Conversion of dTDP-4-keto-6-deoxyglucose to free dTDP-4-keto-rhamnose by the rmlC gene products of Escherichia coli and Mycobacterium tuberculosis


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dTDP-rhamnose is made from glucose-1-phosphate and dTTP by four enzymes encoded by rmlA-D. An Escherichia coli rmlC mutant was constructed and a crude enzyme extract prepared from it did not produce dTDP-4-keto-rhamnose, in contrast to a crude enzyme extract prepared from a wild-type E. coli strain where small amounts of this intermediate were found after incubation with dTDP-glucose in the absence of NADPH. These results showed that dTDP-4-keto-rhamnose, the product of RmlC, exists as a free intermediate. Further, the Mycobacterium tuberculosis rmlC gene was expressed and incubation of the resulting purified M. tuberculosis RmlC enzyme with dTDP-4-keto-6-deoxyglucose resulted in the conversion of approximately 70% of dTDP-4-keto-6-deoxyglucose to dTDP-4-keto-rhamnose. The enzyme also allowed for the incorporation of two deuterium atoms from deuterium oxide solvent into dTDP-4-keto-glucose. Thus the rmlC gene encodes dTDP-4-keto-6-deoxyglucose epimerase capable of epimerizing at both C-3' and C-5'; this enzyme produces free dTDP-4-keto-rhamnose but the equilibrium of the 4-keto sugar nucleotides lies strongly on the side of the gluco configuration.

Keywords: rhamnose, rmlC, mycobacterial cell wall, drug development, dTDP-4-keto-6-deoxyglucose epimerase

INTRODUCTION

A single L-rhamnosyl residue plays a key structural role in the cell wall core of mycobacteria and inhibition of its biosynthesis is likely to lead to mycobacterial cell death (McNeil et al., 1990). In addition a 'rhamnolipid' in Pseudomonas aeruginosa is considered to be essential for virulence (Ochsner & Reiser, 1995) and rhamnose may be important for virulence in Streptococcus suis (Charland et al., 1998). Thus it is now apparent that inhibition of the enzymes involved in the formation of the L-rhamnosyl donor, deoxythymidine diphosphate rhamnose (dTDP-rha), is an important target for drug development in several genera of bacteria. Since L-rhamnose is a common constituent of Gram-negative O antigens, the biosynthesis of dTDP-rha was studied intensively in the late sixties and early seventies from an enzymic standpoint (Gaugler & Gabriel, 1973; Zarkowsky et al., 1970). These studies lead to the conclusion that dTDP-rha is synthesized from thymidine triphosphate (TTP) and glucose-1-phosphate via four enzymes (Fig. 1). It should be noted that the keto products shown in Fig. 1 are designated in this manuscript as follows: RmlB product as dTDP-4-keto-6-deoxyglucose rather than dTDP-6-deoxy-α-xylo-4-hexulose and the RmlC product as dTDP-4-keto-rhamnose rather than dTDP-6-deoxy-α-lyxo-4-hexulose. These names are used as they indicate the relationships between sugar intermediates more clearly.

The biochemistry shown in Fig. 1 was substantiated by genetic studies of O-antigen biosynthesis in which four dTDP-rha formation enzymes were found in the O-antigen synthetic operons in many organisms (Xiang et al., 1993). These genes were originally named rfbA-D; recently the nomenclature has been changed to rmlA-D.

Abbreviations: dTDP-rha, deoxythymidine diphosphate rhamnose; dTDP-glc, deoxythymidine diphosphate glucose.
Fig. 1. The formation of dTDP-rha from glucose-1-phosphate and dTTP as catalysed by the four enzymes encoded by rmlA-D.

(Reeves et al., 1996). The genes encoding the first two enzymes in the pathway, rmlA (α-L-glucose-1-phosphate thymidylyltransferase) and rmlB (dTDP-α-glucose-4',6'-dehydratase) have been cloned and expressed from several different organisms (Ma et al., 1997; Lindquist et al., 1993; Romana et al., 1991; Marumo et al., 1992) and shown to catalyse the expected reactions shown in Fig. 1. However, there are not yet any reports of the last two enzymes having been purified to homogeneity or expressed from their corresponding genes. Indeed, until recently there has been some confusion regarding the assignment of these two genes to their respective enzymes (Stevenson et al., 1994). The putative product of RmlC, dTDP-4-keto-6-deoxyglucose, has never been isolated as such and therefore was thought to exist only as an enzyme-bound intermediate (Melo & Glaser, 1968; Gaugler & Gabriel, 1973). This conclusion was disputed by Wahl & Grisebach (1979) who presented evidence for small amounts of dTDP-4-keto-6-deoxyglucose in incubations containing epimerase but lacking reductase activity as these workers isolated small amounts of dTDP-rha after chemical reduction of the 4-keto group. However, as shown by Naundorf & Klaffke (1996) and by ourselves in initial experiments, 4-keto sugars readily epimerize under the basic conditions used by Wahl & Grisebach (1979) to reduce the 4-keto group and thus the existence of dTDP-4-keto-L-rhamnose, enzyme-bound or not, remains to be demonstrated.

To address these issues we have prepared an Escherichia coli mutant where the rmlC gene is deleted and developed procedures to reduce the 4-keto group of dTDP-4-keto-6-deoxyglucose so that no chemical epimerization takes place. This allowed us to assay for the production of dTDP-4-keto rhamnose by crude enzyme extracts made from bacteria with and without the rmlC gene. In addition, we cloned and overexpressed the Mycobacterium tuberculosis rmlC gene and purified its product. The reaction catalysed by this enzyme was then investigated with and without additional Rml proteins from E. coli and in the presence of D2O. Taken as a whole, the studies showed that RmlC catalyses an equilibrium between dTDP-4-keto-6-deoxyglucose and non-enzyme-bound dTDP-4-keto-rhamnose that lies predominately on the side of dTDP-4-keto-6-deoxyglucose.

METHODS

Plasmids. The plasmid pCANTS7Ndel* was from A. S. Lynch, Harvard Medical School, USA; pCP15 and pCP20 were from W. Wackernagel, University of Oldenburg, Germany; pCR2.1 was from Invitrogen; pGZ119HE was from M. Russel, Rockefeller University, USA; and pPRI1474 was from P. Reeves, University of Sydney, Australia, pLD53 (Metcalf et al., 1996), pSK49AuidA2 (Haldimann et al., 1996), and pSLF52 and pSPORT::merR (Haldimann et al., 1997) were from laboratory stocks. Others are described below. Conditional replication plasmids having the oriR_{R} rep, origin of replication were maintained in the moderate-copy-number (pir+) host BW23473, the high-copy-number (pir-116) host BW23474 or other suitable hosts (Haldimann et al., 1998; Metcalf et al., 1994).

Preparation of E. coli BW24476. The strains used in this study were all derived from E. coli BW24476, a derivative of E. coli K-12. The characteristics of BW24476 are shown in Table 1. BW24476 is a descendent of the BD792-derived strain E. coli BW24476.

Table 1. Key bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>BW24476</td>
<td>F- λ' lacI1  rnlB24476  ΔlacZ Δmfd ΔphoBR580  bsdR514 ΔaraBAD ΔaraBADΔl0rΔrfl-50</td>
</tr>
<tr>
<td>BW24599</td>
<td>As BW24476, except rfbT (rfbT-50 repaired)</td>
</tr>
<tr>
<td>BW24861</td>
<td>As BW24599, except ArmlC::kanR_550</td>
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<tr>
<td>BW24970</td>
<td>As BW24599, except ArmlC::kanR_550</td>
</tr>
<tr>
<td>BW24972</td>
<td>As BW24970, except attR::PTJ09</td>
</tr>
<tr>
<td>BW24973</td>
<td>As BW24861, except attR::PTJ09</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td>Conditional replication (oriR_{R} rep), integration (attP2) plasmid that expresses rmlC behind P_{TAC}</td>
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All bacteria are derivatives of E. coli K-12 (see Methods).
was used in subsequent cloning steps. The PCR fragments binants were selected as described previously (Metcalf elsewhere (Cherepanov nonselectively, after which tetracycline-sensitive recom-
Tetracycline-resistant exconjugants were selected and purified BW2486
al., phen correspond to extensions with a restriction site(s) that chromosonial mutation and to no longer react with 016-
XmnIpolymerase and E.
make the kanamycin-sensitive eliminated by using the FLP plasmid pCP20 as described (Table 1).
Construction of a smooth (rfb) E. coli K-12 strain. The smooth E. coli K-12 strain BW24599 (Table 1) was constructed using allele replacement to correct the rfb-50 mutation, an IS insertion within wbbL [formerly called orf-11 (Reeves et al., 1996)]. To do this, the 2.7 kb PstI fragment corresponding to the wbbL segment in pPR1474 [from the E. coli K-12 strain WG1 (Liu & Reeves, 1994)] was cloned into the allele-replacement vector pLD55 (Haldimann et al., 1996). The resulting plasmid pTJ08-08 was used to transform BW24476. Tetracycline-resistant transformants were selected and purified nonselectively, after which tetracycline-sensitive recombinants were selected as described previously (Metcalf et al., 1996). BW24599 was shown to be rfb- based on its insensitivity to phage P1 and production of O antigen.

Construction of ΔrmIC E. coli K-12 strains. A ΔrmIC mutant of BW24599 was similarly constructed by using pSK95, a derivative of pLD55 having an approximately 3 kb insert with the structure NotI-ΔrmIC-HindIII-FRT-kan-FRT-KpnI-uxx'-BamHI. The insert was assembled by joining upstream rmaA and downstream uxx [formerly called rfx/B (Reeves et al., 1996)] sequences to a kanamycin resistance gene cassette that is flanked by FRT sites for the FLP recombinase. The HindIII-FRT-kan-FRT-KpnI fragment originated from pCP15 (Cherepanov & Wackernagel, 1995). The rmaA and uxx' fragments were generated using PCR with Tag DNA polymerase and E. coli BW24476 chromosomal DNA as template. PCR primers for rmaA were GCGAACTGCGG-GGCCGAT-ATGAAATCGGTAAAGGT and CCGAGAATATCT-CATTATAGCGGCCCCTG; and for uxx' were GCGGCTTATGGCGACAT-ATGAAATCGGCATGGG-GCAA-ACCAGTTATATTTATTC. Sequences preceding a hyphen correspond to extensions with a restriction site(s) that was used in subsequent cloning steps. The PCR fragments were cloned into pCR2.1 and sequenced as described elsewhere (Haldimann et al., 1998). An approximately 0.5 kb MfeI-XmrI fragment was deleted from the 3' end of uxx' in the construction of pSK95. A transformant of BW20767 (Metcalfe et al., 1996) carrying pSK95 was mated with BW24599. Tetracycline-resistant exconjugants were selected and purified nonselectively, after which tetracycline-sensitive recombinants were selected as described previously (Metcalfe et al., 1996). The kanamycin-resistant and P1-sensitive recombinant BW24861 (Table I) was shown to contain the ΔrmIC::kan-gus transposon carried by a bacteriophage and to no longer react with O16-specific antibody. The kanamycin resistance gene was eliminated by using the FLP plasmid pCP20 as described elsewhere (Cherepanov & Wackernagel, 1995) in order to make the kanamycin-sensitive ΔrmICgus mutant BW24970 (Table I).

Construction of an ΔrmIC integration plasmid. The plasmid pTJ09 a conditional replication, integration (attPα) plasmid that expresses rmIC behind Pα was constructed to confirm that properties of strains BW24861 and BW24970 were due to lack of ΔrmIC. The ΔrmIC gene was PCR-amplified with NdeI and BamHI sites using GCGAATTCAT-ATGAAATCGGCATGGG-GCAAACGTTATATTTATTC. The PCR-amplified ΔrmIC gene was subcloned as an Ndel-BamHI fragment into pSPORT::merR and then as an EcoRI-Nhel fragment into pSLF52 (Haldimann et al., 1997). The latter has the 1.5 kbp XhoI-lacF-Pam-BamHI fragment of pG219HE (Russel, 1994) joined to the BamHI-attPα-oriRαcat chloramphenicol resistance SalI fragment of pCANT5Ndel® (Haldimann et al., 1996). Integrants of BW24861 and BW24970 containing a single copy of pTJ09 at attI were made using pINT-ts and verified by PCR as described elsewhere (Haldimann et al., 1996).

Cloning, expression and purification of the protein product from M. tuberculosis rmIC. The sequence of the ΔrmIC gene (Rv 3465) in M. tuberculosis strain H37Rv was obtained directly from the genome sequence (Cole et al., 1998) and can be accessed from http://www.sanger.ac.uk/Projects/M.
tuberculosis/. The putative dTDP-4-keto-6-deoxyglucose 3',5'-epimerase (RmIC) protein of M. tuberculosis and E. coli K-12 are about 38% identical at the amino acid sequence level. The DNA sequence of ΔrmIC in M. tuberculosis H37Rv was found to be very similar but not identical to that of a Korean M. tuberculosis clinical isolate (Lee et al., 1997). The ΔrmIC gene was then cloned from M. tuberculosis H37Rv using the Ligation Independent Cloning (LIC) system (Stratagene). Thus PCR was used to amplify the 5' primer GAGCAGACACAG-ATGAAATCGGTAAAGGT and the 3' antisense primer GCGAGTTATATTGCGGCCCCTG; and for uxx' were CGGAGGCTTAGGCATGGG-GCAAACGTTATATTTATTC. The resulting product was cloned into the LIC plasmid and the inserted DNA was sequenced to ensure proper insertion and PCR fidelity. The gene was then expressed, and the resulting protein purified on a calmodulin-binding column and treated with enterokinase according to the manufacturer's instructions. Both matrix-assisted MS and N-terminal sequencing (City of Hope) showed that the enterokinase cleaved 15 amino acids upstream of the desired cleavage site, resulting in 15 additional amino acids on the N-terminus. The resulting polypeptide nevertheless showed the desired enzyme activity and thus was suitable for the purposes of this study.

Preparation of crude enzyme extracts. Bacteria were harvested and sonicated in 50 mM HEPES (pH 7.6) with 0.1 mM PMFS and ~ 1 mM DTT. The sonicates were then centrifuged at 16000 g for 5–7 min to remove debris.

Preparation of dTDP-4-keto-6-deoxyglucose. dTDP-4-keto-6-deoxyglucose was prepared by incubation of 3 μmol deoxythymidine diphosphate glucose (dTDP-glucose (Sigma) with 23 μg crude E. coli B protein (from crude enzyme extracts) and 5 μmol NADPH in 2 ml of a buffer consisting of 50 mM MOPS (pH 7.8); 3 mM EDTA and 1 mM DTT. After incubation for 30 min at 37 °C, an additional 5 μmol NADPH was added and the incubation continued for 30 min. 5 μmol NADPH was added and the incubation continued for a final 30 min. Three milliliters of ethanol were added, the insoluble proteins removed by centrifugation, the volume reduced to approximately 1 ml and the dTDP-4-keto-6-deoxyglucose purified by HPLC on a Dionex Magnum 9 PA-1 column. The HPLC flow rate was 3 ml min-1 and after a five min elution with 5 mM
NH₄H₂PO₄, a gradient from 5-50 mM NH₄H₂PO₄ over 40 min was applied. The OD₆₅₀-positive fractions were described below; those containing dTDP-rha were combined and desalted on a G-10 column in water. The final product was concentrated to a convenient volume; the identity of the sugar was confirmed to be rhamnose by GC-MS analysis of the resulting alditol acetates (Daffe et al., 1990).

dTDP-4-keto-6-deoxyglucose was prepared by an adaptation of the procedure of Marumo et al. (1992). Thus, the reaction mixture (400 µl) containing 1 µmol dTDP-glc and 4 mg crude soluble protein from E. coli BW24970 in 50 mM HEPES buffer (pH 7.6) was incubated for 1 h at 37 °C. To assay for the completeness of the reaction, a 25 µl aliquot was withdrawn, added to 775 µl of 1 M NaOH and reincubated at 37 °C for 20 min. The absorption was measured at 320 nm (ε₃₂₀ = 4600 M⁻¹·cm⁻¹). The reaction was found to essentially go to completion. Toluene was added and denatured protein was removed by centrifugation at 14000 g for 10 min. The ethanol was removed by evaporation and the crude dTDP-4-keto-6-deoxyglucose reaction applied to a G-10 (2.5 x 120 cm) column at 4 °C. Fractions (5 ml) were collected and analysed for salt by measurement of conductivity and for dTDP-4-keto-6-deoxyglucose by measurement of A₃₂₀. The UV-absorbing fractions were pooled, concentrated to a convenient volume and frozen.

HPLC assay for formation of dTDP-rha. The assay mixture (50 µl) contained 2 nmol TDP-glc, 6 nmol NADPH, enzyme (50 µg crude (10000 g supernatant) protein when testing E. coli strains; 50 µg crude soluble (10000 g supernatant) protein purified RmlC when testing for RmlC complementation], 1 mM MgCl₂, all in 50 mM HEPES buffer (pH 7.6). The reaction was incubated for 1 h at 37 °C, followed by addition of 67 µl ethanol. Denatured protein was removed by centrifugation at 14000 g for 5-10 min and supernatant was introduced on a Dionex PA-100 HPLC column. The column was eluted with 75 mM KH₂PO₄ (isocratic) and detected by measurement of A₂₅₄. Standards of dTDP-rha and dTDP-glc were shown to be separated in this system.

Analysis of enzymically produced TDP-4-keto-6-deoxyhexoses by GC-MS. The assay mixture (35 µl) contained 140 nmol TDP-glc, 330 µg E. coli soluble protein (either from strain BW24970 or BW24599 depending on the experiment) in 50 mM HEPES buffer (pH 7.6). The reaction was incubated for 1 h at 37 °C and then 50 µl ethanol was added, followed by 2 mg sodium borodeuteride (to reduce the 4-keto group). After 2 h, the reaction was terminated with a few drops of glacial acetic acid, 20 µl 10% acetic acid in methanol was added and the sample was dried. The sample was then hydrolysed with 2 M trifluoroacetic acid at 120 °C for 1 h, reduced with sodium borodeuteride (10 mg ml⁻¹ in 1 M ammonium hydroxide), the borate removed by drying the sample after the addition of methanol (this forms volatile methyl borates), and the sample acetylated as described by York et al. (1986). GC-MS was performed on a BPX70 (5% phenyl) column using a Hewlett Packard GC-MS system as described by Daffe et al. (1990), except that the selected ion monitoring mode was used to selectively monitor m/z 172 and 232, and in the deuterium incorporation experiments m/z 217, 218, 231 and 233.

Assay for conversion of dTDP-4-keto-6-deoxyglucose to dTDP-4-keto-6-deoxyglucose by purified M. tuberculosis RmlC protein. dTDP-4-keto-6-deoxyglucose (5 nmol) was incubated either with M. tuberculosis RmlC (7 µg) or without RmlC (control) in HEPES buffer (pH 7.6) for 3-5 h at 37 °C. The resulting 4-keto sugar nucleotides were then derivatized and analysed by GC-MS as described above.

Assay for incorporation of deuterium from solvent into dTDP-4-keto-6-deoxyglucose. The assay mixture (18 µl) contained 1 nmol dTDP-4-keto-6-deoxyglucose, 1·26 µg M. tuberculosis RmlC protein in 50 mM HEPES buffers (pH 7.6). Due to [¹H]₂O in the enzyme the resulting concentration of [¹H]₂O was about 70%. The reaction was incubated for 30 min at 37 °C and then stopped by the addition of ethanol (50 µl). The resulting deuterated 4-keto sugar nucleotides were then reduced by the addition of NaBD₄ (not NaBD₄ as in other experiments) and then derivatized and analysed by GC-MS as described above, except that the second reduction was also with NaBD₄ rather than NaBD₄.

RESULTS

Construction of E. coli ΔrmlC mutants

In E. coli K-12, the rmlC gene lies within the rfb gene cluster that has been well characterized by Reeves and co-workers and is required for O-antigen biosynthesis (Stevenson et al., 1994). We constructed an E. coli K-12 ΔrmlC mutant to aid in the determination of the function of this gene and in the characterization of the M. tuberculosis RmlC protein. As all known laboratory strains of E. coli K-12 are rough (Rfb⁻) due to synthesis of an incomplete lipopolysaccharide structure lacking the O antigen (Liu & Reeves, 1994), this required that

![Fig. 2.](image-url)
Fig. 3. Analysis of the dTDP-4-keto sugars after incubation of dTDP-glc in crude enzyme extracts prepared from E. coli with [BW24599 (trace A)] and without [BW24970 (trace B)] a functional rmlC gene. In both cases, the resultant dTDP-4-keto sugars were analysed after the derivatization shown in Fig. 2 by selected ion GC-MS in which $m/z$ 172 was monitored. Ions with $m/z$ 172 come from a complex loss of 206 atomic mass units from the molecular ion in which hydrogen or deuterium at C-4 (and C-1) is retained. Contaminating 6-deoxyhexoses not deuterated at C-4 yield ions with $m/z$ 171. Peak 1, 1,2,3,4,5-penta-O-acetyl 1,4-dideuterioarabinitol; peak 2, 1,2,3,4,5-penta-O-acetyl 1,4-dideuterio-6-deoxytalitol; peak 3, 1,2,3,4,5-penta-O-acetyl 1,4-dideuteriofucitol; peak 4, 1,2,3,4,5-penta-O-acetyl 1,4-dideuterio-6-deoxyglucitol. Compounds 1 and 2 come from dTDP-4-keto-rhamnose; compounds 3 and 4 result from dTDP-4-keto-6-deoxyglucose (see Fig. 2). In the case of trace A, a substantial amount of the $m/z$ 172 comprising peak 1 is due to the $^{13}$C isotope of $m/z$ 171. Ions with $m/z$ 171 are not found in any other peaks (data not shown) and in the case of peak 1 are from dTDP-rha (non-4-keto compound), which were produced due to small amounts of NADPH that was not removed from the protein preparation of E. coli BW24599 even by dialysis. The calculated value of $m/z$ 172 due to dTDP-4-keto-rhamnose after subtraction of the isotope peak due to dTDP-rha is shown by the dashed line.

Fig. 4. SDS-PAGE analysis of purified M. tuberculosis RmlC. Lane 1, molecular mass standards; lane 2, RmlC.

Fig. 5. HPLC analysis for the production of dTDP-4-keto-rhamnose by enzymes present in crude enzyme extracts of rmlC E. coli BW24970 with no additions (trace A) or after addition of purified M. tuberculosis RmlC (traces B-D). In both instances dTDP-glc and NADPH were added to the extracts and after incubation for varying lengths of time the resulting sugar nucleotides analysed by HPLC. Times of incubations were: A (control), 60 min; B, 10 min; C, 30 min; D, 60 min.

we first make a smooth (Rfb+) E. coli K-12 strain. The rough phenotype results from one of two independent mutations in the rfb gene cluster, rfb-50 or rfb-51. The E. coli K-12 strain EMG2 (Liu & Reeves, 1994), like the recently sequenced strain MG1655 (Blattner et al., 1997), has the rfb-50 mutation, an IS5 insertion in the rhamnosyl transferase (wrbB) gene. The E. coli strains used in this study are descendents of the E. coli K-12 strain BD792 (Haldimann et al., 1997). Both BD792 and MG1655 are immediate and unmutagenized descendents of W1485, which is in turn a descendents of EMG2 (Bachmann, 1996; B. J. Bachmann, personal communication). We were therefore able to repair the rfb mutation by using a standard allele replacement method and a DNA fragment from the E. coli K-12 rfb-51 strain WG1 (Liu & Reeves, 1994). The resulting smooth E. coli K-12 strain BW24599 was shown to produce O antigen by a colony blot using a polyclonal antibody preparation.
known to react against the K-12 O antigen (Stevenson et al., 1994). We then constructed two ΔrmlC derivatives of BW24599: strain BW24861, with a kanamycin resistance gene associated with its ΔrmlC mutation, and strain BW24970, with an unmarked ΔrmlC mutation. We showed that both could be complemented with a plasmid that expresses rmlC* behind P\text{lac} in a single copy at the phage λ attachment site (Table 1). In the course of this study, we also discovered that rough E. coli K-12 strains are sensitive and smooth ones insensitive to the phage P1. This phenotype provided a simple means to test various recombinants. The rfb+ E. coli K-12 strains also proved to be extremely difficult to transform. On several occasions, no or very few transformants were obtained using standard transformation or electroporation techniques (data not shown). These phenotypes may be useful in new studies of genes for polysaccharide biosynthesis. As the rfb+ strains acted normally as recipients in conjugation, particular mutants were constructed using conjugative plasmids.

Analysis of strains for their ability to synthesize dTDP-rha from dTDP-glc

Incubation of crude enzyme extracts of BW24599 with dTDP-glc and NADPH resulted in the formation of dTDP-rha as evidenced by the reduction products (Fig. 6 trace A). The straightforward interpretation of this result is that RmlC protein in the rmlC-containing bacteria was responsible for this conversion.

Cloning, expression and purification of rmlC from M. tuberculosis

To confirm the results reported above and as part of our programme to develop new drugs against M. tuberculosis, the rmlC gene of M. tuberculosis was cloned into the Ligation Independent Cloning (LIC) system of Stratagene. In this commercial system, rmlC is fused at the S' end to DNA encoding a calmodulin-binding peptide with an enterokinase cleavage site directly upstream of the starting ATG of rmlC. After expression, the resulting protein was purified on a calmodulin column and treated with enterokinase to yield the purified polypeptide shown in Fig. 4. Inexplicably, as revealed by N-terminal sequencing, the polypeptide was cleaved 15 amino acids upstream of the enterokinase site and the protein shown in Fig. 4 thus has a 15 amino acid 'tail' at the amino terminus. However, the extra amino acids did not interfere with enzymic activity and thus when RmlC was mixed with the enzymes in a crude enzyme extract prepared from E. coli BW24970, dTDP-rha was readily formed from dTDP-glc as shown in Fig. 5.

Purified RmlC catalyses the conversion of dTDP-4-keto-glucose to dTDP-4-keto-rhamnose

dTDP-4-keto-glucose free of dTDP-4-keto-rhamnose was prepared by incubating dTDP-glc with the enzymes in a particle-free extract prepared from E. coli BW24970 and purification by gel chromatography. GC-MS analysis after reduction, hydrolysis, reduction and acetylation (Fig. 6 trace A) showed the absence of dTDP-4-keto-rhamnose, although care was needed to strictly avoid basic or acidic pH during the purification. When incubated with purified dTDP-4-keto-6-deoxyglucose epimerase and analysed for 4-keto sugar nucleotides, the
Previous workers have shown that crude preparations of dTDP-4-keto-6-deoxyglucose epimerase allow for the incorporation of deuterium at carbons 3 and 5 from [\(^1\)H]O solvent (Melo & Glaser, 1968). We therefore checked the pure dTDP-4-keto-6-deoxyglucose epimerase from \textit{M. tuberculosis} to see if it also was capable of this exchange reaction. As shown in Fig. 7, this turned out to be the case; the single polypeptide catalysed the epimerization at both positions (Fig. 6) and the incorporation of deuterium at both positions (Fig. 7).

**DISCUSSION**

The experiments presented above demonstrate the validity of the prediction that the RmlC protein is a 'di' epimerase acting at both positions 5' and 3' of dTDP-4-keto-6-deoxyglucose. The earlier (Melo & Glaser, 1968; Gaugler & Gabriel, 1973) inability to detect the product, dTDP-4-keto-rhamnose, in a convincing fashion arose from the technical difficulties of analysing the unstable 4-keto sugars and from the fact that the equilibrium with dTDP-4-keto-6-deoxyglucose is such that the 4-keto rhamnose product is present only in small amounts. The equilibrium value is perhaps not unexpected given the inherent stability of equatorial phosphates (i.e. dTDP-4-keto-rhamnose) compared to axial phosphates (i.e. dTDP-4-keto-6-deoxyglucose). The fact that the first step in dTDP-rhamnose formation, the synthesis of dTDP-glucose, is essentially irreversible due to the hydrolysis of the pyrophosphate, and the fact that the equilibrium of the last reaction catalysed by RmlD (Fig. 1) lies strongly on the side of dTDP-rhamnose, results in a committed and efficient pathway in spite of the equilibrium of reactants and products produced by RmlC. It is interesting to compare the biosynthesis of dTDP-rha with that of GDP-fucose. Both 6-deoxyhexose sugar nucleotides are prepared in an analogous fashion with an oxidation/reduction at C-4' and C-6, epimerizations at C-3' and C-5', and finally reduction at C-4'. In the case of GDP-fucose the starting compound is GDP-mannose. An enzyme analogous to RmlB (Gmd) converts GDP-mannose to GDP-4-keto-6-deoxy-mannose (Andrianopoulos et al., 1998; Stevenson et al., 1996). Interestingly, however, the homologues of RmlC and RmlD occur as a single polypeptide as recently shown by Reeves and co-workers (Andrianopoulos et al., 1998). The GDP-4-keto-fucose formed after epimerization also has the phosphate in the equatorial position and would be expected to be less stable than GDP-4-keto-6-deoxyfucose. Combining both the reductase and the di-epimerase in a single polypeptide chain would thus make the complete conversion from GDP-4-keto-6-deoxyfucose to GDP-fucose more efficient. However, clearly two polypeptides are involved in the dTDP-rha story and the epimerase can function independently of the reductase. Nevertheless, our data do not preclude the possibility that in vivo RmlC and RmlD (the putative dTDP-4-keto-rhamnose reductase) interact for a more efficient transformation.

Finally, it should be noted that the inability of the \textit{rmlC} mutant \textit{E. coli} BW24970 to synthesize dTDP-rha establishes the validity of RmlC as a drug target when inhibition of the production of rhamnosyl residues is desired. The \textit{E. coli} strains developed in the course of these studies may be valuable in designing screens for such inhibitors.
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