

Role of Protein Kinase G in Growth and Glutamine Metabolism of *Mycobacterium bovis* BCG

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The survival of pathogenic mycobacteria in macrophages requires the eukaryotic enzyme-like serine/threonine protein kinase G. This kinase with unknown specificity is secreted into the cytosol of infected macrophages and inhibits phagosome-lysosome fusion. The *pknG* gene is the terminal gene in a putative operon containing *glnH*, encoding a protein potentially involved in glutamine uptake. Here, we report that the deletion of *pknG* did not affect either glutamine uptake or intracellular glutamine concentrations. In vitro growth of *Mycobacterium bovis* BCG lacking *pknG* was identical to that of the wild type. We conclude that in *M. bovis* BCG, glutamine metabolism is not regulated by protein kinase G.

The resistance of pathogenic mycobacteria to host innate immune responses has been attributed to their ability to block phagosome-lysosome fusion. This results in their capability to persist inside macrophages, avoiding bactericidal activities of the host macrophages (9, 15, 16). The genome of *Mycobacterium tuberculosis* encodes 11 eukaryotic enzyme-like serine/threonine protein kinases (1, 7). Among these, protein kinase G (PknG) was recently found to promote mycobacterial survival in macrophages. Although a role for PknG in mycobacterial physiology remains to be established, PknG blocks phagosome-lysosome fusion in infected macrophages where it is secreted into the cytosol. Disruption of the *pknG* gene in *Mycobacterium bovis* BCG by homologous recombination, as well as chemical inhibition of PknG by a specific inhibitor, leads to accelerated phagosome maturation and a growth defect in macrophages (19). The *pknG* gene is the last gene in a putative operon that includes *Rv0412c* (encoding an unknown membrane protein) and *glnH* (Fig. 1A) in all mycobacterial genomes sequenced. The *glnH* gene is predicted to encode a glutamine-binding lipoprotein that might be involved in glutamine import through the membrane (1, 6). In *Escherichia coli* and *Bacillus subtilis*, *glnH* is located in the glutamine permease operon including *glnP* and *glnQ*, which together form an ABC transporter. This operon is essential for glutamine import activity (13). In the *M. tuberculosis* genome, the putative glutamine importing system consists of five proteins encoded by genes locating in three different regions (GlnH, Rv2563 and GlnQ, and Rv0072 and Rv0073). GlnQ and Rv0073 are homologous to nucleotide binding proteins, while Rv2563 and

Rv0072 are membrane-spanning proteins. These two pairs of proteins are expected to form one or two separate glutamine transporters, while *glnH* is thought to encode the substrate binding protein (4).

Glutamine biosynthesis is catalyzed by glutamine synthase, which ligates the ammonium group to L-glutamate. The deletion of glutamine synthase in *M. tuberculosis* resulted in L-glutamine auxotrophicity and attenuated growth in human THP-1 macrophages (18). In addition, the glutamine synthase-negative mutant is avirulent in the highly susceptible guinea pig model of pulmonary tuberculosis (18). Depletion of glutamine synthase activity by a specific inhibitor (11) or antisense technology (12) has been shown to effectively inhibit growth of *M. tuberculosis*.

Given the importance of glutamine metabolism for mycobacterial survival and the possible location of *pknG* and *glnH* within the same operon, we analyzed the roles of PknG in glutamine metabolism and growth of *M. bovis* BCG.

Transcription of *glnH* and *ackA* in the presence or absence of *pknG*. To analyze possible coregulation of the genes located surrounding *pknG*, transcription of *glnH* (forward primer, 5'-TCGGGATCAACCTGGACAA-3'; reverse primer, 5'-GAGCACCGTCAGCCACTTG-3'), *ackA* encoding an acetate kinase (forward primer, 5'-GGGCGTCATCAGCTACTTGTG-3'; reverse primer, 5'-CCGCCAACCCCAACATC-3'), and *pknG* (forward primer, 5'-GCCACCGACATCTACACCGT-3'; reverse primer, 5'-GGTGTGCGCCACCAGCAG-3') was measured by RT-PCR. As a control, transcription of the 16S

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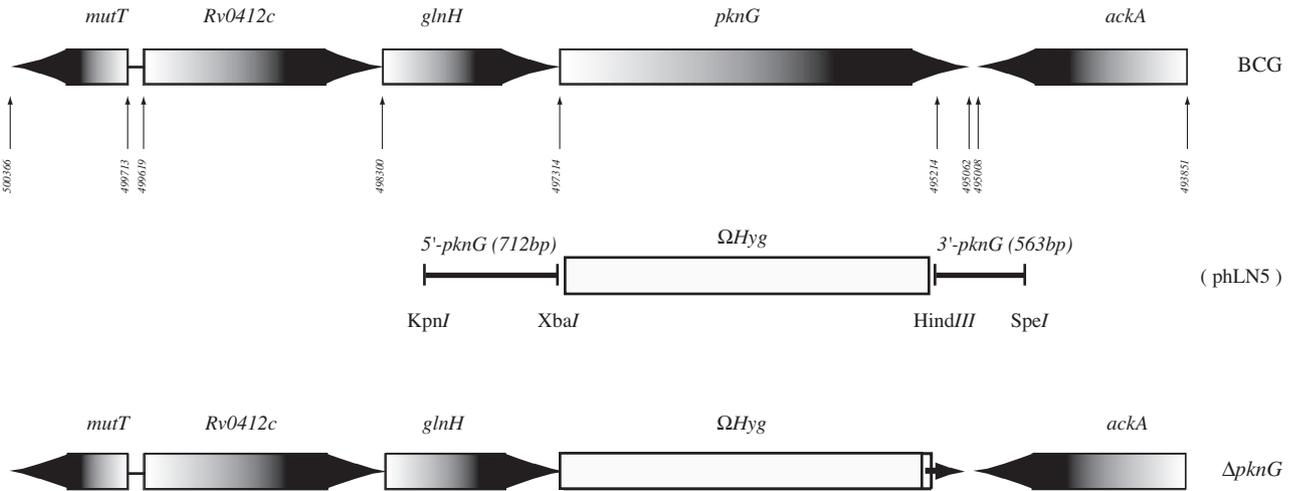
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TABLE 1. Free glutamine/glutamate levels in *M. bovis* BCG and *M. bovis* BCG- $\Delta pknG$ grown in Proskauer-Beck medium

Amino acid	Amino acid level (pmol) (mean \pm SD) of indicated <i>M. bovis</i> strain	
	BCG	BCG- $\Delta pknG$
Glutamine	5.77 \pm 0.24	6.1 \pm 0.21
Glutamate	36.57 \pm 1.11	40.20 \pm 1.17

A



B

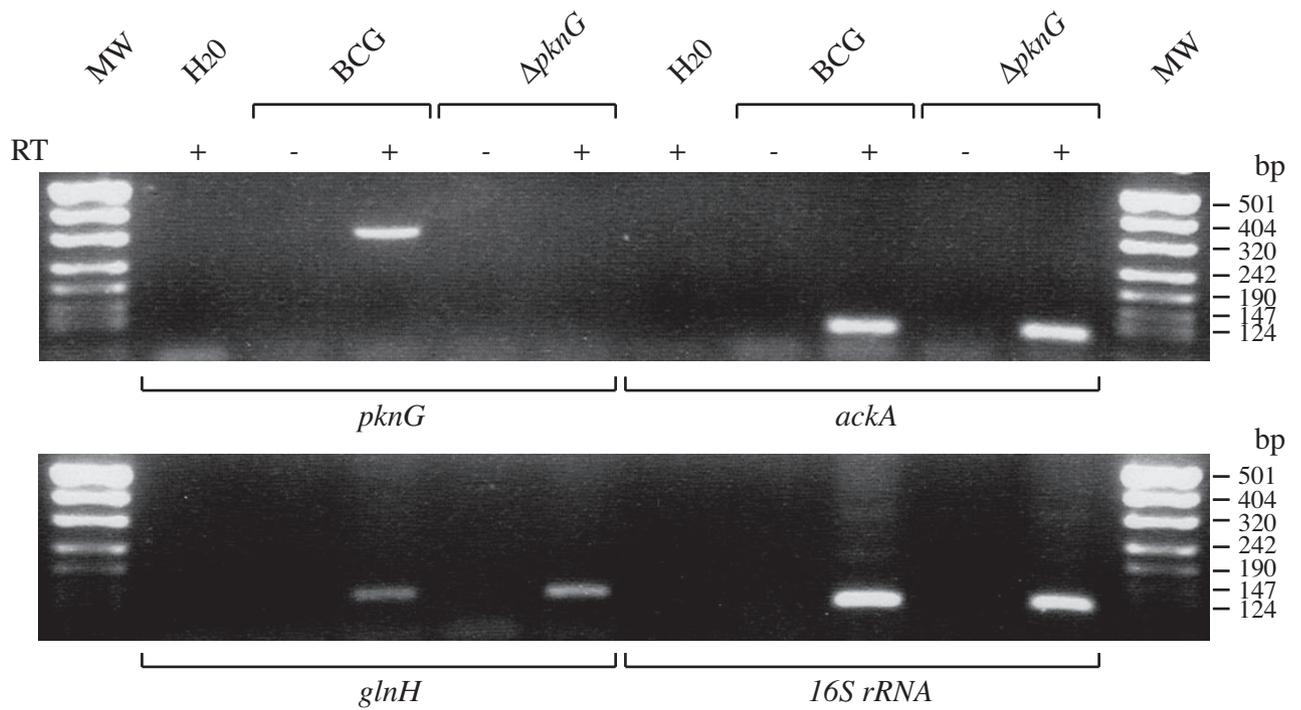


FIG. 1. (A) Genomic organization at the *pknG* locus. (B) RT-PCR analysis for expression of genes *ackA*, *glnH*, and *pknG* predicted to share the same operon. Expression of 16S rRNA genes was used as a control.

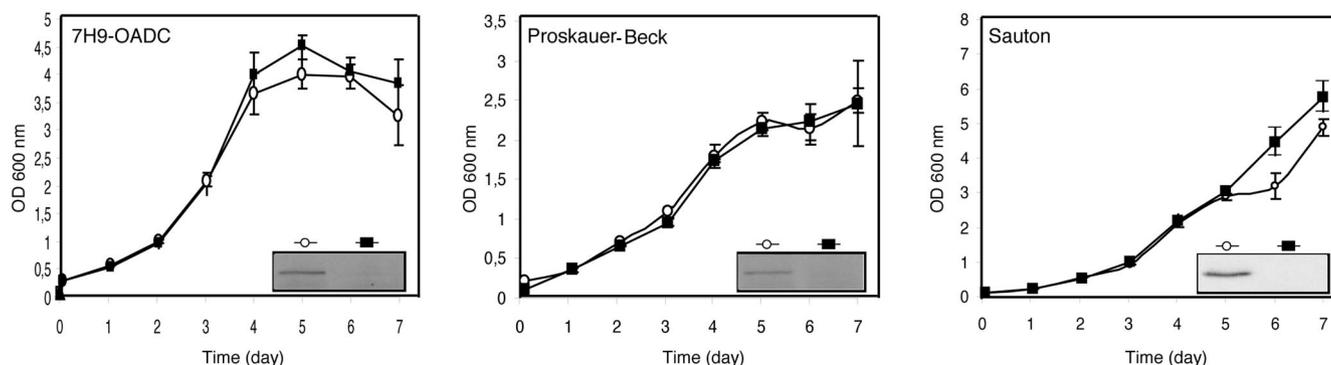


FIG. 2. In vitro growth of *M. bovis* BCG (○) and *M. bovis* BCG- $\Delta pknG$ (■) in 7H9-OADC, Sauton, and PB media. The expression levels of PknG in the different strains were analyzed at day 6 by immunoblotting of lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting (inset).

rRNA gene (forward primer, 5'-ACGAACAACGCGACAAA CC-3'; reverse primer, 5'-CCAGCAGCCGCGTAA-3') was also analyzed. RNA samples were isolated from wild-type *M. bovis* BCG and *M. bovis* BCG $\Delta pknG$ (BCG- $\Delta pknG$) (19) by using the RNeasy midi kit (QIAGEN Inc., Valencia, CA) and treated with DNase for 3 h at 37°C. To analyze transcription, cDNA was synthesized from 1 μ g of total RNA by using Superscript III (Invitrogen Life Technologies, Carlsbad, CA) and random primers (Promega Inc., Madison, WI), followed by amplification of the products by using gene-specific primers (see above).

No *pknG* transcript was detected in *M. bovis* BCG- $\Delta pknG$, whereas transcription of *glnH* and *ackA* was not effected in the $\Delta pknG$ mutant (Fig. 1B), suggesting that PknG does not control the transcription of *glnH* and *ackA*. In addition, this result excludes the possibility that the deletion of *pknG* by homologous recombination in the $\Delta pknG$ mutant resulted in impaired expression of *glnH* and *ackA*.

Effect of PknG depletion on in vitro growth. Changes in glutamine metabolism have been reported to alter the in vitro growth characteristics of mycobacteria (12, 18). To analyze a contribution of PknG to the in vitro growth of *M. bovis* BCG, wild-type *M. bovis* BCG, and *M. bovis* BCG- $\Delta pknG$ (strain Montreal) were grown in 7H9-OADC medium as well as in two different defined media, Sauton and Proskauer-Beck (PB)

media, whose nitrogen sources were based on asparagines, supplemented with 0.05% Tween 80 (14). The in vitro growth of *M. bovis* BCG- $\Delta pknG$ and the growth of its parental strain were monitored by measuring the optical densities of liquid cultures at a wavelength of 600 nm (OD_{600}). Saturated cultures (OD_{600} of 1.5) stored at -70°C were used to inoculate fresh media to the final OD_{600} of 0.1, and cultures were incubated at 37°C with orbital shaking. In none of the media tested was there any detectable alteration in the growth of *M. bovis* BCG- $\Delta pknG$ compared to that of its parental strain (Fig. 2).

Effect of PknG depletion on glutamine uptake. As *glnH*, the gene adjacent to *pknG*, has been suggested to be part of a glutamine transport system (4, 7, 8) and *glnH* and *pknG* may be part of the same operon, the contribution of PknG to glutamine transport was investigated. To that end, the uptake of [3 H]glutamine (Amersham) by *M. bovis* BCG or *M. bovis* BCG- $\Delta pknG$ grown in 7H9-OADC, PB, and Sauton media supplemented with 0.05% Tween 80 was analyzed. Bacterial cultures growing at exponential phase (OD_{600} of 0.5) were washed and diluted to an OD_{600} of 0.2 with fresh medium. Diluted cultures were then distributed in 96-well plates (in triplicate) and incubated for 2 h at 37°C before the addition of 1 μ Ci [3 H]glutamine to each well (final concentration of [3 H]glutamine, 0.1 μ M). After incubation times, cultures from

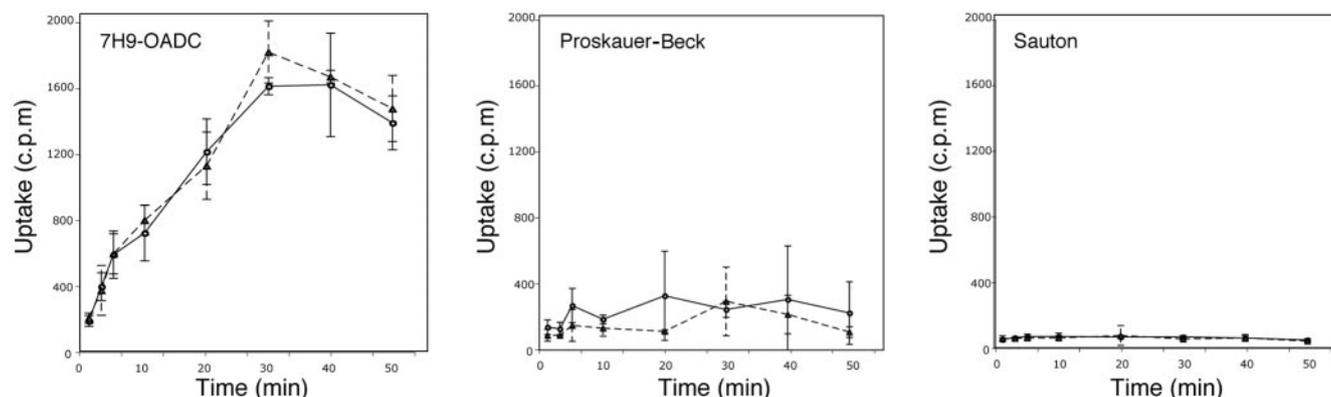


FIG. 3. Effects of PknG depletion on uptake of glutamine. Uptake of [3 H]glutamine by *M. bovis* BCG (○) and *M. bovis* BCG- $\Delta pknG$ (■) in 7H9-OADC, Sauton, and PB media.

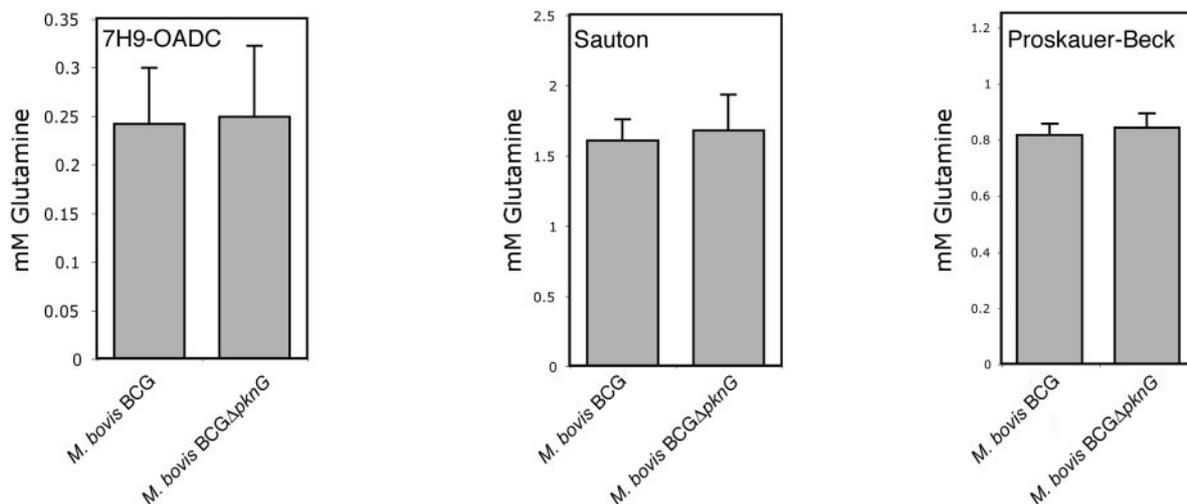


FIG. 4. Glutamine levels in lysates of *M. bovis* BCG and *M. bovis* BCG- Δ pknG as measured via enzymatic deamination by glutaminase.

plates were harvested to UniFilter plates (Packard) and washed to remove extracellular glutamine. Cell-incorporated radioactivity was counted by using the Topcount microplate scintillation counter (Packard). As shown in Fig. 3, no glutamine uptake activity was observed for the bacteria grown in PB and Sauton media. As expected, glutamine was readily internalized by strains grown in 7H9-OADC, reflecting the accelerated extracellular glutamine synthesis and transport when mycobacteria are grown in 7H9, which is supplemented with L-glutamate, the direct substrate of extracellular glutamine synthase (10). However, glutamine uptake was identical in *M. bovis* BCG lacking *pknG* (Fig. 3), indicating that PknG does not contribute to glutamine uptake.

Effect of PknG depletion on intracellular glutamine concentration. An impaired uptake of glutamine would affect the intracellular level of glutamine and other amino acids. To analyze intracellular glutamine levels, *M. bovis* BCG and *M. bovis* BCG- Δ pknG were grown in the different media indicated in Fig. 4 and homogenized, and aliquots equalized for similar protein amounts (bicinchoninic acid; Pierce) were analyzed spectrophotometrically for the presence of glutamine via enzymatic deamination by glutaminase (Sigma). *M. bovis* BCG and *M. bovis* BCG- Δ pknG grown in the different media indicated in Fig. 3 displayed equal levels of glutamine. To obtain an independent assessment of the levels of glutamine in wild-type *M. bovis* BCG and *M. bovis* BCG- Δ pknG, homogenates were either analyzed directly (Tables 1 and 2) or after hydro-

lysis (Table 3), followed by concentration and phenylisothiocyanate derivatization of the amino acids as described previously (2, 3, 5). The levels of the amino acids measured were similar between wild-type and *pknG* mutant bacteria (expressed as percentages of total amino acids determined [Tables 1 and 2]). Importantly, no difference in the levels of glutamine and glutamate was observed, indicating that in *M. bovis* BCG, glutamine does not accumulate upon *pknG* deletion.

Concluding remarks. The availability of glutamine for mycobacteria has been suggested to be crucial for growth and survival both in vitro and within macrophages (18). The positioning of a gene encoding a putative glutamine binding protein, *glnH*, adjacent to *pknG*, has led to the suggestion that in *M. tuberculosis*, one function of PknG is associated with glutamine metabolism (1, 8). While PknG may perform an as yet unknown function in mycobacterial physiology, the results presented here show that the depletion of PknG in *M. bovis* BCG has no effect on in vitro growth. In addition, there was no detectable defect in glutamine uptake or altered intracellular concentration observed in our experiments, suggesting that modulation of glutamine metabolism does not contribute to the intracellular degradation of *M. bovis* BCG lacking PknG (19).

There have been examples suggesting variations in gene functions and pathogenesis among *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG (17). The molecular basis for different roles described for PknG in these bacteria needs to be further characterized.

TABLE 2. Free glutamine/glutamate levels in *M. bovis* BCG and *M. bovis* BCG- Δ pknG grown in Sauton medium

Amino acid	Amino acid level (pmol) (mean \pm SD) of indicated <i>M. bovis</i> strain	
	BCG	BCG- Δ pknG
Glutamine	0.85 \pm 0.09	0.64 \pm 0.10
Glutamate	28.11 \pm 2.81	29.12 \pm 2.00

TABLE 3. Total glutamine levels in *M. bovis* BCG and *M. bovis* BCG- Δ pknG grown in Sauton and Proskauer-Beck media

Medium	Glutamine level (pmol) (mean \pm SD) of indicated <i>M. bovis</i> strain	
	BCG	BCG- Δ pknG
Proskauer-Beck	20.73 \pm 0.19	20.00 \pm 0.13
Sauton	11.28 \pm 0.15	11.20 \pm 0.41

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