroblotting (Ancos). Blots were blocked with 4% skim milk (Difco, Detroit, Mich.) in 1 \times PBS (pH 7.5) overnight at 4°C on a shaker. Blots were washed with 1 \times PBS-0.1% Tween 20 and incubated for 2 h in 1 \times PBS-Tween (pH 7.5) with either mouse monoclonal antiphosphoserine (1/500) (Sigma), mouse monoclonal antiphosphothreonine (1/100) (Sigma), rabbit antiphospho Creb Ser-133 (1/10,000) (New England Biolabs), rabbit antiphospho p38 Tyr-182 (1/10,000) (New England Biolabs), rabbit antiphospho mitogen-activated protein kinase kinase 3 and 6 (MKK3/6) Ser 189-202 (1/10,000), or rabbit anti-ERK1 (1/10,000) (Kinetek Pharmaceuticals, Vancouver, British Columbia, Canada). Blots were washed for 45 min in 1 \times PBS-Tween and incubated with 1/20,000 of either goat anti-rabbit IgG (heavy plus light chain) or goat anti-mouse IgG (heavy plus light chain) HRP-conjugated antibody (Bio-Rad). Blots were incubated in Super Signal reagent (Pierce, Rockford, Ill.) and exposed by using Kodak X-Omat/AR film.

Murine macrophage infection. The mouse macrophage cell line J774.2 (American Type Culture Collection) was seeded and maintained in 100-mm-diameter tissue culture plates in RPMI 1640 medium (Gibco) containing 10 mM HEPES, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS). For reverse transcription (RT)-PCR analysis, approximately 10⁷ cells of *M. tuberculosis* H37Rv cultured in RPMI 1640 medium supplemented with 5% FCS were added to each J774.2 plate and incubated for 24 h. After 24 h, the cells were washed twice with warm RPMI 1640 medium containing 1% FCS. Total RNA was isolated after 24 and 72 h.

Isolation of human alveolar macrophages. Alveolar macrophages were obtained from bronchoalveolar lavage fluid from resected human lungs. Bronchoalveolar lavage fluid was filtered through sterile gauze and centrifuged at 450 \times g at room temperature for 7 min. Erythrocytes were lysed by treatment with distilled water, and cells were washed twice with sterile 1 \times PBS. Cells were resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS. Alveolar macrophages were plated in 60-mm-diameter tissue culture plates at 2 \times 10⁶ cells/ml. Cells were allowed to adhere overnight at 37°C. Nonadherent cells were removed by gently rinsing the plates with warmed RPMI 1640 medium supplemented with 5% FCS. The protocol for using human biological samples was approved by the University of British Columbia Ethics Committee.

cDNA preparation and RT-PCR. RNA was prepared in a P3-level laboratory as follows. Exponentially growing *M. tuberculosis* (H37Rv), J774.2 cells infected with *M. tuberculosis* or adherent alveolar macrophages from BAL fluid, were harvested by centrifugation at 3,000 \times g for 10 min. Pellets were resuspended in 2 ml of Tween-saline (0.8% [wt/vol] NaCl, 0.05% [vol/vol] Tween 80) followed by centrifugation at 17,000 \times g for 1 min. The mycobacterial pellets were resuspended in 1 ml of guanidinium isothiocyanate buffer (5 M guanidinium isothiocyanate, 25 mM sodium acetate [pH 6.0], 1% *N*-lauryl sarcosine, 10 mM DTT), and approximately 1 ml of 0.1-mm-diameter zirconium beads was added. Mycobacteria were then subjected to disruption in a bead-beater device for 3 min. Nucleic acid was prepared from the upper aqueous lysate by a series of chloroform and phenol-chloroform-isoamyl alcohol extractions followed by ethanol precipitation. To remove DNA template, RNA was treated with RNase-free DNase (RQ1; Promega) twice at a concentration of 1 U/ μ g of RNA. DNase was removed by phenol-chloroform-isoamyl alcohol extraction, followed by extraction with chloroform and ethanol precipitation. RNA pellets were resuspended in diethylpyrocarbonate-treated water, and the absence of DNA contamination was confirmed by PCR with specific primers for *pknB*. RNA concentrations were determined by measuring absorbance at 260 nm. Total RNA was heated at 94°C for 3 min before being cooled on ice for 5 min. RNA was reverse transcribed by adding 1 \times PCR buffer, 1.5 mM MgCl₂, 200 μ M each of the four deoxynucleotide triphosphates, 5 nM random hexamers or specific downstream nucleotides, and 50 U of murine leukemia virus reverse transcriptase (Perkin-Elmer). RNA was reverse transcribed at 42°C for 3 h. Murine leukemia virus was inactivated by incubation at 99°C for 3 min. Amplified products were produced routinely with either 20 ng of genomic *M. tuberculosis* DNA, as a positive control, or 5 to 10 ng of cDNA which was added to 20 μ l containing 1 \times PCR buffer, 200 μ M deoxynucleotide triphosphates, 4% dimethyl sulfoxide, 200 pmol of each primer, and 1 U of *Taq* polymerase (Fermentas). The primers for RT-PCR analysis were the same primers used for the gene cloning. The amplification program consisted of preamplification denaturation at 95°C for 3 min, followed by 35 cycles of 60 s of denaturation at 94°C, 60 s of annealing at 58°C, and a 90-s extension at 72°C. Ten percent of the reaction product was run on a 1% agarose gel and visualized by staining with ethidium bromide.

RESULTS

Cloning and expression of the *pknB* gene. Because of the minute quantities of regulatory proteins in bacterial cells and the large volumes of pathogenic *M. tuberculosis* culture needed for purification of proteins, a recombinant expression ap-

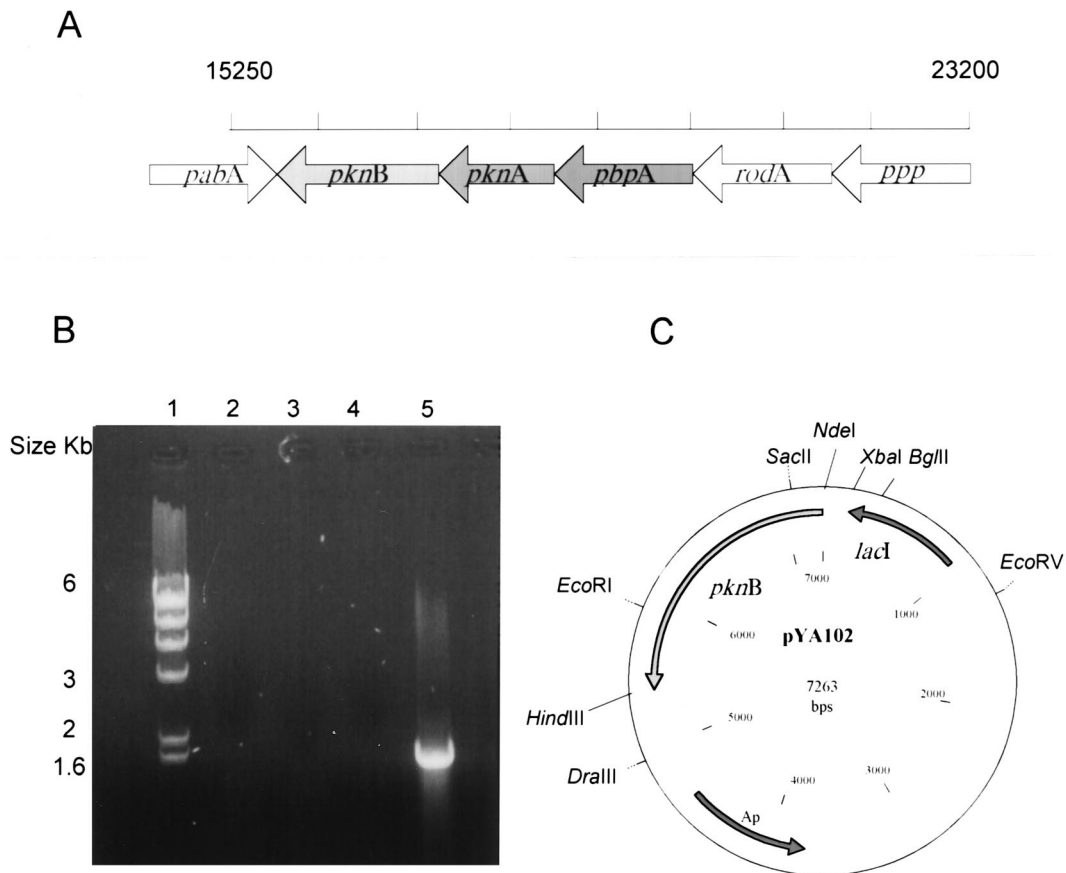


FIG. 1. Chromosomal location and cloning of PknB. (A) Schematic map describing the chromosomal location of the *pknB* open reading frame. (B) PCR amplification of *pknB* by using gene-specific primers as described in Materials and Methods. Lanes: 1, lambda *Hind*III molecular size markers; 2, PCR without 5' primer; 3, PCR without 3' primer; 4, PCR without template DNA; 5, complete reaction using *M. tuberculosis* H37Rv DNA as the template. (C) The expression plasmid pYA102.

proach was taken to characterize PknB at the biochemical level. The *M. tuberculosis pknB* gene was cloned and expressed under the control of a T7 promoter in the *E. coli* expression vector pET-22b. By using the primers described in Materials and Methods, the open reading frame encoding PknB was successfully amplified by PCR from *M. tuberculosis* H37Rv genomic DNA to give a 1,877-bp fragment (Figure 1) and cloned into the T7 expression vector pET-22b. The map of the resulting plasmid described as pYA102 is shown in Fig. 1C.

The *pknB* gene of *M. tuberculosis* was expressed from pYA102 following treatment of exponentially growing pYA102-*E. coli* BL21(DE3) transformed cells with 1 mM IPTG at 37°C for 4 h (Fig. 2). As shown by SDS-PAGE and Coomassie blue staining, IPTG induced a protein approximately 68 kDa in size (Fig. 2A). This expressed band was visible in both whole-cell lysates and postsonication supernatant within 2 h of IPTG induction. Further purification attempts revealed that PknB was present in the form of insoluble inclusion bodies. The inclusion bodies remained as stable insoluble aggregates even following multiple washes with detergent solutions. To verify that the recombinant protein present in the inclusion bodies is identical to the predicted protein encoded by the *M. tuberculosis pknB* 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 *pknB* gene product derived from the *M. tuberculosis* genome sequence database.

Production of polyclonal antibodies against PknB. As described in Materials and Methods, the PknB protein band was excised from an SDS-PAGE gel and used to immunize mice. The polyclonal mouse anti-PknB serum reacted with the PknB inclusion bodies as well as with PknB from all downstream purification steps (Fig. 2B). The polyclonal serum was specific for PknB as it did not react with fractions prepared from *E. coli* BL21 cells containing the expression vector without the *pknB* gene. These anti-PknB antibodies also reacted with cell extracts obtained from *M. tuberculosis* (data not shown) and were able to inhibit PknB activity (Fig. 3C).

Purification and renaturation of PknB from inclusion bodies. Soluble, recombinant PknB in *E. coli* was obtained when bacteria were grown at 28°C. However, the low yield obtained under these conditions and the need for a long purification process prompted us to develop an alternate strategy to obtain active protein from inclusion bodies rather than from the soluble material. A three-step denaturation and purification process was used. As described in Materials and Methods, the approach taken involved solubilization of the inclusion bodies in a highly concentrated solution of urea and DTT followed by dialysis and size-exclusion chromatography (18). The progression of PknB purification and solubilization is shown in Fig. 2A. Dialysis was used to remove urea and DTT, and size-exclusion chromatography was used to further purify refolded PknB in an active form. As shown in Table 1, the purification fold increase for PknB was 58 while the yield of pure active

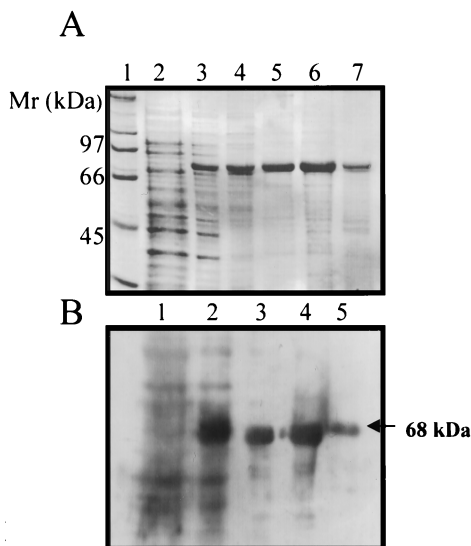


FIG. 2. PknB expression. (A) SDS-PAGE analysis of PknB expression in *E. coli*. Lanes: 1, molecular size markers; 2, negative control pET-22b in *E. coli* BL21 after IPTG induction; 3, cell extract of pYA102 expressing PknB after IPTG induction; 4, pellet of cell extract of pYA102 expressing PknB after IPTG induction; 5, PknB inclusion bodies after washes with detergent; 6, PknB after urea-DTT treatment; 7, PknB after size-exclusion chromatography. (B) Western analysis using mouse anti-PknB polyclonal antibodies. Lanes: 1, negative control pET-22B; 2, pellet of cell extract of pYA102 expressing PknB after IPTG induction; 3, PknB inclusion bodies; 4, PknB after urea-DTT treatment; 5, PknB after size-exclusion chromatography.

PknB through size-exclusion chromatography was 3.48% of the total cell proteins present prior to purification. The 96.52% loss of the protein may be due to the formation of PknB multimers or incompletely renatured PknB molecules that migrate at a different molecular weight than refolded PknB. These values are similar to those obtained for other recombinant proteins that were recovered from inclusion bodies formed in *E. coli* (18).

PknB possesses intrinsic kinase activity. As shown in Fig. 3, in vitro kinase assays revealed that PknB is capable of phosphorylating itself and a conventional in vitro substrate for serine/threonine kinases, MBP from bovine brain. Incorporation of γ - 32 P from ATP into PknB occurred very rapidly, reaching a maximum in 15 min (Fig. 3A). This kinase activity was enhanced in the presence of up to 1 mM Mn^{2+} . Mn^{2+} at concentrations of 10 mM and above inhibits PknB autophosphorylation (Fig. 3B) as well as the phosphorylation of MBP. As shown in Fig. 3C, the mouse anti-PknB polyclonal antibodies block MBP phosphorylation by PknB. The enzyme is completely inactivated when it is incubated with 1/100 dilution of 1:1 (vol/vol) of anti-PknB antibodies.

PknB is autophosphorylated on serine and threonine amino acid residues. To demonstrate that PknB is a eukaryotic-like serine/threonine protein kinase, specific antibodies against phosphoserine and phosphothreonine were used to detect PknB in Western blots. As shown in Fig. 4, specific antiphosphoserine and antiphosphothreonine antibodies recognized PknB, thus confirming that the refolded PknB is phosphorylated on serine/threonine amino acids as suggested by their homology to other serine/threonine kinases. Most interestingly, refolded PknB is also recognized by antibodies against the phosphorylated eukaryotic signaling elements MKK3 and MKK6, P38, and Creb (Fig. 4A). Phosphoamino acid analysis using acid hydrolysis followed by two-dimensional thin-layer

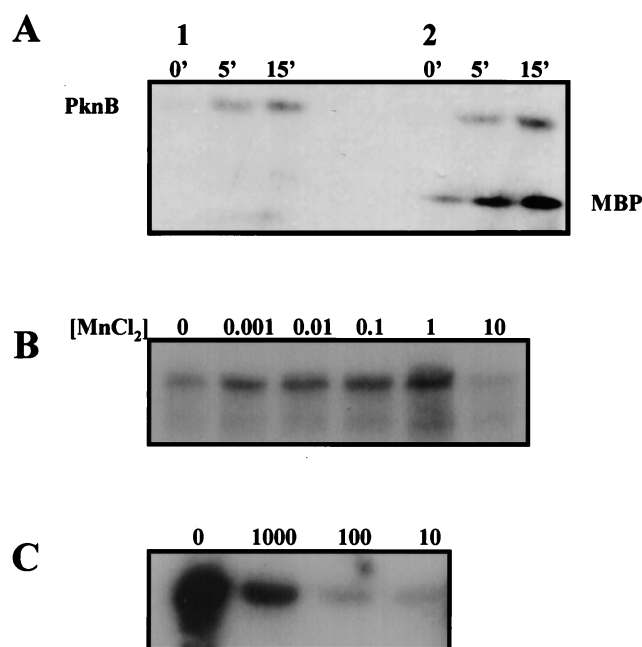


FIG. 3. In vitro kinase assay. SDS-PAGE analysis of PknB labeled with $[\gamma$ - 32 P]ATP. (A) Time course detection of PknB phosphorylation. Lanes: 1, PknB autophosphorylation; 2, MBP phosphorylation mediated by PknB. (B) $MnCl_2$ concentration effect on PknB autophosphorylation. Units are in millimolar concentrations. (C) Effect of serial dilutions of anti-PknB antibodies on MBP phosphorylation by PknB.

chromatography confirmed that PknB is phosphorylated on both serine and threonine residues and not tyrosine (Fig. 4B).

PknB is constitutively transcribed and is detected in alveolar macrophages from a patient with tuberculosis. An important aspect of the life cycle of *M. tuberculosis* is its ability to survive within host macrophages. It was therefore considered pertinent to study whether the expression of *M. tuberculosis pknB* was affected by the transition from an extracellular to an intracellular environment. Hence, the expression of *pknB* in vitro and within host macrophages was examined. RT-PCR analysis of RNA extracted from *M. tuberculosis* growing in culture resulted in the amplification of a fragment of DNA of the expected size (1,600 bp) (Fig. 5A). This indicated that the *pknB* gene is expressed in the organism growing in vitro culture. As shown in Fig. 5, RT-PCR analysis of cells from the murine macrophage cell line J774.2 infected with *M. tuberculosis* resulted in amplification of *pknB* when total RNA was used as a template. No amplification was observed with cDNA

TABLE 1. Recovery of PknB from inclusion bodies^a

Recovery step	Total protein (mg)	Sp act (cpm/min μ g of protein ⁻¹)	Total activity	Yield (%)	Purification (fold)
Cell extract	1,207	4,220	5.10×10^9	100	1
Inclusion bodies	593	4,420	2.63×10^9	51.6	2.03
Macro Prep SE	21	8,460	1.78×10^8	3.48	58

^a One liter of *E. coli* BL21 containing the expression plasmid pYA102 produced 6.77 g (wet weight) of cells after induction with IPTG as described in Materials and Methods. Data shown are those for 1 liter of culture and are representative of several experiments. Specific activity measurements are based upon an in vitro kinase assay using MBP as an alternative to the unidentified natural substrate and thus are artificial and are presented solely for the purpose of purification evaluation.

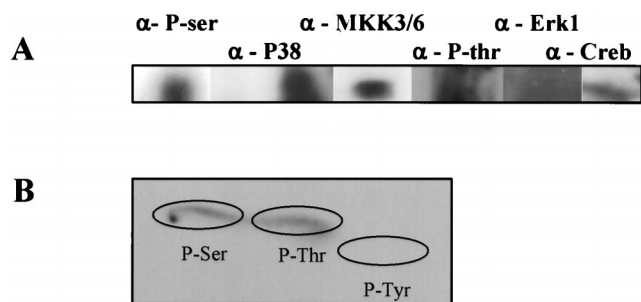


FIG. 4. PknB cross-reactivity with eukaryotic phosphoprotein antibodies and phosphoamino acid analysis. (A) Cross activity of PknB with antibodies against phosphoproteins was determined by SDS-7.5% PAGE analysis followed by Western blot analysis. (B) Phosphoamino acid analysis of autophosphorylated PknB was performed by excision of radioactively labelled PknB from a PVDF membrane followed by acid hydrolysis and two-dimensional thin-layer chromatography. The positions of the unlabeled phosphoamino acids standards are encircled.

prepared from uninfected mouse macrophages. The intensity of specific *pknB* expression within murine macrophages appeared to depend upon the duration of infection. Thus, *pknB* expression was increased between 24 and 72 h of infection (Fig. 5B, lanes 3 and 5). This could suggest either an increase in the expression of *pknB* as a part of adaptation of *M. tuberculosis* to the change in the environment or an increase in the number of bacteria associated with J774.2 cells over the additional period of incubation, given that Mehta et al. (22) showed a fourfold increase in J774-associated *M. tuberculosis* after 3 days of incubation.

Infection with *M. tuberculosis* is usually acquired by inhalation, and it is believed that during the initial establishment of infection organisms reside primarily in alveolar macrophages. The expression of *pknB* in alveolar macrophages taken from a patient with tuberculosis was examined. RT-PCR was performed on cDNA prepared from alveolar macrophages obtained by lung lavage of a patient suffering from tuberculosis. Figure 5C shows RT-PCR amplification of *pknB* in three of four preparations from this patient. No detectable amplification of *pknB* was observed with cDNA prepared from alveolar macrophages obtained from patients suffering from conditions other than tuberculosis.

DISCUSSION

This study characterized PknB, a member of the newly described eukaryotic-like serine/threonine kinase family from *M. tuberculosis* (3, 6). *M. tuberculosis* encodes in its genome 11 putative serine/threonine kinases. In contrast, *E. coli* and other bacteria whose genomes have been completely sequenced thus far do not contain this family of proteins. 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*. Perhaps the question of greatest importance is whether the PSTK genes encode active protein kinases. The findings of the present study show that PknB is indeed a functional protein kinase able to phosphorylate itself as well as model substrates such as MBP. PknB kinase activity and its properties are similar to those first described for the *M. tuberculosis* PSTK, PknD, a putative transmembrane kinase encoded by a gene located in the vicinity of the phosphate-specific transport operon (27).

As emphasized earlier, prokaryotic PSTKs fall into two categories, (i) those that are involved in pathogenicity and (ii) those that are involved in control of developmental processes. The best-studied example of a kinase involved in pathogenicity is provided by *yopO* (*ypkA*) of *Y. pseudotuberculosis* (12). YopO is secreted and targeted to the inner surface of the host cell plasma membrane (12) and is proposed to interfere with the host response by the disruption of cell signaling events. This interference probably occurs by phosphorylation of eukaryotic substrates. We have shown that PknB cross-reacts with certain eukaryotic antiphosphoprotein antibodies such as anti-MKK3/6 and anti-P38. However, there is no evidence for PknB secretion from *M. tuberculosis*, and therefore it seems unlikely that it will interact with eukaryotic signaling elements. Cross-recognition of PknB and eukaryotic kinases suggests that PknB has limited structural homology with these proteins, as suggested by their sequence similarities (3). Nevertheless, given that both P38 and MKK3/6 phosphoproteins are part of a cascade controlling cellular responses to stress and cytokines, it is also possible that PknB and these eukaryotic signaling elements are evolutionary related.

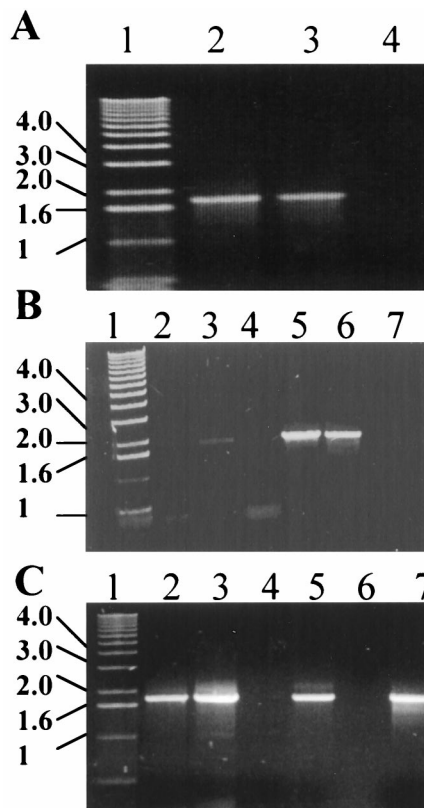


FIG. 5. Transcription analysis of *M. tuberculosis pknB*. (A) RT-PCR using in vitro-grown *M. tuberculosis* RNA. Lanes: 1, 1-kb DNA ladder; 2, *pknB* amplified with cDNA made from *M. tuberculosis* RNA as a template; 3, *pknB* amplified from *M. tuberculosis* genomic DNA preparation; 4, no-DNA-template negative control. (B) Expression upon infection of mouse macrophages. Lanes: 1, 1-kb DNA ladder; 2, cDNA from uninfected control cells after 24 h of incubation; 3, *pknB* amplified from cDNA from *M. tuberculosis* infected cells after 24 h of incubation; 4, cDNA from noninfected control cells after 72 h; 5, *pknB* amplified from cDNA from *M. tuberculosis*-infected cells after 72 h; 6, *pknB* amplified from genomic *M. tuberculosis* DNA; 7, no-DNA template. (C) Expression of *pknB* in alveolar macrophages from a pulmonary tuberculosis patient. Lanes: 1, 1-kb DNA ladder; 2 to 5, *pknB* amplified with cDNA derived from alveolar macrophages from a patient suffering from pulmonary tuberculosis; 6, cDNA from noninfected control cells; 7, *pknB* amplified from genomic *M. tuberculosis* DNA.

As shown in Fig. 1A, PknB is encoded by an open reading frame (Rv 0014c) that is in a cluster with *pknA*, the protein phosphatase *ppp*, and two other open reading frames that are predicted to encode the penicillin-binding protein PbpA and the cell division protein RodA. These open reading frames appear to form an operon structure. In *E. coli*, both *rodA* and *pbpA* are involved in cell structure determination and constitute a single transcriptional unit (20). Clusters of peptidoglycan biosynthesis and cell division genes were identified and sequenced in both gram-positive and gram-negative bacteria (1, 11, 20, 24, 38). These gene clusters are involved in the switching mechanism between cell elongation and division (4, 10, 14, 32). Cell division genes are not usually found coordinately expressed with kinases or phosphatases. The presence of *pknB*, *pknA*, and *ppp* genes within the cell division gene cluster may indicate an unusual regulatory cascade controlling *M. tuberculosis* cell growth.

By using the Kyte-Doolittle algorithm (16) to calculate hydrophilicity and transmembrane regions, PknB is predicted to contain a transmembrane domain between amino acids 332 and 350. Furthermore, fingerprint scan analysis (2) revealed that PknB possesses three of four known domains of the bacterial sensor C-terminal signature. In prokaryotes, histidine protein kinases function as sensors for various external signals (33). However, it was shown recently that *Myxobacteria* and *Streptomyces* contain serine/threonine kinases that are transmembrane proteins that may also serve as receptors for external signals (13, 25, 36). For example, *Streptomyces* AfsK and AfsR are suggested to be a two-component regulatory system involving serine protein kinases (19). Thus, it is reasonable to propose that PknB may serve as a sensor for external signals that translate into cell morphology changes such as division and elongation.

Most of the bacterial eukaryotic-like protein kinases that have been characterized so far have been shown to be involved in the regulation of different developmental states of the bacterium (35, 36, 40, 43). From this point of view, the best-studied organisms are the highly developed bacteria. For example, the Ser/Thr kinase Pkn1 of *Myxococcus xanthus* is expressed exclusively during sporulation and its inactivation inhibits spore formation in this bacterium (40). In a similar manner, inactivation of the *Streptomyces granaticolor* PSTK *pkg2* resulted in changes in the formation of bacterial aerial hyphae (25). Both myxobacteria and *Streptomyces* spp. display developmental characteristics comparable to those of multicellular organisms. In contrast, the unicellular mycobacteria lack a complex developmental life cycle. However, it has been proposed that the *M. tuberculosis* "dormant state" could be considered as a new developmental growth state that is associated with prolonged latent infection and may be analogous to sporulation (9, 26). Interestingly, induction of the mycobacterial stationary phase by low oxygen tension has been shown to result in cell wall thickening (8). Thus, it is possible that PknB may be involved in *M. tuberculosis* progression into the late stationary or dormant stages. The finding that PknB expression is constitutive and present under both in vitro and in vivo growth conditions supports the hypothesis that PknB is a bacterial sensor that transduces signals through the mycobacterial membrane to an undefined accessory protein. Efforts to identify the candidate ligands for PknB are now in progress as well as gene knockout experiments that will attempt to demonstrate a role for PknB in the regulation of *M. tuberculosis* growth and pathogenicity.

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REFERENCES

1. Asoh, S., H. Matsuzawa, F. Ishino, J. L. Strominger, M. Matsushashi, and T. Ohta. 1986. Nucleotide sequence of the *pbpA* gene and characteristics of the deduced amino acid sequence of penicillin-binding protein 2 of *Escherichia coli* K12. *Eur. J. Biochem.* **160**:231–238.
2. Attwood, T. K., M. E. Beck, D. R. Flower, P. Scordis, and J. N. Selley. 1998. The PRINTS protein fingerprint database in its fifth year. *Nucleic Acids Res.* **26**:304–308.
3. Av-Gay, Y., and J. Davies. 1997. Components of eukaryotic-like protein signaling pathways in *Mycobacterium tuberculosis*. *Microb. Comp. Genomics* **2**:63–73.
4. Begg, K. J., and W. D. Donachie. 1998. Division planes alternate in spherical cells of *Escherichia coli*. *J. Bacteriol.* **180**:2564–2567.
5. Brozna, J. P., M. Horan, J. M. Rademacher, K. M. Pabst, and M. J. Pabst. 1991. Monocyte responses to sulfatide from *Mycobacterium tuberculosis*: inhibition of priming for enhanced release of superoxide, associated with increased secretion of interleukin-1 and tumor necrosis factor alpha, and altered protein phosphorylation. *Infect. Immun.* **59**:2542–2548.
6. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaiia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537–544.
7. Cozzone, A. J. 1993. ATP-dependent protein kinases in bacteria. *J. Cell. Biochem.* **51**:7–13.
8. Cunningham, A. F., and C. L. Spreadbury. 1998. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. *J. Bacteriol.* **180**:801–808.
9. DeMaio, J., Y. Zhang, C. Ko, D. B. Young, and W. R. Bishai. 1996. A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **93**:2790–2794.
10. Donachie, W. D., S. Addinall, and K. Begg. 1995. Cell shape and chromosome partition in prokaryotes or why *E. coli* is rod-shaped and haploid. *Bioessays* **17**:569–576.
11. Fsihi, H., E. De Rossi, L. Salazar, R. Cantoni, M. Labo, G. Riccardi, H. E. Takiff, K. Eiglmeier, S. Bergh, and S. T. Cole. 1996. Gene arrangement and organization in a approximately 76 kb fragment encompassing the *oriC* region of the chromosome of *Mycobacterium leprae*. *Microbiology* **142**:3147–3161.
12. Hakansson, S., E. E. Galyov, R. Rosqvist, and H. Wolf-Watz. 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. *Mol. Microbiol.* **20**:593–603.
13. Hanlon, W. A., M. Inouye, and S. Inouye. 1997. Pkn9, a Ser/Thr protein kinase involved in the development of *Myxococcus xanthus*. *Mol. Microbiol.* **23**:459–471.
14. Henriques, A. O., P. Glaser, P. J. Piggot, and C. P. Moran, Jr. 1998. Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. *Mol. Microbiol.* **28**:235–247.
15. Kamps, M. P., and B. M. Sefton. 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilin facilitates the analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* **176**:22–27.
16. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **157**:105–132.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
18. Landman, O., D. Shiffman, Y. Av-Gay, Y. Aharonowitz, and G. Cohen. 1991. High level expression in *Escherichia coli* of isopenicillin N synthase genes from *Flavobacterium* and *Streptomyces*, and recovery of active enzyme from inclusion bodies. *FEMS Microbiol. Lett.* **68**:239–244.
19. Matsumoto, A., S. K. Hong, H. Ishizuka, S. Horinouchi, and T. Beppu. 1994. Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene* **146**:47–56.
20. Matsuzawa, H., S. Asoh, K. Kunai, K. Muraiso, A. Takasuga, and T. Ohta. 1989. Nucleotide sequence of the *rodA* gene, responsible for the rod shape of *Escherichia coli*: *rodA* and the *pbpA* gene, encoding penicillin-binding protein 2, constitute the *rodA* operon. *J. Bacteriol.* **171**:558–560.
21. McDonough, K. A., Y. Kress, and B. R. Bloom. 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* **61**:2763–2773.

22. Mehta, P. K., C. H. King, E. H. White, J. J. Murtagh, Jr., and F. D. Quinn. 1996. Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. *Infect. Immun.* **64**:2673–2679.
23. Moreno, C., A. Mehlert, and J. Lamb. 1988. The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. *Clin. Exp. Immunol.* **74**:206–210.
24. Murray, T., D. L. Popham, and P. Setlow. 1997. Identification and characterization of *pbpA* encoding *Bacillus subtilis* penicillin-binding protein 2A. *J. Bacteriol.* **179**:3021–3029.
25. Nadvornik, R., T. Vomastek, J. Janecek, Z. Technikova, and P. Branny. 1999. Pkg2, a novel transmembrane protein Ser/Thr kinase of *Streptomyces gramineovorus*. *J. Bacteriol.* **181**:15–23.
26. Parrish, N. M., J. D. Dick, and W. R. Bishai. 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol.* **6**:107–112.
27. Peirs, P., L. De Wit, M. Braibant, K. Huygen, and J. Content. 1997. A serine/threonine protein kinase from *Mycobacterium tuberculosis*. *Eur. J. Biochem.* **244**:604–612.
28. Potts, M., H. Sun, K. Mockaitis, P. J. Kennelly, D. Reed, and N. K. Tonks. 1993. A protein-tyrosine/serine phosphatase encoded by the genome of the cyanobacterium *Nostoc*. *J. Biol. Chem.* **268**:7632–7635.
29. Russell, D. G., J. Dant, and S. Sturgill-Koszycki. 1996. *Mycobacterium avium*- and *Mycobacterium tuberculosis*-containing vacuoles are dynamic, fusion-competent vesicles that are accessible to glycosphingolipids from the host cell plasmalemma. *J. Immunol.* **156**:4764–4773.
30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl. 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. *Infect. Immun.* **56**:1232–1236.
32. Signoretto, C., F. Di Stefano, and P. Canepari. 1996. Modified peptidoglycan chemical composition in shape-altered *Escherichia coli*. *Microbiology* **142**:1919–1926.
33. Stock, J. B., A. J. Ninfa, and A. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53**:450–490.
34. Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell. 1994. Lack of acidification in mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**:678–681.
35. Udo, H., M. Inouye, and S. Inouye. 1997. Biochemical characterization of Pkn2, a protein Ser/Thr kinase from *Myxococcus xanthus*, a gram-negative developmental bacterium. *FEBS Lett.* **400**:188–192.
36. Udo, H., J. Munoz-Dorado, M. Inouye, and S. Inouye. 1995. *Myxococcus xanthus*, a gram-negative bacterium, contains a transmembrane protein serine/threonine kinase that blocks the secretion of beta-lactamase by phosphorylation. *Genes Dev.* **9**:972–983.
37. Urabe, H., and H. Ogawara. 1995. Cloning, sequencing and expression of serine/threonine kinase-encoding genes from *Streptomyces coelicolor* A3(2). *Gene* **153**:99–104.
38. Wada, A., and H. Watanabe. 1998. Penicillin-binding protein 1 of *Staphylococcus aureus* is essential for growth. *J. Bacteriol.* **180**:2759–2765.
39. Wang, J. Y., C. H. Li, C. J. Yang, A. Mushegian, and S. G. Jin. 1998. A novel serine/threonine protein kinase homologue of *Pseudomonas aeruginosa* is specifically inducible within the host infection site and is required for full virulence in neutropenic mice. *J. Bacteriol.* **180**:6764–6768.
40. Zhang, C. C. 1996. Bacterial signalling involving eukaryotic-type protein kinases. *Mol. Microbiol.* **20**:9–15.
41. Zhang, C. C., A. Friry, and L. Peng. 1998. Molecular and genetic analysis of two closely linked genes that encode, respectively, a protein phosphatase 1/2A/2B homolog and a protein kinase homolog in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **180**:2616–2622.
42. Zhang, C. C., and L. Libs. 1998. Cloning and characterisation of the *pknD* gene encoding an eukaryotic-type protein kinase in the cyanobacterium *Anabaena* sp. PCC7120. *Mol. Gen. Genet.* **258**:26–33.
43. Zhang, W., M. Inouye, and S. Inouye. 1996. Reciprocal regulation of the differentiation of *Myxococcus xanthus* by Pkn5 and Pkn6, eukaryotic-like Ser/Thr protein kinases. *Mol. Microbiol.* **20**:435–447.

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