

Identification, cloning, purification, and enzymatic characterization of *Mycobacterium tuberculosis* 1-deoxy-D-xylulose 5-phosphate synthase

Ann Marie Bailey, Sebabrata Mahapatra, Patrick J. Brennan, and Dean C. Crick¹

Mycobacterial Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA

Received on April 9, 2002; revised on August 6, 2002; accepted on August 7, 2002

The enzyme encoded by *Rv2682c* in *Mycobacterium tuberculosis* is a functional 1-deoxy-D-xylulose 5-phosphate synthase (DXS), suggesting that the pathogen utilizes the mevalonate-independent pathway for isopentenyl diphosphate and subsequent polyprenyl phosphate synthesis. These key precursors are vital in the biosynthesis of many essential aspects of the mycobacterial cell wall. *Rv2682c* encodes the conserved DRAG sequence that has been proposed as a signature motif for DXSs and also all 13 conserved amino acid residues thought to be important to the function of transketolase enzymes. Recombinant *Rv2682c* is capable of utilizing glyceraldehyde 3-phosphate and erythrose 4-phosphate as well as D- and L-glyceraldehyde as aldose substrates. The enzyme has K_m values of 40 μM , 6.1 μM , 5.6 mM, and 4.5 mM for pyruvate, D-glyceraldehyde 3-phosphate, D-glyceraldehyde, and L-glyceraldehyde, respectively. *Rv2682c* has an absolute requirement for divalent cation and thiamin diphosphate as cofactors. The K_d thiamin diphosphate for the native *M. tuberculosis* DXS activity partially purified from *M. tuberculosis* cytosol is 1 μM in the presence of Mg^{2+} .

Key words: glyceraldehyde/isopentenyl diphosphate synthesis/ pyruvate/transketolase

Introduction

Isopentenyl diphosphate (IPP) is a common precursor in the biosynthesis of all isoprenoid compounds. This five-carbon unit is the building block for over 23,000 identified compounds, diverse in chemistry and structure (Sacchettini and Poulter, 1997). A number of these compounds have been isolated from *Mycobacterium tuberculosis*, and it has long been known that the isoprenoid compound polyprenyl phosphate (Pol-P) is involved in the biosynthesis of bacterial cell walls (Hemming, 1974). It has also been suggested that the levels of Pol-P may be rate limiting for cell wall synthesis in

vivo (Anderson *et al.*, 1972; Baddiley, 1972; Higashi and Strominger, 1970; van Heijenoort, 1996). Our laboratory has shown that Pol-P is instrumental in the synthesis of each component of the covalently linked peptidoglycan-arabinogalactan-mycolic acid complex of mycobacteria, as well as noncovalently associated macromolecules, such as lipomannan and lipoarabinomannan (Besra *et al.*, 1994, 1997; Crick *et al.*, 2001; Mikusova *et al.*, 1996). The enzymes involved in the biosynthesis of several of these cell wall components are known targets for existing antituberculosis drugs (Crick and Brennan, 2000). The importance of Pol-P to the bacteria is highlighted by the fact that *M. tuberculosis* (Rieber *et al.*, 1969) and other *Mycobacterium* spp. (unpublished data) are sensitive to the antibiotic bacitracin, a compound that specifically binds isoprenyl diphosphate intermediates (Storm and Strominger, 1973) involved in Pol-P synthesis. The roles of isoprenoid compounds in mycobacteria are not limited to those played by Pol-P; a number of other isoprenoid and isoprenoid-containing compounds have been identified that play important or essential roles in mycobacterial metabolism, including the electron transporter menaquinone.

In human tissues IPP is synthesized through the acetate/mevalonate pathway, and this was assumed to be true for bacteria as well. However, a series of studies (Duvold *et al.*, 1997; Horbach *et al.*, 1993; Lois *et al.*, 1998; Putra *et al.*, 1998a,b; Rohmer *et al.*, 1993; Sprenger *et al.*, 1997; Zhou and White, 1991) have clearly shown that some bacteria, including *Mycobacterium phlei* (Putra *et al.*, 1998a), use an entirely different pathway for IPP biosynthesis. Through in vivo feeding experiments utilizing *Escherichia coli* (Rohmer *et al.*, 1993), it was demonstrated that the mevalonate-independent pathway utilizes pyruvate and D-glyceraldehyde 3-phosphate (GAP) to form isoprenoid compounds. The first step in this synthesis is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP; Figure 1). The enzyme responsible for catalyzing this reaction in *E. coli* is 1-deoxy-D-xylulose-5-phosphate synthase (DXS). Once DXP has been synthesized a number of other biosynthetic steps are required for the synthesis of IPP (Rohdich *et al.*, 2001).

The emergence of multiple drug-resistant strains of tuberculosis worldwide has increased pressure to develop new antituberculosis drugs (Crick and Brennan, 2000). A novel mevalonate-independent pathway for IPP biosynthesis exists in some bacteria and is absent from mammalian cells, providing attractive and novel drug targets. Results presented here show that pathogenic *M. tuberculosis* is among the bacteria that utilize the mevalonate-independent IPP biosynthetic pathway and that the gene *Rv2682c* encodes a functional DXS in this organism.

¹To whom correspondence should be addressed; E-mail: dean.crick@colostate.edu

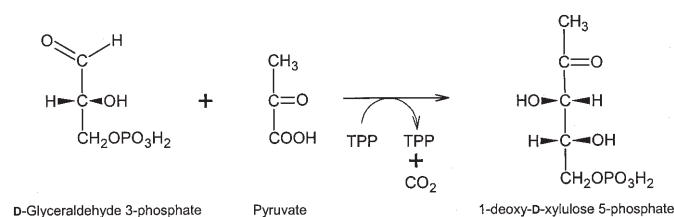


Fig. 1. Reaction catalyzed by DXS.

Results

Initial experiments using cytosolic preparations from *M. tuberculosis* or *Mycobacterium smegmatis* showed that either preparation could enzymatically condense [2-¹⁴C]pyruvate with GAP. Radiolabeled products from these reactions were identified by comigration with authentic DXP on thin-layer chromatography (TLC). Treatment of the putative DXP with bovine alkaline phosphatase for 30 min resulted in a radiolabeled product that comigrated with authentic 1-deoxy-D-xylulose (DX) on TLC. The cytosolic preparations were also capable of forming a radiolabeled compound from [2-¹⁴C]pyruvate and DL-glyceraldehyde that comigrated with authentic DX on TLC plates. These results suggested that *M. tuberculosis* utilizes the mevalonate-independent pathway for IPP biosynthesis. When glyceraldehyde was used as substrate the radiolabeled product, DX could be separated from the [2-¹⁴C]pyruvate by a simple anion exchange step, which provided a facile method to assay the activity.

BLAST searches

BLAST searches of the *M. tuberculosis* genome also supported the hypothesis that *M. tuberculosis* utilized the mevalonate-independent pathway for IPP synthesis. The searches did not reveal any genes that encode proteins with significant homology to known enzymes, such as 3-hydroxy 3-methylglutaryl coenzyme A reductase or mevalonate kinase of the mevalonate pathway for IPP biosynthesis. The searches did identify a number of genes that encode proteins with homology to previously identified enzymes in the nonmevalonate pathway. Two of these genes, *Rv2682c* and *Rv3379c*, encode proteins that are similar in primary structure to the DXS of *E. coli* (38% and 33% identity, respectively) and have a high degree of homology to each other (48.9% identity, Figure 2).

Expression and purification of recombinant Rv2682c

Rv2682c was cloned, expressed, and purified as described in *Materials and methods*. Figure 3 shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the uninduced homogenate (lane 1), the induced homogenate (lane 2), the column flow-through (lane 3), and the pooled fractions eluted from the Talon column (lane 4) containing enzymatically active Rv2682c. The overexpressed Rv2682c was estimated to be at least 80% pure by this analysis, and TLC analysis of the radiolabeled products formed when Rv2682c was incubated with [2-¹⁴C]pyruvate and GAP or glyceraldehyde (GA) showed chromatographic profiles that were identical to those generated in experiments using native enzyme from wild-type *M. tuberculosis* cytosol.

Enzymatic properties of Rv2682c

The enzyme was active over a broad range of pH, with the optimum being between pH 7.0 and 8.0 (data not shown). Both the cytosolic activity and the recombinant Rv2682c were absolutely dependent on divalent cations as neither preparation showed activity in the presence of ethylenediamine tetra-acetic acid (EDTA). Cation exchange chromatography removed enough tightly bound divalent cations from the cytosolic preparation to significantly reduce the enzyme activity, which could be reconstituted by titration with the appropriate divalent cations (Figure 4A) as could activity that had been depleted by addition of EDTA (data not shown). Mg²⁺ and Mn²⁺ supported the partially purified activity from *M. tuberculosis* cytosol equally well; Zn²⁺ supported activity at a lower level and Ca²⁺ was ineffective. The optimum concentration of Mg²⁺ was found to be between 0.5 and 1 mM for the partially purified cytosolic activity. It proved impossible to determine optimal divalent cation concentrations using the recombinant enzyme as removal of the cations resulted in permanent loss of activity.

Similarly, removal of the thiamine diphosphate (TPP) from the recombinant enzyme resulted in permanent loss of activity. However, TPP could be stripped from the cytosolic activity by anion exchange, and the activity could be reconstituted by titration with exogenous TPP. The optimal concentration of TPP was between 5 and 10 μM (Figure 4B) and concentrations as high as 10 mM did not appreciably inhibit the activity.

Activity of the recombinant protein was determined in varying concentrations of GAP and the enantiomers of GA and D-erythrose 4-phosphate in the presence of saturating levels of pyruvate (Figure 5). The activity of the recombinant protein was also determined in varying concentrations of pyruvate in the presence of saturating levels of DL-glyceraldehyde. The K_m^{pyruvate} was determined to be 40 μM. The K_m^{GAP} was three orders of magnitude lower than either the $K_m^{\text{D-glyceraldehyde}}$ or the $K_m^{\text{L-glyceraldehyde}}$ at 6.1 μM (Figure 5). Rv2682c presumably utilizes the L-glyceraldehyde to form 1-deoxy-L-ribulose, although the exact nature of the product seen by TLC was not determined. The enzyme was also able to utilize D-erythrose 4-phosphate as a substrate with a K_m of 121 μM (Figure 5). In this case the product is likely 1-deoxy-D-fructose 6-phosphate. Rv2682c was not able to utilize either D-ribose 5-phosphate or α-ketobutyric acid as substrates.

Discussion

The enzyme encoded by *Rv2682c* in *M. tuberculosis* is a functional DXS, suggesting that *M. tuberculosis* utilizes the mevalonate-independent pathway for IPP synthesis. This hypothesis is supported by the stable isotope labeling experiments using *M. phlei* (Putra *et al.*, 1998a) and the observation that *Mycobacterium* spp. are able to utilize [¹⁴C]DX to synthesize a compound that has the chromatographic properties of menaquinone (data not shown). In addition, the *M. tuberculosis* genome does not appear to contain genes encoding orthologs to the known enzymes of the mevalonate-dependent IPP biosynthetic pathway. Although it has been reported that the *M. tuberculosis* genome does contain a gene encoding an HMG-CoA reductase ortholog (Bellamine *et al.*, 2001; Lamb *et al.*, 1998), our labo-

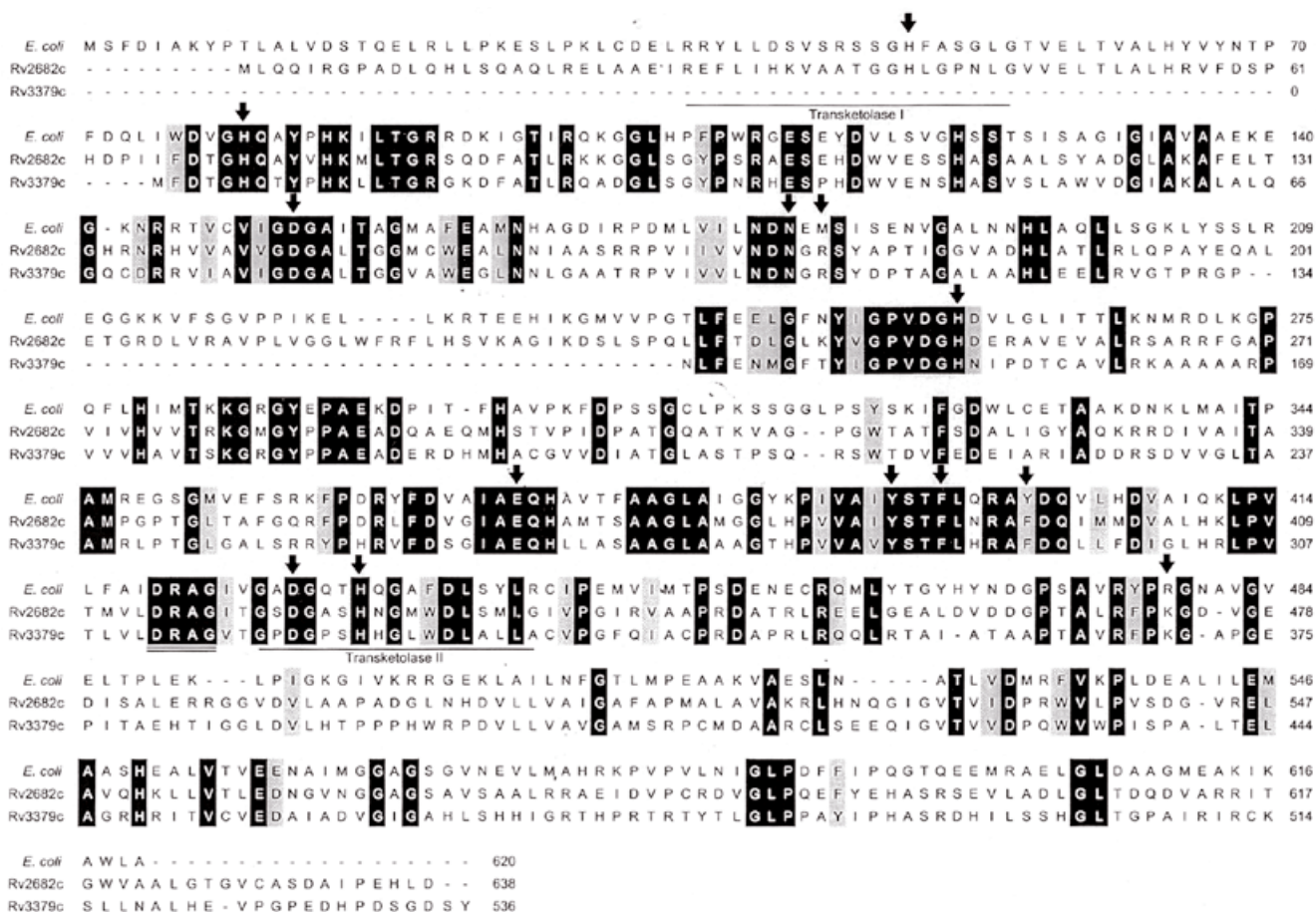


Fig. 2. Multiple alignments of the amino acid sequence of DXS from *E. coli* and two orthologs (Rv2682c and Rv3379c) identified in the *M. tuberculosis* genome. Identities are highlighted in black, conserved residues are highlighted with gray, transketolase signature sequences are underlined, the putative DXS signature sequence (DRAG) is double-underlined, and the 13 amino acids that have been implicated in catalysis or substrate binding are indicated with arrows.

ratory and others (Boucher and Doolittle, 2000) have been unable to identify this gene.

Interestingly, the *M. tuberculosis* genome contains two genes *Rv2682c* and *Rv3379c* that have significant homology at the amino acid level to *E. coli* DXS and to each other. Thus far only two other organisms have been reported to have two homologs of DXS: the photosynthetic *Rhodobacter capsulatus* (Hahn *et al.*, 2001) and the soil bacterium *Streptomyces coelicolor* (Cane *et al.*, 2001). In both cases both proteins were functional. The most obvious differences between the proteins encoded by the genes in *M. tuberculosis* are two apparent deletions (Figure 2). The *N*-terminus of *Rv3379c* appears to have a 65-amino-acid truncation. Inspection of the genome reveals that *Rv3379c* may have been interrupted by an adjacent insertion element (IS6110), which would account for the truncation of the protein. In addition, the amino acids corresponding to positions 193–228 of *Rv2682c* are not found in *Rv3379c*.

The *M. tuberculosis* DXS homologs have a high degree of similarity to previously identified DXS and other transketolase enzymes. The genes both encode the conserved DRAG sequence that has been proposed as a DXS signature sequence (Hahn *et al.*, 2001). *Rv2682c* has all 13 conserved amino acid residues that have been identified as being important in the

function of transketolase enzymes (Hahn *et al.*, 2001), and *Rv3379c* has 12 of the 13 (Figure 2). However, *Rv3379c* is missing a critical histidine residue that corresponds to His49 of the *E. coli* enzyme. His49 of the *E. coli* DXS enzyme has been shown to be essential for catalytic activity (Querol *et al.*, 2001b). This residue aligns with a conserved histidine residue in yeast transketolase (His30) that has also been shown to be involved in catalysis (Wikner *et al.*, 1997). These observations strongly suggest that *Rv3379c* does not encode a functional DXS enzyme. This hypothesis is supported by the observation that when the gene was cloned and expressed (data not shown) under conditions similar to those used for *Rv2682c* the soluble protein is inactive in standard DXS activity assays. However, this is a negative result and, as such, can only be accepted with equivocation.

Although DXP synthases have been cloned from several sources, including *E. coli* (Kuzuyama *et al.*, 2000; Lois *et al.*, 1998; Sprenger *et al.*, 1997), *Streptomyces* spp. (Cane *et al.*, 2001; Kuzuyama *et al.*, 2000), *Rhodobacter capsulatus* (Hahn *et al.*, 2001), *Pseudomonas aeruginosa* (Altincicek *et al.*, 2000), *Synechococcus leopoliensis* (Miller *et al.*, 1999), and several plant species (Lange *et al.*, 1998; Bouvier *et al.*, 1998; Estevez *et al.*, 2000), and there is currently considerable interest in screening for inhibitors of DXS activity as potential

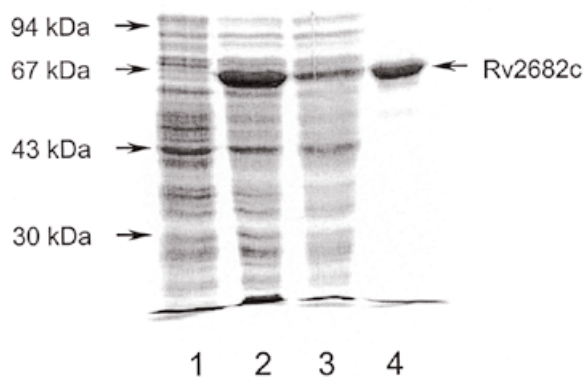


Fig. 3. SDS-PAGE analysis of proteins from recombinant *E. coli* showing purification of Rv2682c. Lane 1, soluble protein (uninduced). Lane 2, soluble protein after induction with isopropylthiogalactoside. Lane 3, flow-through from the Talon column. Lane 4, purified Rv2682c eluted from the Talon column.

antibiotics, herbicides, or antimalarials, little information about the enzymatic properties of these transketolases from pathogens has been published. In general, transketolases from yeast, plants and bacteria can utilize a broad range of substrates, whereas those from mammalian sources are more specific (Schenk *et al.*, 1998). Rv2682c is typical of bacterial transketolases: it acts on a range of substrates and it has kinetic similarities to the other bacterial DXS enzymes for which kinetic data has been reported. However, it also has unique properties. Rv2682c is capable of utilizing D-glyceraldehyde 3-phosphate and D-erythrose 4-phosphate as well as both D- and L-glyceraldehyde as aldose substrates but is not able to utilize D-ribose 5-phosphate. Rv2682c has a K_m^{pyruvate} similar to the values of 96 μM and 65 μM reported for the enzymes from *E. coli* and *Streptomyces* sp. strain CL190 (Kuzuyama *et al.*, 2000) but is considerably lower than the values between 300 μM and 3 mM reported for the enzymes from *R. capsulatus* (Hahn *et al.*, 2001) and *S. coelicolor* (Cane *et al.*, 2001). Rv2682c has a K_m^{GAP} that is 20- to 100-fold lower than the K_m values reported for DXS enzymes from other bacteria (Cane *et al.*, 2001; Hahn *et al.*, 2001; Kuzuyama *et al.*, 2000). This observation is even more notable when one considers that only about 4% of the GAP is in the aldehyde form in solution (Swenson and Barker, 1971). The $K_m^{\text{D-glyceraldehyde}}$ calculated for Rv2682c is similar to the range (10–38 mM) reported for other bacteria (Hahn *et al.*, 2001; Kuzuyama *et al.*, 2000). Interestingly the enzyme encoded by Rv2682c is also capable of utilizing L-glyceraldehyde and D-glyceraldehyde. The ability to utilize L-glyceraldehyde is unusual, because most transketolases are specific for aldoses with a D-configuration at the C₂ atom next to the aldehyde group (Sprenger *et al.*, 1995).

The cofactors Mg^{2+} and TPP can be removed from the native DXS activity and can be reconstituted by addition of exogenous cofactors. However, the purified recombinant could not be reconstituted after removal of either of the cofactors, a property that is most likely due to destabilization of the tertiary structure of the enzyme. The K_d^{TPP} for the native *M. tuberculosis* activity is 1 μM in the presence of Mg^{2+} , which is the same as the value reported for yeast transketolase (Heinrich *et al.*, 1972).

The majority of the DXS assays reported in the literature utilize either TLC or high-performance liquid chromatography

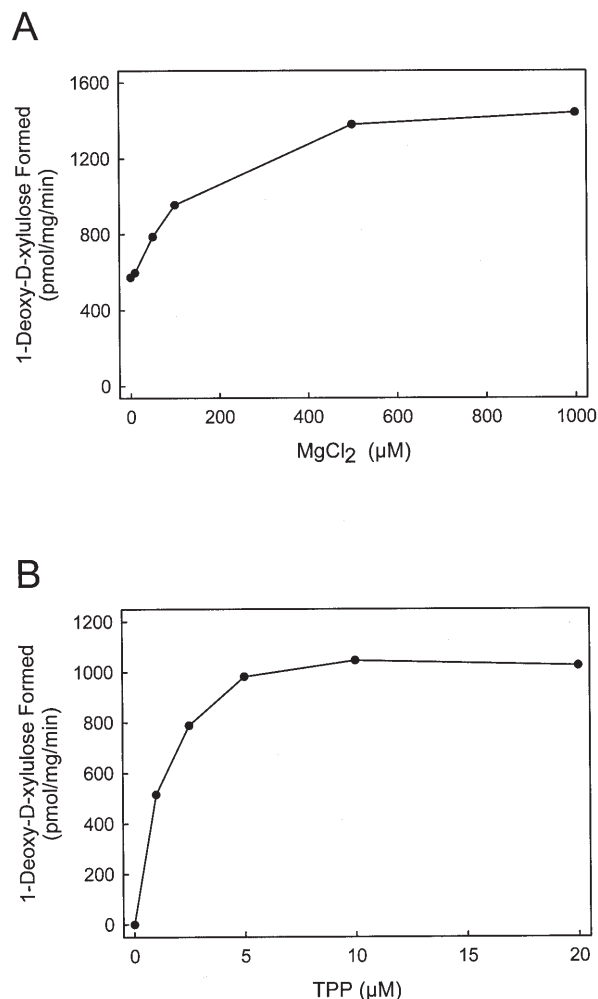


Fig. 4. The effect of MgCl_2 (A) and TPP (B) concentration on the rate of DX synthesis by partially purified DXS from *M. tuberculosis* cytosol. Endogenous divalent cation and TPP were removed from partially purified cytosolic protein as described in *Materials and methods*. Reaction mixtures contained 100 mM MOPS (pH 7.9), 120 μM [¹⁴C]pyruvate, 26 mM DL-glyceraldehyde, and partially purified protein in a final volume of 75 μl . In the experiment shown in A the reaction mixture also contained 5 mM TPP and the indicated concentrations of MgCl_2 . In the experiment shown in B the reaction mixture also contained 1 mM MgCl_2 and the indicated concentration of TPP. Reactions were stopped by the addition of 1 ml of 50% methanol and processed as described in *Materials and methods*.

(HPLC) to quantitate the DXS reaction product (Cane *et al.*, 2001; Estevez *et al.*, 2000; Kuzuyama *et al.*, 2000; Lange *et al.*, 1998; Lois *et al.*, 1998; Sprenger *et al.*, 1997). Both of these methods are time- and labor-intensive and thus have limited usefulness in large-scale screening for inhibitors. A fluorometric assay has been devised for the determination of DXS activity for screening inhibitors with potential antibiotic, herbicidal or antimalarial activity (Querol *et al.*, 2001a). However, the authors report a K_m^{pyruvate} of 2.9 mM for the recombinant *E. coli* DXS, a value 30-fold higher than that reported for recombinant DXS from the same source using an HPLC-based method (Kuzuyama *et al.*, 2000). A coupled spectrophotometric assay has also been reported for rapid screening of DXS activity (Altincicek *et al.*, 2000); the radiometric, ion exchange

Compound	Structure	K _m
D-Glyceraldehyde	$\begin{array}{c} \text{CHO} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	5.6 mM
L-Glyceraldehyde	$\begin{array}{c} \text{CHO} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{CH}_2\text{OH} \end{array}$	4.5 mM
D-Glyceraldehyde 3-Phosphate	$\begin{array}{c} \text{CHO} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OPO}_3\text{H}_2 \end{array}$	6.1 μM
D-Erythrose 4-Phosphate	$\begin{array}{c} \text{CHO} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OPO}_3\text{H}_2 \end{array}$	120 μM
D-Ribose 5-Phosphate	$\begin{array}{c} \text{CHO} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OPO}_3\text{H}_2 \end{array}$	No reaction detected
Pyruvate	$\begin{array}{c} \text{CH}_3 \\ \\ \text{C}=\text{O} \\ \\ \text{COOH} \end{array}$	40 μM

Fig. 5. Kinetic constants of Rv2682c. Reaction mixtures contained 100 or 250 mM MOPS (pH 7.9), 5 mM TPP, 1 mM MgCl₂, 1 μg of purified recombinant protein, varying concentrations of the indicated compounds, and 120 μM [2-¹⁴C]pyruvate or 26 mM DL-glyceraldehyde as appropriate. Reactions were stopped and processed as described in *Materials and methods*. The data was subjected to nonlinear regression analysis using SigmaPlot for Windows version 4.01 (SPSS).

assay method used here is also amenable to high through-put screening for inhibitors and has the advantage of containing a single enzyme in the reaction mixture.

Materials and methods

Materials

[2-¹⁴C]Pyruvate (50 mCi/mmol) was purchased from NEN Life Science Products (Boston, MA). AG1-X2 anion exchange resin (chloride form) and BioRex 70 cation exchange resin were obtained from BioRad (Hercules, CA). DE-52 anion exchange resin was purchased from Whatman (Clifton, NJ). H37Rv genomic DNA was provided by Dr. J. Belisle (through

NIH/NIAID contract N01-AI-75320, Tuberculosis Research Material and Vaccine Testing). Synthetic DXP was purchased from Echelon Research Laboratories (Salt Lake City, UT). DX was generated by treating DXP with bovine alkaline phosphatase (Sigma, St. Louis, MO). DL-glyceraldehyde, D-glyceraldehyde 3-phosphate, and TPP were purchased from ICN (Costa Mesa, CA). D-glyceraldehyde and L-glyceraldehyde were obtained from Acros (Pittsburgh, PA) and D-ribose 5-phosphate and D-erythrose 4-phosphate were from Sigma. Silica gel 60 F254 aluminum-backed TLC plates were purchased from EM Science (Darmstadt, Germany). All other reagents and solvents were of at least analytical grade.

PCR amplification and cloning of Rv2682c from M. tuberculosis H37Rv genome

On the basis of the nucleotide sequence of Rv2682c (Cole *et al.*, 1998) the gene was amplified using the following oligonucleotide primers: 5'-ACTAGCCATATGCTGCAACAGATCCG-3' and 5'-ATGAAGCTTCTTAGTTCGAGATGTTCTGGAA-3' containing a *Hind* III and *Nde* I restriction site (underscored). Polymerase chain reaction (PCR) using a Perkin Elmer GeneAmp 2400 and rTth polymerase (PE Biosystems, Foster City, CA) resulted in the amplification of a single DNA fragment of the expected size (1.6 kb). The PCR fragment was purified from agarose gel after electrophoresis, digested with *Nde* I and *Hind* III, and ligated into pET28a+ vector (Novagen, Madison, WI) that had been similarly digested. Plasmids were propagated in *E. coli* DH5α cells (Life Technologies, Rockville, MD) and were isolated using Qiagen plasmid kits. General molecular biology techniques were done as described earlier (Sambrook *et al.*, 1989).

Expression and purification of the recombinant Rv2682c

E. coli strain ER2566 (New England Biolabs, Beverly, MA) was used as the expression host. Bacteria harboring the plasmid were grown at 37°C in Luria Bertani broth containing kanamycin (50 μg/ml) to an OD₆₀₀ ~ 0.6 with shaking. The culture was allowed to cool on ice, and isopropylthiogalactoside was added to a final concentration of 50 μM, and the culture was incubated overnight at 15°C with shaking. Cells were harvested by centrifugation at 8000 × g for 10 min and stored at -80°C. The frozen cells were resuspended in a lysis buffer (2 ml/g) containing 50 mM 4-morpholine propane sulfonic acid (MOPS) (pH 7.9), 10 mM MgCl₂, 15% glycerol, and 1 mM 2-mercaptoethanol, broken by sonication on ice and then centrifuged for 40 min at 20,000 × g. The volume of the supernatant was adjusted to 30 ml, and imidazole was added to achieve a final concentration of 5 mM. A packed volume of 0.75 ml of Talon metal affinity resin (Clontech, Palo Alto, CA) pre-equilibrated with lysis buffer was added to the resulting solution and rocked at 4°C for 20 min. A small column was packed with the slurry, and the column was washed sequentially with 2 ml lysis buffer followed by 45 ml lysis buffer containing 7 mM imidazole and 750 mM NaCl. The His-tagged Rv2682c was eluted with 2-ml volumes of lysis buffer containing 25 mM, 50 mM, 100 mM, and 200 mM imidazole, and each fraction was analyzed by SDS-PAGE. Although some recombinant protein eluted in all fractions, the majority was found in the 100 mM imidazole fraction. The 100 and 200 mM imidazole fractions were pooled concentrated and desalted on a PD-10 desalting column (Amersham, Piscataway,

NJ). Recombinant Rv2682c was then concentrated by ultrafiltration using a 5000 molecular weight cutoff Centricon (Millipore, Bedford, MA). Glycerol was added to a final concentration of 20% and the resulting mixture was divided into aliquots and stored at -80°C .

Partial purification of native DXS

M. tuberculosis (H37Rv) was grown to mid-log phase in glycerol-alanine-salts medium, harvested by centrifugation and washed with saline. The resulting pellet was irradiated for 18 h at 2315 Rads/min using a JL Shepard instrument with a ^{137}Cs source. This exposure was calculated to kill 100% of the bacteria but retain 90% of enzyme activity. Irradiated H37Rv cells were suspended in ice-cold homogenization buffer containing 50 mM MOPS (pH 7.9), 0.25 M sucrose, 10 mM MgCl_2 , and 5 mM 2-mercaptoethanol and subjected to probe sonication on ice for 10 cycles of 60 s on and 90 s off in a Soniprep 150 (Integrated Services, TCP, Palisades Park, NJ). All steps were done at 4°C . The homogenate was centrifuged at $21,000 \times g$ for 15 min, and the resulting pellet (debris and cell wall) was discarded. The supernatant was then spun at $200,000 \times g$ in a Beckman 70.1 Ti rotor for 1 h. To remove a competing enzymatic activity that interfered with the DXS assay the $200,000 \times g$ supernatant (cytosol) was loaded onto a 15-ml DE-52 anion exchange column pre-equilibrated with homogenization buffer. Protein was eluted with a NaCl gradient from 0–1 M and collected in 1.5-ml fractions. Fractions were assayed for DXS activity as described later, and those containing activity were pooled; this resulted in a twofold increase in the specific activity of DXS and removal of the interfering activity. Glycerol was added to a final concentration of 30%, and the resulting mixture was divided into 100- μl aliquots and frozen at -80°C . Endogenous, tightly bound divalent cations were removed from the partially purified protein by cation exchange on BioRex 70. The anion exchange purification step served to remove bound TPP from the native protein. Attempts to remove divalent cations or TPP from the purified recombinant protein resulted in irreversible loss of enzymatic activity.

In vitro DXS assay using GA

DXS activity was assayed in a mixture containing 100 mM MOPS (pH 7.9), 5 mM TPP, 120 μM $[2-^{14}\text{C}]$ pyruvate, 26 mM GA and 1 μg of recombinant or 50–100 μg of partially purified protein in a final volume of 75 μl . Assays were incubated at 37°C for 30 min and stopped by the addition of 1 ml 50% methanol. Reaction mixtures were then passed over an anion exchange column (1.5 ml bed volume in a Pasteur pipette) of AG1-X2. The radiolabeled product, DX, was eluted with 5 ml 50% methanol. A 1-ml aliquot of eluate was taken and dried under N_2 , resuspended in 250 μl 50% methanol, and an aliquot was subjected to liquid scintillation spectrometry. Aliquots were also spotted on TLC plates (silica gel 60) along with authentic DX, and the plates were developed in n-propanol/ethyl acetate/ H_2O (6:1:3 v/v/v) as previously described (Lois *et al.*, 1998). Radiolabeled product was located with a Bioscan System 200 Imaging System (Bioscan, Washington, DC), and authentic standard DX was located with anisaldehyde spray reagent (Dunphy *et al.*, 1967).

In vitro DXS assay using GAP

To assay activity in the presence of GAP typical reaction mixtures contained 250 mM MOPS (pH 7.9), 5 mM TPP, 120 μM $[2-^{14}\text{C}]$ pyruvate, recombinant or partially purified protein and various concentrations of GAP in a final volume of 75 μl , which were incubated at 37°C for 30 min. In this case reactions were terminated by heating at 80°C . Following centrifugation at $13,000 \times g$ for 5 min, the supernatant was transferred to new tubes and aliquots were counted in a liquid scintillation counter. In addition, aliquots were spotted on silica gel 60 TLC plates along with authentic DXP; radiolabeled enzymatic products were separated from $[2-^{14}\text{C}]$ pyruvate using n-propanol/ethyl acetate/ H_2O (6:1:3) (Lois *et al.*, 1998) and located with the Bioscan imaging system. To confirm the identity of the radiolabeled DXP, some of the radiolabeled enzymatic products were treated with bovine alkaline phosphatase and spotted on silica gel 60 TLC plates along with authentic DX. Standard DXP and DX were located by spraying with anisaldehyde spray reagent (Dunphy *et al.*, 1967).

Enzymatic properties of DXS

Determination of optimal pH was done using the GA-based assay essentially as already described. However, a broad pH range buffer (0.05 M Tris/0.025 M MES/0.025 M acetic acid) adjusted with tetraethylammonium hydroxide was used, and activity was determined at 0.5 pH unit intervals between pH 5.5 and 9.0. To determine the K_m for pyruvate and GA, concentrations of either the $[2-^{14}\text{C}]$ pyruvate and GA were varied while the other was held constant, and the radiolabeled product was assayed as already described. The optimal concentrations of TPP and divalent cation were determined in the same manner; however, partially purified native protein was used.

Other procedures

Protein concentrations were estimated using a BCA protein assay kit (Pierce, Rockford, IL). All enzyme assays were done under conditions that were linear for both time and protein concentration, and the data presented are averages of duplicate assays from representative experiments. Radioactivity was measured in Ecolume (ICN) using a Beckman (Fullerton, CA) LS 6500 liquid scintillation spectrometer. Colorado State University Macromolecular Resources synthesized PCR primers and sequenced DNA. BLAST searches were done at the National Center for Biotechnology Information Web site using standard protein–protein BLAST (blastp). Alignments were done using multiple sequence alignment with hierarchical clustering (Corpet, 1988) using the Multalin interface at the Institut National de la Recherche Agronomique Web site and the resulting alignments were formatted using Multiple Align Show from the Sequence Manipulation Suite available at the University of Alberta Web site. Transketolase signature sequences I and II were identified using ScanProsite on the ExPASy Molecular Biology Server. The GC contents of the Rv2682c and Rv3379c were determined at the *M. tuberculosis* Structural Genomics Consortium Web site.

Acknowledgments

Work was funded by grant AI-46393 provided under the National Cooperative Drug Discovery Groups-Opportunistic

Infections Program from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and grant AI-49151 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Abbreviations

DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; EDTA, ethylenediamine tetra-acetic acid; GA, glyceraldehyde; GAP, D-glyceraldehyde 3-phosphate; HPLC, high-performance liquid chromatography; IPP, isopentenyl diphosphate; MOPS, 4-morpholine propane sulfonic acid; PCR, polymerase chain reaction; Pol-P, polyprenyl phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TPP, thiamine diphosphate.

References

- Altincicek, B., Hintz, M., Sanderbrand, S., Wiesner, J., Beck, E., and Jomaa, H. (2000) Tools for discovery of inhibitors of the 1-deoxy-D-xylulose 5-phosphate (DXP) synthase and DXP reductoisomerase: an approach with enzymes from the pathogenic bacterium *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, **190**, 329–333.
- Anderson, R.G., Hussey, H., and Baddiley, J. (1972) The mechanism of wall synthesis in bacteria. The organization of enzymes and isoprenoid phosphates in the membrane. *Biochem. J.*, **127**, 11–25.
- Baddiley, J. (1972) Teichoic acids in cell walls and membranes of bacteria. *Essays Biochem.*, **8**, 35–77.
- Bellamine, A., Mangla, A.T., Dennis, A.L., Nes, W.D., and Waterman, M.R. (2001) Structural requirements for substrate recognition of *Mycobacterium tuberculosis* 14 alpha-demethylase: implications for sterol biosynthesis. *J. Lipid Res.*, **42**, 128–136.
- Besra, G.S., Morehouse, C.B., Rittner, C.M., Waechter, C.J., and Brennan, P.J. (1997) Biosynthesis of mycobacterial lipoarabinomannan. *J. Biol. Chem.*, **272**, 18460–18466.
- Besra, G.S., Sievert, T., Lee, R.E., Slayden, R.A., Brennan, P.J., and Takayama, K. (1994) Identification of the apparent carrier in mycolic acid synthesis. *Proc. Natl Acad. Sci. USA*, **91**, 12735–12739.
- Boucher, Y. and Doolittle, W.F. (2000) The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol. Microbiol.*, **37**, 703–716.
- Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A., and Camara, B. (1998) Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiol.*, **117**, 1423–1431.
- Cane, D.E., Chow, C., Lillo, A., and Kang, I. (2001) Molecular cloning, expression and characterization of the first three genes in the mevalonate-independent isoprenoid pathway in *Streptomyces coelicolor*. *Bioorg. Med. Chem.*, **9**, 1467–1477.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C. E. III, and others. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, **393**, 537–544. [Published erratum in *Nature* (1998) **396** (6707): 190.]
- Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.*, **16**, 10881–10890.
- Crick, D.C. and Brennan, P.J. (2000) Antituberculosis drug research. *Curr. Opin. Anti-infect. Invest. Drugs*, **2**, 154–163.
- Crick, D.C., Mahapatra, S., and Brennan, P.J. (2001) Biosynthesis of the arabinogalactan-peptidoglycan complex of *Mycobacterium tuberculosis*. *Glycobiology*, **11**, 107R–118R.
- Dunphy, P., Kerr, J.D., Pennock, J.F., Whittle, K.J., and Feeney, J. (1967) The plurality of long chain isoprenoid alcohols (polyprenols) from natural sources. *Biochim. Biophys. Acta*, **136**, 136–147.
- Duvold, T., Bravo, J.M., PaleGrosdemange, C., and Rohmer, M. (1997) Biosynthesis of 2-C-methyl-D-erythritol, a putative C-5 intermediate in the mevalonate independent pathway for isoprenoid biosynthesis. *Tetrahedron Lett.*, **38**, 4769–4772.
- Estevez, J.M., Cantero, A., Romero, C., Kawaide, H., Jimenez, L.F., Kuzuyama, T., Seto, H., Kamiya, Y., and Leon, P. (2000) Analysis of the expression of CLA1, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-C-methyl-D-erythritol-4-phosphate pathway in *Arabidopsis*. *Plant Physiol.*, **124**, 95–104.
- Hahn, F.M., Eubanks, L.M., Testa, C.A., Blagg, B.S., Baker, J.A., and Poulter, C.D. (2001) 1-Deoxy-D-xylulose 5-phosphate synthase, the gene product of open reading frame (ORF) 2816 and ORF 2895 in *Rhodobacter capsulatus*. *J. Bacteriol.*, **183**, 1–11.
- Heinrich, P.C., Steffen, H., Janser, P., and Wiss, O. (1972) Studies on the reconstitution of apotransketolase with thiamine pyrophosphate and analogs of the coenzyme. *Eur. J. Biochem.*, **30**, 533–541.
- Hemming, F.W. (1974) Lipids in glycan synthesis. In Goodwin, T.W. (ed.) *Biochemistry, series 1, volume 4*. Butterworths, London, pp. 39–97.
- Higashi, Y. and Strominger, J.L. (1970) Biosynthesis of the peptidoglycan of bacterial cell walls. XX. Identification of phosphatidylglycerol and cardiolipin as cofactors for isoprenoid alcohol phosphokinase. *J. Biol. Chem.*, **245**, 3691–3696.
- Horbach, S., Sahn, H., and Welle, R. (1993) Isoprenoid biosynthesis in bacteria: two different pathways? *FEMS Microbiol. Lett.*, **111**, 135–140.
- Kuzuyama, T., Takagi, M., Takahashi, S., and Seto, H. (2000) Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces* sp. strain CL190, which uses both the mevalonate and non-mevalonate pathways for isopentenyl diphosphate biosynthesis. *J. Bacteriol.*, **182**, 891–897.
- Lamb, D.C., Kelly, D.E., Manning, N.J., and Kelly, S.L. (1998) A sterol biosynthetic pathway in *Mycobacterium*. *FEBS Lett.*, **437**, 142–144.
- Lange, B.M., Wildung, M.R., McCaskill, D., and Croteau, R. (1998) A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proc. Natl Acad. Sci. USA*, **95**, 2100–2104.
- Lois, L.M., Campos, N., Putra, S.R., Danielsen, K., Rohmer, M., and Boronat, A. (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proc. Natl Acad. Sci. USA*, **95**, 2105–2110.
- Mikusova, K., Mikus, M., Besra, G.S., Hancock, I., and Brennan, P.J. (1996) Biosynthesis of the linkage region of the mycobacterial cell wall. *J. Biol. Chem.*, **271**, 7820–7828.
- Miller, B., Heuser, T., and Zimmer, W. (1999) A *Synechococcus leopoliensis* SAUG 1402-1 operon harboring the 1-deoxyxylulose 5-phosphate synthase gene and two additional open reading frames is functionally involved in the dimethylallyl diphosphate synthesis. *FEBS Lett.*, **460**, 485–490.
- Putra, S.R., Disch, A., Bravo, J.M., and Rohmer, M. (1998a) Distribution of mevalonate and glyceraldehyde 3-phosphate/pyruvate routes for isoprenoid biosynthesis in some gram-negative bacteria and mycobacteria. *FEMS Microbiol. Lett.*, **164**, 169–175.
- Putra, S.R., Lois, L. M., Campos, N., Boronat, A., and Rohmer, M. (1998b) Incorporation of [2, 3-C-13(2)]- and [2, 4-C-13(2)]-D-1-deoxyxylulose into ubiquinone of *Escherichia coli* via the mevalonate-independent pathway for isoprenoid biosynthesis. *Tetrahedron Lett.*, **39**, 23–26.
- Querol, J., Besumbes, O., Maria, Lois L., Boronat, A., and Imperial, S. (2001a) A fluorometric assay for the determination of 1-deoxy-D-xylulose 5-phosphate synthase activity. *Anal. Biochem.*, **296**, 101–105.
- Querol, J., Rodriguez-Concepcion, M., Boronat, A., and Imperial, S. (2001b) Essential role of residue H49 for activity of *Escherichia coli* 1-deoxy-D-xylulose 5-phosphate synthase, the enzyme catalyzing the first step of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid synthesis. *Biochem. Biophys. Res. Commun.*, **289**, 155–160.
- Rieber, M., Imaeda, T., and Cesari, I.M. (1969) Bacitracin action on membranes of mycobacteria. *J. Gen. Microbiol.*, **55**, 155–159.
- Rohdich, F., Kis, K., Bacher, A., and Eisenreich, W. (2001) The non-mevalonate pathway of isoprenoids: genes, enzymes and intermediates. *Curr. Opin. Chem. Biol.*, **5**, 535–540.
- Rohmer, M., Knani, M., Simonin, P., Sutter, B., and Sahn, H. (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem. J.*, **295** (pt 2), 517–524.
- Sacchetti, J.C. and Poulter, C.D. (1997) Creating isoprenoid diversity. *Science*, **277**, 1788–1789.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schenk, G., Duggleby, R.G., and Nixon, P.F. (1998) Properties and functions of the thiamin diphosphate dependent enzyme transketolase. *Int. J. Biochem. Cell Biol.*, **30**, 1297–1318.
- Sprenger, G.A., Schorken, U., Sprenger, G., and Sahn, H. (1995) Transketolase A of *Escherichia coli* K12. Purification and properties of the enzyme from recombinant strains. *Eur. J. Biochem.*, **230**, 525–532.

- Sprenger, G.A., Schorken, U., Wiegert, T., Grolle, S., De Graaf, A.A., Taylor, S.V., Begley, T.P., Bringer-Meyer, S., and Sahl, H. (1997) Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. *Proc. Natl Acad. Sci. USA*, **94**, 12857–12862.
- Storm, D.R. and Strominger, J.L. (1973) Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions. *J. Biol. Chem.*, **248**, 3940–3945.
- Swenson, C.A. and Barker, R. (1971) Proportion of keto and aldehyde forms in solutions of sugars and sugar phosphates. *Biochemistry*, **10**, 3151–3154.
- van Heijenoort, J. (1996) Murein synthesis. In: Neidhardt, F.C. (ed.) *Escherichia coli and Salmonella: cellular and molecular biology*. ASM Press, Washington, DC, pp. 1025–1034.
- Wikner, C., Nilsson, U., Meshalkina, L., Udekwu, C., Lindqvist, Y., and Schneider, G. (1997) Identification of catalytically important residues in yeast transketolase. *Biochemistry*, **36**, 15643–15649.
- Zhou, D. and White, R.H. (1991) Early steps of isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.*, **273** (pt 3), 627–634.