

The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for *in vivo* growth

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Summary

Through examination of one of the fundamental *in vitro* characteristics of *Mycobacterium bovis* – its requirement for pyruvate in glycerol medium – we have revealed a lesion in central metabolism that has profound implications for *in vivo* growth and nutrition. Not only is *M. bovis* unable to use glycerol as a sole carbon source but the lack of a functioning pyruvate kinase (PK) means that carbohydrates cannot be used to generate energy. This disruption in sugar catabolism is caused by a single nucleotide polymorphism in *pykA*, the gene which encodes PK, that substitutes glutamic acid residue 220 with an aspartic acid residue. Substitution of this highly conserved amino acid residue renders PK inactive and thus blocks the ATP generating roles of glycolysis and the pentose phosphate pathway. This mutation was found to occur in other members of the *M. tuberculosis* complex, namely *M. microti* and *M. africanum*. With carbohydrates unable to act as carbon sources, the importance of lipids and gluconeogenesis for growth *in vivo* becomes apparent. Complementation of *M. bovis* with the *pykA* gene from *M. tuberculosis* H37Rv restored growth on glycerol. Additionally, the presence of a functioning PK caused the colony morphology of the complemented strain to change from the characteristic dysgonic growth of *M. bovis* to eugonic growth, an appearance normally associated with *M. tuberculosis*. We also suggest that the glycerol-soaked potato slices used for the derivation of the *M. bovis* bacillus Calmette and Guérin (BCG) vaccine strain selected for an *M. bovis* PK⁺ mutant, a finding that explains the alteration in colony morphology noted during the der-

ivation of BCG. In summary, the disruption of a key step in glycolysis divides the *M. tuberculosis* complex into two groups with distinct carbon source utilization.

Introduction

Bacterial pathogens that replicate *in vivo* must acquire nutrients from their host. Elucidation of how each pathogen exploits this niche therefore promises to shed light on the pathogenic process. However, description of the *in vivo* milieu has proved difficult. The availability of pathogen genome sequences now offers a new approach, with *in silico* reconstruction of metabolic pathways permitting key metabolic lesions to be identified, hence allowing the prediction of *in vitro* and *in vivo* nutrient requirements (Renesto *et al.*, 2003; Somerville *et al.*, 2003). We have applied this approach to the *Mycobacterium tuberculosis* complex as a route to describe the metabolism of these pathogens in the host.

The *M. tuberculosis* complex is a group of pathogens that exact devastating tolls on human morbidity and mortality, and inflict damaging economic losses on world agriculture (Snider *et al.*, 1994; Thoen and Steele, 1995). Members of the complex differ widely in their host specificity (Wayne and Kubica, 1986). *M. tuberculosis* and *M. canettii* cause tuberculosis in humans and appear restricted to the human host. The vole pathogen *M. microti* causes disease in rodents but is attenuated in humans, while *M. africanum* has been isolated from humans and diverse animal species (Hart and Sutherland, 1977; Thorel, 1980; Alfredsen and Saxegaard, 1992). *M. bovis* is reported to have a broad host range, causing disease in a variety of wild and domesticated mammals including cattle and humans. Furthermore, the live-attenuated vaccine strain *M. bovis* bacillus Calmette and Guérin (BCG) was derived from an *M. bovis* isolate and is the most widely used vaccine in the world (Bloom and Fine, 1994).

Progress has been hindered in deciphering intermediary metabolism across the tubercle bacilli due to the inherent difficulties of working with these slow-growing, category III pathogens. In the 1960s and 1970s work focused on carbohydrate metabolism and it became evident that mycobacteria are unusual in their patterns of carbon source utilization, with variation across the genus. This was particularly true with regard to glycerol utilization (Goldman,

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1963; Winder and Brennan, 1966; Andrejew *et al.*, 1976), which gained attention for a number of reasons fundamental to mycobacterial biology. Under laboratory conditions glycerol is the preferred carbon source for copious growth of many mycobacterial species, including *M. tuberculosis* (Ratledge, 1982). However, *M. bovis*, *M. africanum* and *M. microti* are all unable to use glycerol as a sole carbon source, and pyruvate is therefore routinely added to glycerinated media to enable growth (Stonebrink, 1958; Wayne and Kubica, 1986). Indeed, to grow on glycerol *M. bovis* strains need to be 'adapted' by *in vitro* passage on media where glycerol is the sole carbon source; such glycerol-adapted strains include *M. bovis* BCG and the tuberculin production strain *M. bovis* AN5. In the presence of glycerol mycobacteria are reported to produce an excess of lipids and polysaccharides and this may account for the greater mass of bacilli obtained on this substrate per unit volume of culture medium (Tepper, 1965; 1968). Furthermore, as a nutrient glycerol has a pronounced effect on the size and morphology of mycobacterial colonies (Osborn, 1986), with classical methods of differentiation of bovine and human bacilli based on the appearance of glycerol-grown colonies (Wayne and Kubica, 1986). Hence *M. tuberculosis* is said to display 'eugonic' or abundant growth on medium containing glycerol, with a raised and crumbly pattern that develops poorly on glycerol-free medium. On medium containing glycerol, growth of *M. bovis* is 'dysgonic' or sparse and colonies appear flat, moist and glossy. The influences of glycerol on mycobacterial culture may arise from the fact that glycerol and its metabolic derivatives can act as precursors in the biosynthesis of lipids and cell wall constituents.

Considering the dramatic differences in host tropism and levels of virulence between members of the *M. tuberculosis* complex, any *in vitro* variation in phenotype,

such as those observed on glycerol, needs to be explored for clues to *in vivo* phenotypes. Comparison of the genome sequences of *M. bovis* and *M. tuberculosis* revealed a >99.9% identity at the nucleotide level (Garnier *et al.*, 2003). This provides the ideal starting point to decipher the basis for their distinct phenotypes. In this study we used comparative genomics to determine the genetic basis for the difference in glycerol metabolism between *M. tuberculosis* and *M. bovis*. Through this work we reveal the disruption of glycolysis in *M. bovis* because of a single nucleotide polymorphism (SNP) in *pykA*, the gene that encodes pyruvate kinase (PK) (Fig. 1). This lesion also disrupts the link between the pentose phosphate pathway (PPP) and pyruvate, the only other catabolic pathway for sugar utilization available to pathogenic mycobacteria (Jayanthi Bai *et al.*, 1975; Cole *et al.*, 1998), as the Entner–Doudoroff pathway is not present in members of the *M. tuberculosis* complex (Ratledge, 1982; Garnier *et al.*, 2003). Examination of this SNP across members of the *M. tuberculosis* complex showed that *M. africanum* and *M. microti* shared the same *pykA* SNP as *M. bovis*, suggesting that the pyruvate requirement of these strains is also due to the lack of pyruvate kinase activity. Through genetic complementation we produced a recombinant strain of *M. bovis* that both grew on glycerol as a sole carbon source and displayed eugonic growth on solid glycerol-containing medium. The implications of the *pykA* mutation on *in vivo* growth of the bacillus is discussed.

Results

SNP detection and screening

There are 2437 SNPs between *M. bovis* AF2122/97 (spoligotype SB0140; <http://www.mbovis.org>) and *M. tubercu-*

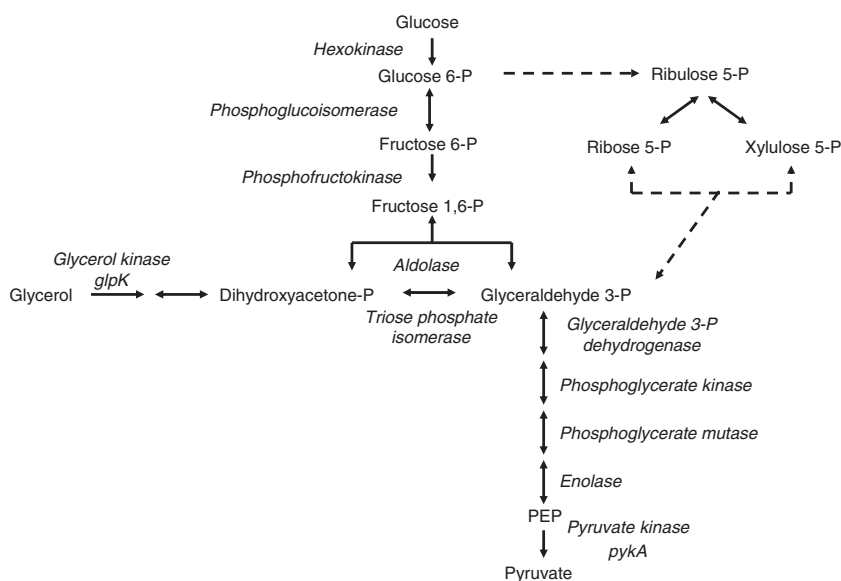


Fig. 1. Glycolysis, the pentose phosphate pathway and glycerol catabolism. Dashed arrows represent multiple enzyme reactions in the pentose phosphate pathway.

losis H37Rv (Garnier *et al.*, 2003). As *M. bovis* differs from *M. tuberculosis* in its ability to utilize glycerol, SNPs in genes predicted to be involved in glycerol catabolism were examined further. The SNPs selected occurred in two genes, *glpK* (codon 191) and *pykA* (codon 220).

glpK encodes glycerol kinase (GK), the enzyme which catalyses the MgATP-dependent phosphorylation of glycerol to yield sn-glycerol 3-phosphate, the rate limiting step in glycerol utilization in *Escherichia coli* (Zwaig *et al.*, 1970). The *M. bovis* AF2122/97 SNP caused a frameshift in the *glpK* gene at codon 191, leading to a truncated coding sequence (Garnier *et al.*, 2003). Further analysis revealed that *M. microti* OV254, the vole bacillus, had the same frameshift mutation in *glpK* as *M. bovis* AF2122/97 (Fig. 2A). Like *M. bovis*, *M. microti* requires pyruvate to be added to glycerinated medium for growth. Noteworthy, the *glpK* frameshift is not evident in the vaccine strain *M. bovis* BCG nor in the bovine tuberculin-production strain *M. bovis* AN5, strains which can grow on glycerol as the

sole carbon source. The *M. bovis* AN5 *glpK* sequence is identical to that of *M. tuberculosis* H37Rv, while in BCG a 2 bp insertion at codon position 191 of the *M. bovis* AF2122/97 *glpK* corrects the frameshift and results in an extra codon with respect to the *M. tuberculosis glpK* (Fig. 2A). This extra codon was found to be present in all *M. bovis* BCG strains tested (Pasteur, Tokyo, Danish, Russia, Tice, Frappier, Sweden). However, further analysis of multiple *M. bovis* strains showed that the *glpK* frameshift is not universally present in *M. bovis* strains; for example, the frameshift was not present in the field isolate *M. bovis* 61/1307/01 (Spoligotype SB0134) despite its inability to grow on glycerol. It is therefore possible that the progenitors of BCG and AN5 had an in frame *glpK* coding sequence.

The second SNP examined was a nucleotide substitution in *pykA*, the gene that encodes PK (Garnier *et al.*, 2003). PK catalyses the final step in glycolysis, the conversion of phosphoenolpyruvate to pyruvate (Fig. 1). The

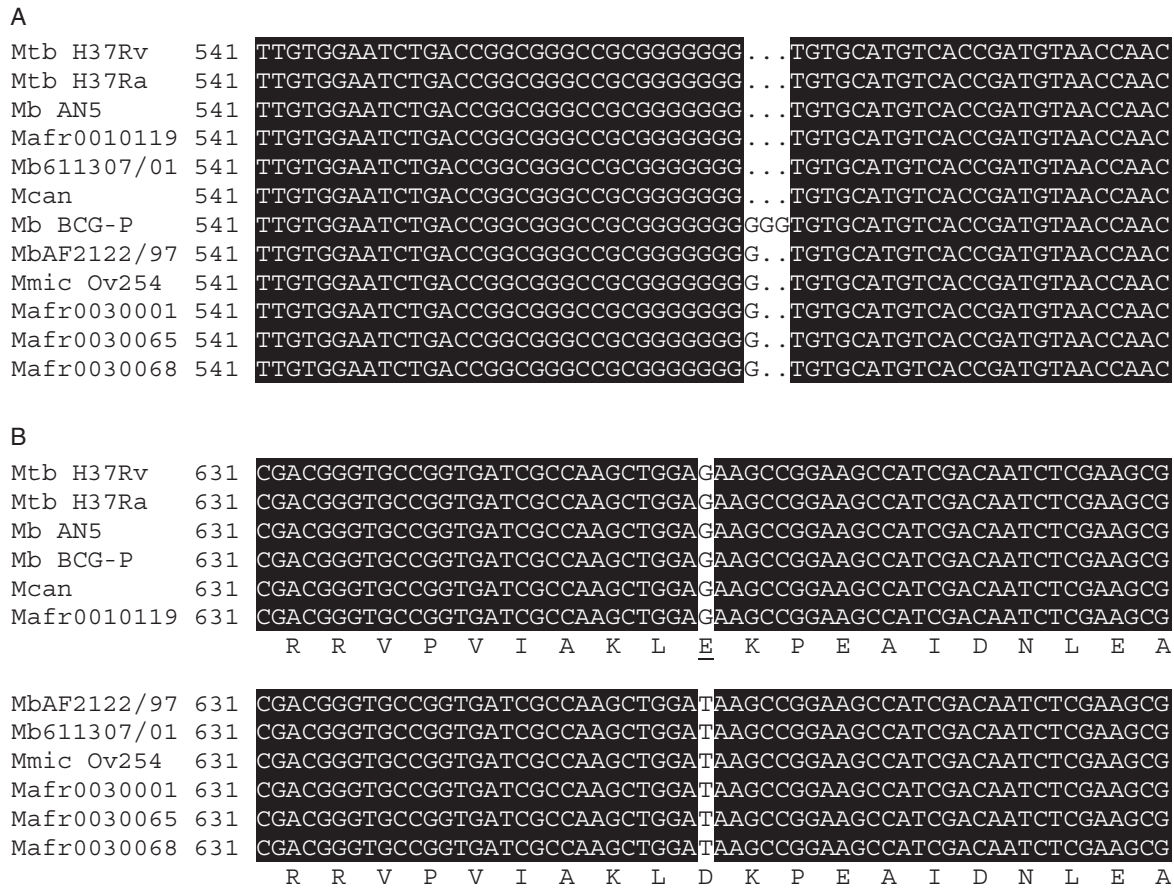


Fig. 2. A. Multiple sequence alignment of the *glpK* gene from several mycobacterial species. The sequences were aligned by Multalin (<http://prodes.toulouse.inra.fr/multalin/multalin.html>), and shading was performed with BoxShade Version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). B. Multiple sequence alignment of the *pykA* gene from several mycobacterial species. The sequences were aligned by Multalin, and shading was performed with BoxShade Version 3.21. The abbreviations used are as follows: Mtb, *M. tuberculosis*; Mb, *M. bovis*; Mmic, *M. microti*; BCG-P, *M. bovis* BCG Pasteur; Mcan, *M. canettii*; Mafr, *M. africanum*.

pykA SNP results in the substitution of Glu220 by Asp (Fig. 2B). This highly conserved glutamic acid residue is predicted to play an important role in the active site of pyruvate kinase and has been associated with cofactor (Mg^{2+}) and substrate [ADP/ATP, phosphoenolpyruvate (PEP)] binding (Munoz and Ponce, 2003). As with the *glpK* SNP, the *pykA* mutation does not occur in the glycerol-adapted laboratory strains *M. bovis* BCG or *M. bovis* AN5. However, the *pykA* SNP was identical across 100 epidemiologically unrelated *M. bovis* strains (VLA Culture Collection) that were tested. All *M. africanum* and *M. microti* strains tested (Table 1) also showed the Glu220Asp mutation, except for *M. africanum* strain 001-0119. This strain belongs to the *M. africanum* type II group described by Niemann *et al.* (Niemann *et al.*, 2000) but reclassified by Niobe-Eyangoh *et al.* (Niobe-Eyangoh *et al.*, 2003) as *M. tuberculosis*; our SNP analysis concurs with the classification of Niobe-Eyangoh *et al.* of this strain as *M. tuberculosis*. An *M. canettii* strain was also tested and it showed the same Glu220 sequence as *M. tuberculosis*.

Detection of glycerol kinase activity

To confirm that the *glpK* SNP disrupts GK activity pro-

tein extracts from strains with and without the mutation were assayed for GK activity at the mid-logarithmic stage of growth (Table 2). As the addition of pyruvate to medium was found to repress GK activity in *M. tuberculosis* (data not shown), extracts were prepared from Middlebrook 7H9 + albumin-dextrose-catalase + Tween 80 (MADCTw) grown cultures; *M. bovis* and *M. microti* did not require the addition of pyruvate to grow in this medium, because of the presence of Tween 80, an oleic acid ester, that can be used as a carbon source by mycobacteria (Wayne, 1994). The organisms in which *glpK* was predicted to be a pseudogene did not possess GK activity, confirming that the mutation disrupted protein function. The additional codon in the *glpK* sequence of *M. bovis* BCG did not have an adverse effect on activity; in fact, the specific activity was higher in *M. bovis* BCG than in *M. bovis* AN5, though this could reflect differential expression. As predicted, the *M. bovis* 61/1307/01 strain with an in-frame *glpK* possessed GK activity. As this strain has a functioning GK, but cannot grow on glycerol as a sole carbon source, the *glpK* lesion is not the sole reason for the inability of *M. bovis* to grow on glycerol.

Table 1. Key mycobacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
<i>M. tuberculosis</i> H37Rv	<i>M. tuberculosis</i> complex Type strain	Cole <i>et al.</i> (1998)
<i>M. tuberculosis</i> H37Ra	Attenuated derivative of the H37Rv strain	Steenken <i>et al.</i> (1935)
<i>M. bovis</i> AN5	Glycerol adapted strain used for bovine tuberculin production	Paterson (1948)
<i>M. bovis</i> AF2122/97	Genome sequenced strain	Garnier <i>et al.</i> (2003)
<i>M. bovis</i> 61/1307/01	Wild-type GK ⁺ field strain of <i>M. bovis</i>	VLA Weybridge Collection
<i>M. microti</i> OV254	Vole bacillus vaccine strain	Brodin <i>et al.</i> (2002)
<i>M. microti</i> 18	Vole bacillus	VLA Weybridge Collection
<i>M. microti</i> 19	Vole bacillus	VLA Weybridge Collection
<i>M. microti</i> 28	Vole bacillus	VLA Weybridge Collection
<i>M. africanum</i> 003 0001	type A1	Niobe-Eyangoh <i>et al.</i> (2003) ^a
<i>M. africanum</i> 003 0065	type A2	Niobe-Eyangoh <i>et al.</i> (2003) ^a
<i>M. africanum</i> 003 0068	type A3	Niobe-Eyangoh <i>et al.</i> (2003) ^a
<i>M. africanum</i> 001 0119	<i>M. africanum</i> type II reclassified as <i>M. tuberculosis</i>	Niemann <i>et al.</i> (2000); Niobe-Eyangoh <i>et al.</i> (2003) ^a
<i>M. canettii</i>	Smooth colony variant of the <i>M. tuberculosis</i> complex	Institute Pasteur, Paris ^b
<i>M. bovis</i> BCG Pasteur	Attenuated vaccine strain	VLA Weybridge Collection
<i>M. bovis</i> /pLK102	<i>M. bovis</i> 61/1307/01 containing a plasmid-borne <i>pykA</i> expressed from the <i>hsp60</i> promoter, Kan ^r	This study
<i>M. bovis</i> attB::I423	<i>M. bovis</i> 61/1307/01 containing an integrated cosmid expressing <i>pykA</i> from its native promoter, Hyg ^r	This study
<i>M. bovis</i> /pSM81	<i>M. bovis</i> 61/1307/01 pSM81vector control, Kan ^r	This study
<i>M. bovis</i> attB::pYUB412	<i>M. bovis</i> 61/1307/01 pYUB412 vector control, Hyg ^r	This study
Plasmids		
pGEMTeasy	<i>E. coli</i> cloning vector	Promega
pLK100	pGEMTeasy vector containing <i>M. tuberculosis pykA</i>	This study
pSM81	Mycobacterial expression vector with a MCS downstream of the <i>M. tuberculosis hsp60</i> promoter	VLA Weybridge Collection
pLK102	pSM81 vector containing <i>M. tuberculosis pykA</i>	This study
pYUB412	Mycobacterial integrating cosmid	Bange <i>et al.</i> (1999)
I423	pYUB412 containing <i>M. tuberculosis</i> genomic DNA, co-ordinates 1786433–1819604	Institute Pasteur, Paris ^b

a. *M. africanum* strains were kind gifts from Dr Veronique Vincent.

b. *M. canettii* strain and I423 were kind gifts from Dr Roland Brosch.

GK⁺, glycerol kinase producing strain. Kan^r and Hyg^r denote resistance to kanamycin and hygromycin respectively.

Table 2. Glycerol kinase activity in various mycobacterial species with and without the *glpK* SNP.

Organism	Glycerol kinase – specific activity
<i>In frame glpK</i>	
<i>M. tuberculosis</i> H37Rv	12.82 ± 2.65
<i>M. tuberculosis</i> H37Ra	14.62 ± 3.32
<i>M. bovis</i> AN5	6.84 ± 2.57
<i>M. bovis</i> BCG	19.78 ± 4.07
<i>M. bovis</i> 61/1307/01	7.72 ± 2.44
<i>Frameshifted glpK</i>	
<i>M. bovis</i> AF2122/97	0.09 ± 0.10
<i>M. microti</i> Ov254	0.03 ± 0.06

Protein extracts from at least two independent mid-log cultures were assayed for glycerol kinase activity by the glycerol 3-phosphate-coupled assay of Pettigrew *et al.* (1998). All values: nmol NADH produced $\text{min}^{-1} \text{mg}^{-1}$ protein ± 95% confidence limits. At least two determinations of activity were carried out on each protein extract.

Detection of pyruvate kinase activity

All pyruvate-requiring strains contained the same *pykA* SNP, prompting an examination of PK activity across the *M. tuberculosis* complex. Extracts were prepared from MADCTw grown cultures, as above, and harvested at the mid-logarithmic stage of growth. PK activity was not detected in *M. bovis* or *M. microti*, strains that harbour the Glu220Asp mutation (Table 3). However, *M. tuberculosis*, *M. bovis* BCG and *M. bovis* AN5 possess the Glu220 residue and have PK activity.

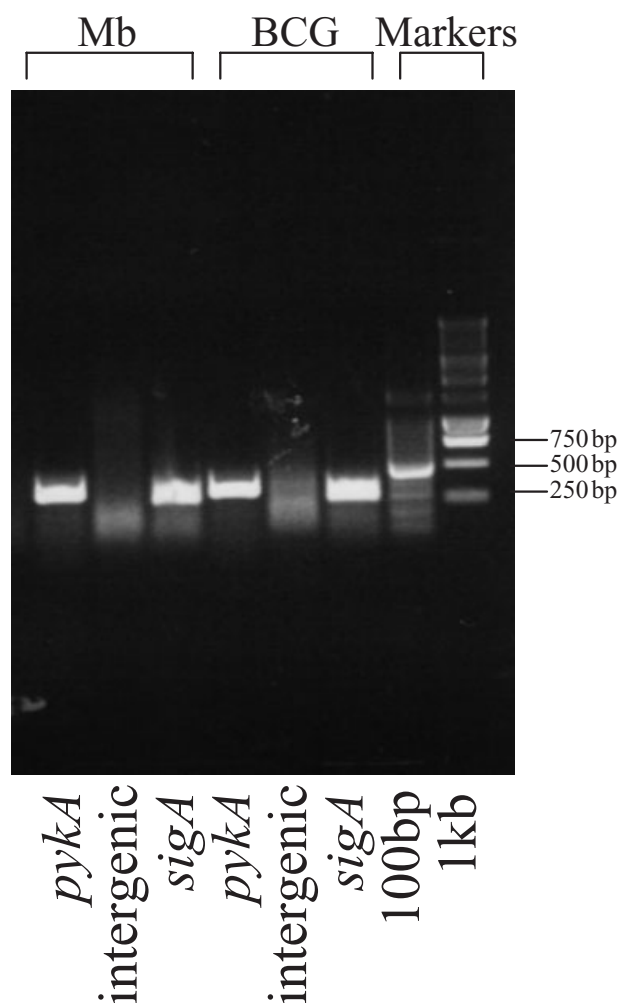
pykA expression levels

To verify that the loss of activity was not attributable to lack of expression, RT-PCR was used. This confirmed *pykA* expression in *M. bovis* (Fig. 3). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out to determine the relative levels of expression of *pykA* mRNA between *M. bovis* and *M. bovis* BCG at the mid-logarithmic stage of growth in MADCTw. Express-

Table 3. Pyruvate kinase activity in various mycobacterial species with and without the *pykA* SNP.

Organism	Pyruvate kinase – specific activity
<i>pykA</i> SNP absent	
<i>M. tuberculosis</i> H37Rv	177.8 ± 45.6
<i>M. tuberculosis</i> H37Ra	238.5 ± 49.2
<i>M. bovis</i> AN5	216.7 ± 50.7
<i>M. bovis</i> BCG	62.7 ± 17.4
<i>M. bovis</i> /pLK102	2163.8 ± 664.3
<i>M. bovis attB::1423</i>	174.1 ± 55.1
<i>pykA</i> SNP present	
<i>M. bovis</i> 61/1307/01	0.70 ± 0.9
<i>M. bovis</i> AF2122/97	0.68 ± 0.8
<i>M. microti</i> Ov254	0.55 ± 0.8

Protein extracts from at least two independent mid-log cultures were assayed for pyruvate kinase activity by the method adapted by Wheeler (1983). All values: nmol NADH consumed $\text{min}^{-1} \text{mg}^{-1}$ protein ± 95% confidence limits. At least two determinations of activity were carried out on each protein extract.

**Fig. 3.** RT-PCR analysis of *pykA* expression in *M. bovis* 61/1307/01 and *M. bovis* BCG Pasteur. RT-PCR was carried out using the Promega Access RT-PCR kit. The resulting amplicons were run on a 1% agarose gel, visualized with ethidium bromide, and sized against Promega 1 kb DNA ladder. The abbreviations used are as follows: Mb, *M. bovis* 61/1307/01; BCG, *M. bovis* BCG Pasteur.

sion levels were normalized to those of *sigA* mRNA and calculated based on the RNA used for reverse transcription. We found that the level of *pykA* mRNA expressed by *M. bovis* is 2.46 (± 0.47, 95% confidence interval) times that expressed by *M. bovis* BCG. We therefore concluded that the inability of *M. bovis* to grow on glycerol as a sole carbon source was attributed to the detrimental effect of the amino acid substitution on the integrity of the enzyme rather than deficiencies in *pykA* expression.

Carbon source utilization

In the absence of a functioning PK *M. bovis* is unable to grow on glycerol or glucose as a sole carbon source. To confirm the disruption of glycolysis by the *pykA* lesion, growth of *M. bovis* on a range of sole carbon sources in

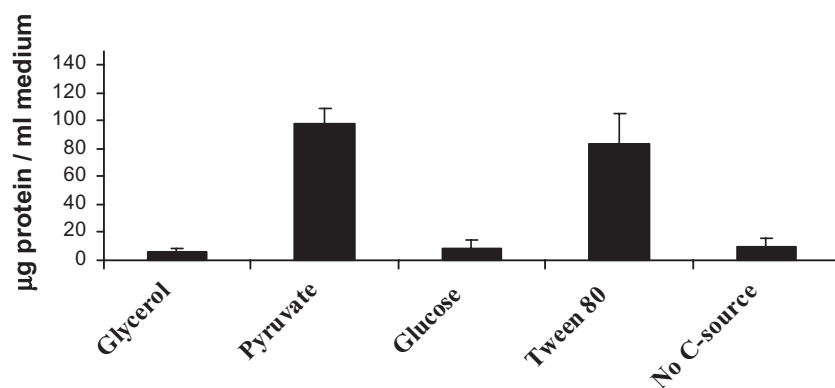


Fig. 4. Growth of *M. bovis* 61/1307/01 in Sauton's medium containing various carbon sources. Cultures were grown for 4 weeks at 37°C in a rolling incubator. Results were obtained from three independent cultures by determining the amount of total cellular protein released from NaOH-treated cells (Meyers *et al.*, 1998). Error bars show the standard deviations of the mean. At least two determinations of protein yield were carried out on each culture.

Sauton's minimal medium was determined (Fig. 4). Bovine serum albumin (BSA) was added to media containing Tween 80 as a sole carbon source as this counteracts the toxic build-up of oleic acid from Tween hydrolysis; BSA does not act as a carbon source for mycobacteria. As predicted *M. bovis* was unable to grow on substrates catabolized through glycolysis (glycerol and glucose) but grew readily on pyruvate (catabolized through the citric acid cycle) and Tween 80 (catabolized through beta-oxidation and the citric acid cycle). Even extended culturing of up to 5 months did not produce growth on glycerol or glucose.

pykA complementation

To confirm the effect of the *pykA* lesion, *M. bovis* 61/1307/01 was complemented with the *pykA* gene from *M. tuberculosis* H37Rv. The *M. bovis* 61/1307/01 strain was used in this study as it has a functioning glycerol kinase. Two strategies for complementation were employed: one used a plasmid construct, pLK102, with *pykA* expression under the *hsp70* promoter, while the other approach employed an integrating cosmid, I423, containing a 33 kb region of H37Rv genomic DNA that includes the *pykA* gene (a gift

from R. Brosch, Institute Pasteur). The resulting complemented strains, *M. bovis*/pLK102 and *M. bovis* attB::I423, produced PK activity (Table 3) and were able to grow on glycerol as the sole carbon source (Fig. 5). However, *pykA* complementation did not restore growth on glucose, indicating that there are additional lesions in glucose utilization in *M. bovis*. For positive and negative controls the ability of the strains to grow on pyruvate or with no carbon source was determined respectively.

Colony morphology

While *M. bovis* field strains have dysgonic colony morphology the laboratory-adapted strains *M. bovis* BCG and *M. bovis* AN5 have been described as eugonic (Paterson, 1948; Guerin, 1980), an appearance normally associated with *M. tuberculosis*. As *M. bovis*/pLK102 and *M. bovis* attB::I423 are glycerol utilizing strains their colony morphology was examined on solid medium, with and without glycerol, to determine whether introduction of a functioning PK into a dysgonic strain conferred eugonic growth on the recombinant strain. This was indeed shown to be the case as restoration of *pykA* by gene knock-in resulted in a change in colony morphology (Fig. 6). The *pykA*-

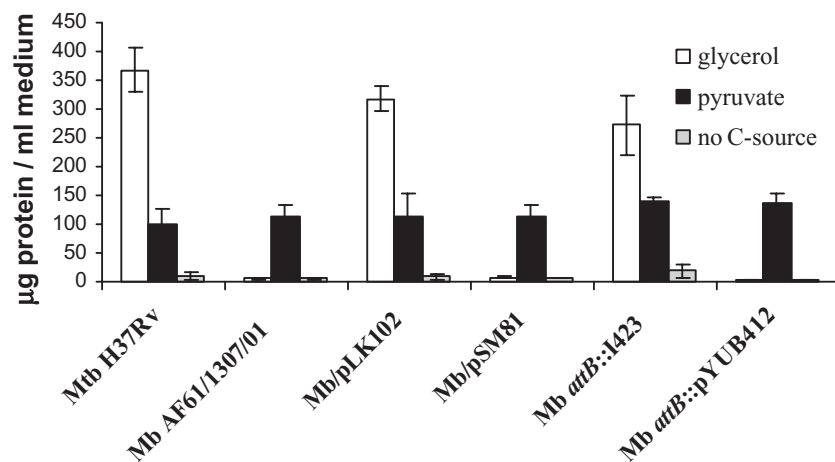


Fig. 5. Growth of GK⁺ wild-type and *pykA* complemented strains in Sauton's medium containing glycerol (□), pyruvate (■) and no carbon source (◻). Cultures were grown with appropriate antibiotics for 4 weeks at 37°C in a rolling incubator. Results were obtained from three independent cultures by determining the amount of total cellular protein released from NaOH-treated cells (Meyers *et al.*, 1998). Error bars show the standard deviations of the mean. At least two determinations of protein yield were carried out on each culture. Mb, *M. bovis*; Mtb, *M. tuberculosis*.

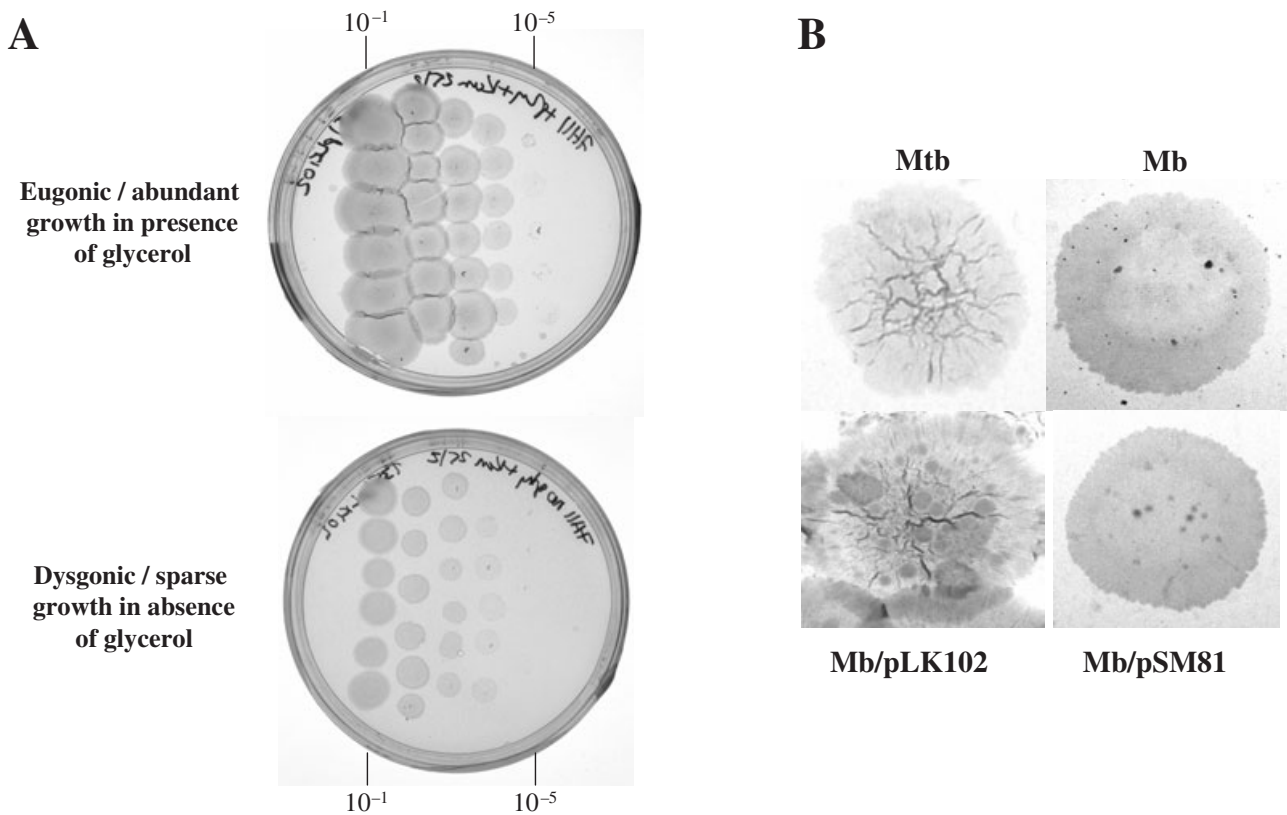


Fig. 6. Colony morphology of eugonic and dysgonic strains.

A. Three week growth of serially diluted *M. bovis*/pLK102 on Middlebrook 7H11 Kan agar plates with and without glycerol.

B. Colony morphology of *M. tuberculosis* H37Rv (Mtb), *M. bovis* 61/1307/01 (Mb), *M. bovis*/pLK102 (Mb/pLK102) and *M. bovis*/pSM81 (Mb/pSM81) after 3 week growth on Middlebrook 7H11 agar plates with 0.5% glycerol and antibiotics as appropriate.

complemented *M. bovis* strains showed more abundant growth in the presence of glycerol (Fig. 6A) and the colonies were raised, crumbly and dry (Fig. 6B), i.e. eugonic growth. This is in contrast to the appearance of the wild-type *M. bovis* and *M. bovis* with either the plasmid background control, *M. bovis*/pSM81 (Fig. 6B) or the cosmid background control, *M. bovis attB::pYUB412* (data not shown), which displayed dysgonic growth, i.e. sparse growth on glycerol-containing medium and a flat, moist glossy morphotype.

Discussion

The *M. tuberculosis* complex is a group of highly genetically related yet phenotypically diverse strains. A range of *in vitro* characteristics can be used to differentiate the members of the complex; for example, unlike *M. tuberculosis*, *M. bovis* is unable to use glycerol as a sole carbon source. It was our aim to define the genetic basis for this *in vitro* phenotype and determine any relevance to *in vivo* biology. Thus, when the *M. bovis* genome was completed it was intriguing to find a number of mutations in genes involved in glycerol metabolism (Garnier *et al.*, 2003).

We focused our analysis on two key genes that showed differences across the *M. tuberculosis* complex, namely *glpK* and *pykA*. Our genetic and biochemical examination of GK in a range of tubercle bacilli showed that the absence of GK activity was not the sole reason for an inability to catabolize glycerol. Our attention therefore turned to *pykA* that encodes PK, which catalyses the final irreversible step in glycolysis, the transphosphorylation of PEP and ADP to pyruvate and ATP. The sequence of this ubiquitous enzyme is highly conserved across prokaryotic and eukaryotic systems and a number of amino acids are essential for catalytic activity (Munoz and Ponce, 2003). Glu220 (Glu271 from the *Oryctolagus cuniculus* rabbit muscle PK) has been shown by Munoz *et al.* (Munoz and Ponce, 2003) to be absolutely conserved among 50 pyruvate kinases analysed. This amino acid residue forms part of the active site and has been associated with the binding of ADP/ATP, PEP (Muirhead *et al.*, 1986) and Mg^{++} (Larsen *et al.*, 1997). A single nucleotide polymorphism in the *M. bovis* sequence results in this glutamic acid residue being substituted with an aspartic acid residue, disrupting the integrity of the enzyme.

The ATP generating role of glycolysis is therefore

defunct in *M. bovis* and glycolytic intermediates are unable to feed into the citric acid cycle or fermentative processes. The PPP cannot be used as an alternative pathway as its metabolic intermediates feed into the lower part of the glycolytic pathway and ultimately to PK. The only other pathway for glucose utilization is the Entner-Doudoroff pathway which is not present in pathogenic mycobacteria (Ratledge, 1982; Garnier *et al.*, 2003). This disruption of glycolysis is not limited to *M. bovis* as the *pykA* mutation is also found in *M. microti* and *M. africanum*, strains that are also defined as requiring pyruvate for growth on glycerinated media (Wayne and Kubica, 1986). This means that *M. microti* and *M. africanum*, like *M. bovis*, cannot catabolize carbohydrates to completion.

Deficiencies in sugar catabolism have been reported in other bacterial pathogens. Most notably *Bordetella* species, also pathogens of the respiratory tract, do not use sugars as carbon sources and lack the genes encoding glucokinase and phosphofructokinase that function in the initial steps of glycolysis (Parkhill *et al.*, 2003). Similarly, the upper glycolytic pathway of *Campylobacter jejuni* is disrupted through the lack of a gene encoding phosphofructokinase, despite having a fully functional PK (Velayudhan and Kelly, 2002). Retention of PK activity indicates a possible catabolic role for the lower part of the glycolytic pathway in this enteric pathogen. In *Salmonella enterica* sv. *typhimurium* glycolysis and the PPP do not appear to play a role in carbohydrate catabolism *in vivo* (Eriksson *et al.*, 2003). Rather, *S. typhimurium* uses the Entner-Doudoroff pathway to metabolize gluconate and related sugars as carbon sources within macrophages. The redundancy of these sugar catabolic pathways suggests a lack of carbohydrate availability within particular *in vivo* environments.

Studies of the *in vivo* environment encountered by *M. tuberculosis* have recurrently found that lipids act as important nutrients. Genes involved in beta-oxidation, the glyoxylate shunt and gluconeogenesis have been shown to be upregulated or required for *in vivo* growth and persistence by a number of groups. McKinney *et al.* (McKinney *et al.*, 2000) showed that the glyoxylate cycle enzyme isocitrate lyase is required for persistence in macrophages and mice. Transcriptome analysis of *M. tuberculosis* in macrophages by Schnappinger and coworkers has demonstrated that genes involved in beta-oxidation and gluconeogenesis were upregulated relative to the *in vitro* condition (Schnappinger *et al.*, 2003). Similarly Timm *et al.* demonstrated upregulated expression of *pckA* (key to gluconeogenesis) and *icl* in the lungs of mice and tuberculosis patients (Timm *et al.*, 2003). Sassetti and Rubin have also shown that inactivation of 15 genes involved in lipid metabolism, and disruption of the gene *glpX* that encodes the key gluconeogenic enzyme fructose 1,6-bisphosphatase, attenuated the mutants for *in vivo*

growth (Sassetti and Rubin, 2003). These studies suggest that in the phagosomal environment, fatty acids are a major carbon source for *M. tuberculosis*. The importance of lipids as sources of carbon to *M. bovis in vivo* is highlighted by the attenuation of *pckA* mutants. PEPCK, encoded by *pckA*, catalyses the first committed step of gluconeogenesis – the decarboxylation and phosphorylation of oxaloacetate to PEP. Collins *et al.* (Collins *et al.*, 2002) concluded from the avirulence of their *pckA* knock-out strains that the gluconeogenic pathway is essential for *M. bovis* virulence in guinea pigs. Similarly *pckA*-deficient *M. bovis* BCG showed attenuated virulence in mice and in macrophages (Liu *et al.*, 2003). The identification of the lesion in *pykA* described in this paper underlines the importance of lipids to mycobacteria and demonstrates that for *M. bovis*, *M. africanum* and *M. microti*, complex lipids are an essential carbon source because of the inability of these strains to catabolize carbohydrates for energy.

When it comes to the importance of carbohydrate catabolism in *M. tuberculosis* the situation appears more complex. There are three enzymes that are key in glycolysis because of the irreversibility of the reactions they catalyse; glucokinase, phosphofructokinase and pyruvate kinase. Glucokinase catalyses the first step of glycolysis, phosphorylating glucose to form glucose 6-phosphate, and is encoded by *ppgK* (Hsieh *et al.*, 1993). By use of a saturation mutagenesis approach *ppgK* was shown to be required for survival of *M. tuberculosis* during infection of mice (Sassetti and Rubin, 2003). The requirement for *ppgK* may signify the use of glucose as an energy source, and/or underline that the end-product of the PpgK catalysed reaction, glucose 6-phosphate, is a critical intermediate metabolite for the synthesis of essential cell wall components of the peptidoglycan-arabinogalactan-mycolic acid network (Tuckman *et al.*, 1997). In contrast to glucokinase, neither of the genes (*pfkA*, *pfkB*) predicted to encode phosphofructokinase were found to be essential to growth *in vivo* by saturation mutagenesis (Sassetti and Rubin, 2003), though this may reflect the compensation of an inactive *pfkA* by a wild-type *pfkB*, and vice versa. It is therefore noteworthy that *pfkB* has been shown by transcriptional analysis to be induced in activated macrophages (Schnappinger *et al.*, 2003). In relation to the last irreversible step in glycolysis, there have been no reports of *pykA* being essential to *in vivo* growth of *M. tuberculosis*, although Rubin and colleagues reported that optimal growth of *M. tuberculosis* in glucose-containing medium required the *pykA* gene (Sassetti *et al.*, 2003). Therefore, in light of the current data the role of carbohydrates as energy sources for *M. tuberculosis* during *in vivo* growth is unclear. This may be reconciled by the dual role of the glycolytic pathway; it degrades glucose to generate ATP and it provides building blocks for synthetic reactions. It is the ATP-generating role that is rendered defunct by

the *pykA* lesion in *M. bovis* and other strains. However, our data underline that fatty acids are the primary source of carbon *in vivo* and in this regard there is consistency across the *M. tuberculosis* complex.

Using chromosomal deletion events Brosch *et al.* have suggested a novel evolutionary scenario for the *M. tuberculosis* complex, with *M. tuberculosis* being closer to the common progenitor of the complex than *M. bovis* (Brosch *et al.*, 2002). A key deletion event, RD9, splits *M. tuberculosis* and *M. canettii* from *M. africanum*, *M. microti* and *M. bovis*. We have found that the *pykA* Glu220Asp point mutation co-segregates with the RD9 deletion event, such that *M. tuberculosis* complex strains that are RD9-deleted also carry the *pykA* Glu220Asp mutation; hence the *pykA* mutation is consistent with the Brosch phylogeny. Clearly, the loss of PK in this branch of the complex shows that this enzyme activity is not required for virulence in *M. africanum*, *M. microti* and *M. bovis*. Taken with the evidence that lipids are the key carbon source for tubercle bacilli *in vivo*, the loss of PK activity may reflect reductive evolution, with the accumulation of mutations in genes whose function is no longer required for *in vivo* survival. The loss of GK activity in some *M. bovis* strains lends further support to this idea. Analysis of the *glpK* SNP across the evolutionary lineages does not show such tight linkage, likely because of the SNP occurring in a homopolymeric tract that is prone to strand-slippage. The key point from the sequencing of *glpK* across strains is that glycerol-utilizing PK⁺ strains retain an in frame *glpK* whereas PK⁻ strains, unable to grow on glycerol, may contain a disrupted *glpK* as its activity is functionally redundant.

The attenuated *M. bovis* strain BCG is the most widely used vaccine in the world, although the exact steps leading to its attenuation remain obscure. Calmette and Guérin derived the BCG vaccine strain by subculturing of an *M. bovis* isolate on a growth medium of potato slices soaked in ox bile and glycerol (Guerin, 1980); hence the carbon source used by *M. bovis* during the subculturing process was glycerol. Paradoxically, *M. bovis* cannot use glycerol as a sole carbon source, so how did the strain grow? As we have shown in this paper, restoration of PK activity allows *M. bovis* to grow abundantly on glycerol, with the colonies of the complemented *M. bovis* appearing raised, dry and crumbly as opposed to the flat, moist colonies of the wild-type strain. Guérin stated that the colony morphology of their *M. bovis* strain during initial passage was 'granular, dry and rough' (Guerin, 1980). The growth on this medium, eugonic colony morphology, and the *pykA* Glu220 genotype of BCG shows that the culture medium they used selected an *M. bovis* mutant that was able to use glycerol as a sole carbon source. The same is also true for *M. bovis* AN5, a strain of *M. bovis* used worldwide for the production of bovine tuberculin that was

adapted to growth on glycerol and presents eugonic growth and PK activity. Hence, our analysis explains the molecular genetic basis for altered colony morphology in glycerol adapted *M. bovis* strains, including BCG.

In conclusion, we have defined the genetic basis for both the pyruvate requirement and the eugonic/dysgonic morphotypes across members of the *M. tuberculosis* complex, *in vitro* phenotypes that have puzzled researchers for decades. Our work also sheds light on the *in vivo* milieu encountered by pathogenic mycobacteria, with the lack of pyruvate kinase activity from *M. bovis* and other RD9-deleted members of the complex showing that lipids are the key *in vivo* nutrient for these pathogens.

Experimental procedures

Bacterial strains and growth conditions

Mycobacteria strains are described in Table 1. The media used included Middlebrook 7H9 medium (Difco) containing 10% (v/v) albumin-dextrose-catalase (ADC, Difco) enrichment, 0.2% glycerol and 0.05% Tween 80 (MADCTw) for cell extract preparation, and Middlebrook 7H11 medium (Difco) containing 10% (v/v) oleic acid-ADC (OADC, Difco) enrichment with and without 0.5% glycerol for colony morphology examination. In addition, a modified Sautons medium was employed as a detergent-free minimal medium for sole carbon source utilization studies, containing (per litre) 4 g of asparagine, 2 g of citric acid, 0.5 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, and 0.05 g of ferric ammonium citrate, pH 7.2. Carbon sources were added to a final concentration of 40 mM for sodium pyruvate, 40 mM for glucose, 0.05% (v/v) for Tween 80 (+0.5% BSA) and 6% (v/v) for glycerol, or omitted as a negative control. *E. coli* DH5 α (Invitrogen) was used as a general purpose cloning host and was grown in Luria–Bertani medium with kanamycin (25 μ g ml⁻¹) or hygromycin (100 μ g ml⁻¹) as appropriate.

SNP analysis

To sequence across the SNP sites in *glpK* and *pykA*, a 240 bp *glpK* PCR fragment was produced with the primers *glpk240F* and *glpk240R*, and a 276 bp *pykA* PCR fragment was amplified with primers *pykasnpF* and *pykasnpR* (Table 4). Amplification reactions for *glpK* were performed using the Advantage-GC Genomic Polymerase Mix kit (Clontech), with thermal cycle conditions of 95°C denaturation, 64°C annealing and 68°C extension, for 30 cycles. Amplification reactions for *pykA* were performed using the HotStar-Taq Master Mix kit (Qiagen), with thermal cycle conditions of 94°C denaturation, 61°C annealing and 72°C extension, for 35 cycles. Sequencing reactions were carried out using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and electrophoresed through an ABI Prism 377 DNA Sequencer. Sixteen *M. bovis* strains of diverse molecular type were checked for the *glpK* SNP, while 100 *M. bovis* strains were checked for the *pykA* SNP (VLA-Weybridge culture collection). SNP analysis was also carried out on various members of the *M. tuberculosis* complex listed in Table 1.

Primer	Sequence
glpk240F	5'-CGGCAAGCTGCAGTGGATCCTGGAA-3'
glpk240R	5'-GGCAGCATGGCCCGAGGTATCGAAA-3'
pykasnpF	5'-CGTTGCCCGGAATGAACGTG-3'
pykasnpR	5'-GGATGGCTCGCTTCTGTACC-3'
pykA355F	5'-CCTGTCGGAGAAGGACATCGAGGATCTCAC-3'
pykA355R	5'-CGAGTTCTCGATCATCGAGTCGAGCATCTG-3'
sigAf	5'-ACGCCGAACCTACCCGCATCC-3'
sigAr	5'-GTCGAGAAACGCCATGCCCC-3'
intraF	5'-TATCGCATCACAGTCGCCAC-3'
intraR	5'-TGATCCGGTCGCCGATCAG-3'
pykaFBamHI	5'-CTGAGGATCCATGACGAGACGCGGGAAAATC-3'
pykaRBamHI	5'-GTCCGGATCCACTAGTCTAGACGTCATCTCCCCGA-3'

Sequences underlined indicate relevant restrictions sites.

Preparation of cell extracts and enzyme assay

Cells were harvested at mid-logarithmic stage and washed twice with 20 mM phosphate buffer pH 6.8 plus 0.05% Tween 80 and resuspended in 20 mM phosphate buffer pH 6.8 plus 5 mM MgCl₂. The chilled cells were disrupted using the Fast Prep® system (Hybaid) for 20 s at speed 6.5 m s⁻¹ twice and the supernatant harvested by centrifugation at 14 000 g for 5 min at 4°C. GK activity was measured at 37°C by the glycerol 3-phosphate-coupled assay of Pettigrew *et al.* (Pettigrew *et al.*, 1998). PK activity was assayed by the method adapted by Wheeler (1983). Extracts were prepared and assayed from at least two independent cultures. When no activity was detected extracts were prepared from four independent cultures for confirmation. Throughout the paper specific activity is expressed as means ± 95% confidence limits. Protein concentration was measured using the Bradford method (Bio-Rad Laboratory) using BSA as standard.

RT-PCR of *pykA*

RNA from mid-logarithmic cultures was extracted using the guanidinium thiocyanate method (Stewart *et al.*, 2002). RT-PCR of *pykA* (355 bp) was carried out using primers pykA355F and pykA355R (Table 4) with the Promega Access RT-PCR kit following the manufacturer's instructions. DNA contamination was checked by using primers for an intragenic region, *intraF* and *intraR*. RT-PCR of the constitutively expressed *sigA* (285 bp) was used as a positive control (*sigAf*, *sigAr*). qRT-PCR was carried out on a Rotor Gene RG3000 Instrument (Corbett Research) with thermal cycle conditions of 95°C denaturation, 60°C annealing and extension, for 45 cycles. cDNA was synthesized using SuperScript II Rnase H⁻ Reverse Transcriptase (Invitrogen). Amplification reactions were performed using the QuantiTect SYBR Green PCR Master Mix kit (Qiagen). The experiment was repeated three times.

Generation of *pykA* plasmid construct

The *pykA* coding region from *M. tuberculosis* H37Rv chromosomal DNA was amplified using primers pykaFBamHI and pykaRBamHI (Table 4). PCR was carried out using Advantage-HF PCR Kit (Clontech) following the manufac-

Table 4. Oligonucleotide primers used in this study.

turer's instructions and thermal cycling conditions of 95°C denaturation, 64°C annealing and 68°C extension, for 30 cycles. The amplified DNA was cloned into the pGEM-T Easy vector (Promega) to generate construct pLK100. After sequencing, the insert was excised from this vector by digestion with *Bam*HI (restriction sites underlined; Table 4) and cloned into *Bam*HI restricted pSM81 (Table 1). This generated the plasmid pLK102 with the *pykA* coding sequence under the control of the *M. tuberculosis hsp70* promoter.

Complementation of *M. bovis*

The integrating cosmid I423, a pYUB412-based construct containing 33 kb that spans the *M. tuberculosis pykA* locus (*M. tuberculosis* genome position 1786–1819 kb), was a gift from Dr Roland Brosch, Pasteur Institute, Paris. pYUB412 and its derivative integrate at the *attB* locus. pLK102 and I423 were transformed into *M. bovis* 61/1307/01 and transformants selected on 7H11 plates containing 25 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ hygromycin respectively. Isolated colonies were selected from the plates, checked for the presence of the plasmid by PCR, and then grown in MADCTw medium containing the appropriate antibiotic to yield stocks of *M. bovis*/pLK102 and *M. bovis attB::I423*.

Determination of growth on various carbon sources

The ability of a carbon source to support growth was determined by the method of Meyers *et al.* (Meyers *et al.*, 1998). This method facilitates the measurement of growth in detergent-free media by quantifying the total cellular protein released from NaOH-treated cells. Results were obtained from three independent cultures.

Colony morphology

Cultures were grown in MADCTw for 8 days, washed, resuspended and serially diluted in PBS/0.05% Tween 80. Twenty-five microlitre samples of each 10-fold dilution were spotted in five or six spots adjacently onto 7H11 agar plates with and without 0.5% glycerol and incubated for 3 weeks at 37°C. Antibiotics were included as required.

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