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Role of Rfe and RfbF in the Initiation of Biosynthesis of D-Galactan I, the Lipopolysaccharide O Antigen from *Klebsiella pneumoniae* Serotype O1

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The 6.6-kb *rfb* gene cluster from *Klebsiella pneumoniae* serotype O1 (rfb_{KpO1}) contains six genes whose products are required for the biosynthesis of a lipopolysaccharide O antigen with the following repeating unit structure: →3-β-D-Gal*f*-1→3-α-D-Gal*p*-1→(D-galactan I). $rfbF_{KpO1}$ is the last gene in the cluster, and its gene product is required for the initiation of D-galactan I synthesis. *Escherichia coli* K-12 strains expressing the $RfbF_{KpO1}$ polypeptide contain dual galactopyranosyl and galactofuranosyl transferase activity. This activity modifies the host lipopolysaccharide core by adding the disaccharide β-D-Gal*f*-1→3-α-D-Gal*p*, representing a single repeating unit of D-galactan I. The formation of the lipopolysaccharide substituted either with the disaccharide or with authentic polymeric D-galactan I is dependent on the activity of the Rfe enzyme. Rfe (UDP-Glc*p*NAc::undecaprenylphosphate Glc*p*NAc-1-phosphate transferase) catalyzes the formation of the lipid-linked biosynthetic intermediate to which galactosyl residues are transferred during the initial steps of D-galactan I synthesis. The $rfbF_{KpO1}$ gene comprises 1,131 nucleotides, and the predicted polypeptide consists of 373 amino acid residues with a predicted M_r of 42,600. A polypeptide with an M_r of 42,000 was evident in sodium dodecyl sulfate-polyacrylamide gels when rfb_{KpO1} was expressed behind the T7 promoter. The carboxy-terminal region of $RfbF_{KpO1}$ shares similarity with the carboxy terminus of RfpB, a galactopyranosyl transferase which is involved in the synthesis of the type 1 O antigen of *Shigella dysenteriae*.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. The hydrophobic lipid A region of LPS forms the outer leaflet of the outer membrane. The core oligosaccharide links lipid A to the O antigen, a polysaccharide which extends from the cell surface and is involved in interactions with the environment. Structural variation in the O side chain polysaccharides in the LPS molecule results in the serological diversity of O antigens.

Several bacterial species, including *Serratia* spp. (4, 43), *Pasteurella haemolytica* (45, 48), and *Klebsiella* spp. (25, 26, 36, 69, 70), produce an O polysaccharide with the following repeat unit structure: →3-β-D-Gal*f*-1→3-α-D-Gal*p*→(D-galactan I). In different *Klebsiella* serotypes, D-galactan I can be expressed alone (69), with an additional O antigen (69, 70), or in a form in which the D-galactan I backbone is modified by either *O*-acetyl (26) or α-D-Gal*p* residues (25, 36). In *Klebsiella pneumoniae*, D-galactan I synthesis is directed by genes located in the *his*-linked *rfb* gene cluster (6, 9, 25, 70). Genetic (6, 9, 25, 70) and chemical (31, 32, 70) analyses both show that D-galactan I is directly linked to the lipid A core.

There are two general mechanisms for O antigen synthesis (reviewed in reference 68). In one pathway (Rfc dependent), individual O repeat units are assembled on lipid-intermediate carriers at the cytoplasmic face of the plasma membrane, translocated across the membrane, and polymerized by the activity of the Rfc enzyme at the periplasmic face of the plasma membrane. Most examples of this mechanism of assembly involve heteropolysaccharides. The second pathway (Rfc independent) is fundamentally different and to date involves only homopolysaccharide O antigens, such as D-galactan I and the polymannose O9 antigen of *Escherichia coli* (27). D-Galactan I is assembled at the cytoplasmic face of the plasma membrane, and polymerization is thought to occur by sequential sugar transfers to a lipid intermediate; no Rfc enzyme is involved. An ATP-binding cassette (ABC) transporter (or traffic ATPase) then translocates polymerized D-galactan I across the plasma membrane prior to its ligation to the lipid A core (6). Homopolymer O antigen synthesis more closely resembles the biosynthesis of group II capsular polysaccharides in *E. coli*, *Haemophilus influenzae*, and *Neisseria meningitidis* (reviewed in references 68 and 71).

Rfe is an *N*-acetylglucosamine (Glc*p*NAc)-1-phosphate transferase which forms lipid I (undecaprenyl pyrophosphoryl Glc*p*NAc) during the biosynthesis of the cell surface polysaccharide known as enterobacterial common antigen (37). In members of the *Enterobacteriaceae*, Rfe can also be involved in both pathways for LPS O antigen synthesis. In the Rfc-dependent pathway, Rfe transfers Glc*p*NAc residues to a lipid intermediate to initiate the formation of each O polysaccharide repeat unit (1, 29, 58, 72). *rfb* gene products then complete the various O antigen repeat units, and after polymerization, Glc*p*NAc residues provide the first monomer in each repeat unit. A different role is played by Rfe in the biosynthesis of some homopolymeric O polysaccharides which, like D-galactan I, lack Glc*p*NAc in their repeating unit structure. In *E. coli* O8 and O9, Rfe primes the synthesis of the mannose homopolymer O polysaccharide by forming an undecaprenyl pyrophos-

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TABLE 1. Bacterial strains and plasmids

E. coli strain or plasmid	Genotype or relevant property	Source or reference
Strains		
AB1133 (2442)	K-12 *thr-1 leuB6* Δ(*gpt-proA*)*66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 supE44*	38
21548	AB1133 derivative; *rfe*::Tn*10*-48	38
CS1883	K-12 *thr-1 leuB6* Δ(*gpt-proA*)*66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 supE44 galE* Δ(*galOPE*::Cm)	53
CWG263	As for CS1883, but with *rfe*::Tn*10*-48	This study
SØ874	K-12 *lacZ trp* Δ*sbcB-rfb upp rel rpsL*	41
C2110	K-12 *gyrA polA1 his rha*	55
HB101	*hsdR serA ara proA lacY galK rpsL xyl mtl supE*	50
JM109DE3	K-12 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15; T7 RNA polymerase under *lac* promoter	63
Plasmids		
pBluescript KS(+)	Cloning vector; Ap^r	Stratagene
pRK404	Cloning vector RK2 derivative; Km^r Tc^r	11
pRK2013	Helper plasmid for mobilization; Km^r Mob$^+$ Tra$^+$ ColE1	12
pTrc99A	Cloning vector; Ap^r	Pharmacia
pHoHo (Tn*3*-HoHoI)	Tn*3* delivery vector *lacIOZYA*$^+$ *tnpA*; Ap^r	55
pSShe	Helper plasmid for Tn*3* mutagenesis; *tnpA*$^+$; Cm^r	55
pJK2363	pGEM-4 carrying *rfpAB* from *S. dysenteriae* type 1; Ap^r	28
pSS37	pACYC184 carrying rfb_{SdI} and *rfpAB* from *S. dysenteriae* type 1; Cm^r	60
pSS37::Tn*lacZ*-T42	pSS37-derivative with *rfpB* inactivated by a Tn*lacZ* insertion; Cm^r Km^r	29
pWQ3	pRK404 containing a 7.2-kbp insert including the rfb_{KpO1} gene cluster cloned from *K. pneumoniae* O1:K20; Tc^r	9
pWQ5	Insert from pWQ3 cloned in pBluescript KS(+); Ap^r	6
pWQ19	pRK404 containing $rfbCDEF_{KpO1}$ cloned on a 5.3-kbp *Pst*I fragment; Tc^r	6
pWQ20	pTrc99A containing $rfbF_{KpO1}$	This study
pWQ5Δ16.1	Deletion derivative from pWQ5, containing a 1.76-kbp insert including $rfbF_{KpO1}$	This study
pWQ3::Tn*3-lacZ91*	pWQ3 derivative containing a Tn*3*-HoHo insertion in $rfbF_{KpO1}$	This study

phoryl-linked intermediate which serves as the acceptor for mannose residues. Mannose monomers are then added sequentially to the nonreducing terminus of the growing O polysaccharide (27). In vitro studies show that the acceptor can be either undecaprenyl pyrophosphoryl glucose or undecaprenyl pyrophosphoryl Glc*p*NAc; the latter is a more efficient acceptor for mannosyl transfer (27). Recent genetic and in vivo labeling studies with an *E. coli* K-12–O8 hybrid strain indicate that Rfe primes O8 polysaccharide synthesis by transfer of Glc*p*NAc-1-P rather than Glc*p*-1-P (49). It remains to be established whether a similar step is involved in the biosynthesis of all homopolymer O antigens.

To better understand the biosynthesis of D-galactan I, the *rfb* cluster of *K. pneumoniae* O1 (rfb_{KpO1}) has been cloned and its nucleotide sequence has been determined. The following studies were performed to investigate the initiation reaction in D-galactan I synthesis. Here, we report the characterization of $RfbF_{KpO1}$, which participates with Rfe in the initiation step.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* CWG263 was constructed by moving the *rfe*::Tn*10*-48 mutation from *E. coli* 21548 to *E. coli* CS1883 by standard transduction methods with P1*vir* (39). Bacterial strains were routinely grown at 37°C in Luria-Bertani broth supplemented as appropriate with antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 15 μg/ml). For examination of Tn*3-lacZ* fusions, Luria-Bertani agar plates were supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml). High-level expression of $RfbF_{KpO1}$ in pWQ20 was achieved by a 1-h induction following the addition of 5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to exponentially growing cultures.

DNA manipulation and analysis. Restriction endonuclease digestion, ligation, and transformation reactions were performed essentially as described by Sambrook et al. (50). Large-scale plasmid DNA preparations were made with Qiagen Inc. (Chatsworth, Calif.) columns according to the manufacturer's instructions. Deletion derivatives were made with Erase-a-base (Promega, Madison, Wis.). The nucleotide sequence was determined by the dideoxynucleotide chain termination method (51) with the Sequenase version 2.0 sequencing kit (United States Biochemicals). Both strands of pBluescript KS(+)-based plasmid templates were sequenced. Custom oligonucleotide primers were synthesized with an Applied Biosystems oligonucleotide synthesizer (Model 391-EP). Sequence data were analyzed with PC/GENE (IntelliGenetics Inc., Mountain View, Calif.). A BLAST (Basic Local Alignment Search Tool) server analysis program (2) was used to search the National Center for Biotechnology Information database.

Isolation of transposon Tn*3-lacZ* insertions in plasmids carrying cloned genes. Tn*3-lacZ* insertions were made with the system described by Stachel et al. (55). The target plasmid, pWQ3, was transformed into *E. coli* HB101 (pHoHoI and pSShe). pHoHo1 carries the transposon Tn*3*-HoHo1 but is defective for transposase activity; pSShe provides *tnpA* activity in *trans*. To select pWQ3::Tn*3-lacZ* derivatives, the strain harboring plasmids pWQ3, pHoHoI, and pSShe was mated with the Nal^r *polA* recipient, *E. coli* C2110. Replication of pHoHoI, pSShe, and pRK2013 is dependent on *polA* function. To facilitate transfer of the mutated pWQ3 derivatives to C2110, the helper plasmid pRK2013 in *E. coli* HB101 was supplied in a triparental plate mating experiment. Independent pWQ3::Tn*3-lacZ* derivatives were purified and used to transform *E. coli* DH5α to screen for the *lacZ* phenotype. The position of the transposon insertion was determined by DNA sequencing with an oligonucleotide primer (5′-GGTCAT CTGAGACCATTA-3′) based on the inverted long repeat sequence of Tn*3*-HoHo1 (55). The LPS phenotypes of strains carrying pWQ3::Tn*3-lacZ* derivatives were established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.

Examination of LPS by SDS-PAGE and Western blotting (immunoblotting). LPS samples were routinely prepared by the SDS-proteinase K whole-cell lysate method (20). Samples were analyzed by SDS-PAGE with equipment and commercially prepared, 1-mm-thick, 10 to 20% gradient Tricine gels from Novex (San Diego, Calif.). The electrophoresis conditions were those recommended by the manufacturer. Gels were silver stained by the method of Tsai and Frasch (65). LPS samples separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Westran; Schleicher and Schuell) by a modification (14) of the original Western blot procedure (64). Rabbit anti-D-galactan I-specific antiserum was raised in New Zealand White rabbits. The rabbits were immunized with whole cells of *K. pneumoniae* CWK37, which contains D-galactan I as the only O antigen (70), and the immune serum was adsorbed with *E. coli* K-12 to remove cross-reacting antibodies. For Western blots, the second antibody was alkaline phosphatase-conjugated goat anti-rabbit $F(ab')_2$ (Jackson Laborato-

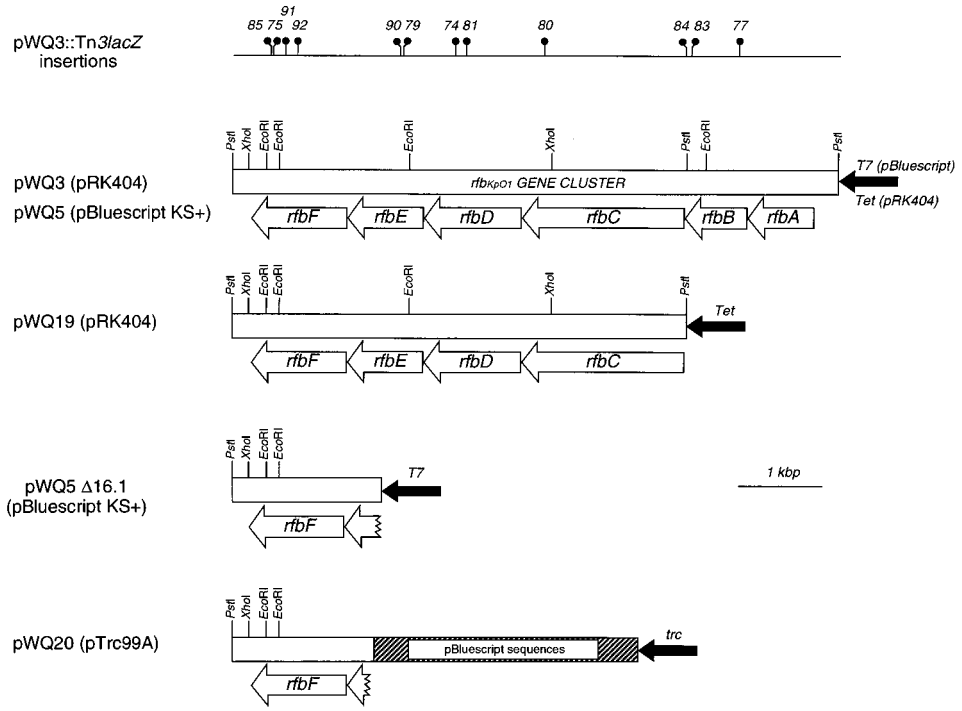

FIG. 1. Genetic organization of the rfb_{KpO1} gene cluster. The complete rfb_{KpO1} cluster is cloned in plasmids pWQ3 and pWQ5. Each of the pWQ3::Tn*3-lacZ* insertion mutations eliminates formation of S-LPS on the surface of the host cell. *E. coli* K-12 strains containing pWQ19 are able to synthesize D-galactan I, but the polymer remains in the cytoplasm; the products of $rfbA_{KpO1}$ and $rfbB_{KpO1}$ form an ABC transporter whose activity is required for transfer of O antigen across the cytoplasmic membrane (6). pWQ5 Δ16.1 was isolated from a deletion series in pWQ5 and was used to identify the $RfbF_{KpO1}$ polypeptide in T7 expression experiments. pWQ20 was constructed by ligation of a fragment that contained $rfbF_{KpO1}$ and that came from a deletion derivative into the expression vector pTrc99A. The vectors and promoters are indicated, but only insert sequences are shown.

ries), and the blots were developed with substrate composed of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

Structural analysis of LPS core oligosaccharides. For chemical analysis, LPS was extracted by a hot phenol-water extraction method (24). To isolate the core oligosaccharides, LPS samples were hydrolyzed in 1.5% acetic acid at 100°C for 2 h. Precipitated lipid A was removed by centrifugation, and the supernatants containing core oligosaccharides were lyophilized. The core oligosaccharide was purified by chromatography on a Sephadex G-50 gel filtration column (2.5 by 90 cm), with 0.05 M pyridinium acetate (pH 4.7) being used as the eluant. Fractions were monitored for hexoses by the phenol-sulfuric acid method (13) and for 2-keto-3-deoxyoctulosonic acid by the method of Aminoff et al. (3). To determine glycose content, polysaccharides were hydrolyzed with 1 M trifluoroacetic acid (for 16 h at 100°C), treated with sodium borodeuteride for 6 h at 37°C, and acetylated. The resulting alditol acetates were then quantitated by gas-liquid chromatography (18). Methylation analysis was performed by the method of Hakomori (19). Core oligosaccharide samples (2 to 5 mg each) were methylated and then hydrolyzed with 1 M trifluoroacetic acid (16 h at 100°C). After removal of the trifluoroacetic acid, the partially methylated glycoses were reduced with sodium borodeuteride, acetylated with Ac_2O, and analyzed by gas chromatography-mass spectrometry. Gas-liquid chromatography analysis was performed with a Hewlett-Packard model P5890 gas chromatograph fitted with a hydrogen-flame ionization detector and a fused-silica capillary column (0.3 mm by 25 m) containing 3% DB-17. The temperature was initially held at 180°C for 2 min, and this was followed by an increase of 4°C/min to 240°C.

Determination of galactopyranosyl (Gal*p*) transferase activity in membrane preparations. Membrane preparations were used as a source of enzyme to determine Gal*p* transferase activity in vitro. Bacteria from 200-ml mid-exponential-phase cultures were harvested, washed, and resuspended in 10 ml of 50 mM Tris-HCl (pH 8.0) containing 2 mM dithiothreitol and 30 mM magnesium acetate. The bacteria were lysed by ultrasonication, and unbroken cells were removed by centrifugation (5,000 × *g* for 10 min at 4°C). Membranes were collected as a pellet by ultracentrifuging the cell-free lysate at 180,000 × *g* for 1 h at 4°C and then were resuspended in 1.0 ml of 50 mM Tris-HCl (pH 8.0) containing 2 mM dithiothreitol and 30 mM magnesium acetate. Incorporation of radioactivity from UDP–D-[^{14}C]Gal*p* into lipid intermediates was measured by incubating samples for 20 min at 37°C. The reactions were performed in a total volume of 0.1 ml containing 50 mM Tris-HCl (pH 8.0), 30 mM magnesium acetate, 2 mM dithiothreitol, 32.5 pmol (25,000 cpm) of UDP-[^{14}C]Gal*p* substrate, and 250 μg of membrane protein. Lipid intermediates were extracted with chloroform-methanol (2:1). The extract was washed in pure solvent upper phase in a procedure described by Osborn et al. (42) and counted in a liquid scintillation counter with Ultima Gold (Canberra Packard).

Nucleotide sequence accession number. The DNA sequence for $rfbF_{KpO1}$ has been entered in GenBank as part of a block comprising $rfbCDEF_{KpO1}$ under the accession number L31762.

RESULTS

$RfbF_{KpO1}$-mediated modification of the LPS core in *E. coli* K-12. The rfb_{KpO1} cluster comprises six genes (Fig. 1). In *E. coli* K-12 strain AB1133(pWQ5), D-galactan I is added to the lipid A core to give a typical ladder of smooth LPS (S-LPS) (Fig. 2). pWQ19 contains the four rfb_{KpO1} genes *C* to *F*, whose products synthesize D-galactan I. In the absence of the ABC transporter composed of $RfbA_{KpO1}$ and $RfbB_{KpO1}$, D-galactan I accumulates in the cytoplasm (6) and *E. coli* AB1133 (pWQ19) retains the rough LPS of the parent strain (Fig. 2). Close inspection of SDS-PAGE gels showed that the lipid A core of *E. coli* AB1133(pWQ19) was more heterogeneous than that of the control, with additional core material migrating just above the predicted lipid A core fraction from AB1133. This extra fraction was absent in both *E. coli* AB1133 and AB1133(pWQ5) (Fig. 2). The gene(s) required for the lipid A core modification was investigated further by using a series of transposon insertion mutations in pWQ3. pWQ3 is a pRK404-based construct containing the same insert as pWQ5 (9), and the LPS phenotypes of *E. coli* AB1133(pWQ3) and AB1133 (pWQ5) are indistinguishable (8). Tn*3*HoHoI insertions located throughout the region cloned in pWQ3 eliminated synthesis of D-galactan I, and the additional LPS core band was not detected; pWQ3::Tn*3-lacZ91* provides a typical example

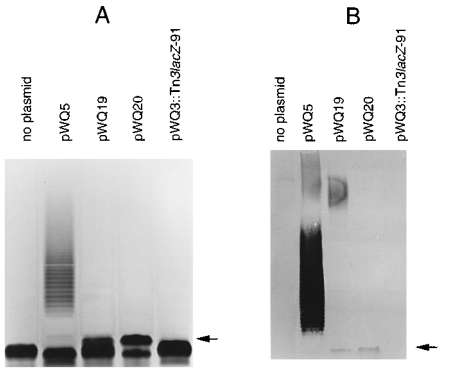

FIG. 2. $RfbF_{KpO1}$-mediated modification of the LPS core oligosaccharide of *E. coli* K-12. Whole-cell lysates were prepared for examination of LPS by SDS-PAGE (A) and Western immunoblotting (B). The plasmids are as indicated above each lane, and the host strain was *E. coli* AB1133. Only AB1133(pWQ5) containing the entire rfb_{KpO1} cluster makes S-LPS. Plasmids containing $rfbC\text{-}DEF_{KpO1}$ (pWQ19) or $rfbF_{KpO1}$ alone (pWQ20) result in an additional band migrating slightly slower than that of the lipid A core of AB1133 (indicated by arrows). Note that this band is not apparent when polymeric D-galactan I is synthesized and ligated to lipid A core in AB1133(pWQ5). In Western immunoblots, the rabbit anti-D-galactan I antiserum reacts with S-LPS in *E. coli* AB1133(pWQ5) and with the modified lipid A core bands. In *E. coli* AB1133(pWQ19), high-molecular-weight D-galactan I accumulates in the cytoplasm and is not ligated to the lipid A core (6). This high-molecular-weight material reacts with the antiserum.

(Fig. 2). The Tn*3-lacZ91* insertion is in the last gene in the cluster, $rfbF_{KpO1}$ (Fig. 1), suggesting that the core modification in *E. coli* AB1133(pWQ19) minimally required the $rfbF_{KpO1}$ gene product.

To directly test the involvement of $RfbF_{KpO1}$ in the core modification reaction, plasmid pWQ20 was constructed with $rfbF_{KpO1}$ cloned behind the *trc* promoter. $rfbF_{KpO1}$ is sufficient for the core modification, which is clearly evident by SDS-PAGE of LPS from *E. coli* AB1133(pWQ20) (Fig. 2). The amount of the additional core band was substantially higher in strains containing pWQ20; this could reflect either increased promoter strength or increased copy number in pWQ20 relative to that of pWQ19. The absence of the modified LPS in *E. coli* AB1133 containing plasmids with transposon insertions upstream of $rfbF_{KpO1}$ is explained by polarity effects on $rfbF_{KpO1}$ and provides direct support for rfb_{KpO1} being a single transcriptional unit.

Since the addition of a sugar or oligosaccharide to the core of *E. coli* K-12 provided an opportunity to potentially resolve the initial steps in D-galactan I synthesis, the properties of $RfbF_{KpO1}$ and the nature of the modification reaction were examined in further detail.

Identification of $RfbF_{KpO1}$ and relationship to RfpB from *Shigella dysenteriae* type 1. The nucleotide sequence for $rfbF_{KpO1}$ (8) shows a potential ATG initiation codon at position 4042 (nucleotide positions are the same as those in GenBank under accession no. L31762) that is preceded by a putative ribosome binding site. The preceding gene in the cluster, $rfbE_{KpO1}$, terminates at position 4029. The $rfbF_{KpO1}$ open reading frame consists of 1,134 nucleotides encoding a predicted polypeptide of 373 amino acids with a molecular mass of 42.6 kDa. The product of $rfbF_{KpO1}$ was identified directly by T7 expression experiments (63) using plasmid pWQ5 Δ16.1, which contains $rfbF_{KpO1}$ as the only intact gene (Fig. 1). A single polypeptide with an apparent M_r of 42,000 was visualized by SDS-PAGE (8), and this size is consistent with the size predicted by nucleotide sequence data.

Although the activity of $RfbF_{KpO1}$ has not been investigated previously, an RfbF homolog has been identified in the *rfb* cluster from *Serratia marcescens* serotype O16 ($RfbF_{SmO16}$) (62). This strain produces an O antigen of D-galactan I, and $RfbF_{SmO16}$ shares 57.6% identity (71.1% similarity) with $RfbF_{KpO1}$ (62). The predicted protein sequence for $RfbF_{KpO1}$ was also compared with known sequences (62), and similarity was detected for $RfbF_{KpO1}$ and RfpB (17), a galactosyl transferase involved in the synthesis of the *S. dysenteriae* type 1 O polysaccharide (15, 17, 29, 59). Both predicted proteins are 377 amino acid residues in length. The highest identity (37.7% identity and 52.4% similarity) was evident over the carboxy-terminal 122 amino acids. These data are consistent with the possibility that RfbF also encodes a galactosyl transferase.

Structure of the LPS core modification in *E. coli* K-12 expressing $RfbF_{KpO1}$. Rabbit anti-D-galactan I antiserum was used to probe Western immunoblots of LPS in whole-cell lysates (Fig. 2). A predictable strong reaction was obtained against D-galactan I-substituted S-LPS in *E. coli* AB1133(pWQ5). No high-molecular-weight immunoreactive material was detected in *E. coli* AB1133(pWQ20), but the modified core band reacted with the antiserum. There was no detectable reaction against the *E. coli* AB1133 host components. In contrast, whole-cell lysates from *E. coli* AB1133(pWQ19) showed a reaction against high-molecular-weight material in addition to the modified core band. The absence of an S-LPS ladder in *E. coli* AB1133(pWQ19) indicates that the high-molecular-weight immunoreactive material is not linked to the lipid A core but is consistent with the O antigen, which is known to accumulate in the cytoplasm when the ABC transporter function provided by $RfbAB_{KpO1}$ is missing (6). Since D-galactan I would not migrate in SDS-PAGE gels without a lipid modification, the most likely explanation for the high-molecular-weight fraction is that it represents undecaprenol-linked polymeric intermediates. SDS-PAGE has previously revealed similar intermediates involved in the biosynthesis of the *E. coli* O9 antigen (61). The immunoblots provide evidence that the modified core band resulting from the expression of $RfbF_{KpO1}$ shares epitopes with D-galactan I, and this was confirmed by chemical analysis.

The structure of the LPS core oligosaccharide from *E. coli* K-12 has been determined (21, 22). The LPSs from *E. coli* AB1133 and AB1133(pWQ20) were extracted, and the core oligosaccharides were purified. Composition analysis of the alditol acetate-derivatized sugars of *E. coli* AB1133 LPS showed the presence of heptitol, glucitol, galactitol, and *N*-acetylglucosaminitol in a molar ratio of 1:1.16:0.17:0.05. In AB1133(pWQ20), the molar ratio was 1:0.77:0.52:0.29, indicating significant increases in the amounts of both Gal and GlcNAc and a decrease in the amount of Glc relative to those of the control. Methylation analysis of the core oligosaccharide from AB1133(pWQ20) gave three additional derivatives which were absent from the control AB1133 sample: 2,3,5,6-tetra-methyl-D-Gal; 2,4,6-tri-methyl-D-Gal; and 2,4,6-tri-methyl-D-GlcNAc. This represents an additional terminal trisaccharide, Gal*f*-(1→3)-Gal*p*-(1→3)-GlcNAc-(1→), in the LPS core of *E. coli* K-12 expressing $RfbF_{KpO1}$. The absence of a detectable methylated GlcNAc derivative in AB1133 is consistent with the small amounts of GlcNAc identified by alditol acetate analysis. Although previous reports have identified terminal Glc*p*NAc residues in the core of *E. coli* K-12, the amount of this substituent is generally low (21, 22). Chemical data, together with the immunochemical reactions described above, indicate that the core oligosaccharide of AB1133(pWQ20) carries a single disaccharide repeat unit of D-galactan I linked to Glc*p*NAc in the trisaccharide structure shown in Fig. 3.

Expression of *rfpB* from *S. dysenteriae* type 1 in *E. coli* K-12

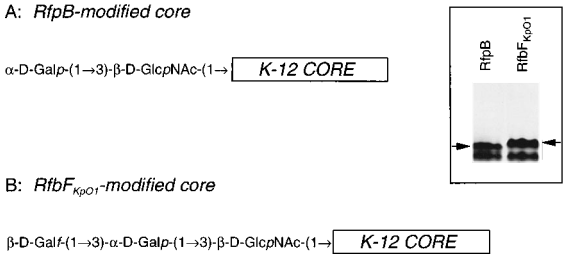

FIG. 3. Comparison of the LPS core modifications in *E. coli* K-12 strains expressing RfpB (A) and RfbF$_{KpO1}$ (B). The core modification in strains containing RfpB has been described elsewhere (15), and the structure of the modification mediated by RfbF$_{KpO1}$ was determined as described in the text. The inset shows SDS-PAGE gels of the core regions of *E. coli* AB1133(pJK2363; *rfpAB*$^+$) and AB1133(pWQ20; *rfbF*$_{KpO1}$$^+$). The difference in the sizes of the modified core bands (indicated by the arrows) is predicted by the chemical structures. Samples for SDS-PAGE were prepared as whole-cell lysates.

has been shown to result in modification of the core oligosaccharide by addition of a single α-D-Gal*p* residue (1→3) linked to β-D-GlcNAc (15) (Fig. 3). The RfpB-modified core therefore differs from the RfbF$_{KpO1}$-modified core by the absence of the terminal D-Gal*f* residue. The difference in one sugar residue is apparent in the sizes of the respective lipid A cores by SDS-PAGE; the upper band of the lipid A core of *E. coli* AB1133(pWQ20) migrates more slowly than that of AB1133 (pJK2363; *rfpAB*$^+$) (Fig. 3).

LPS core modification in *E. coli* K-12 expressing *rfbF*$_{KpO1}$ involves an Rfe-dependent reaction. Formation of D-galactan I is known to be Rfe dependent (9), although the precise role of Rfe in the process has not been established. An Rfe-dependent process is also involved in addition of the Gal*p* residue to the core oligosaccharide in *E. coli* K-12 strains expressing RfpB (29). Rfe is a Glc*p*NAc-1-P transferase (37) and presumably catalyzes the transfer, to a lipid intermediate, of the preceding Glc*p*NAc residue in the type 1 O repeat unit. The similarities in RfbF$_{KpO1}$ and RfpB, and the modification reactions they perform, suggested a similar role for Rfe in D-galactan I initiation. In the absence of Rfe activity, *E. coli* 21548 (*rfe*:Tn*10*-48) containing pWQ20 (*rfbF*$_{KpO1}$$^+$) lacks the LPS core modification (Fig. 4). The RfpB-mediated core modification was also

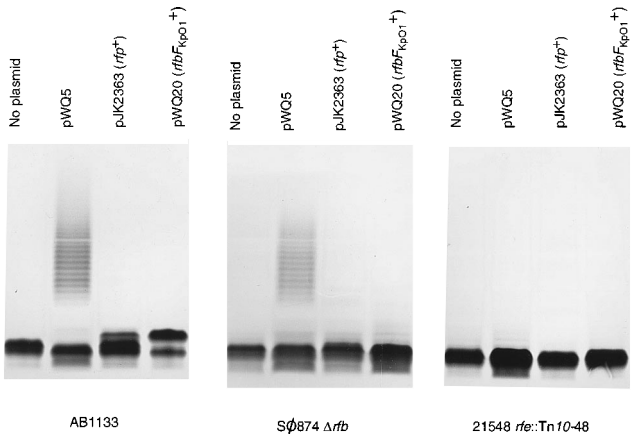

FIG. 4. Role of Rfe and cryptic *E. coli* K-12 *rfb* functions in the modification of the lipid A core mediated by RfbF$_{KpO1}$ and RfpB. LPS samples from whole-cell lysates were examined by SDS-PAGE. The plasmids used are indicated above each lane, and the strains and their important characteristics are given below each panel. Note that RfpB- and RfbF$_{KpO1}$-mediated core modifications are absent in strains SØ874 and 21548. Synthesis of D-galactan I is dependent on Rfe but not on host Rfb proteins.

TABLE 2. Galactopyranosyl (Gal*p*) transferase activity in membranes prepared from *E. coli* CS1883 and its derivatives containing pWQ20 and pJK2363

Strain and relevant property	Plasmid	Gal*p* transferase activity (pmol/mg/20 min)[a]
CS1883 Δ*galE*	None	0.25
CS1883 Δ*galE*	pWQ20	2.56
CS1883 Δ*galE*	pJK2363	1.37
CWG263 Δ*galE rfe*::Tn*10*	None	0.20
CWG263 Δ*galE rfe*::Tn*10*	pWQ20	0.15
CWG263 Δ*galE rfe*::Tn*10*	pJK2363	0.16

[a] Gal*p* transferase activity measures the ability of membrane preparations to incorporate radioactivity from UDP–D-[^{14}C]Gal*p* into lipid intermediates. Reactions were performed in a total volume of 0.1 ml containing 50 mM Tris-HCl (pH 8.0), 30 mM magnesium acetate, 2 mM dithiothreitol, 32.5 pmol (25,000 cpm) of UDP-[^{14}C]Gal*p* substrate, and 250 μg of membrane protein. The reaction mixtures were incubated for 20 min at 37°C. Incorporation of UDP–D-[^{14}C]Gal*p* into the lipid-linked oligosaccharides was determined by extraction with chloroform-methanol (2:1).

absent in the control *E. coli* 21548(pJK2363; *rfpAB*$^+$), as was expected (Fig. 4). The initial steps in the assembly of D-galactan I in *K. pneumoniae* O1 and the type 1 O antigen of *S. dysenteriae* are therefore conserved. The requirement for Rfe is specific and not a requirement for enterobacterial common antigen biosynthesis in general, since other enterobacterial common antigen-deficient strains with *rff* mutations and wild-type *rfe* do show the core modification (8).

The involvement of RfbF$_{KpO1}$ in the formation of lipid-linked intermediates was examined with membrane preparations and UDP–D-[^{14}C]Gal*p* as the labelled precursor. The membrane preparations were made using an *E. coli* CS1883 (*galE*) host strain to eliminate nonspecific background incorporation due to the conversion of UDP–D-[^{14}C]Gal*p* to UDP–D-[^{14}C]Glc*p*. A comparison of control membranes with membranes from *E. coli* CS1883 containing pWQ20 and pJK2363 showed increases in Gal*p* transferase activity of approximately 10- and 5-fold, respectively (Table 2). No Gal*p* transferase activity was detected in membranes from *E. coli* CWG263 (*rfe*::Tn*10*-48) containing either plasmid. These data confirm both the involvement of a lipid-linked biosynthetic intermediate in the synthetic pathway and the specific requirement for a preceding Glc*p*NAc-1-P transfer (catalyzed by Rfe) in the formation of the Gal*p*-containing intermediate.

RfbF$_{KpO1}$ and RfpB both form a lipid intermediate with the structure Gal*p*-(1→3)-Glc*p*NAc-P-P-lipid. In the case of RfbF$_{KpO1}$, this may be a transient product. This suggested that RfbF$_{KpO1}$ might potentially replace RfpB in the synthesis of the *S. dysenteriae* type 1 O antigen. Plasmid pSS37 carrying *rfpAB* and the *rfb* gene cluster from *S. dysenteriae* type 1 was constructed by Sturm and Timmis (60) and was shown to direct formation of type 1 O antigen in *E. coli* K-12 (Fig. 5). In pSS37::Tn*lacZ*-T42, *rfpB* is inactivated and no O antigen is synthesized (29). Introduction of either pWQ20 (*rfbF*$_{KpO1}$$^+$) or the pJK2363 (*rfpAB*$^+$) control into *E. coli* AB1133(pSS37::Tn*lacZ*-T42) restores type 1 O antigen expression (Fig. 5), indicating that the initial reaction catalyzed by RfbF$_{KpO1}$ provides an intermediate which can be utilized by the *S. dysenteriae* type 1 Rfb proteins to complete the formation of the type 1 O antigen.

Modification of the LPS core in *E. coli* K-12 expressing *rfbF*$_{KpO1}$ requires functions encoded by the *E. coli* K-12 *rfb* gene cluster. Formation of D-galactan I polymer occurs in the cytoplasm, and an ABC transporter is required for the export

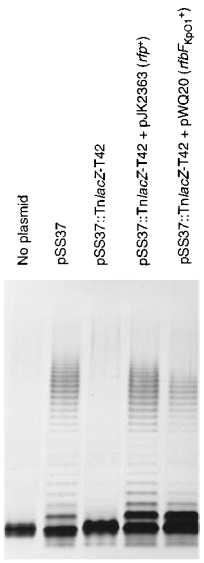

FIG. 5. $RfbF_{KpO1}$ can functionally replace RfpB in the synthesis of *S. dysenteriae* type 1 O antigen. LPS samples from whole-cell lysates were examined by SDS-PAGE. The plasmids used are indicated above each lane, and the host strain was *E. coli* AB1133. The formation of authentic type 1 O antigen in strains with S-LPS was confirmed by reaction with commercial type 1 typing antiserum (8).

of the polymer and subsequent ligation to the lipid A core (6). For the $RfbF_{KpO1}$-mediated core modification to occur, the lipid-linked oligosaccharide intermediate must be available at the periplasmic face for ligation. This was not expected, since there is normally no requirement in the D-galactan I pathway for the transfer of unpolymerized intermediates across the plasma membrane. The possible involvement of host Rfb proteins was therefore assessed in the expression of the Rfe-RfbF-synthesized intermediate. Klena and Schnaitman (29) have previously reported that the RfpB-mediated core modification is reduced in an *E. coli* Δ*rfb* strain, suggesting the involvement of an additional host Rfb protein unrelated to either precursor formation or transferase activity. Plasmid pJK2363 therefore provided a control for the analysis of $RfbF_{KpO1}$ activity. In strain SØ874 Δ*rfb*, all modifications of the core by both $RfbF_{KpO1}$ and RfpB are eliminated (Fig. 4). However, despite the obvious effect of the Δ*rfb* background on these modifications, biosynthesis of complete S-LPS containing polymeric D-galactan I is unaffected in *E. coli* SØ874(pWQ5) (Fig. 4).

DISCUSSION

In the biosynthesis of the type 1 O polysaccharide of *S. dysenteriae*, Rfe transfers the initial sugar (Glc*p*NAc) in the formation of the repeat unit. This is followed by the transfer of an α-D-Gal*p* residue by RfpB and completion of the repeating unit by the remaining *S. dysenteriae* type 1 Rfb proteins (15, 29). Assembly involves glycosyl transfer to a lipid-linked intermediate (17). The pathway for *S. dysenteriae* O polysaccharide biosynthesis therefore resembles the well-documented system described for *Salmonella enterica* (reviewed in reference 68) in which O repeat units are synthesized in blocks attached to undecaprenyl pyrophosphoryl carriers and then polymerized in a reaction which requires Rfc (29, 40).

The biosynthesis pathway for D-galactan I in *K. pneumoniae* O1 is different. The rfb_{KpO1} gene cluster does not encode a homolog of Rfc, and Rfe activity is confined to the initiation of the polymer. Rfb_{KpO1} enzymes then assemble D-galactan I, with lipid-linked Glc*p*NAc being used as a primer. The lipid moiety has not been identified, but in other bacterial systems, the active lipid is undecaprenol phosphate (71). $RfbF_{KpO1}$ represents the first enzyme characterized in the formation of the D-galactan I polymer. The known activity of Rfe (37), structural and immunochemical analysis of $RfbF_{KpO1}$-modified LPS, and in vitro Gal*p* transferase activity in strains containing cloned $rfbF_{KpO1}$ are all consistent with the following reaction sequence: (i) lipid-P + UDP-Glc*p*NAc→Glc*p*NAc-P-P-lipid + UMP; (ii) Glc*p*NAc-P-P-lipid + UDP-Gal*p*→Gal*p*-(1→3)-Glc*p*NAc-P-P-lipid + UDP; and (iii) Gal*p*-(1→3)-Glc*p*NAc-P-P-lipid + NDP-Gal*f*→Gal*f*-(1→3)-Gal*p*-(1→3)-Glc*p*NAc-P-P-lipid + NDP (in which NDP is a nucleoside diphosphate). Reaction i is identical to the initial Rfe transferase reaction in enterobacterial common antigen biosynthesis (37) and proposes a role for Rfe which is similar to that described for the biosynthesis of the O polysaccharides of *E. coli* O8 (49) and O9 (27). Reaction ii is catalyzed by both RfpB and $RfbF_{KpO1}$. Since strains expressing $RfbF_{KpO1}$ synthesize a disaccharide which contains both Gal*p* and Gal*f* residues and which is transferred to the lipid A core, it is likely that Gal*f* residues are added to the lipid intermediate in reaction iii. At present, this additional reaction cannot be demonstrated in vitro, because the identity of the Gal*f* precursor is not known and it is not available for biosynthetic experiments. It is thought that this precursor is UDP-Gal*f* (52). This precursor can be synthesized in *E. coli* K-12 hosts by enzymes coded for by the cryptic *rfb* gene cluster, since the original O antigen of K-12 strains contained Gal*f* residues (58). The *E. coli* K-12 *rfb*-encoded Gal*f* transferase has a different specificity to $RfbF_{KpO1}$ and plays no role in the LPS core modifications reported here. Gal*f* transfer only occurs in strains expressing $RfbF_{KpO1}$ and not in those containing RfpB, despite the common Gal*p*-(1→3)-Glc*p*NAc-P-P-lipid intermediate formed by both enzymes.

The D-galactan I synthesis system shares important features with the *E. coli* O9 and group II capsule systems. First, all require an initiation reaction which involves an enzyme different from that involved in the polymerization steps. In the group II capsules, the initiation step is not known (57), and in the synthesis of the O9 polysaccharide, a discrete mannosyl transferase initiates synthesis by transferring a single mannose residue to the Rfe-activated lipid intermediate (27). Second, all are synthesized at the cytoplasmic face of the plasma membrane by a pathway which requires an ABC transporter to transfer the polymer across the plasma membrane (6, 7, 27, 44, 54). In the case of the O9 polymer (27) and group II capsules (16, 56), synthesis involves a rapid processive addition of glycosyl residues to the growing polymer. The polymerization step(s) has not yet been resolved for D-galactan I, but it is predicted to follow a similar mechanism. This question can only be unequivocally addressed once the functions of the three remaining Rfb_{KpO1} proteins (C, D, and E) are assigned and the Gal*f* precursor is available in quantities sufficient for in vitro synthesis experiments.

The properties of $RfbF_{KpO1}$ suggest that this enzyme may be bifunctional with respect to substrate specificity. In the synthesis of bacterial cell surface polysaccharides, there are a number of enzymes which exhibit more than one activity. Most often, these involve the formation of two or more linkages. Examples include the polysialyltransferase required for assembly of the K92 group II capsule of *E. coli* (57, 66) and mannosyl transferases involved in the polymerization of the *E. coli* O9 O antigen (27). Glycosyl transferase enzymes with dual substrate specificity have been described in the formation of heparin (33–35) and the synthesis of capsular hyaluronic acid polymers in streptococci (10).

Two other galactosyl transferases which are involved in O antigen synthesis have been described. These are the *S. enterica* RfbP ($RfbP_{Se}$) Gal*p* transferases involved in O antigen assembly in *S. enterica* serogroups A, B, and D (23, 67) and RfpB (15, 17) from *S. dysenteriae* type 1. Both of these Gal*p* transferases contain putative membrane-spanning domains. $RfbP_{Se}$ initiates the formation of O antigen repeat units by transferring Gal*p*-1-phosphate residues from UDP-Gal*p* to undecaprenyl phosphate in a reversible reaction. The transmembrane segments of $RfbP_{Se}$ may therefore reflect a requirement to interact directly with undecaprenyl phosphate. Indeed, the secondary structure of $RfbP_{Se}$ has been suggested to resemble that of Rfe, which performs an analogous reaction (29). There is no obvious similarity between the predicted $RfbP_{Se}$ and $RfbF_{KpO1}$ polypeptides (8). The common structure of $RfbF_{KpO1}$ and RfpB reflects the fact that both have Gal*p* transferase activity and both must recognize lipid-P-P-Glc*p*NAc as an acceptor. Although $RfbF_{KpO1}$ lacks transmembrane segments, the active enzyme is membrane associated, and this may be related to the basic pI (8.93) of the predicted protein. $PhoA^+$ fusions indicate that RfpB has three potential transmembrane segments with a cytoplasmic carboxy terminus (17). Given the possible bifunctional activity of $RfbF_{KpO1}$ and the putative transmembrane domains of RfpB, it is perhaps not surprising that the similarity between these two enzymes is limited to the carboxy terminus. The ability of $RfbF_{KpO1}$ to functionally replace RfpB in the synthesis of the *S. dysenteriae* type 1 O polysaccharide indicates that transmembrane segments are not essential for integration into the type 1 biosynthetic enzyme complex. Cooperation among heterologous *rfb* gene clusters has been demonstrated previously by others (30, 72). However, these interactions resulted in novel hybrid O antigens, and the authentic O antigens of both contributors are thought to be synthesized by Rfc-dependent pathways. This is the first example, to our knowledge, of complementation by Rfb enzymes from two mechanistically different synthesis pathways to give an authentic O antigen.

While expression in *E. coli* K-12 facilitates the characterization of $RfbF_{KpO1}$ activity, it is clear that the trans-plasma membrane export reactions for the oligosaccharide in *E. coli* AB1133(pWQ20) differ from those for polymeric D-galactan I that occur when the entire rfb_{KpO1} cluster is present. *E. coli* AB1133 containing the complete rfb_{KpO1} cluster makes S-LPS and no significant band representing the core modified with a single D-galactan I repeat unit (Fig. 2). The $RfbF_{KpO1}$-mediated modification is eliminated in an *E. coli* Δ*rfb* host (Fig. 4), suggesting that transport of the oligosaccharide intermediate is a fortuitous result of recognition by components of the cryptic *rfb* cluster in *E. coli* K-12. Although the gene(s) for the enzyme(s) involved in UDP-Gal*f* synthesis is located in *rfb* (58), determinants for the synthesis of both UDP-Gal*p* and UDP-Glc*p*NAc map outside the *rfb* region (5). The host *rfb* deletion should therefore have no effect on Gal*p* transferase activity. The most likely host component involved in the export process is RfbX; rfb_{Sdl} (29), *S. enterica rfb* clusters (46), and the cryptic *rfb* cluster in *E. coli* K-12 ($rfb_{K\text{-}12}$) (58, 72) all encode an RfbX homolog. RfbX is a transmembrane protein required for the modification of the *E. coli* LPS core by RfpB (29), and the *E. coli* AB1133 $RfbX_{K\text{-}12}$ homolog can functionally replace the $RfbX_{Sdl}$ protein (29). The Δ*rfb* background eliminates all modification of the core by both RfpB and $RfbF_{KpO1}$. Taken together, these observations indicate that host RfbX may also play a similar role in the export of the lipid-linked oligosaccharide in *E. coli* AB1133(pWQ20). In contrast, authentic D-galactan I-substituted S-LPS is transported by an ABC transporter, requires no host *rfb* functions, and is unaffected in *E. coli* Δ*rfb* hosts.

It is interesting that each of the enzymes responsible for the first committed steps of O antigen biosynthesis in *K. pneumoniae* O1, *E. coli* O9 (27), and *S. enterica* (47) is coded for by the last gene in the respective chromosomal *rfb* cluster. The significance of this observation is currently unknown.

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