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Use of a codon alteration strategy in a novel approach to cloning the *Mycobacterium tuberculosis* diaminopimelic acid epimerase

Veeraraghavan Usha[1], Lynn G. Dover[1], David L. Roper[2], Adrian J. Lloyd[2] & Gurdyal S. Besra[1]

[1]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK; and [2]Department of Biological Sciences, University of Warwick, Coventry, UK

Correspondence: Gurdyal S. Besra, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. Tel.: +121 415 8125; fax: +121 414 5925; e-mail: g.besra@bham.ac.uk

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Abstract

Previous attempts to express the diaminopimelate epimerase gene *dapF* of *Mycobacterium tuberculosis* in *Escherichia coli* resulted in undetectable enzyme yields. We used silent mutation of the first 10 codons of the recombinant ORF in an attempt to reduce the formation of secondary structures that might occur near the 5′ end of the mRNA and inhibit translation. This significantly increased the yield of the enzyme, which was purified and characterized biochemically. This strategy could be generally applied to other mycobacterial genes that are difficult to express hetero-specifically and here provided pure *M. tuberculosis* DapF, a good foundation for future research in antimycobacterial agents.

Introduction

Despite a concerted global effort to prevent the spread of the human pathogen *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), approximately 8.9 million new cases and 1.7 million deaths were reported worldwide in 2004 (Dye, 2006). Emerging multidrug resistance (Wright *et al.*, 2006), its synergy with HIV infection (Corbett *et al.*, 2006; Dye, 2006) and its latent infection of around one-third of humanity (WHO, 2003) compound the healthcare problems presented by this pathogen. The isolation and design of novel anti-tuberculosis agents and the identification of new drug targets are of paramount importance.

In addition to defining cell shape and resisting the tremendous turgor pressure developed in the cytoplasm, the peptidoglycan sacculus of *M. tuberculosis* underpins a complex cell wall structure (Dover *et al.*, 2004). The mycolic acids (m), which form the inner leaflet of an outer envelope lipid bilayer, are covalently tethered to the peptidoglycan (PG) via an arabinogalactan (AG) polysaccharide and constitute the mycolyl-arabinogalactan–peptidoglycan (mAGP) complex. The mAGP structure is essential and provides the bacterium with a formidable protective barrier against toxic insult, such as antibiotics and components of the macrophage's bactericidal arsenal (Gao *et al.*, 2003).

Despite the complexity of the *M. tuberculosis* cell wall, the peptidoglycan component is unremarkable and similar to that of *Escherichia coli* (Petit *et al.*, 1969; Wietzerbin *et al.*, 1974; Janczura *et al.*, 1981). However, the muramic acid residues of the glycan moiety can either be glycolylated or acetylated (Azuma *et al.*, 1970; Takayama *et al.*, 1970). A short peptide, L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanine, is attached via an amide linkage to the D-lactyl group of each muramic acid residue and the D-glutamate residue of the stem peptide can be amidated at its carboxyl group (Goffin & Ghuysen, 2002). Like in *E. coli*, adjacent glycan chains are bonded to each other by cross-links in their peptide side-chains, with most occurring between the carboxyl group of the 4th residue, D-alanine, with the free amino group of the diaminopimelate (DAP) residue of the adjacent peptide, although some cross-linking between diaminopimelate residues also occurs (Glauner & Schwarz, 1983) predominantly in stationary phase cultures (Goffin & Ghuysen, 2002).

The provision of the D-amino acids required for peptidoglycan biosynthesis can be facilitated by amino acid racemases and epimerases. D-alanine is formed by alanine racemase (ALR) in a pyridoxal-5-phosphate (PLP)-dependent reaction (Bugg & Walsh, 1992), while the production of *meso*-(DL)DAP and D-glutamate is PLP independent. Two potential biosynthetic routes to *meso*-DAP from

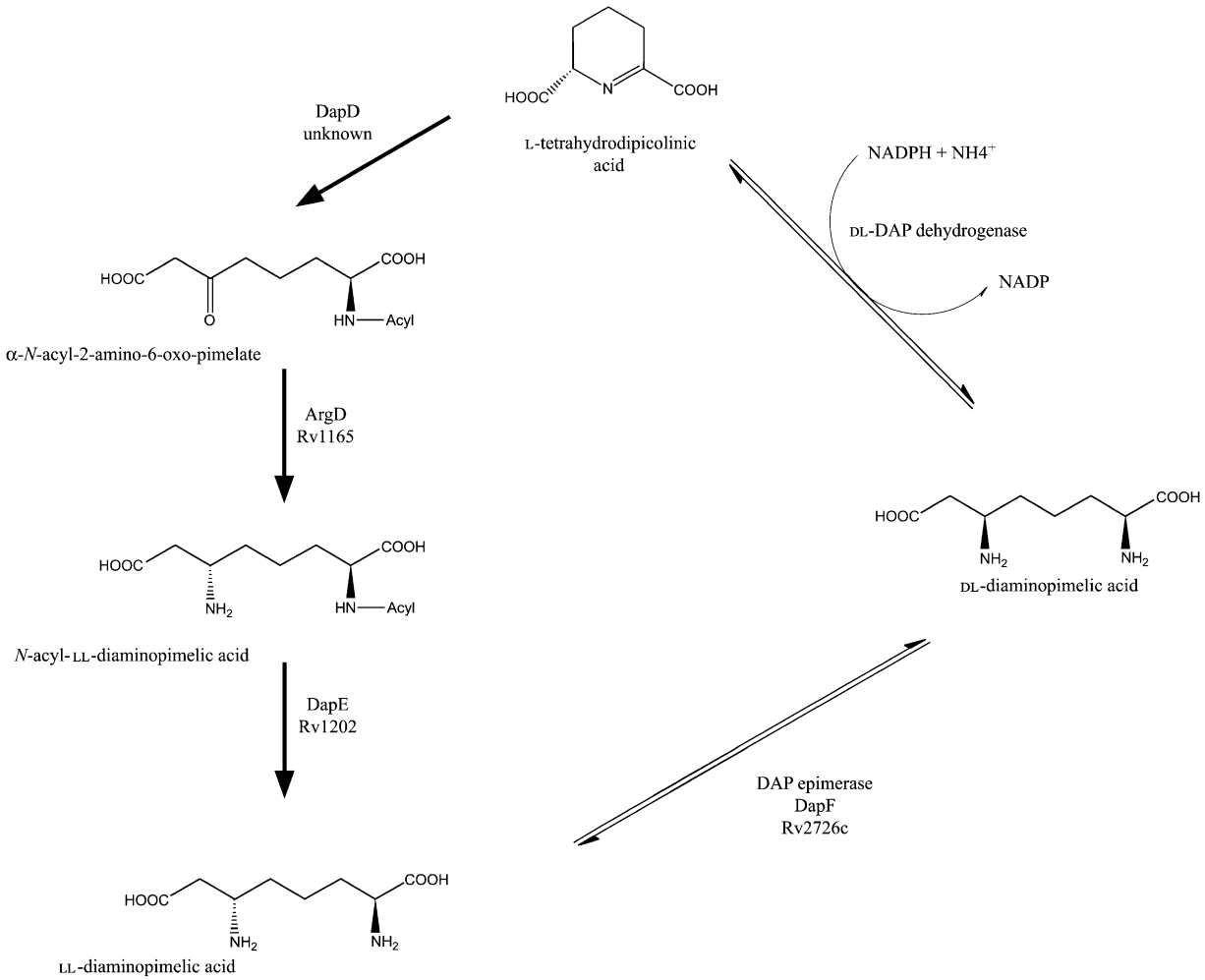

Fig. 1. Potential pathways to *meso*-diaminopimelic acid biosynthesis. The likely pathway to meso-DAP synthesis in *Mycobacterium tuberculosis* occurs via the succinylate pathway, with the final epimerase step being carried out by DapF (Wheeler & Blanchard, 2005). A more direct alternative via *meso*-DAP dehydrogenase is possible but no candidate gene has yet been identified.

L-tetrahydrodipicolinate exist: the conversion to LL-DAP via an acylation-dependent (probably succinate) pathway via DAP-epimerase or by the direct reduction of L-tetrahydrodipicolinate via diaminopimelate dehydrogenase (Cirillo *et al.*, 1994; Scapin *et al.*, 1996); several of the enzymes of the former route have been identified in *M. tuberculosis* (Wheeler & Blanchard, 2005) (Fig. 1).

Mycobacterium tuberculosis has a high genomic G+C content, which is reflected in a strong bias towards C- and G-ending codons for every amino acid. Previously, several strategies have been used to increase the yield of hetero-specifically expressed genes. Some have painstakingly optimized each codon in order to achieve good hetero-specific expression of mycobacterial genes (Ko *et al.*, 2005) and others have used host strains with modified tRNA pools in order to reduce the extent of ribosomal stalling at rare codons during translation (Berger & Knodel, 2003; Korepanova *et al.*, 2005).

Herein, we describe an alternative strategy that we adopted to achieve effective overexpression of a DAP-epimerase gene (*dapF,* Rv2726c) from *M. tuberculosis* in *E. coli* and describe the biochemical characterization of its purified product.

Materials and methods

Restriction enzymes, T4 DNA ligase and Taq DNA polymerase were purchased from New England Biolabs. The expression vector, pET 28b, was obtained from Novagen. *Escherichia coli* Top10 cells (Invitrogen) was used as a host strain for the preparation of plasmid DNA, and *E. coli* C41 (DE3) (Imaxio, France) was used as the expression host for protein preparation. Oligonucleotide PCR primers were synthesized by MWG Biotech, Germany. Plasmid DNA was isolated using the Qiaprep spin miniprep kit (Qiagen). DNA

cleanup from enzymatic reactions and PCR product purification was performed with the Qiaquick gel extraction kit (Qiagen). BCA protein assay reagent was obtained from Pierce.

HiTrap Q FF ion exchange column and Ni^{2+} chelating Sepharose High-performance matrix were obtained from GE Healthcare. DL-Diaminopimelic acid was purchased from Sigma and [2,6-^{3}H] diaminopimelate, 30 Ci mmol^{-1} was obtained from American Radiolabeled Chemicals. Cation exchange resin AG 50W-X8, 200–400 mesh, H^+ form was obtained from BioRad Laboratories. All other chemicals were of analytical grade or better and purchased from Sigma or Fluka. The forward primer for cloning of *M. tuberculosis* DapF gene in *E. coli* was designed based on the codon alteration strategy (Gao *et al.*, 1998).

Cloning of *M. tuberculosis dapF* in *E. coli*

The 870 bp *dapF* ORF was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using oligonucleotide primers DapF reverse, 5′-gatcgatcaagctttcaccccatcgcgttccac-3′ and either DapFca Forward, 5′-gatcgatccatatgatatttg-caaaaggtcatggtacacaaaacgacttcgtgttgctgcctg-3′, or DapF Forward 5′-gatcgatccatatgatcttcgcgaagggcc, which contain recognition sites for *Nde*I and *Hin*dIII, respectively (underlined). The amplified DNA was digested with these endonucleases and ligated with similarly cut pET28b DNA to form pET 28b-*dapFca* and pET 28b-*dapF*. The nucleotide sequence of the inserts was verified by DNA sequencing.

Expression of recombinant *M. tuberculosis dapF*

Escherichia coli C41 (DE3) cells were transformed with pET 28b-*dapFca*. Transformants were selected on Luria–Bertani plates containing 25 μg mL^{-1} kanamycin and a single transformant colony used to inoculate 50 mL of Terrific broth containing 25 μg mL^{-1} kanamycin, which was grown overnight at 37 °C to provide an inoculum for large-scale cultures. Fresh Terrific broth with 25 μg mL^{-1} kanamycin (4 × 1 L) were seeded with a 1% inoculum and cultures were grown at 37 °C with shaking until $OD_{600\,nm} = 0.6$. The culture was cooled to 16 °C and *dapF* expression was derepressed by the addition of isopropyl-thio-β-D-galactopyranoside to 1 mM. Incubation was continued at 16 °C for 16 h with shaking, the cells were harvested by centrifugation and stored temporarily at − 80 °C.

Purification of recombinant *M. tuberculosis* DapF

The method used for purification was based around that used by Lloyd *et al.* (2004) for DapF from *Haemophilus influenzae*. The harvested cell pellet was resuspended in Buffer A containing 50 mM HEPES buffer pH 8, 100 mM NaCl, 5 mM dithiothreitol, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine. The cell suspension was lysed by passing through a cooled (4 °C) French pressure cell three times at 3000 psi and clarified by centrifugation at 27 000 ***g*** and 4 °C for 45 min. The clarified lysate was passed through a 2 mL Ni^{2+}-charged chelating Sepharose High-performance column that had been pre-equilibrated with Buffer A (without the protease inhibitors). The column was extensively washed with Buffer A and adsorbed protein was eluted with a step gradient (50–500 mM) of increasing imidazole concentration in Buffer A. Fractions of eluted material were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and those containing DapF were pooled and dialysed against three changes of Buffer B containing 20 mM Tris-HCl pH 8 and 1 mM dithiothreitol. The preparation was then applied to a 5 mL HiTrap Q Sepharose FF column pre-equilibrated with Buffer B. The column was extensively washed with Buffer B and adsorbed protein was eluted with a linear gradient of Buffer B containing NaCl (25 mM–1 M). The fractions containing DapF were pooled and dialysed against 20 mM Tris-HCl containing 10 mM dithiothreitol to remove NaCl. The dialysed protein was concentrated using 10 kDa cut-off Amicon concentrators, and protein was estimated using the Pierce BCA assay kit with bovine serum albumin as a standard before being stored as aliquots at − 80 °C.

Diaminopimelate epimerase assay

The assay for diaminopimelate epimerase activity reported previously (Wiseman & Nichols, 1984; Richaud *et al.*, 1987) was adapted with minor modifications. The assay measures the release of ^{3}H to H_2O from [2,6-^{3}H] diaminopimelate (Wiseman & Nichols, 1984; Richaud *et al.*, 1987). The standard reaction contained in a final volume of 100 μL comprises of 0.1 M Tris-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 μCi [2.6-^{3}H DAP], 48 μM DL-DAP and 3.5 μg of recombinant *M. tuberculosis* DapF. The assay was initiated by addition of substrate. The reaction mixtures and controls were incubated at 30 °C for 4 h. The reaction was quenched with 500 μL of 10% trichloroacetic acid and applied to a 2 mL AG 50W-X8 ion exchange column (H^+ form) packed in a syringe. The column was washed with 3 × 2 mL of water, the eluates were combined and radioactivity was quantified by liquid scintillation counting. The 'without enzyme control' values were subtracted from the complete reaction values in each case. Assays were run in duplicate. Specific activity is expressed as picomoles of DL-DAP converted per minute per milligram protein.

Results

Cloning, expression and purification of *M. tuberculosis* DapF

Historically, the biosynthesis of the *M. tuberculosis* cell wall has provided good cellular targets for the development of anti-tuberculosis drugs (Takayama *et al.*, 1979; Kilburn & Takayama, 1981; Quemard *et al.*, 1991; Banerjee *et al.*, 1994; Larsen *et al.*, 2002). In order to investigate the potential for drug development in mycobacterial peptidoglycan synthesis, we attempted to clone and characterize the diaminopimelate epimerase, DapF. Our attempts to express *dapF* in *E. coli* using the pET28 expression vector were unsuccessful, with none of its protein product being detected (data not shown).

Mycobacterium tuberculosis is a member of the high-G+C Gram-positive bacteria, with a genomic G+C content of around 65 mol%. This G+C-richness is reflected in a strong bias towards C- and G-ending codons for every amino acid with an overall G+C content at the third position of codons of 83%. However, there is significant variation in codon usage patterns among genes, which appears to be associated with gene expression level (Andersson & Sharp, 1996). Previously, several strategies have been used to increase the yield of hetero-specifically expressed genes. Some have painstakingly optimized each codon in order to achieve good hetero-specific expression of mycobacterial genes (Ko *et al.*, 2005) or of foreign genes in mycobacteria (Kanekiyo *et al.*, 2005), achieving up to 40-fold higher expression than with nonoptimized constructs. Other studies have used host strains with modified tRNA pools in order to reduce the extent of ribosomal stalling at rare codons during translation (Berger & Knodel, 2003; Korepanova *et al.*, 2005).

As our pET28-*dapF* construct was based on the particularly efficient T7 promoter of the pET28 vector, we reasoned that lack of *dapF* expression was most likely related to poor translation or instability rather than inefficient transcription. With this in mind, we compared the codon usage in *E. coli* K12 (Nakamura *et al.*, 2000) and in *M. tuberculosis dapF* (Table 1) to determine whether the gene included many codons rarely used in *E. coli*. Here, we set arbitrary cut-offs for rare codon frequency and looked for discrepancies of greater than 1.5-fold in codon usage frequency between *dapF* and *E. coli* K12 that might lead to depletion of amino acyl-tRNA pools. As codon usage is thought to have coevolved with tRNA populations, we set the cut-off for codon frequency to 10 per thousand codons as this incorporated all those isoaccepting tRNA molecules found to be rare in *E. coli* (Ikemura, 1985). This criterion was met by 41 of the 289 *dapF* codons but, of those, only 18 (6%) corresponded to rare tRNAs. Thirteen codons were overrepresented in *dapF*, but only four of these represented rare codons with a combined frequency of occurrence in *dapF* of only 23 ($< 8\%$).

As we found that rare *E. coli* codons were not grossly overrepresented within *dapF*, and rather than laboriously altering each one, we chose to adopt a codon alteration

Table 1. Comparison of codon usage in *Mycobacterium tuberculosis dapF* and *Escherichia coli* K12 (Nakamura *et al.*, 2000)

Codon	Freq K12*	Freq *dapF*†	No. *dapF*‡	Codon	Freq K12	Freq *dapF*	No. *dapF*	Codon	Freq K12	Freq *dapF*	No. *dapF*	Codon	Freq K12	Freq *dapF*	No. *dapF*
UUU	22.4	3.4	1	*UCU*	*8.5*	*0.0*	*0*	UAU	16.3	0.0	0	*UGU*	*5.2*	*3.4*	*1*
UUC	16.6	24.1	7	*UCC*	*8.6*	*6.9*	*2*	UAC	12.3	13.8	4	***UGC***	***6.4***	***13.8***	***4***
UUA	13.9	0.0	0	*UCA*	*7.1*	*6.9*	*2*	*UAA*	*2.0*	*0.0*	*0*	*UGA*	*0.9*	*3.4*	*1*
UUG	13.7	31.0	9	*UCG*	*8.9*	*10.3*	*3*	*UAG*	*0.2*	*0.0*	*0*	UGG	15.3	13.8	4
CUU	11.0	0.0	0	*CCU*	*7.0*	*3.4*	*1*	CAU	12.9	3.4	1	CGU	21.0	6.9	2
CUC	11.0	13.8	4	*CCC*	*5.5*	*6.9*	*2*	***CAC***	***9.7***	***24.1***	***7***	CGC	22.0	20.7	6
CUA	*3.9*	*0.0*	*0*	*CCA*	*8.5*	*3.4*	*1*	CAA	15.5	0.0	0	*CGA*	*3.5*	*6.9*	*2*
CUG	52.8	37.9	11	CCG	23.3	24.1	7	CAG	28.8	17.2	5	***CGG***	***5.4***	***20.7***	***6***
AUU	30.4	0.0	0	*ACU*	*8.9*	*0.0*	*0*	AAU	17.6	3.4	1	AGU	8.7	6.9	2
AUC	25	3.4	1	**ACC**	**23.4**	**48.3**	**14**	AAC	21.7	20.7	6	AGC	16.0	6.9	2
AUA	*4.3*	*0.0*	*0*	*ACA*	*7.0*	*0.0*	*0*	AAA	33.6	0.0	0	*AGA*	*2.1*	*3.4*	*1*
AUG	27.8	20.7	6	ACG	14.4	10.3	3	AAG	10.2	10.3	3	*AGG*	*1.2*	*3.4*	*1*
GUU	18.4	24.1	7	GCU	15.3	10.3	3	GAU	32.2	24.1	7	GGU	24.9	24.1	7
GUC	**15.2**	**55.2**	**16**	**GCC**	**25.5**	**62.1**	**18**	**GAC**	**19.1**	**55.2**	**16**	**GGC**	**29.4**	**44.8**	**13**
GUA	10.9	10.3	3	GCA	20.3	13.8	4	*GAA*	*39.6*	*6.9*	*2*	***GGA***	***7.9***	***20.7***	***6***
GUG	**26.2**	**65.5**	**19**	**GCG**	**33.7**	**58.6**	**17**	**GAG**	**17.8**	**27.6**	**8**	**GGG**	**11.0**	**37.9**	**11**

Figures in bold represent potential discrepancies§ that might inhibit efficient translation and those in italics represent rare¶ *E. coli* codons.

*Arbitrary index of $> 1.5\times$ more frequent in *dapF* than in *E. coli* K12.

†A cut-off of 10 per thousand was set as this includes all codons corresponding to rare isoaccepting tRNAs.

‡Frequency of codon usage per thousand codons in *E. coli* K12.

§Frequency of codon usage per thousand codons in *M. tuberculosis dapF.*

¶Frequency of codon usage in *M. tuberculosis dapF.*

strategy in which the first 10 codons of the *dapF* mRNA were modified in order to prevent the formation of secondary structures that might inhibit translation. This could be achieved in a single PCR using a long, partly nonhomologous oligonucleotide primer and if successful would represent a rapid and facile approach to increase recombinant protein yield. A similar strategy had been successfully used by Gao *et al.* (1998) to overproduce the extracellular immunoglobulin domain of human CD8α but, to our knowledge, had not been applied to the expression of mycobacterial sequences previously. Briefly, we designed an oligonucleotide primer that would incorporate silent S → W (Liébecq, 1992) single nucleotide substitutions, where possible, in the first 30 nucleotides of the *dapF* ORF, i.e. guanosine and cytosine nucleotides would be replaced by adenosine and thymidine nucleotides without changing product composition (Fig. 2a), in order to disrupt any secondary structure-promoting sequences present. Additionally, as S–S pairs bind more avidly than W–W pairs, these mutations would minimize the impact of any

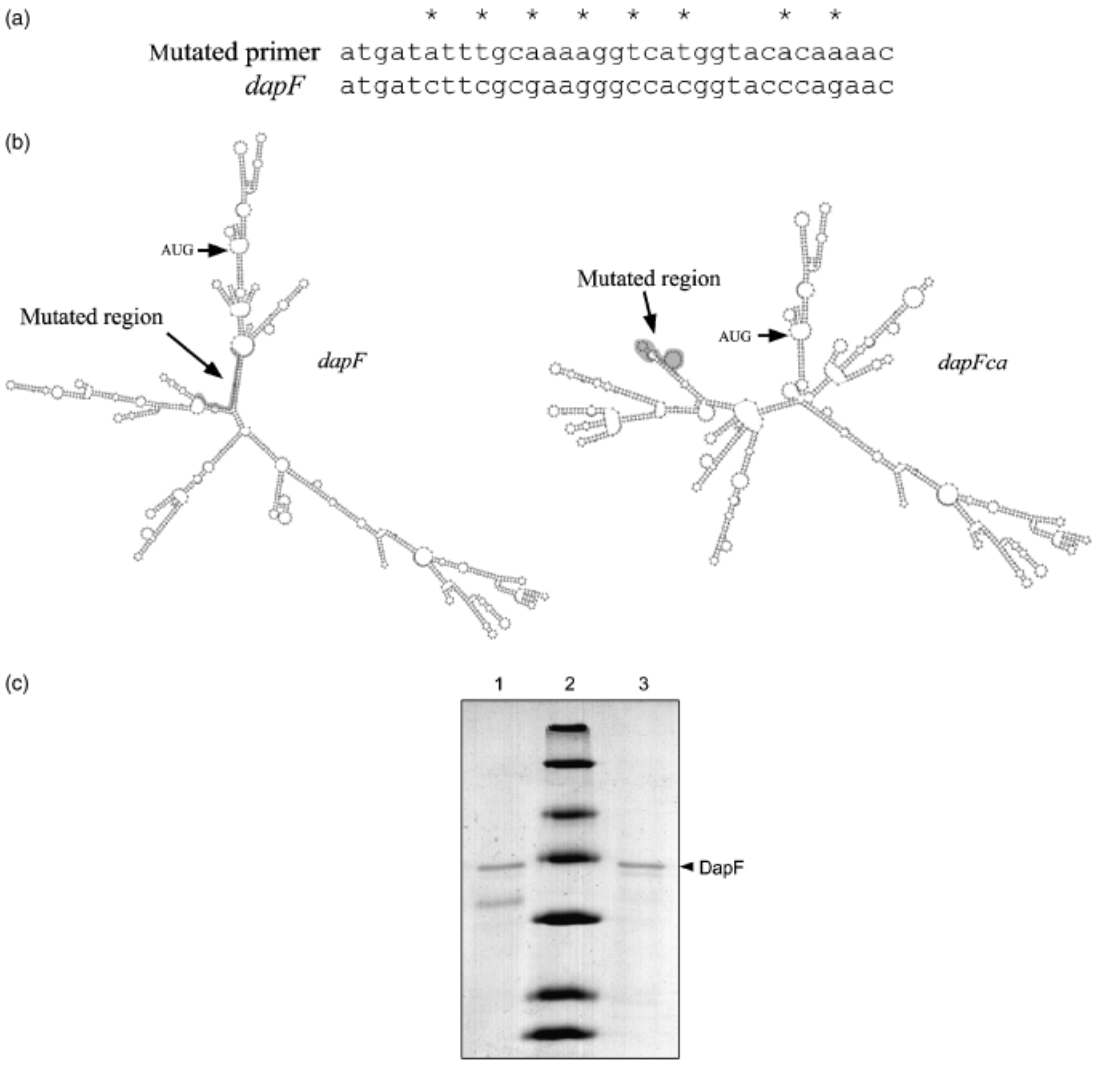

Fig. 2. Synthesis and purification of *Mycobacterium tuberculosis* DapF. (a) Oligonucleotide design used to effect the S → W silent mutations within the first 10 *Mycobacterium*-derived codons of the fusion construct. The sequence of the mutation-bearing region of the DapFca Forward oligonucleotide primer used to amplify *dapFca* is displayed above the sequence of the corresponding native *dapF*; * highlight mutations. (b) Predictions of secondary structure within both *dapF* and *dapFca* mRNA were carried out using the RNAfold program at the Vienna RNA Secondary Structure Prediction website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with default settings and selecting the most stable model. The position of the AUG fMet codon is indicated with an arrow and the mutated region is shaded. (c) A mid-logarithmic phase culture of *Escherichia coli* C41(DE3) pET28-*dapFca* was cooled to 16 °C and gene expression was induced by addition of 1 mM IPTG for 16 h. DapF-containing fractions obtained from chromatography on Ni^{2+}-metal-chelate affinity chromatography and anion exchange chromatography are shown in lanes 1 and 3, respectively; lane 2 contains molecular size standards of 116, 66, 45, 35, 25, 18 and 14 kDa.

interactions that we might generate via this strategy. The 3′ end of the oligonucleotide was designed to be homologous to the *M. tuberculosis* H37Rv genome sequence to promote efficient priming. Predictions of secondary structure within both *dapF* and *dapFca* mRNA were carried out using the RNAfold program at the Vienna RNA Secondary Structure Prediction website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with default settings (Mathews *et al.*, 1999) and selecting the most stable model (Fig. 2b). Comparison of these predicted structures highlighted the disruption of a long duplex near the centre of this representation, which might facilitate improved messenger translation.

In contrast to our earlier experiments, our initial trials using *E. coli* C41(DE3) pET28-*dapFca* transformants confirmed that a large quantity of an insoluble ~30 kDa protein, consistent with the 309 amino acid residue recombinant DapF and presumably present as misfolded aggregates, was produced on isopropyl β-D-thiogalactosidase (IPTG)-induced gene expression (data not shown). Similarly, when we cooled cultures to 16 °C before derepression of *dapF* transcription, the majority of protein was present as insoluble aggregates, but enough soluble protein was afforded to allow its purification and analysis.

The soluble fraction was applied to a Ni^{2+}-primed chelating Sepharose column, and DapF-containing eluate fractions were dialysed and further purified by anion exchange chromatography on a HiTrap Q Sepharose FF column as described in Materials and methods (Fig. 2c). This second chromatography step yielded an electrophoretically homogenous preparation of recombinant *M. tuberculosis* DapF representing a yield of 1 mg L^{-1} culture.

Standardization of diaminopimelate epimerase assay

The recombinant *M. tuberculosis* DapF was assayed for diaminopimelate epimerase activity by following the generation of ^3H_2O using the established assay described in Materials and methods with the intention of screening inhibitors as it had been reported previously that sodium borohydride and some inhibitors interfered with the alternate assay of diaminopimelate epimerase (Wiseman & Nichols, 1984; Lam *et al.*, 1988).

In a time course experiment, diaminopimelate epimerization was monitored over 6 h with samples being taken at 1 h intervals. Control reactions without enzyme at each time point were analysed in parallel. The exchange of ^3H with water demonstrated that diaminopimelate epimerization progressed at a constant rate for 4 h (Fig. 3a), which we then established as our standard sampling point for subsequent assays. Next, we compared the progress of diaminopimelate epimerization at three temperatures showing that the recombinant *M. tuberculosis* DapF is almost 50% more active at 30 °C than at 25 °C (Fig. 3b). As the activity observed at 37 °C was slightly less than at 30 °C, we chose the latter for subsequent assays. The rate of diaminopimelate epimerization was dependent upon the amount of enzyme added to the reaction (Fig. 3c); although with higher enzyme concentrations the effect was not linear.

The profile of diaminopimelate epimerase activity under various conditions of pH showed a typical bell-shaped response. The recombinant *M. tuberculosis* DapF was active over the range pH 6.5–9.0 and showed maximum activity at pH 7.5 (Fig. 3d). A similar bell-shaped pH dependence has been reported for the diaminopimelate epimerase of *H. influenzae* (Koo & Blanchard, 1999). Unlike its *E. coli* counterpart, which is denatured on freezing (Wiseman & Nichols, 1984; Higgins *et al.*, 1989), the recombinant *M. tuberculosis* DapF could be stored at −80 °C without significant loss of activity for over a month.

Determination of K_M for DL-DAP

We varied the initial substrate concentration in the assays between 8 and 320 μM *meso*-DAP in order to determine the K_M for this substrate. The generation of ^3H_2O responded in direct proportion to the substrate concentration applied across the full range (Fig. 4) and allowed us to estimate a Michaelis constant, $K_M = 1217$ μM, for *meso*-DAP, a figure that is consistent with reported values for diaminopimelate epimerase from other organisms (Wiseman & Nichols, 1984; Lam *et al.*, 1988; Koo & Blanchard, 1999).

Discussion

The immense global burden of *M. tuberculosis* infection, its interaction with HIV and the emergence of multidrug resistant strains have demanded that the basic physiology of this pathogen is thoroughly researched in order to identify novel drug targets. Previous studies have firmly established the mycobacterial cell wall as an interesting target for potential new antituberculosis agents (Takayama *et al.*, 1979; Kilburn & Takayama, 1981; Quemard *et al.*, 1991; Banerjee *et al.*, 1994; Larsen *et al.*, 2002). Genetic studies have determined that many of the genes known to direct the synthesis of this unique structure have no human orthologues and are essential for the growth of the organism (Mikusova *et al.*, 2000; Kremer *et al.*, 2001; Mills *et al.*, 2004; Alderwick *et al.*, 2006). Recently, we have turned our attention towards the characterization of enzymes involved in the biosynthesis of peptidoglycan in this organism. DapF represented an attractive target for study as it has no human orthologue. In order to initiate a program of research into inhibitor design, we sought to clone and express *M. tuberculosis dapF* hetero-specifically in *E. coli*. Our initial attempts were hampered by poor expression;

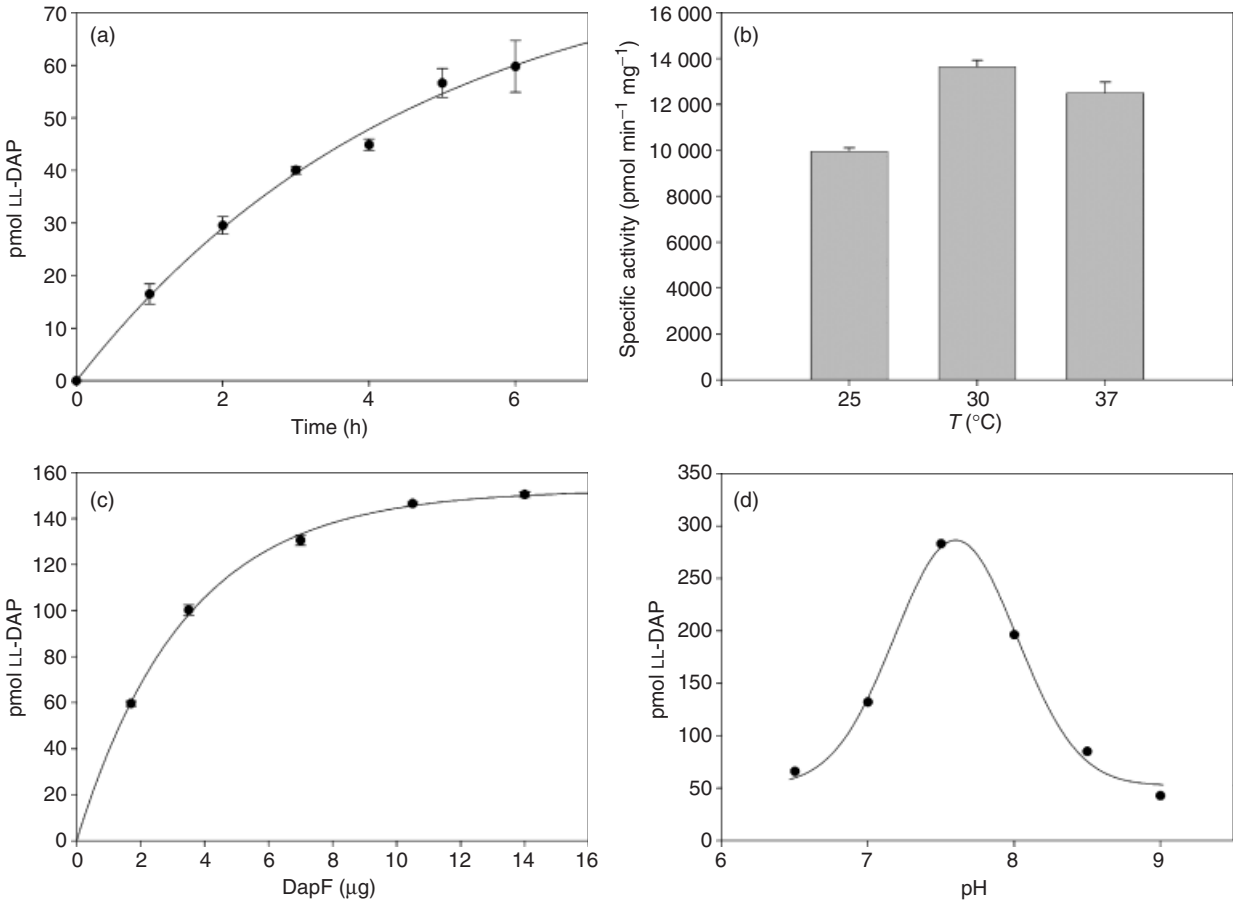

Fig. 3. Diaminopimelic acid epimerase activity. ^{3}H exchange between *meso*-DAP and water was followed as an index of epimerization. The panels illustrate (a) a time course of diaminopimelate epimerization, (b) temperature dependence, (c) recombinant protein dose response and (d) pH dependence of diaminopimelate epimerization. Eight nanomoles (0.5 μCi) [^{3}H]DAP was used in each assay. The extent of ^{3}H exchange was quantified after 4 h apart from in (a). All assays were run in duplicate; each data point is modified by the subtraction of identical no enzyme controls.

production of a hexa-histidine-tagged DapF fusion using a pET28-based construct could not be detected using immunochemical detection or sensitive and specific stains for polyhistidine tags (data not shown). As our analysis suggested that *dapF* mRNA did not contain any codons that were particularly uncommon in *E. coli*, we did not pursue the strategies used by others in which all of the codons were optimized for particularly efficient translation within the expression host. Rather, we surmised that the poor expression of *dapF* might be related to mRNA secondary structure, which might be particularly detrimental to translation if it were to occur towards the 5′ end of the messenger strand. We chose to modify the first 30 nucleotides of those encoding the mycobacterial elements of the fusion protein, introducing silent S → W substitutions wherever possible. Subsequent expression of this allele in *E. coli* led to dramatically increased yields of recombinant protein, so much so that we were forced to reduce the temperature at which gene expression was induced in order to recover soluble protein rather than insoluble aggregates.

The protein was readily purified using a two-column protocol and we were able to demonstrate diaminopimelate epimerization activity using an established radiometric assay. We have optimized the assay and biochemically characterized the mycobacterial enzyme. The data reported here demonstrate that *M. tuberculosis* DapF displays a K_M for *meso*-DAP similar to other bacterial diaminopimelate epimerases, suggesting that the recombinant protein we have produced is correctly folded and is a suitable tool for a drug development study. The enzyme is currently in crystallization trials and efficient inhibitors of the enzyme are being sought for development as antimycobacterial lead compounds. Furthermore, we suggest that this codon alteration strategy might be generally applicable to other mycobacterial genes that are poorly expressed in *E. coli* and have gained encouraging results with several other genes that will be reported elsewhere.

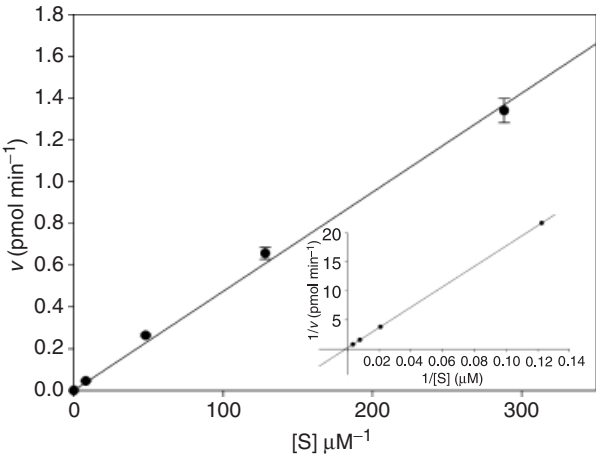

Fig. 4. Kinetic analysis of *meso*-DAP epimerization. The effect of *meso*-DAP concentration on the rate of diaminopimelate epimerization was monitored using the standard assay detailed in Materials and methods. ^{3}H exchange between *meso*-DAP and water was followed as an index of epimerization. Up to 0.5 μCi [^{3}H]DAP was used in each assay. All assays were run in duplicate; each data point is modified by the subtraction of identical no enzyme controls.

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