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Distribution of the *rol* Gene Encoding the Regulator of Lipopolysaccharide O-Chain Length in *Escherichia coli* and Its Influence on the Expression of Group I Capsular K Antigens

CHRISTINE DODGSON, PAUL AMOR, AND CHRIS WHITFIELD*

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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The *rol* (*cld*) gene encodes a protein involved in the expression of lipopolysaccharides in some members of the family *Enterobacteriaceae*. Rol interacts with one or more components of Rfc-dependent O-antigen biosynthetic complexes to regulate the chain length of lipopolysaccharide O antigens. The Rfc-Rol-dependent pathway for O-antigen synthesis is found in strains with heteropolysaccharide O antigens, and, consistent with this association, *rol*-homologous sequences were detected in chromosomal DNAs from 17 different serotypes with heteropolysaccharide O antigens. Homopolymer O antigens are synthesized by a pathway that does not involve either Rfc or Rol. It was therefore unexpected when a survey of *Escherichia coli* strains possessing mannose homopolymer O8 and O9 antigens showed that some strains contained *rol*. All 11 *rol*-positive strains coexpressed a group IB capsular K antigen with the O8 or O9 antigen. In contrast, 12 *rol*-negative strains all produced group IA K antigens in addition to the homopolymer O antigen. Previous research from this and other laboratories has shown that portions of the group I K antigens are attached to lipopolysaccharide lipid A-core, in a form that we have designated K_{LPS}. By constructing a hybrid strain with a deep rough *rfa* defect, it was shown that the K40 (group IB) K_{LPS} antigen exists primarily as long chains. However, a significant amount of K40 antigen was surface expressed in a lipid A-core-independent pathway. The typical chain length distribution of the K40 antigen was altered by introduction of multicopy *rol*, suggesting that the K40 group IB K antigen is equivalent to a Rol-dependent O antigen. The prototype K30 (group IA) K antigen is expressed as short oligosaccharides (primarily single repeat units) in K_{LPS}, as well as a high-molecular-weight lipid A-core-independent form. Introduction of multicopy *rol* into the K30 strain generated a novel modal pattern of K_{LPS} with longer polysaccharide chains. Collectively, these results suggested that group IA K_{LPS} is also synthesized by a Rol-dependent pathway and that the typically short oligosaccharide K_{LPS} results from the absence of Rol activity in these strains.

The cell surface polysaccharides of *Escherichia coli*, i.e., capsular polysaccharides (K antigens), and the side-chain polysaccharides (O antigens) of lipopolysaccharides (LPSs) are important virulence determinants (reviewed in reference 53). LPS O antigens play a key role in avoidance of the host complement system, and O-polysaccharide length, distribution, and chemical structure all influence the bacterial cell's resistance to serum. Although some K antigens may also contribute to serum resistance, capsules are generally considered to play more important roles in evasion of phagocytosis.

The K antigens of *E. coli* are divided into two groups (reviewed in references 18 and 53). The thermostable group I K antigens are coexpressed with a limited range of O antigens, predominantly serotype O8 and O9 LPS. Group I K antigens are further subdivided on the basis of the absence (IA) or presence (IB) of amino sugars in the K-antigen polysaccharide repeat unit. Group IA K antigens resemble structures found in *Klebsiella* spp., but there are no obvious counterparts for the group IB K antigens. Thermolabile group II K antigens are expressed with many different O antigens. Group I and II K antigens are distinguished by two additional important characteristics: (i) mode of linkage of the K antigen to the cell surface and (ii) chromosomal location of the respective gene clusters for K-antigen biosynthesis. *E. coli* O9:K30 is a prototype strain with a group IA K antigen. It produces a low-molecular-weight K30 antigen oligosaccharide (termed K_{LPS} [30]) that is attached to the cell surface by means of lipid A-core and a high-molecular-weight K30 antigen that forms a capsular structure and whose surface expression is LPS independent. Other group IA strains also produce K_{LPS} with short chains (30). K_{LPS} is also found in *E. coli* O8:K40 (19), a prototype strain with a group IB K antigen. However, substantially longer group IB K-antigen polysaccharide chains are linked to lipid A-core in K40 (19, 30) and other K-antigen serotypes of this group (30). In contrast, group II capsules have *sn*-L-α-glycerophosphatidic acid as the terminal lipid residue. The organization and functions encoded by *serA*-linked *kps* genes that are responsible for expression of group II K antigens have been researched quite extensively (reviewed in references 6, 18, 46, and 55). However, other than the fact that the *cps* genes for biosynthesis of group IA K antigens map near the *his* and *rfb* (O-antigen biosynthesis) loci (29, 45, 54), little is known about their organization or the functions of their gene products. The *cps* genes for group IB strains have not yet been mapped or characterized.

Although the mechanisms involved in the biosynthesis of the group IA and IB K antigens are unknown, the proximity of the *cps* (IA) locus to *rfb* and the lipid A-core linkage of at least some forms of group IA and IB K antigens suggest that their

* Corresponding author. Phone: (519) 824-4120, ext. 3478. Fax: (519) 837-1802. Electronic mail address: cwhitfie@micro.uoguelph.ca.

synthesis may resemble that of O antigens. There are two mechanisms for O-antigen biosynthesis in members of the family *Enterobacteriaceae* (reviewed in reference 52). These pathways differ in the mechanism of polymerization as well as the cellular location of the polymerization process. The two pathways are termed Rfc dependent and Rfc independent.

Salmonella enterica serovars, *Shigella* species, and certain *E. coli* serotypes use the Rfc-dependent mechanism for O-antigen polymerization. These strains produce heteropolymer O antigens, and individual O-antigen repeat units (O units) are synthesized on a lipid carrier at the cytoplasmic face of the plasma membrane. The lipid-linked O units are then translocated across the membrane by a currently unknown mechanism. Polymerization of the lipid-linked O polysaccharide and its subsequent ligation to lipid A-core both occur at the periplasmic face of the plasma membrane (33). The *rfc* gene product is known to be required for polymerization and is considered to be a polymerase enzyme. The product of the *rol* (regulator of O-chain length) gene (3), also known as *cld* (chain length determinant) (1), is also involved in the polymerization of some heteropolysaccharide O antigens. This gene was first described for *E. coli* O75 by Batchelor et al. (2, 3) and is located near the *rfb* (O-antigen biosynthesis cluster) region. Mutants which lack *rol* have unregulated O-antigen polymerization and exhibit LPS banding patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels which differ from the modal pattern of the wild type. Rol homologs with related primary amino acid sequences and conserved secondary structure have now been described for *E. coli* O111 (1), *S. enterica* serovar Typhimurium (1, 2), and *Shigella* species (25, 37).

The Rfc-independent pathway involves assembly of the O antigen by a processive glycosyl transfer mechanism occurring at the cytoplasmic face of the plasma membrane (52). There is no blockwise synthesis of individual lipid-linked O units, and no Rfc homologs are involved. Once the polymer chain is complete, it is translocated across the plasma membrane by an ABC transporter in a manner first described for biosynthesis of group II K antigens in *E. coli* (40, 41, 47) and other bacteria (14, 27). Bacteria which have been shown to use this pathway of O-antigen synthesis include *Klebsiella pneumoniae* O1 (7), *Serratia marcescens* O16 (49), *E. coli* O8 and O9 (24), and *Yersinia enterocolitica* O:3 (56). To date, this pathway is confined to homopolymer O antigens, although use of a similar assembly pathway for heteropolysaccharide group II K antigens suggests that the pathway may not necessarily be restricted by O-antigen structure. Expression from cloned gene clusters of the *E. coli* O9 (20) and *K. pneumoniae* O1 (9) antigens is unaffected in the *rol*-deleted background of *E. coli* SØ874. These cloned *rfb* clusters do not contain *rol* homologs, and it is therefore clearly established that the activity of Rol is confined to the Rfc-dependent pathways.

Since group I K antigens are coexpressed with Rol-independent O8 and O9 O antigens, the *rol* status of such strains is unclear. The objective of these studies was to investigate whether strains with group I K antigens possess a *rol* gene and to determine whether Rol plays any role in expression of the group IA and IB K_{LPS} antigens. The data presented here establish that group I K-antigen synthesis occurs by a pathway that can interact with Rol.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells were routinely cultured in Luria-Bertani broth or M9 salts defined medium containing 0.2% D-glucose and, when appropriate, amino acids (40 μg/ml), thiamine and nicotinamide (1 μg/ml), and uracil (40 μg/ml) (36). Antibiotics were added when required at the following concentrations: chloramphenicol, 25 μg/ml; streptomycin, 200 μg/ml; and ampicillin, 100 μg/ml. Incubation was at 37°C.

E. coli CWG283 was constructed by transferring the Δ*rfaGPBI* mutation from PR3339 by conjugation to *E. coli* 2775. Counterselection on M9 defined medium was based on the auxotrophic markers in PR3339, and the Δ*rfa* defect was selected by chloramphenicol resistance. Details of the strategy were reported previously (30).

Recombinant plasmids. Plasmid pRAB10 contains the *rol* gene cloned from *E. coli* O75 into the vector pACYC184 (2, 3). The 2.9-kbp *Nco*I-*Eco*RI fragment containing *rol* was excised from pRAB10 and cloned into pBR329 to produce plasmid pWQ30. This construct was made to provide a compatible plasmid for cotransformation with pSS37. Plasmid pSS37 is pACYC184 carrying the *rfb* gene cluster and *rfpAB* from *Shigella dysenteriae* type I (48). The functions determined by pSS37 are sufficient for expression of the Rol-dependent *S. dysenteriae* type I O antigen in *E. coli* K-12. All transformations were made by electroporation (5) with a Gene Pulser from Bio-Rad Laboratories.

Amplification of the *rol* gene and Southern hybridization. PCR amplification reactions were performed with a Coy Temp-Cycler model 60 thermocycler. Oligonucleotide primers for PCR amplification were designed from the nucleotide sequence of *rol* from *E. coli* O75 (2). Primer 1 (5′-GTCAGTTAGGGTAA TGATGA-3′) begins at nucleotide 121 of the deposited sequence (GenBank accession number M89934) and contains the start codon (underlined ATG) of *rol*. The reverse primer, primer 2 (5′-CGTCGGTTTCATTACATAGC-3′) is complementary to nucleotides 988 to 969. The *rol* open reading frame terminates with a TAA at nucleotide 1112. Each cycle consisted of a denaturing step at 95°C for 1 min, an annealing step at 52°C for 2 min, and a polymerization step at 72°C for 2 min. Amplification was carried out for 25 cycles with *Taq* DNA polymerase from Boehringer Mannheim.

For hybridization experiments, agarose gel-separated DNA fragments were depurinated, denatured, and neutralized prior to Southern transfer (44). The fragments were transferred by overnight capillary blotting to positively charged nylon membranes (Boehringer Mannheim) by using 10× SSC (1.5 M NaCl, 150 mM Na citrate [pH 7.0]) and cross-linked to the membrane with a Stratagene UV cross-linker. Probe labeling and detection were performed by using the digoxigenin labeling kit from Boehringer Mannheim. Prehybridization and hybridization steps were carried out at 37°C, and all other conditions were those recommended by the manufacturer.

Gel electrophoresis of LPS. LPS from proteinase K-digested whole-cell lysates was isolated from stationary-phase cultures as described by Hitchcock and Brown (17). Samples were examined by SDS-PAGE using equipment and commercially prepared, 1-mm-thick, 10 to 20% acrylamide gradient Tricine gels from Novex (San Diego, Calif.). The electrophoresis conditions were those recommended by the manufacturer, and silver staining was done according to the protocol of Tsai and Frasch (51).

Detection of K30 and K40 antigen expression by Western immunoblotting. Polyclonal antisera were raised against the K30 (group IA) and K40 (group IB) K antigens. Formalin-killed whole cells of *E. coli* strains CWG44 (O^-:K30) and 2775 (O8:K40) were used to immunize New Zealand White rabbits. To prepare K30-specific antibodies, immune serum was absorbed with whole cells of *E. coli* CWG57. The resulting anti-K30 serum did not recognize antigens from *E. coli* CWG57, 2775, or AB1133 in either agglutination or Western immunoblot experiments. Cross-reactive antibodies in the anti-K40 serum were removed by absorbing the immune serum with E. Coli F1321 cells, and after this treatment, no reaction against *E. coli* F1321, CWG44, CWG57, or AB1133 was detected.

For Western blots, SDS-PAGE-separated LPS samples were transferred to BioTrace NT membrane (Gelman Sciences) by a modification (11) of the method of Towbin et al. (50). Rabbit antisera were used at dilutions of 1:1,000. The second antibody was alkaline phosphatase-conjugated goat anti-rabbit $F(ab')_2$ (Sigma), and the development system was nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim).

RESULTS

Distribution of *rol* sequences in *E. coli* strains with heteropolysaccharide O antigens. Examination of the distribution of *rol* sequences was initially performed by Southern hybridization (Fig. 1). To produce a *rol*-specific probe, PCR was used to amplify an 867-bp fragment containing the *rol* gene of *E. coli* O75 (2, 3). *rol*-homologous sequences were detected in chromosomal DNAs isolated from 17 *E. coli* serotypes with heteropolymer O antigens (12), including O75. Most of these strains contained group II K antigens, such as K1, K5, and K13 (18). The hybridization result is consistent with the association of Rol activity with synthesis of heteropolysaccharide O antigens. On the basis of the number and sizes of DNA fragments detected by the probe, there appears to be extensive polymor-

TABLE 1. *E. coli* strains used in this study

Strain	Genotype and/or description	Serotype[a]	Source or reference
DH5α	K-12 ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) U169 F^-		44
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*		35
SØ874	K-12 *lacZ trp* Δ*sbcB-rfb upp rel rpsL*		38
CLM4	SØ874 but Δ(*recA-srl*)*306*		31
F1321	*his pro pyr rha met* Str^r	O8:K^-	34
E69	Prototroph	O9:K30:H12 (IA)	F. Ørskov
CWG44	rfb_{O9} *his trp lac rpsL*	O^-:K30 (IA)	32
CWG57	CWG44 derivative; rfb_{O9} cps_{K30} *his trp lac rpsL*	O^-:K^-	8
PR3339	Hfr derivative; *azi-15 fhuA21 lacY1 supE44* λ^- *rfbD1 thi-1* Δ*rfaGBPI*::Cml^r[b]	O^-:K^-	30
2775	Prototroph	O8:K40:H9 (IB)	B. Jann
CWG283	2775 but Δ*rfaGBPI*::Cml^r[c]	O^-:$K40^+$	This study
2146		O9:K26:H^- (IA)	B. Jann
E56b		O8:K27:H^- (IA)	F. Ørskov
K14a		O9ab:K28:H^- (IA)	F. Ørskov
Bi161-42		O9:K29:H^- (IA)	F. Ørskov
Su3973-41		O9:K31:H^- (IA)	F. Ørskov
E75		O9:K34:H^- (IA)	F. Ørskov
2150		O9:K37:H^- (IA)	B. Jann
2151		O9:K39:H9 (IA)	B. Jann
A295b		O8:K42:H^- (IA)	F. Ørskov
2178		O8:K43:H11 (IA)	B. Jann
N24c		O9:K55:H^- (IA)	F. Ørskov
G3404-41		O8:K8:H^- (IB)	F. Ørskov
2667		O9:K9:H^- (IB)	B. Jann
2176		O8:K41:H11 (IB)	B. Jann
2179		O8:K44:H^- (IB)	B. Jann
2167		O8:K45:H^- (IB)	F. Ørskov
2181		O8:K46:H4 (IB)	B. Jann
2182		O8:K47:H2 (IB)	B. Jann
2184		O8:K49:H21 (IB)	B. Jann
2185		O8:K50:H9 (IB)	B. Jann
D227		O8:K87:H19 (IB)	F. Ørskov
U5-41		O1:K1:H7	F. Ørskov
Su1242		O2:K2:H1	F. Ørskov
U4-41		O4:K3:H5	F. Ørskov
2147-59		O6:K13:H49	F. Ørskov
		O7:K1:H^-	M. Valvano
Bi8337-41		O10:K5:H4	F. Ørskov
P4		O16:K?:H48	F. Ørskov
RS218		O18ac:K1:H^-	R. P. Silver
F8188-41		O19ab:K?:H7	F. Ørskov
E47a		O25:K19:H12	F. Ørskov
5306-56		O26:K?:H46	F. Ørskov
K42		O45:K1:H23	F. Ørskov
4106-54		O52:K?:H45	F. Ørskov
C993-81		O55:K?:H?	F. Ørskov
P10a		O71:K?:H12	F. Ørskov
RS501		O75:K100	M. Valvano
H40		O87:K?:H12	F. Ørskov

[a] The group designation for the K antigen (IA or IB) in parentheses is from reference 21.
[b] The construction of Δ*rfaGBPI*::Cml^r is described in reference 39.
[c] PR3339 was mated with 2775 with selection for chloramphenicol resistance; auxotrophic markers were used for counterselection.

phism in this region of the chromosome, as might be expected given the diversity of the adjacent *rfb* regions.

Distribution of *rol* sequences in *E. coli* strains with homopolysaccharide O antigens. *E. coli* O8 and O9 strains with group I K antigens were also screened for *rol*-homologous sequences by Southern hybridization using the same *rol*-specific probe. The detection of *rol* sequences in DNA from O8 and O9 isolates was variable (Fig. 2A), and there was no correlation between the presence of *rol* sequences and expression of either O8 or O9 LPS. However, there was a definite correlation between the presence of *rol* sequences and strains with group IB K antigens. In an expanded analysis, all of the strains in our collection with group IB K antigens (11 strains in all) were found to contain *rol* sequences (Fig. 2B). In contrast to the heterogeneity obtained for strains with heteropolysaccharide O antigens (Fig. 1), most (9 of 11) strains with group IB K antigens contained single *rol*-homologous fragments of the same apparent size. To confirm that the hybridization probe was indeed detecting *rol*, these findings were verified by PCR amplification using the primers based on the published *E. coli* O75 *rol* sequence (2). The predicted 867-bp fragment was amplified from the positive control *E. coli* O75 and all of the group IB strains (11 in total) (Fig. 3).

No hybridization with the *rol* probe was detected in DNAs

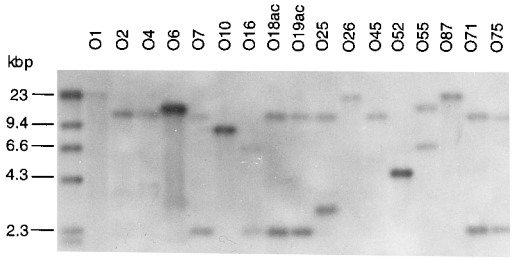

FIG. 1. Detection of *rol*-homologous sequences in chromosomal DNA from *E. coli* O serotypes producing heteropolysaccharide O antigens. The *rol* probe consists of an 867-bp fragment amplified from the *rol* gene of *E. coli* O75 by PCR. Chromosomal DNAs were digested with *Pst*I.

from a collection of strains with group IA K antigens (12 in all) (10), and no DNA fragments were amplified by PCR (Fig. 3). In strains with heteropolymer O antigens such as *E. coli* O75 (2, 3), *E. coli* O111 (1), and *S. enterica* serovar Typhimurium (1), *rol* is located between the chromosomal *his* and *gnd* loci and the gene order is *his-rol-gnd-rfb*. *E. coli* O9:K30 produces a prototype group IA K antigen, and in this strain the O9 *rfb* gene cluster is instead located between *his* and *gnd* (10, 20). The *rfb* cluster in another O9 strain has a similar location (24). Nucleotide sequence analysis reveals no *rol* gene in this region (10, 24), consistent with the inability to detect *rol* sequences by Southern hybridization or PCR amplification in strains with group IA K antigens.

Absence of Rol activity in a prototype strain with a group IA capsule. Although hybridization and nucleotide sequence data indicate the absence of *rol* sequences in the *his-rfb* region of strains with group IA K antigens, this does not preclude Rol activity from a gene with a divergent nucleotide sequence,

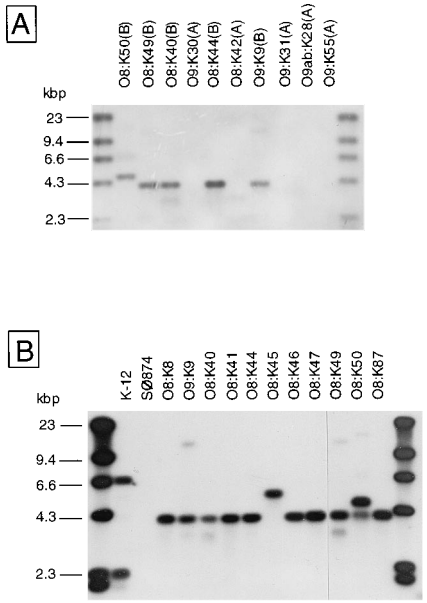

FIG. 2. Detection of *rol*-homologous sequences in chromosomal DNA from *E. coli* O8 and O9 serotypes producing group IB K antigens. The *rol* probe consists of an 867-bp fragment amplified from the *rol* gene of *E. coli* O75 by PCR. Chromosomal DNAs were digested with *Pst*I. (A) Random selection of strains. For each, the K-antigen subgroup (IA or IB) is indicated in parentheses after the serotype. (B) Collection of 11 serotypes with group IB K antigens. *E. coli* K-12 (DH5α) and SØ874 (Δ*rfb-rol*) provide positive and negative controls, respectively.

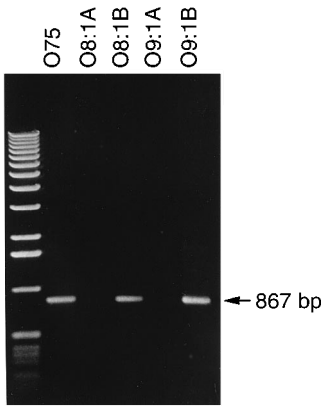

FIG. 3. PCR amplification of a *rol* gene fragment from *E. coli* serotypes with group IB K antigens. A fragment of the predicted size (867 bp) was amplified from chromosomal DNAs of the control strain, *E. coli* O75, and from each of 11 strains with group IB K antigens. No amplification product was obtained from strains with group IA K antigens (12 strains), confirming the results of Southern hybridization experiments. Only one representative is shown for strains from each K-antigen subgroup with either O8 or O9 antigens.

potentially located elsewhere on the chromosome. To examine this possibility, an assay to detect Rol function was developed.

Generation of a specific smooth LPS (S-LPS) pattern in SDS-PAGE requires the parental *rfb* and *rol* gene products. However, heterologous Rol proteins can interact with Rfc to modify the extent of O-chain polymerization (1, 3), so that a Rol-dependent *rfb* cluster carried on a plasmid can be used to probe for chromosomal Rol activity. The *S. dysenteriae* type 1 *rfb* cluster, carried on plasmid pSS37, determines synthesis of the Rol-dependent type 1 O antigen (48). When pSS37 was used to transform an *E. coli* K-12 strain containing chromosomal *rol* (AB1133), the resulting LPS exhibited a modal distribution of O-polysaccharide chain length (Fig. 4A). A high-molecular-weight fraction of S-LPS was clearly evident. This high-molecular-weight fraction was not present when pSS37

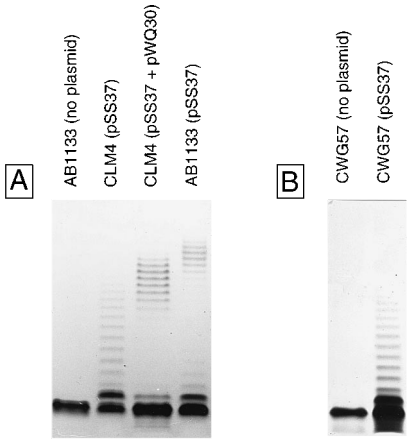

FIG. 4. An activity assay demonstrates the absence of Rol activity in a prototype strain with a group IA K antigen. (A) Controls for the Rol activity assay. *E. coli* AB1133 provides a Rol^+ genetic background, and CLM4 has an *rfb-rol* deletion. Plasmid pSS37 encodes the Rol-dependent O-antigen biosynthesis system from *S. dysenteriae* type I. In AB1133(pSS37) the S-LPS shows the typical modal distribution. The modal distribution is lost in CLM4(pSS37), but the defect can be complemented by introduction of pWQ30 containing the *rol* gene from *E. coli* O75. (B) LPS from *E. coli* CWG57 ($O9^-$:$K30^-$) and nonmodal distribution of S-LPS produced when this strain is transformed with pSS37. All LPS samples consisted of SDS-proteinase K whole-cell lysates.

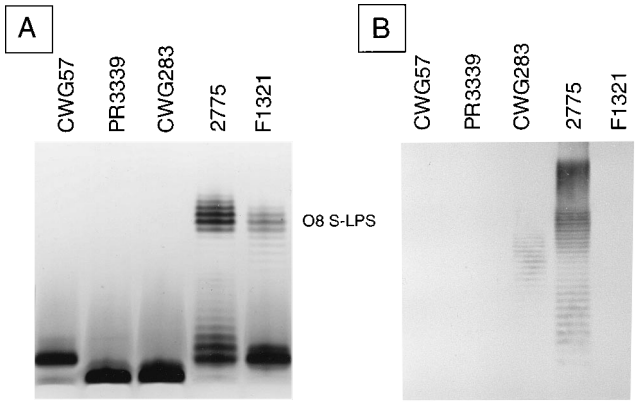

FIG. 5. Lipid A-core association of group IB K40 antigen. (A) Silver-stained SDS-PAGE profile; (B) corresponding Western immunoblot with polyclonal anti-K40 serum. *E. coli* 2775 (O8:K40) contains a cluster of high-molecular-weight silver-stained O8 S-LPS bands with mobility identical to that of those in the *E. coli* O8 reference strain F1321; these are indicated beside panel A. *E. coli* PR3339 is the donor strain for the Δ*rfaGPBI* mutation, and its deep-rough LPS comigrates with that of CWG283, the deep-rough mutant derived from 2775. The O8 S-LPS bands are eliminated in *E. coli* CWG283. Strain CWG57 contains a complete R1 LPS core. As shown in panel B, the K40 antiserum detected K_{LPS} bands throughout the gel profile of *E. coli* 2775. All LPS samples consisted of SDS-proteinase K whole-cell lysates.

was used to transform the Δ*rol-rfb* derivative (SØ874); only low-molecular-weight S-LPS with unregulated O-chain length was detected. The modal distribution was restored when the *E. coli* O75 *rol* gene was added in *trans* by using plasmid pWQ30 (Fig. 4A).

To test for Rol activity in a prototype *E. coli* strain with a group IA K antigen (CWG57 [$O9^-$:$K30^-$]), plasmid pSS37 was introduced. The LPS of the transformants was indistinguishable from that produced in the *E. coli* K-12 Δ*rol* background (Fig. 4B). Attempts to obtain stable double transformants with an additional plasmid containing *rol* were unsuccessful in this background. This may be due to the presence of several endogenous plasmids within CWG57. These results confirm that the absence of hybridization with the *rol* gene probe reflects the absence of Rol activity in a prototype strain with a group IA K antigen.

Lipid A-linked group IB K antigen (K_{LPS}) in *E. coli* O8:K40. A portion of the group IB K antigen of the prototype strain *E. coli* O8:K40 has been shown to be covalently linked to lipid A-core (19). Lipid A-core, therefore, acts as a surface anchor for both the homopolysaccharide O8 antigen and the heteropolysaccharide K40 K_{LPS} antigen in *E. coli* O8:K40. Previous research on expression of the group IA K30 antigen showed that the K30 antigen could be expressed as K_{LPS} and as lipid A-core-independent high-molecular-weight K30 polysaccharide (30). To determine whether attachment to lipid A-core was essential for production of group IB K antigen, a core-deficient *E. coli* O8:K40 strain was constructed by moving a characterized *rfa* deletion from *E. coli* K-12 into *E. coli* O8:K40. LPSs from the wild-type strain (*E. coli* 2775 [O8:K40]) and its Δ*rfa* derivative (CWG283) were analyzed by SDS-PAGE (Fig. 5A). The absence of the O8-substituted S-LPS bands and the presence of a lower-molecular-weight lipid A-core, comigrating with that of the *E. coli* K-12 donor PR3339, confirmed the core deficiency in CWG283. The extensive high-molecular-weight K40 antigen in the Western blot of *E. coli* 2775 LPS showed a distinct modality, with two areas of higher intensity (Fig. 5B). Discrete bands were evident in the lower parts of the Western blot profile for *E. coli* 2775. In contrast, *E. coli* CWG283 lacks most of the high-molecular-weight K40 antigen, suggesting that the majority of the K40 antigen detected in SDS-PAGE exists as K_{LPS}. However, a small amount of immunoreactive material with a distinct ladder-like banding pattern was still observed in *E. coli* CWG283 (Fig. 5B). The remaining K40 antigen was surface exposed in *E. coli* CWG283, since whole cells gave a positive agglutination reaction in anti-K40 serum (10). This is consistent with earlier reports which showed that a significant amount of K40 antigen lacks terminal lipid A-core (19). Therefore, as reported for the group IA K30 antigen (30), some K40 polysaccharide is exported to the cell surface in an LPS lipid A-core-independent pathway.

Effect of introduction of multicopy Rol on group IB K antigen in *E. coli* O8:K40. The profile of K_{LPS} in *E. coli* 2775 is radically altered following introduction of pRAB10, containing the cloned *rol* gene from *E. coli* O75. Introduction of multicopy *rol* resulted in the appearance of novel silver-stained bands migrating ahead of the O8 S-LPS (Fig. 6A). In the corresponding Western blot, additional copies of *rol* caused a dramatic reduction in the amount of very-high-molecular-weight K_{LPS} and the remaining material showed a much reduced average chain length; a new cluster of K_{LPS} bands was evident migrating slightly faster than the lower-molecular-weight modal cluster of the wild-type strain. Rol can therefore interact with the biosynthetic system for K40 (and presumably other group IB K antigens) to modulate the extent of polymerization.

Effect of introduction of multicopy Rol on expression of group IA K_{LPS} in *E. coli* O^-:K30. One possible explanation for the shorter chain length of the group IA K_{LPS}, relative to that of group IB K_{LPS}, is the absence of *rol* in strains with group IA K antigens. To address this possibility, pWQ30 was used to introduce the *E. coli* O75 *rol* gene into *E. coli* CWG44 ($O9^-$:K30). Normally, the only CWG44 K_{LPS} band visible in silver-stained SDS-PAGE gels consists of a single repeat unit of K30 antigen attached to lipid A-core (30), which migrates slightly more slowly than the rough lipid A-core band (Fig. 7A). Introduction of *rol* resulted in production of novel higher-molecular-weight bands in SDS-PAGE. To confirm the identity of this high-molecular-weight material, LPS samples were probed by Western blotting using K30-specific antiserum (Fig. 7B). As

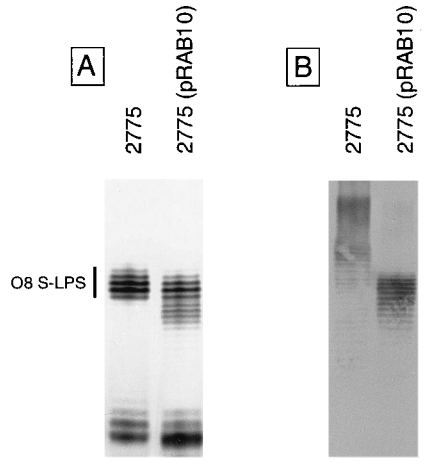

FIG. 6. Effect of multicopy *rol* on expression of the group IB K40 antigen. *E. coli* 2775 (O8:K40) is the prototype strain for the group IB K antigen. pRAB10 contains the *rol* gene from *E. coli* O75. (A) Silver-stained SDS-PAGE gel; (B) corresponding Western immunoblot with polyclonal anti-K40 serum. The migration of molecules comprising O8 S-LPS is indicated beside panel A. All LPS samples consisted of SDS-proteinase K whole-cell lysates.

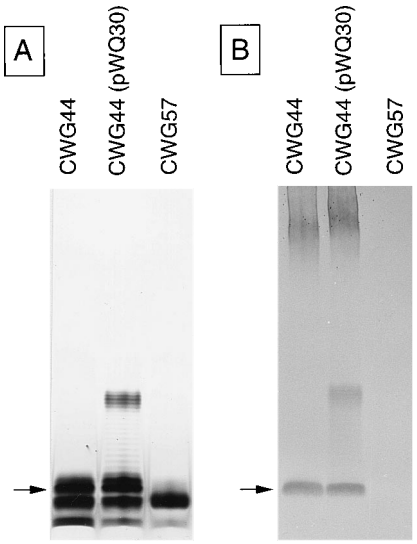

FIG. 7. Effect of multicopy *rol* on expression of the group IA K30 antigen. *E. coli* CWG44 (O^-:K30) is the prototype strain for the group IA K antigen. pWQ30 contains the *rol* gene from *E. coli* O75. Strain CWG57 contains a complete R1 LPS core. (A) Silver-stained SDS-PAGE gel; (B) corresponding Western immunoblot with polyclonal anti-K30 serum. The arrows indicate a band of K_{LPS} containing one repeat unit of K30 antigen (30). All LPS samples consisted of SDS-proteinase K whole-cell lysates.

expected, *E. coli* CWG44 contained a single band of immunoreactive K_{LPS}, as well as a smear of high-molecular-weight K30 antigen. The high-molecular-weight K30 antigen was also detected in *E. coli* PR3358 (10); PR3358 is a $K30^+$ encapsulated strain with a Δ*rfa* mutation, leading to a deep rough LPS phenotype (30). Therefore, the high-molecular-weight K30 fraction seen in Western immunoblots represents the LPS-free form of K30 antigen. The additional bands in the SDS-PAGE profile of CWG44(pWQ30) reacted with K30-specific antisera in Western blots (Fig. 7B), confirming the presence of a ladder of K_{LPS} with an apparent modal distribution, in addition to the smear of high-molecular-weight K30 polysaccharide (Fig. 7B). Thus, although there is no endogenous Rol activity in the prototype *E. coli* strain with the group IA K30 antigen, the biosynthesis and modality of the K_{LPS} are responsive to Rol. However, unlike the case with the parallel experiment using *E. coli* O8:K40 (Fig. 6), introduction of multicopy *rol* did not have any detectable effect on formation of the high-molecular-weight LPS-free K30 antigen.

To examine whether this effect was typical of other group IA K_{LPS}s, two additional *E. coli* O9 strains with group IA K antigens were transformed with pRAB10. In serotypes O9:K26 and O9:K31, K_{LPS} exists as single repeat units of K antigen attached to lipid A-core (30). For each transformed strain, the SDS-PAGE LPS profile showed a series of new silver-stained bands migrating between the lipid A-core and the region comprising the unaffected O9 S-LPS (Fig. 8). The new bands are interpreted as reflecting an increase in the degree of polymerization of K_{LPS}. Although K-specific antisera to directly confirm the identity of the novel K_{LPS} bands in these additional serotypes are not available, the absence of any effect of *rol* on expression of the O9 LPS and the data presented above for CWG44 are all in strong agreement with this interpretation.

DISCUSSION

The product of the *rol* (*cld*) gene has been shown to control the extent of polymerization of LPS O antigens in a variety of bacteria of the family *Enterobacteriaceae*, including *E. coli* serotypes (1–3), *Shigella* species (25, 37), and *S. enterica* (1, 2). The predicted Rol proteins are conserved and have similar hydropathy plots. Rol gene products are transmembrane proteins with a large hydrophilic central region and membrane-spanning segments at the amino- and carboxy-terminal ends (37). It has been suggested that Rol interacts with either the O-antigen polymerase (Rfc) (1) or the O-antigen ligase (RfaL) (37), both of which are also transmembrane proteins, to produce O chains with strain-dependent chain length distributions (1). However, there is no direct experimental evidence to support either alternative hypothesis. All of the Rol-dependent O antigens described to date are heteropolysaccharides whose polymerization occurs via an Rfc-dependent process. Since many of these O antigens are known virulence determinants and O-chain length is known to influence serum resistance in *E. coli* (22, 23, 53), Rol may have a direct impact on virulence.

The detection of *rol* in strains which coexpress group IB K antigens and Rol-independent O antigens led us to consider the possibility that *rol* was involved in regulating the length of other cell surface polysaccharides, specifically the group IB K antigen. There is clearly no chemical feature in a polysaccharide which would define it as either O antigen or K antigen (53). The current distinctions generally reflect an operational definition in serology, wherein (longer) K antigens often mask (shorter) underlying O antigens. Although serologically classified as K (capsular) antigens, the group IB K antigens are better considered O antigens for several reasons: (i) a significant amount of group IB K antigen in *E. coli* O8:K40 is attached to lipid A-core, giving a ladder of high-molecular-weight K_{LPS} in SDS-PAGE (19; also this study); (ii) introduction of multicopy *rol* alters the normal chain length distribution of K_{LPS} in a prototype strain with a group IB K antigen (K40), providing evidence for the involvement of a Rol-dependent pathway; and (iii) there are several strains for which serological results classify a given polysaccharide as an O antigen in one strain and a (group IB) K antigen in another. Examples include K87 = O32, K85 = O141, and K9 = O104 (18). This could simply be explained by the absence of an O8 or O9 antigen in certain strains, leaving the group IB K_{LPS} as the only major lipid A-core associated antigen.

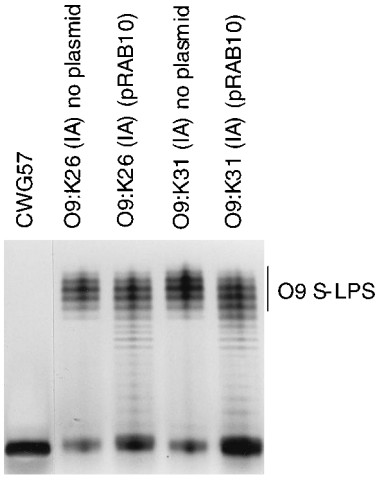

FIG. 8. Effect of multicopy *rol* on expression of the group IA K26 and K31 antigens. Introduction of multicopy *rol* by transformation with pRAB10 results in the appearance of novel bands in the silver-stained SDS-PAGE profile. The tight cluster of O9 S-LPS bands (indicated beside the gel) is unchanged. Strain CWG57 contains a complete R1 LPS core. All LPS samples consisted of SDS-proteinase K whole-cell lysates.

The effect of multicopy *rol* on the distribution of K30 antigen in immunoblots of SDS-PAGE gels is different from that observed with the K40 polysaccharide. Specifically, multicopy *rol* results in the reduction of high-molecular-weight K antigen levels in K40 but not in K30. This could reflect differences in attachment of the respective polysaccharides to the cell surface, differences in the biosynthetic systems, or more likely the artificial nature of the multicopy system. It is known from previous work with O antigens that strain-specific O-antigen chain length distribution requires interaction between the authentic Rol and Rfb proteins (1, 3). Both the heterologous nature of the introduced Rol and the multicopy situation make interpretation of the different effects in K30 and K40 impossible. However, the interaction of Rol with biosynthetic systems for both group IA and IB K antigens and the known association of Rol with Rfc-dependent (blockwise) repeat unit polymerization provide the first indication of the type of pathway by which these cell surface polymers are synthesized.

As this work was being completed, independent studies in P. Reeves's laboratory showed that Rol influenced the modal distribution of molecules presumed to be K_{LPS} in two *E. coli* strains with group IB K antigens (serotypes K9 and K87) (13). The presence of *rol* in these strains was confirmed by determination of the nucleotide sequences of the *rol* homologs and demonstration of similarity to *rol* homologs of known function, including that from *E. coli* O75 (2, 3).

The lack of *rol* in all tested strains with group IA K antigens is intriguing. It seems unlikely that each of these strains independently lost *rol*. The simplest explanation is that *rol* was initially lost from a common progenitor strain. K-antigen diversity may then have occurred by successive recombination events among and between different gene clusters for polysaccharide synthesis, following the loss of *rol*. Analogous recombination events have been proposed to have generated the observed diversity in the O antigens of *Salmonella* spp. (43).

As previously found with the group IA K30 antigen, export of the K40 polysaccharide to the cell surface does not follow a single pathway. Although most material is evidently attached to lipid A-core as K_{LPS}, smaller amounts of additional LPS-free material are evident. Similar export of LPS-free and LPS-associated material has been seen in the O antigens of *Proteus mirabilis* O6 (4) and in *E. coli* serotypes O55 and O127 (42). The best-studied example of this phenomenon known as "O-antigen capsules" is *E. coli* O111 (15, 16, 42), for which it has been shown that surface expression of O111 antigen occurs in strains with *rfaL* (O-antigen ligase) defects (26). In contrast, O antigen is detected only in the lipid A-core-attached form in *Salmonella* serovars Typhimurium and Minnesota (42), and the molecular basis for the "relaxed" export systems in other bacteria is unknown. For *E. coli* CWG283, some ladders of K antigen are still detected in SDS-PAGE, despite the absence of a complete lipid A-core for attachment of the K40 antigen. Such migration in SDS-PAGE generally reflects attachment to a lipid. The identity of the potential lipid moiety in these molecules remains to be established. The only other situation in which different lipids are used as "anchors" for the same polysaccharide occurs with enterobacterial common antigen (ECA). ECA can be attached to lipid A-core in some *E. coli* strains as ECA_{LPS} or to *sn*-L-α-glycerophosphatidic acid as ECA_{PG} (28). From the results of studies of group I K antigens and the data for O antigens and ECA, it is clear that the precise manner in which polysaccharides are presented on the cell surface of *E. coli* is much more complicated than was originally anticipated.

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