RESEARCH LETTER

Mycobacterium smegmatis biofilm formation and sliding motility are affected by the serine/threonine protein kinase PknF

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Received 2 August 2007; accepted 8 October 2007.
First published online December 2007.

DOI:10.1111/j.1574-6968.2007.00989.x

Editor: David Clarke

Keywords
mycobacteria; serine/threonine kinase; GPLs; sliding motility; biofilm;cell morphology.

Abstract

Eighteen 'eukaryotic-like' serine/threonine kinases are present in the *Mycobacterium smegmatis* genome. One of them encoded by the ORF 3677 demonstrates high similarity to the *Mycobacterium tuberculosis* protein kinase PknF. A merodiploid strain was generated, which showed reduced growth associated with irregular cell structure. The merodiploid strain displayed altered colony morphology, defective sliding motility and biofilm formation. These data indicate a role for PknF in biofilm formation, possibly associated with alterations in glycopeptidolipid composition.

Introduction

Serine/threonine protein kinases (STPKs) are highly conserved key mediators of cellular signaling in eukaryotes and have a similar role in many prokaryotes (Cozzone, 2005). While the genome of *Mycobacterium tuberculosis* contains 11 putative STPKs (Cole *et al.*, 1998), the genome of *Mycobacterium smegmatis* is predicted to encode 18 putative STPKs (www.tigr.org) (supplementary Table S1). Most of the annotated STPKs in *M. tuberculosis* have several homologues in *M. smegmatis* and a few kinases in *M. smegmatis* do not have homologues in *M. tuberculosis*. MSMEG3677 of *M. smegmatis* was identified as the closest homologue (64%) to PknF of *M. tuberculosis* (Fig. 1).

In this study, an attempt was made to decipher the role of *M. smegmatis pknF*. *Mycobacterium tuberculosis* PknF has been shown to be involved in cell growth, septum formation and glucose transport (Deol *et al.*, 2005) and to phosphorylate fork head associated domains of the proteins encoded by the ORFs Rv1747 and Rv0020 (Molle *et al.*, 2004; Grundner *et al.*, 2005). Sequence comparison showed that Ms-PknF possesses the previously unnoticed GXGXXGEV subdomain I of kinases, which are capable of covering and anchoring the nontransferable phosphates of ATP. We have cloned the gene encoded by ORF, MSMEG3677 (designated as Ms-*pknF* in this study), and overexpressed the protein in *M. smegmatis* mc^{2}155 under the control of an inducible acetamidase promoter, and studied the effect on growth and cell morphology of the merodiploid strain. The observed differences in colony morphology prompted studies on sliding motility and biofilm formation. This is the first report on the functional analysis of a putative STPK from *M. smegmatis* and provides new information about the control of biofilm formation by kinases in mycobacteria.

Materials and methods

Molecular biological procedures

Standard molecular biology techniques were followed for the DNA manipulations as described earlier (Sambrook *et al.*, 1989). Electroporation in mycobacteria was carried

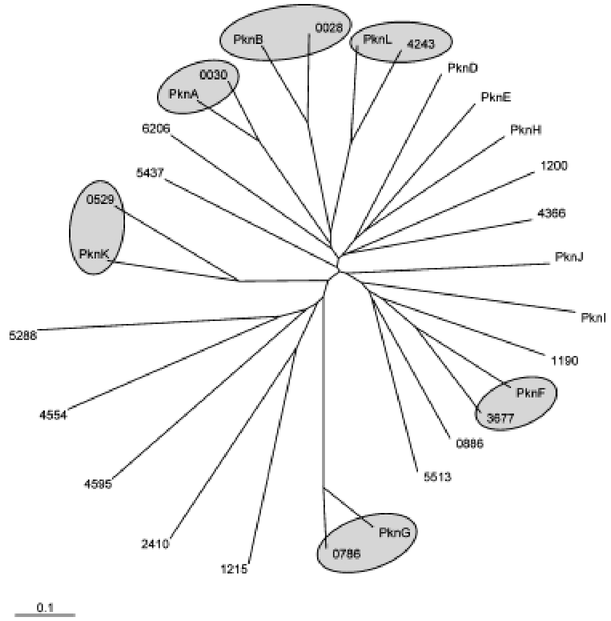

Fig. 1. Dendrogram of a multiple sequence alignment of all the STPKs from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Sequences of *M. tuberculosis* (designated as Pkn's) and *M. smegmatis* (designated by locus number) kinases obtained from the Tuberculist and TIGR database respectively were aligned using CLUSTALW and the dendrogram displayed using TREEVIEW (version 1.6.6). The scale bar indicates a distance of 0.1 amino acid substitutions per position in the sequence. Closest homologues between *M. tuberculosis* and *M. smegmatis* kinases are shaded in gray.

out following the published protocols (Snapper *et al.*, 1990). All restriction and modifying enzymes were purchased from New England Biolabs and all chemicals were procured from Sigma (Sigma-Aldrich). The DNA sequence of all PCR products was confirmed by automated sequencing (Applied Biosystem Genetic Analyzer, model 3100).

Bacterial strains and growth conditions

Mycobacterium smegmatis mc^{2}155 was grown in Luria–Bertani (LB) broth with 0.05% Tween80 and plated in the same medium with 1.5% agar supplemented with appropriate antibiotics (100 $\mu g\,mL^{-1}$ of hygromycin and 20 $\mu g\,mL^{-1}$ of kanamycin, wherever applicable) unless otherwise indicated. *Escherichia coli* DH5α or TOP10 cells (Invitrogen Inc.) were grown in LB medium with appropriate antibiotics (150 $\mu g\,mL^{-1}$ of hygromycin and 40 $\mu g\,mL^{-1}$ of kanamycin, wherever applicable).

DNA manipulations

The 1.4-kb coding region of MSMEG3677 was PCR amplified from the genomic DNA of *M. smegmatis* mc^{2}155 using oligomer pair 5′-CGCGGATCCGCGATGCCACTGGCC GCTGGGGAGA-3′ (with *Bam*HI overhang) and 5′-GGG CAGGCCGTTGCTGCGAC-3′ and *Tgo* DNA polymerase (Roche). The amplified product was purified and cloned into the BamHI and EcoRV site of pSD26 (Daugelat *et al.*, 2003), an *E. coli–Mycobacterium* shuttle vector containing the acetamidase promoter to create pRGS5. The MSMEG3677 gene was cloned without its stop codon in frame to the His-tag preceding the stop codon in pSD26. Replacement of the ATP-binding domain lysine (K) at position 41 to methionine (M) was performed using a Quik-Change site directed mutagenesis kit (Stratagene) following the supplier's instructions. The primers used for creating lysine to methionine mutation were 5′-GATGC GCTCATGGTGCTGCC-3′ and 5′-GGCAGCACCATGAGC GCATC-3′. The resulting plasmid was designated as pRGS5-K41M. The MSMEG3677 gene with acetamidase promoter was subcloned from pRGS5 and pRGS5-K41M into mycobacterial integrative vector pMV306 (Stover *et al.*, 1991) to create pRGS7 and pRGS7-K41M, respectively. The control plasmid pAce-306 was created by introducing the acetamidase promoter into pMV306.

Overexpression of Ms-PknF by acetamide induction

The plasmids (Table 1) were introduced into *M. smegmatis* mc^{2}155 by electroporation and strains were designated as indicated. For the induction experiments, 0.2% acetamide (final concentration) was added to broth or solid medium containing 0.05% Tween80. Lysate was prepared using glass beads (106 μm and finer) as described previously (Hatfull & Jacobs, 2000). The concentration of total cellular protein was estimated with a BCA protein assay reagent kit (Pierce) and equal amounts (0.5 μg) of protein was separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Blots were incubated with 1 : 5000 anti-His C-terminal horseradish peroxidase antibody for 1 h at room temperature. Positope was included as the positive control for the antibody (Invitrogen Inc.). The blots were processed using the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) following the manufacturer's instructions.

In vitro growth kinetics of *M. smegmatis* strains

Fresh mid log-phase cultures of control, strains overexpressing Ms-PknF and its kinase mutant Ms-PknF-K41M were diluted in LB (supplemented with 0.05% Tween80) to an initial $OD_{600\,nm}$ of 0.05. One set of cultures was induced with acetamide and the other set was left untreated. Aliquots of 1 mL were removed at 3 h intervals up to 24 h and growth was monitored by measuring OD_{600} values. The experiment was performed in triplicate.

Table 1. List of plasmids and strains used in this study

Plasmid/Strain	Description	Source
Plasmids		
pMV306	Integration proficient *E. coli–Mycobacterium* shuttle vector	Stover *et al*. (1991)
pSD26	*E. coli– Mycobacterium* shuttle vector carrying acetamidase promoter.	Daugelat *et al*. (2003)
pRGS5	pSD26 harbouring 1482-bp Ms-PknF	This study
pRGS5-K41M	pSD26 harbouring 1482-bp Ms-PknF bearing a K to M mutation at position 41	This study
pAce-306	pMV306 derivative harbouring acetamidase promoter from *M. smegmatis*	This study
pRGS7	pMV306 carrying the acetamidase promoter driving Ms-PknF	This study
pRGS7-K41M	pMV306 carrying the acetamidase promoter driving Ms-PknF bearing a K to M mutation at position 41	This study
Strains		
mc^2155	Laboratory strain	Snapper *et al*. (1990)
mc^24808	*M. smegmatis* bearing integrated pAce-306; Control strain	This study
mc^24809	*M. smegmatis* bearing integrated pRGS7 overexpressing Ms-PknF	This study
mc^24816	*M. smegmatis* bearing integrated pRGS7-K41M overexpressing Ms-PknF-K41M	This study

Scanning electron microscopy (SEM)

Strains overexpressing Ms-PknF and Ms-PknF-K41M as well as control strain grown in LB medium (supplemented with 0.05% Tween80) with or without acetamide were subjected to SEM as described earlier (Vilcheze *et al*., 2000). The samples were examined at $6000\times$ in a JEOL-JSM6400 SEM (Peabody, MA) using an accelerating voltage of 10 kV.

Colony morphology of merodiploid strains

Control, strains overexpressing Ms-PknF and Ms-PknF-K41M were grown in 7H9 media (supplemented with 1% glucose and 0.05% Tween80) and 10 μL of each strain was spotted onto 7H9 agar plates with or without acetamide (supplemented with 0.5% glycerol and 0.05% Tween80). Plates were incubated at 37 °C for 2–3 days.

Sliding motility of mycobacteria

The three *M. smegmatis* strains (control and strains overexpressing Ms-PknF and Ms-PknF-K41M) grown on 7H9 agar plates with acetamide were inoculated via sterile toothpicks onto 7H9 plates containing 0.3% agarose without any carbon source. The plates were incubated at 37 °C for 2–3 days.

Determination of biofilm formation

Mycobacterium smegmatis strains were inoculated as 10 μL of a saturated culture onto 1 mL of M63 medium supplemented with 0.5% Casamino acids, 1 mM $MgSO_4$ and 0.7 mM $CaCl_2$ with acetamide. Cells were grown in a 24-well flat bottom plate (polystyrene) and incubated at 30 °C without disturbance for 4–5 days. Strains overexpressing Ms-PknF, and Ms-PknF-K41M, and control were grown and induced in 24-well plates and stained with crystal violet (CV) as described previously. Quantitation of biofilm was carried out by extracting the CV with 95% ethanol and measuring $OD_{570\,nm}$ (Recht *et al*., 2000).

Results and discussion

PknF of *M. tuberculosis* was shown to be involved in glucose transport and cell division (Deol *et al*., 2005). In this work, we identified MSMEG3677 gene from *M. smegmatis* as a homologue to *pknF* of *M. tuberculosis* (Fig. 1). We have analyzed the role of Ms-PknF by overexpressing it in *M. smegmatis* under the control of an inducible promoter and studied its physiological role.

Cloning and overexpression of the *M. smegmatis pknF* homologue

To study the physiological role of MSMEG3677 in mycobacteria, we have cloned and overexpressed the MSMEG3677 gene and a K41M kinase dead mutant in *M. smegmatis* mc^2155 using an inducible acetamidase promoter. We used a K41M point mutation because this lysine residue, located in subdomain II of STPKs, is highly conserved and is essential for the kinase activity (Hanks *et al*., 1988). Loss of the kinase function by a mutation of lysine to methionine has been described earlier (Molle *et al*., 2003; Deol *et al*., 2005) and we included this strain to establish whether the observed phenotype was due to the kinase function or overexpression of protein. Expression of the recombinant *M. smegmatis* PknF homologue was confirmed by visualization of an ~56-kDa band by Western blotting against the C-terminal His-tag of the recombinant protein (Fig. 2a).

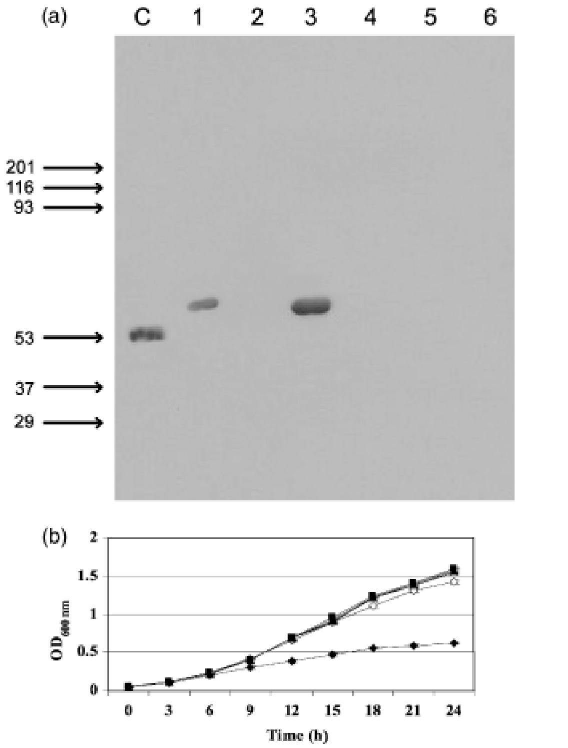

Fig. 2. Overexpression of Ms-PknF in *M. smegmatis* mc[2]155 and its *in vitro* growth characteristics. (a) Western blot with Anti-His C antibody shows expression of cloned Ms-PknF gene. Equal amounts (0.5 μg) of cell lysates were separated on 10% SDS-PAGE and electroblotted. Blots were probed with anti-HisC antibody. Lane C, positope (Invitrogen Inc.) was included as a positive control for the antibody; Lanes 1, 2, strain harboring acetamidase driven Ms-PknF induced (Ace+) or uninduced (Ace −); Lanes 3, 4, strain harboring acetamidase driven Ms-PknF-K41M induced (Ace+) or uninduced (Ace −); Lanes 5, 6, Control induced (Ace+) or uninduced (Ace −). Numbers indicate molecular weight standards. (b) *In vitro* growth kinetics of merodiploid strain. Strains harboring acetamidase driven Ms-PknF, Ms-PknF-K41M and control were inoculated at a final OD_{600nm} of 0.05 into fresh medium and either induced with 0.2% acetamide or left uninduced. Aliquots of 1 mL were taken out from control uninduced (□) and induced (■), merodiploid strain harboring Ms-PknF uninduced (◇) and induced (◆) as well as merodiploid strain harbouring Ms-PknF-K41M uninduced (Δ) and induced (▲) every 3 h up to 24 h and measured for OD_{600nm}. Values plotted are the averages and SD from triplicate experiments.

In vitro growth kinetics of the merodiploid strain

Mycobacterium smegmatis merodiploid strain expressing recombinant Ms-PknF showed growth retardation compared with control and the strain overexpressing the K41M mutant of Ms-PknF upon induction with acetamide. The difference in growth in liquid media was prominent as soon as 9 h after acetamide treatment and increased with time. The retardation of growth started at early exponential phase (9 h after induction), indicating that PknF interferes with early cell growth during the period of active cell division. Following 24 h of induction, a more than twofold reduction in growth was observed, with the induced cells overexpressing Ms-PknF compared with Ms-PknF-K41M and parental strain controls (Fig. 2b). In *M. tuberculosis*, overexpression of PknF resulted in slow growth, and the strain bearing a lysine to methionine mutation in subdomain II failed to show any phenotype associated with the merodiploid strain (Deol *et al.*, 2005).

Clumping of cells was observed with Ms-PknF overexpression, but not with Ms-PknF K41M, when grown beyond 24 h. Hence, growth was plotted only during the time that cultures were dispersed. Extensive clumping observed with sustained acetamide induction suggested a possible role for Ms-PknF in fatty acid metabolism and/or other pathways involved in the regulation of cell structure. Mycobacterial mutants that were defective in the synthesis of glycopeptidolipids (GPLs) have been shown to be associated with extensive clumping in liquid culture (Etienne *et al.*, 2002).

Morphology of merodiploid strains

Possible morphological variations, due to overexpression of Ms-PknF, were studied as individual cells and colonies. SEM was carried out on cells following 21 h of induction and compared with bacteria grown without induction. The strain overexpressing Ms-PknF following acetamide induction had irregular cell structure marked with bulb-like protrusions along the length or the ends of the cell, indicating abnormal or defective cell shape. However, neither induced nor uninduced cells expressing Ms-PknF-K41M showed such phenotypes (Fig. 3). Similarly, PknF of *M. tuberculosis* when disrupted or overexpressed had a comparatively small cell size with bulbous structures at one or both ends of the cells, indicating cell-deviant cell division (Deol *et al.*, 2005). This further indicated a functional homology among these kinases. However, further studies are required to delineate the stimuli these kinases perceive *in vivo*.

On acetamide induction, the strain overexpressing Ms-PknF revealed different colony morphology on agar media, showing partially rough cells that tend to cluster towards the center of the colony. In contrast, strains overexpressing Ms-PknF-K41M and control showed smooth well-spread colonies (Fig. 4a).

Analysis of sliding motility and biofilm formation of merodiploid strains

Previous studies have shown that GPL-defective mutants like *mps* [coding for nonribosomal peptide synthetase (Billman-Jacobe *et al.*, 1999; Recht *et al.*, 2000)] and *mtf1* [coding for methyl transferase (Recht & Kolter, 2001)] had rough colony

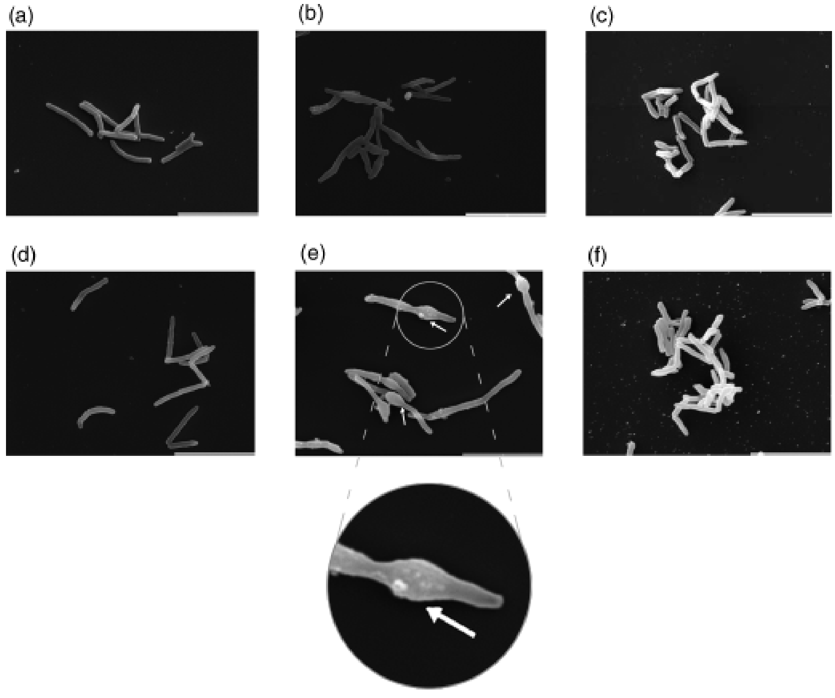

Fig. 3. SEM-Cultures of control, and strains overexpressing Ms-PknF and Ms-PknF-K41M were grown in absence (a–c) or presence (d-f) of 0.2% acetamide (uninduced and induced, respectively) for 21 h and samples were fixed and processed for electron microscopy as mentioned in Materials and methods. Cells were visualized and photographed at $\times$ 6000 in SEM. Arrows indicate the 'bulb' structure due to overexpression of MSMEG3677. The scale bar represents 5 μm. The 'bulb' from image 'e' is magnified $\times$ 3 and shown as an inset.

morphology. Differences in colony morphology were also shown to be associated with sliding motility (Martinez *et al.*, 1999; Recht & Kolter, 2001; Etienne *et al.*, 2005) and biofilm formation (Recht & Kolter, 2001). We examined the same by assessing the surface spreading assay and attachment of mycobacterial cells to polystyrene surface. The motility of the strain overexpressing Ms-PknF was reduced when compared with the strain overexpressing Ms-PknF-K41M and control strain (Fig. 4b). The measurement of the diameter of the halo formed on 0.3% agarose plates indicated a reduction in the sliding motility of the strain overexpressing Ms-PknF compared with control strains (Table 2). Colonies of Ms-PknF overexpressing strain grown on rich media showed no reduction in diameter (Fig. 4a) as compared with growth on minimal media (no carbon source) (Fig. 4b). Interestingly, overexpression of *gtf3*, a glycosyl transferase, had colonies grown on rich media displaying slightly increased diameter with rough morphology. However, the same study showed a reduction in sliding motility (Deshayes *et al.*, 2005). GPLs are found on the cell surface and are important for sliding on minimal media, and no such difference is observed when grown in rich media.

Using an assay to determine biofilm formation (Recht & Kolter, 2001), we found that *M. smegmatis* was able to cover polystyrene surface and formed biofilms on media with acetamide. However, *M. smegmatis* overexpressing Ms-PknF showed clumping and failed to spread on the surface of the polystyrene plate following acetamide induction (Fig. 4c). CV staining revealed that strains overexpressing Ms-PknF fail to form biofilm while the control strains are spread on the surface of the polystyrene plate (Fig. 4d). Quantitative estimation of CV staining also showed a significant reduction with Ms-PknF overexpression compared with Ms-PknF-K41M overexpression and control (Fig. 4e).

Ms-PknF overexpression was associated with reduced sliding motility and defective biofilm formation, which is an intermediate phenotype but not a complete nonsliding phenotype. Supporting this observation was a report on *atf1* mutant (*atf1* coding for a protein involved in GPL acetylation) that displayed intermediate phenotypes for sliding motility, biofilms and colony morphology (Recht & Kolter, 2001). A previous study proposed a model for the role of GPLs in sliding motility and biofilm formation based on interaction between the mycobacterial cell surface and either hydrophilic (agarose) or hydrophobic (polystyrene) surfaces (Recht *et al.*, 2000). Preliminary studies using thin layer chromatography showed an alteration in the GPL profile in strains overexpressing Ms-PknF (data not shown), suggesting a possible role for Ms-PknF in GPL metabolism. Ms-PknF being a kinase with an extracellular domain could sense the external signals and play a role in altering GPLs, resulting in observed phenotypes that are normally associated with the absence/alterations of GPLs. However, it has yet to be determined whether the slow growth and altered cell morphology observed is the direct result of changes in GPL composition or attributable to some other function of the kinase. This has also

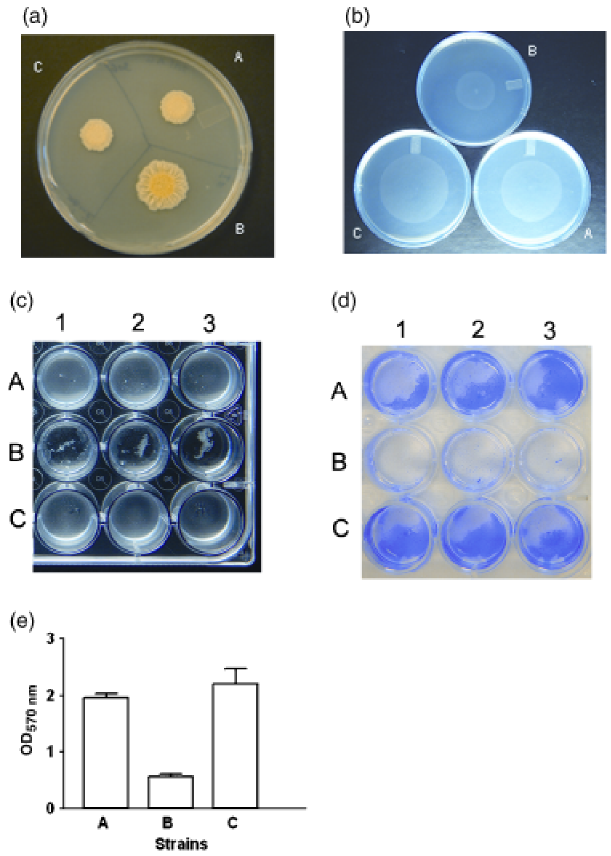

Fig. 4. Phenotypes associated with overexpression of Ms-PknF using acetamide induction. (a) Colony morphology: 10μL of the control, and strains overexpressing Ms-PknF and Ms-PknF-K41M (A, B and C, respectively) were spotted on 7H9-glycerol-Tween plates with acetamide and incubated at 37 °C. (b) Sliding motility: cells were spotted on 7H9 media with 0.3% agarose plates from 7H9-glycerol-Tween plates with acetamide using a sterile toothpick and incubated at 37 °C. Control, and strains overexpressing Ms-PknF and Ms-PknF-K41M are indicated as A, B and C, respectively. (c) Analysis of biofilm formation. Biofilm formation was analyzed by growing the strains in modified M63 media with acetamide in triplicate columns 1, 2 and 3. Control (row A), strain overexpressing Ms-PknF (row B) and strain overexpressing Ms-PknF-K41M (row C) were grown in triplicate in a 24-well plate. (d) CV staining. Control (row A), strain overexpressing Ms-PknF (row B) and strain overexpressing Ms-PknF-K41M (row C) were grown in triplicate (1, 2 and 3) in a 24-well plate. Cells were stained with 1% CV, washed and observed for crystal ring formation. (e) Estimation of CV uptake. Control (A), strain overexpressing Ms-PknF (B) and strain overexpressing Ms-PknF-K41M (C) were stained with 1% CV and washed with water a couple of times and CV dissolved using 95% ethanol. OD_{570nm} was measured to calculate the amount of CV uptake. The experiment was carried out in triplicate and the graph indicates the mean value with SE bars.

been reported earlier for the PknF of *M. tuberculosis* (Deol *et al.*, 2005).

In conclusion, our results indicated that overexpression of Ms-PknF affected cell structure in *M. smegmatis* and controlled its sliding motility and biofilm formation. The sliding properties reported in our study are pivotal for pathogenic mycobacterial species for colonizing surfaces in the environment and host under nutrient depletion conditions. Cell wall architecture gains importance in this regard for pathogenesis of the mycobacteria. Further work lies in understanding the GPLs involved in this Ms-PknF signaling and environment cues perceived by this kinase to bring about alterations in GPLs.

Table 2. Sliding motility of strains overexpressing Ms-PknF, Ms-PknF-K41M and control strains

Strains	Diameter (mm)
Control	40 ± 3
Strain overexpressing Ms-PknF	24 ± 2
Strain overexpressing Ms-PknF-K41M	46 ± 2

The experiment was performed in triplicate and the table represents the mean values of the readings.

Acknowledgements

R.G. was partly supported by the Council of Scientific and Industrial Research (CSIR), India; AECOM AIDS International Training and Research Program (D43 TW 001403; P. I. V. Prasad); and ICER grant of NIH to Tuberculosis Research Centre. Funding for this research was provided by the Canadian Institute of Health Research (CIHR) Grant # MOP-68857 (Y.A.) and the British Columbia TB Veterans Association.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. List of kinases from *M. smegmatis*.

Fig S1. Alignment of PknF kinase from mycobacteria.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00989.x. (This link will take you to the article abstract).

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