Determination of the pathway for rhamnose biosynthesis in mycobacteria: cloning, sequencing and expression of the *Mycobacterium tuberculosis* gene encoding α -D-glucose-1-phosphate thymidyltransferase

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The mycobacterial cell wall core consists of an outer lipid layer of mycolic acids connected, via arabinogalactan polysaccharide, to an inner peptidoglycan layer. An α -L-rhamnopyranosyl residue has been shown to be a key component linking the mycolated arabinogalactan to the peptidoglycan and, therefore, the biosynthesis of L-rhamnose (Rha) in mycobacteria was investigated as the first step of developing inhibitors of its biosynthesis. Biochemical assays were used to show that dTDP-Rha was synthesized in *Mycobacterium smegmatis* from α -D-glucose 1-phosphate (α -D-Glc-1-P) and dTTP by the same four enzymic steps used by *Escherichia coli* and other bacteria. PCR primers based on consensus regions of known sequences of the first enzyme in this series, α -D-Glc-1-P thymidyltransferase (RfbA) were used to amplify *rfbA* DNA from *M. tuberculosis*. The entire *rfbA* gene was then cloned and sequenced. The deduced amino acid sequence revealed a 31362 Da putative protein product which showed similarity to RfbA proteins of other bacteria (59% identity to that found in *E. coli*). Sequencing of DNA flanking the *rfbA* gene did not reveal any of the other *rfb* genes required for dTDP-Rha biosynthesis. Therefore, the four Rha biosynthetic genes are not clustered in *M. tuberculosis*. The enzymic activity of the sequenced gene product was confirmed by transformation of *E. coli* with pBluescript KS(–) containing the *rfbA* gene from *M. tuberculosis*. Analysis of enzyme extracts prepared from this transformant revealed an 11-fold increase in α -D-Glc-1-P thymidyltransferase activity.

Keywords: rhamnose, *rfb*, mycobacterial cell wall, drug development, thymidyltransferase

INTRODUCTION

The mycobacterial cell wall consists of a lipid mycolic acid layer (Brennan & Nikaido, 1995; McNeil & Brennan, 1991) attached, by means of the polysaccharide

arabinogalactan (AG), to the peptidoglycan layer. This attachment, via AG, is fundamental to the structural integrity of the wall and thus required for mycobacterial viability. For example, ethambutol, which inhibits the attachment of the peptidoglycan layer to the mycolic acid layer by inhibiting the formation of the arabinan region of AG (Takayama *et al.*, 1979; Takayama & Kilburn, 1989; Deng *et al.*, 1995; Mikusova *et al.*, 1995), stops the growth of mycobacteria and is an effective drug. When AG is examined in molecular detail, it is evident that an L-rhamnosyl (L-Rha) residue plays an essential structural role as it is part of the 'linker' region, a specialized unit that links the AG proper with

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Abbreviations: AG, arabinogalactan; Glc, glucose; Glc-1-P, glucose 1-phosphate; Rha, rhamnose (rhamnosyl); 6-dTal, 6-deoxytalose.

The EMBL/GenBank/DDBJ accession number of the *rfbA* nucleotide sequence reported in this paper is U55242.

Fig. 1. Structure of the mycobacterial cell wall emphasizing the position and role of the L-Rha-containing linker disaccharide.

peptidoglycan (McNeil *et al.*, 1990), as shown in Fig. 1. Thus, it is attractive to hypothesize that inhibition of the biosynthesis of L-Rha would be lethal to the mycobacteria by making formation of the linker unit impossible. Therefore, we have begun studies on its biosynthesis in mycobacteria.

In Gram-negative bacteria, L-Rha residues are often present in LPS and are synthesized at the sugar nucleotide level, dTDP-Rha being the nucleotide product (Shibaev, 1986). dTDP-Rha then functions as the Rha donor in all bacteria studied (Shibaev, 1986) and, indeed, in a recent report it has been shown to donate the Rha residue for the linker region in mycobacteria (Mikusova *et al.*, 1996). The pathway for dTDP-Rha biosynthesis has been studied extensively in Gram-negative bacteria (Shibaev, 1986) and dTDP-Rha has been shown to be produced from TTP and glucose 1-phosphate (Glc-1-P) as shown in Fig. 2. The genes encoding these four biosynthetic transformations (known as *rfbA*, *rfbB*, *rfbC* and *rfbD* in Gram-negative organisms) have been cloned and sequenced in many organisms (Minnikin, 1982; Stevenson *et al.*, 1994; Rajakumar *et al.*, 1994). They have invariably been found in a biosynthetic operon with the genes usually in the order *rfbBDAC* (Stevenson *et al.*, 1994).

Here we present evidence that mycobacteria produce dTDP-Rha by the same sequence of reactions shown in Fig. 2. We also report the cloning, sequencing and expression in *Escherichia coli*, of the first of the

Fig. 2. The biosynthetic pathway for formation of dTDP-Rha as found in Gram-negative bacteria and, as shown here, in mycobacteria. RfbA, α -D-Glc-1-P thymidyltransferase; RfbB, dTDP-Glc 4,6-dehydratase; RfbC, dTDP-6-deoxy-4-ketoglucose epimerase; RfbD, dTDP-Rha synthase.

Mycobacterium tuberculosis Rha biosynthetic genes, namely the *rfbA* gene which encodes α -D-Glc-1-P thymidyltransferase (RfbA). Sequence data in the vicinity of *rfbA* show that, in contrast to Gram-negative organisms, the *rfbA* gene is not clustered with the remaining Rha genes.

METHODS

Chemicals, biochemicals and enzymes. All chemicals, enzymes and biochemicals were purchased from Sigma unless otherwise indicated. dTDP-[U- 14 C]glucose (dTDP-[14 C]Glc), specific activity 257 mCi mmol $^{-1}$ (9.5×10^9 Bq mmol $^{-1}$), was purchased from ICN and is unfortunately no longer available. Restriction enzymes were obtained from Boehringer Mannheim and used according to the supplier's instructions.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *M. smegmatis* ATCC 8548 and *M. tuberculosis* H37Rv were grown on glycerol-alanine-salts medium (Takayama *et al.*, 1975). *E. coli* DH5 α and S ϕ 874 were grown on LB medium (Life Technologies). When necessary, antibiotics were used at

Table 1. Properties and sources of relevant bacterial strains and plasmids

Species/strain	Relevant properties	Source*
Bacteria		
<i>M. smegmatis</i> 8548	Fast growing; non-pathogenic	ATCC
<i>M. tuberculosis</i> H37Rv		ATCC
<i>E. coli</i> DH5 α	F ⁻ <i>deoR recA hsdR17</i> (r_k^-, m_k^+) <i>sup44</i> λ^- <i>thi-1 gyrA96 recA1</i>	Gibco BRL
<i>E. coli</i> S ϕ 874	Δ (<i>sbcB-rfb</i>)	CGSC (Bachmann, 1987)
Plasmids		
pBlueT7 T	PCR product cloning vector	Stratagene
pBluescript KS(-)	Sequencing and expression vector	Stratagene
pGC1	Vector for <i>M. tuberculosis</i> cosmid library	This study
pYM1	<i>M. tuberculosis</i> H37Rv 495 bp <i>rfbA</i> PCR product in pT7Blue T vector	This study
pYMc1	30–40 kb of <i>M. tuberculosis</i> DNA containing <i>rfbA</i> in pGC1 vector	This study
pYM2	5.7 kb of <i>rfbA</i> containing <i>XhoI</i> fragment from pYMc1 in pBluescript KS(-)	This study
pYM3	8.0 kb of <i>rfbA</i> containing <i>EcoRV</i> fragment from pYMc1 in pBluescript KS(-)	This study
pYM4	5.2 kb of <i>rfbA</i> containing <i>PstI</i> – <i>EcoRI</i> fragment from pYMc1 in pBluescript KS(-)	This study
pYM2d1–d9	Deletion plasmids from pYM2	This study

* ATCC, American Type Culture Collection, Rockville, MD, USA; CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Connecticut, USA.

the following final concentrations: ampicillin, 50 μ g ml⁻¹; kanamycin, 30 μ g ml⁻¹; tetracycline, 12.5 μ g ml⁻¹.

Cell-free assays using *M. smegmatis*. *M. smegmatis* ATCC 8548 was prepared as a cell-free extract by sonicating the cells at 4 °C with a 4710 series Cole Parmer Ultrasonic homogenizer in a buffer solution containing 50 mM HEPES, pH 7.6, 10 mM MgCl₂, 5 mM MnCl₂, 0.2 mM PMSF and 1 mM DTT. The cell homogenate was then centrifuged at 12000 *g* for 10 min and the protein concentration of the resulting supernatant was measured using the Bio-Rad Protein Assay.

To assay for RfbA activity, 0.5 μ Ci (1.85 $\times$ 10⁴ Bq) dTDP-[¹⁴C]Glc was incubated in a total volume of 110 μ l, with the final concentrations of reagents as follows: cold dTDP-Glc, 0.1 mM; MgCl₂, 5 mM; NaF, 5 mM; tetrasodium pyrophosphate, 1 mM; crude *M. smegmatis* protein extract, 0.5 mg ml⁻¹. The sample was incubated for 45 min at 30 °C and then injected onto an HPLC system consisting of a 4 $\times$ 250 mm Partisil 10-SAX anion exchange HPLC column (Whatmann). The sample was eluted with a gradient of NH₄H₂PO₄ from 5 to 185 mM over a period of 75 min with a flow rate of 1 ml min⁻¹. Seventy 1 ml fractions were then counted for radioactivity using liquid scintillation. A control reaction was run in the same fashion except the PP_i and NaF were omitted.

The assay for RfbB activity was performed as described by Wang & Gabriel (1969) except for the use of 50 mM HEPES, pH 7.6, instead of Tris buffer.

The assay for the combination of RfbB, RfbC and RfbD enzymes was done in a 100 μ l volume and the final concentrations of reagents were as follows: dTDP-[¹⁴C]Glc, 0.5 μ Ci (1.85 $\times$ 10⁴ Bq); cold dTDP-Glc, 0.5 mM; NADPH, 10 mM; NAD⁺, 5 mM; *M. smegmatis* enzyme extract prepared as described above, 7.5 mg protein ml⁻¹. The tube was incubated for 45 min at 30 °C and was made 50% with respect to ethanol resulting in a soluble crude sugar nucleotide fraction. After

centrifugation at 12000 *g* for 5 min, the clear supernatant was dried, hydrolysed with 2 M trifluoroacetic acid for 1 h at 120 °C and dried. The hydrolysate was then injected onto a Dionex HPLC System with a Dionex Carbpak PA1 column (4 $\times$ 250 mm) with isocratic elution with 10 mM NaOH. Thirty 1 ml fractions were then counted for radioactivity in a liquid scintillation system.

DNA techniques. *M. tuberculosis* DNA was prepared using the protocol described for *M. avium* (Belisle *et al.*, 1991). DNA was isolated from agarose gels with a GeneClean II kit (Bio101). Purified plasmids were prepared using a QIAGEN plasmid kit according to the manufacturer's instructions.

PCR and sequencing of PCR products. Amino acid sequence alignment of known bacterial α -D-Glc-1-P thymidyltransferases [*Shigella flexneri* (Rajakumar *et al.*, 1994), *E. coli* (Stevenson *et al.*, 1994), *Yersinia enterocolitica* (Zhang *et al.*, 1993) and *Streptomyces griseus* (Distler *et al.*, 1987)] revealed highly conserved sequences. Two degenerated primers were designed based on the conserved TRLYPIT and AVTGLYFY stretches shown in Figs 6 and 7, and the *M. tuberculosis* codon usage preference (Hatfull & Sarkis, 1993). The sense primer was 5' AC(GC)CG(GC)CT(GC)TACCC(GC)ATCAC 3' and the antisense primer was 5' GTAGAAGTA(GC)AG(AGCT)-CC(GC)GT(GC)AC(GC)GC 3'. PCR was performed in a total volume of 50 μ l using a Thermal Cycler (Perkin Elmer). The reaction mixture contained 1.25 units AmpliTaq DNA polymerase (Perkin Elmer), buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂), 0.2 mM each deoxynucleotide triphosphate (Gibco BRL), 10 pmol each primer and 0.4–1.0 μ g *M. tuberculosis* H37Rv genomic DNA. The PCR conditions consisted of 1 cycle of denaturation (94 °C, 5 min) followed by 35 cycles of amplification consisting of denaturation (94 °C, 1 min), annealing (65 °C, 1 min) and primer extension (72 °C, 1 min). After the final cycle there was a 7 min incubation at 72 °C. A PCR product of correct size (495 bp) was cloned into the pBlueT7 T vector (Stratagene) resulting in plasmid pYM1.

The cloned PCR product was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (USB). The BLAST algorithm was used to search protein databases for similarity (Altschul *et al.*, 1990).

DNA probe preparation and DNA hybridization. A 546 bp *XbaI*–*EcoRI* restriction fragment (containing the entire *rfbA* PCR product and a small amount of DNA on each end from the pBlueT7 T vector) from pYM1 was prepared and a digoxigenin non-radioactive probe made from it using a DNA labelling kit, Genius 2 (Boehringer Mannheim). For Southern blotting, 1 µg *M. tuberculosis* H37Rv genomic DNA was digested with 10 units *Bam*HI, *Cl*aI, *Eco*RI, *Eco*RV, *Pst*I and *Xho*I, respectively, and separated by electrophoresis through a 0.7% agarose gel. The gel was treated for 10 min with 0.25 M HCl and then for 30 min with 0.6 M NaCl/0.4 M NaOH. DNA was transferred to a Maximum Strength Nytran Plus membrane (Schleicher & Schuell) and immobilized by UV cross-linking. The membrane was pre-hybridized at 42 °C for 2 h in pre-hybridization buffer containing 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim) and 50% deionized formamide. Following this treatment, the digoxigenin-labelled 546 bp *XbaI*–*EcoRI* *rfbA* probe was denatured, added to the membrane and incubated at 42 °C overnight. The membrane was washed three times at room temperature with 2 × SSC/0.1% SDS and then twice at 65 °C with 0.5 × SSC/0.1% SDS. Colorimetric detection was carried out using a nucleic acid detection kit, Genius 3 (Boehringer Mannheim).

Construction and screening of a cosmid library. A cosmid library of *M. tuberculosis* genomic DNA was constructed in pGC1, an *E. coli*–*Mycobacterium* shuttle cosmid. For this study, pGC1-based cosmid clones were used merely as a *M. tuberculosis* H37Rv DNA library source and the shuttle properties of the cosmid were not utilized. Vector pGC1 was constructed by ligation of a 2044 bp *Bam*HI–*Pst*I fragment containing *λcos* from pYUB18 (Belisle *et al.*, 1991) with pEP2 (Radford & Hodgson, 1991) digested with *Bam*HI–*Pst*I. The *M. tuberculosis* cosmid library was constructed with 30–40 kb fragments generated by a partial *Sau*3A digestion of *M. tuberculosis* DNA followed by ligation to *Bam*HI-digested pGC1. The ligation mixture was packaged *in vitro* with Gigapack II XL Packaging Extracts (Stratagene) and introduced into DH5α cells (Gibco BRL). To locate cosmids containing *rfbA*, *E. coli* recombinant colonies were lifted onto Optitran membranes (Schleicher & Schuell), lysed and then hybridized with the 546 bp *XbaI*–*EcoRI* probe (Maniatis *et al.*, 1982).

Southern analysis of five positive cosmid clones digested with a variety of restriction enzymes and probed with the 546 bp *XbaI*–*EcoRI* *rfbA* DNA probe yielded blots identical to those prepared with genomic DNA. Therefore, one of the five cosmid clones, pYMc1, was used to subclone smaller fragments containing *rfbA*. Thus, 5.7 kb *Xho*I, 8 kb *Eco*RV and 5.2 kb *Eco*RI–*Pst*I fragments were prepared from pYMc1 and individually subcloned into pBluescript II KS(–) (Stratagene) to produce pYM2, pYM3 and pYM4, respectively.

DNA sequencing and analysis. Sequences of double-stranded plasmid DNA were determined using the Taq FS Ready Reaction Kit (Applied Biosystems) on a GeneAmp PCR system 9600 (Perkin Elmer) and run on an ABI 377 PRISM Sequencer (Applied Biosystems) at the Macromolecular Resource Facility at Colorado State University, USA. To sequence *rfbA*, plasmids pYM2, pYM3, pYM4 and pYMc1 were all used as sources of template DNA. Initial sequencing was done using

the PCR primers as primers. Additional primers were then synthesized as needed using newly obtained sequence data. Both strands of the *rfbA* DNA were fully sequenced and these two sequences were in complete agreement. Comparisons of sequences with the database were done using the BLAST program (Altschul *et al.*, 1990).

To sequence the 5' and 3' flanking regions of *rfbA*, the nested deletion sequencing method was used. Thus, the pYM2 plasmid was digested with *Sac*I and *Xba*I. The resulting linear DNA was deleted using the Erase-a-Base System (Promega). The fragments resulting from nine different digestion times were re-circularized to form plasmids pYM2d1–9. These cosmids were sequenced as above using a primer based on the M13(–20) site present on the pBluescript II KS(–) vector. pYM2d1 was deleted such that sequencing commenced approximately 2 kb downstream from the 3' end of *rfbA*, pYM2d9 was situated so that sequencing began approximately 2 kb upstream from the 5' end of *rfbA* and the sequencing commenced on the remaining plasmids in between these two extremes. For these experiments only a single strand of DNA was sequenced and the sequences were then subjected to BLAST searches. The sequence of *rfbA* itself was found in pYM2d5 and pYM2d6 but no sequence corresponding to any other *rfb* genes was obtained.

Expression of α-D-Glc-1-P thymidyltransferase in *E. coli* and measurement of its activity. *E. coli* Sφ874 was transformed with pYM2 and with pBluescript II KS(–) only, as a control. Single colonies of each transformant were grown in 10 ml LB broth with ampicillin. The resulting cultures were transferred into 1 litre of the same medium and grown to an OD₆₀₀ of 0.6 after which IPTG was added to a final concentration of 1 mM. Cells were harvested after an additional 3 h of incubation. One half gram of each of the transformed cell preparations was resuspended in 1 ml breaking buffer (0.1 mM PMSF, 1 mM DTT, 50 mM HEPES, pH 7.4), sonicated with a 4710 series Cole Palmer ultrasonics homogenizer and centrifuged for 20 min at 16000 g. The protein concentration of the supernatants containing the crude enzyme was determined using the Bio-Rad Protein Assay.

The crude enzymes prepared from the two *E. coli* strains were then assayed for α-D-Glc-1-P thymidyltransferase activity. The assay was based on the formation of Glc-1-P as described for the *M. smegmatis* enzyme; however, the Glc-1-P was detected by a linked enzyme assay which produced NADPH rather than by HPLC. The enzyme assays were done in a volume of 1 ml containing 184 mM HEPES, pH 7.4, 4 mM tetrasodium pyrophosphate, 5 mM MgCl₂, 2 mM NADP, 1 mM TDP-Glc (or 1 mM UDP-Glc to determine α-D-Glc-1-P uridylyltransferase activity), 0.02 mM Glc 1,6-diphosphate, 0.2 units phosphoglucomutase, 1 unit Glc-6-P dehydrogenase and 148 µg crude enzyme. The rate of formation of NADPH was determined spectrophotometrically at 340 nm. A parallel reaction was done in the absence of PP_i.

RESULTS

Demonstration of RfbA, B, C and D activities in mycobacteria

We have previously shown that the cell wall core of *M. tuberculosis* and *M. smegmatis* contain the same linker disaccharide (Fig. 1) and that the arabinosyl and galactosyl residues of their respective AGs are arranged identically as determined by ¹H and ¹³C NMR analyses (Daffé *et al.*, 1993). Therefore, it is highly likely that the

Fig. 3. Inorganic PP_i -dependent conversion of dTDP-[^{14}C]Glc to α -D-Glc-1-P by α -D-Glc-1-P thymidyltransferase present in enzyme extracts of *M. smegmatis*. HPLC profiles of reaction products from dTDP-[^{14}C]Glc and enzyme extract without (a) and with (b) added PP_i . Authentic α -Glc-1-P eluted in 25 min under the conditions used; the heterogeneity seen around 25 min in (b) probably results from further conversion of Glc-1-P into other sugar phosphates.

cell wall cores, including the Rha residues, are synthesized in the same fashion in both species. We therefore decided for practical reasons to conduct biosynthetic assays using extracts from the fast-growing and non-pathogenic *M. smegmatis* but, since our ultimate aim is to design new drugs against tuberculosis, we conducted cloning experiments using *M. tuberculosis* DNA. Thus, we first looked for the presence α -D-Glc-1-P thymidyltransferase (RfbA) activity in a crude extract of *M. smegmatis* utilizing the reverse direction reaction (see Fig. 2). dTDP-[^{14}C]Glc was incubated with *M. smegmatis* enzyme extracts in the absence and presence of PP_i as this is needed as a co-substrate for the reaction. HPLC analysis of the products showed that in the absence of PP_i (Fig. 3a) no reverse reaction forming Glc-1-P took place. However, when PP_i was added to this system (Fig. 3b), dTDP-Glc was cleaved to yield TTP (data not shown) and radioactive Glc-1-P (Fig. 3b).

dTDP-Glc 4,6-dehydratase (RfbB) was assayed spectrophotometrically (Wang & Gabriel, 1969) as the product of dTDP-Glc dehydrogenase, dTDP-6-deoxy-4-ketoglucose, yields an UV-active compound after treatment with base. Incubation of varying amounts of crude enzyme prepared from *M. smegmatis* with dTDP-Glc followed by base treatment gave A_{318} values that were fourfold greater than those of controls with no dTDP-Glc in the incubation. At a protein concentration of $70 \mu\text{g ml}^{-1}$ the *M. smegmatis* enzymes converted $97 \text{ nmol dTDP-Glc to dTDP-6-deoxy-4-ketoglucose min}^{-1} (\text{mg protein})^{-1}$.

Fig. 4. Conversion of dTDP-[^{14}C]Glc to [^{14}C]Rha by treatment with an enzyme extract of *M. smegmatis* followed by acid hydrolysis. HPLC analysis of the resultant sugars is presented. Most of the radioactive Glc was converted to Rha with a small amount being converted to 6-dTal. The elution positions of all three sugars (determined using standards) are indicated.

RfbB activity was confirmed and evidence for the activities of dTDP-6-deoxy-4-ketoglucose epimerase (RfbC) and dTDP-Rha synthase (RfbD) obtained by the conversion of dTDP-Glc to a Rha-yielding product (presumably dTDP-Rha). Thus dTDP-[^{14}C]Glc was treated with a *M. smegmatis* enzyme extract, the resultant sugar nucleotides hydrolysed and the free sugars analysed by Dionex anion exchange chromatography (Fig. 4). The results demonstrated the enzymic conversion of the Glc unit to Rha with a small amount of Glc being converted to 6-deoxytalose (6-dTal) (see Discussion).

Amplification, cloning and sequencing of *rfa* DNA from *M. tuberculosis*

The α -D-Glc-1-P thymidyltransferase amino acid sequences from *Sh. flexneri* (Rajakumar *et al.*, 1994), *E. coli* (Stevenson *et al.*, 1994), *Y. enterocolitica* (Zhang *et al.*, 1993) and *S. griseus* (Distler *et al.*, 1987) were aligned. PCR primers were designed based on sequence similarities, mycobacterial codon bias and melting temperatures (see Fig. 6 for primer positions). Using *M. tuberculosis* H37Rv DNA as a template, a PCR product of the correct size was amplified. The PCR product was cloned into pT7Blue T vector (see Table 1), resulting in pYM1, and a partial sequence determined. A BLAST search (Altschul *et al.*, 1990) showed significant homology with *rfa* from several species. Analyses by Southern blotting of genomic *M. tuberculosis* DNA cut with various restriction enzymes using a 546 bp probe containing *rfa* sequence generated from pYM1 all yielded single bands (Fig. 5), suggesting that only a single copy of the gene is present in the *M. tuberculosis* genome.

To isolate the entire *rfa* gene, the 546 bp *rfa* probe was used to screen the *M. tuberculosis*:pGC1 cosmid library by colony blot hybridization. A cosmid, pYMc1, obtained from a positive colony was subsequently analysed by Southern blot analysis with the same probe. The hybridization pattern was identical to that shown in Fig. 5 for genomic DNA as expected. Thus, three

Fig. 5. Southern blot analysis of *M. tuberculosis* DNA digested with various enzymes and hybridized with the *rfbA* 546 bp probe. Lanes: A, *Xho*I; B, *Pst*I; C, *EcoRV*; D, *EcoRI*; E, *Cla*I; F, *Bam*HI.

subclones, pYM2, pYM3 and pYM4, were prepared from pYMc1 by ligation of its 5.7 kb *Xho*I, 8.0 kb *EcoRV* and 5.2 *Pst*I–*EcoRI* restriction fragments, respectively, into pBluescript II KS(–) (Table 1).

The complete sequence of *rfbA* was then obtained using plasmids pYMc1, pYM2, pYM3 and pYM4 as templates for double-stranded automated sequencing. The sequence is presented in Fig. 6. The G+C content was found to be 61% and a putative ribosome binding site was detected. The DNA encoded a protein of 288 aa with a molecular mass of 31362 kDa. The deduced amino acid sequence is compared with α -D-Glc-1-P thymidyltransferase amino acid sequences from other Gram-negative bacteria and one Gram-positive bacterium in Fig. 7. In addition, the sequence flanking the *rfbA* gene present in the pYM2 subclone was obtained by the nested deletion method. Approximately 2 kb on each side of *rfbA* were sequenced. BLAST searches of these sequences resulted in the recognition of no other genes except *rfbA*; it was expected that the other *rfb* genes should have readily been detected if they flanked *rfbA* as in other bacteria.

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-121 CCCAAGACCACGTCGCATCGGTTTCCGGCACGAAGACTGGCGTCGGCACTGACGACCACCGAA
                                     RBS      M R G
-56 CTCACCGAGGACGATTGGTCGATTGAGGTCCGGCGAACGGCAGTTAGGAGCCAGTTATGCGCGGG
                                     sense primer
10  I I L A G G S G T R L Y P L T M G I S K Q L
   ATCATCTTGGCCGGCGTTTCGGGCACCCGGCTGTACCCGATCACCATGGGGATCAGCAAGCAGCT
75  L P V Y D K P M I Y Y P L T T L M M A G I R
   GCTGCCGGTCTACGACAAACCGATGATCTACTACCCGCTACCCAGCTGATGATGGCTGGGATCC
140  D I Q L I T S P H D A P G F H R L L G D G
   GAGACATTGATGATCACCTCCCGCATGACGCGCCGGCTTTCATCGACTCCTGGGCGACGGC
205  A H L G V N I S Y A T Q D Q P D G L A Q A F
   GCGCACTTGGGAGTGAACATCAGCTACGCCACCCAGGATCAGCCTGACGGTCTGGCGCAGGCGTT
270  V I G A N H I G A D S V A L V L G D N I F Y
   CGTCATTGGGCCAACACATCGGCGCCGATTTCGGTGGCATTGGTGTGGGGGACAAATCTTCT
335  G P G L G T S L K R F Q S I S G G A I F A
   ACGGCCAGGCTGGGGACAGCCTGAAGCGCTTCAATCCATCAGTGGTGGAGCAATTTTCGCC
400  Y W V A N P S A Y G V V E F G A E G M A L S
   TATTGGGTAGCAACCCGTCGGCTATGGTTCGTGAGTTCGGCGCCGAGGGCATGGCGCTGC

                                     antisense primer
465  L E E K P V T P K S N Y A V P G L Y F Y D N
   TCTGGAGGAGAACCGGTGACCCCGAAGTCGAATTACCGGTGCCGGGCTGTATTCTATGACA
530  D V I E I A R G L K E I S A R G V R D H R
   ACGATGTGATCGAAATCGCCAGGGGTTTAAAGGAAATCAGCGCGGGGAGTACGAGATCAGCA
595  G Q Q V Y L N Q G R L A V E V L A R G T A W
   GGTCAACAGGCTTACCTCAATCAGGTAGGTGGCGGTGAGGTGCTGGCCCGGGGACAGCGTG
660  L D N G T F D S L L D A D F V R T L E R R
   GCTGGACAAACGGACATTCGACTCGCTGGACGCCCGGATTCGTCCGACCTGGAGCGTC
725  Q G L K V S I P E E V A W R M G W I D D E
   GGCAGGGCCTGAAGGTACGATCCCGAAGTGGCGTGGCGCATGGGCTGGATCGACGACGAG
790  Q L V Q R A R A L V K S G Y G N Y L L E L L
   CAGCTGGTCAGCGAGCCCGTCTGTGGTCAAGTCCGGATATGGTAACCTACTGCTGGAGTTGT
855  E R N *
   GGAGCGCAACTGATTTTCGGCGGTTATTGTCGGTGATTATGGAACCCCTGGTAGGCGCTCTGG
920  ATGAGCAGCCACCGGACCGCA

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Fig. 6. DNA sequence and deduced amino acid sequence of *M. tuberculosis rfbA* encoding α -D-Glc-1-P thymidyltransferase. The start codon (ATG), putative ribosome binding site (RBS), location of the PCR primers and stop codon (*) are indicated.

Expression of *rfbA*

E. coli strain S ϕ 874, a strain where the entire *rfb* region is deleted, was chosen to express the *M. tuberculosis rfbA* gene. It was recognized that this strain would express low levels of α -D-Glc-1-P thymidyltransferase activity due to the presence of a functional homologue of *rfbA* (Marolda & Valvano, 1995), but it was expected that this background level of activity would still be considerably less than that of a wild-type *E. coli*. Thus, *E. coli* S ϕ 874 was transformed with pYM2 and, as a control, with pBluescript KS(–) vector only. The transformants were grown to mid-exponential phase, treated with IPTG, harvested, disrupted by sonication, centrifuged and enzyme assays for Glc-1-P thymidyltransferase (RfbA) and Glc-1-P uridylyltransferase were conducted. As expected, the control bacteria showed some Glc-1-P thymidyltransferase activity (Table 2). However, bacteria containing the *Xho*I subclone showed an approximately 11-fold elevation of Glc-1-P thymidyltransferase activity per mg protein.

DISCUSSION

The results of the biochemical studies strongly suggest that dTDP-Rha is formed by the same pathway in mycobacteria as it is in other bacteria (Fig. 2). In addition, the biosynthesis of 6-dTal was also shown to occur in *M. smegmatis* (Fig. 4) by use of RfbA–C followed by reduction of the keto sugar (Fig. 2) by the

Sf	MKTRKGIIILAGSGGTRLYPVMTAVSKQLLEIYDKPMIYYPLSTLMLAGIRDILIIISTPQDTPRFQQLLGDGSQ	73
Ec	MKMRKGIIILAGSGGTRLYPVMTAVSKQLLEIYDKPMIYYPLSTLMLAGIRDILIIISTPQDTPRFQQLLGDGSQ	70
Ye	M---KGIVLAGGAGTRLHPITRGVSKQLLPVYDKPMIYYEISVLMLAGIQDILIIISTPDLPSFKRLLDGDSQ	69
Sg	MKAL---VLAGGTGTRLRPIHTISAKQIVPVANKFVLFYGLAIRAAGIIDVG-IVVGDTADEIVAAVCDGSR	73
Mt	M--R-GIILAGSGGTRLYPITMGTISKQLLPVYDKPMIYYPIITLMMAGIRDITQLITSFHPDAPGTHRLLDGDGH	70
Sf	WGLNLQYKVQPSPDGLAQAFITIGEEFIGGDDCALVLGDNIFYGHDLPKLMDTAVNRESGATVFAYHYNDHERY	146
Ec	WGLNLQYKVQPSPDGLAQAFITIGEEFIGGDDCALVLGDNIFYGHDLPKLMEEAVNKEGATVFAYHYNDHERY	143
Ye	FCIRLOYAKQPSPDGLAQAFITIGEEFIGAGERCALVLGDNIFYGQSFCKOLREVASRNDGATVFGYQVDAERF	141
Sg	FGLKVSYPQSKELGLAHCVLISRDFLEEDDFIMYLGDNFVVGVVED-SVREFRAARPD AHLMLTRVPEHRSF	146
Mt	LGVNISYATQDQPDGLAQAFVIGANHIGASVALVLGDNIFYGPGLGTSIKRFQSSISGCAI-FAYWVANPSAY	142
Sf	GVVEFDKNGTATISLEEKPEPKSNYAVTGLYFYDNDVVMMAKNLKP-SARGELEITDINRIYMDQGRLSVAMM	218
Ec	GVVEFDKNGTATISLEEKPEPKSNYAVTGLYFYDNDVVMMAKNLKP-SARGELEITDINRIYLEQGRLSVAMM	215
Ye	GVVEFDENFNLISLEEKPEPKSDWAVTGLYFYDKDVVEMAKEIKP-SERGELEITTLNEMYIAKGLRVELL	213
Sg	GVAELSDSGOVLGLEEKPAHPKSDLALVGVLFSIPAIEAFAAITP-SWGELEITDAVQWLIDAGRDVRSTV	218
Mt	GVVEFGAEGMALISLEEKPEVTPKSNYAVPGLYFYDNDVITETARGLKEISARGVRDHRGQQV-VLNQGRLEAVEVL	214
Sf	GRGYAWLDTGTH-QSLIEASNFATIE-----ERQGLKVSCE-EETIAHRKCFIDAEQVKVLAEPILKNAY	281
Ec	GRGYAWLDTGTH-QSLIEASNFATIE-----ERQGLKVSCE-EETIAFRKCFIDVEQVRKLAVPLIKNNY	278
Ye	GRGFAWLDTGTH-DSLIDASLTHTIE-----KROGKVACL-EETIAYONQLSRKILNELAEALNKTY	287
Sg	ISGY-WKDTGNVDTMLEVNRLVLETTPEPCDGLVDRSDLIGRVLVEEAGEVRNSRVMPYVIGAGTRVTSNY	281
Mt	ARCTAWLDNGTF-DSLIDAADEVRTIE-----RROGLKVSIP-EEVAVRMGWIDDEQLVQRARALVSGY	277
Sf	GOYLLKMIK-----GY	292
Ec	GOYLYKLTkd-----SN	289
Ye	GOYLLKAKE-----S	355
Sg	VGPFTSLAEDCVVEDSEVEFSIVLRGASISGVRRIEASLIGRHVQVTSAPFVPHAHRLVLGDHSRAQISS	293
Mt	GNYLLELLER-----N	288

Fig. 7. Comparison of the deduced amino acid sequence for α -D-Glc-1-P thymidyllyltransferase (RfbA) from *M. tuberculosis* with the amino acid sequence of RfbA from three Gram-negative and one Gram-positive bacteria. The shaded areas are those where the sequence from *M. tuberculosis* is identical to one or more of the other sequences. The conserved putative activator binding site (Thorson *et al.*, 1994; Parsons & Preiss, 1978) at K-24 (*M. tuberculosis*) is underlined as is the conserved postulated α -Glc-1-P binding site (Thorson *et al.*, 1994) at K-159 (*M. tuberculosis*). Sf, *Sh. flexneri*; Ec, *E. coli*; Ye, *Y. enterocolitica*; Sq, *S. griseus*; Mt, *M. tuberculosis*.

Table 2. Cell-free assays for α -D-Glc-1-P thymidyllyltransferase and α -D-Glc-1-P uridylyltransferase activities in crude extracts of *E. coli* strain S ϕ 874 transformed with pYM2 or with the pBluescript KS(–) vector only

Plasmid	Enzyme	Activity [nmol min ⁻¹ (mg protein) ⁻¹]
pYM2	α -D-Glc-1-P thymidyltransferase	20.0
pYM2	α -D-Glc-1-P uridyltransferase	74.9
pBluescript II KS (—)	α -D-Glc-1-P thymidyltransferase	1.8
pBluescript II KS (—)	α -D-Glc-1-P uridyltransferase	56.9

enzyme dTDP-6-dTal synthase forming the L-*talo* configuration (Gaugler & Gabriel, 1973). *M. smegmatis* utilizes 6-dTal as a component of glycopeptidolipids produced by *M. smegmatis* (McNeil *et al.*, 1989). It should be noted that since *M. tuberculosis* is not known to produce any 6-dTal-containing molecules, dTDP-6-dTal synthase is unlikely to be present in *M. tuberculosis*.

The fact that the Southern analysis of various restriction fragments produced from genomic *M. tuberculosis* DNA using a fragment of *rfbA* as a probe showed only single bands (Fig. 5) suggests that *M. tuberculosis* has only one copy of *rfbA*. Although the PCR approach

described here was successful in amplifying the *rfbA* gene, similar approaches to amplifying the other three *rfb* genes were unsuccessful. Also, surprisingly and disappointingly, the remaining *rfb* genes were not found to be clustered with *rfbA* as in other bacteria (Minnikin, 1982; Stevenson *et al.*, 1994; Rajakumar *et al.*, 1994). Indeed, although we sequenced approximately 2 kb on either side of *rfbA*, no sequences with substantial homology to those found in the database were found in these areas. Confirming these results, Dr Tae-Yoon Lee (Yeungnam University, Taegu, Korea), in the context of examining RFLP patterns of various *M. tuberculosis* strains, isolated and sequenced a region of DNA which showed high homology to *rfbB* and *rfbC* but not to

rfbA (Tae-Yoon Lee, personal communication). Several different 30–40 kb cosmids from the *M. tuberculosis* pGC1 cosmid library containing the DNA sequences homologous to *rfbB* and *rfbC* did not hybridize with the *rfbA* probe (T. Y. Lee, Y. Ma & M. McNeil, unpublished results) as expected if *rfbA* is not linked to *rfbB*/*rfbC*.

The deduced amino acid sequence of the RfbA protein is very similar to that of other bacteria (Fig. 7). Somewhat surprisingly it is more similar to the Gram-negative bacterial proteins (e.g. 59% identity to the *E. coli* protein) than to the single Gram-positive α -D-Glc-1-P thymidyltransferase protein sequence (32% identity). However, it should be noted that the *S. griseus* protein is not involved in Rha biosynthesis but rather catalyses the formation of dTDP-Glc as the first step in streptose biosynthesis. The *M. tuberculosis* α -D-Glc-1-P thymidyltransferase contains the highly conserved lysine at residue 24 (Fig. 7) seen in most, if not all enzymes which transfer a nucleoside phosphate to Glc-1-P (Thorson *et al.*, 1994; Parsons & Preiss, 1978) and postulated to be part of an activator binding site. It also contains the Glu-Lys-Pro (158–160) sequence which is postulated to be part of the Glc-1-P-binding region (Thorson *et al.*, 1994) and is fully conserved, amongst α -D-Glc-1-P nucleoside transferases (Thorson *et al.*, 1994).

Enzymic activity from the cloned RfbA *M. tuberculosis* protein was readily detected (Table 2) even in the presence of some *E. coli* background. Although the mechanism of transcription and translation of pYM2 in *E. coli* was not studied, we assume that the vector *lacZ* promoter was responsible for transcription and the putative ribosome binding site 6 bases upstream from the *rfbA* start codon (Fig. 6) was used for translation. *E. coli* transformed with pYM2 (Table 2) also showed a slight (1.3-fold) increase in α -D-Glc-1-P uridylyltransferase activity. This is not unexpected as α -D-Glc-1-P thymidyltransferase is known to utilize UDP-Glc as a substrate (Bernstein & Robbins, 1965; Melo & Glaser, 1965).

α -D-Glc-1-P thymidyltransferase is a potential drug target since it is likely to be an essential protein involved in mycobacterial cell wall linker synthesis and since its enzymic product, dTDP-Glc, is not found in humans. However, care must be exercised targeting this enzyme as potential inhibitors must necessarily not inhibit the very similar α -D-Glc-1-P uridylyltransferase needed in human metabolism. It thus remains important to clone the genes for the three remaining dTDP-Rha biosynthetic proteins in mycobacteria as well as the transferase gene which utilizes dTDP-Rha to transfer the Rha residue to the three positions of the linker GlcNAc residue (McNeil *et al.*, 1990; Mikusova *et al.*, 1996) for the purpose of drug targeting.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Bachmann, B. J. (1987). Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1190–1219. Edited by F. C. Neidhardt, J. L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC; American Society for Microbiology.
- Belisle, J. T., Pascopella, L., Inamine, J. M., Brennan, P. J. & Jacobs, W. R. (1991). Isolation and expression of a gene cluster responsible for the biosynthesis of the glycopeptidolipid antigens of *Mycobacterium avium*. *J Bacteriol* **173**, 6991–6997.
- Bernstein, R. L. & Robbins, P. W. (1965). Control aspects of uridine 5'-diphosphate glucose and thymidine 5'-diphosphate glucose synthesis by microbial enzymes. *J Biol Chem* **240**, 391–397.
- Brennan, P. J. & Nikaido, H. (1995). The envelope of mycobacteria. *Annu Rev Biochem* **64**, 29–63.
- Daffé, M., McNeil, M. & Brennan, P. J. (1993). Major structural features of the cell wall arabinogalactans of *Mycobacterium*, *Rhodococcus*, and *Nocardia* spp. *Carbohydr Res* **249**, 383–398.
- Deng, L., Mikusova, K., Robuck, K. G., Scherman, M., Brennan, P. J. & McNeil, M. (1995). Recognition of multiple effects of ethambutol on the metabolism of the mycobacterial cell envelope. *Antimicrob Agents Chemother* **39**, 694–701.
- Distler, J., Ebert, A., Mansouri, K., Pissowotzki, K., Stockmann, M. & Piepersberg, W. (1987). Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. *Nucleic Acids Res* **15**, 8041–8056.
- Gaugler, R. W. & Gabriel, O. (1973). Biological mechanisms involved in the formation of deoxy sugars. VII. Biosynthesis of 6-deoxy-L-talose. *J Biol Chem* **248**, 6041–6049.
- Hatfull, G. F. & Sarkis, G. J. (1993). DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. *Mol Microbiol* **7**, 395–405.
- McNeil, M. & Brennan, P. J. (1991). Structure, function, and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis, and drug resistance; some thoughts and possibilities arising from recent structural information. *Res Microbiol* **142**, 451–463.
- McNeil, M., Chatterjee, D., Hunter, S. W. & Brennan, P. J. (1989). Mycobacterial glycolipids: isolation, structures, antigenicity, and synthesis of neoantigens. *Methods Enzymol* **179**, 215–242.
- McNeil, M., Daffé, M. & Brennan, P. J. (1990). Evidence for the nature of the link between the arabinogalactan and peptidoglycan components of mycobacterial cell walls. *J Biol Chem* **265**, 18200–18206.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marolda, C. L. & Valvano, M. A. (1995). Genetic analysis of the dTDP-rhamnose biosynthesis region of the *Escherichia coli* VW187 (O7:K1) *rfb* gene cluster: identification of functional homologs of *rfbB* and *rfbA* in the *rff* cluster and correct location of the *rffE* gene. *J Bacteriol* **177**, 5539–5546.

- Melo, A. & Glaser, L. (1965). The nucleotide specificity and feedback control of thymidine diphosphate-D-glucose pyrophosphorylase. *J Biol Chem* **240**, 398–405.
- Mikusova, K., Slayden, R. A., Besra, G. S. & Brennan, P. J. (1995). Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob Agents Chemother* **39**, 2484–2489.
- Mikusova, K., Mikus, M., Besra, G., Hancock, I. & Brennan, P. J. (1996). Biosynthesis of the linkage region of the mycobacterial cell wall. *J Biol Chem* **271**, 7820–7828.
- Minnikin, D. E. (1982). Lipids: complex lipids, their chemistry, biosynthesis and roles. In *The Biology of the Mycobacteria*, vol. I, pp. 95–184. Edited by C. Ratledge & J. L. Stanford, London: Academic Press.
- Parsons, T. F. & Preiss, J. (1978). Biosynthesis of bacterial glycogen: incorporation of pyridoxal phosphate into the allosteric activator site and an ADP-glucose-protected pyridoxal phosphate binding site of *Escherichia coli* B ADP-glucose synthase. *J Biol Chem* **253**, 6197–6202.
- Radford, A. J. & Hodgson, A. L. M. (1991). Construction and characterization of a *Mycobacterium*–*Escherichia coli* shuttle vector. *Plasmid* **25**, 149–153.
- Rajakumar, K., Jost, B. H., Sasakawa, C., Okada, N., Yoshikawa, M. & Adler, B. (1994). Nucleotide sequence of the rhamnose biosynthetic pathway operon *Shigella flexneri* 2a and role of lipopolysaccharide in virulence. *J Bacteriol* **176**, 2362–2373.
- Shibaev, V. N. (1986). Biosynthesis of bacterial polysaccharide chains composed of repeating units. *Adv Carbohydr Chem Biochem* **44**, 277–339.
- Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley, M., Redmond, J. W., Lindquist, L. & Reeves, P. R. (1994). Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene cluster. *J Bacteriol* **176**, 4144–4156.
- Takayama, K. & Kilburn, J. O. (1989). Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **33**, 1493–1499.
- Takayama, K., Schnoes, H. K., Armstrong, E. L. & Boyle, R. W. (1975). Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis*. *J Lipid Res* **16**, 308–317.
- Takayama, K., Armstrong, E. L., Kunugi, K. A. & Kilburn, J. O. (1979). Inhibition of ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **16**, 240–242.
- Thorson, J. S., Kelly, T. M. & Liu, H. W. (1994). Cloning, sequencing, and overexpression in *Escherichia coli* of the alpha-D-glucose-1-phosphate cytidylyltransferase gene isolated from *Yersinia pseudotuberculosis*. *J Bacteriol* **176**, 1840–1849.
- Wang, S. F. & Gabriel, O. (1969). Biological mechanisms involved in the formation of deoxy sugars. V. Isolation and crystallization of thymidine diphosphate-D-glucose oxidoreductase from *Escherichia coli* B. *J Biol Chem* **244**, 3430–3437.
- Zhang, L., al-Hendy, A., Toivan, P. & Skurnik, M. (1993). Genetic organization and sequence of the *rfb* gene cluster of *Yersinia enterocolitica* serotype O:3: similarities to the dTDP-L-rhamnose biosynthesis pathway of *Salmonella* and to the bacterial polysaccharide transport systems. *Mol Microbiol* **9**, 309–321.

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