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Relationships between *rfb* Gene Clusters Required for Biosynthesis of Identical D-Galactose-Containing O Antigens in *Klebsiella pneumoniae* Serotype O1 and *Serratia marcescens* Serotype O16

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The lipopolysaccharide O antigens of *Klebsiella pneumoniae* serotype O1 and *Serratia marcescens* serotype O16 both contain a repeating unit disaccharide of [→3)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→]; the resulting polymer is known as D-galactan I. In *K. pneumoniae* serotype O1, the genes responsible for the synthesis of D-galactan I are found in the *rfb* gene cluster (rfb_{KpO1}). We report here the cloning and analysis of the *rfb* cluster from *S. marcescens* serotype O16 (rfb_{SmO16}). This is the first *rfb* gene cluster examined for the genus *Serratia*. Synthesis of D-galactan I is an *rfe*-dependent process for both *K. pneumoniae* serotype O1 and *S. marcescens* serotype O16. Hybridization experiments with probes derived from each of the six rfb_{KpO1} genes indicate that the cloned rfb_{SmO16} cluster contains homologous genes arranged in the same order. However, the degree of homology at the nucleotide sequence level was sufficiently low that hybridization was detected only under low-stringency conditions. $rfbAB_{SmO16}$ genes were subcloned and shown to encode an ABC-2 (ATP-binding cassette) transporter which is functionally identical to the one encoded by the corresponding *rfb* genes from *K. pneumoniae* serotype O1. The amino acid sequences of the predicted RfbA and RfbB homologs showed identities of 75.7% (87.9% total similarity) and 78.0% (86.5% total similarity), respectively. The last gene of the rfb_{KpO1} cluster, $rfbF_{KpO1}$, encodes a bifunctional galactosyltransferase which initiates the formation of D-galactan I. $RfbF_{KpO1}$ and $RfbF_{SmO16}$ are 57.6% identical (with 71.1% total similarity), and both show similarity with RfpB, the galactosyltransferase involved in the synthesis of *Shigella dysenteriae* type I O-polysaccharide. The G+C contents of the *rfbAB* genes from each organism are quite similar, and values are lower than those typical for the species. However, the G+C content of $rfbF_{SmO16}$ (47.6%) was much higher than that of $rfbF_{KpO1}$ (37.3%), despite the fact that the average for each species (52 to 60%) falls within the same range.

Lipopolysaccharide (LPS) is located in the outer leaflet of gram-negative bacteria and is responsible for the biological activity of endotoxin. In enteric bacteria, this molecule consists of three regions: lipid A, the core oligosaccharide, and the O polysaccharide. Lipid A forms the outer leaflet of the outer membrane bilayer. The core oligosaccharide region is attached to lipid A, and the O polysaccharide extends outward from the core region. O polysaccharides are composed of repeating oligosaccharide units with variable numbers of sugar residues. There is extensive structural diversity within O polysaccharides (22, 64). The composition, sequence, linkage, and substitution of the individual monomer residues within a repeating unit is characteristic for a given LPS and the parental bacterial strain. Thus, the O polysaccharide gives rise to epitopes (O antigens) which contribute to the immunological specificity of LPS.

Klebsiella pneumoniae serotype O1 produces a high-molecular-weight smooth LPS (S-LPS). The high-molecular-weight S-LPS contains two structurally distinct O polysaccharide domains composed of the repeating units [→3)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→] (D-galactan I) and [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→] (D-galactan II) (26, 27, 63). Genetic (63) and chemical (26) data indicate that D-galactan I is attached to lipid A-core, with D-galactan II attached to the distal end of a proportion of available D-galactan I chains. LPS O polysaccharides may contribute to the virulence of a bacterium by enabling the bacterium to resist serum killing (18, 56). D-Galactan II is responsible for the serum resistance of *K. pneumoniae* O1 (34, 63). *K. pneumoniae* O1 mutants that produce only D-galactan I were found to be serum sensitive (34). Sensitivity either can be attributed to loss of D-galactan II or may be a function of altered O-chain length in the mutant. Polysaccharide structures similar to those in O1 are found in a number of serotypes of *Klebsiella* and other bacteria. In *K. pneumoniae* O8, O acetylation of D-galactan I creates a novel antigenic type (20). Strains belonging to serotype O2 produce D-galactan I either as the sole O polysaccharide (serotype O2a) or in combination with an additional polymer (O2a, 2c) (62). Variants of D-galactan I with side chain D-galactose substituents are found in other O2 subgroups and in serotype O9 (19, 30). The D-galactan I polymer is also produced by *Pasteurella haemolytica* serogroups 4 (41) and 10 (46), *Serratia marcescens* serotypes O16 and O20 (40), and *Serratia plymuthica* (3).

In *K. pneumoniae* serotype O1, the enzymes involved in biosynthesis of D-galactan I are encoded by genes in the *rfb* cluster (7). This gene cluster is found near the *his* genes, as are other *rfb* clusters in enteric bacteria (31, 50, 64). The *rfb* cluster from *K. pneumoniae* serotype O1 contains six genes. $rfbA_{KpO1}$ and $rfbB_{KpO1}$ encode an ATP-binding cassette (ABC) transporter required for transport of polymerized O polysaccharide across the cytoplasmic membrane (5). $RfbA_{KpO1}$ is the integral membrane component, and $RfbB_{KpO1}$ contains an ATP-binding site motif. Synthesis of D-galactan I therefore occurs in the cytoplasm, and ligation to lipid A-core occurs following trans-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant property	Source or reference
S. marcescens		
O16	Production of D-galactan I and an additional Rib*f*-containing polymer	40
O20	Production of D-galactan I	40
E. coli		
DH5α	K-12 ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 F^-	48
LE392	K-12 *hsdR514* ($r_K^- m_K^-$) *supE44 supF58 lacY1* or Δ(*lac proAB*)*6 galK2 galT22 metB1 trpR55* λ^- F^-	48
JM109 DE3	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	55
CLM4	K-12 *lacZ trp* Δ*sbc-B-rfb upp rel rpsL* Δ(*recA-srl*)*306*	33
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*	36
21548	AB1133 but *rfe*::Tn*10*-48	36
Plasmids		
pEMBLCos4	Cosmid cloning vector; Ap^r	12
pBluescript SK(+)	Cloning vector; Ap^r	Stratagene
pWQ5	pBluescript KS(+) containing a 7.2-kbp insert including the rfb_{KpO1} gene cluster cloned from *K. pneumoniae* O1:K20; Ap^r	5
pWQ25	31-kbp pEMBLCos 4 cosmid clone containing *S. marcescens* O16 DNA and including rfb_{SmO16}; Ap^r	This study
pWQ26	pBluescript SK(+) containing partial rfb_{SmO16} cluster contained on an 8-kbp *Pst*I fragment; Ap^r	This study
pWQ27	pBluescript SK(+) containing complete rfb_{SmO16} cluster contained on a 9.6-kbp *Xho*I fragment; Ap^r	This study
pWQ28	pBluescript SK(+) containing $rfbAB_{SmO16}$ on a 4.05-kbp *Bam*HI fragment, cloned from pWQ27; Ap^r	This study

location to the periplasmic face of the cytoplasmic membrane. This pathway is therefore fundamentally different from the well-characterized system used to assemble LPS in strains of *Salmonella enterica*. In *S. enterica*, both polymerization and ligation of O antigen occur at the periplasmic face of the cytoplasmic membrane (44, 50, 64).

The relationships and diversity among *rfb* gene clusters are intriguing. Of particular interest is the question of evolutionary origin of *rfb* clusters in different bacterial species which produce identical O antigen structures. To address this question, we have cloned the *rfb* gene cluster from *S. marcescens* serotype O16 (rfb_{SmO16}) and compared its genetic organization with that of the *rfb* cluster from *K. pneumoniae* serotype O1 (rfb_{KpO1}).

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely grown in Luria-Bertani medium. Media were supplemented, where required, with ampicillin (100 μg/ml). Cultures were grown at 37°C. The type strains for *S. marcescens* O16 and O20 (40) are those used for O antigen structure determinations. These strains were obtained from H. Auken (Central Public Health Laboratory, London, United Kingdom). Bacteriophage Ffm lyses *Escherichia coli* K-12 strains which contain rough LPS (R-LPS) core (65).

Cloning of the rfb_{SmO16} gene cluster from *S. marcescens* serotype O16. A cosmid gene library was constructed with *Sau*3A-digested chromosomal DNA obtained from a *S. marcescens* serotype O16 cultures. Size-fractionated 35- to 40-kbp DNA fragments were ligated with *Pvu*II-*Bam*HI-digested pEMBLCos4 (12). The ligation mixtures were packaged into lambda particles by using a commercial packaging extract (Packagene; Promega, Madison, Wis.), and the resultant recombinant phages were used to infect *E. coli* LE392. pWQ25, which contains the putative rfb_{SmO16} gene cluster, was identified by Ffm selection as described previously for the *rfb* clusters from *K. pneumoniae* O1 (7) and *E. coli* O9 (17). *E. coli* LE392 has R-LPS, and the bacteriophage Ffm recognizes the LPS core, resulting in cell lysis. If the R-LPS is sufficiently masked by O polysaccharide, the bacteria are resistant to Ffm. The gene library in *E. coli* LE392 was screened by dilution plating to obtain single colonies on plates seeded with 0.1 ml of Ffm stock (approximately 1×10^9 PFU/ml). Ffm-resistant strains were isolated and examined for whole-cell agglutination with the monoclonal antibody (MAb) O1-2.6, which is specific for D-galactan I epitopes (63). The S-LPS phenotype was then confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

LPS analysis and SDS-PAGE. LPS from proteinase K-digested whole-cell lysates was isolated from stationary-phase cultures, as described by Hitchcock and Brown (15). The SDS-PAGE gel composition and electrophoretic conditions were those described by Darveau and Hancock (8). LPS gels were silver stained by using the procedure of Tsai and Frasch (57).

DNA manipulation. Chromosomal DNA was prepared from *S. marcescens* by the method described by Hull et al. (16). To eliminate the endogenous nuclease activity in this bacterium, cells were first resuspended in 10 mM NaCl to an A_{578} of 6.0 and heated for 2.5 min at 65°C (42). After being heated, the cells were washed twice in 10 mM NaCl and the DNA was then extracted. Plasmid DNA was purified by an alkaline lysis method (48). Cosmid pWQ25 and all other subclones were transferred into *E. coli* strains by transformation (32) or by electroporation, using a Bio-Rad Gene pulser (4).

In hybridization experiments, DNA fragments in agarose gels were depurinated, denatured, and neutralized prior to Southern transfer (48). DNA fragments were transferred by overnight capillary blotting onto positively charged nylon membranes (Boehringer Mannheim), with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer. DNA fragments were cross-linked to the membranes with a Stratagene UV Stratalinker 1800. Prehybridization was performed with a solution of 5× SSC, 2% blocking reagent (Boehringer Mannheim), 30% formamide (low-stringency conditions) or 50% formamide (high-stringency conditions), 0.1% *N*-lauroylsarcosine, and 0.02% SDS. Incubation was conducted at either room temperature (low-stringency conditions) or 42°C (high-stringency conditions), for 3 h. Hybridization was carried out overnight. The membranes were washed under either low-stringency conditions (three 20-min washes in 2× SSC–0.1% SDS at room temperature) or high-stringency conditions (three 5-min washes in 2× SSC–0.1% SDS at room temperature followed by two 15-min washes in 0.1× SSC–0.1% SDS at 68°C).

DNA fragments for gene probes were isolated from agarose gels and were purified with GENE CLEAN (Bio/Can Scientific, Mississauga, Ontario, Canada). DNA probes were digoxigenin labeled with a kit from Boehringer Mannheim. PCR amplification was used to make specific gene probes from each gene in the rfb_{KpO1} cluster. Ten nanograms of plasmid pWQ5 DNA was used as the template. Twenty nanograms of each oligonucleotide primer (Table 2) was used. Digoxigenin-labeled deoxynucleoside triphosphates from the Boehringer Mann-

TABLE 2. Oligonucleotide primers used in PCR amplification for the generation of gene probes

Primer	Sequence[a]	Description	Size of amplified product (bp)
P3	5′-CCTCATTACCGGCTTGT	$rfbA_{KpO1}$ forward	
P12	5′-CATCAGCATAATCCCCA	$rfbA_{KpO1}$ reverse	332
ORF2-1	5′-AGGCTTTCATCCTGAACT	$rfbB_{KpO1}$ forward	
ORF2-2	5′-GCTAACAAACAATATTGT	$rfbB_{KpO1}$ reverse	301
DBON 14	5′-ATGATATCGTCGTACTC	$rfbC_{KpO1}$ forward	
DBON 17	5′-CAATCGAAGCCGCTAAC	$rfbC_{KpO1}$ reverse	796
DBON 6	5′-TTGCTGATCCACAAAACT	$rfbD_{KpO1}$ foward	
DBON 5	5′-ATTCTCGGCCAGTGACA	$rfbD_{KpO1}$ reverse	600
DBON 12	5′-TCACAGCTGGATAAGCG	$rfbE_{KpO1}$ forward	
DBON 4	5′-GTAGTCAAGACTGGTCG	$rfbE_{KpO1}$ reverse	223
DBON 2	5′-TTGTATTGTTTGCCAGC	$rfbF_{KpO1}$ forward	
DBON 1	5′-GTGCCTTTAGGATTGCTA	$rfbF_{KpO1}$ reverse	421

[a] The DNA sequence on which these primers are based is available in GenBank under accession numbers L31775 ($rfbA_{KpO1}$-$rfbB_{KpO1}$) and L31762 ($rfbC_{KpO1}$-$rfbF_{KpO1}$).

heim labeling kit were also included at a final concentration of 1 mM. Amplification involved 40 cycles, each consisting of (i) a denaturation step of 2 min at 95°C, (ii) an annealing step of 2 min at 42°C, and (iii) a polymerization step of 2 min at 72°C. The PCR product was precipitated with ethanol and used directly in the hybridization experiments.

Hybridization reactions were developed with 3-(2′ spiroadamantane)-4-methoxy-4-(3″ phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) chemiluminescent substrate used according to the manufacturer's recommendations.

Plasmid DNA sequencing was performed either by the dideoxy chain termination method (49) with Sequenase version 2.0 (U.S. Biochemical Corp.) or by automated sequencing with an Applied Biosystems machine (performed by Mobix, McMaster University, Hamilton, Ontario, Canada). Both strands were sequenced. Custom oligonucleotide primers were synthesized using an Applied Biosystems oligonucleotide synthesizer (model 391-EP) or were purchased from either Amitof (Boston, Mass.) or Mobix.

Identification of plasmid-encoded gene products. DNA containing either rfb_{KpO1} or rfb_{SmO16} was cloned into pBluescript and transformed into *E. coli* JM109 DE3. This strain contains the T7 RNA polymerase gene under the control of the *lac* promoter. Exclusive expression of *rfb* genes was obtained by inducing the T7 RNA polymerase with IPTG (isopropyl-β-D-thiogalactopyranoside), in the presence of rifampin to inhibit host RNA polymerase. Rfb proteins were labeled with [^{35}S]methionine in vivo. The methods were modified from those of Tabor and Richardson (55) and are described elsewhere (5). Samples were separated by SDS-PAGE with a 12% acrylamide resolving gel used as described by Laemmli (28). Gels were fixed, treated with Amplify (Amersham International), and dried prior to autoradiography.

Sequence analysis. Computer analysis of nucleotide and amino acid sequences was performed with the NALIGN, PALIGN, and CLUSTAL programs supplied with PC/GENE version 6.7 (Intelligenetics, Inc., Mountain View, Calif.). A BLAST (Basic Alignment Search Tool) server analysis program (2) was used to search the nucleotide and protein sequence databases of the National Center for Biotechnology Information.

Nucleotide sequence accession number. The nucleotide sequence reported here for $rfbF_{SmO16}$ is available as accession number L34167 from GenBank. The sequence for the region containing $rfbA_{SmO16}$ and $rfbB_{SmO16}$ is entered as accession number L34166.

RESULTS AND DISCUSSION

Cloning of the rfb_{SmO16} cluster from *S. marcescens* serotype O16. The O polysaccharides of *S. marcescens* O16 and O20 both contain D-galactan I (40). Three fragments spanning the rfb_{KpO1} gene cluster from *K. pneumoniae* serotype O1 were used to probe genomic DNAs from *S. marcescens* serotypes O16 and O20 to detect homologous sequences. An internal 3.2-kbp *Eco*RI fragment from pWQ5 contained only rfb_{KpO1} DNA, whereas two other probes (5.6- and 1.85-kbp *Pst*I fragments) contained some additional DNA flanking the cluster (Fig. 1). The genomic DNAs were digested with several restriction enzymes, and the digests were examined by hybridization with the rfb_{KpO1} probes. No signals were detected in Southern hybridization experiments performed at high stringency (54). However, at low stringency, all three probes detected an 8-kbp *Pst*I fragment from *S. marcescens* serotype O16 and a 7-kbp *Pst*I fragment from *S. marcescens* serotype O20 (Fig. 2). These results suggest that the *rfb* genes in *S. marcescens* are clustered on the chromosome.

To clone the rfb_{SmO16} cluster, fragments from *Sau*3A partial digests of *S. marcescens* serotype O16 genomic DNA were ligated with pEMBLCos4 and packaged into LE392. This cloning procedure generated 2,500 colonies. Colonies were isolated and exposed to bacteriophage Ffm. It was anticipated that recombinant cosmids carrying the rfb_{SmO16} genes would express O polysaccharide and therefore would be Ffm resistant. This approach has been used previously to clone the *rfb* clusters from *K. pneumoniae* O1 (7) and *E. coli* O9 (17). Several Ffm-resistant recombinants were obtained, and these tested positive for expression of D-galactan I by whole-cell agglutination with MAb O1-2.6, which is specific for D-galactan I (63). Cosmid DNA (pWQ25) from one recombinant was selected for further analysis. pWQ25 was used to retransform *E. coli* K-12 recipients. In SDS-PAGE, *E. coli* LE392 produces R-LPS consisting of only lipid A core. Transformation of *E. coli* LE392 with pWQ25 resulted in a typical S-LPS ladder pattern, indicating that the cosmid carried genes which determine O polysaccharide biosynthesis (54).

Cosmid pWQ25 has a total size of approximately 31 kbp, which would be near the lower limit for packaging in lambda. This plasmid may have undergone a deletion event after packaging, but this aspect has not been pursued further. The inserted *S. marcescens* DNA in pWQ25 includes a *Pst*I fragment of 8 kbp, the size predicted by the hybridization experiments. The 8-kbp fragment was therefore cloned in pBluescript as pWQ26 and transformed into *E. coli* DH5α. However, the resulting transformants did not agglutinate in the presence of MAb O1-26 and, by SDS-PAGE, only R-LPS was observed. These observations indicated that the rfb_{SmO16} genes were not fully contained within the 8-kbp *Pst*I fragment. Therefore, a larger 9.6-kbp *Xho*I fragment (which contained the 8-kbp *Pst*I fragment) was subcloned into pBluescript as pWQ27. *E. coli* DH5α transformed with pWQ27 agglutinated in the presence of MAb O1-26, and the S-LPS profile was evident by SDS-PAGE (54).

E. coli K-12 strains retain some *rfb* functions (29, 52, 66) that can interact with the products of cloned genes and therefore participate in the expression of O polysaccharides (25, 66). To determine whether pWQ27 contained a complete functional *rfb* cluster, these plasmids were introduced into *E. coli* CLM4 (Δ*rfb*). Figure 3A shows that *E. coli* CLM4 harboring pWQ27 synthesizes S-LPS. *S. marcescens* O16 produces two neutral O

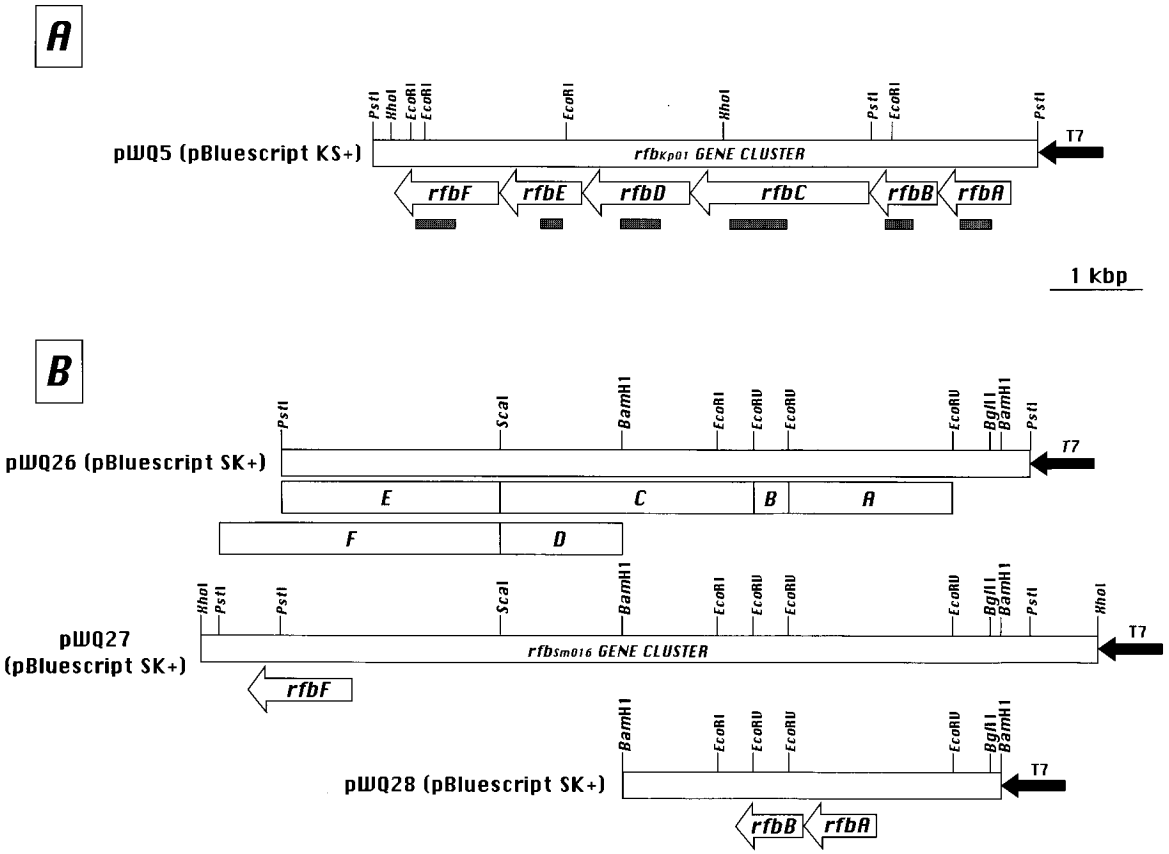

FIG. 1. (A) Genetic organization of the *rfb* gene clusters from *S. marcescens* O16 (B) and *K. pneumoniae* O1 (A). The rfb_{KpO1} cluster contains six genes and is cloned in pWQ5 (5). The sequence of rfb_{KpO1} is available in GenBank under accession numbers L31775 (*rfbA-rfbB*) and L31762 (*rfbC-rfbF*). (B) Physical map of the *rfb* region from *S. marcescens* O16. The intact cluster is cloned in pWQ27. Internal gene probes from the *K. pneumoniae* serotype O1 *rfb* gene cluster were used to identify homologous rfb_{SmO16} regions in pWQ27 by hybridization. Each shaded box beneath pWQ5 indicates the location of an internal gene probe. The corresponding boxes between pWQ26 and pWQ27 illustrate the fragments identified by hybridization to each gene probe. The locations of $rfbAB_{SmO16}$ and $rfbF_{SmO16}$ were established by sequencing. The orientation of insert DNA relative to the T7 promoter is indicated (arrows), but vector DNA is not shown.

polysaccharides. One is D-galactan I, and the other is a polymer containing Rib*f* residues (40). Only D-galactan I is encoded by rfb_{SmO16} (61). This situation therefore resembles that for *K. pneumoniae* O1, where rfb_{KpO1} determines synthesis of D-galactan I and additional genes required for synthesis of D-galactan II are located outside rfb_{KpO1} (7).

Hybridization of rfb_{KpO1} genes with rfb_{SmO16}. Cloning and sequencing of rfb_{KpO1} revealed that six genes (*rfbA* to -*F*) are responsible for the synthesis and export of D-galactan I (Fig. 1). $rfbA_{KpO1}$ and $rfbB_{KpO1}$ encode enzymes that comprise an ABC transporter which is required for transport of the O polysaccharide across the cytoplasmic membrane (5). The remaining four genes are involved in synthesis of the D-galactan I polymer. $rfbF_{KpO1}$ is a bifunctional galactosyltransferase capable of transferring both Gal*p* and Gal*f* residues (6). The precise functions of $RfbC_{KpO1}$, $RfbD_{KpO1}$, and $RfbE_{KpO1}$ are unknown.

To investigate whether the rfb_{SmO16} gene cluster contains a similar complement of homologous genes, probes consisting of internal fragments from each rfb_{KpO1} gene were used to map the rfb_{SmO16} genes on pWQ27 DNA. Each probe hybridized strongly at low stringency, indicating that rfb_{SmO16} contains genes homologous to *K. pneumoniae* serotype O1. On the basis of physical mapping, these genes appear to be arranged in the same order (Fig. 1). However, the degree of homology at the nucleotide sequence level is sufficiently low that hybridization was not detected under high-stringency conditions (54). In *K.*

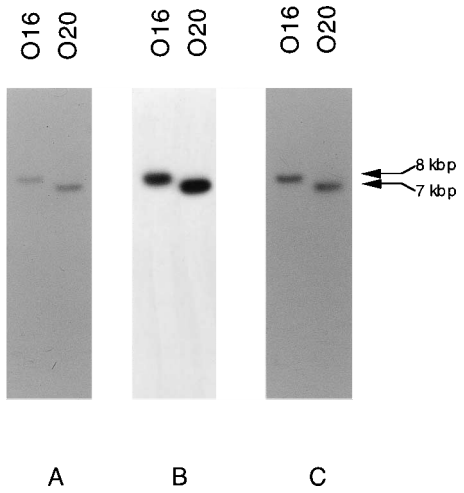

FIG. 2. Hybridization of rfb_{KpO1} probes with *Pst*I-digested chromosomal DNA from *S. marcescens* serotypes O16 and O20. Hybridization was performed under low-stringency conditions as described in Materials and Methods. Autoradiograms of Southern blots of *Pst*I-digested chromosomal DNA probed with the 5.6-kbp *Pst*I fragment (A), the 1.85-kbp *Pst*I fragment (B), and the 3.2-kbp *Eco*RI fragment (C) from pWQ5 are shown. The sizes of the fragments (arrows) are indicated on the right.

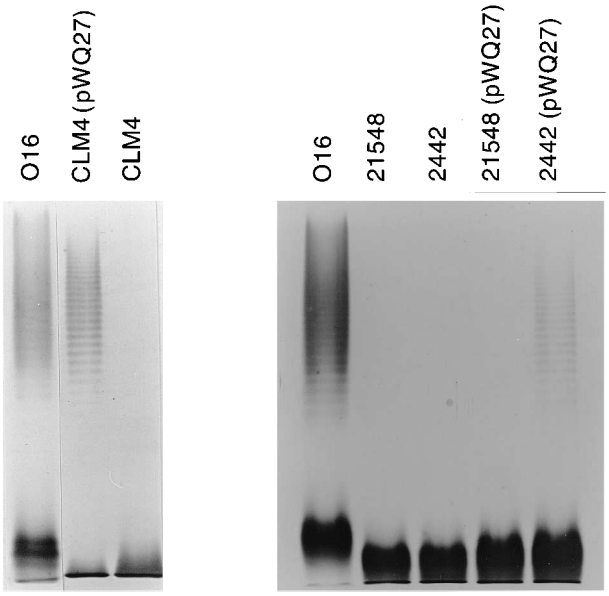

FIG. 3. Expression of S-LPS containing D-galactan I in *E. coli* K-12 strains containing pWQ27. (A) Expression of O polysaccharide by *S. marcescens* serotype O16 and by *E. coli* CLM4 Δ*rfb* transformed with pWQ27. Without the plasmid, *E. coli* CLM4 produces R-LPS. (B) *rfe*-dependent synthesis of D-galactan I. Introduction of pWQ27 into *E. coli* K-12 2442 (also known as strain AB1133) results in formation of S-LPS containing D-galactan I. *E. coli* 21548 (*rfe*::Tn*10*-48) is derived from strain 2442. The absence of S-LPS in *E. coli* 21548(pWQ27) indicates that synthesis of D-galactan I involves an *rfe*-dependent pathway. LPS samples were prepared from whole-cell lysates (15). For all samples, the presence of D-galactan I was confirmed by a whole-cell agglutination reaction with D-galactan I-specific MAb O1-2.6.

pneumoniae, there are at least three different clonal groups of *rfb* gene clusters responsible for the synthesis of either D-galactan I or a modified version of D-galactan I. One group is represented by serotype O1 (7, 20), and another is represented by O8 (20). Strains belonging to serotype O9 and some subgroups of O2 do not hybridize to gene probes from either O1 or O8 under high-stringency conditions (21). Gene probes for O8 also recognize rfb_{SmO16}, but only under low-stringency conditions (54).

Synthesis of *S. marcescens* serotype O16 D-galactan I O polysaccharide is *rfe* dependent. The synthesis of D-galactan I O polysaccharide by *K. pneumoniae* serotype O1 requires Rfe in addition to the Rfb proteins (6, 7). Rfe is a Glc*p*NAc-1-phosphate transferase (UDP-Glc*p*NAc::undecaprenolphosphate Glc*p*NAc-1-phosphate transferase), which forms the first lipid-linked intermediate in the synthesis of enterobacterial common antigen (35, 36). Rfe plays two different roles in O antigen biosynthesis. In some heteropolysaccharide O antigens of *Shigella* species (24, 66), the cryptic O antigen of *E. coli* K-12 (52), and several *E. coli* O serotypes (1), synthesis of each repeating unit begins by Rfe transferring Glc*p*NAc-1-P to undecaprenol phosphate. Rfb proteins then complete the formation of the repeating unit, and the units are polymerized. Rfe is also involved in the synthesis of some O antigen homopolymers which lack Glc*p*NAc in their repeating unit structures. In *E. coli* O8 and O9 and *K. pneumoniae* O1, an Rfe-dependent reaction transfers a sugar phosphate to undecaprenol phosphate. This transfer provides a primer to which mannosyl (O8 and O9) or galactosyl (O1) residues are sequentially transferred, forming the respective homopolymers. In vitro experiments with *E. coli* O9 suggest the primer is undecaprenol pyrophosphoryl glucose (60). However, recent in vivo analyses of *E. coli* O8–K-12 hybrids (47) and *E. coli* K-12–*K. pneumoniae* O1 hybrids (6) indicate that the lipid is modified with Glc*p*NAc in a reaction that is identical to that involved in biosynthesis of enterobacterial common antigen and other hetero-O-polysaccharides. The reason(s) for the anomalies between the in vivo and in vitro data is unclear but may reflect either strain-specific differences or altered specificity of transferases examined in vitro.

To examine whether D-galactan I biosynthesis directed by the rfb_{SmO16} gene cluster is also *rfe* dependent, pWQ27 was electroporated into *E. coli* 2442 and 21548 strains. *E. coli* 21548 is an *rfe*::Tn*10* derivative of *E. coli* 2442 (36). Only *E. coli* 2442 harboring pWQ27 produced an O polysaccharide (Fig. 3B), indicating that D-galactan I biosynthesis directed by the *S. marcescens* serotype O16 *rfb* cluster is a further example of *rfe*-dependent biosynthesis. The similarities between the rfb_{KpO1} and rfb_{SmO16} (described below) are such that it is expected that the Rfe enzyme plays the same role in biosynthesis of both O antigens.

Characterization of $rfbA_{SmO16}$ and $rfbB_{SmO16}$ encoding an ABC-2 transporter for O polysaccharide export. In *K. pneumoniae* O1, assembly of the O polysaccharide occurs at the cytoplasmic face of the cytoplasmic membrane. An ABC-2 transporter (45) or a traffic ATPase (9) is required for export of O polysaccharide across the cytoplasmic membrane to the periplasmic face, where ligation to lipid A-core occurs (5). The D-galactan I transport system consists of $RfbAB_{KpO1}$ and is similar to that required for export of group II capsular polysac-

TABLE 3. Comparison of *rfb* genes and their predicted products from *K. pneumoniae* O1 with those from *S. marcescens* O16

Gene	Nucleotide sequence data			Predicted polypeptide data		
	No. of nucleotides	% G+C	% Homology[a]	No. of amino acid residues	Molecular mass (Da)	% Identity (% total similarity)[b]
$rfbA_{KpO1}$	768	37.4		255	29,576	
$rfbA_{SmO16}$	768	39.2	71.7	255	30,029	75.7 (87.9)
$rfbB_{KpO1}$	741	44.2		246	27,446	
$rfbB_{SmO16}$	741	45.6	69.0	246	27,169	78.0 (86.5)
$rfbF_{KpO1}$	1,134	37.3		377	43,060	
$rfbF_{SmO16}$	1,143	47.6	60.4	380	42,600	57.6 (71.1)

[a] In comparison with the prototypes from *K. pneumoniae* O1, calculated by NALIGN (39). The *rfbA* and *rfbB* sequences were aligned without breaks. An optimal alignment for *rfbF* required introduction of breaks in the sequence.

[b] In comparison with the prototypes from *K. pneumoniae* O1, calculated by PALIGN (39). The RfbA and RfbB sequences were aligned without breaks (Fig. 4). An optimal alignment for RfbF (Fig. 7) required the introduction of breaks in the predicted sequence. Amino acids considered similar are A, S, T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, W.

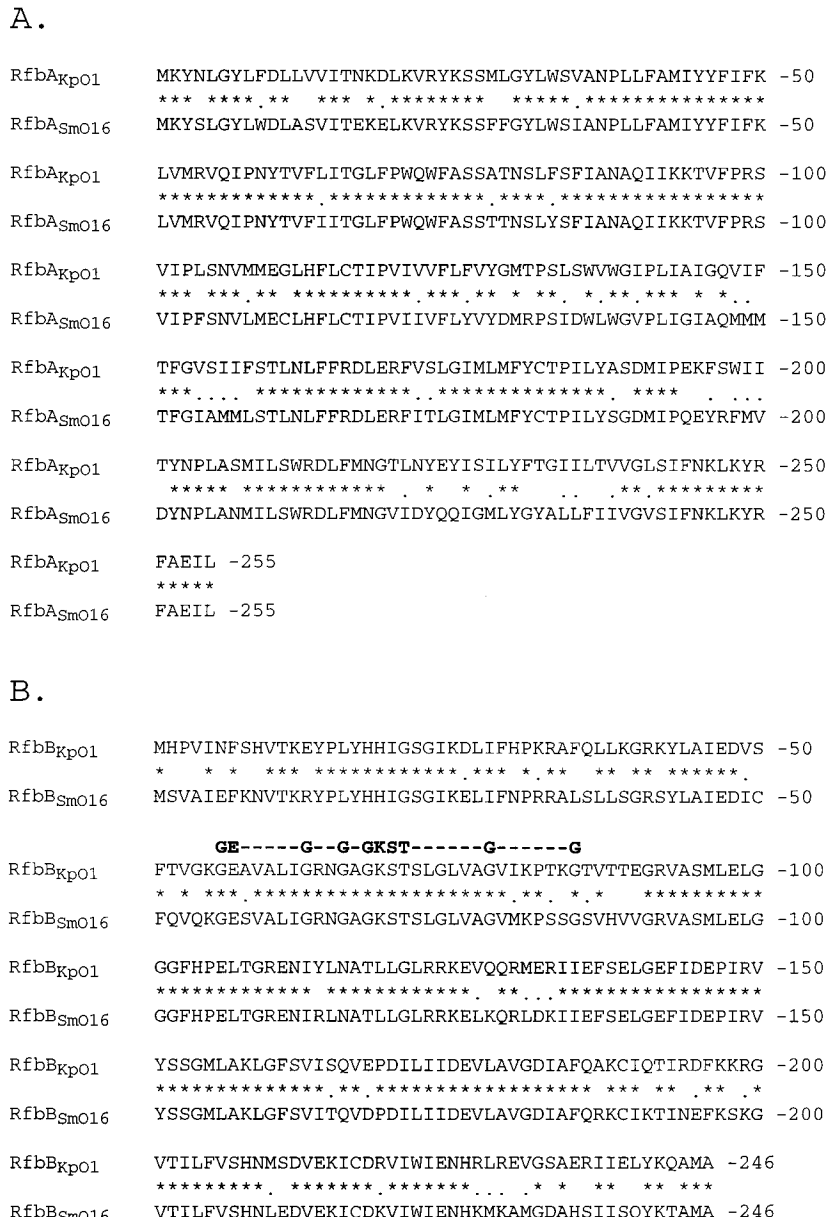

FIG. 4. Alignment of predicted RfbA (A) and RfbB (B) homologs from *K. pneumoniae* serotype O1 and *S. marcescens* serotype O16. Alignment was performed with PALIGN (39). Amino acids considered similar (dots) are A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, and W. RfbA is the transmembrane component of the ABC-2 transporter. RfbB is proposed to be the ATP-binding component, and the consensus ATP-binding motif (59) is shown in boldface type. Identical amino acids are indicated by asterisks. Sequence position numbers are marked on the right.

charides in *E. coli*, *Neisseria meningitidis*, and *Haemophilus influenzae* (reviewed in reference 64). To determine if a similar pathway is used for assembly of D-galactan I in *S. marcescens* O16, the putative *rfbAB* region identified by hybridization in pWQ27 was sequenced. Two open reading frames with approximately 70% identity with and sizes equivalent to those of the nucleotide sequences for *rfbA*$_{KpO1}$ and *rfbB*$_{KpO1}$ were detected (Table 3). The predicted proteins were very highly conserved (Table 3), and the relationships were clearly evident in protein alignments (Fig. 4). A protein with an apparent molecular mass of 29.0 kDa, consistent with the predicted size (27,169 Da) for RfbB$_{SmO16}$, was expressed by the *rfb*$_{SmO16}$ cluster. (Fig. 5; also, see the section on comparison of gene products below).

To confirm the function of the putative transporter, pWQ28 was constructed. This plasmid contains only *rfbAB*$_{SmO16}$ and a truncated *rfbC*$_{SmO16}$ (Fig. 1). pWQ28 was transformed in *E. coli* DH5α with pWQ19. pWQ19 contains *rfbCDEF*$_{KpO1}$, and strains containing pWQ19 accumulate cytoplasmic D-galactan I and make R-LPS. Formation of S-LPS results when the absent transporter is complemented in *trans* by a plasmid carrying *rfbAB*$_{KpO1}$ (5), indicating that the ABC-2 transporter predicted by the sequence data for *rfbAB*$_{SmO16}$ was functional for export of heterologous D-galactan I (Fig. 6).

Identification of *rfbF*$_{SmO16}$ and comparison with *rfbF*$_{KpO1}$. In *K. pneumoniae* O1, the last gene of the *rfb* cluster is *rfbF* (6). To locate the end of the *rfb*$_{SmO16}$ cluster, *rfbF*$_{SmO16}$ was iden-

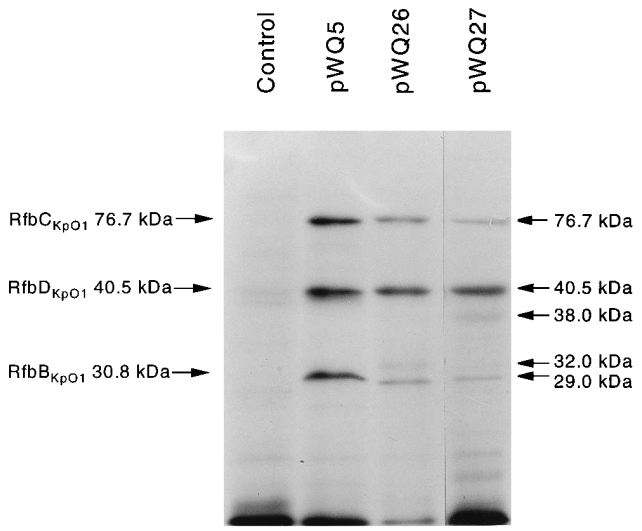

FIG. 5. Comparison of *K. pneumoniae* serotype O1 *rfb* gene products from pWQ5 with the *S. marcescens* serotype O16 *rfb* gene products from pWQ26 and pWQ27. The labeled proteins shown (arrows) are from *E. coli* K-12 strain JM109 DE3 transformants containing pBluescript SK(+) (Control), pWQ5, pWQ26, and pWQ27. The Rfb$_{KpO1}$ proteins are indicated on the left of the figure. The sizes of polypeptides encoded by pWQ26 and pWQ27 are indicated on the right.

tified by hybridization and by the different LPS phenotypes conferred by pWQ26 and pWQ27. The smaller subclone, pWQ26, is unable to direct synthesis of D-galactan I. Since additional DNA is present upstream of *rfbA*$_{SmO16}$ in pWQ26 and hybridization places *rfbF*$_{SmO16}$ at the end of the cloned fragment in pWQ26, the R-LPS phenotype most likely results from a truncation at the end of the cluster defined by *rfbF*$_{SmO16}$ (Fig. 1). In *K. pneumoniae* O1, mutations in *rfbF* eliminate

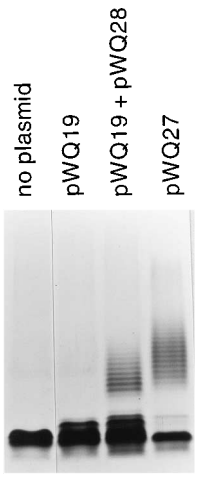

FIG. 6. Functional demonstration of the ABC-2 transporter encoded by *rfbAB*$_{SmO16}$. Plasmids were transformed into *E. coli* AB1133, and the resulting LPS was examined by SDS-PAGE. pWQ19 contains *rfbCDEF*$_{KpO1}$. Strains containing this plasmid make R-LPS and accumulate intracellular D-galactan I because of the absence of the transporter (5). The transport defect is complemented in *trans* by plasmid pWQ28, which contains *rfbAB*$_{SmO16}$. The size distributions of the D-galactan-substituted S-LPS differ in strains containing pWQ19 plus pWQ28 and pWQ27 (complete *rfb*$_{SmO16}$ cluster) alone. This difference may reflect altered stoichiometry of the proteins involved in synthesis (lower-copy-number pRK404 construct) versus transport (higher-copy-number) pBluescript construct).

D-galactan I formation (6). Sequence analysis confirmed the *Pst*I site within the putative *rfbF*$_{SmO16}$ gene.

RfbF$_{KpO1}$ is a galactosyltransferase which initiates formation of D-galactan I. In *E. coli* K-12, RfbF$_{KpO1}$ transfers galactosyl residues to the Glc*p*NAc-containing lipid intermediate formed by Rfe (6). RfbF$_{KpO1}$ modifies the *E. coli* K-12 LPS core by addition of a disaccharide [β-D-Gal*f*-(1→3)-α-D-Gal*p*-1→] (6). RfbF$_{KpO1}$ shares homology with RfpB (11), a plasmid-encoded galactopyranosyl transferase (10, 24) involved in the biosynthesis of the *Shigella dysenteriae* type 1 O polysaccharide (6).

The predicted RfbF$_{SmO16}$ protein contains 380 amino acid residues and has a molecular mass of 43,060 Da. RfbF$_{KpO1}$ is slightly smaller; it contains 377 residues and has a molecular mass of 42.6 kDa (6). In the RfbF homologs, 57.6% of the amino acids are identical and a further 13.5% have similarity (Table 3). The proteins can be aligned without gaps, and both RfbF homologs share similarity with RfpB from *S. dysenteriae* type I (Fig. 5). When RfbF$_{SmO16}$ and RfpB sequences are aligned, 25.2% of the amino acids are identical (38.5% total similarity). A consensus sequence generated by alignment of the two RfbF homologs with RfpB contained 19.1% identical amino acid residues, and a further 37.7% residues are similar (Fig. 7). The sequence relationships support the conclusion that RfbF$_{SmO16}$ and RfbF$_{KpO1}$ are functionally identical galactosyltransferase enzymes. Although RfbF$_{KpO1}$, RfpB, and RfbF$_{SmO16}$ are (probably) all galactosyltransferases, the highest identity exists between RfbF$_{SmO16}$ and RfbF$_{KpO1}$. This relationship reflects the novel bifunctional nature described for RfbF$_{KpO1}$ (6). RfpB has been reported to have three predicted transmembrane segments and a cytoplasmic carboxyl tail (11). There are no predicted transmembrane segments in either RfbF protein, and the highest consensus similarity in the three galactosyltransferases is located in the carboxyl-terminal regions.

Comparison of *rfb*$_{KpO1}$ and *rfb*$_{SmO16}$ gene products. The T7 promoter expression system was used to identify Rfb$_{SmO16}$ proteins and compare them with Rfb$_{KpO1}$. Rfb$_{SmO16}$ protein expression was detected only when the T7 promoter was upstream of the fragment hybridizing to the *rfbA*$_{KpO1}$ probe (Fig. 1), providing further evidence for the similarity in genetic organization of *rfb*$_{SmO16}$ and *rfb*$_{KpO1}$ (54). An autoradiogram with the putative *rfb*$_{SmO16}$ gene products of pWQ26 and pWQ27 alongside the gene products of *rfb*$_{KpO1}$ from pWQ5 is shown in Fig. 5.

Three detectable proteins are produced from pWQ5. These have apparent molecular masses by SDS-PAGE of 76.7, 40.5, and 30.8 kDa and correspond to RfbC$_{KpO1}$, RfbD$_{KpO1}$, and RfbB$_{KpO1}$, respectively (5). RfbA$_{KpO1}$ is not visualized by this system, and the remaining gene products are produced in very small amounts (5). pWQ27 produced proteins of 76.7 and 40.5 kDa which comigrate with Rfb$_{KpO1}$ proteins. The smallest protein (29.0 kDa) may be the *S. marcescens* equivalent to RfbB$_{KpO1}$, which is consistent with results for *K. pneumoniae* O1 (5) and with the sequence data described above. The 43-kDa RfbF$_{KpO1}$ protein is detected only when the *rfbF*$_{KpO1}$ structural gene is cloned in isolation (6). However, pWQ27 encodes an additional polypeptide of 38.0 kDa, consistent with the size predicted for RfbF$_{SmO16}$. The 38.0-kDa polypeptide is absent in pWQ26 recombinants, and a new protein of approximately 32.0 kDa is evident. This product is a result expected from truncation of *rfbF*$_{SmO16}$ and fusion with vector sequences. Overall, the profiles of polypeptides encoded by the *rfb*$_{KpO1}$ and *rfb*$_{SmO16}$ gene clusters and those visible in the T7 expression system are comparable.

Comparison of *rfb* sequence homology and G+C content in

FIG. 7. Alignment of $RfbF_{SmO16}$, $RfbF_{KpO1}$, and RfbB. Alignment of $RfbF_{SmO16}$ and $RfbF_{KpO1}$ was performed with PALIGN (39). The consensus is an alignment of all three proteins by using the CLUSTAL program (14). Gaps (−) have been introduced to optimize the alignment. An asterisk indicates that two aligned residues are identical, and a dot indicates that two aligned residues are similar. Amino acids considered similar are A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, and W. Potential transmembrane regions in RfpB (11) are underlined.

***K. pneumoniae* O1 and *S. marcescens* O16.** The degree of relatedness among *rfb* clusters is variable. In some bacterial species, highly homologous *rfb* genes encode similar functions. The best-documented examples are found among serovars of *S. enterica* (43). There are also reports of high degrees of *rfb* homology in different species with shared Rfb functions, for example, *S. enterica* serovar Typhimurium and *Yersinia pseudotuberculosis* (23). The same is true of *E. coli* O7 and *Shigella boydii* type 12 (58), but these bacteria are sufficiently closely related to be considered a single species (44). In contrast, *K. pneumoniae* (20, 21), *E. coli* O101 (13), and *S. boydii* type 12 (58) provide documented examples of a low degree of *rfb* homology among bacteria belonging to the same or closely related serotypes.

The *rfbAB* genes from *K. pneumoniae* O1 and *S. marcescens* O16 are conserved and have comparable G+C values. The *rfbAB* genes are relatively AT rich, compared with the average values typical for each species (*K. pneumoniae*, 56 to 58 mol%; and *S. marcescens*, 57.5 to 60 mol%) (38). To date, most genes associated with LPS synthesis have been found to have low-percentage G+C relative to the species average. One interpretation is that this reflects acquisition, by lateral gene transfer,

of genes from a progenitor with A+T-rich DNA (43). However, other explanations for the AT bias seen in genes encoding biosynthetic enzymes for different cell surface polysaccharides have been suggested (37, 51, 64). One possibility is the maintenance of low levels of translation. The similar sequences and G+C compositions for the *rfbAB* homologs are consistent with a common evolutionary history. Any lateral transfer acquisition event is presumed to be relatively recent, since there is no evidence for the *rfb* genes from one source being more closely equilibrated to the chromosomal G+C average than those from the other bacterium. However, these arguments are difficult to maintain when the results for RfbF homologs are considered. The $rfbF_{KpO1}$ and $rfbF_{SmO16}$ nucleotide sequences show only 60.4% identity. When the sequences are aligned without breaks, 89 of the 381 codons are identical and 118 have alterations in the third position. Surprisingly, the G+C compositions of $rfbF_{KpO1}$ and $rfbF_{SmO16}$ are quite different, with values of 37.3 and 47.6%, respectively. The difference in G+C contents between the homologs could reflect different periods that the respective *rfb* clusters have resided in each organism, leading toward varied amounts of equilibration of G+C content to the chromosomal average for the species (53). However, if this argument is followed, the distance from a common *rfbF* progenitor is greater than that for the *rfbAB* genes and this is not consistent with simultaneous lateral transfer of the entire *rfb* cluster. It is conceivable that the amount of genetic drift (after transfer) in *rfbAB* is limited by a highly stringent structural requirement imposed by the function of the ABC-2 transporter.

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