JOURNAL OF BACTERIOLOGY, June 1994, p. 3126–3139
0021-9193/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 176, No. 11

Cloning and Analysis of Duplicated *rfbM* and *rfbK* Genes Involved in the Formation of GDP-Mannose in *Escherichia coli* O9:K30 and Participation of *rfb* Genes in the Synthesis of the Group I K30 Capsular Polysaccharide

PADMAN JAYARATNE,[1]† DOROTHEA BRONNER,[1] P. RONALD MACLACHLAN,[1]‡ CHRISTINE DODGSON,[1] NOBUO KIDO,[2] AND CHRIS WHITFIELD[1]*

Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada,[1] and Biological Systems, Department of Natural Science Informatics, School of Informatics and Sciences, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan[2]

Received 24 November 1993/Accepted 20 March 1994

The rfb_{O9} gene cluster, which is responsible for the synthesis of the lipopolysaccharide O9 antigen, was cloned from *Escherichia coli* O9:K30. The *gnd* gene, encoding 6-phosphogluconate dehydrogenase, was identified adjacent to the rfb_{O9} cluster, and by DNA sequence analysis the gene order *gnd-rfbM-rfbK* was established. This order differs from that described for other members of the family *Enterobacteriaceae*. Nucleotide sequence analysis was used to identify the *rfbK* and *rfbM* genes, encoding phosphomannomutase and GDP-mannose pyrophosphorylase, respectively. In members of the family *Enterobacteriaceae*, these enzymes act sequentially to form GDP-mannose, which serves as the activated sugar nucleotide precursor for mannose residues in cell surface polysaccharides. In the *E. coli* O9:K30 strain, a duplicated $rfbM^2$-$rfbK^2$ region was detected approximately 3 kbp downstream of $rfbM^1$-$rfbK^1$ and adjacent to the remaining genes of the rfb_{O9} cluster. The *rfbM* isogenes differed in upstream flanking DNA but were otherwise highly conserved. In contrast, the *rfbK* isogenes differed in downstream flanking DNA and in 3′-terminal regions, resulting in slight differences in the sizes of the predicted RfbK proteins. $RfbM_{O9}$ and $RfbK_{O9}$ are most closely related to CpsB and CpsG, respectively. These are isozymes of GDP-mannose pyrophosphorylase and phosphomannomutase, respectively, which are thought to be involved in the biosynthesis of the slime polysaccharide colanic acid in *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium. An *E. coli* O^-:K30 mutant, strain CWG44, lacks $rfbM^2$-$rfbK^2$ and has adjacent essential rfb_{O9} sequences deleted. The remaining chromosomal genes are therefore sufficient for GDP-mannose formation and K30 capsular polysaccharide synthesis. A mutant of *E. coli* CWG44, strain CWG152, was found to lack GDP-mannose pyrophosphorylase and lost the ability to synthesize K30 capsular polysaccharide. Wild-type capsular polysaccharide could be restored in CWG152, by transformation with plasmids containing either $rfbM^1$ or $rfbM^2$. Introduction of a complete rfb_{O9} gene cluster into CWG152 restored synthesis of both O9 and K30 polysaccharides. Consequently, *rfbM* is sufficient for the biosynthesis of GDP-mannose for both O antigen and capsular polysaccharide in *E. coli* O9:K30. Analysis of a collection of serotype O8 and O9 isolates by Southern hybridization and PCR amplification experiments demonstrated extensive polymorphism in the *rfbM-rfbK* region.

Many bacterial cell surface polysaccharides contain the sugar mannose, and GDP-mannose serves as the activated precursor for the biosynthesis of these polysaccharides (51). In enteric bacteria, GDP-mannose is synthesized in a three-step reaction as follows: fructose-6-phosphate $\overset{1}{\leftrightarrow}$ mannose-6-phosphate $\overset{2}{\rightarrow}$ mannose-1-phosphate $\overset{3}{\rightarrow}$ GDP-mannose. Reaction 1 is catalyzed by phosphomannose isomerase, reaction 2 is catalyzed by phosphomannomutase, and reaction 3 is catalyzed by GDP-mannose pyrophosphorylase (mannose-1-phosphate guanyl transferase) (14).

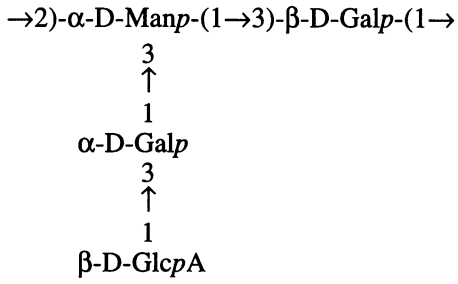
Phosphomannose isomerase is encoded by *manA*, which is located outside *rfb* (O-polysaccharide biosynthesis) and *cps* (capsular polysaccharide [CPS] biosynthesis) gene clusters in enteric bacteria (1, 47). ManA catalyzes a reversible reaction which is also required for metabolism of mannose via fructose. In *Salmonella enterica* serovar Typhimurium, mannose is present in the repeating unit structure of the serogroup B lipopolysaccharide (LPS) O polysaccharide. The phosphomannomutase and GDP-mannose pyrophosphorylase activities are encoded by *rfbK* and *rfbM*, respectively; these genes are located in the *rfb* gene cluster (24, 41, 42). The *rfb* gene clusters from *Escherichia coli* O7:K1 (36) and *E. coli* $O9:K^-$ (strain F719) (53) also contain *rfbM* and *rfbK* genes. In *S. enterica* serovar Typhimurium, the *cps* gene cluster is located approximately 9 kbp from *rfb* (52). Enzymes encoded by *cps* genes in *S. enterica* are responsible for the synthesis of colanic acid or M antigen, a non-serotype-specific slime exopolysaccharide produced by many enteric bacteria (35). Although colanic acid lacks mannose, it does contain fucose, and the precursor GDP-fucose is synthesized from GDP-mannose. The *cps* cluster in *S. enterica* serovar Typhimurium contains additional structural genes for phosphomannomutase (*cpsG*) and GDP-mannose pyrophos-

* Corresponding author. Mailing address: Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Phone: (519) 824-4120 ext. 3478. Fax: (519) 837-1802. Electronic mail address: cwhitfie@uoguelph.ca.

† Present Address: Microbiology Section, Department of Laboratory Medicine, Hamilton General Division, Hamilton, Ontario L8L 2X2, Canada.

‡ Present Address: Veterinary Infectious Diseases Organization, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada.

A



B

→3)-α-D-Manp-(1→3)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→

FIG. 1. Repeating unit structures of the capsular K30 antigen (A) and the LPS O9 antigen (B) from *E. coli*.

phorylase (*cpsB*), presumably to catalyze intermediate steps in GDP-fucose synthesis (52). While the enzyme activities appear to be identical, the CpsB and RfbM amino acid sequences show only 57% identity. CpsG and RfbK have only 19% identity (52). Phosphomannomutase and GDP-mannose pyrophosphorylase isozymes vary among different *S. enterica* serovars. For example, when RfbK from serovar Montevideo (group C1) is compared with phosphomannomutase isozymes from serovar Typhimurium (group B), more identity is found with CpsG than with RfbK (31).

Research in this laboratory is focused on the synthesis and expression of the group I (20) CPSs (K antigens) of *E. coli*. Group I CPSs are most frequently expressed with serotype O8 and O9 LPSs. The O8 and O9 polysaccharides are both homopolymers of mannose (11). *E. coli* O9:K30 produces a prototype group IA K antigen (23), and the K30 CPS repeating unit also contains mannose residues (Fig. 1). The genes required for biosynthesis of the O9 and K30 polysaccharides are designated rfb_{O9} and cps_{K30}, respectively. These genes are closely linked and are located near the *his* locus on the chromosome; linkage experiments suggest that the gene order is *his*-rfb_{O9}-cps_{K30} (30, 50, 58). *E. coli* strains with group IA K antigens do not produce colanic acid (23), and the cps_{K30} gene cluster appears to be allelic with cps_{K-12} genes required for colanic acid production in *E. coli* K-12 (26). These *cps* gene clusters also have common regulatory elements (15, 23, 26).

The role of GDP-mannose as the precursor for mannose residues in the O9 polymer has been clearly established (21). The rfb_{O9} cluster cloned from an unencapsulated mutant of *E. coli* (O9:K31$^-$) contains *rfbK* and *rfbM* genes (53).

On the basis of findings with other systems, it is assumed that K30 polysaccharide assembly also requires GDP-mannose (51). However, it is not clear whether duplication of phosphomannomutase and GDP-mannose pyrophosphorylase activities occurs in *E. coli* or whether genes in one locus (rfb_{O9} or cps_{K30}) are responsible for the provision of GDP-mannose precursor for both polymers. Mutations which eliminate expression of either O9 or K30 polysaccharide but which do not affect the other polymer have been described (37, 58). Other mutations eliminate synthesis of both K30 and O9 simultaneously (56). However, the relationship, if any, between rfb_{O9} and cps_{K30} remains unclear.

The studies described here were initiated to address two objectives: first, to examine possible interaction between the pathways of biosynthesis of the O9 and K30 polysaccharides and second, to investigate the relationships among genes involved in GDP-mannose formation in *E. coli* strains with group I CPS.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* E69 is the type strain for the group IA K30 capsular K antigen. *E. coli* CWG44 is a mutant with a wild-type K30 capsule but lacking the serotype O9 LPS O-polysaccharide side chains; this strain is described elsewhere as B187 (37). *E. coli* CWG152 is one of a collection of spontaneous capsule-deficient mutants isolated from CWG44. The mutants were selected by resistance to coliphage K30, as described previously (58). The type strains for other group I K antigens were generously provided by B. Jann, K. Jann, and F. Ørskov and have been described previously (23). All strains were routinely grown in either Luria-Bertani medium (38) or M9 minimal medium containing 0.2% D-glucose. Plasmid-encoded 6-phosphogluconate dehydrogenase activity was determined by complementation of the defect in *E. coli* DF710 *gnd-1*. The parental strain and transformants were tested for Gnd enzyme activity by being plated on gluconate bromothymol blue indicator plates (60). Media were supplemented when required with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), tetracycline (15 μg/ml), amino acids (40 μg/ml), sugars (2 mg/ml), uracil (40 μg/ml), nicotinamide (10 μg/ml), and thiamine-HCl (1 μg/ml). Cultures were routinely grown at 37°C.

Bacteriophage Ffm (59) lyses *E. coli* strains with rough LPS (R-LPS) (49). Coliphages O9-1 (37) and K30 (57) are specific for *E. coli* strains expressing serotype O9 LPS and K30 CPS, respectively.

Cloning of the rfb_{O9} gene cluster from *E. coli* E69. A cosmid gene bank was constructed by using chromosomal DNA obtained from *E. coli* E69. DNA fragments were size fractionated after partial digestion with *Hin*dIII. DNA fragments of 20 to 25 kbp were ligated to *Hin*dIII-digested pVK102 vector, and the ligation mixtures were packaged into lambda particles. The packaged recombinant cosmids were transduced into *E. coli* LE392. pWQ607, containing the rfb_{O9} gene cluster, was isolated by screening for clones which were resistant to bacteriophage Ffm, as described previously (8). *E. coli* LE392 produces R-LPS and is lysed by Ffm. Ligation of O polysaccharide to

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

Strain or plasmid	Genotype, serotype, and/or relevant property(ies)	Source or reference(s)
Strains		
LE392	F$^-$ *hsdR514* (r_K^- m_K^-) *supE44 supF58 lacY1* or Δ(*lac-proAB*)*6 galK2 galT22 metB1 trpR55* λ$^-$	46
DH5α	K-12 F$^-$ φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_k^- m_k^-) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169	46
DF710	*fhuA22*? *edd-1 gnd-1 tyrA2 relA1*? *rpsL125 pit-10*? *spoT1*? *thi-1* λ$^-$?	44
SØ874	K-12 *lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL*	40
G3404-41	Serotype O8:K8:H$^-$; group IB K antigen	F. Ørskov
2667	Serotype O9:K9:H$^-$; group IB K antigen	B. Jann
2146	Serotype O9:K26:H$^-$; group IA K antigen	B. Jann
E56b	Serotype O8:K27:H$^-$; group IA K antigen	F. Ørskov
K14a	Serotype O9ab:K28:H$^-$; group IA K antigen	F. Ørskov
Bi161-42	Serotype O9:K29:H$^-$; group IA K antigen	F. Ørskov
Su3973-41	Serotype O9:K31:H$^-$; group IA K antigen	F. Ørskov
E75	Serotype O9:K34:H$^-$; group IA K antigen	F. Ørskov
2150	Serotype O9:K37:H$^-$; group IA K antigen	B. Jann
2151	Serotype O9:K39:H9; group IA K antigen	B. Jann
2775	Serotype O8:K40:H9; group IB K antigen	B. Jann
2176	Serotype O8:K41:H11; group IB K antigen	B. Jann
A295b	Serotype O8:K42:H$^-$; group IA K antigen	F. Ørskov
2178	Serotype O8:K43:H11; group IA K antigen	B. Jann
2179	Serotype O8:K44:H$^-$; group IB K antigen	B. Jann
2167	Serotype O8:K45:H$^-$; group IB K antigen	F. Ørskov
2181	Serotype O8:K46:H4; group IB K antigen	B. Jann
2182	Serotype O8:K47:H2; group IB K antigen	B. Jann
2183	Serotype O8:K48:H9; group IB K antigen	B. Jann
2184	Serotype O8:K49:H21; group IB K antigen	B. Jann
2185	Serotype O8:K50:H9; group IB K antigen	B. Jann
N24c	Serotype O9:K55:H$^-$; group IA K antigen	F. Ørskov
D227	Serotype O8:K87:H19; group IB K antigen	F. Ørskov
E69	Serotype O9:K30:H12; group IA K antigen; prototroph	I. Ørskov
CWG44	E69 but O$^-$:K30:H12; rfb_{O9} *his trp lac rpsL*	37
CWG28	E69 but O9:K$^-$:H12; cps_{K30} *his trp lac rpsL*	58
CWG152	CWG44 but O$^-$:K$^-$:H12 *rfbM*	This study
Plasmids		
pVK102	Cosmid vector; Tc^r Km^r	27
pRK2013	Helper plasmid; RK2 derivative; Km^r Mob$^+$ Tra$^+$ ColE1	10, 13
pUC19	Cloning vector; Ap^r	61
pBR325	Cloning vector; Ap^r Tc^r Cm^r	6
pWQ607	pVK102 derivative containing a 24.5-kbp *Hin*dIII fragment from the chromosome of *E. coli* E69; contains *gnd* and the rfb_{O9} gene cluster	This study
pWQ625	pUC19 derivative containing a 7.7-kbp *Sal*I fragment from pWQ607; contains *gnd*, $rfbM_{O9}^1$, and the 5′ end of $rfbK_{O9}^1$	This study
pWQ629	pUC19 derivative containing a 6.0-kbp *Sal*I fragment from pWQ607; contains $rfbM_{O9}^2$, the 5′ end of $rfbK_{O9}^2$, and the 3′ end of $rfbK_{O9}^1$	This study
pWQ622	pUC19 derivative containing a 6.2-kbp *Sal*I fragment from pWQ607; contains the 3′ end of $rfbK_{O9}^2$	This study
pWQ616	pBR325 derivative containing a 3.4-kbp *Sal*I-*Cla*I fragment from pWQ625; contains the 5′ end of *gnd*	This study
pWQ620	pBR325 derivative containing a 4.2-kbp *Sal*I-*Cla*I fragment from pWQ625; contains the 3′ end of *gnd*	This study
pNKB26	pACYC184 containing the rfb_{O9} gene cluster from *E. coli* F719 O9:K$^-$	53
pBHC1-Sc6	pBluescript SK(+) derivative containing a 4.25-kbp *Sac*I-*Hin*dIII fragment from pNBK26; *rfbM*$^+$ *rfbK*$^+$	N. Kido
pBHC4-K9	pBluescript SK(+) derivative containing a 2.82-kbp *Hin*dIII-*Kpn*I fragment from pNBK26; *rfbM rfbK*$^+$	N. Kido

lipid A core masks the Ffm receptor and confers resistance to the bacteriophage. The gene library in *E. coli* LE392 was screened en masse by dilution plating onto plates seeded with approximately 10^7 PFU of phage Ffm. Plasmids from Ffm-resistant strains were isolated and used to transform *E. coli* DH5α The LPS was then examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA manipulation. Plasmid DNA was purified by an alkaline lysis method (46). Recombinant plasmids constructed in the vector pVK102 were mobilized by triparental plate matings with the helper plasmid pRK2013 in *E. coli* HB101. All other subclones were transferred by transformation (34) or electroporation with a Bio-Rad Gene Pulser (4).

Plasmid DNA sequencing was performed by the dideoxy chain termination method (48) with Sequenase version 2.0 (U.S. Biochemicals); both strands were sequenced. Custom oligonucleotide primers were synthesized with an Applied Biosystems oligonucleotide synthesizer (model 391-EP).

TABLE 2. Oligonucleotide primers used in this study[a]

Primer	Sequence	Description
PJ102	5′-ACGTGGAATACGGTAG	$rfbK_{O9}$ flanking, forward
CHD4	5′-TCAATAATAAAGCCACG	$rfbK_{O9}^2$ flanking, reverse
CHD6	5′-TTGCGCAATCAGGACAC	$rfbK_{O9}^1$ flanking, reverse
PJ114	5′-CCACCGCGAGATTTACC	$rfbM_{O9}$ internal, forward
PJ115	5′-GATCCGCTCCTTGATGA	$rfbK_{O9}$ internal, reverse
CHD10	5′-GGTCACCTTCGGTTACG	$rfbM_{O9}$ internal, forward
CHD11	5′-TTCACGTCCTGGGAACG	$rfbM_{O9}$ internal, reverse
CHD17	5′-AGCAGAGATAGCGCTGA	$rfbM_{O9}^1$ flanking, forward
DBK7	5′-ATTGATGATATGGTCGG	$rfbM_{O9}^2$ flanking, forward
CHD22	5′-TCTCTGCTTTTAGGTTTT	$rfbM_{O9}$ flanking, reverse
CHD9	5′-ATGAACCGATGGTCATC	$rfbM_{O9}$ gene probe, forward
PJ100	5′-CCGACTGGATCTCCAGC	$rfbM_{O9}$ gene probe, reverse
DBK3	5′-GTGCATCACCAGCCGGA	$rfbK_{O9}$ gene probe, forward
DBK6	5′-CGGCCGCCGGCTCATAC	$rfbK_{O9}$ gene probe, reverse

[a] The fragments amplified by PCR with these primers are shown in Fig. 6A.

In hybridization experiments, DNA fragments in agarose gels were depurinated, denatured, and neutralized prior to Southern transfer (46). The fragments were transferred by overnight capillary blotting onto positively charged nylon membranes (Boehringer Mannheim, Laval, Québec, Canada) 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. Prehybridization was performed with a solution of 5× SSC–2% blocking reagent (Boehringer Mannheim)–50% formamide–0.1% *N*-lauroyl-sarcosine–0.02% SDS at 42°C for 3 h. Hybridization was carried out overnight at high stringency. 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. DNA fragments used for probes were separated by agarose gel electrophoresis, excised from the gel, and purified by using GeneClean (Bio/Can Scientific, Mississauga, Ontario, Canada). DNA fragments were digoxigenin labeled by using a kit from Boehringer Mannheim. Reactions were developed by using the Boehringer Mannheim 3-(2′-spiroadamantane)-4-methoxy-4-(3″phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) chemiluminescent substrate, according to the manufacturer's recommendations.

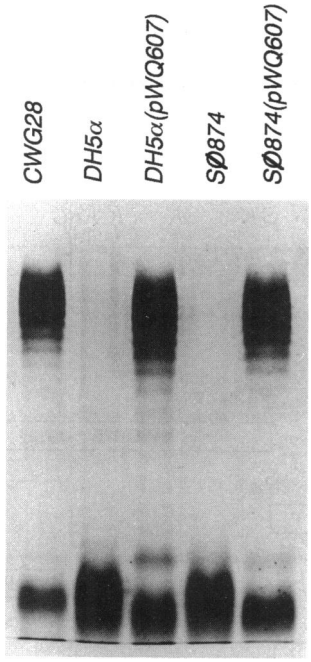


FIG. 2. SDS-PAGE of LPS samples from *E. coli* strains expressing serotype O9 S-LPS. *E. coli* DH5α is a K-12 derivative with R-LPS. Following transformation with pWQ607, *E. coli* DH5α produces an S-LPS profile identical to that of the O9 prototype strain, CWG28. A similar profile of S-LPS was also evident when *E. coli* SØ874 (Δ*rfb*) was transformed with pWQ607.

Enzymes were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Boehringer-Mannheim and used as recommended by the manufacturer.

PCR amplification was carried out by using 10 ng of chromosomal or plasmid DNA template and 20 ng of each oligonucleotide primer. 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 the appropriate temperature for each primer pair, and (iii) a polymerization step of 2 min at 72°C. The nucleotide sequences for the primers used are listed in Table 2.

DNA analysis was performed by using PC/Gene (Intelligenetics Inc., Mountain View, Calif.). Multiple sequence alignments and dendrograms were made by using CLUSTAL (17) with the default parameters.

LPS analysis and SDS-PAGE. Proteinase K-digested whole-cell lysates were prepared from stationary-phase cultures as described by Hitchcock and Brown (18). The gel conditions were those described by Darveau and Hancock (9), and LPS gels were silver stained by using the procedure of Tsai and Frasch (54).

Nucleotide sequence accession number. The DNA sequences for *gnd-rfbM*^{1}-*rfbK*^{1} (5,367 bp) and *rfbM*^{2}-*rfbK*^{2} (3,426 bp) reported here have been entered in GenBank under accession numbers L27646 and L27632, respectively.

RESULTS

Cloning of the rfb_{O9} gene cluster from *E. coli* O9:K30. Recombinant plasmids carrying rfb_{O9} were isolated from a gene library in *E. coli* LE392 by taking advantage of the observation that expression of smooth LPS (S-LPS) from cloned *rfb* genes in *E. coli* K-12 hosts makes the transformant resistant to the R-LPS-specific phage Ffm (8). All of the Ffm-resistant transformants were lysed by O9 S-LPS-specific coliphage O9-1. To confirm that O9 S-LPS was being synthe-

TABLE 3. Comparison of $RfbM_{O9}{}^1$ from *E. coli* O9:K30 with GDP-mannose pyrophosphorylase isozymes from *Enterobacteriaceae*

Organism	Protein	Size (kDa)	No. of amino acids	No. of identical amino acids	% Identity to $RfbM_{O9}{}^1$	Function(s)	Reference
E. coli O9 (E69)	$RfbM_{O9}{}^1$	52.6	471	471	100	O9 antigen, K30 antigen	This study
	$RfbM_{O9}{}^2$	52.6	471	470	99.8	O9 antigen, K30 antigen	This study
E. coli O9 (F719)	RfbM	52.8	471	464	98.5	O9 antigen	53
S. enterica serovar Typhimurium	CpsB	53.3	480	295	62.6	Colanic acid	52
E. coli K-12	CpsB	53.0	478	292	62.0	Colanic acid	Aoyama and Reeves (GenBank no. L11721)
S. enterica serovar Montevideo	RfbM	52.6	471	281	59.7	Group C1 O antigen	31
S. enterica serovar Typhimurium	RfbM	54.0	479	275	58.4	Group B O antigen	24
S. enterica serovar Muenchen	RfbM	52.9	473	269	57.1	Group C2 O antigen	7
E. coli O7	RfbM	52.6	464	217	46.8	O7 antigen	36

sized, recombinant plasmids were isolated and used to transform *E. coli* DH5α. The transformants were retested for bacteriophage sensitivities, and their LPSs were examined by SDS-PAGE. The profiles showed ladder patterns of S-LPS that were identical to that of an authentic O9 LPS standard (Fig. 2). *E. coli* K-12 strains produce R-LPS but carry remnants of a chromosomal *rfb* gene cluster (32). These host genes can participate with cloned genes in expression of O polysaccharides and, in some instances, have been reported to modify the structure of O polysaccharides produced in recombinant bacteria (16, 28). To avoid this problem, plasmids carrying rfb_{O9} were used to transform *E. coli* SØ874 (Δ*rfb*). *E. coli* SØ874 recombinants produced S-LPS with an SDS-PAGE profile similar to that of the authentic O9 strain (Fig. 2), indicating that the complete rfb_{O9} gene cluster was cloned and that synthesis of the O9 polysaccharide did not require any additional host-encoded *rfb* functions. Several plasmids carrying rfb_{O9} were isolated, but most were found to contain an identical single *Hin*dIII fragment. One plasmid, termed pWQ607, was selected for further study, and the physical maps of pWQ607 and subcloning derivatives are shown in Fig. 3.

Plasmid pWQ607 carries the *gnd* gene from *E. coli* O9:K30. In various serovars of *S. enterica* (45), *E. coli* serotypes O2 (39), O7 (36), and O75 (3), and *Shigella flexneri* (33), the *rfb* gene cluster is located adjacent to *gnd*, the structural gene for 6-phosphogluconate dehydrogenase. The location of the gnd_{O9} gene on pWQ607 was therefore determined, in order to define one end of the rfb_{O9} gene cluster. All of the subclones shown in Fig. 3 were tested for the ability to complement the *gnd-1* mutation in *E. coli* DF710. Two plasmids, pWQ607 and pWQ625, complemented *gnd-1*; no complementation was detected with either pWQ622 or pWQ616. The precise location, shown in Fig. 3, was then determined by DNA sequencing (22).

Identification of *rfbM* and *rfbK* genes on pWQ607. To

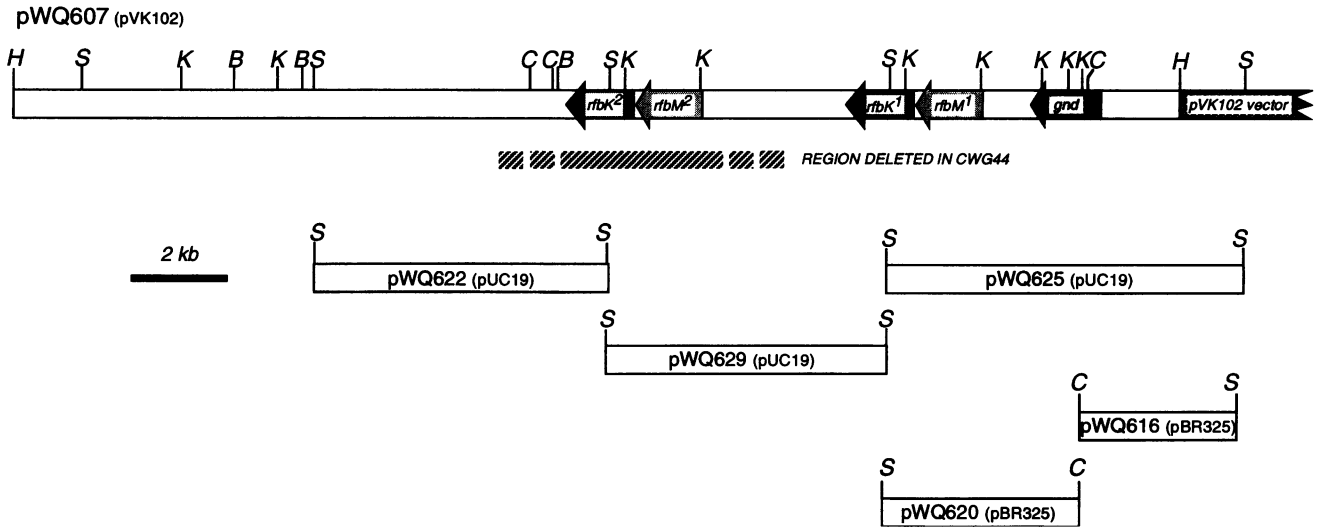


FIG. 3. Physical map of the *gnd-*rfb_{O9} region cloned in pWQ607. Plasmid pWQ607 contains a 24.5-kbp *Hin*dIII fragment from the chromosome of *E. coli* E69 (O9:K30) in the cosmid vector pVK102. Subclones and the vectors used are indicated, but only the insert DNA is shown, unless otherwise indicated. The positions of *gnd* and the duplicated *rfbM* and *rfbK* genes were determined by DNA sequencing (for details, see text). The hatched bar below pWQ607 shows the region that is deleted in *E. coli* CWG44 (O^-:K30). The enzymes used were *Hin*dIII (*H*), *Sal*I (*S*), *Kpn*I (*K*), *Bam*HI (*B*), and *Cla*I (*C*).

TABLE 4. Comparison of $RfbK_{O9}{}^1$ from *E. coli* O9:K30 with phosphomannomutase isozymes from *Enterobacteriaceae*

Organism	Protein	Size (kDa)	No. of amino acids	No. of identical amino acids	% Identity to $RfbK_{O9}{}^1$	Function(s)	Reference
E. coli O9 (E69)	$RfbK_{O9}{}^1$	50.4	456	456	100	O9 antigen, K30 antigen	This study
	$RfbK_{O9}{}^2$	51.0	460	450	98.7	O9 antigen, K30 antigen	This study
E. coli O9 (F719)	RfbK	50.8	460	449	98.5	O9 antigen	53
S. enterica serovar Montevideo	RfbK	49.9	456	348	76.3	Group C1 O antigen	31
E. coli K-12	CpsG	50.4	456	347	76.1	Colanic acid	Aoyama and Reeves (GenBank no. L11721)
S. enterica serovar Typhimurium	CpsG	49.9	456	344	75.4	Colanic acid	52
E. coli O7	RfbK	49.9	453	334	73.3	O7 antigen	36
S. enterica serovar Typhimurium	RfbK	52.0	477	76	16.7	Group B O antigen	24
S. enterica serovar Muenchen	RfbK	52.8	478	55	12.1	Group C2 O antigen	7

localize *rfbM* on pWQ607, an *rfbM*-specific gene probe was isolated from plasmid pNKB26, which contains the rfb_{O9} gene cluster from *E. coli* F719 (53). The probe consisted of an approximately 0.5-kbp *Kpn*I-*Stu*I internal fragment from *rfbM*. The probe hybridized to a large region of pWQ607 and, surprisingly, to both pWQ625 and pWQ629 (22). This was subsequently explained by the existence of an *rfbM* duplication in pWQ607 (see below). Hybridization to pWQ620 indicated that $rfbM_{O9}$ was located in a region downstream of gnd_{O9}, and the nucleotide sequence was therefore determined for a 3.8-kbp region downstream of gnd_{O9}. Two open reading frames were detected, and the predicted proteins had significant levels of sequence identity with characterized RfbM and RfbK proteins from *E. coli* and *Salmonella* serovars. These genes are designated $rfbM_{O9}{}^1$ and $rfbK_{O9}{}^1$ (Fig. 3) because of the duplication. The predicted $RfbM_{O9}{}^1$ protein contained the same number of amino acid residues as RfbM, previously characterized from *E. coli* F719 (53), and the two homologs differed at only 7 amino acid residues (Table 3). $rfbK_{O9}{}^1$ was located downstream of $rfbM_{O9}{}^1$, and the two open reading frames are separated by a gap of 27 nucleotides. The predicted $RfbK_{O9}{}^1$ was again nearly identical to its counterpart from *E. coli* F719. Although the predicted F719 protein was 4 amino acids longer than $RfbK_{O9}{}^1$, the RfbK proteins from these two O9 isolates share 449 identical amino acids (Table 4).

There is a gap of 1,021 bp between $rfbM_{O9}{}^1$ and gnd_{O9}, but this region contains no significant open reading frames.

The *rfbM* and *rfbK* genes are duplicated in *E. coli* O9:K30

```
rfbMO9 1        GCT-----------TTTGCCGTT-ACGCA--CCACC---CCGTCA--GTAGCTG--AACA
rfbMO9 2        T::CTGCTTTTAGG:::T:A:GAC:::--TT::G::AGA:T::::TT::T::C:CTG::T
rfbMO9 F719     T::CTGCTTTTAGG:::T:A:GAC::::GTT::G::CGG:T::::TT:::::C:CTG::T

rfbMO9 1        GGAGGGACAGCTGATAGAAACAGAAGCCACTG-GAGCACCTCAAAAACACCATCATACAC
rfbMO9 2        :AAC:AT:T::C::C:::--C:T:::::G:AA:A:G:TGG:G:TT:G---:::TG-GAG:::
rfbMO9 F719     :AAC:AT:T::C::C:::--C:T:::::G:AA:A:G:TGG:GTTT:G---:::TG-GAG:::

rfbMO9 1        TAAATCAGTAAG----TTGGCAGCATCA-CCTCTTTTAAGAATTTGCCATATTTATACTT
rfbMO9 2        GGG:G:::::C:CATT::::TT::GA::G::AA:GGCGGT:GCG::::TC:AAA:GG:CA
rfbMO9 F719     GGG:G:::::C:CATT::::TT::GA::G::AA:GGCGGT:GCG::::TG:AAA:GG:CG

rfbMO9 1        CTGTATATCTATAATATTCTGCTTCTGGCCTCAGAATATATCTTGGCGTTTAATAACAGG
rfbMO9 2        :G------:---------:AC----::A:GGTC::T:GAT:GA:A:TAAAAT:A:G::AT
rfbMO9 F719     T:------:---------:AC:--:A:A:GGTC::T:GAT:GA:A:TAAAAT:A:G::AT

rfbMO9 1        GTTTTAATTAAAGGAAATTAATATGTTACTTCCTGTAATTATGGCTGGCGGTACCGGCAG
rfbMO9 2        -:A::T:::T:::::TC:C:T:ATG:::::::::::::::::::::::::::::::::
rfbMO9 F719     -:A::T:::T::::::T:C:T:ATG:::::C:::::::::::::::::T::::::::::

RfbMO9 1                                MetLeuLeuProValIleMetAlaGlyGlyThrGlySer
RfbMO9 2                                 :  :  :  :  :  :  :  :  :  :  :  :  :
RfbM F719                                :  :  :  :  :  :  :  :  :  :  :  :  :
```

FIG. 4. Comparison of the upstream DNA sequences flanking $rfbM_{O9}{}^1$ and $rfbM_{O9}{}^2$ alleles from *E. coli* O9:K30 strain E69 and *rfbM* from *E. coli* O9:K^- strain F719. The multiple alignment was made by using CLUSTAL. Breaks introduced to optimize alignments are indicated by dashes. Colons in the sequences represent positions where the nucleotides or amino acid residues are identical to those of the prototype, $rfbM_{O9}{}^1$ from E69. The characters in boldface indicate the initiation codons.

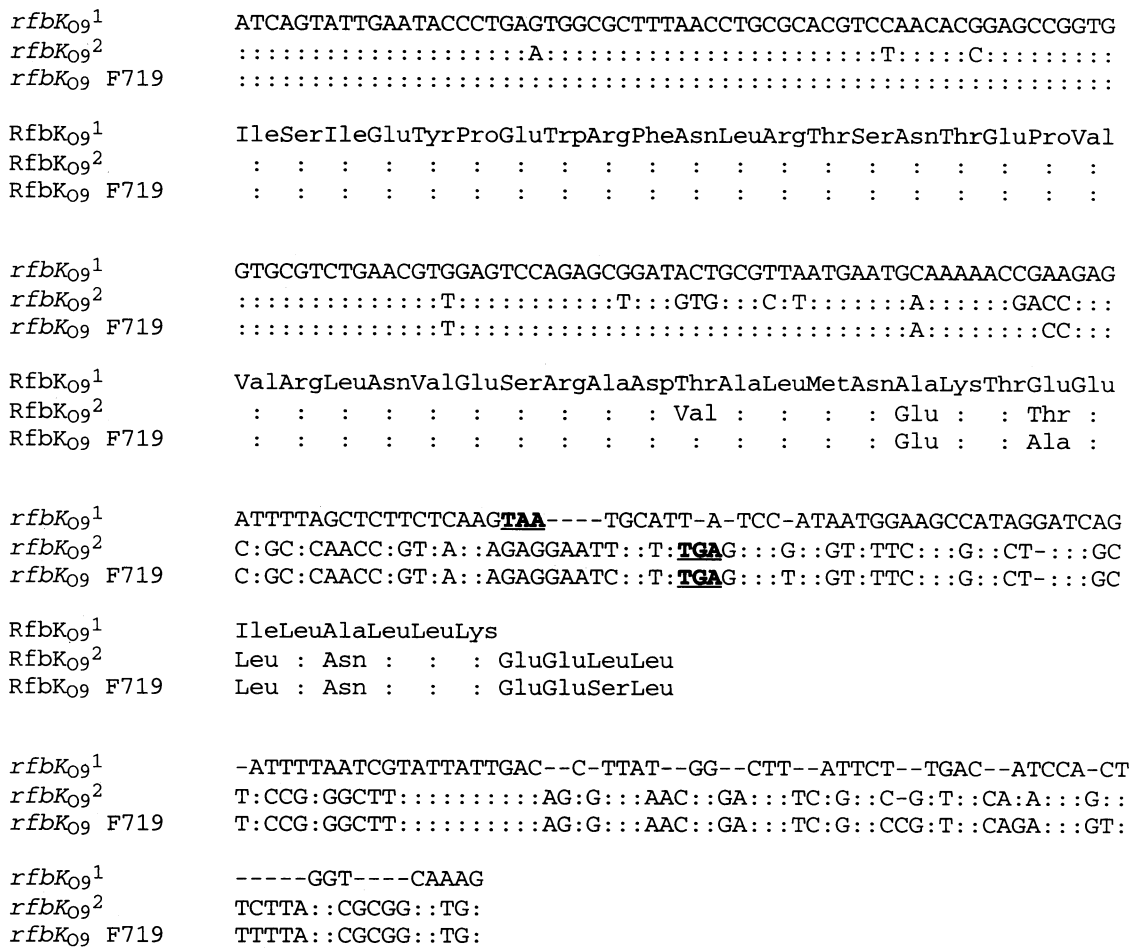


FIG. 5. Comparison of the downstream DNA sequences flanking the $rfbK_{O9}{}^1$ and $rfbK_{O9}{}^2$ alleles from *E. coli* O9:K30 strain E69 and *rfbK* from O9:K$^-$ strain F719. The multiple alignment was made by using CLUSTAL. Breaks introduced to optimize alignments are indicated by dashes. Colons in the sequences represent positions where the nucleotides or amino acid residues are identical to those of the prototype, $rfbK_{O9}{}^1$. The characters in boldface indicate the termination codons.

strain E69. As indicated above, hybridization studies showed that the adjacent DNA fragments cloned in plasmids pWQ625 and pWQ629 contained homologous sequences. In addition, both plasmids contained identically sized *Kpn*I-*Sal*I and *Kpn*I-*Kpn*I fragments, which in pWQ625 resulted from restriction sites within *rfbM* and *rfbK* (Fig. 3). DNA sequence analysis clearly established a duplication of *rfbM-rfbK* in pWQ607. The coding region of the second copy of *rfbM* ($rfbM_{O9}{}^2$) was almost identical to that of $rfbM_{O9}{}^1$ and highly similar to that of the *rfbM* gene from *E. coli* F719. This is reflected in identities of 98.5% ($RfbM_{O9}{}^1$ and $RfbM_{F719}$) and 99.8% ($RfbM_{O9}{}^1$ and $RfbM_{O9}{}^2$) for the predicted RfbM proteins (Table 3). Despite similarities in the coding regions, nucleotide sequences immediately upstream of $rfbM_{O9}{}^1$ and $rfbM_{O9}{}^2$ showed significant divergence (Fig. 4). Comparison of the nucleotide sequences upstream of the three *rfbM* alleles from *E. coli* O9 strains indicated that the *E. coli* F719 *rfbM* gene corresponds to the $rfbM_{O9}{}^2$ version from *E. coli* E69 (Fig. 4).

A duplicate copy of *rfbK* ($rfbK_{O9}{}^2$) was identified downstream of $rfbM_{O9}{}^2$, with an intervening gap between the two open reading frames that was identical to that between $rfbM_{O9}{}^1$ and $rfbK_{O9}{}^1$. Identity between the $rfbK_{O9}{}^1$ and $rfbK_{O9}{}^2$ DNA sequences degenerated at the 3′ end of the *rfbK* open reading frames, and the flanking DNA downstream of each *rfbK* gene also differed (Fig. 5). Differences at the 3′ ends of the coding sequences resulted in an extension of 4 amino acid residues at the carboxyl terminus of $RfbK_{O9}{}^2$. Comparison of nucleotide sequences at the 3′ termini of the *rfbK* genes from

TABLE 5. Complementation of defects affecting synthesis of O9 and K30 polysaccharides by using cloned rfb_{O9} genes

Plasmid	Relevant gene(s)	Cell surface polysaccharide phenotype[a] in:	
		CWG44	CWG152
None		O$^-$:K30	O$^-$:K$^-$
pWQ607	rfb_{O9}	O9:K30	O9:K30
pWQ625	$rfbM_{O9}{}^1$	O$^-$:K30	O$^-$:K30
pWQ620	$rfbM_{O9}{}^1$	O$^-$:K30	O$^-$:K30
pWQ622		O$^-$:K30	O$^-$:K$^-$
pWQ629	$rfbM_{O9}{}^2$	O$^-$:K30	O$^-$:K30
pNKB26[b]	rfb_{O9}	O9:K30	O9:K30
pBHC1-Sc6[b]	*rfbM-rfbK*	O$^-$:K30	O$^-$:K30
pBHC4-K9[b]	*rfbK*	O$^-$:K30	O$^-$:K$^-$

[a] The presence of S-LPS containing the O9 antigen was determined by SDS-PAGE. K30 capsule was detected by sensitivity to coliphage K30 (57) and agglutination in MAb4-15A (19).
[b] Plasmid carrying DNA from *E. coli* F719 (53).

TABLE 6. Summary of analysis of *rfbM* and *rfbK* types from *E. coli* O8 and O9 strains

Strain	Serotype	K antigen group	PCR analysis		Restriction fragment(s) (kbp)[a] hybridizing to:	
			rfbM type(s)	*rfbK* type(s)	*rfbM* probe	*rfbK* probe
E69	O9:K30	IA	1, 2	1, 2	1.6	4.4, 7.3
CWG44	O$^-$:K30	IA	1	1	NT[b]	NT
K14a	O9ab:K28	IA	2	2	1.6	4.4, 7.3
2146	O9:K26	IA	2	2	1.6	5.5, 7.3
Bi161-42	O9:K29	IA	2	2	1.6	5.5, 7.3
E75	O9:K34	IA	2	2	1.6	7.3
2150	O9:K37	IA	2	2	9.0	9.5
2151	O9:K39	IA	2	2	9.0	9.5
Su3973-41	O9:K31	IA	Unknown	Unknown	9.0	9.5
N24c	O9:K55	IA	Unknown	2	6.0, 9.0	6.1, 9.5
2181	O8:K46	IB	Unknown	Unknown	1.6	9.5
G3404-41	O8:K8	IB	Unknown	Unknown	1.6	9.5
2667	O8:K40	IB	Unknown	Unknown	1.6	9.5
2185	O8:K50	IB	1	Unknown	1.6	9.5
2183	O8:K48	IB	1	1	1.6	9.5
2184	O8:K49	IB	1	1	1.6	9.5
2176	O8:K41	IB	2	Unknown	1.6	9.5
2182	O8:K47	IB	2	Unknown	1.6	9.5
D227	O8:K87	IB	2	Unknown	1.6	9.5
E56b	O8:K27	IA	2	1	1.6	3.9, 9.5
2167	O8:K45	IB	2	Unknown	1.6, 6.6	6.6, 9.5
2179	O8:K44	IB	1	Unknown	1.6, 9.5	7.8, 9.5
A295b	O8:K42	IA	Unknown	1	5.5, 8.0, 11.0	5.5, 8.0, 11.0
2178	O8:K43	IA	Unknown	1	5.5, 8.0, 11.0	5.5, 8.0, 11.0

[a] *Kpn*I-digested chromosomal DNA.
[b] NT, not tested.

E. coli E69 and *rfbK* from F719 indicated that the F719 version was most similar to $rfbK_{O9}{}^2$ (Fig. 5), and these genes encoded similar predicted proteins (Table 4).

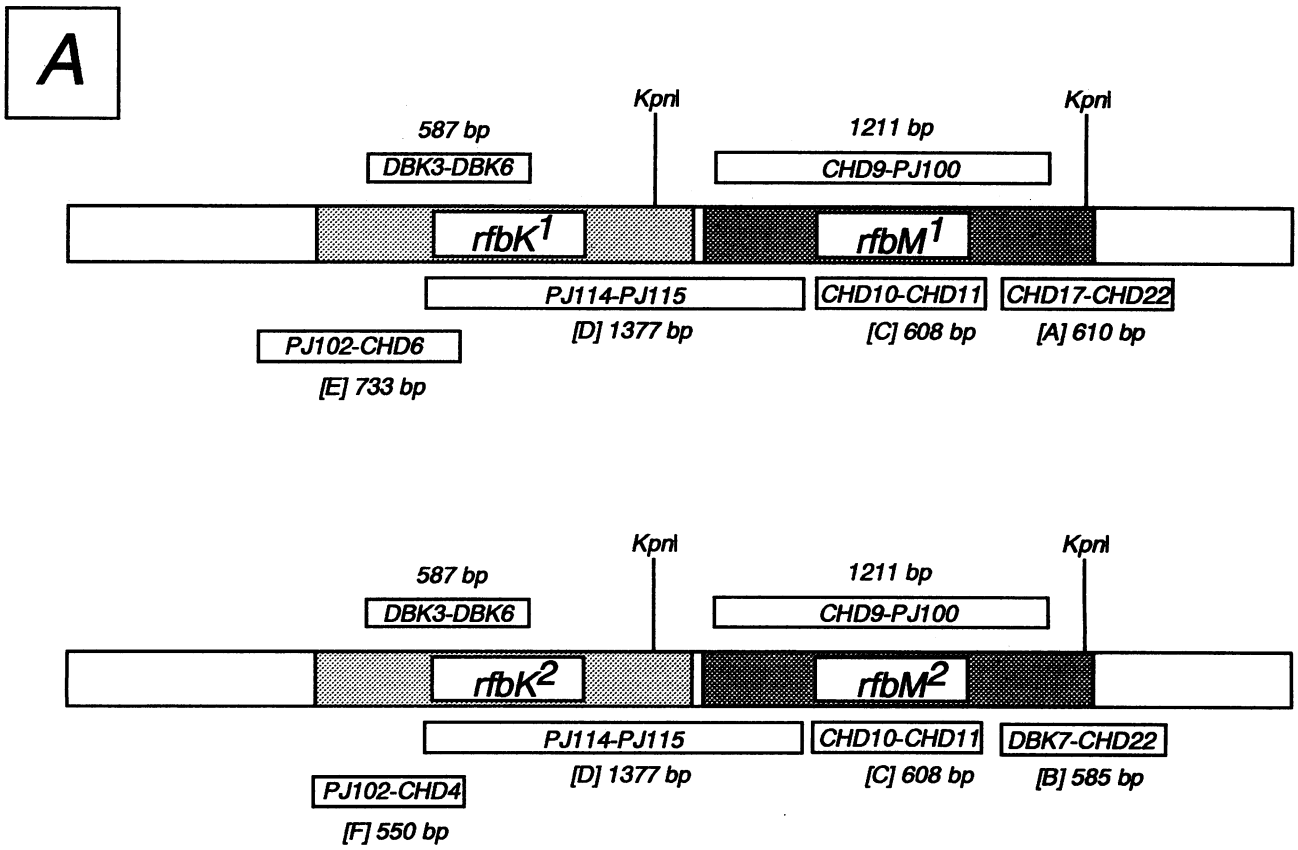
The duplicated regions of $rfbM_{O9}{}^1$-$rfbK_{O9}{}^1$ and $rfbM_{O9}{}^2$-$rfbK_{O9}{}^2$ are separated by approximately 3 kbp, and this region has not been analyzed further.

To confirm that the *rfbM-rfbK* duplication in pWQ607 was not a cloning artifact, the corresponding chromosomal region of *E. coli* O9:K30 was mapped by using gene probes derived from pWQ607. Mapping by hybridization was complicated by the duplicated regions, but, as far as could be determined, the physical map of the chromosome in strain E69 appeared to be identical to that of pWQ607 (22). The differences at the 5′ end of $rfbM_{O9}$ and the 3′ end of $rfbK_{O9}$ could be exploited to identify the individual copies and confirm the duplication, by using PCR amplification. The primer pairs used in these experiments and the strategy and expected amplification products are shown in Fig. 6A. As shown in Fig. 6B, *E. coli* E69 contained the same duplication in $rfbM_{O9}$ and $rfbK_{O9}$ that was identified in pWQ607.

***E. coli* CWG44 O$^-$:K30 has a deletion including $rfbM_{O9}{}^2$-$rfbK_{O9}{}^2$.** The same PCR strategy was used to examine the *rfbM-rfbK* chromosomal region of *E. coli* CWG44 (O$^-$:K30). Strain CWG44 was isolated as a spontaneous mutant with a defect in O9 polysaccharide expression. This strain has a wild-type K30 capsule and has been used as a prototype strain in our previous work on synthesis and expression of K30 CPS (23, 26). Interestingly, *E. coli* CWG44 contains $rfbM_{O9}{}^1$-$rfbK_{O9}{}^1$ but lacks $rfbM_{O9}{}^2$-$rfbK_{O9}{}^2$, as indicated by the absence of amplification products B and F (Fig. 6B). The O9 defect in CWG44 is due to deletion of DNA sequences adjacent to $rfbM_{O9}{}^2$-$rfbK_{O9}{}^2$ (22). The O9 defect in CWG44 is not complemented by introduction of $rfbM_{O9}{}^2$ alone but is complemented by pWQ607 and pNKB26, which contain complete *rfb* clusters (Table 5). The approximate location and extent of the deletion in the *rfb* region of strain CWG44 from preliminary Southern hybridization analyses are shown in Fig. 3, but precise deletion endpoints have not been determined.

***E. coli* CWG152 lacks GDP-mannose pyrophosphorylase.** It is not clear whether genes required for GDP-mannose synthesis in *E. coli* O9:K30 are found in both the *rfb* and *cps* gene clusters, as in *S. enterica* serovar Typhimurium (52). In an attempt to address this question, a collection of 20 independent spontaneous K30 CPS-deficient mutants isolated from *E. coli* CWG44 was screened to see if any of the defects affected phosphomannomutase or GDP-mannose pyrophosphorylase activity. All of the mutants tested were resistant to coliphage K30, which is specific for the K30 capsular antigen. No phosphomannomutase-deficient mutants were identified, but *E. coli* CWG152 was found to contain a defect in GDP-mannose pyrophosphorylase. The K30 synthesis defect in CWG152 was complemented by plasmids carrying either $rfbM_{O9}{}^1$ or $rfbM_{O9}{}^2$, suggesting that both $rfbM_{O9}{}^1$ and $rfbM_{O9}{}^2$ are potentially functional. However, it is possible that a promoter(s) for these cloned genes is provided by vector sequences. The *rfbM* gene from *E. coli* F719 (53) also complemented CWG152 (Table 5). No complementation was obtained with a plasmid (pBHC4-K9) containing *rfbK* alone. As expected, introduction of pWQ607 and pNKB26 into CWG152 restored synthesis of both K30 CPS and O9 O antigen, indicating that *rfbM* is sufficient to support synthesis of both polysaccharides. pWQ607 was not able to restore K30 CPS in any of the 19 additional CPS-deficient mutants tested (22).

Polymorphism in the *rfbM* and *rfbK* region in *E. coli* O8 and O9 serotypes with other group I CPSs. A series of *E. coli* strains with other group I K antigens was studied in an attempt to determine whether the *rfbM-rfbK* duplication is a common event. All of the strains produced O8 and O9 S-LPS. Both of



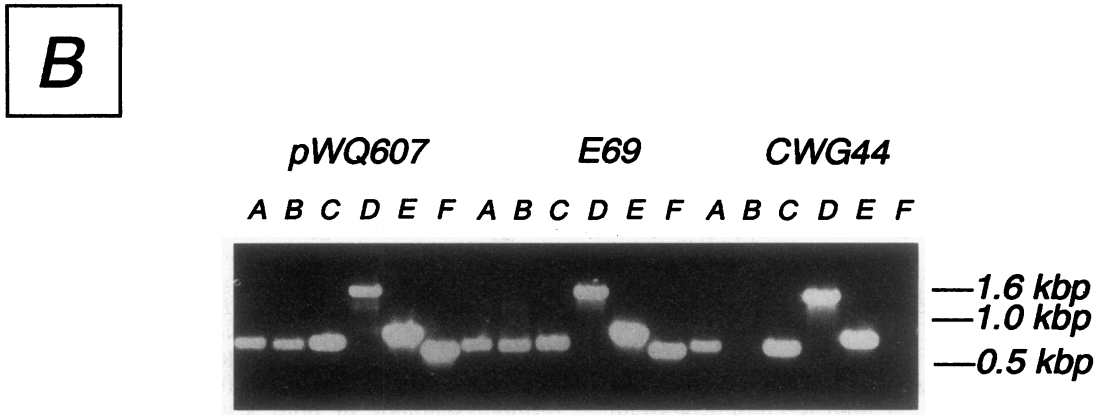


FIG. 6. Strategy for the PCR-based discrimination of $rfbM_{O9}{}^{1}$-$rfbK_{O9}{}^{1}$ and $rfbM_{O9}{}^{2}$-$rfbK_{O9}{}^{2}$. (A) Physical maps and primer pairs. The fragments produced by CHD9-PJ100 and DBK3-DBK6, shown above the physical map, were used only to generate $rfbM_{O9}$- or $rfbK_{O9}$-specific internal probes, respectively, for Southern hybridization experiments. The three primer sets shown below each of the physical maps were used to characterize individual $rfbM_{O9}$ and $rfbK_{O9}$ isogenes. The expected PCR fragments are designated with a letter code (A to F), and the size of each fragment is indicated. (B) Characterization of pWQ607 and chromosomal DNA from *E. coli* E69 and CWG44. Strain E69 gave the expected amplification products with each primer set, indicating that it contains the $rfbM_{O9}$-$rfbK_{O9}$ duplication. In contrast, fragments B and F were not generated from DNA of strain CWG44, indicating that this strain contains only $rfbM_{O9}{}^{1}$-$rfbK_{O9}{}^{1}$.

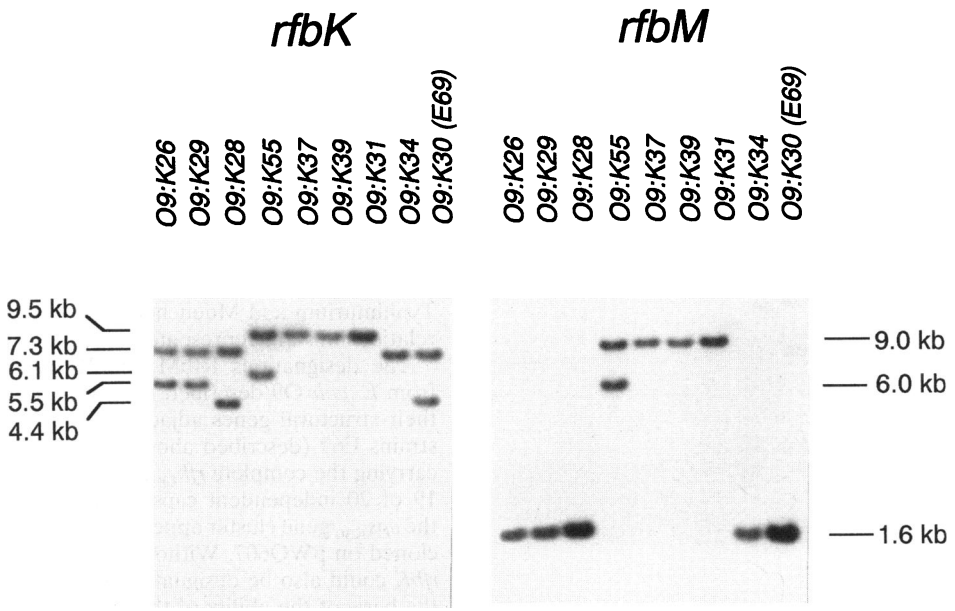


FIG. 7. Southern hybridization of the *rfbK* and *rfbM* regions in representative *E. coli* serotype O9 isolates with different K antigens. The internal probes for *rfbK* and *rfbM* were isolated from pWQ607 by PCR, and the locations of DNA fragments used as gene probes are shown above the physical map in Fig. 6A. Chromosomal DNAs from the strains indicated were digested with *Kpn*I. The physical map of pWQ607 (Fig. 3) predicts that the *rfbK* probe would detect two fragments with sizes of 7.3 and 4.4 kbp, from $rfbK_{O9}{}^2$ and $rfbK_{O9}{}^1$, respectively. The *rfbM* probe should detect only one fragment (1.6 kbp) duplicated in $rfbM_{O9}{}^1$ and $rfbM_{O9}{}^2$.

these O polysaccharides are linear homopolymers of mannose, but they differ in the size of the repeating unit, defined by a single linkage change (11). It has been shown previously that the *rfbM-rfbK* regions from representatives of serotypes O8 and O9 have homology identified in Southern hybridization experiments (53). The PCR approach used to analyze *E. coli* E69 and CWG44 as described above was applied to the additional strains. The results of these analyses are summarized in Table 6.

Eight additional serotype O9 strains were examined. On the basis of characteristic PCR products, six of these strains contained $rfbM_{O9}{}^2$ and $rfbK_{O9}{}^2$. The representative of serotype O9:K55 contained $rfbK_{O9}{}^2$ but gave no amplification products for *rfbM*. Surprisingly, the only O9 strain which gave no amplification products with either of the diagnostic terminal primer sets was *E. coli* O9:K31. This strain has the same serotype as the parent of *E. coli* F719, but, other than serotype, the relationships between these individual isolates are unknown. On the basis of PCR data, none of the additional O9 strains appeared to contain the *rfbM-rfbK* duplication seen in *E. coli* E69. However, minor sequence variation within the regions selected for primers would influence this result. Significantly, we also tested amplification of *rfbM* and *rfbK* internal fragments with the internal primer sets (generating fragments C and D in Fig. 6A), but the formation of amplification products varied from strain to strain (22), supporting the possibility of sequence variation. Therefore, internal DNA probes for *rfbK* and *rfbM* were made in order to examine the other strains by Southern hybridization. The probes were obtained from pWQ607 by PCR and are shown above the physical maps of *rfbM-rfbK* in Fig. 6A. Hybridization experiments revealed extensive polymorphism within the *rfbM* and *rfbK* regions of O9 isolates. Figure 7 shows the results for *Kpn*I-digested chromosomal DNA, but similar results were obtained by using *Pst*I (22). These results are summarized in Table 6. One strain, O9:K28, produced a hybridization pattern identical to that of E69 with both *Kpn*I and *Pst*I-digested chromosomal DNA.

The PCR results for 13 O8 strains were even more complex, and the $rfbM_{O9}$ and $rfbK_{O9}$ types were not correlated (Table 6). In the five strains for which the *rfbK* type could be identified, it was always $rfbK_{O9}{}^1$. Although the *rfbM* type could not be determined for five O8 strains, representatives containing $rfbM_{O9}{}^1$ (four strains) and $rfbM_{O9}{}^2$ (five strains) were found. No O8 strain contained both $rfbM_{O9}{}^1$ and $rfbM_{O9}{}^2$. Two O8 strains (O8:K48 and O8:K49) gave PCR results which predict $rfbM_{O9}{}^1$ and $rfbK_{O9}{}^1$. The O8:K27 representative contains $rfbM_{O9}{}^2$ and $rfbK_{O9}{}^1$. No O8 strain gave amplification products diagnostic for $rfbK_{O9}{}^2$. Finally, three strains gave no diagnostic amplification products with any of the terminal primer pairs. As with the O9 isolates, some strains gave no amplification reactions with the internal primers, perhaps because of minor sequence variation in the regions selected for primers. Therefore, internal *rfbM* and *rfbK* gene probes were used in Southern hybridization experiments to confirm that all of the O8 strains tested contained homologous *rfbM* and *rfbK* sequences. Four different hybridization patterns were detected in the *Kpn*I digests with each of these probes, again indicating polymorphism in the region (Fig. 8). Interestingly, the O8:K42 and O8:K43 strains contained similar patterns, and both gene probes hybridized to three DNA fragments (11.0, 8.0, and 5.5 kbp) which are each substantially larger than the size of the probe. One interpretation of this result is that there is a duplication of *rfbK* and *rfbM* in these strains, but there are no further data to support this possibility, and PCR analyses with these strains identified only $rfbK_{O9}{}^1$ (Table 6).

DISCUSSION

In enteric bacteria, most of the GDP-mannose pyrophosphorylase and phosphomannomutase enzymes that have been described are the products of *rfb* genes. However, in *S. enterica*

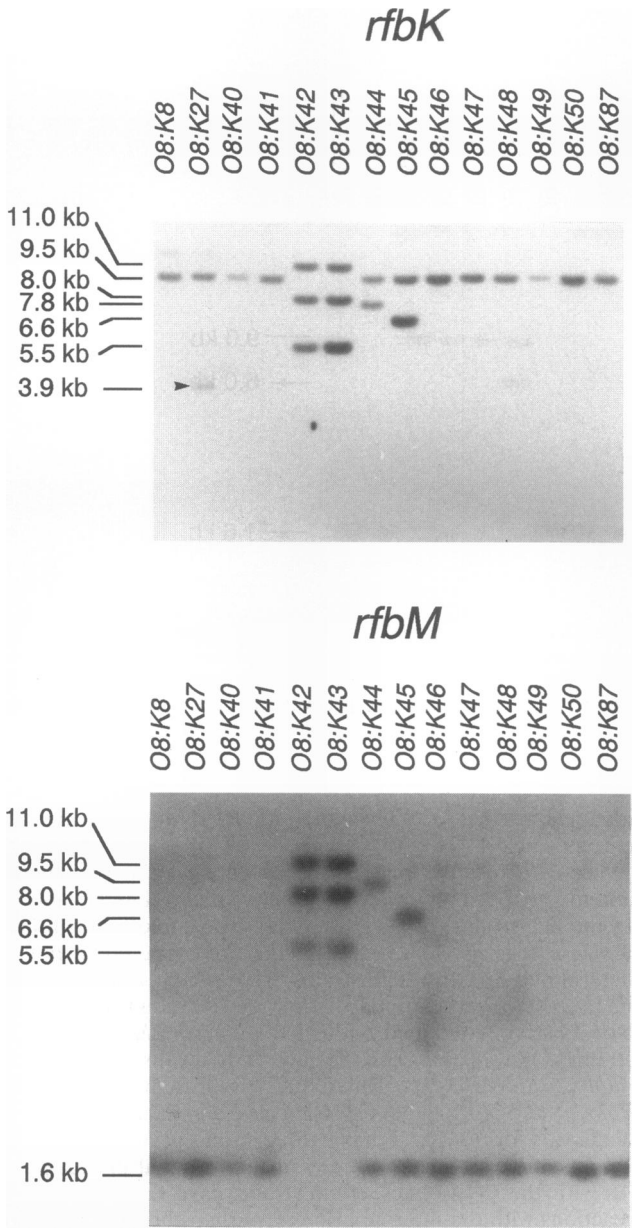


FIG. 8. Southern hybridization of the *rfbK* and *rfbM* regions in representative *E. coli* serotype O8 isolates with different K antigens. The internal probes for *rfbK* and *rfbM* were those used for Fig. 7 and described in Fig. 6A.

serovar Typhimurium, there are two isozymes each of GDP-mannose pyrophosphorylase (RfbM and CpsB) and phosphomannomutase (RfbK and CpsG) (52). As with *rfbM* and *rfbK*, *cpsB* and *cpsG* are located adjacent to one another. It has been proposed that CpsG and CpsB form part of a pathway leading to the formation of GDP-fucose, via GDP-mannose, for biosynthesis of the exopolysaccharide colanic acid (52). Homologs of CpsB and CpsG have also been identified by P. Reeves' group in *E. coli* K-12 (GenBank accession number L11721). Both CpsB homologs showed significant identity (62 and 62.6%) to $RfbM_{O9}$[1] (Table 3). Less identity in the RfbM proteins from *S. enterica* serovars and *E. coli* serotype O7 was detected (Table 3). The overall relationships among the GDP pyrophosphorylase isozymes examined are shown in the dendrogram in Fig. 9. Comparison of the $RfbK_{O9}$ proteins with other phosphomannomutase isozymes showed a broad range of identity values, consistent with the observations of others (36). The highest identity was with the CpsG isozymes from *E. coli* K-12 and *S. enterica* serovar Typhimurium and with the RfbK proteins from *E. coli* O7 and *S. enterica* serovar Montevideo. The relationships of the serovar Montevideo RfbK protein to both CpsG (31) and *E. coli* O7 RfbK (36) have been reported by others. Substantially lower levels of identity between $RfbK_{O9}$[1] and RfbK isozymes from *S. enterica* serovars Typhimurium and Muenchen were detected (Table 4). These relationships are represented schematically in Fig. 9.

The designations RfbM and RfbK given to the enzymes from *E. coli* O9 described here are based on the locations of their structural genes adjacent to the remaining *rfb* genes in strains E69 (described above) and F719 (53). Since plasmids carrying the complete rfb_{O9} cluster are unable to complement 19 of 20 independent capsule-deficient mutants tested here, the cps_{K30} gene cluster appears to be located outside the region cloned on pWQ607. Without these additional data, *rfbM* and *rfbK* could also be designated *cps* genes in *E. coli* O9:K30 on the basis of the ability of these enzymes to contribute to CPS synthesis.

The $cps_{K\text{-}12}$ gene cluster in *E. coli* K-12 is responsible for the synthesis of the slime polysaccharide colanic acid (15, 35). DNA homologous to *S. enterica* serovar Typhimurium *cpsB* and *cpsG* has been detected in *E. coli* K-12, and hybridizing sequences are absent in a *cps* deletion mutant, providing support for the contention that both *cpsB* and *cpsG* are present in *E. coli* K-12 (52); the sequences of CpsB and CpsG are now available in GenBank. *E. coli* strains with group II (25, 26) and group IB (23) K antigens can also produce colanic acid. In contrast, no colanic acid is synthesized by *E. coli* strains with group IA CPSs. The *cps* genes for synthesis of colanic acid and the group IA K30 CPS are allelic (26) and respond to common regulatory systems (23, 26). The mutation in *E. coli* CWG152 eliminates the production of K30 CPS and is complemented by plasmids carrying $rfbM_{O9}$. Introduction of rfb_{O9} clusters containing one (pNKB26) or two (pWQ607) copies of $rfbM_{O9}$ restored synthesis of both O9 and K30 polysaccharides in CWG152. Therefore, RfbM is sufficient to provide GDP-mannose precursor for both the O9 and K30 polysaccharides. There is no evidence from hybridization analyses (22) or complementation experiments for an additional functional isozyme of GDP-mannose pyrophosphorylase (e.g., one analogous to CpsB) in *E. coli* CWG44. This situation differs from that for *S. enterica* serovar Typhimurium, in which the GDP-mannose pyrophosphorylase isozymes RfbM and CpsG both provide the cell with GDP-mannose (52).

On the basis of data obtained with *E. coli* CWG44 and CWG152, K30 CPS cannot be synthesized in strains lacking *rfbM*. Consequently, this capsule type would be confined to strains with O polysaccharides containing mannose residues or their derivatives. If this situation occurs in other strains with group IA CPSs, this provides one possible explanation for the observation that group I CPSs, which often contain mannose and fucose (11), are predominantly coexpressed with serotype O8 or O9 LPS (20). In contrast, colanic acid is coexpressed in *E. coli* strains with group IB K antigens (23) and in strains with group II K antigens and a wide variety of O serotypes (20, 23, 25, 26). Many of these polysaccharides lack mannose or fucose residues, and this would place a selective pressure for maintenance of *cpsB* and *cpsG* in the colanic acid biosynthesis gene cluster. The situation in *cps* clusters may therefore resemble that seen in the enterobacterial common antigen biosynthesis

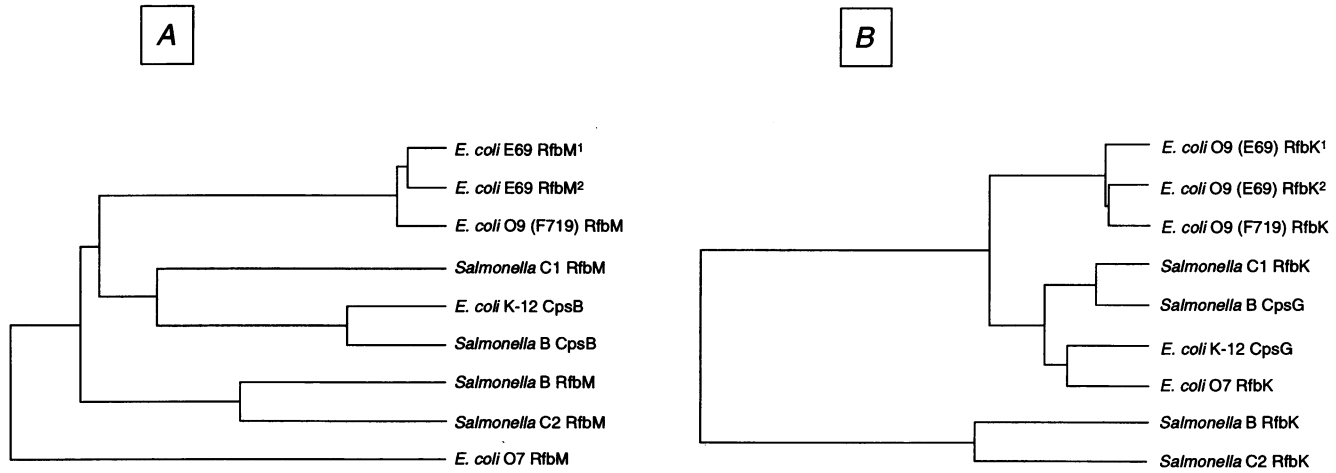


FIG. 9. Dendrograms showing the relationships between GDP-mannose pyrophosphorylase isozymes (A) and phosphomannomutase isozymes (B) from members of the *Enterobacteriaceae*. The multiple alignments were made by using CLUSTAL. Quantitative analyses are shown in Tables 3 and 4.

gene clusters of *S. enterica* serovars, in which the presence of structural genes for enzymes involved in the formation of TDP-4-keto-6-deoxy-D-glucose in the enterobacterial common antigen cluster is variable, depending on whether the *rfb* genes present encode isozymes for the same function (29).

The *gnd* region of *E. coli* has been shown to be highly polymorphic (5, 12), and it is proposed that intragenic recombination and lateral transfer of the entire *gnd* gene have occurred in *E. coli*, to generate the observed polymorphism (5). Of the Gnd sequences examined (Swiss Prot release 26), the predicted Gnd_{O9} protein has the greatest identity (457 of 468 residues) with Gnd from the *E. coli* reference strain ECOR16 from the collection of Ochman and Selander (43). The DNA sequences directly upstream of *gnd* are particularly variable (2). Therefore, DNA sequences containing the 5′ end of the gnd_{O9} coding region and upstream flanking DNA were compared with 14 other *gnd* alleles. The highest similarity score (71.6%) from the multiple alignment was again obtained with *gnd* from the ECOR16 reference strain (22).

The *rfb* regions adjacent to *gnd* are also highly variable. Analysis of different isolates of *E. coli* O8 and O9 highlighted the polymorphism in this region. Although there appeared to be more conservation within the O9 serotype with respect to *rfbM* and *rfbK*, the patterns within O8 were heterogeneous. There was also no correlation between the *rfbM* and *rfbK* patterns and the K antigen group (IA or IB). Group IA and IB CPSs differ in chemical composition; group IB CPSs contain amino sugars, whereas group IA CPSs do not (20). Also, group IB strains are able to produce colanic acid in addition to the group IB K antigen (23), and analysis of *rfbM* and *rfbK* in such strains is therefore potentially complicated by the presence of *cpsB* and *cpsG*. Taken together, the PCR and Southern hybridization data suggest that there are several clonal groups in serotypes O8 and O9, based on polymorphic internal *rfbM* and *rfbK* DNA sequences or due to differences in DNA flanking the *rfbM* and *rfbK* genes. Detailed analysis of the DNA sequences of *rfb* gene clusters in serovars of *S. enterica* has led to the proposal that the diversity of O antigens has arisen because of lateral transfer, and recombination into *rfb*, of genes from other sources (45). Each *rfb* gene cluster is

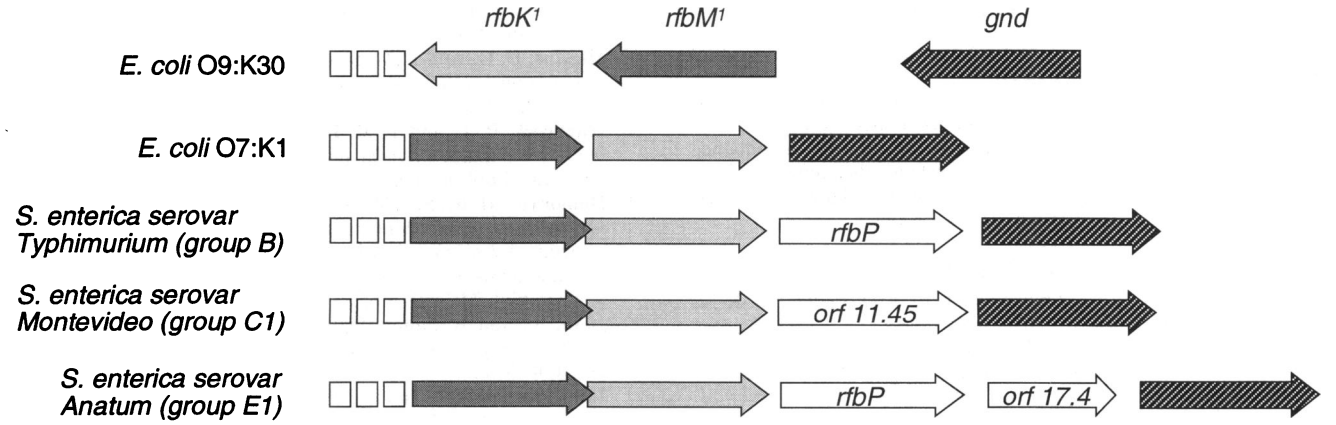


FIG. 10. Arrangement of the *gnd*, *rfbM*, and *rfbK* genes in members of the *Enterobacteriaceae*. Gene homologs are indicated by similar shading. The $gnd\text{-}rfbM_{O9}{}^{1}\text{-}rfbK_{O9}{}^{1}$ region of *E. coli* O9:K30 is compared with the corresponding regions from *E. coli* O7:K1 (36) and from *S. enterica* serovars Typhimurium (24), Montevideo (31), and Anatum (55). The *S. enterica* chromosome has one or two additional *rfb* genes between the GDP-mannose synthesis genes and *gnd*. The organization in *S. enterica* serovar Typhimurium (group B) is conserved in serogroups A, D, and C2 (45). The remaining *rfb* genes are identified by the short open boxes.

therefore proposed to be a mosaic of genes, some of which evolved independently in other strains of the same species or, in some cases, in other bacterial species. In the studies reported here, the majority of O9 isolates contained $rfbM_{O9}{}^{2}$ and $rfbK_{O9}{}^{2}$. The additional presence of $rfbM_{O9}{}^{1}$ and $rfbK_{O9}{}^{1}$ in *E. coli* E69 could be explained by transfer of genes from a related strain, but why this transfer would lead to duplication rather than allelic replacement is not clear. Furthermore, the possible selection pressure for maintaining duplicated genes in *E. coli* E69 is also unclear. The polymorphism in this region makes unequivocal determination of the true extent of *rfbM* and *rfbK* duplication in other *E. coli* O8 and O9 strains impossible without sequencing each of the appropriate regions.

In addition to nucleotide sequence variation, the arrangement of *rfbM* and *rfbK* varies in different members of the *Enterobacteriaceae*. For example, $rfbM_{O9}{}^{1}$ and $rfbK_{O9}{}^{1}$ are separated by 27 bp in *E. coli* E69 and by 25 bp in the serotype O9 strain F719 (53). In *E. coli* O7, *rfbM* and *rfbK* are also separated, but in this case the gap is 33 bp (36). This is different from the situation in various *S. enterica* serovars, in which the two genes overlap (24). However, the most striking difference between *E. coli* E69 and the other members of the *Enterobacteriaceae* examined is the position of *rfbM-rfbK* relative to *gnd* (Fig. 10). In all cases, transcription is in the direction *rfbM*→*rfbK*, but with the exception of that of *E. coli* O9, transcripts originate upstream of *rfbM* within the *rfb* cluster. In *S. enterica* serovar Typhimurium (24) and *E. coli* O7:K1 (36), there are no identifiable promoter sequences preceding either *rfbM* or *rfbK*, suggesting that these genes form part of a larger transcriptional unit. In *E. coli* serotype O9 strain F719, the orientation of $rfbM_{O9}$-$rfbK_{O9}$ relative to other rfb_{O9} genes is reversed (53), and $rfbM_{O9}$ and $rfbK_{O9}$ appear to be transcribed independently of the other rfb_{O9} genes. Although we have no data on the directions of transcription of the remaining rfb_{O9} genes in *E. coli* O9:K30, the arrangement of $rfbM_{O9}$ and $rfbK_{O9}$ relative to remaining rfb_{O9} genes appears to be the same in *E. coli* F719 and E69. This is based on the position of *gnd* and the observation that deletions of DNA downstream of $rfbK_{O9}{}^{2}$ in pWQ607 eliminate synthesis of O9 LPS. In *E. coli* E69, the location of $rfbM_{O9}$-$rfbK_{O9}$ relative to *gnd* is also reversed compared with those of the other characterized *gnd-rfb* regions (Fig. 10). The results presented here for *E. coli* E69 therefore provide the most extreme example of polymorphism in the *rfb* region reported to date.

ACKNOWLEDGMENTS

This work was financially supported by a grant from the Medical Research Council of Canada to C.W.

We are grateful to B. Jann, K. Jann, F. Ørskov, and I. Ørskov for providing the *E. coli* O8 and O9 strains examined here. We thank M. A. Valvano for generously providing strains and DNA sequence data prior to publication. Finally, we acknowledge the contributions of W. Moreno for some of the PCR analyses, and we acknowledge M. A. Valvano and M. Szabo for stimulating discussions.

REFERENCES

1. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54**:130–197.
2. **Barcak, G. J., and R. E. J. Wolf.** 1988. Comparative nucleotide sequence analysis of growth-rate-regulated *gnd* alleles from natural isolates of *Escherichia coli* and from *Salmonella typhimurium*. J. Bacteriol. **170**:372–379.
3. **Batchelor, R. A., P. Alifano, E. Biffali, S. I. Hull, and R. A. Hull.** 1992. Nucleotide sequences of the genes regulating O-polysaccharide chain length (*rol*) from *Escherichia coli* and *Salmonella typhimurium*: protein homology and functional complementation. J. Bacteriol. **174**:5228–5236.
4. **Binotto, J., P. R. MacLachlan, and P. R. Sanderson.** 1991. Electrotransformation of *Salmonella typhimurium* LT2. Can. J. Microbiol. **37**:474–477.
5. **Bisercic, M., J. Y. Feutrier, and P. R. Reeves.** 1991. Nucleotide sequences of the *gnd* genes from nine natural isolates of *Escherichia coli*: evidence of intragenic recombination as a contributing factor in the evolution of the polymorphic *gnd* locus. J. Bacteriol. **173**:3894–3900.
6. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene **4**:121–136.
7. **Brown, P. K., L. K. Romana, and P. R. Reeves.** 1992. Molecular analysis of the *rfb* gene cluster of *Salmonella* serovar muenchen (strain M67): the genetic basis of the polymorphism between groups C2 and B. Mol. Microbiol. **6**:1385–1394.
8. **Clarke, B. R., and C. Whitfield.** 1992. Molecular cloning of the *rfb* region of *Klebsiella pneumoniae* serotype O1:K20: the *rfb* gene cluster is responsible for synthesis of the D-galactan I O polysaccharide. J. Bacteriol. **174**:4614–4621.
9. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium*. J. Bacteriol. **155**:831–838.
10. **Ditta, G., D. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
11. **Dutton, G. G. S., and L. A. S. Parolis.** 1989. Polysaccharide antigens of *Escherichia coli*, p. 223–240. *In* I. C. M. Dea and S. S. Stivola (ed.), Recent developments in industrial polysaccharides: biological and biotechnological advances. Gordon and Breach Science Publishers, New York.
12. **Dykhuizen, D. E., and L. Green.** 1986. DNA sequence variation, DNA phylogeny and recombination in *E. coli*. Genetics Abstr. **113**:s71.
13. **Figurski, D. H., and D. R. Helinksi.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
14. **Gabriel, O.** 1987. Biosynthesis of sugar residues for glycogen, peptidoglycan, lipopolysaccharide, and related systems, p. 504–511. *In* F. C. Niedhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
15. **Gottesman, S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12. Mol. Microbiol. **5**:1599–1606.
16. **Haraguchi, G. E., U. Zähringer, B. Jann, K. Jann, R. I. Hull, and S. I. Hull.** 1991. Genetic characterization of the O4 polysaccharide gene cluster from *Escherichia coli*. Microb. Pathog. **10**:351–361.
17. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73**:237–244.
18. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
19. **Homonylo, M. K., S. J. Wilmot, J. S. Lam, L. A. MacDonald, and C. Whitfield.** 1988. Monoclonal antibodies against the capsular K antigen of *Escherichia coli* (O9:K30:H12): characterization and use in analysis of K antigen organization on the cell surface. Can. J. Microbiol. **34**:1159–1165.
20. **Jann, B., and K. Jann.** 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol. Immunol. **150**:19–42.
21. **Jann, K., and B. Jann.** 1984. Structure and biosynthesis of O-antigens, p. 138–186. *In* E. T. Reitschel (ed.), Handbook of endotoxin, vol. I. Chemistry of endotoxin. Elsevier Science Publishers, B.V., Amsterdam.
22. **Jayaratne, P., D. Bronner, P. R. MacLachlan, C. Dodgson, and C. Whitfield.** Unpublished results.
23. **Jayaratne, P., W. J. Keenleyside, P. R. MacLachlan, C. Dodgson,**

and C. Whitfield. 1993. Characterization of *rcsB* and *rcsC* from *Escherichia coli* O9:K30:H12 and examination of the role of the *rcs* regulatory system in expression of group I capsular polysaccharides. J. Bacteriol. **175:**5384–5394.

24. **Jiang, X.-M., B. Neal, F. Santiago, S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar typhimurium (strain LT2). Mol. Microbiol. **5:**695–713.
25. **Keenleyside, W. J., D. Bronner, K. Jann, B. Jann, and C. Whitfield.** 1993. Coexpression of colanic acid and serotype-specific capsular polysaccharides in *Escherichia coli* strains with group II K antigens. J. Bacteriol. **175:**6725–6730.
26. **Keenleyside, W. J., P. Jayaratne, P. R. MacLachlan, and C. Whitfield.** 1992. The *rcsA* gene of *Escherichia coli* O9:K30:H12 is involved in the expression of the serotype-specific group I K (capsular) antigen. J. Bacteriol. **174:**8–16.
27. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an Agrobacterium Ti plasmid. Plasmid **8:**45–54.
28. **Kogan, G., G. Haraguchi, S. I. Hull, R. A. Hull, A. Shashkov, B. Jann, and K. Jann.** 1993. Structural analysis of O4-reactive polysaccharides from recombinant *Escherichia coli*. Changes in the O-specific polysaccharide induced by cloning of the *rfb* genes. Eur. J. Biochem. **214:**259–265.
29. **Kuhn, H.-M., U. Meier-Dieter, and H. Mayer.** 1988. ECA, the enterobacterial common antigen. FEMS Microbiol. Rev. **54:**195–222.
30. **Laakso, D. H., M. K. Homonylo, S. J. Wilmot, and C. Whitfield.** 1988. Transfer and expression of the genetic determinants for O and K antigen synthesis in *Escherichia coli* O9:K(A)30:H12 and *Klebsiella* sp. O1:K20, in *Escherichia coli* K-12. Can. J. Microbiol. **34:**987–992.
31. **Lee, S. J., L. K. Romana, and P. R. Reeves.** 1992. Sequence and structural analysis of the *rfb* (O antigen) gene cluster from a group C1 *Salmonella enterica* strain. J. Gen. Microbiol. **138:**1843–1855.
32. **Liu, D., and P. R. Reeves.** 1994. *Escherichia coli* K12 regains its O antigen. Microbiology **140:**49–57.
33. **MacPherson, D. F., P. A. Manning, and R. Morona.** 1994. Characterization of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*. Mol. Microbiol. **11:**281–292.
34. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**154.
35. **Markovitz, A.** 1977. Genetics and regulation of bacterial capsular polysaccharide biosynthesis and radiation sensitivity, p. 415–462. *In* I. W. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, Inc., New York.
36. **Marolda, C. L., and M. A. Valvano.** 1993. Identification, expression, and DNA sequence of the GDP-mannose biosynthesis genes encoded by the O7 *rfb* gene cluster of strain VW187 (*Escherichia coli* O7:K1). J. Bacteriol. **175:**148–158.
37. **McCallum, K. L., D. H. Laakso, and C. Whitfield.** 1989. Use of a bacteriophage-encoded glycanase enzyme in the generation of lipopolysaccharide O side chain deficient mutants of *Escherichia coli* O9:K30 and *Klebsiella* O1:K20: role of O and K antigens in resistance to complement-mediated serum killing. Can. J. Microbiol. **35:**994–999.
38. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Neal, B. L., G. C. Tsiolis, M. W. Heuzenroeder, P. A. Manning, and P. R. Reeves.** 1991. Molecular cloning and expression in *Escherichia coli* K-12 of chromosomal genes determining the O antigen of an *Escherichia coli* O2:K1 strain. FEMS Microbiol. Lett. **82:**345–352.
40. **Neuhard, J., and E. Thomassen.** 1976. Altered deoxynucleotide pools in P2 eductants of *Escherichia coli* K-12 due to the deletion of the *dcd* gene. J. Bacteriol. **126:**999–1001.
41. **Nikaido, H., M. Levinthal, K. Nikaido, and K. Nakane.** 1967. Extended deletions in the histidine-rough-B region of the *Salmonella* chromosome. Proc. Natl. Acad. Sci. USA **57:**1825–1832.
42. **Nikaido, H., K. Nikaido, and P. H. Mäkelä.** 1966. Genetic determination of enzymes synthesizing specific sugars of *Salmonella* lipopolysaccharide. J. Bacteriol. **91:**1126–1135.
43. **Ochman, H., and R. K. Selander.** 1984. Standard reference strains of *Escherichia coli* from natural populations. J. Bacteriol. **157:**690–693.
44. **Peyru, G., and D. G. Fraenkel.** 1968. Genetic mapping of loci for glucose-6-dehydrogenase, gluconate-6-phosphate dehydrogenase, and gluconate-6-phosphate dehyrdrase in *Escherichia coli*. J. Bacteriol. **95:**1272–1278.
45. **Reeves, P. R.** 1993. Evolution of *Salmonella* O antigen variation by interspecific gene transfer on a large scale. Trends Genet. **9:**17–22.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sanderson, K. E., and J. R. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition 7. Microbiol. Rev. **52:**485–532.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
49. **Schmidt, G., B. Jann, and K. Jann.** 1974. Genetic and immunochemical studies on *Escherichia coli* O14:K7:H$^-$. Eur. J. Biochem. **42:**303–309.
50. **Schmidt, G., B. Jann, K. Jann, I. Ørskov, and F. Ørskov.** 1977. Genetic determinants of the synthesis of the polysaccharide capsular antigen K27(A) of *Escherichia coli*. J. Gen. Microbiol. **100:**355–361.
51. **Shibaev, V. L.** 1986. Biosynthesis of bacterial polysaccharide chains composed of repeating units. Adv. Carbohydr. Chem. Biochem. **44:**277–339.
52. **Stevenson, G., S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. The *cps* gene cluster of *Salmonella* strain LT2 includes a second mannose pathway: sequence of two genes and relationship to genes in the *rfb* gene cluster. Mol. Gen. Genet. **227:**173–180.
53. **Sugiyama, T., N. Kido, T. Komatsu, M. Ohta, K. Jann, B. Jann, A. Saeki, and N. Kato.** 1994. Genetic analysis of *Escherichia coli* O9 *rfb*: identification and DNA sequence of phosphomannomutase and GDP-mannose pyrophosphorylase genes. Microbiology **140:**59–71.
54. **Tsai, G. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
55. **Wang, L., L. K. Romana, and P. R. Reeves.** 1992. Molecular analysis of a *Salmonella enterica* group E1 *rfb* gene cluster: O antigen and the genetic basis of the major polymorphism. Genetics **130:**429–443.
56. **Whitfield, C.** Unpublished results.
57. **Whitfield, C., and M. Lam.** 1986. Characterization of coliphage K30, a bacteriophage specific for *Escherichia coli* serotype K30. FEMS Microbiol. Lett. **37:**351–355.
58. **Whitfield, C., G. Schoenhals, and L. Graham.** 1989. Mutants of *Escherichia coli* O9:K30 with altered synthesis and expression of the capsular K antigen. J. Gen. Microbiol. **135:**2589–2599.
59. **Wilkinson, R. G., P. Gemski, and B. A. D. Stocker.** 1972. Nonsmooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70:**527–554.
60. **Wolf, R. E. J., and F. M. Shea.** 1980. Combined use of strain construction and affinity chromatography in the rapid, high-yield purification of 6-phosphogluconate dehydrogenase from *Escherichia coli*. J. Bacteriol. **138:**171–175.
61. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.