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Acinetobacter calcoaceticus Glucose-1-phosphate Thymidylyltransferase: Cloning, Sequencing, and Expression in *E.coli*

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dTDP-rhamnose is synthesized from dTTP and glucose-1-phosphate by four enzymatic steps in the gram-negative bacteria. By using a homologous PCR product, a gene cluster encoding four genes (*rfbA, rfbB, rfbC, rfbD*) involved in L-rhamnose biosynthesis by *Acinetobacter calcoaceticus* was isolated and sequenced. The four genes were clustered on the biosynthetic operon in the order of *rfbB, D, A, C*. A gene, *rfbA*, encoding glucose-1-phosphate thymidylyltransferase (*RfbA*), was cloned from *A.calcoaceticus* pathogenic and encapsulated in the gram-negative bacterium. This enzyme catalyzes the formation of dTDP-D-glucose from α-D-glucose-1-phosphate and dTTP. *RfbA* was amplified by PCR and inserted into the T_7 expression system. The activity of *RfbA* was determined by the capillary electrophoresis. The K_m values for dTTP and α-D-glucose-1-phosphate were calculated to be 1.27 mM and 0.80 mM, respectively by using the Line-Weaver Burk plot. *RfbA* is inactivated by diethylpyrocarbonate.

Keywords: *Acinetobacter calcoaceticus*, Rhamnose, Glucose-1-phosphate thymidylyltransferase.

Introduction

Acinetobacter species are frequently found in many different environments; including sewage, soil, food, and animals. In particular, the frequency of the bacteria present is increasing in hospital intensive care units. This causes many serious problems, such as septicemia and respiratory-tract infection. (Haseley *et al.*, 1997) *A. calcoaceticus* is a heavily encapsulated gram-negative bacterium. A compositional analysis of the intact and carboxyl-reduced capsular polysaccharide of *A. calcoaceticus* showed that it consists of L-rhamnose, D-glucose, D-glucuronic and D-mannose in molar ratios of 4 : 1 : 1 : 1. (Kaplan *et al.*, 1985). In eukaryotic and prokaryotic cells, saccharides are important surface components, which are known to play roles in cell recognition. Lipopolysaccharide (LPS), a major cell surface component, is a crucial virulence factor of Gram-negative bacteria; including *Shigella, Salmonella, Escherichia coli,* etc. In most cases, its function in bacterial pathogenecity seems to be the mediation of the bacterial resistance to the major host defense. LPS is a favored antigen for the production of vaccines against some of these pathogens. (Sturm *et al.*, 1986). LPS is composed of three parts: lipid A, the core region, and the O-antigen. The O-antigen constitutes of repeating oligosaccharide units, which extend outwards from the cell surface. The differences in their structure are the basis for the serotype variation. The genes involved in the biosynthesis of the basic O-antigen unit are located in the *rfb* region. This region is constant in all serotypes, but different specificities arise owing to the modification of this basic structure by temperate bacteriophage (Macpherson *et al.*, 1994). In gram-negative bacteria, L-rhamnosyl residues are often present in LPS, and they are transferred by dTDP-rhamnose. The pathway for dTDP-rhamnose biosynthesis, which uses dTTP and glucose-1-phosphate as substrates, has been studied extensively in gram-negative bacteria. The genes encoding enzymes for the four biosynthetic transformations (known as *rfbA, rfbB, rfbC,* and *rfbD* in gram-negative organisms) have been cloned and sequenced in *Shigella flexineri* (Macpherson *et al.*, 1994), *Streptococcus pneumoniae* (Munoz *et al.*, 1997), *Mycobacterium tuberculosis* (Arino and Vandecasteel, 1996) and *Streptococcus mutans*. The genes are clustered on a biosynthetic operon in the order of *rfbB, D, A, C,* with the exception of those in *Mycobacterium tuberculosis* (Arino *et al.*, 1996) and *Streptococcus mutans* (Sturm *et al.*, 1986). The first step in the biosynthesis of dTDP-rhamnose is the formation of dTDP-glucose from glucose-1-phosphate and dTTP. Glucose-1-phosphste thymidylyltransferase, encoded by *rfbA*, is the enzyme that catalyzes the reaction. The enzyme catalyzes a reversible bimolecular group transfer reaction, which is known to be a 'ping-pong' mechanism (Klena and Schnaitman, 1994). However, there is no information about

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the enzyme from *A. calcoaceticus*. In this study, we present cloning and sequencing of the *rfb* gene cluster, and the expression of *Acinetobacter rfbA* in *Escherichia coli*, as well as the characterization of glucose-1-phosphate thymidylyltransferase encoded by *rfbA*.

Materials and methods

DNA manipulations Total *A. calcoaceticus* DNA was prepared using the protocol described in Sambrook *et al.* (1989). Purified plasmids were prepared using a Qiaprep Spin Miniprep Kit, according to the manufacturers instructions.

PCR and sequencing of PCR products The Amino acid sequence alignment of known bacterial dTDP-glucose 4,6-dehydratase revealed highly conserved sequences. Three degenerated primers were designed based on the conserved sequence. The sense primer was 5' G(AGCT)GG(AGTC)GC(AG TC)GG(AGTC)TT(TC)AT(ATC)GG 3', the antisense primer was 5' (AG)TC(AG)TG(AGTC)CC(AGTC)GG(AGTC)C(GT)(AG)TC 3' and the antisense primer for nested PCR was 5' AC(AG)TG (AGTC)GA(CT)(TC)TC(AGTC)-GC(AGTC)GC 3. 'PCR was performed in a total volume of 50 μl using a thermal cycler (Techne). The reaction mixture contained 1 unit *Taq* DNA polymerase, 5 μl 10× *Taq* DNA polymerase buffer, 0.2 mM each dNTP, 100 pmole of each primer, and 0.2 μg *A. calcoaceticus* genomic DNA. PCR was performed by 30 amplifications on the condition, denaturation (95°C, 1 min), annealing (55°C, 1 min), and primer extension (72°C, 1 min.). After the final cycle, the reaction mixture was kept for 7 min at 72°C. The nested PCR was performed with the first PCR products as a template with the same condition. The PCR products were run on a 8% polyacrylamide gel (PAGE). An expected PCR product (243 bp) was eluted from the gel, as described previously (Sambrook *et al.*, 1989), and cloned into the pCR 2.1 vector through the Original TA Cloning Kit (Invitrogen, Netherlands). The plasmid containing the PCR product was prepared and sequenced with ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA)

DNA probe preparation and DNA hybridization The plasmid was radiolabeled with [α-^{32}P]dCTP using the Prime-1 Labeling system (Promega, USA). For Southern blot hybridization, 1 μg of DNA was digested with 10 units of *Eco*RI, *Xba*I, *Sal*I, and two of the restriction enzymes respectively, then run by electrophoresis on a 0.8% agarose gel.

Screening of λ library A Lambda library of *A. calcoaceticus* was previously made in this laboratory (Koo *et al.*, 1997). To locate λ containing *rfbB*, λ plaques were lifted onto Hybond N+ membranes (Amersham Pharmacia Biotech, Sweden), lysed, and then hybridized with a [^{32}P]-labeled probe (Manniatis *et al.*, 1982). From a Southern analysis of four positive λ clones, a 4.3-kb *Eco*R1-digested fragment was hybridized with the labeled probe, and subcloned into pBluescript KS. (Stratagene, USA)

DNA sequencing and analysis Sequences of double-stranded plasmid DNA were determined using a Dye Terminator Cycle Sequencing Ready mixture (Perkin Elmer, USA) on a PCR system 2400 and ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA). To sequence rhamnose synthesis genes, subcloned plasmids were used as sources of template DNA. Initial sequencing was done using the M13 reverse primer, M13 forward (-20) primer as primers. Additional primers were then synthesized as needed using newly obtained sequence data. Comparisons of sequences were done using the BLAST program (Altschul *et al.*, 1990)

Expression of *rfb*A gene in *E. coli* The vector pRSET (A) (Invitrogen, Netherlands) was digested with *Eco*RI and *Bam*HI. The *rfb*A among the rhamnose synthesis genes were prepared by PCR from cloned genes using 5' primers containing *Bam*HI, and 3' primers containing *Eco*RI at each end respectively. The amplification product was digested with *Bam*HI and *Eco*RI. The insert and vector were ligated, and the recombinant plasmid was introduced into *E. coli* XL1-Blue MRF. The resulting construct encoded a protein with a N-terminal His-tag. The recombinant plasmid was introduced into the *E. coli* BL21 (DE3) pLysS competent cell. The cultured cell was induced with 1mM IPTG and incubated at 23°C for 12 h. The cell culture was harvested and sonicated. The HisPBind resin and HisPBind Buffer Kit were used to purify enzymes. The enzyme purification step was carried out according to the manufacturer's instructions. Concentrations of the protein were determined by the BCA method (Smith, 1985).

Activity assay of *RfbA* *RfbA* activity was determined by measuring the change of concentration of dTTP and dTDP-glucose by CE analysis. The direction of formation of dTDP- glucose from α-D-glucose 1-phosphate and dTTP was used in a standard assay protocol. The reaction mixture (15 μmol Tris/HCl pH 8.0, 3.6 μmoles $MgCl_2$, 7.2 μmol α-D-glucose 1-phosphate, 1.8 μmol dTTP, 1.8 U inorganic pyrophosphatase and an appropriate aliquot of *RfbA* in total volume of 300 μl) was incubated at 37°C. Samples (30 μl) were withdrawn at timed intervals for up to 20 min. The samples was immediately mixed with 1.00 ml 50 mM potassium phosphate pH 3.0 in order to terminate the reaction (Lindquist, *et al.*, 1993). The diluted samples were stored at 4°C until analysis by CE. CE was performed using the automated P/ACE system 5500 with the cooling capability for the column and samples (Beckman). The capillary cartridge contained a fused-silica capillary 50 cm × 50 μm i.d. Detection was accomplished by an on-column UV-absorbency detector at 254 nm. Runs were performed using a 50 mM Tris-HCl buffer (pH 8.0), under the following condition: T_{samp} = 4°C, T_{cap} = 20°C, V = 20 kV, I = 90mA, with forward polarity and separated for 5 min. The column was sequentially pretreated with degassed water for 3 min, and 0.1 N NaOH for 3 min. Finally, the capillary was equilibrated with the buffer used for the analysis for 1 min. Standard solutions of dTDP-D-glucose in a concentration range from 20-1000 mM were prepared in a 50 mM Tris-HCl buffer (pH 8.0) and injected by pressure for 3 sec.

K_m and k_{cat} measurement For determination of kinetic constants, the assay was modified. Inorganic pyrophosphatase was not included in the reaction mixture. The standard reaction mixture for the CE assay contained the following component in a final volume of 30 μl : 15 μmol Tris/HCl (pH 8.0), 3.6 μmoles $MgCl_2$, and an 1 μg of *RfbA*. The experiments were designed to provide data by

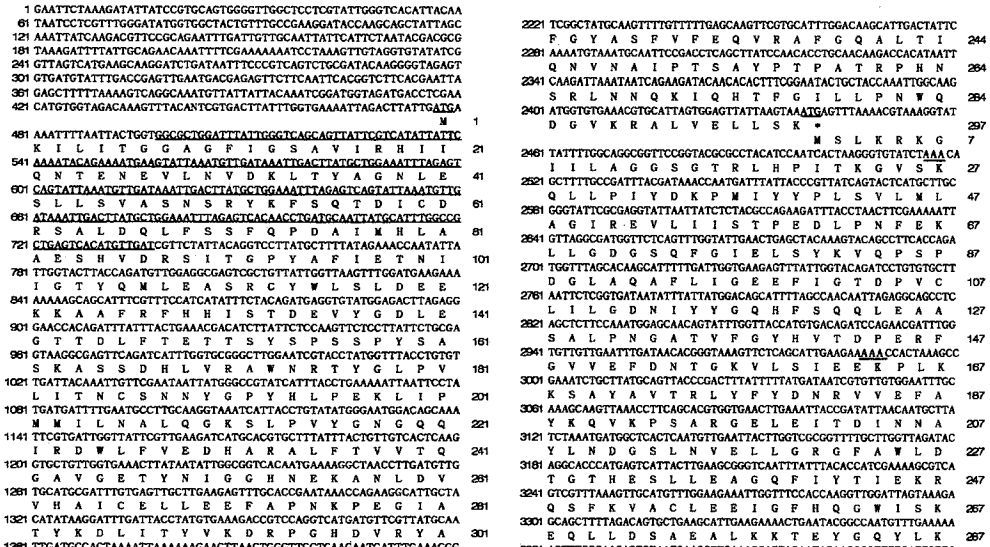

Fig. 1. DNA sequence and deduced amino acid sequence of the *A. calcoaceticus rfb* gene cluster. The start codon (ATG) and *RfbB* probe sequence are indicated. The conserved putative activator binding site at K-27 (*A. calcoaceticus*) is underlined as is the conserved postulated α-D-glucose-1-phosphate binding site at K-164.

varying the concentration of dTTP at a fixed concentration of -D-glucose 1-phosphate and vice versa. For the K_m and k_{cat} measurement of dTTP, the concentration of glucose 1-phosphate was fixed as 7.2 μmol. The concentration of dTTP was varied as 0.050, 0.0333 0.025, 0.0167, and 0.0125 μmol. For the Km and kcat measurement of glucose 1-phosphate, the concentration of dTTP was fixed as 1.8 μmol. The concentration of glucose 1-phosphate was varied as 0.100, 0.050, 0.0333 0.025, 0.0020, 0.0167, 0.0143, 0.0125, and 0.0111 μmol. The amount of dTDP-D-glucose formed was plotted as a function of the reaction time. The initial velocity was measured by drawing the tangent to the product accumulation curve at the initial stage of the reaction. The initial velocity was expressed in enzyme units, where 1 unit is defined as the amount catalyzing the formation of 1 μmol dTDP-D-glucose/min.

Chemical modification The inactivation of the enzyme by PCMB was performed at 25°C for 10 min in the dark by addition of a reagent into the enzyme solution in a 50 mM Tris-HCl buffer (pH 8.0). The final concentrations of PCMB and the enzyme were 0.1 mM and 10 μM, respectively. The inactivation of the enzymes by NEM and iodoacetamide was performed at 25°C for 10 min by addition of reagents into the enzyme solution in a 50 mM Tris-HCl buffer (pH 8.0). The final concentrations of NEM, iodoacetamide, and the enzyme were 1 mM, 0.1 mM and 10 μM, respectively, as mentioned previously (An *et al.*, 1999) The inactivation of the enzyme by DEPC was performed at 25°C for 10 min in the dark by addition of the reagent into the enzyme solution in a 50 mM Tris-HCl buffer (pH 8.0). The final concentrations of DEPC and the enzyme were 0.1 mM and 10 μM, respectively. After the inactivation of the enzyme with chemical reagents, aliquots were withdrawn for the assay of activity.

Results

Cloning and sequencing of dTDP-rhamnose biosynthetic genes from *A. calcoaceticus* The entire genes of the Rhamnose biosynthesis were cloned. At the beginning, degenerate PCR primers were designed based on the highly conserved dTDP-D-glucose 4,6 dehydratase amino acid sequence. Using *A. calcoaceticus total* DNA as a template, the PCR product of approximately 250bp was amplified. It was cloned into the TA cloning vector (Promega, USA), and the

Fig. 2. Comparison of the deduced amino acid sequence for (A) glucose-1-phosphate thymidylyltransferase (*RfbA*), (B) dTDP-glucose 4,6-dehydratase (*RfbB*), (C) TDP-6-deoxy-4-keto-glucose epimerase (*RfbC*), and (D) dTDP-rhamnose reductase (*RfbD*) from *A. calcoaceticus* with the amino acid sequence of other bacteria. The shaded areas are those where the sequence from *A. calcoaceticus* is identical to all of the other sequences and arrows, which indicate the degenerate primer sites of RfbB. The conserved putative activator-binding site at K-27 (*A. calcoaceticus*) is underlined, as is the conserved postulated α-D-glucose-1-phosphate binding site at K-164 (*A. calcoaceticus*). Ac, *A. calcoaceticus*; Aa, *A. actinomycetemcomitans*; Ec, *E. Coli*; St, *S. typhimurium*

sequence was determined. A BLAST search showed significant homology with *rfbB* from several species. To isolate the entire rhamnose synthesis genes, approximately a 250 bp *rfbB* probe was used to screen the *A. calcoaceticus*

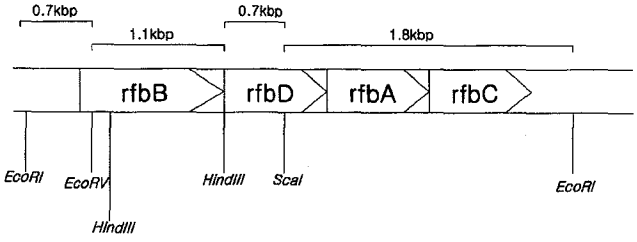

Fig. 3. Restriction map of the *rfb* gene cluster in *A. calcoaceticus.*

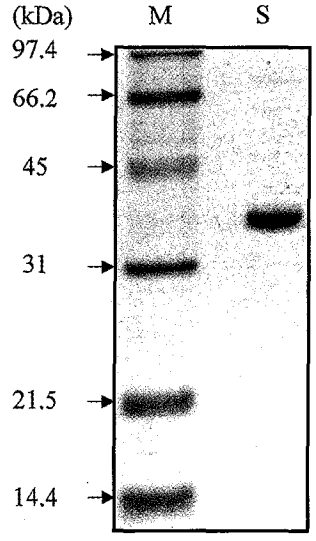

Fig. 4. SDS/PAGE analysis of *RfbA* purified from the transformed *E. coli.* Lane M was loaded with the following molecular mass standards: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trysin inhibitor (20 kDa). Lane S was loaded with the enzyme purified from the His · Bind column.

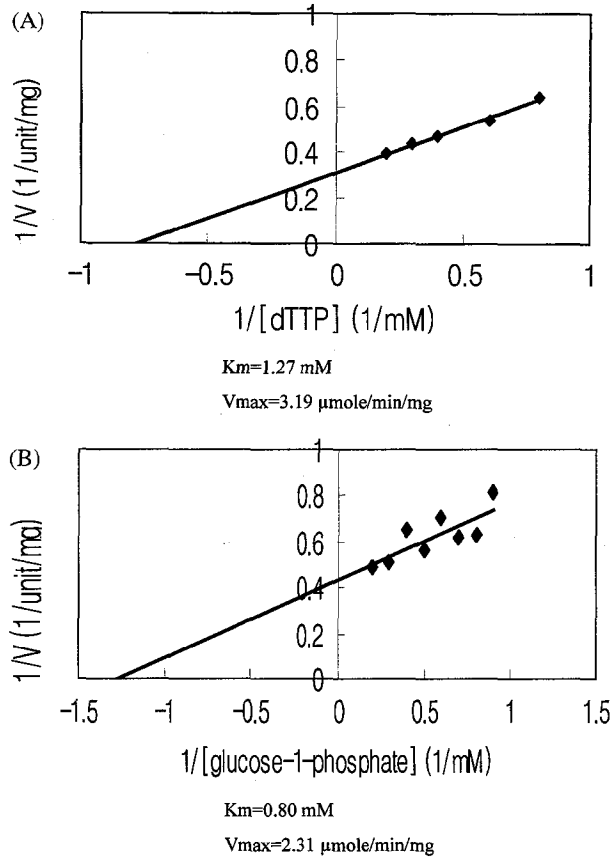

Fig. 5. Lineweaver-Burk plot of the initial reaction velocity for glucose-1-phosphate thymidylyltransferase against various concentrations of dTTP (A) and glucose-1-phosphate (B).

DNA library by the plaque blot hybridization. Several plaques were subsequently analyzed by a Southern blot analysis with the same probe. The fragments from the positive plaque were subcloned and prepared for sequencing. The complete sequence of rhmanose synthesis genes was obtained and presented in Fig. 1. The deduced amino acid sequence of the *RfbA* protein is similar to that of *A. Actinobacillus actinomycetemcomitans* (69.2% identical residues), *E. coli* (67.6%), and *Salmonella typhimurium* (66.1 %) (Fig. 2A). The deduced amino acid sequence of the *RfbB* protein that was encoded by *rfbB* is similar to that of A. actinomycetemcomitans (74.4% identical residues), *E. coli* (70.3%), and Salmonella typhimurium (69.5 %) (Fig. 2B). The deduced amino acid sequence of the *RfbC* protein that was encoded by *rfbC* is similar to that of Shigella flexneri (60.0%), Salmonella enterica (58.0%), and *E. coli* (58.0%) (Fig. 2C). The deduced amino acid sequence of the *RfbD* protein that was encoded by *rfbD* is similar to that of *E. coli* (57.0%), Shigella flexneri (57.0%), and Salmonella typhimurium (56.0 %) (Fig. 2D).

Expression of *rfbA* Plasmid pRSET (A) that contained the predicted ORF was used in the T7 expression system to prepare the *rfbA* gene product. The over-expressed protein was purified using His bind resin, and run on 15% SDS-PAGE (Fig. 4). The size (36 kDa) of the purified protein was correlated to the prediction from the nucleotide sequence data.

Assay of *RfbA* On the 100 mM borate buffer (pH 8.25), electro-osmotic flow (EOF) appeared within 1.5 min and all peaks appeared within 5 min. Each peak was confirmed with spikes of dTTP and dTDP- D-glucose. The dTDP-D-glucose peak appeared at 2 min and the dTTP peak appeared at 3 min. There was a linear relationship between the initial rate and the amount of the enzyme. The total absorbance area of dTTP and dTDP-D -glucose was constant at the range from 0% to over 30% formation of dTDP-D-glucose. This indicates that the molar absorbance coefficients of dTTP and dTDP-D glucose are similar.

***K_m* and *V_{max}* measurement** By increasing the dTTP and α-D-glucose-1-phosphate, the formation of dTDP-D-glucose was increased. With the variation of dTTP and the α-D-glucose-1-phosphate concentration, K_m and V_{max} were measured by a Lineweaver-Burk plot (Fig. 5). K_m and the V_{max}

values of dTTP were 1.27 mM and 3.19 μmole/min/mg. The approximate K_m and V_{max} values of α-D-glucose-1-phosphate were 0.80 mM and 2.31 μmole/min/mg.

Chemical modification In the presence of thiol group specific reagents, such as p-chloromrtcuribenzoic acid (PCMB), N-ethylmalemide (NEM), and iodoacetamide, no inhibition of the enzyme activity was observed. However, diethyl pyrocarbonate (DEPC) strongly inhibited the activity of the enzyme. The inhibition by DEPC depended on its concentration (residual activity, %; 90 at 0.5 mM, 80 at 1 mM, 50 at 1.5 mM, 10 at 2 mM, respectively). This suggests that lysine residues, conserved in the active site, may be essential for its catalysis.

Discussion

In many gram-negative bacteria, rhamnose for the capsular polysaccharide was formed through dTDP-D-glucose, dTDP-D-*xylo*-hexulose, dTDP-L-*lyxo*-hexulose, and dTDP-rhamnose, in that order. In this study, a gene cluster that encoded the four enzymes that were related to the synthesis of dTDP-rhamnose was isolated from *A. calcoaceticus*. That gene cluster was cloned and sequenced, and one of them was expressed in *E.coli*. The sequencing homology studies suggested that the four genes (*rfbA, B, C, D*) were clustered on a biosynthetic operon in the order of *rfb BDAC*. Each enzyme has encoding genes that are quite similar to those of other gram-negative bacteria (Fig. 2). *A. calcoaceticus RfbA* contains highly conserved lysine at residue 27, it appears in most enzymes that transfer nucleoside phosphate to α-D-glucose 1-phospghate. It was postulated to be a part of an activator-binding site (Thorson *et al.*, 1994). It also contains the Glu-Lys-Pro (163-165) sequence, which is postulated to be a part of the α-D-glucose-1-phosphate binding region. This sequence is also conserved in most of α-D-glucose-1-phosphate nucleoside transferases (Thorson *et al.*, 1994). The *RfbA* was inhibited by DEPC, indicating that histidine, or the lysine residue, is essential for this enzyme catalysis. However, histidine residues are not found in the putative activator binding site, or the α-D-glucose-1-phosphate binding site. It is postulated that lysine 27 or 164, conserved in various enzymes, may play an essential role. *RfbA* is a potential drug target since it is likely to be an essential protein involved in the mycobacterial cell wall linker synthesis. Also, its enzymatic product, dTDP-D-glucose, is not found in humans. Therefore, the information on this enzyme from pathogenic *A. calcoaceticus* may be applied for this purpose. However, care must be taken since this enzyme is quite similar to glucose-1-phosphate uridylyltransferase that is needed in human metabolism.

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