CHARACTERIZATION OF THE C-TERMINAL DOMAIN OF PROTEIN KINASE G OF *Mycobacterium tuberculosis*

Rossana Jaspe, Elba Guerrero and Leiria Salazar

SUMMARY

Mycobacterium tuberculosis *uses several strategies to evade the innate immune response. It has been suggested that the secretion of serine protein kinase G (PknG) inhibits the phagosome-lysosome fusion, allowing the survival of the mycobacteria. The protein PknG of* M. tuberculosis *harbor, in addition to the kinase domain, two thioredoxin (Trx) motifs and one tetratricopeptide (TPR) motif. In this work a* M. tuberculosis *PknG mutant (*M. tuberculosis *pknGΔCt::hyg) was constructed which has an interruption at the C-terminal region of* pknG *gene. No significant difference was observed during the exponential growth phase between the mutant and parental strains. However, survival of the mutant strain was much lower than that shown by the wild type strain during the stationary phase. This phenotype could not be complemented by the wild type PknG, probably due to the accumulation of the PknGΔCt mutant protein in the bacterial cell. Alteration of colony morphology in the mutant strain was also observed. Finally, using a bacterial two-hybrid system, the C-terminal domain of PknG was shown to be involved in its dimerization and interactions with its substrate.*

Introduction

PknG is one of the 11 serine/threonine protein kinases (STPK) present in *Mycobacterium tuberculosis* (Cole *et al*., 1998), which is involved in pathogenesis and metabolism. PknG interferes immune response in the normal host during mycobacterial infection by inhibiting the phagosome-lysosome fusion (Walburger *et al*., 2004). It is secreted into the macrophage cytosol (Walburger *et al*., 2004) where it selectively downregulates the mammalian PKC-α protein (Chaurasiya and Srivastava, 2009). The PknG protein of *M. tuberculosis* and *Corynebacterium glutamicum* is also involved in glutamate metabolism (Cowley *et al*., 2004; Niebisch *et al*., 2006; O'Hare *et al*., 2008); it phosphorylates the GarA protein that, in its non-phosphorylated state, inhibits the activity of a central enzyme complex involved in the tricarboxylic acid cycle (Niebisch *et al*., 2006).

The *pknG* gene is present in pathogenic and non-pathogenic mycobacteria with a high sequence homology in the kinase domain and the C-terminal region, but shows some variations at the N-terminus (Houben *et al*., 2009). The kinase domain of rhe PknG of *M. tuberculosis*, located at the center of the protein, is inactive being isolated and its total activity depends of the flanking domains (Tiwary *et al*., 2009). Mutations of the cysteine residues of the thioredoxin (Trx) motifs or deletion of the 73 amino acids N-terminus, containing the Trx motifs, result in a significant diminution of the PknG activity and reduction in the survival of mycobacteria in a host tissue (Scherr *et al*., 2007; Tiwary *et al*., 2009). The elimination of the last 330 aminoacids of the C-terminal region also leads to the reduction of the kinase activity (Tiwary *et al*., 2009). The PknG protein of *M. tuberculosis* has in this region a simple tetratricopeptide (TPR) motif. It is know that the TPR motifs mediate protein-protein interactions and often support the assembly of multiprotein complexes (Das *et al*., 1998). Unlike other protein kinases (Mieczkowski *et al*., 2008) the PknG of *M. tuberculosis* dimerizes via its TPR domain (Scherr *et al*., 2007) and not through of the kinase domain.

In the present work, a mutant strain of *M. tuberculosis* was constructed and characterized which has an interruption in the TPR motif coding sequence of the *pknG* gene and possibly produced a C-terminus truncated PknG protein (PknGΔCt). This interruption leads to decreased survival of the mutant strain at the stationary phase and alters colonial morphology in all phases of *in vitro* growth. Also, the mutant protein does not form dimers nor interacts with its substrate.

Materials and Methods

Bacterial strains, media, culture conditions and plasmids

Mycobacterium strains were cultured in Middlebrook 7H9 broth (Difco) supplemented with 0.5% glycerol (Difco), 0.05% Tween 80 and 10% of OADC (Difco). Growth on solid media was performed using Middlebrook 7H10 agar supplemented with 10% OADC. The cultures were incubated at 37°C for 3-4 weeks and were added 50μg/ml hygromycin B (Hyg), 25μg/ml (or 12.5μg/ml in allelic exchanged) kanamycin (Km), and 2% sucrose when necessary. *M. tuberculosis* strains were grown in a biosafety level 3 laboratory. *Escherichia coli* XL1-Blue was used for cloning and plasmid propagation. BTH101 strain (Euromedex, EUB001) was used during two-hybrid assays and grown on supplemented MacConkey agar base medium.

KEYWORDS / Deletion / Kinase / *M. tuberculosis* / Mutant / TPR Motifs /

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CARACTERIZACIÓN DEL DOMINIO C-TERMINAL DE LA PROTEÍNA QUINASA G DE *Mycobacterium tuberculosis*

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RESUMEN

Mycobacterium tuberculosis *utiliza varias estrategias para evadir la respuesta inmune. Se ha sugerido que la secreción de la proteína serina quinasa G (PknG) inhibe la fusión fagosoma-lisosoma, lo que permite la supervivencia de las micobacterias en el macrófago. La proteína PknG de* M. tuberculosis *posee, además del dominio quinasa, dos motivos tioredoxina (Trx) y un motivo tetratricopéptido sencillo (TPR). En este trabajo se construyó una mutante de PknG de* M. tuberculosis *PknG (*M. tuberculosis *pknGΔCt:: hyg) por interrupción de la región C-terminal del gen* pknG. *No se observó diferencias significativas durante la fase exponencial de crecimiento de las cepas mutante y parental. Sin embargo, la supervivencia de la cepa mutante fue mucho menor que la mostrada por la cepa de tipo salvaje durante la fase estacionaria. Este fenotipo no pudo ser complementado por la proteína PknG silvestre, probablemente debido a la acumulación de la proteína mutante PknGΔCt en la célula bacteriana. También se observó alteración de la morfología de las colonias de la cepa mutante. Por último, utilizando un sistema de dos híbridos bacterianos, se observó que el dominio C-terminal de PknG está involucrado en su dimerización y en las interacciones con su sustrato.*

CARACTERIZAÇÃO DO DOMINIO C-TERMINAL DA PROTEÍNA QUINASE G DE *Mycobacterium tuberculosis*

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RESUMO

Mycobacterium tuberculosis *utiliza várias estratégias para evadir a resposta imune. Tem sido sugerido que a secreção da proteína serina quinase G (PknG) inibe a fusão fagosoma--lisosoma, o que permite a sobrevivência das micobactérias no macrófago. A proteína PknG de M. tuberculosis possui, além do domínio quinase, dois motivos tioredoxina (Trx) e um motivo tetratricopéptide simples (TPR). Neste trabalho se construiu um mutante de PknG de* M. tuberculosis *PknG (*M. tuberculosis *pknGΔCt:: hyg) por interrupção da região C-terminal do gen pknG. Não se observou diferenças significativas durante a fase exponencial de crescimento das cepas mutante e parental. No entanto, a sobrevivência da cepa mutante foi muito menor que a mostrada pela cepa de tipo selvagem durante a fase estacionaria. Este fenótipo não pode ser complementado pela proteína PknG silvestre, provavelmente devido à acumulação da proteína mutante PknGΔCt na célula bacteriana. Também se observou alteração da morfologia das colônias da cepa mutante. Por último, utilizando um sistema de dois híbridos bacterianos, se observou que o domínio C-terminal de PknG está envolvido em sua dimerização e nas interações com seu substrato.*

Construction of a M. tuberculosis pknGΔCt::hyg *mutant and its complemented strain*

The coding sequence of the *pknG* gene of *M. tuberculosis* H37Rv has 2253bp in length and the TPR motif coding sequence is located between nucleotides 1602 and 1704 (Cole *et al*., 1998). In order to interrupt the TPR motif of PknG, the method of gene replacement by homologous recombination described by Parish and Stocker (2000) was used. A 3030bp fragment containing the *pknG* gene and 780bp of the upstream flanking gene, *ackA*, was PCR amplified using the FTrxyM (5´-CCCAAGCTTATGGCCAAAGCGTCAG-3´) and RTPR (5´-ACGGCGGCACGGAGTTTCTAGAGC-3´) primers and genomic DNA as template. The resulting PCR product was cloned in the *Hind*III/*Not*I sites of the plNIL vector. The *ΔpknG::hyg* allele was constructed by insertion of the hygromycin resistance cassette (*hyg*) of the pIJp63 plasmid (Salazar *et al*., 1996) in the *Kpn*I site of *pknG* gene, resulting in the partial deletion of the TPR domain. Next, a genetic market from pGOAL17 that contains the counter-selectable marker *sacB* was cloned into the *Pac*I unique site of the recombinant plasmid to produce the suicide p*PknGΔTPR::hyg* plasmid, which was used to electroporate *M. tuberculosis* H37Rv. Isolates double cross-over recombinants were termed *M. tuberculosis pknGΔCt::hyg*.

A complementary strain was also constructed by integration of an intact *pknG* gene of *M. tuberculosis* into the *atΔtB* site (Dussurget *et al*., 1999) of *M. tuberculosis pknGΔCt::hyg*. The wild type *pknG* gene was PCR amplified using the FcomplG (5´CGCAAGCTTGGCACCTGGAAC3´) and RcomplG (5´CGAAGCTTCACACTCGGGCG3´) primers and genomic DNA from *M. tuberculosis* H37Rv. The amplicon obtained was cloned into the *Hind*III site of pGFP22-4-*int* (Salazar *et al*., 2003) resulting in the pCompl-*pknG* plasmid, where the *dnaA* promoter of *M. tuberculosis* controls the expression of *pknG*. This plasmid was electroporated into *M. tuberculosis pknGΔCt::hyg* cells and the resulting strain was named *M. tuberculosis CompleG*, which was confirmed by PCR and Southern blot analysis.

Southern blot analysis

Chromosomal DNA (3µg) of the wild type, single crossover, mutant and complemented strains was digested with *Bgl*I, analyzed by electrophoresis in 0.8% agarose gel and transferred to a Nylon Immobilon-Ny^{+} membrane (Millipore). Southern blot was performed following standard protocols and using PCR fragments as probes processed for chemiluminescent detection using the 'ECL Direct Nucleic Acid Labelling and Detection System' kit (Amersham).

mRNA quantification of pknG

RNA was isolated as previously described (Salazar *et al*., 2003) from *M. tuberculosis* H37Rv and *M. tuberculosis pknGΔCt::Hyg* cultures grown until exponential and stationary growth phases (OD_{600nm}= 1.0-1.5 and 2.5-3.0, respectively). Total RNA was treated twice with DNAse (Promega) at 37ºC for 45min. Elimination of DNA was verified by PCR amplifica-

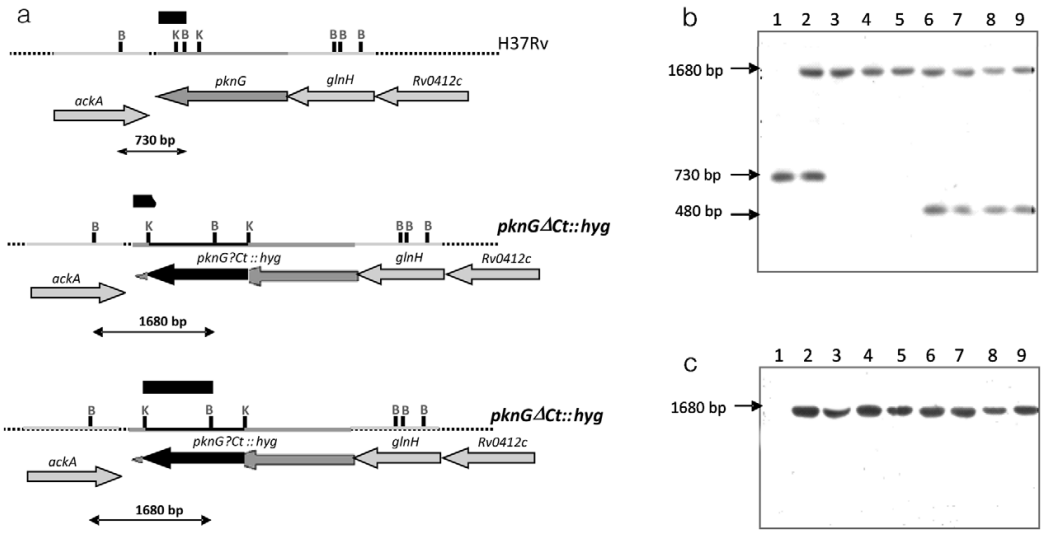

Figure 1. Evaluation of *Mycobacterium tuberculosis pknGΔCt::hyg* mutant strain. a: Schematic representation of the *pknG* locus of H37Rv (Cole *et al.,* 1998) and the corresponding *pknGΔCt::hyg* mutant allele. b: Southern blot analysis of *Bgl*I-digested genomic DNA probed with the 410bp fragment containing the *pknG* region. c: Southern blot analysis of *Bgl*I-digested genomic DNA probed with the 1000bp fragment containing the 3' *hygromycin* region. In b and c lanes are 1: *M. tuberculosis* H37Rv, lane 2: parental single cross-over recombinant, lanes 3 to 5: *pknGΔCt::hyg* mutants, lanes 6 to 8: complemented *pknGΔCt::hyg* mutants.

tion using specific primers. Total RNA (5ng·µl^{-1}; DNA-free) was denatured at 65ºC for 10min, chilled in ice-water and added to the reaction mixture of a final concentration containing 10mM each dNTP, 2.5µM of ramdom hexamers (Promega), 5mM $MgCl_2$, 1X AMV RT buffer, 20 U ribonuclease inhibitor (RNAsin, Promega) and 50 U AMV (Promega) reverse transcriptase, and incubated at 42ºC for 1h. The cDNA obtained was used as a template for PCR together with gene-specific primers (0.625µM of each one) and SYBR Green (New England BioLabs). Quantitative PCR was performed in a DNA Engine OPTICON, Continuous Fluorescence Detector (MJ Research, USA). The primers used were Rq*pknG*: 5´CGACCGACTGGGACTGAAGATC3´ (upstream to TPR domain coding sequence) and 16S-F: 5´ATGACGGCCTTCGGGTTGTAA3´/16S-R:5´CGGCTGCTGGCACGTAG TTG 3´ (Shi *et al.*, 2003). A standard curve was performed for each pair of primers using serial dilutions of genomic DNA of *M. tuberculosis* H37Rv (10^6 to 10^3 molecules). The melting curve of each amplicon was determined at the end of each experiment.

In vitro growth studies

Seven-day cultures of the parental, mutant (*pknGΔCt::*Hyg) and complemented (*CompleG*) strains were diluted in Middlebrook 7H9-ADC fresh broth until they reached $O.D_{600nm}$= 0.0054, and incubated with gentle shaking at 37°C in order to follow the growth by cell density and to determine the colony forming units. Pictures of the colonies obtained on 7H10-OADC plates were taken using a Nikon 2× lens and a digital camera.

Production of polyclonal antibodies against PknG of M. tuberculosis

PknG of *M. tuberculosis* H37Rv was over-expressed as a chimeric protein (glutathione-S-transferase-PknG, GST-PknG) in *E. coli* XL1-Blue transformed with the plasmid pGEX-pknG, which was gently provided by Yogendra Singh (Centre for Biochemical Technology, Mall Road, Delhi, India). Purified GST-PknG chimeric proteins (2mg) where used to immunize two rabbits (Chemicon International Inc., Millipore). IgG antibodies were purified from serum obtained four weeks after immunization, using Protein A Sepharose Agarose Fast Flow (Upstate) following the manufacturer's recommendations.

Immunofluorescence microscopy

Aliquots of *M. tuberculosis* H37Rv and *M. tuberculosis pknGΔCt::hyg* cultures grown until early exponential, exponential and stationary growth phases (OD_{600nm}= 0.5, 1.0-1.5 and 2.0-3.0, respectively) were treated following the protocol described by Cimino *et al.* (2006) without the permeabilization step, with the following specifications: the primary antibody IgG α PknG-GST (1:100) and the secondary antibody IgG α-rabbit conjugated with Cy3 (Chemicon 1:200) were used in cell staining.

BACTH assay

The BACTH (bacterial adenylate cyclase-based two-hybrid) assay was performed using the BATCH system Kit (Euromedex). The *pknG*, *garA* and *pknGΔCt::hyg* genes were PCR amplified using genomic DNA of *M. tuberculosis* H37Rv or the p*PknGΔTPR::hyg* plasmid, according to the case. The amplified genes were cloned in-frame at the C-terminal end (in the pKT25 and pUCT18C plasmids) or the N-terminal region (in the pKNT25 and pUT18 plasmids) of the T25 or T18 domain coding sequence of the bacterial adenylate cyclase. Plasmids were introduced by electroporation into BTH101 cells and the transformed cells were assayed for β-galactosidase activity.

Results

A M. tuberculosis pknG *mutant with a truncated-C-terminal region*

The *pknG* gene from *M. tuberculosis* H37Rv was replaced by homologous recombination (Parish and Stoker, 2000) with the *pknGΔCt::hyg* interrupted gene, in which *pknG* has a partial deletion of the TPR coding sequence and insertion of a hygromycin resistance cassette. Sequencing of PCR products corresponding to mutant *pknG* gene in *M. tuberculosis pknGΔCt::hyg,* revealed the presence of a stop codon at nucleotide position 1776, suggesting that the PknG mutant protein (65.7kDa) has a truncated C-terminal domain (PknGΔTPR). As expected, there were no changes in the nucleotide sequence of the Trx and kinase domains. Based on *in silico* analysis of the *pknG* mutated gene, the translated PknGΔTPR protein contains the first 541 amino acids of the wild PknG protein, including the first six amino acids of the TPR domain, and 51 amino acids corresponding to the HygR cassette.

For genetic complementation analysis, a wild type copy of the *pknG* gene, transcriptionally fused to the *M. tuberculosis dnaA* promoter region, and cloned in the vector pGFP22-4-*int*, was electroporated into the mutant strain to generate the complemented strain *M. tuberculosis CompleG*. The mutant and complemented strains were analyzed and confirmed genotypically by Southern blot hybridization (Figure 1).

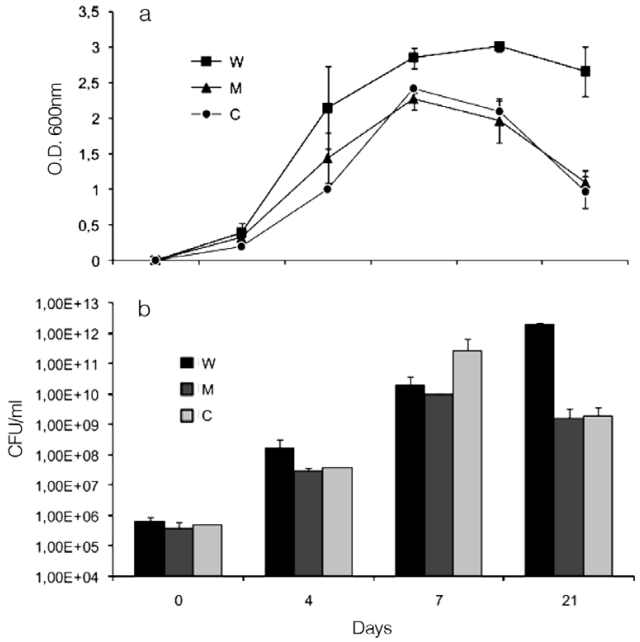

Figure 2. *In vitro* growth and survival of *M. tuberculosis pknGΔCt::hyg* mutant versus the parental and complemented strains. a: Growth in supplemented 7H9 media measured as OD_{600nm}. b: Viable bacterial counts are expressed as the number of colony counts/ml. W: *M. tuberculosis* H37Rv, M: *M. tuberculosis pknGΔCt::hyg* mutant, and C: complemented strain. Each point is the mean of three different experiments.

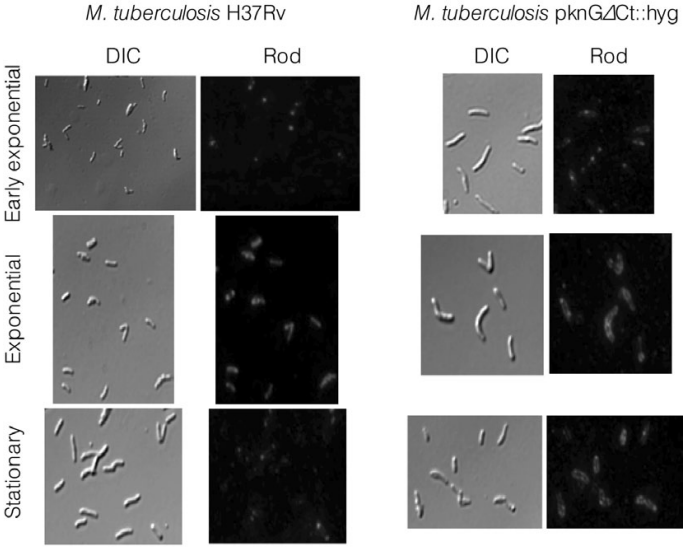

Figure 3. Localization of PknG in cells of *Mycobacterium tuberculosis pknGΔCt::hyg* mutant *vs* the parental strain using inmunofluorecense. Aliquots of the cultures were taken at early exponential, exponential and stationary growth phases. The cells were hybridized with IgG-anti-PknG and anti-rabbit coupled to Cy3 as primary and secondary antibodies. Photographs are displayed using DIC (differential interference contrast) filters and Cy3 (Rod). These images present the result of one typical experiment. Bar= 3μm.

In vitro growth and colony morphology of M. tuberculosis *pknGΔCt::hyg*

The growth of the mutant and complemented strains was evaluated and compared with that of the wild type strain. As seen in Figure 2a, the deletion of the C-terminal domain of PknG did not significantly affect the *in vitro* growth of *M. tuberculosis* during exponential phase, as both wild type and mutant strains showed similar growth patterns. However, after achieving stationary growth (at 12 days), the mutant strain showed a significant decrease in optical density, and the CFU/ml registered at 21 days was three logs of magnitude below that shown by the parental strain (Figure 2b).

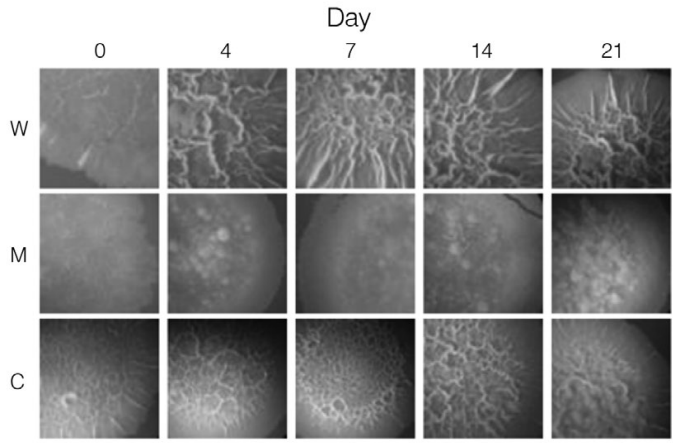

Figure 4. Colony morphology of *M. tuberculosis pknGΔCT::hyg* mutant *vs* the parental and complemented strains on Middlebrook 7H10 agar. Using a Nikon 2x magnifying lens and a digital camera, pictures were taken of the colonies of undiluted cultures of *M. tuberculosis* H37Rv (W), *M. tuberculosis pknGΔCt::hyg* mutant (M), and complemented strains (C) grown on 7H10 agar.

Immunofluorescence microscopy using an IgG α-PknG-GST showed a lower abundance of PknG in the mycobacteria envelope of the wild type strain in stationary phase, as compared to the one registered during the exponential phase. In contrast, in the *M. tuberculosis pknGΔCt::hyg* strain, PknG-ΔCt abundance was elevated during both phases (Figure 3). An increase of pknG transcription during stationary phase was discarded by analyzing mRNA levels of *pknG* by RTq-PCR (data not shown). Contrary to expectation, the introduction of an intact copy of the *pknG* gene in the mutant strain did not restore growth (Figures 2a and 2b).

To evaluate the effect of the PknG C-terminal domain deletion on colony morphology, undiluted aliquots of 0, 4, 7, 14 and 21 days old cultures (Figure 4) of the wild type, mutant and complemented strains were grown on solid medium. An altered morphology (flatter and less wrinkled colony) was observed with the mutant strain, which was partially restored to the wild type phenotype in the complemented strain.

Dimerization of PknG and interaction with its substrate

The TPR domain is implicated in protein-protein interactions, and in PknG it has been shown to be involved in the formation of dimers during the crystallization of the protein (Scherr *et al.*, 2007). In order to determine if the deletion of the C-terminal domain of PknG, which contains the TPR motif, affects the dimerization and interaction with its substrate (GarA; O' Hare *et al.*, 2008), the mutated and wild *pknG* and *garA* genes were amplified by PCR and cloned into the plasmids of the BACTH system. As seen in Figure 5, the removal of the C-terminal domain eliminated the dimerization of PknG and the interaction with its substrate GarA.

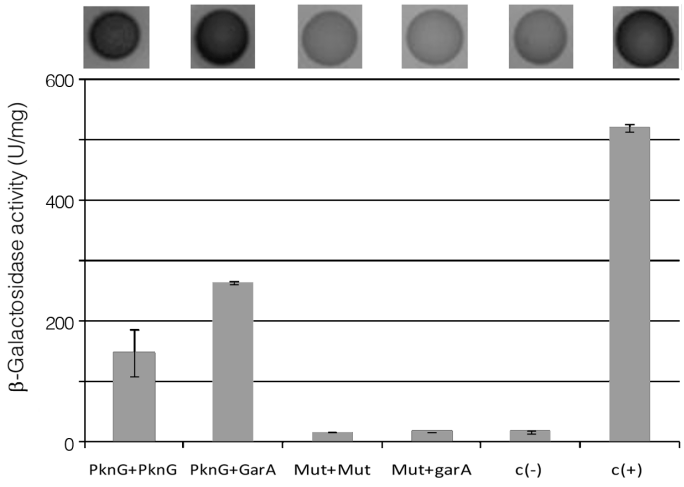

Figure 5. Dimerization of PknG and its interaction with GarA. The efficiencies of functional complementation between the indicated hybrid proteins were quantified by measuring β-galactosidase activities in suspensions of toluene-treated *E. coli* BTH101 cells harboring the corresponding plasmids. Each bar represents the mean value for at least three independent cultures. The first genes of each pair were cloned into the plasmid pUT18c and the second ones into pKT25. Mut: PknGΔCt; c(-): negative control (cells carrying pKNT25 and pUT18 plasmids); c(+): positive control (cells carrying pKT25-zip and pUT18c-zip).

Discussion

Comparative analysis of amino acid sequences of mycobacterial PknG proteins has shown that they have similar kinase, Trx and TPR domains (Houben *et al.*, 2009). In addition, the activity of the kinase domain of PknG of *M. tuberculosis* is regulated by the Trx and TPR domains (Tiwary *et al.*, 2009).

In order to determine the role of the C-terminal domain TPR motif in the function of the PknG protein, a mutant of *M. tuberculosis*, called *M. tuberculosis pknGΔCt::hyg* was constructed by inserting the hygromycin resistance cassette into the TPR motif (Figure 1). This mutant strain appears to produce a PknG protein without the C-terminal domain (PknGΔCt). Comparing the growth of the mutant strain with the wild type it was observed that the mutant bacterial cells have decreased survival during the stationary phase. On the other hand, the introduction of an intact copy of the *pknG* gene in this mutant strain does not restore the growth. However, this could be due in this case to that pknG expression being under the control of a heterologous promoter. The decreased survival of the mutant strain during the stationary phase is possibly due to the fact that in the absence of the C-terminal domain the PknGΔCt protein is accumulated in the cell, as suggested by the strong PknG signal observed in the mycobacteria cell envelope of the mutant cells during the stationary phase (Figure 3). The accumulation of this protein might be toxic, and this would explain the cellular death observed during *in vitro* growth (Figure 2a).

The behavior exhibited by the mutant strain *M. tuberculosis PknGΔCt::hyg* is different to that observed in *M. tuberculosis* lacking a complete PknG gene (Cowley *et al.*, 2004), which grows in 7H9 medium at a rate significantly lower than the parental strain, and whose wild type phenotype can be restored upon complementation.

The colonial morphology of the mutant strain *M. tuberculosis PknGΔCt::hyg* is altered (flatter and less wrinkled) in comparison to the wild type strain (Figure 4). As the deletion of the C-terminal domain of PknG leads to a decrease in the kinase activity (Tiwary *et al.*, 2009), it could be that some phosphorylation cascade involving PknG is altered, and this in turn modifies the cell envelope structure.

The lack of interaction observed in this work between the mutant protein PknG-ΔCt with itself, and with its substrate GarA (Figure 5), confirms that the TPR motif found in the C-terminal domain is required for both interactions. However, since PknG had been previously proposed to interact with GarA through phosphothreonine residues located in the N-terminal domain of the protein (O'Hare *et al.*, 2008; Scherr *et al.*, 2009), the lack of interaction between PknG-ΔCt and GarA could be result of conformational changes in the mutant protein, brought about by deletion of the C-terminus, which would prevent the interaction of its N-terminal domain with GarA. Alternatively, removal of the C-terminal domain of PknG has been shown to reduce the kinase activity of the protein (Tiwary *et al.*, 2009), and this in turn may affect the PknG autophosphorylation, a step probably needed by PknG to modulate its interaction with substrates. Thus the lack of interaction between PknGΔCt and GarA could be due to inadequate auto-phosphorylation of the mutant PknG.

The present results show that deletion of the C-terminal domain of the PknG protein of *M. tuberculosis*, which contains the TPR motif, stimulates the accumulation of the protein in the cell envelope. PknGΔCt induces decreased survival of the strain and alters bacterial colonies morphology. Furthermore, the C-terminal domain is important for dimerization of PknG and the interaction with its substrate, and therefore this domain must be required to PknG function.

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