**pckA-deficient Mycobacterium bovis BCG shows attenuated virulence in mice and in macrophages**

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Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the reversible decarboxylation and phosphorylation of oxaloacetate (OAA) to form phosphoenolpyruvate (PEP). In this study, the regulation of the PEPCK-encoding gene pckA was examined through the evaluation of green fluorescent protein expression driven by the pckA promoter. The results showed that pckA was upregulated by acetate or palmitate but downregulated by glucose. Deletion of the pckA gene of *Mycobacterium bovis* BCG led to a reduction in the capacity of the bacteria to infect and survive in macrophages. Moreover, mice infected with ΔpckA BCG were able to reduce the bacterial load much more effectively than mice infected with the parental wild-type bacteria. This attenuated virulence was reflected in the degree of pathology, where granuloma formation was diminished both in numbers and degree. The data indicate that PEPCK activity is important during establishment of infection. Whether its role is in the gluconeogenic pathway for carbohydrate formation or in the conversion of PEP to OAA to maintain the TCA cycle remains to be determined.

**INTRODUCTION**

Organisms growing on fatty acids as their limiting carbon source have been shown to mobilize the glyoxylate cycle to avoid loss of carbon as carbon dioxide and feed it, in the form of oxaloacetate (OAA), into gluconeogenesis. Phosphoenolpyruvate carboxykinase (PEPCK) catalyses reversibly the formation of phosphoenolpyruvate (PEP) and carbon dioxide from OAA and ATP or GDP, and is required to direct carbon flux into the gluconeogenic pathway in virtually all organisms.

The physiological roles of PEPCK vary in different organisms. For instance, in *Rhizobium* species (Osteras et al., 1991) and *Staphylococcus aureus* (Scovill et al., 1996), PEPCK functions as a key gluconeogenic enzyme. However, in *Plasmadium*, PEPCK catalyses the reverse reaction, decarboxylation of OAA, and is thought to produce succinate, aspartate and glutamate whilst bypassing the TCA cycle. In *Trypanosoma cruzi* (Cymeryng et al., 1995), *Ascaris suum* (Rohrer et al., 1986), *Ruminococcus flavefaciens* (Schocke & Weimer, 1997) and *Treponema pallidum* (Barbieri et al., 1981), PEPCK functions in the anaplerotic pathway, where it forms OAA from PEP. In addition, in *Escherichia coli*, PEPCK, together with the NAD- and NADP-dependent malic enzymes (MaeA and MaeB) and phosphoenolpyruvate synthase (PPS), is important for growth on C4 carbon sources (Hou et al., 1995). Clearly PEPCK fulfils many diverse roles in carbon metabolism in many organisms. We had shown previously that *Mycobacterium tuberculosis* in which the gene encoding the glyoxylate cycle enzyme isocitrate lyase (ICL) was disrupted was attenuated for maintenance of a persistent infection in mice and in activated but not resting macrophages (McKinney et al., 2000). We therefore wished to examine the role of PEPCK as a possible ‘downstream’ enzyme to the glyoxylate cycle.

*Mycobacterium bovis* Bacille Calmette–Guérin (BCG) is an attenuated strain of *M. bovis*, which is the causative agent of tuberculosis of cattle and belongs to the *M. tuberculosis* complex. An analysis of the whole genome sequence of *M. tuberculosis* shows that this bacterium possesses a putative GTP-dependent PEPCK encoded by the gene pckA. Mukhopadhyay et al. (2001) showed that PEPCK from *Mycobacterium smegmatis* preferred the gluconeogenesis/glycerogenesis direction. Recently, we confirmed that disruption of pckA in *M. smegmatis* inhibited the growth of the bacteria on acetate and palmitate (unpublished data). These data all suggest an important role for PEPCK in *Mycobacterium* species during metabolism of fatty acids. To study the role of PEPCK in this group of bacteria, the pckA gene of BCG was mutated by homologous recombination. The wild-type BCG (BCG-WT) and pckA mutant BCG (ΔpckA) strains were compared and the results demonstrate that expression of PEPCK is regulated by carbon source, that PEPCK is required for growth on fatty acids and that mutants defective in PEPCK expression are attenuated in both macrophages and mice.

**Abbreviations:** GFP, green fluorescent protein; ICL, isocitrate lyase; Kan, kanamycin; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase.
 METHODS

Bacterial strains and growth conditions. Mycobacterium bovis BCG (Pasteur) was obtained from Barry Bloom (Harvard University School of Public Health, Boston). Bacteria were maintained in Middlebrook (MB) 7H9 media (Difco) with 10% (v/v) OADC enrichment (100 g glucose, 8-5 g NaCl, 0-6 ml oleic acid, 50 g BSA) and 0-05% Tween 80 at 37˚C. For selective culture, BCG was maintained in minimal medium (LB) medium (Fisher Biotech) was used in all experiments.

Construction of plasmids. The plasmid for the disruption of the pckA gene was constructed as follows. pck-U primer (5'-CGCAGTAGTACCTCAGCGACCATCCC-3') was designed from the first nucleotide of the ORF of the pckA gene of M. tuberculosis, pck-D (5'-CGCACATGATGGCTTCGATCTGCGAC-3') was taken from 360 bp after the pckA stop codon, and a SpeI site was added to both primers. BCG chromosomal DNA was amplified and the product subcloned into pGEM-T vector (Promega). The kanamycin (Kan) gene cassette was excised from pUC4K with EcoRI and the 1281 bp Kan cassette was inserted in an EcoRI site located 940 bp into the pckA ORF. The recombination vector was constructed in pPR23 (Pelicic et al., 1996) by cloning the SpeI fragment from pGEM/pckA/Kan. The new construct was designated pPR/pck/Kan (Fig. 1).

To study regulation of pckA expression, a plasmid was constructed to express green fluorescent protein (GFP) driven by the pckA promoter. Genomic DNA of M. tuberculosis was amplified with primer 5'-CCGCTCTAGATGACACGAGATGTTGAGATCCG-3' and primer 5'-AATGGATCCCGACGACACGAGATCCG-3' to obtain 800 bp upstream of the 5'-end and 73 bp ORF of pckA. The PCR product was cloned in plasmid pMV262, generating a gene encoding a fusion protein between PckA and GFP. The construct was designated pMV/pck::gfp.

Electrotransformation and allelic exchange. Electroporation cells of BCG were prepared as described by Pelicic et al. (1996) with minor modifications. BCG was cultured in 200 ml MB 7H9 medium to an OD600 of 0.6. Then the culture was washed three times with 10% glycerol at room temperature (RT) in a volume of 45, 20 and 10 ml, respectively. The pellet was resuspended in 0.5 ml 10% glycerol. One hundred microlitres of cells was mixed with 1 μg (2.5 μl) plasmid and incubated for 2 min without shaking at RT, then electrotoperated at 25 μF, 2.5 kV and 1000 ohms in 0.2 cm cuvettes for a single pulse. Then 5 ml fresh MB was added to the tube and incubated for 24 h before plating to allow antibiotic resistance expression.

To select pckA mutants, the BCG wild-type strain was transformed with plasmid pPR/pck/Kan. After 24 h incubation at 32˚C, the cells were centrifuged at 3000 r.p.m. for 10 min at RT, then the pellet was plated onto fresh MB-agar-Kan (25 μg ml-1)-sucrose (2%). After incubation at 32˚C for 4 weeks, the colonies were picked and transferred to 200 μl MB-Kan (25 μg ml-1)-sucrose (2%) medium and incubated at 32˚C overnight. Then, 100 μl of the bacteria was streaked on the MB-agar-Kan-sucrose plate and incubated at 39˚C for 3–4 weeks. The colonies were picked up and cultured in MB-Kan medium for further analysis.

Southern blot. The DNA from the putative pckA mutant of BCG was digested with restriction enzyme BamHI, separated on a 0-8% agarose gel, and blotted onto a nylon membrane. The DNA was hybridized with a probe consisting of a 940 bp fragment of pckA fused to the lacZ gene of E. coli.

Fig. 1. Strategy for allelic exchange of the pckA gene. (a) Shuttle vector used for positive selection of gene replacement. (b) The fragment of the pckA ORF and 360 bp downstream sequence was amplified by the primers pck-U, designed from the first nucleotide of the ORF of M. tuberculosis pckA, and pck-D, 360 bp downstream of the pckA ORF, subcloned into the SpeI site of plasmid pPR23 to generate plasmid pPR/pck. A Kan gene cassette was subcloned into the EcoRI (E) site of plasmid pPR/pck to generate pPR/pck/Kan. (c) After transformation of BCG by plasmid pPR/pck/Kan, crossover recombination occurred between chromosomal pckA and plasmid pckA DNA, and the pckA gene in the chromosome of M. tuberculosis was interrupted by the insertion of the Kan gene. EcoRI (E) (940 bp) and BamHI (B) (309, 1240 and 2173 bp) sites in the PCR fragment are shown. Kan-F (5'-AGGATCTTCCGACGACACGCCGGAC-3') and Kan-R (5'-GATAAGTGTGCGACCCAGTGGCC-3') were the primers used for PCR to check the gene replacement.
agrose gel and transferred onto nylon membrane as reported by Liu et al. (2000). The DNA probe was prepared by PCR labelling with DIG (Roche). The primers for the pckA fragment were the same as those used in allelic exchange vector construction. The primers used for Kan gene cassette amplification were as follows: Kan-Up, 5'-GGCCGCGATTAAATTCCAACATGG-3'; and Kan-Dn, 5'-GGTGTGTTGTCGACTCATACCCGGC-3'. For sacB gene probe PCR, plasmid pCDV442 was used as template and amplified by the primers 5'-GGCCGCGATTAAATTCCAACATGG-3'; and 5'-AATTGGCATAATTGGATCTCGG-3'. Prehybridization was performed at 42 °C for 4 h, followed by hybridization overnight at 42 °C with 20 ng ml⁻¹ denatured DNA probe in hybridization solution. After stringent washing, anti-DIG antibody was added and detected using a DIG luminescent detection kit as instructed (Roche). For rehybridization, the membrane was washed at 60°C in a water bath for 20 min, then at 37°C for 2 × 20 min in 0.2 M NaOH, 0.1% SDS.

**Macrofage infection.** Bone-marrow-derived macrophages (BMMΦ) were isolated from BALB/c mice and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 5% horse serum and 20% L cell-conditioned medium (IM) and the antibiotics penicillin (10000 units ml⁻¹) and streptomycin (10000 μg ml⁻¹) at 37°C for 5 days. Then BMMΦ were resuspended into a 24-well plate at 2 × 10⁶ cells per well in 1 ml IM and incubated at 37°C overnight. Prior to infection, BCG was cultured for about 1 week to exponential phase. At the time of infection, bacteria were washed twice with PBS-0.05% Tween 80, pH 7.4 (PBS-T), and dispensed by multiple passages through a tuberculin syringe with a 25G needle. Clumps were removed by centrifugation at 200 g for 10 min. The supernatant was recovered and bacteria were counted on a haemocytometer. The macrophages were infected with mycobacteria at 1:10 in a volume of 1 ml. After incubation for 2 h, the wells were washed with IM once, then 1 ml new IM was added. After incubation at 37°C with 6% CO₂, for specified intervals, the supernatant was removed and 250 μl 0.1% saponin was added to lyse the cells. The bacteria were plated on an MB 7H10 agar plate and counted.

**Infection of mice.** Female BALB/c mice were purchased from Charles River. A BCG culture with an OD₆₀₀ of 0.8 was washed with PBS-0.01% Tween 80 and homogenized as described above. Bacteria were adjusted to a final density of 1 × 10⁶ cells ml⁻¹ and the mice were injected via the lateral tail vein with 0.01 ml PBS-T. The suspension was dispensed by multiple passages through a tuberculin syringe with a 25G needle. Clumps were removed by centrifugation at 200 g for 10 min. The supernatant was recovered and bacteria were counted on a haemocytometer. The macrophages were infected with mycobacteria at 1:10 in a volume of 1 ml. After incubation for 2 h, the wells were washed with IM once, then 1 ml new IM was added. After incubation at 37°C with 6% CO₂, for specified intervals, the supernatant was removed and 250 μl 0.1% saponin was added to lyse the cells. The bacteria were plated on an MB 7H10 agar plate and counted.

**Histology.** A small piece of the left dorsal lobe of each of the lungs, livers and 1/4 of spleens from the mice infected with BCG were fixed with 10% PBS-buffered formalin for 24 h. The pieces were dehydrated in graded ethanol solutions and embedded in paraffin. Paraffin sections were cut on a rotary microtome in three sections, separated by 100 μm, and were stained with haematoxylin and eosin and acid-fast stains (Ziehl–Neelsen), respectively. Histological change of the tissues was documented by using an Axioskop 2 plus microscope, a × 63 oil immersion objective, and AxioCam Zeiss camera. Mycobacteria were checked on ZN slides.

**Fluorescence assay.** BCG transformed with plasmid pMVpck::gfp was cultured in MB and harvested from the exponential growth phase (7 days). The cells were washed and homogenized as described above, then resuspended to a final concentration of 1 × 10⁶ cells ml⁻¹ and cultured in MB medium in 96-well microtiter plates in triplicate at 37°C for 48 h. The units of fluorescence were measured using a spectrophotometer at 538 nm. For fluorescence microscopy examination, cells were centrifuged at 6000 r.p.m. in an Eppendorf centrifuge, then washed once with PBS, mixed with antifade at 1:5, spotted on the slides, covered with cover glasses and fixed for 1 h at 37°C. The slides were observed with an oil lens under fluorescence microscopy.

**Statistical analysis.** Differences among the results generated by the BCG-WT and ΔpckA strains were evaluated for statistical significance by the pooled variance t test for two-tailed P value. Probability values of <0.05 were considered significant.

**RESULTS**

**Regulation of pckA in extracellular bacteria**

In *M. tuberculosis*, expression of both ICL and malate synthase is modulated by the carbon source (Smith et al., 2003; McKinney et al., 2000). If PEPCK is also involved in modulation of the carbon flux in response to nutrient availability, its gene expression should be regulated by different carbon sources. Moreover, the upstream promoter regions of both *icl* (encoding ICL) and *glcB* (encoding malate synthase) conferred appropriate, regulated expression on these genes. In comparable experiments, BCG wild-type was transformed with the plasmid pMVpck::gfp, in which GFP expression was driven by the pckA promoter. The transformed BCG was cultured in minimal media with glucose, acetate or palmitate as the limiting carbon source.

The results showed that the transformants revealed stronger fluorescence with acetate or palmitate than with glucose (Fig. 2). GFP expression was quantified by measurement of the fluorescence intensity at 538 nm. After subtraction of background fluorescence from wild-type bacteria the values obtained from comparable growth-stage cultures reproducibly exhibited ratios of approximately 1:4:7 (0.5% glucose : 0.1% palmitate : 0.1% acetate). These data indicate that the expression of the *pckA* gene is modulated by carbon source, and that acetate is the strongest inducer of *pckA* expression.

**Disruption of pckA**

To further investigate the function of PEPCK, we disrupted the *pckA* gene by homologous recombination. To verify the gene replacement, DNA of BCG-WT and ΔpckA strains was used as template for PCR using the primers designed from the *pckA* gene of *M. tuberculosis*. BCG-WT generated a PCR product of 2 kb, as predicted from the genome sequence of *M. bovis*. However, strains transformed by pPR/pckA/Kan generated a band at 3-2 kb, demonstrating that *pckA* was interrupted by insertion of the Kan gene (Fig. 3). The disruption of the *pckA* gene from *M. bovis* BCG was further confirmed by Southern blotting (Fig. 4).

**Survival of the ΔpckA mutant in macrophages**

To test whether disruption of the *pckA* gene had any effects on the growth of BCG, murine bone-marrow-derived macrophages were infected with BCGΔpckA clone 11. The macrophages were harvested at 6, 24, 48 and 72 h...
post-infection and the survival of bacteria was documented by counting the c.f.u. There was a clear disparity in survival at early time points between the wild-type BCG and BCG\textsuperscript{D}pck\textsubscript{A} clones, where the BCG\textsuperscript{D}pck\textsubscript{A} mutant was clearly attenuated. However, from 24 h onwards, both the BCG-WT and Δpck\textsubscript{A} clones maintained similar growth patterns (Fig. 5a).

To analyse these early time points further, macrophages were infected for 1, 2, 5 and 8 h and bacterial survival was scored. The results showed that although the number of BCG used to infect macrophages was comparable ($P > 0.4$), there was an obvious difference in the ability of the BCG\textsuperscript{D}pck\textsubscript{A} mutant to establish an infection (Fig. 5b). Even at 1 h the survival of BCG\textsuperscript{D}pck\textsubscript{A} was significantly less than that of the wild-type strain ($P < 0.015$), and at 8 h, the difference was more marked ($P < 0.001$). The results indicate that the \textit{pckA} gene is particularly important in the initiation of BCG infection in the macrophages. Consistent with this phenotype was the observation that bacteria transformed with pMVP\textit{pck}:\textit{gfp} were highly fluorescent on infection in macrophages (Fig. 6).

**Growth of BCG\textsubscript{ΔpckA} in mice**

To determine the effects of disruption of the \textit{pckA} gene on the growth of BCG \textit{in vivo}, mice were infected with $1 \times 10^6$ c.f.u. of BCG\textsuperscript{ΔpckA} clone 11. The bacterial load in the spleen was evaluated by a c.f.u. count. In week 1, the c.f.u. count was significantly lower in the spleens of mice infected with BCG\textsuperscript{ΔpckA} compared with the wild-type BCG control and this attenuation was observed up to week 5 post-infection ($P < 0.005$) (Fig. 7). However, from 5 week onwards, the infecting bacteria in both BCG-WT- and BCG\textsuperscript{ΔpckA}-infected mice maintained a comparable growth pattern. This observation is consistent with our previous data from the macrophage infection experiments.

The differential bacterial burden was reflected in the pathology of the mice. There was no obvious histological change in the spleens from mice infected with either BCG-WT or the Δpck\textsubscript{A} clone at 6 h infection. However, from week 2, BCG-WT-infected mice had clear granulomas in the white pulp of the spleen that persisted for the length of the experiment. The appearance of similar pathology was less marked in the mice infected with BCG\textsuperscript{ΔpckA} mutants. Analysis of the lung, liver and spleen at day 20 post-infection confirmed the pattern observed in the spleen. BCG-WT-infected mice exhibited distinct granulomas comprising macrophages, epithelioid cells, neutrophils and lymphocytes, and perivascular and peribronchiolar lymphocytic accumulation. The overall extent of inflammation was heavier in the lung tissue of BCG-WT-infected mice than in the BCG\textsuperscript{ΔpckA}-infected mice (data not shown).

**DISCUSSION**

The gene \textit{pckA} encodes the enzyme PEPCK, which is capable of conversion of OAA to PEP, or the reverse reaction. The directionality of this reaction determines the role of the
enzyme. The carbon flux mediated by PEPCK can lead to either replenishment of TCA cycle intermediates (anaplerosis) or to the initial step of gluconeogenesis. PEPCK is thought to be of particular importance when organisms are exploiting fatty acids as their major carbon source. Previous work had demonstrated that ICL, the gating enzyme into the glyoxylate shunt pathway that leads to production of malate from fatty acids, played a key role in maintenance of infection in activated macrophages or an immunocompetent host (McKinney et al., 2000). In the current study, we wished to ascertain if PEPCK expression and function mirrored those of ICL.

Our GFP reporter assays indicated that the pckA promoter was active when fatty acids were provided as the limiting carbon source. Previous studies had shown that icl expression and, to a lesser extent, glcB expression were upregulated when Mycobacterium was placed in acetate or palmitate as a primary carbon source. These data indicate that PEPCK, ICL and GlcB are all mobilized under similar growth conditions.

The phenotype of icl-deficient mutants of M. tuberculosis was quite marked and the attenuation was only observed when the bacteria were present in activated macrophages, or after the development of an immune response in infected mice (McKinney et al., 2000). However, ΔpckA mutants were attenuated in the initial stages of infection of resting macrophages, suffering the greatest drop in bacterial numbers in the first 3 h of infection. This phenotype differs from that observed in the icl-deficient M. tuberculosis, suggesting that the roles played by the two enzymes do not lie solely in the same metabolic pathway. PEPCK can function to direct carbon in the form of OAA into the pathway of gluconeogenesis; however, it can also catalyse the reverse reaction, converting PEP into OAA for the replenishment of the TCA cycle. We speculate that the attenuation observed shortly after infection by ΔpckA is due to a reduced capacity to respond to the changing environment within the macrophage, possibly when OAA is needed to fulfil an anaplerotic role in the TCA cycle. Anaplerosis facilitates entry of carbon into the TCA cycle from sources other than via citrate synthase, thus bypassing the oxidation of carbon and allowing it to be channelled into other routes. More analysis is required to determine the balance of gluconeogenic and anaplerotic activities during infection.

Collins et al. (2002), in a study designed to evaluate metabolic mutants of M. bovis as potential vaccine candidates, reported that bacteria deficient in PEPCK expression were avirulent in guinea pigs and failed to induce a protective immune response. This attenuation in animal infection experiments was also observed in our studies on mice, where we found that the survival of mycobacteria was similar between BCG-WT- and ΔpckA-infected mice at early time points post-infection. However, starting from week 5 to the end of experiments (8 weeks), the bacterial number in the spleens of mice infected with ΔpckA was significantly less than that in the spleens of mice infected with the BCG-WT control, and this attenuation was reflected in the reduced pathology observed in the infected animals. The difference is unlikely to be due to different growth rates of the two BCG strains as there was no difference in the growth rates of the strains in vitro in MB medium (data not shown). The ΔpckA mutants are clearly less fit in the complex environment within both the host macrophage and the mouse but the basis of this attenuation remains to be elucidated.
Analysis of intermediate metabolism in whole organisms remains complex because of the potential pathways of redundancy and compensation, nonetheless these data are vital to our appreciation of infection by intracellular microbes and the development of new antimicrobial agents.

**Fig. 5.** Survival of BCG in macrophages. Murine bone-marrow macrophages were infected with BCG wild-type (□) or the ΔpckA strain (●) at a multiplicity of 10:1 (bacteria per macrophage) in triplicate wells. After incubation for 2 h, the wells were washed and fresh medium was added. Samples were collected at different time points by lysing infected cells with 0.1% saponin. Lysates were diluted in PBS-T and plated on 7H10 agar. Colonies were scored after 3 weeks at 37°C and results were expressed as means ± standard error of the mean. The time zero point refers to the bacteria used for the infection, at which time no difference exists between the two strains for both (a) and (b) (P>0.1). (a) Samples were collected at 6, 24, 48 and 72 h time points. The difference between the two strains was significant at 6 h (P<0.02). From that time point on, the curve patterns for both strains were similar. (b) Samples were collected at 1, 2, 5 and 8 h time points post-incubation. The difference between the two strains was significant at 1 h (P<0.015), 2 h (P<0.03), 5 h (P<0.02) and 8 h (P<0.001).

**Fig. 6.** Level of GFP expression 48 h post-infection. Macrophages were plated onto coverslips in a 24-well plate and infected with wild-type BCG transformed with the plasmid pMVpck::gfp. The coverslips were harvested at different time points, washed with PBS and then fixed in 4% paraformaldehyde. The above illustration shows the expression of PckA::GFP 48 h following infection.

**Fig. 7.** Mycobacterial burden in the spleens of BALB/c mice. Mice were infected with BCG-WT (□) or ΔpckA (●) at 10^6 cells ml\(^{-1}\) in PBS via the tail vein. The spleens were collected at 5, 10, 20, 35 and 56 days post-infection and subjected to colony assay. Results are expressed as means ± standard error of the mean from three mice per strain per time point. Time zero refers to the bacterial c.f.u. used for infection, at which time no difference existed (P>0.1) between the two strains. The difference between the two strains was significant at days 35 and 56 (P<0.005).

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