Characterization of a β -hydroxybutyryl-CoA dehydrogenase from *Mycobacterium tuberculosis*

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The lipid-rich cell wall of mycobacteria is essential not only for virulence but also for survival. Whilst anabolic pathways for mycobacterial lipid biosynthesis have been well studied, there has been little research looking into lipid catabolism. The genome of *Mycobacterium tuberculosis* encodes multiple enzymes with putative roles in the β -oxidation of fatty acids. In this report we explore the functionality of FadB2, one of five *M. tuberculosis* homologues of a β -hydroxybutyryl-CoA dehydrogenase, an enzyme that catalyses the third step in the β -oxidation cycle. Purified *M. tuberculosis* FadB2 catalysed the *in vitro* NAD⁺-dependent dehydration of β -hydroxybutyryl-CoA to acetoacetyl-CoA at pH 10. Mutation of the active-site serine-122 residue resulted in loss of enzyme activity, consistent with the function of FadB2 as a fatty acyl dehydrogenase involved in the β -oxidation of fatty acids. Surprisingly, purified FadB2 also catalysed the reverse reaction, converting acetoacetyl-CoA to β -hydroxybutyryl-CoA, albeit in a lower pH range of 5.5–6.5. Additionally, a null mutant of *fadB2* was generated in *Mycobacterium smegmatis*. However, the mutant showed no significant differences from the wild-type strain with regard to lipid composition, utilization of different fatty acid carbon sources and tolerance to various stresses; the absence of any phenotype in the mutant strain could be due to the potential redundancy between the five *M. smegmatis* *fadB* paralogues.

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, is estimated to kill 1.6 million people every year (WHO, 2007). With the evolution of drug-resistant strains and the recent report of a strain resistant to all known drugs, there is an urgent need for effective new anti-tuberculosis therapies and novel drug targets (Velayati *et al.*, 2009). Presently, some of the most successful anti-tubercular treatments currently used target lipid metabolism, for example isoniazid, which targets mycolic acid biosynthesis (Takayama *et al.*, 1972). The importance of lipids in mycobacteria is seen by the unusually high lipid content of the cell wall and the vast number of genes involved in lipid metabolism, as revealed by analysis of the complete genome sequence (Cole *et al.*, 1998).

Despite lipid biosynthesis having been well studied in mycobacteria (Bhatt *et al.*, 2007; Heath & Rock, 2004; Takayama *et al.*, 1972), the extensive field of lipid degradation has not yet been investigated with equal intensity. The chemistry of the β -oxidation pathway is similar to the reverse of lipid biosynthesis. Saturated long-chain fatty acids

are first activated by CoA attachment and subsequently undergo oxidation by an acyl-CoA dehydrogenase. This is followed by hydration catalysed by an enoyl-CoA hydratase to produce β -hydroxyacyl-CoA. The hydroxy group is further oxidized to form a β -ketoacyl-CoA, which is then finally cleaved by a β -ketoacyl-CoA thiolase, releasing acetyl-CoA and a saturated fatty acid that is two carbons shorter (Black & DiRusso, 1994). While playing a general role in fatty acid turnover, in mycobacteria fatty acid β -oxidation may have a specific relevance for the physiology of the bacterium during survival within the hypoxic niche of a granuloma, a critical site of chronic infection. The glyoxylate cycle, which utilizes acetyl-CoA (produced by β -oxidation) to generate glucose by conserving the two carbon atoms lost as 2 CO₂ in the TCA cycle, is crucial for the persistence of *M. tuberculosis* in the mouse model of infection (McKinney *et al.*, 2000). Additionally, several studies have indicated that host-derived fatty acids could be a key source of carbon for the bacteria (Bloch & Segal, 1956; Bishai, 2000; Sassetti & Rubin, 2003), suggesting that infecting mycobacteria favour exploiting the lipid-rich environment of host tissues over synthesizing their own lipids *de novo* (Wheeler *et al.*, 1990; Wheeler & Ratledge, 1994).

The third step in β -oxidation is carried out by a β -hydroxyacyl-CoA dehydrogenase [EC 1.1.1.35], which

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Abbreviation: CD, circular dichroism.

converts the β -hydroxy group to a β -keto group using NAD^+ as a cofactor. Unlike in *Escherichia coli*, where the *fadA* and *fadB* genes form a single operon encoding the two proteins of the β -oxidation multi-enzyme complex (DiRusso, 1990), in *M. tuberculosis* there are five discrete *fadB* genes and six *fadA* genes. Recently, *Mt-FadA5* has been shown to catalyse the thiolysis of cholesterol, although the function(s) of the other five *fadA* genes are yet to be elucidated (Nesbitt *et al.*, 2009). *FadB1* is thought to perform the second step in the pathway, involving conversion of the *trans*-2-enoyl to the β -hydroxy group, but the function(s) of *FadB2*, *B3*, *B4* and *B5* remain unknown. This may suggest a certain degree of redundancy amongst the *FadB* enzymes, especially as only *FadB3* has been described as essential (Sasseti *et al.*, 2003). The *Mt-FadB2* sequence is homologous to the products of the *Mycobacterium smegmatis* gene *MSMEG0912* and the *Mycobacterium leprae* gene *ML_2461*, and to sections of the *E. coli* and *Pseudomonas fragi* *FadB* enzymes (Fig. 1A, B). It is possible that the *FadB* enzymes are chain length specific, like the short-, medium- and long-chain β -hydroxyacyl-CoA dehydrogenases found in mammalian mitochondria (El-Fakhri & Middleton, 1982; McGuire *et al.*, 1990). One interesting feature of the *fadB2* gene is that it is conserved across mycobacteria, and is located in the genome adjacent to *icl1* (Fig. 1B). This gene encodes the glyoxylate shunt enzyme isocitrate lyase, which has been shown to promote persistence of *M. tuberculosis* cells in an infected host (McKinney *et al.*, 2000). Prior to the sequencing of the first *M. tuberculosis* genome, *H37Rv*, a β -hydroxyacyl-CoA dehydrogenase enzyme from *Mycobacterium smegmatis* had also been characterized using a purified protein fraction containing β -hydroxyacyl-CoA dehydrogenase activity (Shimakata *et al.*, 1979). This and a previous study by the same group led to the conclusion that the enzyme was involved in the acetyl-CoA-dependent elongation of fatty acids, but failed to provide evidence for its involvement in fatty acid β -oxidation (Shimakata *et al.*, 1977).

This present study describes the cloning of *M. tuberculosis* β -hydroxyacyl-CoA dehydrogenase, *Mt-FadB2*, its overexpression in *E. coli* and its purification, which has allowed for detailed protein characterization and kinetic studies. In parallel, a mutant of the *fadB2* homologue in the surrogate *M. smegmatis* was also generated, in order to explore the functional role of *fadB2*.

METHODS

In silico analysis of *Mt-FadB2*. The *FadB2* nucleotide and protein sequences were obtained from TubercuList (<http://genolist.pasteur.fr/TubercuList/>). CLUSTAL W (Labarga *et al.*, 2007) and ESPript (Gouet *et al.*, 1999) were used to produce multiple sequence alignments of β -hydroxyacyl-CoA dehydrogenase from various species. The ProtParam tool from the Swiss Institute of Bioinformatics ExPASy Server was used for primary sequence analysis (Gasteiger *et al.*, 2005)

and GOR IV was used to predict the secondary protein structure (Garnier *et al.*, 1996).

Plasmids and DNA manipulation. The *E. coli*-compatible vector pET28b (Novagen) containing a T7 promoter and encoding both N- and C-terminal 6-histidine tags was used for the overexpression of *Mt-FadB2*. All PCR amplifications were executed using Vent DNA polymerase (New England Biolabs) and *M. tuberculosis* H₃₇Rv chromosomal DNA. *Mt-FadB2* PCR amplification was performed using the upstream primer 5'-GATCGATCCATATGGTGAGCGA-TGCGATCCAGCGG-3' and the downstream primer 3'-GATC-GATCAAGCTTGTACGTGTAGAAACCTCGACC-5', which contain *NdeI* and *HindIII* restriction sites respectively (underlined). The 858 bp PCR product was then doubly digested with *NdeI/HindIII* and ligated into a similarly cut pET28b plasmid, yielding pET28b-*Mt-FadB2*. DNA sequencing was used to verify the recombinant gene.

Construction of the *Mt-FadB2* S122A mutant. The Stratagene QuikChange Multi Site-Directed Mutagenesis kit was used to create a pET28b-*Mt-fadB2*(S122A) plasmid. The following primers were used to introduce the mutation into the pET28b-*Mt-fadB2* plasmid template: 5'-TGGCGTCGAATACCGCCAGCATCCCGATC-3' and 5'-GATCGGGATGCTGGCGGTATTTCGACGCCA-3'. The ensuing PCR and transformation were performed according to the kit manual. Sequencing was used to confirm that the mutation had been made.

Overexpression of wild-type and S122A *Mt-FadB2*. The pET28b-*Mt-fadB2* plasmid was transformed into *E. coli* C43 (DE3) cells and incubated on LB agar containing 25 $\mu\text{g ml}^{-1}$ kanamycin overnight at 37 °C. One colony was used to inoculate LB broth (5 ml) containing kanamycin (25 $\mu\text{g ml}^{-1}$) and cultures were incubated on a rotary incubator at 37 °C overnight. This mini-culture was used to inoculate Terrific broth (1 l) containing kanamycin (25 $\mu\text{g ml}^{-1}$) and was incubated on a rotary incubator (180 r.p.m.) at 37 °C until the OD₆₀₀ had reached ~0.6. At this point, the culture was induced by the addition of 1 mM IPTG and incubated with shaking for 16 h at 16 °C. The cells were harvested by centrifugation at 4500 g for 12 min and stored at -20 °C for purification. Overexpression of the His-tagged *Mt-FadB2* was confirmed by Western blot analysis using mouse monoclonal Penta-His antibody (Qiagen).

Purification of wild-type and S122A *Mt-FadB2* protein. Pelleted cells of the wild-type and the *Mt-FadB2* S122A mutant were resuspended in buffer A (0.02 M sodium phosphate, pH 7.9, 0.5 M NaCl) and lysed by probe sonication (10 cycles of 30 s pulses followed by 30 s cooling intervals). The crude lysate was centrifuged at 27 000 g for 40 min at 4 °C to remove the insoluble components and the resulting clarified lysate was applied to a Ni²⁺-charged 5 ml HisTrap HP affinity column (GE Healthcare). The column was washed with 10 column volumes of buffer A and the protein was eluted with a stepwise imidazole gradient of 50 mM (50 ml), 130 mM (10 ml) and 1 M (10 ml). The fractions were analysed by 12% SDS-PAGE followed by Coomassie staining. A large single band at 30 kDa on SDS-PAGE demonstrated the purity of the protein and *Mt-FadB2* was correctly identified by trypsinization digest and mass spectrometry. The fractions containing pure *Mt-FadB2* protein were dialysed, first against 50 mM potassium phosphate (pH 7.9), 50 mM NaF, with 1 mM EDTA added to remove any leached Ni²⁺, and then twice against 50 mM potassium phosphate (pH 7.9), 50 mM NaF at 4 °C. The potassium phosphate/NaF buffer was chosen because it provided optimal conditions for circular dichroism experiments. *Mt-FadB2* concentration was determined using a BCA Protein Assay kit (ThermoScientific); a total of 18 mg protein could be obtained from a 1 l culture. The purified protein was stored at -80 °C both with and without the addition of 10% glycerol.

Fig. 1. Sequence alignments. (A) Multiple sequence alignment of *M. tuberculosis* FadB2 with mycobacterial homologues and other *M. tuberculosis* Fad proteins. The amino acid sequence of *M. tuberculosis* FadB2 (Mt_FadB2, accession no. CAA17423) is compared with those of homologues in *M. smegmatis* (Ms_0912, accession no. ABK71785), *M. leprae* (ML_2461, accession no. CAC31978), *E. coli* (Ec_FadB, accession no. AAC76849 residues 297–624) and *Pseudomonas fragi* (Pf_FadB accession no. 2D3T_A) as well as the other *M. tuberculosis* FadB-annotated proteins: FadB1 (accession no. CAA17666 residues 310–633) and FadB3 (accession no. CAE55415). The figure was generated using CLUSTAL W and ESPript (available from <http://expasy.ch/>), and data for the corresponding secondary structure for *P. fragi* FadB (residues 270–575) were obtained from the Protein Data Bank. Red-filled boxes indicate residues conserved across all the species aligned and blue-boxed residues are those of high similarity. The triangles indicate the putative NAD⁺-binding site and the stars highlight residues of the hypothetical catalytic triad. (B) Maps of the genomic regions of *M. tuberculosis* H₃₇Rv *fadB2*, *M. smegmatis* mc²155 MSMEG0912 and *M. leprae* TN *fadB2*.

Enzyme assay for wild-type and S122A *Mt-FadB2*. The activity assay for *Mt-FadB2* was based on that previously described (Nemeria *et al.*, 2001). The forward *Mt-FadB2*-dependent reduction of NAD⁺ to NADH was monitored spectrophotometrically at 340 nm using a Jenway 6-10 spectrophotometer. The standardized 1 ml reaction contained 100 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (pH 9.5), 50 µM NAD⁺, 50 µM β-hydroxybutyryl-CoA and 7 µg purified *Mt-FadB2* protein. The reaction mixture minus the protein was incubated for 2 min at 30 °C before adding the enzyme to start the reaction. The reverse reaction was observed similarly. The standard 1 ml reaction contained 100 mM potassium phosphate buffer (pH 7.0), 50 µM NADH, 50 µM acetoacetyl-CoA and 7 µg pure *Mt-FadB2* protein. All controls were performed to ensure that neither the potassium phosphate/NaF buffer nor any of the assay buffers or substrates interfered with the assay results. All reactions were monitored for a long enough period to obtain an accurate rate. The kinetic parameters K_m , V_{max} and k_{cat} for each substrate and cofactor were ascertained by varying their concentration and fitting the data to the Michaelis–Menten equation and using SigmaPlot 8.0. Each reaction was performed in triplicate.

Circular dichroism (CD) spectroscopy. All CD far-UV spectra were obtained at 25 °C using a Jasco J-715 spectropolarimeter and a 0.01 cm pathlength cell. The wild-type and S122A-mutant *Mt-FadB2* proteins were made to 1.1 mg ml⁻¹ as determined by the BCA Protein Assay kit. To compare the folding of the wild-type and mutant protein, 50 mM potassium phosphate (pH 7.9), 50 mM NaF buffer was used. To assess protein folding at different pH values, 50 mM potassium phosphate (pH 6, 7 or 8), 50 mM NaF or 50 mM CAPS (pH 9, pH 10 or pH 11), 50 mM NaF was used. Each condition was scanned eight times over a wavelength of 197–245 nm and a bandwidth of 2 nm. The results were normalized by subtracting the baseline buffer spectrum from each.

Construction of the *M. smegmatis fadB2* deletion mutant. The *M. smegmatis* knockout mutant was generated using the method previously described (Bardarov *et al.*, 2002). A 1000 bp sequence of the upstream region of *Ms-fadB2* and an 800 bp sequence of the downstream region of *Ms-fadB2* were PCR amplified using *M. smegmatis* mc²155 genomic DNA, Phusion DNA polymerase (Finnzymes) and the following primers, each containing a *Dra*III restriction site at the 5' end (underlined): *Ms-fadB2_LL* (5'-TTTTTTTTCACAAAGTGCTGGAGCCCTGCATCGCGCG-3'), *Ms-fadB2_LR* (5'-TTTTTTTTCACCTTCGTGCGGGATGCTCGACGTGTTCG-3'), *Ms-fadB2_RL* (5'-TTTTTTTTCACAGAGTGCTGCTCTCGCAATCCGCAT-3') and *Ms-fadB2_RR* (5'-TTTTTTTTCACCTTGCTGCTTCCCAACCCTGCAGGCGG-3'). The PCR fragments were cloned in the vector p0004S to generate the allelic-exchange plasmid pΔ*Ms-fadB2*, which was then *PacI*-digested and packaged into the mycobacteriophage phAE159 to generate the knockout phage phΔ*Ms-fadB2*. High-titre phage particles were produced and transduced into wild-type *M. smegmatis* mc²155 cells as previously described (Bardarov *et al.*, 2002). Hygromycin-resistant colonies obtained after transduction were confirmed as *Ms-fadB2*-deletion mutants by Southern blotting.

Construction of the complemented Δ*Ms-fadB2* strains. The *E. coli*–*Mycobacterium* shuttle vector pMV261 was used for complementation (Stover *et al.*, 1991). *Ms-fadB2* was amplified from *M. smegmatis* mc²155 chromosomal DNA using Phusion DNA polymerase. The upstream primer (5'-GATCGATCGGATCCAGTGAGCAATGCAATCGAACGA-3') contained a *Bam*HI restriction site (underlined) and the downstream primer (3'-GATCGATCAAGC-TTTCAGTAGGTATAGAAGCCCTT-5') a *Hind*III restriction site (underlined). *Mt-fadB2* PCR amplification was performed using *M. tuberculosis* H₃₇Rv genomic DNA, the upstream primer 5'-GATCGATCGGATCCAGTGAGCGATGCGATCCAGCGG-3' and the

downstream primer 3'-GATCGATCAAGCTTATCAGTACGTGTAG-AAACCTCG-5', which contain *Bam*HI and *Hind*III restriction sites respectively (underlined). The PCR products were then digested with *Bam*HI and *Hind*III and ligated into a similarly cut pMV261 plasmid, yielding pMV261-*Ms-fadB2* and pMV261-*Mt-fadB2*. DNA sequencing was used to verify the recombinant genes. Electrocompetent Δ*Ms-fadB2* mutant cells were electroporated with pMV261-*Ms-fadB2*, pMV261-*Mt-fadB2* or pMV261 plasmid and transformants were selected for using hygromycin (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹).

Growth analysis of the *M. smegmatis fadB2* deletion mutant.

Wild-type *M. smegmatis*, Δ*Ms-fadB2*, and pMV261-*Ms-fadB2*- and pMV261-*Mt-fadB2*-complemented transformants were grown to saturation in minimal medium (MM) plus 0.1 % glucose. The MM used was based on that of Chang *et al.* (2009), containing (per litre) 1.5 g KH₂PO₄, 1.0 g NH₄Cl, 0.2 g MgSO₄·7H₂O, 20.0 mg CaCl₂·2H₂O, 1.2 mg ferric ammonium citrate, 0.85 g NaCl and 8.99 g Na₂HPO₄·12H₂O. The cultures were then washed and resuspended in PBS before streaking on MM agar plates supplemented with either 0.1 % glycerol, glucose, acetate or propionate, or 0.002 % caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, oleic acid or linoleic acid. MM containing cholesterol (0.2 mM) was prepared according to Chang *et al.* (2009).

RESULTS AND DISCUSSION

In silico analysis of *Mt-FadB2*

Mt-FadB2 has a higher than average proportion of charged amino acids (24.1 %) compared to the entire *M. tuberculosis* proteome (20.0 %), the majority of which being negatively charged (Asp + Glu, 13.3 %) (Luthra *et al.*, 2008). Similarly, there are more hydrophobic residues (34.1 %) than polar residues. The high percentage of charged and polar residues suggests that the protein is soluble and therefore not an integral cell membrane protein. The theoretical pI of the native protein is calculated as 5.2, whereas that of the recombinant protein used here is calculated as 6.1 (Gasteiger *et al.*, 2005).

Alignment of β-hydroxyacyl-CoA dehydrogenases from mycobacteria and other species highlights the conserved residues of the hypothetical catalytic triad as identified by Barycki *et al.* (1999) (Fig. 1A). The residues annotated with triangles are consistent with the sequence of a Rossmann fold, a structural motif known to bind NAD⁺. This is supported by the computed GOR IV secondary structure (Garnier *et al.*, 1996), which predicts a β-sheet–α-helix–β-sheet motif at the C terminus, a feature that is also seen in the overlaid secondary structure from the *Pseudomonas fragi* FadB protein. *Mt-FadB2* has high identity to *Mt-FadB3* and part of the *Mt-FadB1* protein (alignment scores 444 and 485 respectively; data not shown). The identity between *Mt-FadB2*, -FadB4 and -FadB5 from *M. tuberculosis*, however, is much less (alignment scores 13 and 0 respectively) and *Mt-FadB4* and -FadB5 do not share the same NAD⁺-binding site or hypothetical catalytic triad residues (data not shown). This may indicate redundancy between *Mt-FadB1*, -FadB2 and -FadB3 but alternative role(s) for *Mt-FadB4* and -FadB5.

***Mt*-FadB2 is an enzymically active β -hydroxybutyryl-CoA dehydrogenase**

In an effort to demonstrate enzymic activity of *Mt*-FadB2, a spectrophotometric assay was developed, which involved monitoring the reduction of the cofactor NAD⁺ at 340 nm (Fig. 2A–E). The kinetic parameters calculated from the data using SigmaPlot 8.0 are displayed in Table 1. The activity for the NAD⁺-dependent dehydrogenase reaction was tested over a range of temperatures and pH values and the optima were found to be 37 °C and pH 10 (CAPS buffer), respectively (Fig. 3A). While it was not unexpected to find that *Mt*-FadB2 was most active at the physiological temperature of the *M. tuberculosis* mammalian host, it was more surprising that the enzyme appeared to work best in alkaline conditions, given the acidic nature of the bacteria-containing phagolysosomal compartment in macrophages. However, the same result has been obtained by Shimakata *et al.* (1979), who purified a protein fraction containing β -hydroxyacyl-CoA dehydrogenase activity from *M. smegmatis*. CD spectra revealed that the protein is more susceptible to unfolding when in acidic conditions than

at high pH values; the tertiary structure is maintained even at pH 10 (Fig. 5B). Nevertheless, it is likely that the optimal pH for *in vivo* activity may be different from those obtained with purified protein, especially knowing that the pI values of the native and recombinant protein are different.

Furthermore, *Mt*-FadB2 NAD⁺-reducing activity was found to be non-metal-dependent; however, various uni- and divalent cations were tested to see if their presence inhibited or activated the protein. Mg²⁺ and Ca²⁺ enhanced the activity of the enzyme, whereas Zn²⁺, Ni²⁺ and Co²⁺ had an inhibitory effect (Fig. 4). The enzyme was not active when NADP⁺ was used as a cofactor, even in the presence of CaCl₂ (data not shown).

Liu *et al.* (2004) found that by substituting the Ser-137 residue with an alanine in rat mitochondrial β -hydroxyacyl-CoA dehydrogenase the activity was greatly reduced, demonstrating that Ser-137 was a key catalytic residue (Fig. 1). Generation of the equivalent mutation in *Mt*-FadB2 (S122A) abolished all activity, supporting the idea

Fig. 2. *Mt*-FadB2 enzyme assay. (A) *Mt*-FadB2 (β -hydroxyacyl-CoA dehydrogenase) catalyses the NAD⁺-dependent reduction of β -hydroxyacyl-CoA to β -oxoacyl-CoA. The reaction velocities were ascertained using increasing concentrations of substrate, β -hydroxybutyryl-CoA (B, D) and acetoacetyl-CoA (C, E). The data were fitted to the Michaelis–Menten equation using SigmaPlot 8.0 (B, D) and by linear regression in the Lineweaver–Burk plot (D, E). Points are the mean $\pm$ SE of three replicates.

Table 1. Kinetic parameters of *Mt*-FadB2 as determined by spectrophotometric assay and double-reciprocal plots

The standardized 1 ml forward reaction contained 100 mM CAPS buffer (pH 9.5), 50 μ M NAD⁺, 50 μ M β -hydroxybutyryl-CoA and 7 μ g purified *Mt*-FadB2 protein. The reverse reaction (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 50 μ M NADH, 50 μ M acetoacetyl-CoA and 7 μ g pure *Mt*-FadB2 protein. In all cases the reaction mixture minus the protein was incubated for 2 min at 30 °C before adding the enzyme to start the reaction and NADH or NAD⁺ production, respectively, was monitored at 340 nm. Values are means of three replicates with the standard error shown in parentheses.

	K_m (mM)	V_{max} (nmol min ⁻¹ mg ⁻¹)	k_{cat} (min ⁻¹)
β -Hydroxybutyryl-CoA	43.5 (5.82)	188.3 (7.4)	0.723 (0.0284)
Acetoacetyl-CoA	65.6 (4.78)	126.6 (8.6)	0.677 (0.0330)
NAD ⁺	29.5 (2.78)	63.1 (1.7)	0.242 (0.00653)
NADH	50.0 (16.8)	2588.0 (200)	11.36 (0.768)

that *Mt*-FadB2 is indeed a β -hydroxyacyl-CoA dehydrogenase. CD far-UV (197–245 nm) spectra of the wild-type and mutant protein were obtained to confirm the correct folding of both proteins (Fig. 5A). As well as showing that the mutation did not cause any changes to the secondary structure, this result strongly indicates that Ser-122 is a key active-site residue of *Mt*-FadB2 that is directly involved in catalysis.

The reverse reaction

We also investigated whether *Mt*-FadB2 could catalyse the reverse reaction, converting acetoacetyl-CoA to β -hydroxybutyryl-CoA, using NADH as the cofactor (Fig. 2C, E, Table 1). Shimakata *et al.* (1979) could not conclude that their protein with β -hydroxyacyl-CoA dehydrogenase activity was involved in fatty acid β -oxidation, but their results did show that the dehydrogenase was involved in the fatty acid elongation system. Wheeler *et al.* (1991) also implied the same conclusions in mycobacteria by showing that other enzymes of the β -oxidation pathway could catalyse both forward and reverse reactions. We tested this reverse reaction over a range of pH values and the optimum was found to be pH 5.5–6.5 (potassium phosphate buffer) (Fig. 3B), a result that has been previously described for β -hydroxyacyl-CoA dehydrogenase (Shimakata *et al.*, 1979). The enzyme kinetics for *Mt*-FadB2 indicate that the protein has a slightly greater affinity for β -hydroxybutyryl-CoA than acetoacetyl-CoA, in other words, the fatty acid catabolism direction. However, the fact that the NADH-oxidation reaction was found to be optimum at the pH inside the macrophage vacuole could suggest that this 'reverse' reaction is favoured *in vivo* and the dehydrogenase is utilized by the bacteria to synthesize, or elongate, fatty acids rather than degrade them. We also found that whereas the forward reaction could not use NADP⁺ as the cofactor, the reverse reaction was able to use NADPH as well as NADH. This therefore also indicates a role for *Mt*-FadB2 in lipid synthesis and fatty acid elongation.

M. smegmatis *fadB2* deletion mutant analysis

The *Ms-fadB2* knockout mutant was generated using specialized transduction (Bardarov *et al.*, 2002) and confirmed by Southern blotting (data not shown). This verified that the gene is not essential in *M. smegmatis*, as suggested by transposon data reported for *M. tuberculosis* (Sasseti *et al.*, 2003). No obvious differences were observed either in colony morphology or lipid profiles between the

Fig. 3. Effect of pH on the *Mt*-FadB2-catalysed reaction rate. Taking either β -hydroxybutyryl-CoA (A) or acetoacetyl-CoA (B) as the substrate, the reactions were assayed spectrophotometrically as described in the text. For pH values with low activity, the reaction was monitored for a long enough period to obtain a rate. Points are the mean $\pm$ SE of three replicates.

Fig. 4. Effect of different uni- and divalent cations on the rate of reaction. Using β -hydroxybutyryl-CoA and NAD⁺ as the substrate and cofactor respectively, different uni- and divalent cations were added to the reaction mix at a concentration of 1 mM or 10 mM. Bars represent the mean $\pm$ SE of three replicates.

mutant and wild-type *M. smegmatis* strain mc²155 (data not shown). Growth of the Δ *Ms-fadB2* mutant on minimal medium containing a variety of carbon sources was compared to that of the wild-type and complemented strains. No difference in growth was observed between any of the strains when grown on agar media containing glycerol, glucose, acetate, propionate, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, oleic acid or linoleic acid as the sole carbon source. Following the recent report on a mycobacterial FadA enzyme, which acts as a thiolase in cholesterol metabolism, we also tested the growth of the *Ms-fadB2* deletion mutant on minimal medium containing cholesterol as the sole carbon source. However, in this case too, no differences were observed in the growth of the wild-type and mutant strains.

Previously it has been shown that *Mt-fadB2* is upregulated upon acid shock and SDS stress (Fisher *et al.*, 2002; Manganelli *et al.*, 2001). To test whether FadB2 conferred tolerance to these stresses, the Δ *Ms-fadB2* mutant was grown in media of various pH values or containing different concentrations of SDS. We found, however, that the deletion had no effect on cell growth rate and survival at physiological pH, and under acid and SDS stress.

Given the presence of an additional four putative *fadB* genes in the genome of *M. smegmatis* (homologues of the *M. tuberculosis* genes *fadB1*, *B3*, *B4* and *B5*), it was quite likely that the effects of loss of *Ms-fadB2* were masked due to a redundancy between orthologous *Ms-fadB* genes. Under these circumstances, the generation of double or multiple *fadB* knockout strains would be ideal for studying

Fig. 5. CD spectra (A) Superimposed CD spectra of wild-type *Mt-FadB2* and S122A-mutated *Mt-FadB2* in 50 mM potassium phosphate (pH 7.9), 50 mM NaF buffer. (B) Superimposed CD spectra of wild-type *Mt-FadB2* in different pH buffers [50 mM potassium phosphate (pH 6, 7 or 8), 50 mM NaF or 50 mM CAPS (pH 9, 10 or 11), 50 mM NaF]. Protein samples (1.1 mg ml⁻¹) were scanned in a 0.01 cm pathlength cell using a Jasco-J-715 spectropolarimeter. Spectra are averages of eight scans over a wavelength range of 197–245 nm, with a bandwidth of 2 nm. The results were normalized by subtracting the baseline buffer spectrum from each.

the roles of these genes in the biology of mycobacteria and is currently under way.

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