Characterization of dTDP-4-dehydrorhamnose 3,5-Epimerase and dTDP-4-dehydrorhamnose Reductase, Required for dTDP-1-rhamnose Biosynthesis in Salmonella enterica Serovar Typhimurium LT2*

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The thymidine diphosphate-1-rhamnose biosynthesis pathway is required for assembly of surface glycoconjugates in a growing list of bacterial pathogens, making this pathway a potential therapeutic target. However, the terminal reactions have not been characterized. To complete assignment of the reactions, the four enzymes (RmlABCD) that constitute the pathway in Salmonella enterica serovar Typhimurium LT2 were overexpressed. The purified RmlC and D enzymes together catalyze the terminal two steps involving NAD(P)H-dependent formation of dTDP-1-rhamnose from dTDP-6-deoxy-D-xylo-4-hexulose. RmlC was assigned as the thymidine diphosphate-4-dehydrorhamnose 3,5-epimerase by showing its activity to be NAD(P)H-independent. Spectrofluorometric and radiolabeling experiments were used to demonstrate the ability of RmlC to catalyze the formation of dTDP-6-deoxy-1-lyxo-4-hexulose from dTDP-6-deoxy-D-xylo-4-hexulose. Under reaction conditions, RmlC converted approximately 3% of its substrate to product. RmlD was unequivocally identified as the thymidine diphosphate-4-dehydrorhamnose reductase. The reductase property of RmlD was shown by equilibrium analysis and its ability to enable efficient biosynthesis of dTDP-1-rhamnose, even in the presence of low amounts of dTDP-6-deoxy-D-xylo-4-hexulose. Comparison of 23 known and predicted RmlD sequences identified several conserved amino acid residues, especially the serine-tyrosine-lysine catalytic triad, characteristic for members of the reductase/epimerase/dehydrogenase protein superfamily. In conclusion, RmlD is a novel member of this protein superfamily.

Bacterial cell-surface glycoconjugates are essential for survival of pathogenic bacteria and interactions between bacteria and host. Consequently, there is reason to believe that inhibitors directed against target reactions in surface glycoconjugate assembly may provide viable alternate therapeutic approaches. However, bacterial cell surface glycoconjugates show remarkable structural diversity due to variations of the sugar components, linkages, and substitutions. A successful strategy requires identification of enzymes and pathways unique to bacteria, yet present within a wide spectrum of bacterial species. One such target is the synthesis of the activated form of l-rhamnose, dTDP-1-l-rhamnose. l-Rhamnose is found in polysaccharides from strains of important human pathogens such as Salmonella, Shigella, Burkholderia, and streptococci, as well as in plant-associated bacteria including Xanthomonas and Rhizobium. The primary structures of many of these glycoconjugates have been reported (see the Complex Carbohydrate Structure Data base). l-Rhamnose is also found in many surface layer glycoproteins from Bacillaceae (1), in the linkage unit that joins the mycolylarabinogalactan complex to peptidoglycan in mycobacteria (2) and in some mycobacterial glycopeptidolipids (3). Examination of gene data bases also indicates the presence of the structural genes for enzymes involved in dTDP-1-rhamnose synthesis in strains where the rhamnose-containing structure is not necessarily resolved, for example, in Enterococcus faecalis (4), Leptospira interrogans serovar Copenhageni (5), and some members of the archaea.

The pathway for the biosynthesis of dTDP-1-rhamnose from glucose 1-phosphate and thymidine triphosphate was proposed in the early 1960’s by Glaser and Kornfeld (6, 7) although the enzymes were not specifically identified. Genetic data indicates that the pathway requires four genes, rmlABCD with the prototypes being identified in the lipopolysaccharide O-antigen biosynthesis (rfb) gene cluster from Salmonella enterica serovar Typhimurium LT2 (8).

The reaction steps and the genes required for dTDP-1-rhamnose biosynthesis (9, 10) were: 1) dTTP + d-Glc-1-P → dTDP- d-Glc + PP, (glucose-1-phosphate thymidyltransferase, RmlA: EC 2.7.7.24); 2) dTDP-d-Glc → dTDP-6-deoxy-d-xylo-4-hexulose (dTDP-d-glucose 4,6-dehydratase, RmlB: EC 4.2.1.46); 3) dTDP-6-deoxy-d-xylo-4-hexulose → dTDP-6-deoxy-1-lyxo-4-

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The abbreviations used are: dTDP, thymidine diphosphate; RmlA, glucose-1-phosphate thymidyltransferase; RmlB, dTDP-6-glucose 4,6-dehydratase; RmlC, dTDP-4-dehydrorhamnose 3,5-epimerase; RmlD, dTDP-4-dehydrorhamnose reductase; HPARE/CED, high performance anion exchange chromatography-pulsed electrochemical detection; PAGE, polyacrylamide gel electrophoresis; LB, Luria-Bertani; RED family, reductase/epimerase/dehydrogenase protein superfamily; GMER, GDP-4-keto-6-deoxy-d-mannose epimerase/reductase.
dTMP-L-rhamnose (dTDP-4-dehydroharmnose 3,5-epimerase, RmlC: EC 5.1.3.13); 4) dTDP-6-deoxy-D-xylo-4-hexulose + NAD(P)H → dTDP-L-rhamnose + NAD(P)+ (dTDP-4-dehydroharmnose reductase, RmlD: EC 1.1.1.133).

Recently, the first two enzymes in the pathway, glucose-1-phosphate thymidylytransferase (RmlA) (11) and dTDP-D-glucose 4,6-dehydratase (RmlB) (12) were analyzed in detail. Overexpressed gene products were used for enzymatic synthesis of dTDP-6-deoxy-D-xylo-4-hexulose and dTMP-L-rhamnose with varying yield (12, 13). Although RmlC and D are required for the conversion of dTDP-6-deoxy-D-xylo-4-hexulose to dTMP-L-rhamnose, the definitive assignment of individual activities has not been done, and the mechanism has not been elucidated. This is a clear limitation for studies where inhibitor development is the ultimate goal.

In this report we describe the purification, characterization, and unequivocal assignment of dTDP-4-dehydroharmnose 3,5-epimerase (RmlC) and dTDP-4-dehydroharmnose reductase (RmlD) using the overexpressed enzymes from S. enterica serovar Typhimurium LT2. The following reaction scheme for the conversion of dTDP-6-deoxy-D-xylo-4-hexulose to dTMP-L-rhamnose by RmlC and D is proposed (Reaction 1).

**EXPERIMENTAL PROCEDURES**

**Materials**

Thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose, ADP-D-glucose, NADH, NADP+, NAD+, diethiothreitol, glucose 1-phosphate, and Cibacron blue-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). NADPH was from Biomol (Hamburg, Germany). Mono Q HR 5/5, D-glucose, ADP-D-glucose, GDP-D-glucose, NADH, NADP, thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose, thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose, thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose, thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose, thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), dTDP-D-glucose, CDP-D-glucose, UDP-D-glucose.

**Analytical Techniques**

Nucleotide-activated sugars were analyzed on a CarboPac PA-1 column as described previously (15). Determination of the molecular weight of native proteins was done on a Bioanalytical Systems (Indianapolis, IN) size-exclusion column (430,000), chymotrypsinogen A (25,000), and RNase A (13,700) were used as reference proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using slighlty modifications (16) of the original method of Laemmli (17). Non-denaturing PAGE was performed in 50 mM Tris/citrate buffer. Electrophoresis was performed using a constant voltage (200 V) at 10 °C for 4 h. Gels were silver-stained using a method described elsewhere (18). Protein concentration was determined by the method of Bradford (22). Fluorescence spectroscopy was performed at 25 °C in a Hitachi F-2000 spectrofluorometer and at excitation and emission wavelengths as indicated. Radioactivity counting was done in Ultima Gold liquid scintillation mixture (Packard Instrument Co., Meriden, CT) on a Packard Tri-Carb 1900 CA liquid scintillation counter.

**Bacterial Strains and Growth Conditions**

Plasmids were routinely maintained in Escherichia coli DH5α (K-12 F′ φ80d lacZAM15 endA1 recA1 hisD17 (rK mK) supE44 thi-1 gyrA96 relA1 ΔlacZΔ8T-hisD2007; HfrH) (20). For enzyme overexpression, E. coli BL21 (DE3) (F′ ompT hsdSB (rK mK) gal dcm (DE3); Novagen, Madison, WI) was used. All strains were routinely grown in Luria-Bertani (LB) medium. Media were supplemented, where required, with kanamycin (Km; 30 µg/ml). Cultures were grown at 37 °C with or without agitation.

**Construction of Plasmids**

DNA sequence for the complete rfb gene cluster from S. enterica strain LT2 was obtained from the GenBank (Ref. 8; accession number X56793). The order of the dTMP-L-rhamnose biosynthesis genes is rmlB, rmlA, rmlD. The four open reading frames were individually amplified by polymerase chain reaction using primers designed to introduce a unique Ncol or NdeI site (underlined below) overlapping the initiating ATG codon. A downstream SstI site was introduced to facilitate cloning of the amplified fragment. Primers were synthesized with the following sequences at the Guelph Molecular Supercentre: RMLBl1 (Forward), 5′-TGGATATAGAGCTGAGATACCT-3′ (NcoI); RMLB2 (reverse), 5′-TTCTACgTCACCgCgAAACTCCT-3′ (SstI); RMLD1 (forward), 5′-GAAgGACCGCAcatATGAAATACCT-3′ (NdeI); RMLD2 (reverse), 5′-TTgcgtCTcATCTCgTcATcA-3′ (SstI); RMLA1 (forward), 5′-AGAAGAgTGAAATgGgAACgGTaAG-3′ (NdeI); RMLA2 (reverse), 5′-TTgacTcTCgtATgATCGACT-3′ (SstI). Lowercase letters indicate nucleotides introduced to construct appropriate restriction sites (in parentheses); the restriction sites are underlined.

Polymerase chain reaction amplifications were performed using a GeneAmp polymerase chain reaction system 2400 (Perkin-Elmer, Norwalk, CT). Chromosomal DNA from S. enterica LT2 (1 µg/ml) was obtained from Dr. Wendy L. Koenkleyse. For the amplification reactions, 2.5 units of Pwo DNA polymerase (Roche Molecular Biochemicals), 0.5 µg of template DNA, 200 µmol of each deoxynucleotide triphosphate, and 25 µmol of the corresponding synthetic nucleotide primers were used. Twenty cycles were used. Optimal MgSO4 concentration and reaction temperatures and times were individually determined for each polymerase chain reaction product. The product sizes for rmlA (943 base pairs), rmlB (1, 222 base pairs), rmlC (627 base pairs), and rmlD (966 base pairs) were those predicted from their respective nucleotide sequences.

The amplified fragments were digested with the appropriate restriction endonucleases, according to the manufacturer’s recommendations and cloned in plasmids pET-28a(+) (rmlB) or pET-30a(+) (rmlA,C,D) (Novagen, Madison, WI). The procedures for manipulation of DNA and for ligations were described by Sambrook et al. (20). Electrophorograms of E. coli DH5α and BL21 (DE3) were performed using published protocols (21) with a Gene Pulser apparatus (Bio-Rad). Transformants were selected on LB/Km plates. Plasmids were column-purified using Qiagen spin columns (Qiagen, Chatsworth, CA) according to the manufacturers instructions. The sequence of each cloned rml gene was determined and confirmed to be identical to the authentic chromosomal copy. Plasmid DNA sequencing was performed by automated sequencing at the Guelph Molecular Supercentre.

**Sequence Analyses**

Nucleotide and protein sequences were analyzed using online analysis tools, including BLAST (Basic local alignment search tool; Ref. 22) and Clustal W 1.6 (Refs. 23 and 24) using default parameter settings. For protein family analysis and motif analysis, the Prosite data base (25) was used.
Expression of Genes in the rml Operon

The pET vector-based constructs place the cloned rml genes under the control of a T7-promoter. For overexpression of the rml gene products, an isopropyl-1-thio-β-D-galactopyranoside-inducible T7 RNA polymerase is supplied by the host, E. coli BL21 (DE3). Isopropyl-1-thio-β-D-galactopyranoside induction of E. coli BL21 (DE3) transformed with pET constructs carrying each rml gene, led to expression of RmlA, RmlB, RmlC, and RmlD as the predominant proteins in the induced culture. For large-scale enzyme preparations, up to 500 ml of LB/EK medium was inoculated with 1% of an overnight culture and cultivated at 37 °C on a rotatory shaker. After 3 h, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and incubation was continued for 3 h. The bacterial cells were then collected by centrifugation at 7,000 rpm for 6 min at 4 °C. The cell pellets were washed twice with cold 10 mM Tris-HCl buffer, pH 8.0. Before sonication, the buffer was adjusted by addition of dithiothreitol and MgCl2 to final concentrations of 1 and 10 mM, respectively. Sonication on ice was carried out 6 times for 10 s with intervals of 20 s. Large debris were removed by centrifugation at 5,000 rpm for 10 min at 4 °C. The cell-free supernatants were carefully removed and centrifuged at 60,000 rpm for 40 min at 4 °C in a Ti 70.1 rotor in a Beckman LE-80 ultracentrifuge. Supernatants were stored at −20 °C following addition of glycerol to a final concentration of 44%.

Enzyme Assays

Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24, RmlA) and dTDP-β-glucose 4,6-dehydratase (EC 4.2.1.46, RmlB) activities were assayed as described by Kornfeld and Glaser (6) and Vara and Hutchinson (26), respectively.

The specific activity for determination of RmlD typically contained 45 mM potassium phosphate buffer, pH 7.0, 9 mM MgCl2, 0.18 mM dTDP-6-deoxy-x-ylo-4-hexulose, 0.07 mM NAD(P)/H, a 20-fold molar excess of RmlD for determination of RmlC, and an appropriate volume of the reaction mixture containing 10 and 10 mM, respectively. Sonication on ice was carried out 6 times for 10 s with intervals of 20 s. Large debris were removed by centrifugation at 5,000 rpm for 10 min at 4 °C. The cell-free supernatants were carefully removed and centrifuged at 60,000 rpm for 40 min at 4 °C in a Ti 70.1 rotor in a Beckman LE-80 ultracentrifuge. Supernatants were stored at −20 °C following addition of glycerol to a final concentration of 44%.

Equilibrium Analysis

For determination of the thermodynamic equilibrium constants of the reaction described in reaction steps 3 and 4, the reverse reaction starting from dTDP-L-rhamnose was used. Assays contained 0.05 to 0.18 mM dTDP-L-rhamnose, 0.03 to 0.04 mM NADH, 0.16 mM MgCl2 in 50 mM potassium phosphate buffer, pH 7.0, or 50 mM ethanolamine-HCl buffer, pH 9.0. The assays were performed with, and without, addition of RmlC (0.6 μM). Equilibrium constants were calculated as: $K_{eq,RmlD} = \frac{[NAD][dTDP-L-rhamnose]}{[NADPH][dTDP-6-deoxy-L-xylulose][RmlC]}$ and $K_{eq,RmlC} = \frac{[NAD][dTDP-6-deoxy-L-xylulose]}{[NADPH][NADH][dTDP-L-rhamnose]}$.

pH Optima

For determination of the pH dependence of enzyme activity, RmlC and RmlD were assayed in different buffers ranging from pH 5.5 to 11.0 (50 mM potassium phosphate buffer, pH 5.5–8.5, and 50 mM ethanolamine-HCl buffer, pH 8.5–11.0) without addition of MgCl2. To determine the profile of enzyme stability versus pH, enzymes were dialyzed in various buffers (potassium phosphate buffer, pH 5.5–8.0) and incubated at 37 °C for 20 min. Residual activities were determined at pH 7.0 after exchange of the buffer.

Kinetic Analyses

Measurements of kinetic data were performed using the spectrophotometric and spectrofluorometric assays described above. The kinetic constants, $K_m$ and $v_{max}$, were obtained by fitting the experimental data to the equation (27),

$$v = \frac{v_{max}[A][K_m + [A]]}{(K_m + [A])}$$

using the program Sigma Plot (Jandel, Erkrath, Germany). $v_{max}$ (μmol/liters) was calculated from the maximum initial velocity $v_{max}$ (μmol/liters) as,

$$v_{max} = k_{cat}E$$

where $E$ is the total enzyme concentration (μmol/liter). A molecular mass of 20.6 and 32.6 kDa was used for RmlC and RmlD, respectively. For RmlC, the initial velocities were recorded for the concentration range 0.036 to 0.36 mM dTDP-6-deoxy-β-xylo-4-hexulose. The RmlC concentration was 16.6 mM and RmlD was used in 20-fold molar excess. For RmlD, NADH and NADPH concentrations were 0.0015 to 0.109 mM and dTDP-6-deoxy-β-xylo-4-hexulose concentration was 0.18 mM. The RmlD concentration was 7.5 mM and RmlC was used in 100-fold molar excess. Activities at NADPH concentrations below 0.01 mM were measured spectrofluorometrically.

For the analysis of the reaction mechanism of RmlD the concentrations of NADPH and dTDP-6-deoxy-β-xylo-4-hexulose were varied between 0.007 and 0.11 mM, and 0.091 and 0.36 mM, respectively. The experimental data were fit to

$$v_{max} = \frac{[NAD][NADH][hexulose]}{K_{hexulose} + [NAD][NADH][hexulose]} + \frac{[NAD][NADH][hexulose]}{K_{hexulose} + [NAD][NADH][hexulose]}$$

for a sequential reaction mechanism (27, 28). For an ordered mechanism, $k_2$ represents the dissociation constant of the first substrate. The correlation coefficients of nonlinear regression were usually 0.98 or better. Variance analysis and other statistics provided by the program showed that Equation 3 adequately fits the data.

### Purification of dTDP-4-dehydroxylrhamnose 3,5-Epimerase

To prevent oxidative damage of proteins during the purification process, all buffers contained 0.5 mM dithiothreitol. Fractions were collected on ice.

### Step 1: Hydroxyapatite Chromatography

Cell-free lysates were applied to a Bio-Gel H10 column (1.8 × 10 cm) at a flow rate of 1 ml/min. Proteins were eluted using the following gradient: 0–20 ml, 10 mM potassium phosphate buffer, pH 6.8; 20–100 ml, 0–100% 200 mM potassium phosphate buffer, pH 6.8. Two-ml fractions were collected. Fractions showing enzyme activity eluted at approximately 0.06 M potassium phosphate and these were combined and adjusted to 1 mM ammonium sulfate.

### Step 2: Hydrophobic Interaction Chromatography

Pooled enzyme fractions from step 1 were applied to phenyl-Superose HR 5/5 column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM ammonium sulfate. Proteins were eluted at a flow rate of 0.5 ml/min using the following gradient: 0–10 ml, 1 mM ammonium sulfate; 10–30 ml: 0–1 mM ammonium sulfate. One-ml fractions were analyzed for enzyme activity. Enzymatically active fractions eluted at 0.2 M ammonium sulfate and these were pooled and dialyzed overnight at 4 °C against several exchanges of 20 mM Tris-HCl buffer, pH 7.7.

### Step 3: Ion Exchange Chromatography

The enzyme preparation from step 2 was applied to a Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl buffer, pH 7.7. Proteins were eluted at a flow rate of 1 ml/min using the following gradient: 0–10 ml, 20 mM Tris-HCl buffer, pH 7.7; 10–30 ml: 0 to 0.5 mM KCl. Fractions showing enzyme activity eluted at 0.28 mM KCl. These were combined, adjusted to 44% glycerol, and stored at −20 °C until use. The purified RmlC enzyme preparation (86% yield; approximately 12.5 mg/liter culture) showed a specific activity of approximately 21 units/mg.

### Purification of dTDP-4-dehydroxylrhamnose Reductase

#### Step 1: Cibacron Blue-Sepharose Chromatography

Cell-free lysates—Cell-free lysates (stored without addition of glycerol) were dialyzed 1:1 in 50 mM Tris-HCl buffer, pH 7.7, and up to 10 ml of fractions were applied to a Cibacron blue-Sepharose CL-4B column (1.5 × 5 cm) at a flow rate of 2.5 ml/min. The column was washed with 50% ethylene glycol in 50 mM Tris-HCl buffer, pH 7.7, and 0.3 mM KCl in the same buffer. Most of the proteins in the lysate did not bind to the matrix, while RmlD was absorbed and subsequently eluted in 1.5 mM KCl. The active fractions were dialyzed overnight as described.

#### Step 2: Ion Exchange Chromatography

This was performed as described...
Synthesis of Nucleotide Activated Monosaccharides

Reaction mixtures for synthesis of dTDP-6-deoxy-D-xylo-4-hexulose (12, 13) contained approximately 20 μmol of dTDP-β-glucose and 2 units of RmlB in 1 ml of 20 mM Tris–HCl buffer, pH 7.7. Incubation was performed at 25 °C for 1 h. The reaction was stopped by addition of 1 ml of absolute ethanol and precipitated proteins were removed by centrifugation. The product was desalted on a Sephadex G-10 column (1.5 × 120 cm), lyophilized, and stored at -20 °C until use. UV spectroscopy at 320 nm performed in 0.1 M NaOH was used to determine concentration of dTDP-6-deoxy-D-xylo-4-hexulose. Quantitation is based on the characteristic absorption of the 4-keto group. Following reduction with NaB[3H]4 (100 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO). Following hydrolysis and HPAEC/PED of the resulting monosaccharides, fractions (0.25 ml) were analyzed by high performance anion exchange chromatography with pulsed electrochemical detection (HPAEC/PED) (15).

For synthesis of dTDP-6-deoxy-D-lyxo-4-hexulose, 1 μmol dTDP-β-glucose was incubated at 25 °C for 30 min with 1 unit each of RmlB and RmlC in 500 μl of 50 mM Tris–HCl buffer, pH 7.0, at 25 °C for 30 min. The enzymes were removed by ultrafiltration and the reaction mixture containing both dTDP-6-deoxy-D-xylo-4-hexulose and dTDP-6-deoxy-D-lyxo-4-hexulose was desalted as described above. The amount of dTDP-6-deoxy-D-lyxo-4-hexulose in the resulting preparation was determined by conversion to dTDP-β-ribofuranosyluracil in the presence of RmlD and spectrophotometric measurement of the decrease of NADH. Direct proof for the presence of dTDP-6-deoxy-D-lyxo-4-hexulose was obtained by reducing the 4-keto group with NaB[3H]4 (100 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO). Following hydrolysis and HPAEC/PED of the resulting monosaccharides, fractions (0.25 ml) were analyzed for radioactivity.

dTDP-β-rhamnose was synthesized from dTDP-β-glucose using RmlB, RmlC, and RmlD in the presence of low amounts of NAD+. NADH was regenerated using NADH-dependent formate dehydrogenase from Candida boidinii (ASA Spezialenzyme GmbH, Braunschweig, Germany). A typical reaction mixture contained 20 μmol of dTDP-β-glucose, 1 μmol of NAD+, 100 μmol of ammonium formate, 1 unit of each RmlB, RmlC, and RmlD, and 3.5 units of formate dehydrogenase in a total volume of 5 ml of 0.1 M Tris–HCl buffer, pH 7.0. After reaction at 25 °C for 2 h, proteins were removed by ultrafiltration in an Amicon model 8050 cell using a Millipore PLGC 10-kDa ultrafiltration membrane. dTDP-β-rhamnose was purified by anion exchange chromatography on a DEAE-Sephael column as described previously (15) and desalted as described above.

RESULTS

Expression of the Cloned Protein Products in E. coli—Isopropyl-β-D-thiogalactopyranoside-induction of E. coli BL21 (DE3) cells, transformed with pET-derivatives carrying each of the rmlABC genes, led to expression of RmlA, RmlB, RmlC, and RmlD as the predominant proteins in the induced cultures (Fig. 1). Limited amounts of these proteins were also present in the noninduced cultures (not shown). The majority of each Rml protein was found in the cell-free supernatant fraction. This, together with their activities (see below), indicates that these proteins are located in the cytoplasm. The residual amounts of RmlC and RmlD were sedimented during removal of cellular debris from the ultrasonicated cell lysate. Electron microscopy of ultrathin-sectioned intact cells confirmed formation of inclusion bodies (not shown), presumably containing the enzyme confined to the particulate fraction. Since sufficient amounts of soluble protein was obtained, inclusion bodies were not pursued further for 1 h.

The expressed Rml enzymes showed in SDS-PAGE apparent molecular masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively. These values correspond closely to the predicted transmembrane masses of 31.8, 42.9, 23.4, and 34.8 kDa, respectively.
To prove the ability of RmlC to produce dTDP-6-deoxy-L-lyxo-4-hexulose, dTDP-D-glucose was reacted with RmlB and RmlC. The purified reaction mixture contained both dTDP-6-deoxy-D-lyxo-4-hexulose and dTDP-6-deoxy-L-lyxo-4-hexulose in a ratio of approximately 97:3, as shown by a spectrofluorometric assay. Consistent with this result, radiolabeling of the 4-keto group with NaB\(^{3H}\), followed by hydrolysis of the labeled hydrolyzed product. The expected 6-deoxytalose product was not detected and probably coelutes with one of the other components. The arrows indicate maximum PED response of the respective monosaccharides. Rhamnose was detected by HPAEC/PED only when added as an internal standard (see inset).

dTDP-6-deoxy-D-lyxo-4-hexulose.

To prove the ability of RmlC to produce dTDP-6-deoxy-L-lyxo-4-hexulose, dTDP-D-glucose was reacted with RmlB and RmlC. The purified reaction mixture contained both dTDP-6-deoxy-D-lyxo-4-hexulose and dTDP-6-deoxy-L-lyxo-4-hexulose in a ratio of approximately 97:3, as shown by a spectrofluorometric assay. Consistent with this result, radiolabeling of the labeled nucleotide as evidenced by the single peak in HPAEC analysis on a CarboPac PA-1 column (Fig. 3, inset). Identity of dTDP-L-rhamnose was demonstrated by monosaccharide analysis of the hydrolyzed product.

**Fig. 2.** HPAEC/PED analysis of a reduced (NaB\(^{3H}\)) and hydrolyzed mixture of dTDP-6-deoxy-D-lyxo-4-hexulose and dTDP-6-deoxy-L-lyxo-4-hexulose, synthesized by incubation of dTDP-D-glucose with RmlB and RmlC. Fractions (0.25 ml) were collected and analyzed for incorporated radioactivity. The major peaks represent fucose (1) and 6-deoxyglucose (2) derived from dTDP-6-deoxy-D-lyxo-4-hexulose, the minor peak represents rhamnose (3) from dTDP-6-deoxy-L-lyxo-4-hexulose. The expected 6-deoxytalose product was not detected and probably coelutes with one of the other components. The arrows indicate maximum PED response of the respective monosaccharides.

**Fig. 3.** High performance liquid chromatography analysis of enzymatic synthesis of dTDP-L-rhamnose. A, plot showing substrates dTDP-D-glucose and NAD\(^{+}\) before addition of enzyme. B, reaction products obtained after addition of RmlB, RmlC, and RmlD to substrates and using formate dehydrogenase for regeneration of NADH. C, purified reaction product, dTDP-L-rhamnose.

To prove the substrate specificity of the three enzymes converting dTDP-D-glucose to dTDP-L-rhamnose (RmlB, RmlC, and RmlD), UDP-D-glucose, CDP-D-glucose, ADP-D-glucose, and GDP-D-glucose were used as substrates instead of dTDP-D-glucose. The activities with all these substrates were lower than 1% of the activity with dTDP-D-glucose.

**Distinction between dTDP-4-dehydrorhamnose 3,5-Epimerase and dTDP-4-dehydrorhamnose Reductase—**The existing assignments of RmlC and RmlD to specific enzymatic activities are based on sequence data and there is some ambiguity in the literature. Direct assays for the separate measurement of RmlC activity or RmlD activity are not available. However, by using a coupled assay in which RmlC and RmlD are utilized together, and by varying the conditions in the assay, the activities of RmlC and RmlD were clearly distinguished. Quantitation in the assays is based on the consumption of NADPH by the reductase, which is conveniently monitored by a time-dependent decrease in the absorbance at 340 nm. When RmlD was used in 20-fold molar excess over RmlC, the initial reaction velocity of RmlC, determined with 0.18 mM dTDP-6-deoxy-D-lyxo-4-hexulose, is not dependent on the NADPH concentration in a range of 0.007–0.109 mM. This result identified RmlC as the epimerase and RmlD as the reductase. Accordingly, with RmlC being used in 100-fold molar excess, the activity of RmlD can be specifically measured.

**Conditions for Enzyme Assays and Synthesis of dTDP-activated Monosaccharides—**Under the assay conditions employed, RmlC shows maximum activity at pH 7.5 and is stable over a wide pH range. Its pH activity profile is similar to that of RmlD, indicating that their inclusion in a coupled RmlCD assay is not compromised by pH considerations. The pH optimum of RmlD is 6.5, but stability is highly dependent on the pH conditions. Even at pH 7.0 approximately 75% of RmlD activity is lost during incubation at 37 °C for 20 min. However, RmlD is more stable at temperatures below 30 °C (data not available).
shown). Treatment with chelators such as EDTA (up to 0.3 mM) causes a reversible loss of up to 70% of enzyme activity for both RmlC and RmlD. Upon addition of MgCl₂ in concentrations exceeding that of EDTA full activity is restored.

Kinetic Constants for RmlC and Its Potential Interaction with RmlD—For determination of RmlC activity, the assay was coupled with RmlD (20-fold molar excess) producing NADP⁺ from NADPH. The $K_m$ value of RmlC for dTDP-6-deoxy-d-xylo-4-hexulose was $0.71 \pm 0.17$ mM and $k_{cat}$ was $39 \pm 6.6$ s⁻¹ (Table I). There is clear evidence for product formation from dTDP-6-deoxy-d-xylo-4-hexulose by RmlC acting in the absence of RmlD. However, dTDP-6-deoxy-d-lyxo-4-hexulose was extremely unstable, decomposing too rapidly to allow its isolation on a preparative scale. Therefore, no kinetic data were obtained for the reverse reaction of RmlC.

One explanation for the very low amount of dTDP-6-deoxy-l-lyxo-4-hexulose, if RmlC is examined in isolation, is that RmlC and RmlD form a complex, converting dTDP-6-deoxy-d-xylo-4-hexulose to dTDP-L-rhamnose. This was proposed in the early work of Melo and Glaser (30), in a scenario where only dTDP-6-deoxy-d-lyxo-4-hexulose bound to the enzyme complex can be reduced by the reductase. dTDP-6-deoxy-l-lyxo-4-hexulose would therefore not exist in free form. To address this hypothesis, mixtures of RmlC and RmlD, with and without substrate, were examined in nondenaturing anionic PAGE. No evidence was found for tight complexes of both enzymes, even in overloaded silver-stained gels (Fig. 4). Additionally, enzyme assays were performed in the presence of Ficoll. Ficoll can act as a macromolecular crowding agent to inhibit the diffusion of proteins (31, 32) and should strengthen the interaction of any macromolecular assemblies involving a dTDP-6-deoxy-l-lyxo-4-hexulose/RmlC complex. Enzyme activity was not influenced by addition of Ficoll at concentrations of up to 30%, making a complex of RmlC and RmlD, formed during catalysis, very unlikely.

Kinetic Measurements for RmlD—The pH optimum for the reduction of dTDP-6-deoxy-l-lyxo-4-hexulose by RmlD is 6.5. At pH values above 9.0 NAD(P)⁺-dependent oxidation of dTDP-L-rhamnose was detectable. The activity for the reverse reaction was less than 1% of the activity of the reduction reaction, with a maximum at pH 10.0. In the presence and absence of RmlC, similar initial reaction velocities were detected, although the equilibrium levels of NADH were different. The equilibrium constant of substrates and products formed in the absence of RmlC allowed calculation of the $K_{eq,RmlC}$ to be $3.6 \times 10^{-13}$ (± $1.5 \times 10^{-13}$, $n = 6$) in reaction 4. When RmlC was added, the calculated $K_{eq,RmlC} \times K_{eq,RmlD}$ value was $4.5 \times 10^{-13}$ (± $1.1 \times 10^{-13}$, $n = 6$). From these results $K_{eq,RmlC}$ was estimated to be 0.013. This value is in agreement with the detection of low amounts of free dTDP-6-deoxy-l-lyxo-4-hexulose in equilibrium with dTDP-6-deoxy-d-xylo-4-hexulose.

Since isolation of dTDP-6-deoxy-d-lyxo-4-hexulose was not feasible due to instability of the product, this intermediate was generated in situ for kinetic analysis of RmlD using a 100-fold molar excess of RmlC. Apparent $K_m$ and $k_{cat}$ values for NADH and NADPH were determined with a constant concentration of dTDP-6-deoxy-d-xylo-4-hexulose (Table I). RmlD shows dual coenzyme specificity for NADH and NADPH with a slight preference for NADH. The initial velocities were determined with several fixed concentrations of dTDP-6-deoxy-d-xylo-4-hexulose and varying concentrations of NADPH. Double-reciprocal plots obtained from these data showed an intersecting pattern (not shown), indicating a sequential, ternary complex mechanism of RmlD. A fit of the experimental data to Equation 3 by nonlinear regression is shown in Fig. 5. The lines drawn represent a fit of the experimental data to Equation 3.
NAD(P)⁺ and dTDP-l-rhamnose were used sequentially for fluorescence titration they showed additive quenching effects.

**RmlD Resembles the Reductase/Epimerase/Dehydrogenase (RED) Protein Superfamily**—The search for conserved motifs rather than overall sequence identity may reveal family relationships, even when overall primary sequence identity/similarity is not higher than 15–20%. To find conserved regions in RmlD sequences, a multiple sequence alignment was performed using sequences from 23 different bacteria and archaea (Fig. 6). The sequences used for *Shigella flexneri* 2a (33), *Xanthomonas campestris* (34), and *Saccharopolyspora erythrea* (35) have been described as dTDP-4-dehydrorhamnose 3,5-epimerases but are, according to sequence similarities, the corresponding reductases. Some of the sequences have not yet been definitively designated as RmlD or dTDP-4-dehydrorhamnose reductase, but have distinct homologies to the *S. enterica* LT2 RmlD sequence. All of the RmlD sequences contain a strictly conserved (Y-X₃-K) motif that is characteristic for the single domain reductase/epimerase/dehydrogenase protein superfamily (RED family, see Refs. 36 and 37). The RED family includes the short-chain dehydrogenases/reductases (38). The (Y-X₃-K) motif is located within a larger conserved domain, identified in Fig. 6 as “motif 1.” Furthermore, the typical coenzyme-binding Rossman fold, containing a modified Wierenga motif (G-X₂-G), was identified previously in the amino-terminal part of the RmlD sequence (39). The same motif is present in all other members of this protein family. The crystal structures of two functionally related members of the RED family, UDP-galactose epimerase (40, 41) and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (42, 43), have been elucidated recently. The active site of both enzymes was shown to consist of a catalytic triad of serine, tyrosine, and lysine (40, 43). The catalytic serine residue is located upstream of the Y-X₃-K loop.

### DISCUSSION

L-rhamnose is a common constituent of different bacterial glycoconjugates. The biosynthetic precursor, dTDP-L-rhamnose, is formed by the sequential action of the RmlABCD enzymes. Detailed mechanisms have been proposed for synthesis of dTDP-l-glucose by RmlA (11) and its conversion to dTDP-6-deoxy-o-xylene by RmlB (12). The final two steps are catalyzed by RmlC and RmlD and convert dTDP-6-deoxy-o-xylene to dTDP-l-rhamnose. These activities are now assigned by the studies reported here.

Using purified enzymes, dTDP-6-deoxy-o-xylene and dTDP-l-rhamnose were synthesized enzymatically from...
dTDP-d-glucose for further analyses of RmlC and RmlD. Although the intermediate product, dTDP-6-deoxy-L-lyxo-4-hexulose, was detected, it could not be isolated in significant amounts when synthesized either from dTDP-6-deoxy-d-xylo-4-hexulose with RmlC, or from dTDP-L-rhamnose in the reverse reaction of RmlD.

Melo and Glaser (30) postulated that the epimerase and reductase might form a complex, so that dTDP-6-deoxy-L-lyxo-4-hexulose is only reduced by RmlD when the substrate is bound to RmlC. From the experiments described here, we found no data consistent with such a complex. In addition, in a separate study using the E. coli mutant strain Y10, defective in RmlD, Wahl and Grisebach (44) reported that approximately 1% of 3H-labeled dTDP-6-deoxy- d-xylo-4-hexulose is converted to dTDP-6-deoxy-L-lyxo-4-hexulose. In agreement with these data, we detected conversion of about 3% of dTDP-6-deoxy-d-xylo-4-hexulose by RmlC from S. enterica LT2. The yield of free dTDP-6-deoxy-L-lyxo-4-hexulose is explained by the relatively small equilibrium constant for RmlC alone. In a coupled system comprising RmlC and RmlD, the reductase nature of RmlD enables efficient biosynthesis of dTDP-L-rhamnose. A similar biosynthetic pathway is known for the de novo biosynthesis of GDP-L-fucose. The last two steps in this pathway are catalyzed by a bifunctional epimerase/reductase (42, 43). Probably due to the instability of the intermediate, strategies have evolved to keep its concentration low. In the case of dTDP-L-rhamnose, the instability of the intermediate product, dTDP-6-deoxy-L-lyxo-4-hexulose, was detected, it could not be isolated in significant amounts when synthesized either from dTDP-6-deoxy-d-xylo-4-hexulose with RmlC, or from dTDP-L-rhamnose in the reverse reaction of RmlD.

The sequences used for S. flexneri 2a (33), X. campestris (34), and S. erythraea (35) have been described as dTDP-4-dehydro-L-rhamnose 3,5-epimerases. Other sequences have not yet been definitively designated as dTDP-4-dehydro-L-rhamnose reductase, dTDP-6-deoxy-L-rhamnose dehydrogenase, or dTDP-L-rhamnose synthetase, but according to sequence similarities they all are RmlD homologues. The consensus sequences identified here facilitate clarification of these enzyme activities. RmlD proteins should contain: (i) a Rossman fold in the NH2-terminal region (motif 1); (ii) motif 2; and (iii) the conserved Y-X3-K loop within an extended motif 3. Possession of motif 2 distinguishes RmlD from other members of the RED family. RmlD shows some similarities to GalE (40, 41) and GMER (42, 43). The structures of these proteins have been elucidated and allowed their assignment to the RED protein superfamily. RmlD shows homology to GalE and GMER in those amino acids known to have catalytic function, but GalE and GMER lack the distinctive motif 2 that characterizes RmlD homologues. In conclusion, RmlD is a novel member of the RED protein superfamily.

Kinetic analysis of RmlC and RmlD, including pH optima and requirement of specific ions led to the development of a novel method for synthesis of dTDP-L-rhamnose from dTDP-d-glucose. Highly efficient enzymatic synthesis of dTDP-L-rhamnose was achieved with a mixture of RmlB, RmlC, RmlD, and by including an internal system to recycle NAD+ to NADH (Fig. 3). After removal of the proteins and salts, the reaction supernatant contained approximately 5% of NADH, which should not be inhibitory for most of the reactions dTDP-L-rhamnose is used for (e.g. rhamnosyltransferases). Following purification, the yield of dTDP-L-rhamnose was 80%. Such a reaction system is ideal for generating substrates for studies of rhamnosyltransferase activities and the use of these enzymes for enzymatic synthesis of complex carbohydrates.

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Addendum—While this paper was in the final stages of the review process, data for the action of RmlC in E. coli and Mycobacterium tuberculosis was published by another research group (47). Their findings are consistent with the results described here.

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