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Clonally Diverse *rfb* Gene Clusters Are Involved in Expression of a Family of Related D-Galactan O Antigens in *Klebsiella* Species

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Klebsiella **species express a family of structurally related lipopolysaccharide O antigens which share a common backbone known as D-galactan I. Serotype specificity results from modification of D-galactan I by addition of domains of altered structure or by substitution with *O*-acetyl and/or α-D-Gal*p* side groups with various linkages and stoichiometries. In the prototype, *Klebsiella* serotype O1, the *his*-linked *rfb* gene cluster is required for synthesis of D-galactan I, but genes conferring serotype specificity are unlinked. The D-galactan I part of the O polysaccharide is O acetylated in *Klebsiella* serotype O8. By cloning the *rfb* region from *Klebsiella* serotype O8 and analyzing the O polysaccharide synthesized in *Escherichia coli* K-12 hosts, we show that, like rfb_{O1}, the rfb_{O8} region directs formation of unmodified D-galactan I. The *rfbAB* genes encode an ATP-binding cassette transporter required for export of polymeric D-galactan I across the plasma membrane prior to completion of the lipopolysaccharide molecule by ligation of the O polysaccharide to lipid A-core. Complementation experiments show that the *rfbAB* gene products in serotypes O1 and O8 are functionally equivalent and interchangeable. Hybridization experiments and physical mapping of the *rfb* regions in related *Klebsiella* serotypes suggest the existence of shared *rfb* genes with a common organization. However, despite the functional equivalence of these *rfb* gene clusters, at least three distinct clonal groups were detected in different *Klebsiella* species and subspecies, on the basis of Southern hybridization experiments carried out under high-stringency conditions. The clonal groups cannot be predicted by features of the O-antigen structure. To examine the relationships in more detail, the complete nucleotide sequence of the serotype O8 *rfb* cluster was determined and compared with that of the serotype O1 prototype. The nucleotide sequences for the six *rfb* genes showed variations in moles percent G+C values and in the values for nucleotide sequence identity, which ranged from 66.9 to 79.7%. The predicted polypeptides ranged from 64.3% identity (78.4% total similarity) to 94.3% identity (98.0% similarity). The results presented here are not consistent with dissemination of the *Klebsiella* D-galactan I *rfb* genes through recent lateral transfer events.**

Lipopolysaccharide (LPS) is a major component of the outer membranes of gram-negative bacteria. LPS is a complex molecule and is divided into three distinct regions. Lipid A forms the outer leaflet of the outer membrane and is attached through the core oligosaccharide to the O-antigenic side chain polysaccharide. In members of the family *Enterobacteriaceae*, the O polysaccharide extends from the cell surface to form a discrete layer. The O polysaccharide is composed of polymerized oligosaccharide repeat units, and structural variation in this region of LPS results in serological heterogeneity of O antigens.

Klebsiella species can be isolated from clinical and environmental (soil, water, and plant) sources. Clinical and environmental strains share some common O and K (capsular) antigens and are generally difficult to separate by biochemical tests. There are at least five species in the genus: *K. pneumoniae* (which is further separated into *K. pneumoniae* subspecies *pneumoniae*, *ozaenae*, and *rhinoscleromatis*), *K. oxytoca*, *K. planticola*, *K. terrigena*, and *K. mobilis* (reviewed in reference 12). *K. pneumoniae* and, to a lesser extent, *K. oxytoca* are important nosocomial pathogens which cause bacteremia, pneumonia, and urinary tract infections in humans (31, 56). However, those *Klebsiella* species generally considered to be environmental isolates (*K. planticola* and *K. terrigena*) are also frequently identified as opportunistic pathogens among clinical isolates. Members of the genus produce characteristically mucoid colonies on agar plates because of the synthesis of capsular polysaccharides (K antigens). K-antigen serotyping is well established for *Klebsiella* species, and there are 77 recognized K antigens (36). In contrast, the exact number of structurally unique O antigens is unknown, but they are fewer than the K antigens.

We are interested in a subset of *Klebsiella* O antigens which consist of homopolymers of galactose; the prototype member of this family of structurally related O polysaccharides is serotype O1 (Fig. 1). The O1 polysaccharide is composed of two discrete repeat-unit structures (23, 54) designated D-galactan I and D-galactan II (54). Chemical data predict that D-galactan I chains are linked directly to the core oligosaccharide and that D-galactan II is attached at the distal end of D-galactan I (22). D-Galactan II is confined to the highest-molecular-weight LPS molecules (54), and some D-galactan I chains lack the terminal D-galactan II (22, 54). This structural organization is consistent with the isolation of mutants which lack D-galactan II and produce O chains containing only D-galactan I (30, 54). Additional members of the family of related D-galactan O polysaccharides are described in Fig. 1. The O8 polysaccharide is structurally similar to O1, and O acetylation of D-galactan I in serotype O8 provides the only chemical basis for serological distinction between these two serotypes (18). The serology of the serotype O2 group is complex, with antigenic factors and subfactors described in an extended antigenic formula (35). Our structural analyses have shown that the 2a antigen is D-galactan I and that O2(2a) strains contain D-galactan I as the only O polysaccharide in their LPS (53). Serotype O2(2a,2c) LPS resembles O1 in that it contains an O polysaccharide with

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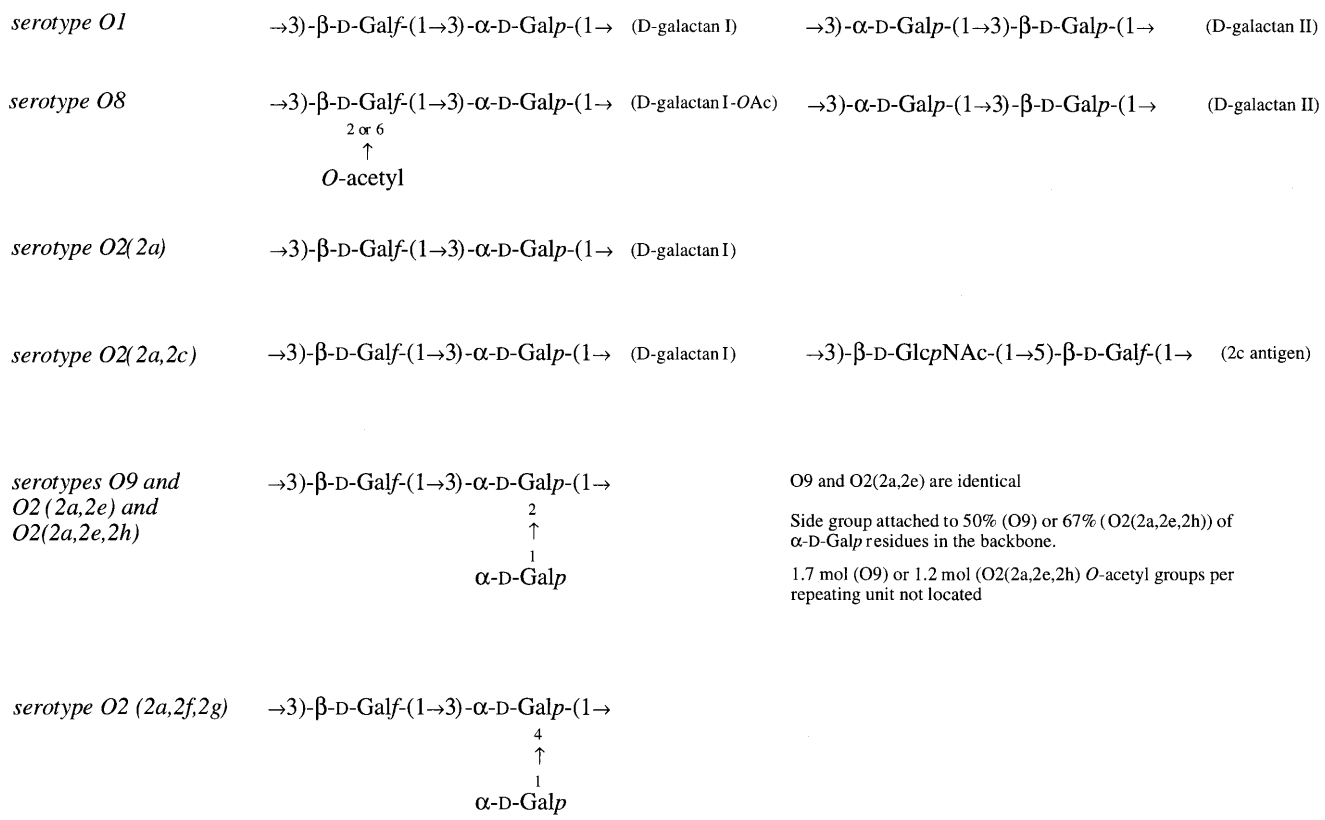

FIG. 1. Repeat-unit structures of D-galactan I-based O polysaccharides in the LPSs of *Klebsiella* species. The determinations of the structures of the O1 (23, 54), O2(2a) (53), O2(2a,2c) (53), O8 (18), O9 (28), and O2(2a,2e), O2(2a,2e,2h), and O2(2a,2f,2g) (17) polysaccharides are described elsewhere.

two repeat-unit structures; the unique 2c antigen is a heteropolymer of D-Gal*f* and D-Glc*p*NAc (53). Serotype O2(2a,2f,2g) has an O polysaccharide which is stoichiometrically modified by (1→4)-linked α-D-Gal*p* residues on each repeat unit of D-galactan I (17). Serological identification of the 2a antigen in this serotype is difficult to interpret because its O polysaccharide contains no unsubstituted D-galactan I and the LPS is not recognized by D-galactan I-specific antibodies (19). Serological cross-reactivity in this strain (35) may be due to additional, non-LPS, factors. The O9 polysaccharide is a derivative of D-galactan I which is O acetylated and nonstoichiometrically substituted with (1→2)-linked α-D-Gal*p* residues (28). The O-polysaccharide repeat units of serotypes O2(2a,2e) and O2(2a,2e,2h) are essentially the same as those of O9 and differ only in the extent of α-D-Gal*p* modification of D-galactan I (17). Previously reported antigenic differences in these strains (35) are not due to O polysaccharides, and there is no apparent basis for the separation of O9, O2(2a,2e), and O2(2a,2e,2h) into different O serotypes from either determined O-repeat structures or reaction with D-galactan I-specific antisera (17).

D-Galactan-substituted LPS is produced by *Escherichia coli* K-12 strains harboring the cloned *rfb* (O-antigen biosynthesis) genes from *K. pneumoniae* O1 (8). The rfb_{O1} gene cluster is clearly required for expression of both D-galactans I and II, but while it is sufficient for D-galactan I synthesis, an additional unmapped locus (or loci) is also required for the formation of D-galactan II (8, 54). In a similar fashion, the *Klebsiella* serotype O9 *rfb* region encodes genes required for D-galactan I synthesis, and an unlinked locus directs addition of the α-D-Gal*p* side groups which provide the O9 epitope (17). The biosynthesis of D-galactan I in serotype O1 is dependent on the activity of Rfe (7), and like in other examples of the Rfc-independent O-antigen synthesis pathway (52), its surface expression requires a dedicated ATP-binding cassette (ABC) transporter (6).

Relationships between *rfb* gene clusters responsible for the expression of structurally related O antigens have been studied extensively for serovars of the bacterial species *Salmonella enterica* (reviewed in reference 41). In the *S. enterica* clusters, genes encoding shared enzymes are very similar, if not identical. Despite the structural similarities in the D-galactan I family of O polysaccharides, the relationships among *rfb* gene clusters from these strains are complex. Hybridization analyses have shown that the *rfb* genes from serotypes O1 and O8 form distinct clonal groups at the DNA level (18). In this paper, we show that serotype O9 forms a third clonal group. To examine the relationships in more detail, the serotype O8 *rfb* cluster was cloned and shown to be functionally equivalent to that of serotype O1. The basis for clonal differences between the O1 and O8 *rfb* gene clusters was established by comparison of the nucleotide sequences of the two clusters and alignment of their respective predicted Rfb proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Klebsiella* strains used in this study are described in Table 1. The extended serotype designation for the O2 strain is that of Ørskov (35). Strains were routinely grown and maintained by using Luria-Bertani broth, and incubation was at 37°C. When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; and tetracycline, 15 μg/ml.

LPS extraction and purification and analysis of O polysaccharides. Large-scale LPS preparations were made by using a modified hot-phenol extraction method. Detailed protocols for the isolation and purification of O polysaccharides and for their structural analysis by chemical and nuclear magnetic resonance (NMR) methods are reported elsewhere (54).

SDS-PAGE. LPS samples for sodium dodecyl sulfate-polyacrylamide gel elec-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Bacterial strains		
K. pneumoniae subspecies *pneumoniae*		
CWK2	Serotype O1:K$^-$; produces D-galactan I and D-galactan II; capsule-deficient mutant of strain 889/50 (O1:K20)	54
CWK37	Mutant of CWK2, producing only D-galactan I	54
CWK55	Serotype O2(2a,2f,2g):K$^-$; capsule-deficient mutant of strain 6613 (O2:K27)	17
K. pneumoniae subspecies *ozaenae* CWK47	Serotype O8:K$^-$; capsule-deficient mutant of strain 889 (O8:K69)	18
K. oxytoca CWK48	Serotype O9:K$^-$; capsule-deficient mutant of strain 1205 (O9:K72)	28
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$^-$	45
SØ874	K-12 *lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL*	34
Plasmids		
pWQ5	pBluescript KS(+) derivative carrying the rfb_{O1} region from strain CWK2; Ap^r	6
pWQ19	pRK404 containing $rfbCDEF_{O1}$ cloned on a 5.3-kbp *Pst*I fragment; Tc^r	6
pWQ210	pBluescript KS(+) derivative carrying an 8.1-kbp *Hin*dIII fragment of chromosomal DNA containing the rfb_{O8} region from strain CWK47; Ap^r	This study
pWQ209	pBluescript KS(+) derivative carrying a 2.8-kbp *Pst*I fragment containing $rfbAB_{O8}$; Ap^r	This study
pWQ211	pBluescript KS(+) derivative carrying an internal 1.8-kbp *Pst*I fragment from rfb_{O8}, used as the O8-specific probe; Ap^r	This study

trophoresis (SDS-PAGE) were obtained either by phenol extraction or from SDS-proteinase K whole-cell lysates by the procedure of Hitchcock and Brown (15). The conditions used for electrophoresis (9) and silver staining (49) are described elsewhere.

Antisera. Monoclonal antibody O1-2.6 is specific for D-galactan I and was raised against the LPS from *K. pneumoniae* serotype O1 (54).

DNA methods. Genomic DNA was extracted by the method of Hull et al. (16), and plasmid DNA was extracted by an alkaline lysis method and then purified on columns from Qiagen Inc. (Chatsworth, Calif.). Electroporation was performed by procedures described elsewhere (4). rfb_{O1} gene probes were derived from plasmid pWQ5 in two ways. Larger restriction fragments were excised from low-melting-point agarose gels and purified by using GeneClean (Bio 101 Inc., La Jolla, Calif.). Internal fragments from each rfb_{O1} gene were generated by PCR amplification. The primers used and the predicted size of each product are described elsewhere (48). Gene probes were labeled by using the digoxigenin-labeling kit from Boehringer Mannheim (Laval, Québec, Canada). For PCR-generated probes, the amplification and labeling reactions were performed simultaneously. DNA fragments in agarose gels were depurinated, denatured, and neutralized prior to Southern transfer (45). The 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 by using a Stratagene UV Stratalinker 1800. For low-stringency conditions, prehybridization was performed with a solution of 5× SSC, 2% blocking reagent (Boehringer Mannheim), 30% formamide, 0.1% *N*-lauroyl-sarcosine, and 0.02% SDS at 37°C for 3 h. For high-stringency conditions, the prehybridization step was carried out at 42°C for 3 h and the solution was adjusted to 50% formamide. Hybridization was carried out overnight. Low-stringency washes consisted of three 20-min washes in 2× SSC–0.1% SDS at room temperature. High-stringency washes consisted of 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. Reactions were developed by using 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)-phenyl-1,2-dioxetane as the substrate. DNA sequencing was done by Mobix (McMaster University, Hamilton, Ontario, Canada) on an Applied Biosystems machine and with custom oligonucleotide primers (Mobix).

Nucleotide sequence accession numbers. The sequences of the rfb_{O8} genes are available in GenBank under accession number L41518. The sequences of the *rfbAB* and *rfbCDEF* genes from serotype O1 are available under accession numbers L31775 and L31762, respectively.

RESULTS

Three distinct clonal groups among *rfb* regions from *Klebsiella* strains with galactose-homopolymer O polysaccharides. Previously, we demonstrated that *rfb* clusters in *Klebsiella* serotypes O1 and O8 belong to substantially different clonal groups, as determined from DNA homology (18). *rfb* probes from serotype O1 strains recognized DNAs from other O1 isolates and from O2(2a) and O2(2a,2c) strains which also produced D-galactan I. In contrast, serotype O8 was detected only under low-stringency Southern hybridization conditions. To investigate the relationships between *rfb* regions in strains containing α-D-Gal*p* side-chain-modified D-galactan I O polysaccharides and in those clonal groups already defined by serotypes O1 and O8, hybridization analyses were expanded to include serotypes O9 and O2(2a,2f,2g) (Fig. 2). At low stringency, the O1 probe hybridized to DNAs from all serotypes tested, although the fragments detected in each serotype varied in size. The intensities of the signal obtained with O1 and O2(2a,2f,2g) DNAs were appreciably stronger than those from other DNAs tested. This is consistent with the results of hybridization carried out under high-stringency conditions, when only the DNAs from serotypes O1 and O2(2a,2f,2g) were recognized.

To determine whether the *rfb* gene clusters from serotypes O8 and O9 formed a single homogeneous clonal group, a gene probe from rfb_{O8} was used for hybridization. Previous analyses resulted in a chromosomal map of the region containing the rfb_{O8} gene cluster (18). The 1.8-kbp *Pst*I fragment located within the rfb_{O8} region was identified by using a gene probe from the rfb_{O1} cluster and cloned in plasmid pWQ211. To confirm the identity of the rfb_{O8} fragment, the termini of the insert were sequenced by using commercial T3 and T7 primers, and the predicted protein sequence was compared with that reported for rfb_{O1} (see below). From these analyses, the rfb_{O8} probe spans the junction of *rfbC* and *rfbD* (see Fig. 5). This confirmation that fragments identified by using rfb_{O1} gene probes at low stringency do indeed contain *rfb* sequences provides validation of results obtained by examination of heterologous *rfb* clusters under low-stringency hybridization conditions. The rfb_{O8} probe detected all chromosomal DNAs at low stringency, although the O8 DNA gave a much stronger signal. At high stringency, however, only the O8 chromosomal DNA was detected with the O8 probe. The serotype O9 *rfb* cluster therefore represents a third and distinct clonal group.

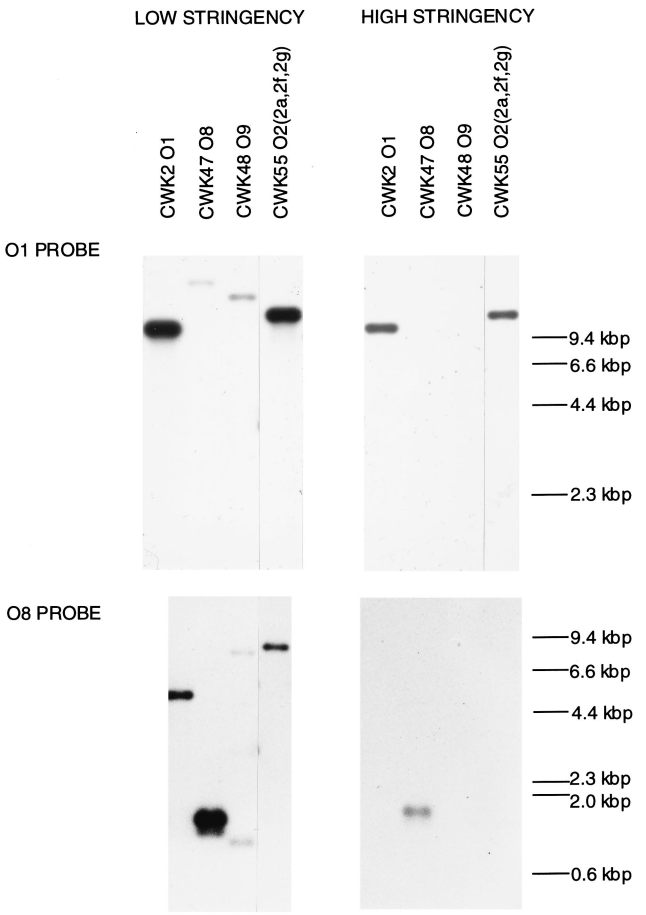

FIG. 2. Southern hybridization analysis reveals a third clonal group in the *rfb* genes required for expression of D-galactan I-based O polysaccharides in *Klebsiella* spp. Each chromosomal DNA was tested with O1- and O8-specific *rfb* probes at both high and low stringencies. (Upper panels) *Sac*I-digested chromosomal DNAs from the strains indicated were analyzed by using probe 1 from the rfb_{O1} cluster (Fig. 3). Only the DNAs from serotypes O1 and O2(2a,2f,2g) are recognized under high-stringency conditions. (Lower panels) The same DNAs were digested with *Pst*I and probed with an internal fragment from rfb_{O8} (Fig. 5). The O8 probe does not detect DNA from serotype O1, O2(2a,2f,2g), or O9 under high-stringency conditions.

By using low-stringency Southern hybridization and fragments of rfb_{O1} as probes, the physical maps of the *rfb* gene clusters from serotypes O9 and O2(2a,2f,2g) were established. The maps, shown in Fig. 3, demonstrate that the clusters are organized with homologous fragments arranged in the same order. Previous analyses of the *rfb* maps from *Klebsiella* strains within a single serotype (O1) indicated that the physical maps within a clonal group are highly polymorphic (8, 18). Given this fact and the low degree of homology shown in hybridization, the observation that the restriction sites were not conserved was to be expected.

Internal probes were made for each of the six rfb_{O1} genes and used to probe chromosomal DNAs of representative strains in dot blots, to determine whether the same genes were present and if the low degree of homology of *rfb* genes was confined to specific genes within the clusters (Fig. 4). Consistent with the Southern hybridization results described above, serotype O2(2a,2f,2g) DNA was recognized by each of the six rfb_{O1} gene probes at high stringency. DNAs from serotypes O8 and O9 were detected by rfb_{O1} probes only at low stringency; no reaction was detected with any of the probes at high stringency. When the dot blot results for serotypes O1 and O2(2a,2f,2g) are compared with those for the low-homology group (O8 and O9) under low-stringency conditions, it is evident that the amount of homology between the *rfbE* and *rfbF* genes in different serotypes is lower than that for *rfbABCD*. This is reflected in signals with reduced intensity. Each of the samples contained equal amounts of chromosomal DNA. The consistent signal intensity seen with each rfb_{O1} probe tested against the O1 and O2(2a,2f,2g) DNAs indicates that the results cannot be explained by variations in either the concentrations or labeling efficiencies of the different probes.

From this analysis, it is clear that there are at least three clonal groups of *rfb* genes which encode enzymes required for synthesis of D-galactan I. Serotypes O1, O8, and O9 serve as prototypes for these groups. Serotype O2(2a,2f,2g) belongs to the same clonal group as O1, indicating that the presence of an O antigen containing an α-D-Gal*p* side chain cannot be used to predict the *rfb* clonal group.

Cloning and functional analysis of the *rfb* genes from *Klebsiella* serotype O8. To address the clonal relationships in more detail, the rfb_{O8} region was cloned in plasmid pWQ210 (Fig. 5) on an 8.1-kbp *Hin*dIII fragment. Plasmid pWQ210 was electroporated into *E. coli* DH5α, and the recombinant strain produced smooth O-substituted LPS with an SDS-PAGE profile similar to that of authentic D-galactan I-substituted LPS (Fig. 6). A similar profile was evident when plasmid pWQ210 was introduced into SØ874 (Δ*rfb*) (19), indicating that the plasmid contained the complete rfb_{O8} cluster. The recombinant LPS reacted with antibodies specific for D-galactan I (19). However, no antiserum that can discriminate between D-galactan I (from O1) and its O-acetylated form (from O8) is available to us. Therefore, ^{1}H NMR was used resolve the precise identity of the O polysaccharide, since the presence of resonances in the 2.0- to 2.2-ppm region unambiguously identifies the methyl protons of the *O*-acetyl groups (18). No such resonances were detected, and the ^{1}H-NMR profile for *E. coli* DH5α(pWQ210) O polysaccharide was identical to that of authentic unacetylated D-galactan I (19). The genes required for O acetylation are therefore not located within rfb_{O8}, consistent with our observations on the determinants for other structural decorations on D-galactan I in serotypes O1 (8) and O9 (17).

Nucleotide sequence analysis shows that rfb_{O8} contains the six genes identified in rfb_{O1}, organized in a similar fashion (Fig. 5). In serotype O1, *rfbAB* encode an ABC-2 transporter (42) or traffic ATPase (10) which exports nascent O polysaccharide across the plasma membrane to the periplasmic face where ligation to lipid A-core occurs (6). RfbA is the transmembrane component, and RfbB is a cytoplasmic protein with consensus ATP-binding motifs. To confirm the function of the putative rfb_{O8} transporter, plasmid pWQ209 containing *rfbAB* and a truncated *rfbC* gene was electroporated into *E. coli* DH5α containing pWQ19. pWQ19 carries *rfbCDEF* from serotype O1. *E. coli* strains harboring pWQ19 are able to synthesize D-galactan I, but in the absence of the cognate transporter, the polymer accumulates in the cytoplasm (6). SDS-PAGE analysis (Fig. 7) illustrates that the transport functions from serotype O8 can substitute for those from serotype O1. ABC transporters are used for the transport of group II capsular polysaccharides in *E. coli*, *Haemophilus influenzae*, and *Neisseria meningitidis* (reviewed in references 5, 46, and 55). In the *E. coli* capsule system, for which polysaccharide ABC transporters are best described, the transporter can export heterologous polysaccharides with different repeat-unit structures. However, we were unable to reconstitute D-galactan I export with the

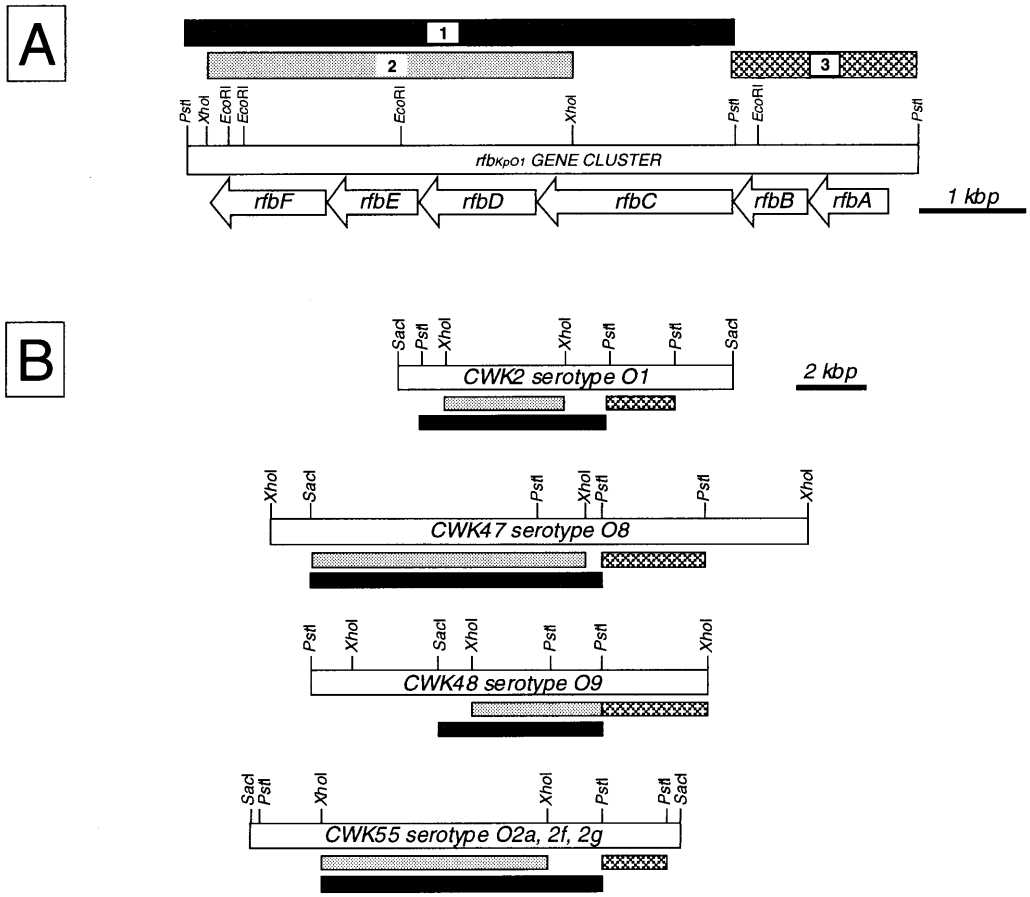

FIG. 3. Gene probes (A) and physical maps of the chromosomal *rfb* regions from *Klebsiella* serotypes O1, O8, O9, and O2(2a,2f,2g) (B). Physical maps were established by Southern hybridization experiments involving chromosomal DNAs digested by using one or more restriction endonucleases. The gene probes (1 to 3) were fragments from pWQ5 containing the rfb_{O1} gene cluster. The boxes under each map in panel B indicate the extents of the sequences detected with each probe. The serotype O1 cluster from strain CWK2 is taken as the prototype and is shown for reference. The rfb_{O8} region map was published previously (18), although the position of one *Pst*I site in the O8 map was subsequently found to be incorrect; the corrected form is shown here.

ABC transporter from *E. coli* K1 (6). From the results presented here, it is clear that O acetylation is not a prerequisite for export of D-galactan I by RfbAB from *Klebsiella* serotype O8.

Sequence comparisons of the *rfb* gene clusters from *Klebsiella* serotypes O1 and O8. The *rfb* genes from serotypes O1 and O8 exhibit a great deal of similarity (Table 2). The amount of identity at the nucleotide sequence level ranges from 66.9 to 79.7%, and there is no obvious pattern of drift in homology across the cluster. The lowest degrees of homology and the largest differences in respective moles percent G+C values were seen between the *rfbE* and *rfbF* homologs, as expected from the hybridization data presented above (Fig. 4). The predicted Rfb proteins were highly conserved. With the exception of RfbC, homologs from serotypes O1 and O8 were composed of identical numbers of amino acid residues. Interestingly, $RfbC_{O8}$ is one residue smaller than $RfbC_{O1}$ because of a single codon deletion. The third codon from $RfbC_{O1}$ (encoding an asparagine residue) is missing from $RfbC_{O8}$. The appropriate regions from both clusters were resequenced to confirm that the change was not due to a sequencing error. Optimal alignment of the C-terminal 60 residues of the RfbC homologs also required the introduction of single breaks into each sequence. The remaining five predicted Rfb proteins were readily aligned without breaks. The overall similarities ranged from 78.4% (64.3% identical) to 97.6% (93.4% identical). The greatest identity was found in the RfbA and RfbB homologs.

DISCUSSION

D-Galactan I-based O polysaccharides are found in a number of *Klebsiella* O serotypes, with serotype-specific distinctions resulting from decoration of a common backbone (Fig. 1). The type of structural variation in the D-galactan I family of polymers differs from that in, for example, the polymannose O polysaccharides of *Klebsiella* serotypes O3 and O5. In the mannose homopolymers, the size and antigenicity of the repeat unit vary because of alterations in the sequence of linkages in a linear backbone (20). The situation reported here resembles more closely that seen for LPS O-antigen factors in some *S. enterica* serovars. *S. enterica* serogroups A, B, and D share a common trisaccharide repeat-unit backbone, but they differ in the identity of the side group dideoxyhexose attached to the backbone. The dideoxyhexose addition is encoded by genes in the *rfb* cluster (41). Within each serogroup, nonstoichiometric additions of α-D-glucosyl and *O*-acetyl groups further alter the serospecificity and give rise to new O factors (26). It is now apparent that the serotype-specific modifications of D-galactan I in *Klebsiella* species are encoded by genes located outside *rfb*, as is the case for the nonstoichiometric α-D-glucosylation and

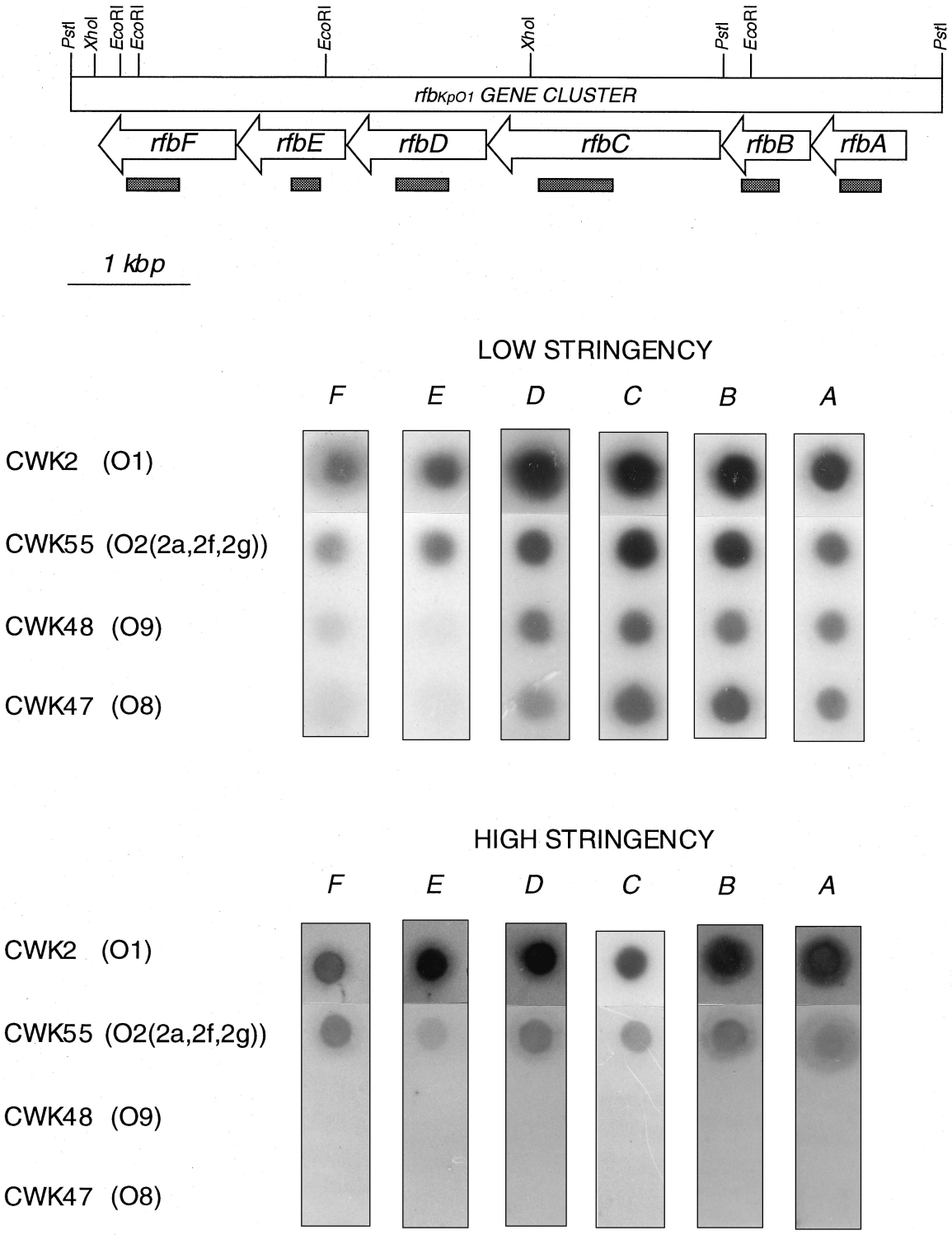

FIG. 4. Detection of sequences having homology with individual rfb_{O1} genes by dot blot hybridization. Gene probes were generated by PCR amplification, and their sizes and locations are shown under the physical map of the rfb_{O1} gene cluster. Chromosomal DNAs from the strains indicated were examined at both high and low stringencies; identical amounts of DNA were applied in each DNA sample. Only O1 and O2(2a,2f,2g) are uniformly detected at high stringency, but each DNA sample gives a positive result with all six gene probes at low stringency.

O acetylation of *S. enterica* O polysaccharides (29). These unlinked loci in *S. enterica* often result from lysogenic bacteriophages.

Although there are a number of reports of comparisons of *rfb* homologs in different bacteria, there are often problems in the unequivocal interpretation of the *rfb* clonal relationships. This is because the detailed structures for the O polysaccharides are often not known or because the precise structure and

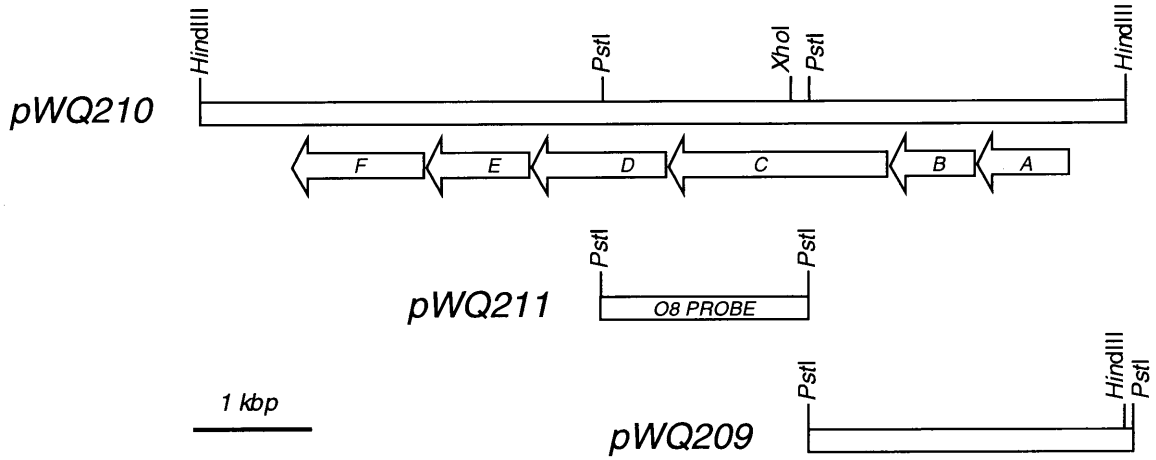

FIG. 5. Physical map of plasmid pWQ210 and its subclones. A single *Hin*dIII fragment containing the complete O8 *rfb* region is cloned in pWQ210. Plasmid pWQ211 contains the region that was used as an O8-specific probe in the hybridization experiments whose results are shown in Fig. 2.

function of the *rfb* clusters have not been determined. To our knowledge, there are only two systems for which *rfb* comparisons have been made and these essential requirements have been met. The two systems are the O polysaccharides of some *S. enterica* serogroups, studied by P. Reeves and his colleagues (41), and the D-galactan I O antigens of *Klebsiella* species reported here. These systems provide interesting comparison, since the studies of *S. enterica* examine serovars within a species and the results for *Klebsiella* species deal with different species within a genus. The clonal relationships detected in *rfb* genes in these two systems are quite different. The *S. enterica* serogroup A, B, and D gene clusters differ only in the genes for the dideoxyhexose precursor synthesis pathway (41). Common genes in the clusters are very similar, if not identical, and the restriction maps for conserved regions are essentially identical (51). Changes in the dideoxyhexose pathway genes are proposed to have arisen by the (relatively) recent introduction of genes into the serogroup B cluster, by lateral transfer, to generate serogroup D, followed by mutation of one gene in serogroup D to give rise to serogroup A (41). From a broader study, with a range of strains belonging to 45 different serogroups of *S. enterica*, it was concluded that *rfb* genes within the same serogroup are very similar or identical (57). When *rfb* genes for shared enzymes in *S. enterica* serovars with structurally diverse O antigens are compared, the levels of homology are quite variable (57).

There are two other reports of clonal diversity based on different levels of *rfb* homology within a bacterial species or serogroup. In *E. coli* O101, two clonal groups have been identified (14). However, despite there being no reports of serological variants in O101, LPS SDS-PAGE profiles from individual strains appear remarkably variable and inconsistent with an homogeneous structure; no detailed chemical analysis has been performed. In *Shigella boydii* type 12, two *rfb* clonal groups were found in isolates whose O antigens showed some differences in immunogenicity (50). The chemical basis for the antigenic differences in *S. boydii* type 12 O antigens is also unknown.

When less closely related strains have been compared, the levels of similarity between the *rfb* genes have been quite variable. High levels of homology have been reported for *rfb* genes that encode enzymes responsible for the synthesis of precursors for shared 3,6-dideoxyhexose O-polysaccharide constituents in *S. enterica* serovar Typhimurium (serogroup B) and serotypes of *Yersinia pseudotuberculosis* (21). Similarly, *E. coli* O7 and *S. boydii* type 12 synthesize structurally similar O polysaccharides, and the respective *rfb* genes also show high levels of homology (50). In contrast, there are also situations in

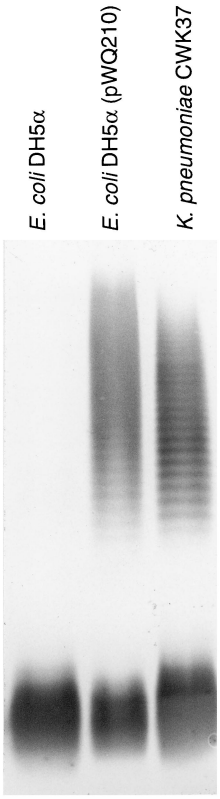

FIG. 6. Plasmid pWQ210 contains the complete *rfb* region from *Klebsiella* serotype O8 and encodes enzymes required for expression of D-galactan I in an *E. coli* K-12 host. SDS-PAGE was used to examine the LPSs of *E. coli* DH5α, *E. coli* DH5α(pWQ210), and *Klebsiella* strain CWK37, a reference strain producing smooth LPS containing only D-galactan I. The identification of D-galactan I in *E. coli* DH5α(pWQ210) was made by using specific antisera and by NMR analysis of the purified O polysaccharide. Samples for SDS-PAGE were prepared by the SDS-proteinase K whole-cell lysate method (15).

FIG. 7. *rfbAB*$_{O8}$ encode an ABC transporter capable of complementing a transport defect in the serotype O1 cluster. Plasmid pWQ19 contains *rfbC-DEF*$_{O1}$, and strains containing this plasmid can synthesize D-galactan I but produce rough LPS because the O polysaccharide is not exported across the plasma membrane to facilitate its ligation to lipid A-core (6). pWQ209 contains *rfbAB*$_{O8}$, encoding the ABC transporter. When both pWQ209 and pWQ19 functions are expressed in *E. coli* K-12, smooth D-galactan I-substituted LPS is formed.

which common Rfb enzymes are used in O-antigen biosynthesis in different bacterial species, despite low levels of DNA homology in the respective structural *rfb* genes. This is exemplified by the *rfb* genes from *E. coli* O111 and O55 and from *S. enterica* serovar Greenside, whose O antigens contain the sugar colitose (3).

The finding of clonal diversity in *Klebsiella* species with functionally identical *rfb* loci, together with the observation that many *Klebsiella* O-antigen structures are quite similar (20), is particularly interesting. However, D-galactan I-based O antigens are not confined to *Klebsiella* species. *Serratia marcescens* O16 and O20 (38) and serotypes 4 (39) and 10 (43) of *Pasteurella haemolytica* also produce D-galactan I. *S. marcescens* O24 (37) produces a carbohydrate backbone identical to that of *Klebsiella* serotype O2(2a,2f,2g), and *Serratia plymuthica* contains an O antigen containing both D-galactan I and D-galactan II (2). Sequence comparisons, functional analyses, and complementation experiments are consistent with conserved *rfb* functions in strains of *Klebsiella* (reference 17 and this paper) and *S. marcescens* (48). Significantly, when the *rfb* genes from *S. marcescens* O16 were analyzed, the cluster showed an organization similar to that of the *Klebsiella* serotype O1 *rfb* genes (48). The predicted *S. marcescens* O16 RfbA (RfbA$_{SmO16}$) and RfbB$_{SmO16}$ proteins were highly conserved compared with the *Klebsiella* O1 prototypes. The similarity values were 87.9% (75.7% identical) and 86.5% (78.0% identical), respectively. In comparison, the RfbF$_{SmO16}$ homolog had only 71.1% similarity (57.6% identity). The higher level of conservation in the transporter proteins is consistent with the findings for *Klebsiella* serotypes O1 and O8. The reasons for this are unclear, although it is possible that stringent requirements for function of the ABC transporter accomodate less sequence drift. RfbD is needed for Gal*f* precursor formation and has UDP-galactopyranose mutase activity (24). A protein with a similar amino acid sequence and enzymatic activity has been identified in *E. coli* K-12 (32). RfbF is known to be an initiating galactosyltransferase enzyme capable of transferring one residue each of Gal*p* and Gal*f* to a primed lipid intermediate (7). Preliminary evidence indicates that RfbC and -E are also required for galactosyl transferase activity to complete the D-galactan I polymer (24). From the comparison of the protein sequences, it appears that galactosyltransferase enzymes can accomodate more sequence variation without compromising activity and specificity. Overall, the homologs from different *Klebsiella* clonal groups show a closer relationship than that exhibited between *Klebsiella* species and *S. marcescens*, as might be expected.

It has been proposed that O-antigen diversity in *S. enterica* reflects a series of genetic exchanges between different organisms with different G+C contents (40). In *E. coli*, the consequences of such lateral transfer events are evident from the finding of diverse *rfb* regions in three isolates which, aside from *rfb*, form a single clonal group (27). In other members of the *Enterobacteriaceae*, the *gnd* (6-phosphogluconate dehydrogenase) structural gene is located adjacent to *rfb*. This location also occurs in *Klebsiella* serotype O1 (8) and has been suggested for at least some other *Klebsiella* strains (33). Lateral intergeneric transfer and recombination of *rfb* gene regions have been invoked to explain the significant differences in the *gnd* sequences among *E. coli* strains. This gene is expected to be highly conserved because of the important metabolic function of its product (33). Nelson and Selander have suggested that the surprisingly high rate of recombination in *gnd* could be attributed to its cotransfer along with adjacent loci such as *rfb*, whose activities are subject to diversifying selection in connection with the avoidance of host defense systems (33). Effectively, sequence differences in alleles of *gnd* within *E. coli* are proposed to arise from their frequent cotransfer with the *rfb* region of strains producing different O-specific polysaccharides. Nelson and Selander performed a sequence analysis of

TABLE 2. Comparison of *rfb* genes and their predicted products from *K. pneumoniae* serotypes O1 and O8

Gene	Nucleotide sequence			Predicted polypeptide sequence	
	No. of nucleotides	Mol% G+C	% Identity between O1 and O8 genes	No. of amino acid residues	% Identity (total % similarity) between O1 and O8 polypeptides
rfbA$_{O1}$	777	37.4	79.7	259	93.4 (97.6)
rfbA$_{O8}$	777	36.6		259	
rfbB$_{O1}$	738	44.2	77.4	246	94.3 (98.0)
rfbB$_{O8}$	738	45.6		246	
rfbC$_{O1}$	1,893	38.7	72.6	631	77.6 (85.4)
rfbC$_{O8}$	1,890	39.8		630	
rfbD$_{O1}$	1,152	40.9	75.8	384	85.2 (89.6)
rfbD$_{O8}$	1,152	44.3		384	
rfbE$_{O1}$	891	42.5	66.9	297	64.3 (78.4)
rfbE$_{O8}$	891	40.4		297	
rfbF$_{O1}$	1,131	37.3	72.4	377	78.3 (84.4)
rfbF$_{O8}$	1,131	40.6		377	

the *gnd* loci of a variety of members of the family *Enterobacteriaceae*, including *Klebsiella* strains belonging to different species (33). Evidence which supports lateral transfer between *Klebsiella* species and *E. coli* was presented. The *gnd* sequences within the *Klebsiella* genus were found to be clonally diverse, as we report here for the adjacent *rfb* loci. Significantly, among the strains examined by Nelson and Selander (33) are *Klebsiella* strains 7380 [O2(2a,2b)], which has a high degree of homology to serotype O1 (18), and CWK53 [O2(2e,2h)], which is a member of the O9 group (19). The *gnd* alleles in these strains were genetically quite distant (33). Given that the *Klebsiella rfb* clusters studied here are all involved in expression of the same O-antigen structure, D-galactan I, high levels of conservation in the *rfb* genes might be expected if they were transferred in a (relatively) recent lateral transfer event. Although the moles percent G+C values for different *Klebsiella* species are not dramatically different, hybridization studies indicate that the individual species are not highly related (25, 44). If both *gnd* and *rfb* sequences exhibit these variations, the directional mutation theory would predict that they have been together in the same genetic background for some time (47). Consequently, the *rfb* diversity argues against frequent and recent transfer of *gnd* and *rfb* regions between *Klebsiella* strains to disseminate the D-galactan I antigen. This may be a feature which distinguishes *Klebsiella* species from *E. coli* and *Salmonella* species.

The stable maintenance of *rfb* regions encoding D-galactan I could be explained if the D-galactan I structure provided significant advantages in virulence and survival to those bacteria which produce it. The presence of bacteria expressing galactan polymers in the normal human intestinal flora is considered to be responsible for the high normal concentration (1%) of anti-(1→3)-α-D-Gal antibodies in circulating immunoglobulin G (11). The (1→3)-α-D-Gal epitope is cryptic in humans but is widespread in other mammals, and the immune response to this epitope is linked to inflammation, tissue damage, and in some cases, autoimmune phenomena (reference 11 and references therein). Although reaction of the human anti-(1→3)-α-D-Gal antibody with either D-galactan I or modified D-galactan I has not been shown directly, it is known that binding of the antibody to the surface of an uncharacterized strain of *Serratia* in some way provides resistance to complement-mediated lysis via the alternative pathway (13). D-Galactan I-based O polysaccharides are common in *Serratia* species (2, 37, 38). Thus, there is at least some indirect evidence to support an advantage of D-galactan I expression. Selective pressures may still favor diversification of O antigen structure in *Klebsiella* species, but the ability to alter antigenicity through modifications of a basic D-galactan I polymer by using unlinked genetic loci may offer advantages over recombination events within the gene cluster. Some, or all, of these observations may provide a partial explanation for the conservation of D-galactan I and its derivatives.

However, there is one particularly interesting alternative explanation for diversity in the *gnd* locus. In *Klebsiella* species, the LPS O antigens are masked by large amounts of capsular K antigens. It is conceivable that the high level of structural and serological diversity seen in the K antigens (20, 36) may be more important in niche adaptation than the O antigens in these bacteria. Significantly, the *gnd* locus maps adjacent to the *cps* genes for K-antigen biosynthesis in *Klebsiella* species (1, 8), and it is possible that lateral transfer of *cps* genes plays a much more significant role in *gnd* diversity in *Klebsiella* species.

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