JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 1047–1052
0021-9193/96/$04.00+0

Vol. 178, No. 4

Galactofuranose Biosynthesis in *Escherichia coli* K-12: Identification and Cloning of UDP-Galactopyranose Mutase

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Received 31 July 1995/Accepted 5 December 1995

We have cloned two open reading frames (*orf6* and *orf8*) from the *Escherichia coli* K-12 *rfb* region. The genes were expressed in *E. coli* under control of the T7*lac* promoter, producing large quantities of recombinant protein, most of which accumulated in insoluble inclusion bodies. Sufficient soluble protein was obtained, however, for use in a radiometric assay designed to detect UDP-galactopyranose mutase activity (the conversion of UDP-galactopyranose to UDP-galactofuranose). The assay is based upon high-pressure liquid chromatography separation of sugar phosphates released from both forms of UDP-galactose by phosphodiesterase treatment. The crude *orf6* gene product converted UDP-[α-D-U-^{14}C]-galactopyranose to a product which upon phosphodiesterase treatment gave a compound with a retention time identical to that of synthetic α-galactofuranose-1-phosphate. No mutase activity was detected in extracts from cells lacking the *orf6* expression plasmid or from *orf8*-expressing cells. The *orf6* gene product was purified by anion-exchange chromatography and hydrophobic interaction chromatography. Both the crude extract and the purified protein converted 6 to 9% of the UDP-galactopyranose to the furanose form. The enzyme was also shown to catalyze the reverse reaction; in this case an approximately 86% furanose-to-pyranose conversion was observed. These observations strongly suggest that *orf6* encodes UDP-galactopyranose mutase (EC 5.4.99.9), and we propose that the gene be designated *glf* accordingly. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified UDP-galactopyranose mutase revealed one major band, and analysis by electrospray mass spectrometry indicated a single major species with a molecular weight of 42,960 ± 8, in accordance with that calculated for the Glf protein. N-terminal sequencing revealed that the first 15 amino acids of the recombinant protein corresponded to those expected from the published sequence. UV-visible spectra of purified recombinant enzyme indicated that the protein contains a flavin cofactor, which we have identified as flavin adenine dinucleotide.

The thermodynamically disfavored furanose form of galactose occurs infrequently in nature, though it is found in a wide range of macromolecules, including bacterial O antigens (2, 18), fungal exopolysaccharides (10, 22), protozoal glycoproteins (12, 13), and the cell walls of mycobacteria (1, 4, 8).

The first polysaccharide found to contain galactofuranose was galactocarolose, an extracellular β-D-(1→5)-linked polygalactofuranose produced by *Penicillium charlesii* (9). Early experiments suggested that intact *P. charlesii* cells could not utilize exogenous galactose for galactocarolose biosynthesis (7). Cell extracts of this organism were subsequently found to incorporate either glucose or glucose-1-phosphate into galactocarolose (21). In the presence of Zn^{2+} or F^- ions, polysaccharide synthesis was inhibited and UDP-galactofuranose was found to accumulate, suggesting that this nucleotide sugar might be the activated galactofuranose precursor in galactocarolose biosynthesis.

Galactofuranose also occurs in the T1 antigen of *Salmonella typhimurium*, in which it has been established that α-D-galactofuranose is synthesized from a derivative of galactopyranose, and it is known that the pyranose-to-furanose ring contraction does not take place at either the free-sugar or sugar phosphate level (14, 17). These observations led to speculation that interconversion might occur at the UDP-sugar level, with UDP-galactofuranose providing activated galactofuranose units for T1 antigen biosynthesis, paralleling the reported role of this sugar nucleotide in *P. charlesii*. Unfortunately, little progress has been made in defining the steps involved in the biosynthesis and utilization of galactofuranose since these early reports, despite the key role played by this unusual sugar residue in important pathogens such as *Mycobacterium tuberculosis* and *Leishmania donovani.*

Modern laboratory strains of *Escherichia coli* K-12 contain mutations in the O antigen (*rfb*) gene cluster which block O antigen biosynthesis (for a review, see reference 15). It is only recently that complementation experiments with genes from strains carrying different mutations in *rfb* have allowed the structure of the K-12 O antigen to be determined (11, 18), revealing that the repeat unit contains β-D-galactofuranose. The deduced sequence of the original K-12 *rfb* region contains 11 open reading frames (11, 18, 25), and functions for most of the corresponding proteins have been identified either experimentally or by homology to known enzymes from other species. Two remaining genes (*orf6* and *orf8*) were tentatively assigned roles in synthesis and transfer of the galactofuranose residue to the O antigen, these being the only biosynthetic steps that remained undesignated after assignation of the other genes. It was proposed that galactofuranose might be synthesized in a single step from UDP-galactopyranose, with a second enzyme responsible for the transfer of UDP-galactofuranose to the O antigen repeat unit (18). The identification of a consensus βαβ dinucleotide binding motif in the amino acid sequence encoded by *orf6* (18, 24) fuelled speculation that it encodes the UDP-galactofuranose biosynthetic enzyme (UDP-galactopyranose mutase, EC 5.4.99.9), this structural

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element being more common in nucleotide-sugar biosynthetic enzymes than in transferases.

It is apparent from the rate of occurrence of this rare sugar residue in pathogenic fungi and bacteria that a fuller understanding of the biosynthesis of galactofuranose and its incorporation into biopolymers might be exploited to combat infectious disease. We describe here the cloning and expression of *orf6* and *orf8* from the K-12 *rfb* cluster and our efforts to establish which, if either, of these two genes is responsible for galactofuranose biosynthesis in *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents. *E. coli* BL21 (DE3) [F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm met* (DE3) (19)] and NovaBlue {*endA1 hsdR17*($r_{K-12}^- m_{K-12}^-$) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB* $lacI^qZ\Delta M15$::Tn*10* (Tc^r)] (19)} and plasmid pET-11a (6, 20) were purchased from Novagen. *E. coli* strains were grown in Luria-Bertani (LB) medium or on LB agar (16) supplemented with ampicillin (80 μg/ml) when required for plasmid selection. IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added to growth media when required. UDP-[^{14}C]-galactopyranose (309.6 mCi/mmol) and UDP-[^{3}H]-galactopyranose (10.6 Ci/mmol) were from DuPont. α-D-Galactofuranose-1-phosphate was synthesized in house by Andrew Whittington according to the method of De Lederkremer et al. (5). Centricon C30 ultrafilters were supplied by Amicon. Manual DNA sequencing was carried out with T7 polymerase (T7 sequencing kit; Pharmacia); reactions were run on a Bethesda Research Laboratories S2 apparatus. Automated sequencing reactions were performed by Applied Biosystems DyeDeoxy methodology on an Applied Biosystems 373 sequencer.

Cloning of *orf6* and *orf8* from the *E. coli* K-12 *rfb* region. K-12 *rfb* region *orf6* and *orf8* were amplified from *E. coli* C600 genomic DNA by PCR with the primers K12orf6.1 (5′-TGTTTTGCTGAGGATCATATGTACGATTATATCA TT-3′), K12orf6.2 (CACTAATTACAAGGATCCTCATTAATCCGTACTC AT-3′), K12orf8.1 (CCAGAAAATAAAGTGCATATGTATTTTTTGAATG AT-3′), and K12orf8.2 (CCACAGAGACCGGATCCTTTAGCGAGTTTTAA GATG-3′). PCR mixtures contained 10 ng of genomic DNA; 500 ng of each oligonucleotide; 200 μM dATP, dCTP, dGTP, and dCTP; 2 mM $MgCl_2$; 2.5 U of *Taq* polymerase (Promega); 10 mM Tris-HCl; 50 mM KCl; and 0.1% Triton X-100 (pH 9.0) in a total volume of 100 μl. PCR was performed for 25 cycles of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C, followed by a further 10 min at 72°C. Amplified DNA fragments were digested with *Nde*I and *Bam*HI, purified, and ligated into pET-11a between the *Nde*I and *Bam*HI sites to give pORF6 and pORF8, respectively. Cloning work was carried out with the *E. coli* K-12 strain NovaBlue. The integrity of cloned sequences in pORF6 and pORF8 was checked by sequencing in both directions.

Expression of recombinant *orf6* in *E. coli*. Ten nanograms of pORF6 DNA was used to transform 20 μl of *E. coli* BL21 (DE3) cells to ampicillin resistance. Several clones were checked for the presence of intact pORF6 by restriction mapping.

Five milliliters of LB medium containing ampicillin was inoculated with a single colony of BL21 (DE3)(pORF6) and incubated overnight at 37°C. This culture (0.5 ml) was used to inoculate 50 ml of LB medium-ampicillin, and the resulting solution was shaken at 250 rpm at the designated temperature until an A_{600} value of 0.4 to 0.5 was reached. Protein production was induced by the addition of IPTG to a final concentration of 0.5 mM; incubation was continued for 3 h, and cells were harvested by centrifugation (15 min, 3,000 × *g*, 4°C). Larger batches of cells (up to 8 liters) were grown in LB medium-ampicillin to an A_{600} of 0.6 and induced with IPTG (0.5 mM final concentration) for 3 h at 37°C. Cells were harvested by centrifugation (10,000 × *g*, 15 min at 4°C).

Preparation of cell extracts and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells from cultures of BL21 (DE3) (pORF6) were suspended in ice-cold extraction buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) at a concentration of 0.5 g of cells per ml of buffer. Suspended cells were sonicated on ice for 15 s with a 90-s cooling period (using a Branson Sonifier), a procedure repeated 12 times. Cell debris was pelleted by centrifugation at 20,000 × *g* for 15 min at 4°C; the supernatant was transferred to a clean tube and used in cell-free assays.

Protein content of cell extracts was assessed by the Bradford method (3) and analyzed by SDS-PAGE.

Assay of UDP-galactopyranose mutase. UDP-[α-D-U-^{14}C]-galactose (4 μl) was added to a microcentrifuge tube containing 2.5 μl of 10 mM NAD^+. The mixture was evaporated to dryness in a vacuum centrifuge, 12.5 μl of *E. coli* extract (protein concentration, approximately 25 mg/ml) was added, and the reaction mixture was incubated for 15 min at 30°C. Extraction buffer (200 μl) was added, and the mixture was spun through a Centricon C30 ultrafilter to remove protein. Extraction buffer (40 μl) containing 0.025 U of phosphodiesterase I (P3134; Sigma) was added to the filtrate and incubated for 1 h at 37°C. Samples were applied directly to a Dionex Carbopak PA1 column (240 by 10 mm) preequilibrated in 100 mM sodium acetate–10 mM NaOH. Sugar phosphates were eluted with a linear gradient of 100 to 200 mM sodium acetate in 10 mM NaOH and applied over a 20-min period at a flow rate of 1 ml/min. The retention times of α-galactofuranose-1-phosphate and α-galactopyranose-1-phosphate standards were determined prior to each series of assays.

Synthesis and purification of UDP-[U-^{14}C]-galactofuranose. UDP-[U-^{14}C]-galactopyranose (5 μCi) was evaporated to dryness. Ten microliters of BL21 (DE3)(pORF6) extract (protein concentration, 40 mg/ml) was added, and the mixture was incubated for 15 min at 30°C. Protein was removed by ethanol precipitation, and the sample was applied across 4 cm of Whatman 3MM filter paper. Paper chromatography was performed with 1 M ammonium acetate (pH 7.5) in 95% (vol/vol) ethanol until the mobile phase reached the end of the paper. The paper was dried completely and then run a second time by using the same mobile phase. The paper was dried and divided into 1-cm-long strips perpendicular to the direction of separation, and a 2-mm-long fragment of each strip was analyzed by liquid scintillation counting. UDP-[U-^{14}C]-galactofuranose ran approximately 1 cm in front of UDP-[U-^{14}C]-galactopyranose. UDP-[U-^{14}C]-galactofuranose was eluted from the paper with 20 mM HEPES, pH 7.5. The purity of the recovered material was determined by treating a small sample with phosphodiesterase I and analyzing the sugar phosphates released by high-pressure liquid chromatography (HPLC). The purified UDP-[U-^{14}C]-galactofuranose contained approximately 10% UDP-[U-^{14}C]-galactopyranose.

Reverse reaction assay. Fifteen microliters (5,000 cpm) of the purified UDP-[U-^{14}C]-galactofuranose was incubated with 30 μl of BL21 (DE3)(pORF6) extract (protein concentration, 40 mg/ml) at 30°C for 15 min. The sample was diluted to 95 μl with water and passed through a Centricon C30 ultrafilter. Phosphodiesterase I (0.02 U) was added, and the mixture was incubated for 45 min at 37°C. Protein was removed from the sample by ethanol precipitation, and sugar phosphates were analyzed by HPLC as described for the forward assay.

Purification of recombinant UDP-galactopyranose mutase. (i) Anion-exchange chromatography. An extract was prepared from 6.7 g of BL21 (DE3) (pORF6) cells as described above, with the inclusion of 1 μg of leupeptin (Boehringer Mannheim) per ml in the extraction buffer. A 6-ml Pharmacia Resource Q column was equilibrated in buffer A (50 mM HEPES [pH 7.6], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol), and crude extract containing 150 mg of protein was applied to the column. After washing the column with 6 ml of buffer A, protein was eluted with a gradient of 0 to 500 mM NaCl in buffer A, which was applied over a 40-min period at a flow rate of 3 ml/min. Fractions (3 ml) were collected, and corresponding fractions from three successive runs were pooled. Protein was detected with an on-line detector to monitor A_{280}, and fractions were assayed for UDP-galactopyranose mutase activity. See Fig. 3A for a typical column profile.

(ii) Hydrophobic interaction chromatography. Fractions from the ion-exchange column with enzyme activity were pooled and dialyzed for 4 h against buffer B (50 mM HEPES [pH 7.6], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 1 M $(NH_4)_2SO_4$ and applied at 1 ml/min to a hydrophobic interaction chromatography column (5 by 30 mm) containing Toyopearl HICPAK-Butyl resin preequilibrated with buffer B. After washing the column with 6 ml of buffer B containing 1 M $(NH_4)_2SO_4$, a 20-ml linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer B was applied. One-milliliter fractions were collected. Enzyme activity was detected in six fractions containing a total of 3 mg of protein. See Fig. 3B for a typical elution profile.

Characterization of the assay product. Products from the equivalent of 40 forward assay mixtures were pooled, and sugar phosphates were separated by HPLC as described above. Fractions with the same retention time as the α-D-galactofuranose-1-phosphate standard were pooled (2.2 μCi, total count) and diluted 50-fold in 10 mM MES (morpholineethanesulfonic acid; pH 6.0). The diluted material was applied to a column (5 by 20 mm) containing Q-Sepharose (Pharmacia) to remove the HPLC buffer; sugar phosphates were eluted with 3 ml of 100 mM sodium citrate, pH 5.0. Acid phosphatase (5 U) was added to 1 ml of the material, and the sample was incubated at 37°C for 1 h. Protein was removed by ultrafiltration through a Centricon C30 ultrafilter. Neutral sugar analysis of the filtrate was performed on a Dionex PA1 column (240 by 10 mm). Samples were applied to a column preequilibrated in 10 mM NaOH and eluted isocratically with reagent grade water over a 9-min period at a flow rate of 1 ml/min. The system was calibrated with authentic labelled and unlabelled sugar standards with radiometric (Canberra Packard) and pulsed amperometric (Dionex) detectors. Fifty microliters (6.8×10^4 dpm) of the phosphatase-treated test material was injected onto the column for analysis with 1 μg of unlabelled galactose added to the sample to act as an internal standard.

UV-visible light scanning spectroscopy. The UV-visible light spectrum of purified UDP-galactopyranose mutase between 300 and 500 nm was recorded with a Perkin-Elmer Lambda 7 UV/VIS spectrophotometer.

Identification of the flavin cofactor. Purified enzyme (0.9 mg) was boiled for 10 min, and the denatured protein was pelleted in a microcentrifuge (5 min, 20,000 × *g*). The supernatant was filtered (Centricon C30 filter), and 200 μl was applied to a C_{18} reverse-phase HPLC column (300 by 4.6 mm) equilibrated in 5 mM ammonium acetate, pH 6.0. Elution was achieved with a 30-ml linear gradient of 15 to 40% methanol in 5 mM ammonium acetate, pH 6.0. The elution times of flavin mononucleotide and flavin adenine dinucleotide standards were determined in separate experiments.

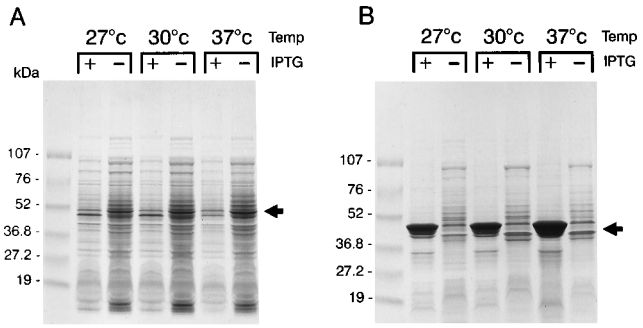

FIG. 1. Expression of *orf6* by pORF6 in *E. coli* BL21 (DE3). (A) Soluble cell extract. (B) Insoluble inclusion bodies. Expression experiments were performed at 27, 30, and 37°C as indicated; uninduced (− IPTG) samples are included for comparison. Arrows indicate the position of the *orf6* protein. Temp, temperature.

RESULTS

Cloning and expression of *orf6* and *orf8*. The two open reading frames (*orf6* and *orf8*) were amplified from *E. coli* K-12 genomic DNA by PCR and cloned into pET-11a (6) to create plasmids pORF6 and pORF8, containing *orf6* and *orf8*, respectively. In *E. coli* BL21 (DE3), both plasmids expressed large quantities of recombinant protein in the presence of the inducer IPTG. In each case most of the overproduced protein accumulated in insoluble inclusion bodies when cells were grown at 37°C, though the protein product of *orf6* could also be observed by SDS-PAGE in soluble extracts of induced BL21 (DE3)(pORF6). Lowering the temperature at which pORF6-containing cells were grown had little effect on the quantity of soluble recombinant protein produced in expression experiments, though the yield of insoluble protein was reduced under these conditions (Fig. 1).

Assay conditions for UDP-galactopyranose mutase. A radiometric assay was developed to detect UDP-galactopyranose mutase activity (interconversion of UDP-galactopyranose and UDP-galactofuranose). Cell extracts were incubated with radiolabelled UDP-galactose, and after removal of protein, the reaction products were treated with phosphodiesterase. Sugar phosphates derived from the furanose and pyranose forms of UDP-galactose were separated by HPLC. Detection of a radioactive product which eluted with the same retention time as an authentic α-D-galactofuranose-1-phosphate standard indicated the presence of UDP-galactopyranose mutase activity.

When tested in the assay, crude extracts containing the *orf6* gene product showed UDP-galactopyranose mutase activity. Routinely, 6 to 9% of the UDP-α-D-galactopyranose was converted to the furanose form. A typical HPLC assay trace is shown in Fig. 2. Equilibrium between UDP-galactopyranose and UDP-galactofuranose was attained very quickly (<4 min) under the reaction conditions described, though reaction mixtures were routinely incubated for 15 min for ease of sample handling. Extracts from cells containing pORF8 or from untransformed *E. coli* BL21 (DE3) showed no activity in the assay. Retention times for the sugar phosphates in the HPLC system showed some day-to-day variation, possibly because of temperature fluctuations. For this reason, the system was calibrated prior to each series of assays with synthetic α-galactofuranose-1-phosphate and α-galactopyranose-1-phosphate standards.

UDP-galactopyranose is also a substrate for the *galE* gene product, UDP-glucose epimerase. Epimerase activity can be detected in our assay by the appearance of a peak corresponding to glucose-1-phosphate. Control extracts from *E. coli* K-12 strain C600 showed significant epimerase activity (data not shown), but we were unable to detect glucose-1-phosphate in assays with BL21 (DE3) extracts. BL21 (DE3) is an *E. coli* B *gal* derivative (19), and the absence of UDP-glucose epimerase activity suggests that it contains a *galE* mutation.

Characterization of the reaction product. The limited extent of conversion of UDP-galactopyranose forestalled attempts to accumulate sufficient product for structural analysis by nuclear magnetic resonance. Nevertheless, it was clearly important to obtain corroborative evidence that the product of the reaction was indeed UDP-galactofuranose. To this end, the products from a series of assays were pooled, and the suspected furanose components were isolated by HPLC. This material was dephosphorylated, and the resulting compound was analyzed by HPLC. By comparison with a series of neutral sugar standards, the dephosphorylated product was identified as galactose, supporting the evidence from HPLC analysis that the assay product itself is galactofuranose-1-phosphate.

Purification of UDP-galactopyranose mutase. The recombinant protein was purified to confirm that a single enzyme was capable of catalyzing the observed pyranose-to-furanose conversion. Most of the *orf6* gene product accumulated in insoluble inclusion bodies within the expression host when cells containing pORF6 were induced with IPTG, but attempts to solubilize inclusion bodies with urea and refold this material to recover active enzyme were unsuccessful. We therefore concentrated on purifying the recombinant protein from crude extracts, after the removal of insoluble material.

Preliminary experiments indicated that the protein bound to anion-exchange resins at an alkaline pH. Resource Q, a strong quaternary ammonium anion-exchange resin, was chosen for the first purification step. For the reported data, several small-scale purification runs (performed on a 6-ml Resource Q column) were pooled. Recently we have used a larger (23-ml) column in single runs with no loss of resolution. UDP-galactopyranose mutase activity eluted from the Resource Q column at 80 to 120 mM NaCl (Fig. 3A). From a crude sample containing 426 mg of protein, active fractions were pooled to

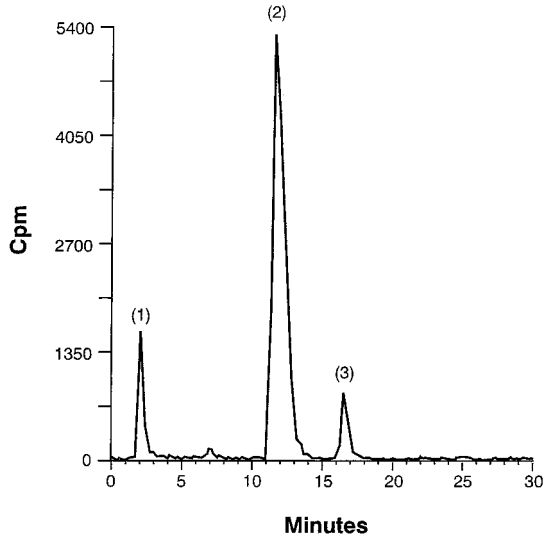

FIG. 2. UDP-galactopyranose mutase HPLC assay trace. BL21 (DE3) (pORF6) extract (12.5 μl) was used in an assay with 20 mM UDP-[U-^{14}C]-galactopyranose as the substrate. Peak assignments: (1), galactose; (2), α-D-galactopyranose-1-phosphate; and (3), α-D-galactofuranose-1-phosphate.

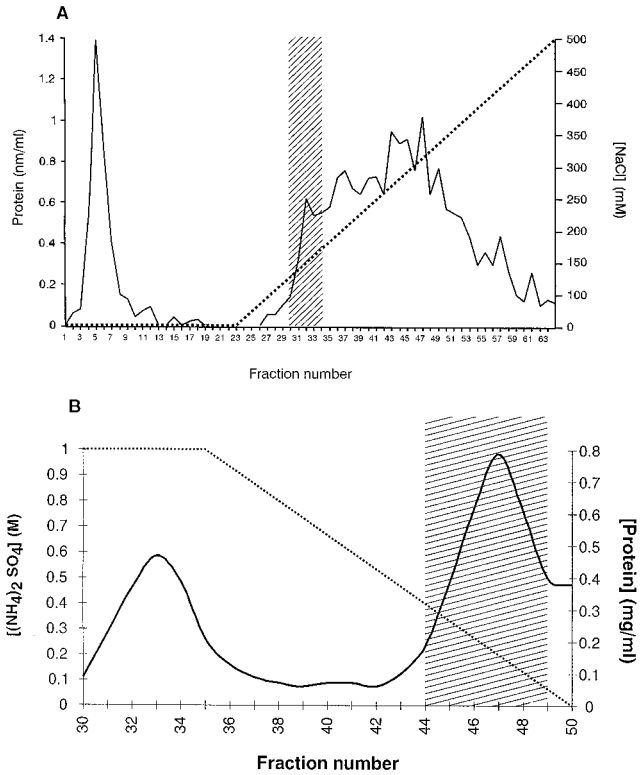

FIG. 3. Resource Q column chromatography (A) and butyl hydrophobic interaction column chromatography (B) profiles. Solid lines indicate protein concentration, and dotted lines represent the concentrations of NaCl (A) and $(NH_4)_2SO_4$ (B). Hatched areas correspond to fractions containing UDP-galactopyranose mutase activity.

yield 18 mg of protein. A contaminating phosphatase activity coeluted with the UDP-galactopyranose mutase. Depletion of sugar phosphates from the assay mixture by this contaminant made detection of galactopyranose mutase activity in the column eluant difficult, though sufficient activity was observed to identify active fractions. Analysis by SDS-PAGE revealed four protein bands, with the major component running very close to the 45-kDa molecular mass marker.

We evaluated several hydrophobic interaction chromatography resins for the second purification step and finally selected Toyopearl HICPAK-Butyl resin. Partially purified enzyme was applied to the column in 1 M ammonium sulfate. UDP-galactopyranose mutase activity eluted at 0.58 M ammonium sulfate (Fig. 3B). Pooled active fractions showed a single major band by SDS-PAGE. The contaminating phosphatase activity was removed by this purification step. Figure 4 shows SDS-PAGE analysis of enzymes from each step of the purification procedure.

Characterization of the recombinant enzyme. Analysis of the purified protein by SDS-PAGE revealed a single protein with a molecular mass of approximately 42 kDa (calculated by SDS-PAGE). N-terminal sequence analysis indicated the protein to be greater than 95% pure with the N-terminal amino acid sequence MYDYIIVGSFLFGAV, identical to the first 15 amino acids encoded by *orf6*. Electrospray mass spectroscopic analysis of the purified protein indicated its molecular weight to be 42,960 ± 8, in agreement with the predicted value of 42,967. During purification it was noted that active fractions were yellow. UV-visible light spectroscopy (Fig. 5) revealed peaks at A_{382} and A_{450}, with a shoulder at A_{465}, indicating that

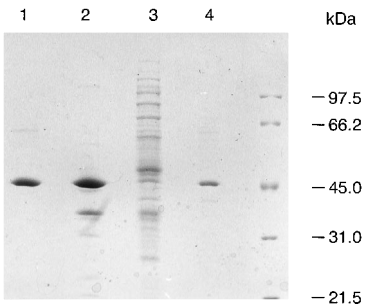

FIG. 4. SDS-PAGE analysis of UDP-galactopyranose mutase purification. Lane 1, after analysis in Resource Q and butyl hydrophobic interaction chromatography columns; lane 2, after analysis in a Resource Q column; lane 3, crude extract from BL21 (DE3) containing pORF6; lane 4, crude insoluble material for comparison.

the yellow coloration is due to the presence of a flavin cofactor. Thermal denaturation of the protein released the cofactor, which was identified as flavin adenine dinucleotide by comparative reverse-phase HPLC.

The equilibrium facilitated by UDP-galactopyranose mutase lies strongly (approximately 11.1) in favor of the pyranose form, i.e., the starting material in the assay described here. Specific activity and kinetic measurements requiring preequilibrium determinations of the extent of pyranose-to-furanose conversion would necessitate accurate quantitation of galactofuranose-1-phosphate and galactopyranose-1-phosphate under conditions in which significantly less than 9% of the starting material has been consumed. It was apparent that our assay was not sufficiently sensitive to make such measurements reliably; the determination of specific activity and kinetic data for UDP-galactopyranose mutase is likely to present difficulties until significant quantities of UDP-galactofuranose can be obtained for use as a substrate. NAD^+ appeared to improve the reproducibility of assays with crude extracts containing mutase activity, though this may not reflect a requirement of UDP-galactopyranose mutase itself, as similar effects were not ob-

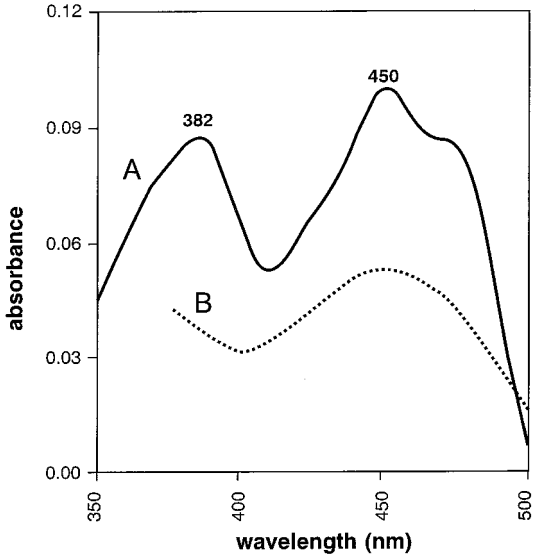

FIG. 5. UV-visible light absorption spectra of the UDP-galactopyranose mutase enzyme (100 μg) (line A) and the cofactor released from 100 μg of the purified enzyme by boiling (line B).

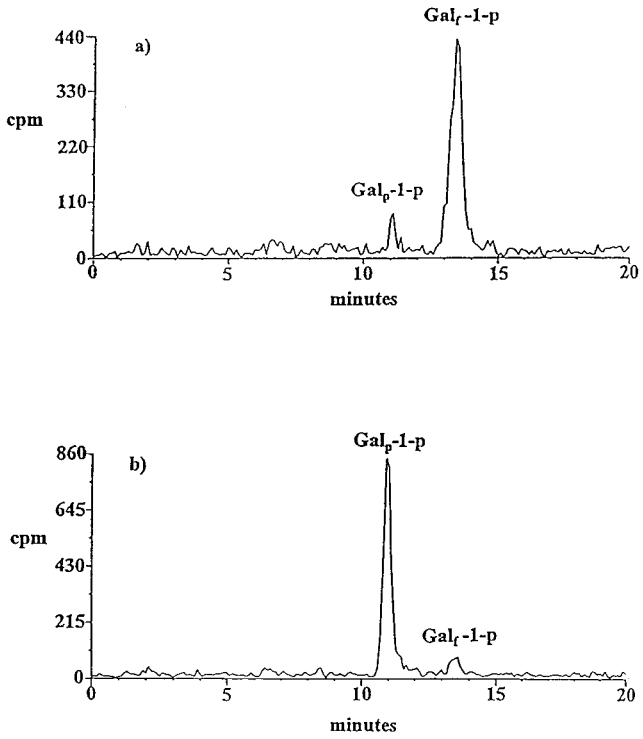

FIG. 6. Conversion of UDP-galactofuranose to UDP-galactopyranose. HPLC traces show products of phosphodiesterase treatment of purified UDP-galactofuranose before (A) and after (B) treatment with UDP-galactopyranose mutase.

served with purified enzyme. The enzyme appears to be relatively unstable; storage of the purified protein at 4°C for prolonged periods (>24 h) resulted in a complete loss of activity. The addition of glycerol (10%) to enzyme preparations had little stabilizing effect. No mutase activity could be detected in enzyme purified in the absence of protease inhibitors (phenylmethylsulfonyl fluoride and leupeptin; see Materials and Methods).

Reverse assay. Further confirmation that the *orf6* gene product catalyzes the reversible interconversion of UDP-galactopyranose and UDP-galactofuranose was sought by attempting the reverse (i.e., furanose-to-pyranose) reaction. Labelled UDP-galactofuranose was generated by incubating UDP-[U-^{14}C]-galactopyranose with purified enzyme and purified by paper chromatography. A small sample of the purified material treated with phosphodiesterase I and analyzed by HPLC indicated that it contained about 90% UDP-[U-^{14}C]-galactofuranose (Fig. 6A). Purified UDP-galactofuranose was incubated with extract from BL21 (DE3)(pORF6), and the reaction product was treated with phosphodiesterase I and analyzed by HPLC. Eighty-six percent of the UDP-galactofuranose was found to have been converted back to the pyranose form (Fig. 6B), after allowing for the residual amount (approximately 10%) of UDP-galactopyranose present in the sample. These findings clearly demonstrate that the reaction catalyzed by UDP-galactopyranose mutase is reversible.

DISCUSSION

Our findings indicate that *orf6* of the *E. coli* K-12 *rfb* region encodes the enzyme UDP-galactopyranose mutase, which catalyzes the interconversion of UDP-galactopyranose and UDP-galactofuranose, and we propose that the gene be designated *glf* accordingly. The chromosomal location of *glf* within the *rfb* cluster reflects its role in O antigen biosynthesis, in which galactopyranose mutase presumably serves to provide activated galactofuranose residues in the form of UDP-galactofuranose, which is required for repeat-unit synthesis. *E. coli* UDP-galactopyranose mutase is the first enzyme from any organism shown to catalyze the conversion of a galactopyranose derivative to the furanose form, though it shows significant sequence similarity (38.8% identity over 358 amino acids) to the hypothetical RfbD protein (derived from the nucleotide sequence, GenBank accession number L31762) of the related species *Klebsiella pneumoniae*, which also produces a galactofuranose-containing O antigen (23). It seems likely, therefore, that a similar biosynthetic route to galactofuranose also operates in *K. pneumoniae.*

The enzyme contains flavin adenine dinucleotide, verifying predictions that the protein may contain a flavin or nicotinamide cofactor (25), predictions which were made on the basis that the primary sequence of the protein has a consensus nucleotide diphosphate binding motif at its N terminus (24). Our experimental finding thus lends weight to the predictive value of motif identification, though the mechanistic role of flavin adenine dinucleotide in the interconversion of the two forms of UDP-galactose is unclear. It has been suggested (18) that the reaction may proceed via a 2-keto intermediate, in a manner analogous to the reaction catalyzed by UDP-galactose epimerase, but it is not obvious how an oxidation-reduction pathway operating at this position would effect the observed pyranose-to-furanose interconversion. The role of the cofactor thus remains to be established.

The equilibrium facilitated by UDP-galactopyranose mutase is biased strongly in favor of the pyranose isomer (as is the uncatalyzed equilibrium between the corresponding forms of free galactose). It seems likely, therefore, that the second galactofuranose-handling enzyme, namely, the transferase which incorporates it into the O antigen repeat unit, has a high-level affinity for UDP-galactofuranose. Given that our results affirm the predictions of Stevenson et al. (18) and Yao and Valvano (25) regarding the function of *orf6*, we believe that the remaining *rfb* cluster gene (*orf8*) must now be considered a strong contender for the role of galactofuranose transferase.

ACKNOWLEDGMENTS

We thank Andrew Whittington for synthesis of α-D-galactofuranose-1-phosphate, Steven Graham and Neil Freeman for N-terminal sequence analysis, and Terry Haley and Richard Dennis for performing electrospray mass spectrometry.

This work was supported in part by funds provided by Public Health Service grant NIAID, NIH (AI-33706).

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