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The Ser/Thr Protein Kinase PknB Is Essential for Sustaining Mycobacterial Growth▽

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The receptor-like protein kinase PknB from *Mycobacterium tuberculosis* is encoded by the distal gene in a highly conserved operon, present in all actinobacteria, that may control cell shape and cell division. Genes coding for a PknB-like protein kinase are also found in many more distantly related gram-positive bacteria. Here, we report that the *pknB* gene can be disrupted by allelic replacement in *M. tuberculosis* and the saprophyte *Mycobacterium smegmatis* only in the presence of a second functional copy of the gene. We also demonstrate that eukaryotic Ser/Thr protein kinase inhibitors, which inactivate PknB in vitro with a 50% inhibitory concentration in the submicromolar range, are able to kill *M. tuberculosis* H37Rv, *M. smegmatis* mc^{2}155, and *Mycobacterium aurum* A+ with MICs in the micromolar range. Furthermore, significantly higher concentrations of these compounds are required to inhibit growth of *M. smegmatis* strains overexpressing PknB, suggesting that this protein kinase is the molecular target. These findings demonstrate that the Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth and support the development of protein kinase inhibitors as new potential antituberculosis drugs.

Mycobacterium tuberculosis is the causative agent of tuberculosis, a major world health problem that is responsible for the deaths of over two million people every year. A better understanding of the biology of the tubercle bacillus, with the goal of unveiling and validating new therapeutic targets, is an imperative requirement for improving the control and treatment of tuberculosis. The study of signaling elements, in particular Ser/Thr protein kinases (STPKs), is of outstanding interest in this context, given their likely important roles in mycobacterial physiology and virulence, as well as the available expertise on the design of specific inhibitors for eukaryotic STPKs, which currently represent one of the most actively studied groups of drug targets.

Signal transduction in prokaryotes is conducted primarily by two-component regulatory systems, basically consisting of a sensor histidine kinase and a response regulator (45). The *M. tuberculosis* genome encodes 11 complete two-component systems (11, 19), several of which contribute to the virulence of *M. tuberculosis* (33, 34, 36, 38, 49), but only one system (MtrA, MtrB) was found to be essential for cell growth (48). The relatively low number of two-component systems is offset by alternative signal transduction mechanisms involving Ser/Thr phosphorylation (2) that are generally less common in bacteria than in eukaryotes (26). *M. tuberculosis* has genes for one phospho-Ser/Thr phosphatase (*pstP*) and as many as 11 STPKs (*pknA* to *pknL*) (11). In mycobacteria with larger genomes, such as *Mycobacterium marinum* or *Mycobacterium smegmatis*, STPKs outnumber two-component systems, suggesting that the bulk of signal transduction is via Ser/Thr (de)phosphorylation.

Paradoxically, most of these STPKs do not appear to control essential physiological processes. *Mycobacterium leprae*, a closely related species that has undergone extensive gene decay (12), has retained only four STPKs, and orthologs of just three of them (*pknA*, *pknB*, and *pknG*) were found to be required for optimal growth of *M. tuberculosis* using saturation transposon mutagenesis (39). Furthermore, inactivation of the *pknG* gene in *M. tuberculosis* was reported to decrease viability both in vitro and upon infection of BALB/c mice (13), although independent work showed that the in vitro growth of *Mycobacterium bovis* BCG lacking *pknG* was identical to that of the wild type (29). In a similar way, wild-type-like growth was observed for *M. tuberculosis* strains lacking either the *pknD* or the *pknH* gene (32, 37), and downregulation of PknF protein synthesis in *M. tuberculosis* using an antisense strategy also confirmed a viable phenotype, with faster-growing and shorter cells than the wild-type strain (15).

In this work, we focus on the *pknB* gene, which is part of an operon that is strictly conserved in all known mycobacterial genomes and some related actinomycetes. Genes coding for a PknB-like protein kinase are also found in a large number of gram-positive bacteria (9). PknB is a receptor-like transmembrane protein, with an extracellular signal sensor domain and an intracellular kinase domain (4), that shares striking similarity in protein fold, catalytic machinery, and kinase regulation mechanism with eukaryotic STPKs (9, 31, 47). Here, we report the inactivation of the *pknB* gene in *M. tuberculosis* H37Rv and *M. smegmatis* mc^{2}155 and provide the first direct evidence of the essentiality of a STPK gene in mycobacteria. We also present the effects of several known ATP-competitive inhibitors on PknB phosphorylation activity in vitro and demonstrate that strong PknB inhibitors can prevent the growth of slow-

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and fast-growing mycobacterial species, highlighting the potential of STPKs as therapeutic targets for the development of new antituberculosis drugs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* XL1-blue, used for cloning experiments, was routinely propagated in LB broth (Difco) at 37°C. *M. smegmatis* mc^2155 (41) was grown at 30, 37, or 42°C in LB broth supplemented with 0.05% Tween 80 or in 7H9 broth (Difco) supplemented with 0.05% Tween 80 and albumin-dextrose-catalase (ADC). *M. tuberculosis* H37Rv was grown in 7H9 broth supplemented with 0.05% Tween 80 and ADC or on 7H11 agar medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) at 30, 37, or 39°C. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; hygromycin, 50 μg/ml. When required, 10% (*M. smegmatis*) or 2% (*M. tuberculosis*) sucrose was added to the solid medium.

Cloning procedures and Southern blot analysis. (i) Construction of pRBexint-*pknB* and pOMK-*pknB*. Preparation of *E. coli* and mycobacterial electrocompetent cells, extraction of chromosomal DNA, Southern blotting, and cloning were carried out as described previously (24, 35). *M. smegmatis* genomic sequences were obtained from TIGR (http://www.tigr.org).

M. tuberculosis pknB and flanking regions were PCR amplified and cloned into the SpeI restriction site of the replicative plasmid pOMK (22) to obtain pOMK-*pknB* or were amplified using primers harboring SpeI and HpaI restriction sites and cloned into pRBexint (a kind gift from R. Brosch), an integrative cosmid vector derived from pYUB412 (5), to generate pRBexint-*pknB*. All constructs were verified by sequencing. Details of the primers are available upon request.

(ii) Construction of the *M. tuberculosis* and *M. smegmatis pknB* mutant. The essentiality of the *pknB* gene in *M. tuberculosis* and *M. smegmatis* was investigated following a standard strategy based on the construction of merodiploid strains (23). The ts-*sacB* method was used to achieve allelic replacement at the *pknB* locus of *M. tuberculosis* (23, 35), and pRBexint-*pknB* was integrated to perform complementation experiments. A unique HindIII restriction site was created in the *M. tuberculosis pknB* gene by using a QuikChange site-directed mutagenesis kit (Stratagene) into which the kanamycin resistance cassette from pUC4K (Amersham Biosciences) was inserted. The resulting *pknB*::Km gene was then cloned into pPR27 with the *xylE* colored marker (35) to obtain pPR27pknB, the construct used for allelic replacement.

The essentiality of the *M. smegmatis pknB* gene was investigated using a two-step homologous recombination procedure to achieve allelic replacement at the *pknB* locus (23) and pRBexint-*pknB* to perform complementation experiments. The *M. smegmatis pknB* gene and flanking regions were PCR amplified, and a disrupted allele, *pknB*::Km, was then obtained as described above. The construct used for allelic replacement, pJQpknB, was obtained by cloning *pknB*::Km into pJQ200-XylE (23).

Protein kinase assays. PknB and GarA were expressed in *E. coli* and purified as described previously (9, 43). Kinase assays were carried out in 15 μl of kinase buffer (50 mM HEPES [pH 7.0], 1 mM dithiothreitol, 0.01% Brij35, 5% glycerol, 2 mM $MnCl_2$). All reactions were started with the addition of ATP and conducted at 30°C for 20 min. Myelin basic protein (MBP) phosphorylation assays were carried out with an enzyme:substrate molar ratio of 1:20 in the presence of ATP (0.1 mM containing 1 μCi of [γ-^{33}P]ATP). The reactions were stopped by adding EDTA (15 mM, final concentration), and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For GarA phosphorylation, a 2.25 μM final concentration of ATP (containing 1 μCi of [γ-^{33}P]ATP) and an enzyme:substrate ratio of 1:2,000 were used. The reactions were stopped by heat inactivation of the enzyme, and 5 μl of the reaction was transferred by pipette onto P81 paper (phosphocellulose; Whatman). The P81 papers were washed with 1% phosphoric acid three times for 5 min, rinsed with acetone, and allowed to dry on a sheet of aluminum foil. The radiolabeled spots of both one-dimensional gels and P81 papers were visualized by autoradiography, and the incorporation of radiolabeled ATP was quantified using a PhosphorImager system (Storm; Molecular Dynamics). In inhibition experiments, each compound (100 μM in MBP phosphorylation assays; 10 μM in GarA phosphorylation assays) was preincubated for 30 min at 4°C in the reaction mixture without ATP. All compounds tested (A-3, calphostin C, GF109203X, H-7, H-8, H-9, H-89, HA-1004, HA-1077, hypericin, K-252-a, K-252-b, KN-62, KT-5720, KT-5823, ML-7, ML-9, and staurosporine) were purchased from BIOMOL Research Laboratories, Inc. For determination of the 50% inhibitory dose (IC_{50}), serial twofold dilutions were started at 10 μM (K-252-a and K-252-b) or 20 μM (staurosporine), and the IC_{50} values and standard errors were calculated using KaleidaGraph (Synergy Software).

Resazurin microtiter assays. Resazurin sodium salt powder (Sigma) was prepared at 0.01% (wt/vol) in distilled water and filter sterilized. The assays were performed at 37°C in 7H9 medium containing 0.2% glycerol and OADC. Serial twofold dilutions of each inhibitor were prepared in 100 μl of medium directly in 96-well plates at concentrations of 40 to 0.31 μM for K-252-a and K-252-b and 200 to 1.56 μM for staurosporine. Growth controls containing no inhibitor and sterility controls without inoculation were also included. The inoculum was prepared from fresh cultures adjusted to an optical density at 600 nm of 0.1 in the medium described above and diluted 1:10, and 100 μl was added per well (~10^6 cells). The plates were incubated at 37°C in a 5% CO_2 incubator for 10 days (for *M. tuberculosis*) or 4 days (for *M. smegmatis*). Thirty microliters of resazurin solution was then added to control wells containing no inhibitor or medium alone, incubated overnight at 37°C, and assessed for color development. A change from blue to pink indicates the reduction of resazurin and, therefore, bacterial growth. If a color change occurred, resazurin was added to all of the wells. The MIC was defined as the lowest drug concentration that prevented the color change.

RESULTS

Genomics and experimental rationale. An inspection of the available genome sequences of mycobacteria, corynebacteria, and streptomycetes (1, 8, 11, 12, 20) revealed a conserved operon, near the chromosomal origin of replication, comprising five or six genes that may be involved in signal transduction pathways and cell division. In *M. tuberculosis*, this operon, in which the termination codon of each gene overlaps the initiation codon of its follower, begins with Rv0019, coding for forkhead-associated (FHA) protein B. This is followed by an in-frame mycobacterial intergenic repetitive unit (42) which precedes *pstP*, encoding phosphoserine/threonine protein phosphatase (9), and the *rodA* and *pbpA* genes before the operon ends with the STPK genes, *pknA* and *pknB* (Fig. 1). Except for the presence of the mycobacterial intergenic repetitive unit, which is confined to *M. tuberculosis*, the same gene arrangement occurs in all sequenced actinobacteria, and the operon is transcribed counter to the direction of replication. In *Streptomyces coelicolor*, there is one less STPK gene, as *pknA* appears to have been lost (7). It has been speculated that these STPK genes may be essential and control cell division (3), although there is no formal evidence. To substantiate this claim, we have undertaken genetic and biochemical analyses of *pknB* which, as the distal gene in the operon, should be readily inactivated, without polar effects, unless it is essential.

Inactivation of *pknB* in *M. tuberculosis*. Attempts were made by allelic replacement to disrupt *pknB* in the pathogenic species, *M. tuberculosis* H37Rv, using the ts-*sacB* methodology (23, 35). However, despite our having performed three independent experiments, PCR analysis of clones corresponding to putative double crossover recombinants showed that none of them exhibited the expected pattern for allelic exchange mutants. To investigate whether the failure to disrupt the *pknB* gene could be due to its essentiality, we next performed an allelic replacement experiment using a *M. tuberculosis* merodiploid strain. This strain was constructed by integrating a functional copy of *pknB* into the chromosome of *M. tuberculosis* H37Rv using the integrative cosmid vector pRBexint-*pknB*. The integration of this cosmid at the $tRNA^{Gly}$ site of the chromosome was confirmed by PCR and Southern blotting. Gene replacement experiments were carried out as described above. Analysis by PCR and Southern blotting of clones corresponding to putative double crossover recombinants showed that, in most cases, the allelic exchange occurred at the inte-

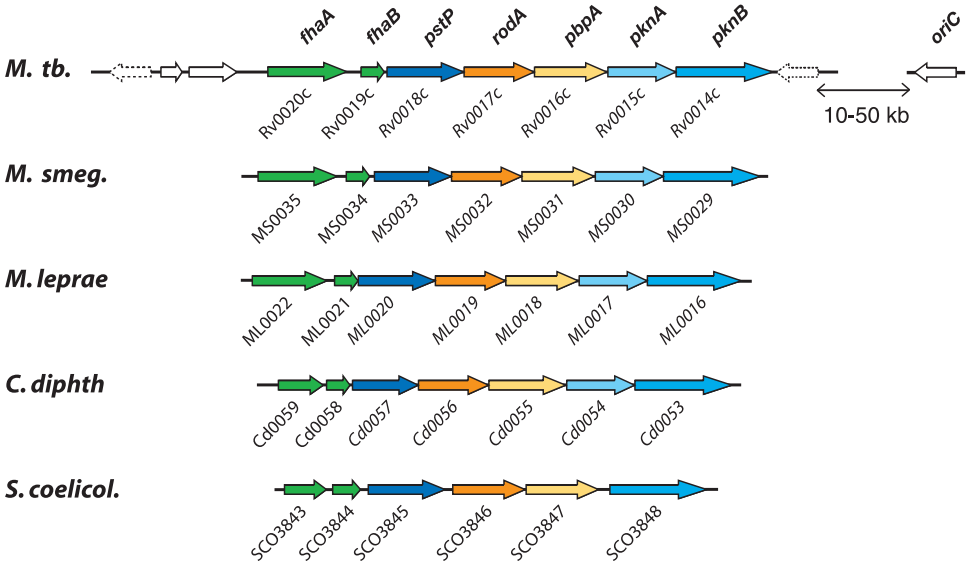

FIG. 1. Conserved structure of the *fhaB-pknB* gene cluster in actinobacteria. This cluster comprises six genes, except in *Streptomyces coelicolor* where *pknA* is missing: *fhaA*, encoding FHA protein A; *fhaB*, FHA protein B; *pstP*, phosphoserine/threonine protein phosphatase; *rodA*, cell division protein; *pbpA*, peptidoglycan biosynthesis protein; *pknA-pknB*, STPK. In all five bacteria, the cluster is situated near *oriC* but transcribed convergently with respect to the direction of replication. Abbreviations: *M. tb.*, *M. tuberculosis*; *M. smeg.*, *M. smegmatis*; *C. diphth.*, *Corynebacterium diphtheriae*; *S. coelicol.*, *Streptomyces coelicolor.*

grated *pknB* locus. However, we were also able to isolate clones in which the gene replacement had taken place at the wild-type *pknB* locus (Fig. 2a). Therefore, the expression of the wild-type *pknB* gene from pRBexint-*pknB* was sufficient to rescue a *M. tuberculosis pknB* knock-out mutant, indicating that this gene is essential for *M. tuberculosis*.

Inactivation of *pknB* in *M. smegmatis*. To investigate whether the *pknB* gene is also required for *M. smegmatis* growth, a kanamycin-disrupted copy of the *pknB* gene, *pknB*::Km, was inserted into the *sacB* suicide vector pJQ200-XylE and introduced into wild-type *M. smegmatis* mc^{2}155 by electroporation. Kanamycin-resistant mc^{2}pJQpknB transformants were selected with LB-kanamycin (LB-Km) plates at 30°C, and Southern blot analyses of eight of them indicated that all resulted from a single crossover event at the *pknB* locus (data not shown). Subsequently, two transformants, mc^{2}pJQpknB.2 and mc^{2}pJQpknB.5, were grown in LB-Km broth and then plated onto LB-Km-sucrose plates to select for clones that had undergone a second intrachromosomal crossover. Allelic exchange mutants are expected to carry the disrupted allele *pknB*::Km and to have lost the *sacB* and *xylE* genes carried by pJQpknB. However, the spraying of thousands of kanamycin-sucrose resistant colonies with catechol (to test for XylE$^-$ strains) revealed that none of them exhibited the expected phenotype. Instead, these clones had probably undergone mutations in the *sacB* gene that conferred sucrose resistance.

To investigate whether the failure to disrupt *pknB* was due to its essentiality, we transformed the single crossover strains mc^{2}pJQpknB.2 and mc^{2}pJQpknB.5 with either pRBexint or pRBexint-*pknB*. Allelic exchange mutants were selected on LB-Km-hygromycin B-sucrose plates at 30°C as described above. The phenotype of approximately 1,000 Km^r-Hyg^r-Suc^r colonies was tested for both types of transformants plated. No allelic exchange mutant (Km^r-Hyg^r-Suc^r-XylE$^-$ colony) was found when mc^{2}pJQpknB.2/pRBexint or mc^{2}pJQpknB.5/pRBexint was plated, confirming our previous results. In contrast, when mc^{2}pJQpknB.2/pRBexint-*pknB* and mc^{2}pJQpknB.5/pRBexint-*pknB* were plated, 30% of Km^r-Hyg^r-Suc^r colonies were found to be XylE negative. A Southern blot analysis of a recombinant revealed that it had undergone gene replacement at the *pknB* locus (Fig. 2b), indicating that *pknB* is also an essential gene in *M. smegmatis*.

PknB inhibitors prevent mycobacterial growth. We next looked for strong PknB inhibitors and tested their effect on mycobacterial growth. Since the physiological substrate of PknB is unknown, the initial kinase assays to identify PknB inhibitors (within a panel of 18 commercially available compounds known to inactivate different eukaryotic STPKs) used MBP as a surrogate substrate (4, 9). As shown in Fig. 3a, significant inhibitory effects were observed, in particular for K-252-a and K-252-b, two natural products that contain the indole carbazole chromophore and are thought to target the ATP-binding site. It should be noted that, although the PKA inhibitor H-7 has been previously reported to inhibit PknB (16), we observed no effect of this compound on PknB, whereas it was able to inactivate mouse PKA at a 5 μM concentration in the present assay (data not shown).

Although PknB phosphorylates MBP in at least five different sites (17), MBP is a poor PknB substrate; a high enzyme: substrate molar ratio (1:20), which is probably unrepresentative of the physiological situation, has to be used in the kinase assay. We therefore used a proteomic approach to identify putative physiological substrates of PknB and identified the protein GarA, an FHA domain-containing protein, as the optimal PknB substrate in a soluble protein extract from *M. tuberculosis* (43). Based on the apparent kinetic constants, a new kinase assay was established using a PknB:GarA molar ratio of 1:2,000 and an ATP concentration of 2.25 μM, the

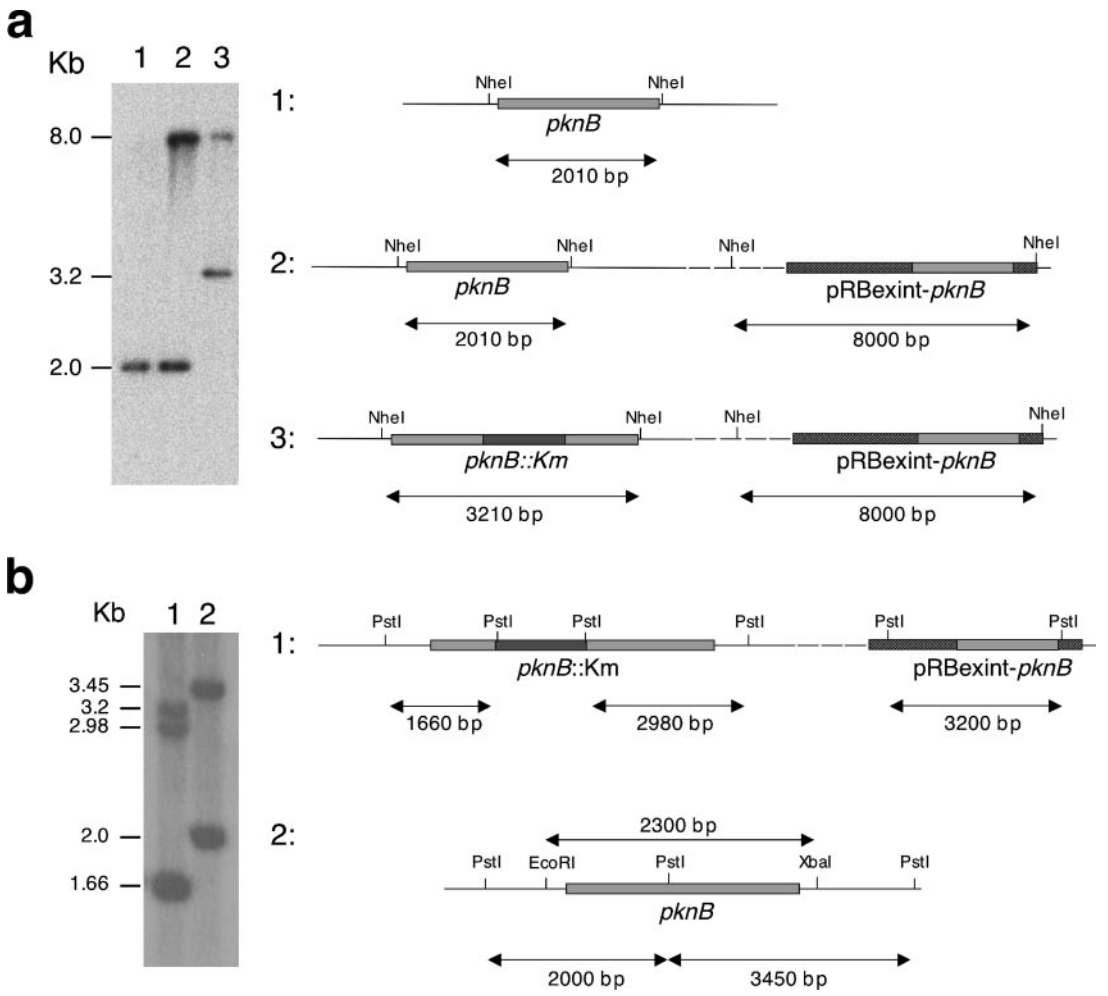

FIG. 2. (a) Allelic replacement at the *M. tuberculosis pknB* locus. Southern blot analysis and corresponding NheI restriction profiles of DNA from H37Rv (lane 1), DNA from the initial diploid strain carrying the pRBexint-*pknB* integrated plasmid (lane 2), and DNA from the resulting allelic replacement mutant strain (lane 3) are shown. The probe was a 1.9-kb PCR fragment carrying the *pknB* gene. (b) Allelic replacement at the *M. smegmatis pknB* locus. Southern blot analysis and expected hybridization profiles of a *pknB* allelic exchange mutant carrying the pRBexint-*pknB* rescue plasmid (lane 1) and mc^{2}155 (lane 2) are shown. Chromosomal DNA was digested with PstI. The probe used to perform the hybridization corresponds to the wild-type *pknB* gene and flanking regions obtained by PCR (2.3-kb EcoRI/XbaI restriction fragment).

apparent Km value for ATP. A screening of the panel of inhibitors using this assay confirmed the previous results, showing K-252-a, K-252-b, and staurosporine to be the strongest inhibitors (Fig. 3b). The IC_{50} values for these compounds were determined by quantification and graphical analysis of radiolabeled spots from serial twofold dilutions (Fig. 3c) and found to be 96 ± 7 nM for K-252-a (Fig. 3d), 106 ± 6 nM for K-252-b, and 0.6 ± 0.05 μM for staurosporine.

Using a colorimetric, resazurin microtiter assay (28), the antibacterial activity of compounds displaying a strong inhibitory effect in vitro was then assayed against *M. tuberculosis* H37Rv, *M. smegmatis* mc^{2}155, and *Mycobacterium aurum* A+. The latter species is generally very sensitive to antitubercular drugs. The results of the susceptibility tests (Table 1) demonstrate that K-252-a inhibited the growth of both *M. tuberculosis* H37Rv and *M. smegmatis* mc^{2}155 at a concentration of 20 μM and that of *M. aurum* A+ at 5 μM. As a control, staurosporine also showed inhibitory effects on *M. tuberculosis* H37Rv (25 μM < MIC < 50 μM). In contrast, K-252-b failed to inhibit the growth of all mycobacterial species at the highest concentration tested (40 μM), perhaps due to the low permeability of the envelope to this compound.

To further assess the correlation between PknB inactivation and the inhibition of mycobacterial growth, the *M. tuberculosis pknB* gene and flanking regions were cloned into pOMK, a mycobacterial replicative vector. The resulting plasmid, pOMK-*pknB*, was introduced into *M. smegmatis* mc^{2}155, and the overexpression of PknB was assessed by Western blotting (data not shown). As a control, *M. smegmatis* mc^{2}155 was also transformed with the empty pOMK vector, and the K-252-a resistance of the mycobacterial transformants was then assayed as described above. Two independent experiments showed that the MICs of the *M. smegmatis* mc^{2}155 strains transformed with pOMK-*pknB* were twofold that of the control strain (Table 1), further suggesting that PknB is the actual molecular target in vivo.

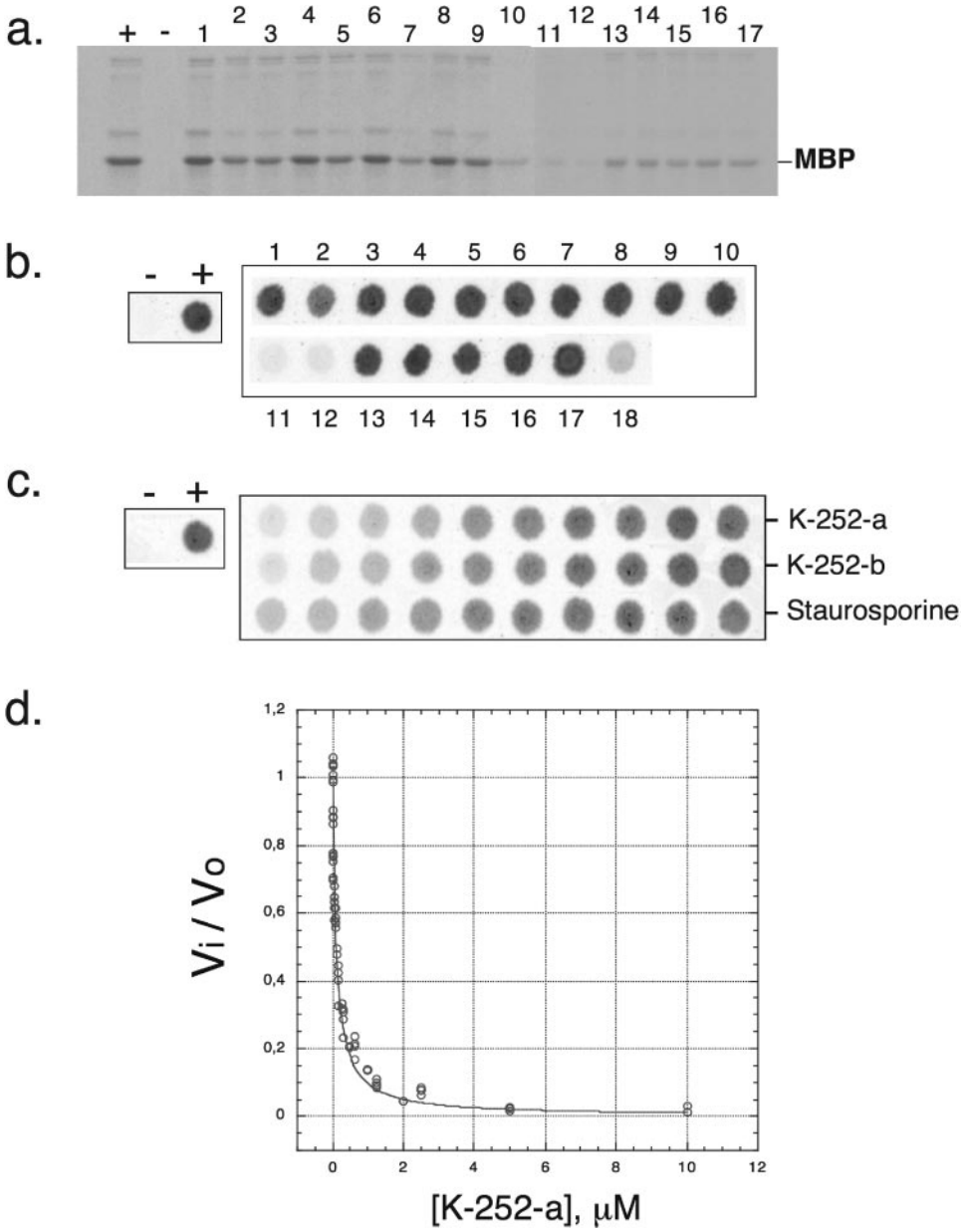

FIG. 3. PknB inhibition assays. (a) Autoradiography of a sodium dodecyl sulfate-polyacrylamide electrophoresis gel showing PknB phosphorylation of MBP and the effect of different inhibitors at 100 μM. (b) Autoradiography of labeled spots on phosphocellulose paper showing the effect of the same inhibitors at 10 μM using a more sensitive phosphorylation assay (PknB phosphorylation of GarA). (a and b) Tested inhibitors were: lane 1, A-3; 2, calphostin C; 3, GF109203X; 4, H-7; 5, H-8; 6, H-9; 7, H-89; 8, HA-1004; 9, HA-1077; 10, hypericin; 11, K-252-a; 12, K-252-b; 13, KN-62; 14, KT-5720; 15, KT-5823; 16, ML-7; 17, ML-9; and 18, staurosporine. Positive (+) and negative (−) controls are shown. (c) Autoradiography of labeled spots on phosphocellulose paper showing GarA phosphorylation after serial twofold dilutions, starting at left from 10 μM (K-252-a and K-252-b) or 20 μM (staurosporine). (d) Dose-response plot of PknB fractional activity from five independent experiments as a function of K-252-a concentration. Similar plots were obtained for K-252-b and staurosporine. Vi and Vo, velocities in the presence and absence of inhibitors, respectively.

DISCUSSION

Among the signaling elements in *M. tuberculosis*, only the MtrA response regulator has been previously found to be essential for growth (48). Although rare, other essential genes encoding two-component systems have been identified for gram-positive bacteria, in particular orthologs of *Bacillus subtilis* YycG-YycF (18) that are likely involved in cell division (21). However, despite the widespread occurrence of STPK genes in bacteria (2), no enzyme of this family has been demonstrated to be essential for bacterial viability. Our study now provides the first direct evidence that a eukaryotic-like Ser/Thr protein kinase, PknB, is essential for growth of both the pathogen *M. tuberculosis* and the saprophyte *M. smegmatis*.

What is the essential process(es) regulated by PknB? This kinase was first implicated as a potential regulator of cell growth and division, because of its localization close to the

TABLE 1. Antibacterial effect of selected PknB inhibitors

Bacterial strain[b]	MIC (μM)[a]		
	K-252-a	K-252-b	Staurosporine
M. tuberculosis H37Rv	20 (8)	>40 (2)	25 < MIC < 50 (3)
M. smegmatis mc²155	20 (5)	>40 (2)	
M. aurum A+	5 (3)	>40 (2)	
M. smegmatis mc²155 + pOMK	20 (2)		
M. smegmatis mc²155 + pOMK-*pknB*	40 (2)		

[a] MICs were determined using the resazurin microtiter assay (28), and values in parentheses indicate the number of independent experiments.
[b] + pOMK and + pOMK-*pknB*, strain transformed with empty pOMK vector and with pOMK-*pknB*, respectively.

chromosomal origin of replication and since the operon also includes genes known to be important for these processes (*rodA* and *pbpA*) (3). More recent results demonstrated that PknB is expressed predominantly during exponential growth (25) and upon infection of THP-1 human macrophages (40). Furthermore, the depletion or overexpression of PknB (and PknA) alters cell morphology, lending further support to its involvement in cell shape and cell division control (25). However, the physiological substrate(s) of PknB are currently unknown. The optimal PknB substrate in soluble protein extracts from *M. tuberculosis* was identified as GarA (43), an FHA domain-containing protein that has been linked both to glycogen degradation during exponential growth of *M. smegmatis* (6) and to regulation of the tricarboxylic acid cycle in *Corynebacterium glutamicum* (30). However, other putative PknB substrates that could be involved in downstream signaling events have also been proposed, such as penicillin-binding protein PbpA (14) or Rv1422 (25), and further biological studies are clearly required to elucidate the actual signaling pathway(s). Similar uncertainty concerns the signal sensed by the PknB extracellular ligand-binding region, which comprises four copies of the recently described penicillin-binding protein and serine/threonine kinase-associated (PASTA) domain. It has been speculated that PASTA domains could bind unlinked peptidoglycan (46) but, to our knowledge, no experimental evidence is currently available to support this claim.

PknB is conserved not only in actinobacteria (7)—a PknB-like protein kinase also is found in a large number of more distantly related gram-positive bacteria (9). In *B. subtilis*, the *prkC* (*pknB*-like) gene has also been proposed to control developmental processes, since disruption of the gene impairs sporulation efficiency and reduces biofilm formation (27). However, the mutant lacking *prkC* showed no differences in growth and morphology when compared with the wild-type strain, in contrast with our present results for mycobacteria. These observations suggest that *pknB*-like genes could have an ancient evolutionary origin in gram-positive bacteria, so that the biological processes that they control have significantly diverged in response to specific bacterial adaptation to the environment. Alternatively, PknB and PrkC may be functionally unrelated, as *prkC* is neither located in an operon resembling that found in actinobacteria nor situated close to *oriC*.

To further assess PknB essentiality, we tested the effect of ATP-competitive STPK inhibitors on bacterial growth. Two out of 18 compounds tested (K-252-a and K-252-b) were found to inhibit PknB in vitro with an IC_{50} in the 100 nM range. The MIC of K-252-a against different slow- and fast-growing mycobacterial strains was found to be 5 to 20 μM. To address the question of whether PknB was the actual molecular target, we transformed *M. smegmatis* mc²155 with a multicopy replicative plasmid expressing *M. tuberculosis* PknB. This overexpressor exhibited a twofold increase in resistance to K-252-a compared with the control transformant, supporting the hypothesis that PknB inactivation is related to the inhibition of bacterial growth.

Eukaryotic protein kinases have become one of the most important groups of drug targets (10), and large chemical libraries of specific protein kinase inhibitors are currently available. Thus, the evidence that PknB, a structurally and mechanistically eukaryotic-like STPK (31, 47), is an essential enzyme in *M. tuberculosis* and that PknB inhibitors, despite being selected from a small test panel, may have a significant antibacterial effect strongly suggests that STPKs may represent new therapeutic targets for antituberculosis drug design. We anticipate that further inhibitor screenings followed by compound optimization may lead to novel antibiotics of value against tuberculosis and note that other STPKs, such as PknG (44), may also be tractable targets.

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