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 as follows: RmlB product as dTDP-4-keto-6-deoxyglucose rather than dTDP-6-deoxy-~-xylo-4-hexulose and the RmlC product as dTDP-4-keto-rhamnose rather than dTDP-6-deoxy-1-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.
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; pGZI19HE was from M. Russel, Rockefeller University, USA; and pPRI474 was from P. Reeves, University of Sydney, Australia, pLD55 (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*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.

**Table 1.** Key bacterial strains and plasmids

All bacteria are derivatives of *E. coli* K-12 (see Methods).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>BW24476</td>
<td>F- lacI1 araB176 galK107 malA2 apfB8</td>
</tr>
<tr>
<td>BW24599</td>
<td>As BW24476, except rfb* (rfb-50 repaired)</td>
</tr>
<tr>
<td>BW24861</td>
<td>As BW24599, except <em>rmlC::kan</em></td>
</tr>
<tr>
<td>BW24970</td>
<td>As BW24599, except <em>rmlC::kan</em></td>
</tr>
<tr>
<td>BW24972</td>
<td>As BW24970, except <em>att</em>:pTJ09</td>
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<tr>
<td>BW24973</td>
<td>As BW24861, except <em>att</em>:pTJ09</td>
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<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pTJ09</td>
<td>Conditional replication (<strong>oriRrep</strong>), integration (<strong>attP2</strong>) plasmid that expresses <em>rmlC</em> behind <em>P</em>&lt;sub&gt;lac&lt;/sub&gt;.</td>
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was used in subsequent cloning steps. The PCR fragments were cloned into pCR2.1 and sequenced as described elsewhere (Metcalf 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 rifb+ based on its sensitivity to phage P1 and production of O antigen.

Cloning of a smooth (rifb) E. coli K-12 strain. The smooth E. coli K-12 strain BW24599 (Table 1) was constructed using allele replacement to correct the rifb-50 mutation, an 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 rifb+ based on its sensitivity to phage P1 and production of O antigen.

Construction of ΔrmfC A. coli K-12 strains. A ΔrmfC mutant of BW24599 was similarly constructed by using pSK95, a derivative of pLD55 having an approximately 3-kbp insert with the structure NotIΔrmfA-HindIII-FRT-kan-FRT-KpnI-uxz'-BamHI. The insert was assembled by joining upstream rma and downstream uxz [formerly called rfxB (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 rma and uxz fragments were generated using PCR with Taq DNA polymerase and E. coli BW24476 chromosomal DNA as template. PCR primers for rma were GCAAGAGCTTGGCCGGCAGCAT-ATGAAATACGTAAGGGT and GCAAGACTTAGATC-TATATTGACATGACCTGC; and for uxz were GCAGTAGTCCGGCACAT-ATGATAATCGAGATAATTATTC and GCAGAGCCGATCCATCTTTAGAACAACGACATTATTTATTC. 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 uxz in the construction of pSK95. A transformant of BW20767 (Metcalf 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 (Metcalf et al., 1996). The kanamycin-resistant and P1-sensitive recombinant BW24861 (Table 1) was shown to contain the ΔrmfC::kan7385 cosmochromosomal mutation 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 ΔrmfC7385 mutant BW24970 (Table 1).
NH₄H₂PO₄, a gradient from 5–50 mM NH₄H₂PO₄ over 40 min was applied. The OD₆₅₀-positive fractions were analysed for dTDP-rha using an analytical HPLC column as 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 μg 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 0.1 M NaOH and reincubated at 37 °C for 20 min. The absorption was measured at 320 nm (ε₃₂₀ = 4600 M⁻¹ cm¹). 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 × 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 A₆₅₀. 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 prepared from E. coli BW24970 and 2 μg M. tuberculosis 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 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. The scheme used to derivatize dTDP-4-ketosugars for GC-MS analysis as illustrated for dTDP-4-keto-6-deoxyglucose (a) and for dTDP-4-keto-rhamnose (b). The resulting alditol acetates are numbered according to their elution order (see Fig. 3). D, deuterium; Ac, acetate.](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-dideuteriohamnitol; 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 13C 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 rhamnolysyl transferase (wbbL) 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 descendent 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 shown in Fig. 6. In contrast incubation of extracts of strains BW24970 and BW24861 with dTDP-glc and NADPH resulted in dTDP-glc, NADPH and no dTDP-rha, as expected of these rmlC mutants (HPLC data not presented except for strain BW24970 in Fig. 5 trace A).

Incubation of enzymes present in a crude enzyme extract of E. coli strain BW24970 with dTDP-glc yields dTDP-4-keto-6-deoxyglucose but not dTDP-4-keto-rhamnose

To assay for the presence of 4-keto sugar nucleotides, the 4-keto group of the enzymically produced compounds resulting from the incubation of dTDP-glc with E. coli BW24970 was reduced with NaB\text{[2H]}\text{4} in 1 M NH\text{2}OH and the resulting sugar nucleotide diastereomers treated with acid, NaB\text{[2H]}\text{4} and acetic anhydride to prepare the corresponding alditol acetates (Fig. 2). These alditol acetates were analysed by GC-MS and compounds derived from both dTDP-4-keto-6-deoxyglucose and from small amounts of dTDP-4-keto-rhamnose were detected. When the conditions of the first chemical reduction were changed by quenching the enzymic reaction with ethanol and adding NaB\text{[2H]}\text{4} as a powder (or dissolved in ethanol) directly to the ethanol-soluble sugar nucleotides in the absence of the commonly included NH\text{2}OH (York et al., 1986), only the alditol acetates derived from dTDP-4-keto-6-deoxyglucose were produced (Fig. 3 trace A). However, when the crude enzyme extract was prepared from E. coli BW24599 it could be seen that a small amount of the dTDP-4-keto-6-deoxyglucose was converted to dTDP-4-keto-rhamnose as evidenced by the reduction products (Fig. 3 trace B). 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 5′ 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

Fig. 6. Formation of dTDP-4-keto-rhamnose by purified M. tuberculosis RmlC. The enzyme (trace A), or buffer control (trace B) was incubated with dTDP-4-keto-6-deoxyglucose and the resultant 4-keto-sugar nucleotides derivatized as in Fig. 2 and analysed by selected ion mass spectrometry as in Fig. 3. The numbered peaks correspond to those identified in Fig. 3.
with mlz 217 and 231 in (b); this is in part due to the fact that from *H,O solvent for the per-O-acetylated 6-deoxyglucitol derivative (peak 4, Fig. 3) is shown. No deuterium incorporation is seen in the control except that all reductions were done with NaB[*H]O, rather than NaB[*H]O. The structure of the resulting 6-deoxyglucitol pentactetate with no deuteration and with 3,5 deuteration and the expected fragment ions are illustrated. Only the trace for the per-O-acetylated 6-deoxyglucitol derivative (peak 4, Fig. 3) is shown. No deuterium incorporation is seen in the control but incorporation at both positions 3 (mlz 218) and 5 (mlz 233) occurred in the presence of enzyme. The deuterium incorporation was not complete as seen by the presence of ions with mlz 217 and 231 in (b); this is in part due to the fact that [*H]O was present in the reaction mixture to the extent of 30%, but it is also likely that exchange between the solvent and substrate was not complete.

**Fig. 7.** Incorporation of deuterium into dTDP-4-keto-6-deoxyglucose as catalysed by dTDP-4-keto-6-deoxyglucose epimerase (RmlC) from *M. tuberculosis*. After incubation in the absence (a) and presence (b) of dTDP-4-keto-glucose epimerase (RmlC) in deuterium oxide, the resultant 4-keto-sugar nucleotides were converted into alditol acetates as in Fig. 2 except that all reductions were done with NaB[*H]O rather than NaB[*H]O. The structure of the resulting 6-deoxyglucitol pentactetate with no deuteration and with 3,5 deuteration and the expected fragment ions are illustrated. Only the trace for the per-O-acetylated 6-deoxyglucitol derivative (peak 4, Fig. 3) is shown. No deuterium incorporation is seen in the control but incorporation at both positions 3 (mlz 218) and 5 (mlz 233) occurred in the presence of enzyme. The deuterium incorporation was not complete as seen by the presence of ions with mlz 217 and 231 in (b); this is in part due to the fact that [*H]O was present in the reaction mixture to the extent of 30%, but it is also likely that exchange between the solvent and substrate was not complete.

**Table 1.** The ratio of peaks with mlz 218 and 233 at various times in (a) and (b).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>mlz 218</th>
<th>mlz 233</th>
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<tbody>
<tr>
<td>10.6</td>
<td>10.8</td>
<td>10.6</td>
</tr>
<tr>
<td>10.8</td>
<td>10.6</td>
<td>10.8</td>
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**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-deoxymannose (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-deoxy mannose. Combining both the reductase and the di-epimerase in a single polypeptide chain would thus make the complete conversion from GDP-4-keto-6-deoxymannose 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 *rmlC* mutant *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 *E. coli* strains developed in the course of these studies may be valuable in designing screens for such inhibitors.
ACKNOWLEDGEMENTS

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