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Expression of the O9 Polysaccharide of *Escherichia coli*: Sequencing of the *E. coli* O9 *rfb* Gene Cluster, Characterization of Mannosyl Transferases, and Evidence for an ATP-Binding Cassette Transport System

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The *rfb* gene cluster of *Escherichia coli* O9 directs the synthesis of the O9-specific polysaccharide which has the structure →2-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→3)-α-Man-(1→3)-α-Man-(1→. The *E. coli* O9 *rfb* cluster has been sequenced, and six genes, in addition to the previously described *rfbK* and *rfbM*, were identified. They correspond to six open reading frames (ORFs) encoding polypeptides of 261, 431, 708, 815, 381, and 274 amino acids. They are all transcribed in the counter direction to those of the *his* operon. No gene was found between *rfb* and *his*. A higher G+C content indicated that *E. coli* O9 *rfb* evolved independently of the *rfb* clusters from other *E. coli* strains and from *Shigella* and *Salmonella* spp. Deletion mutagenesis, in combination with analysis of the in vitro synthesis of the O9 mannan in membranes isolated from the mutants, showed that three genes (termed *mtfA*, *-B*, and *-C*, encoding polypeptides of 815, 381, and 274 amino acids, respectively) directed α-mannosyl transferases. MtfC (from ORF274), the first mannosyl transferase, transfers a mannose to the endogenous acceptor. It critically depended on a functional *rfe* gene (which directs the synthesis of the endogenous acceptor) and initiates the growth of the polysaccharide chain. MtfB (from ORF381) then transfers two mannoses into the 3 position of the previous mannose, and MtfA (from ORF815) transfers three mannoses into the 2 position. Further chain growth needs only the two transferases MtfA and MtfB. Thus, there are fewer transferases needed than the number of sugars in the repeating unit. Analysis of the predicted amino acid sequence of the ORF261 and ORF431 proteins indicated that they function as components of an ATP-binding cassette transport system. A possible correlation between the mechanism of polymerization and mode of membrane translocation of the products is discussed.

Lipopolysaccharides (LPS) of enteric bacteria consist of lipid A, the core oligosaccharide, and the O-specific polysaccharide moiety, which are joined through glycosidic linkages (17). Whereas lipid A mediates endotoxic activities of LPS, the polysaccharide moiety is responsible for serological O specificity (O antigen) (17, 38a). In *Escherichia coli*, there are in excess of 170 structurally and serologically distinct O antigens. The O8 and O9 polysaccharides are mannans, the repeating unit of the O8 polysaccharide being →3)-β-Man-(1→2)-α-Man-(1→2)-α-Man-(1→ and that of the O9 polysaccharide being →2)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→3)-α-Man-(1→3)-α-Man-(1→ (18, 21, 40). Many *Klebsiella* strains have α-mannans or α-galactans as O-specific polysaccharides (18, 55), and the O3- and O5-specific polysaccharides of *Klebsiella* strains are identical to the O8 and O9 polysaccharides of *E. coli* (8, 18, 29). Mannans of algal origin were found to exert antitumor activity (37). Such an activity could later be attributed also to the mannan-containing LPS of *E. coli* and *Klebsiella* strains (13, 37).

The genetics of LPS biosynthesis in enteric bacteria is well documented in recent reviews (33, 43, 48, 55). Two mechanisms, block and monomeric, have been described for O-polysaccharide synthesis (49). In the block mechanism, observed for *Salmonella typhimurium* and related *Salmonella* serotypes, the oligosaccharide repeating units are assembled on undecaprenol phosphate (antigen carrier lipid [ACL]) under the direction of *rfb* genes. The first sugar transferred was found to be galactose-1-phosphate, and the corresponding transferase gene was termed *rfbP* (48). The repeating units are polymerized under the direction of the *rfc* gene, which may be located outside of or within the *rfb* gene cluster (41). The chain length is controlled by the *rol* gene, located between *gnd* and *his* (3, 4).

The monomeric mechanism, experimentally proven only for *E. coli* O8 and O9 (18, 55), consists of the direct and sequential transfer of the monosaccharide residues from their nucleotide-activated precursors to the nonreducing end of the growing polysaccharide chain.

The synthesis of some O polysaccharides requires the *rfe* gene. According to this requirement, LPS biosynthesis can also be divided into *rfe*-dependent and *rfe*-independent pathways. The *rfe* gene, first described by Mäkelä et al. (31), was found to be essential for the synthesis of the O polysaccharide in *Salmonella* strains of O groups C1 and L, and *E. coli* O8 and O9 (18, 33) and more recently in *E. coli* O4, O7, O18, O75, and O111 (1, 23a). It was reported to determine the tunicamycin-sensitive transfer of *N*-acetylglucosamine (GlcNAc)-1-phosphate from UDP-GlcNAc to undecaprenol monophosphate

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli F719	O9:K31$^-$:H$^-$	This laboratory
E. coli K-12		
HU1190	Δ(*sbcB-rfb*) *hsdR4 recA56 srl*::Tn*10*	51
21547	*rfe*-negative AB1133 derivative	36
Plasmids		
pBluescript II SK(+)	Cloning vector; Ap^r	Toyobo
pACYC184	Cloning vector, Tc^r Cp^r	51
pNKB26	Derivative of a cloning vector pACYC184 carrying *E. coli* O9 *rfb*	51
pXX195	Derivative of pNKB26 carrying ORF261, ORF431, ORF708, *mtfA*, *mtfB*, and *mtfC*	51
pBHA6	Derivative of pNKB26 carrying ORF431, ORF708, *mtfA*, *mtfB*, and *mtfC*	51
pBHA6-Ea1	Derivative of pNKB26 carrying ORF708, *mtfA*, *mtfB*, and *mtfC*	This study
pBRV13	ORF708 in pBluescript II SK(+)	This study
pBSC25	*mtfA* and *mtfB* in pBluescript II SK(+)	This study
pSLB1	*mtfB* and *mtfC* in pBluescript II SK(+)	This study
pBSC25-St1	*mtfA* in pBluescript II SK(+)	This study
pBHA33-SlSc1	*mtfB* in pBluescript II SK(+)	This study
pBBB6-H1	*mtfC* in pBluescript II SK(+)	This study
pABB2	*mtfC* in pACYC184	This study

with the formation of GlcNAc-pyrophosphorylundecaprenol (35). Whereas the *rfbP* dependence of an O-polysaccharide synthesis is an indication of a block mechanism, such a clear situation does not exist for *rfe*-dependent syntheses. In the *rfe*-dependent polymerization of the *Shigella dysenteriae* O1 antigen, the gene corresponding to *rfc*, determining block polymerization, was located to the *rfb* gene cluster (25).

In continuation of previous studies on the *E. coli* O8 and O9 antigens (11, 17–20, 23, 24, 26, 51–55), we wanted to characterize the *rfb* genes determining the synthesis of the O9 antigen and to elucidate the mechanism of polymerization in more detail. Two of these genes (*rfbK* and *rfbM*) were recently found to determine phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMP) homologous to the corresponding genes of *S. typhimurium* (51). Here we report the complete sequence of the *E. coli* O9 *rfb* gene cluster, the analysis of genes for a putative ATP-binding cassette (ABC) transporter, as well as the characterization of the O9-specific mannosyl transferases and their *rfe* dependence.

MATERIALS AND METHODS

Chemicals. GDP-mannose was obtained from Sigma (Deisenhofen, Germany), and GDP-[^{14}C]mannose was obtained from Amersham Buchler (Braunschweig, Germany). TSK HW40(S) was purchased from Merck (Darmstadt, Germany). The glycosyl derivatives [α- and β-glucosyl-, α-*N*-acetylglucosaminyl-, α-galactosyl-, α-glucuronyl-, and α-mannosyl-(1→3)-α-glucosyl] of pyrophosphorylmoraprenol were prepared as described previously (20). Moraprenol is an undecaprenol from mulberry leaves that differs from bactoprenol only in the ratio of *cis* and *trans* double bonds (20, 49).

Bacterial strains and plasmids. The *E. coli* strains and plasmids used are shown in Table 1. *E. coli* F719 is a strain from which the *rfb* genes were isolated (24, 51). The bacteria were grown in L broth with 0.5% glucose. Ampicillin (100 μg/ml) and/or chloramphenicol (30 μg/ml) were added for the cultivation of bacteria harboring plasmids.

DNA methods. Preparations of plasmid and single-stranded DNA, endonuclease digestion, ligation, transformation of bacterial competent cells, and electrophoresis of DNA were done as described by Sambrook et al. (45).

The dideoxynucleotide chain-terminating method of Sanger et al. (46) was used for DNA sequencing of M13 phage and pBluescript II SK(+) phagemid recombinant subclones as described previously (51). For isolation of deleted derivatives of M13 and pBluescript II SK(+) subclones, a deletion kit (Kilo-Sequence deletion kit; Takara Shuzo Co.) was used. Single-strand phage was prepared from subclones of M13 and pBluescript II SK(+) and used for sequencing with the Sequenase sequencing kit (U.S. Biochemical, Cleveland, Ohio). Both strands were completely sequenced. Sequence data were analyzed by using the SDC-GENETYX system (Software Development Co., Tokyo, Japan) and the ODEN program of the National Institute of Genetics, Mishima, Japan (51).

Preparation of membranes. Membranes were prepared as described previously (11, 17, 19, 23). In brief, the bacteria were grown in liquid culture to the late logarithmic phase and collected by centrifugation (10,000 × *g*, 10 min, 4°C). They were washed once in 50 mM Tris-HCl (pH 8.0)–2 mM dithiothreitol–30 mM magnesium acetate (T buffer), resuspended in T buffer (approximately 1/20 of the original volume), and disrupted by two passages of the suspension through a French pressure cell (Aminco) at 75 kg/cm^2 (11,000 lb/in^2). After removal of bacterial fragments by centrifugation, the membranes were obtained from the homogenate by ultracentrifugation (180,000 × *g*, 60 min) as a sediment. They were suspended in T buffer to a concentration of 10 mg/ml and kept at −80°C.

Preparation of butanol extracts and suspensions of glycosyl derivatives of diphosphomoraprenol. For the isolation of lipid-linked intermediates, membranes were extracted twice with butan-1-ol (1 to 2 volumes) at room temperature. After phase separation by centrifugation, the organic phases were combined and washed twice with water. Prior to use in incubation tests, the extracts were mixed with an equal volume of water and evaporated to near dryness. This process was repeated several times until all of the butanol was removed and a turbid aqueous suspension was obtained.

To methanolic solutions of glycosyl derivatives of pyrophosphorylmoraprenol (20 to 60 μl), chloroform (200 μl) and 0.9% saline (75 μl) were added, and after shaking, the phases were separated. The lower phase was transferred into siliconated glass tubes, and after the addition of butan-1-ol (50 μl), the mixture was concentrated in vacuo to about 50 μl and washed with water (50 μl). The resulting butanol solution was converted to an aqueous solution by repeated concentration with water as described above.

Assay of mannose incorporation. The incubation mixtures contained membrane suspension (50 μl, 50 to 100 μg of protein), GDP-[^{14}C]mannose (4 μM), and, if required, butanol extract or a pyrophosphorylmoraprenol derivative in 50 mM Tris buffer (pH 7.5). After incubation at 37°C for 10 min, the reaction was stopped by the addition of 1 ml of ice-cold 12% acetic acid. The mixture was filtered through a cellulose acetate filter (0.45-μm pore size; Sartorius, Göttingen, Germany). The filter was washed with 12% acetic acid, dried, and counted in a Beckman liquid scintillation counter.

Product analysis by gel permeation chromatography. The incubation products were liberated from the membranes by treatment with 0.25 N hydrochloric acid (100°C, 10 min). The denatured membranes were removed by centrifugation, and the supernatants were applied to a column (17 by 780 mm) of TSK HW40(S) and eluted with 0.1 N acetic acid. Fractions were counted in a Beckman liquid scintillation counter. The column was standardized with mannose, cellobiose, raffinose, stachyose, and the O9 polysaccharide, as isolated from the LPS by mild acid hydrolysis and purification on Sephadex G-50.

Smith degradation. Fractions from the TSK-gel permeation chromatography, detected by counting in a Beckman liquid scintillation counter, were pooled and treated with 0.2 M sodium metaperiodate for 24 h at room temperature in the dark. The products were reduced with sodium borohydride and desalted by gel permeation chromatography on TSK HW40(S). Pooled fractions were hydrolyzed (12) with aqueous 2% acetic acid (100°C, 2 h) and rechromatographed, and fractions were detected by counting in a Beckman liquid scintillation counter.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D43637.

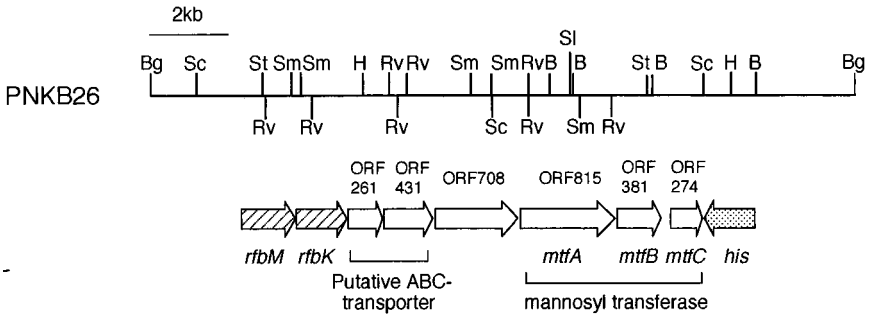

FIG. 1. Physical map and gene structure of *E. coli* O9 *rfb*. ORF positions are determined on the basis of the nucleotide sequence. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; RV, *Eco*RV; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; St, *Stu*I.

RESULTS

Nucleotide sequence analysis. The *E. coli* O9 *rfb* region of plasmid pNKB26 has previously been found to encode at least seven proteins, two of which were characterized as PMM and GMP. Nucleotide sequences of the corresponding genes, *rfbK* and *rfbM*, were determined (51). We determined the nucleotide sequence of the remaining region and identified six open reading frames (ORFs) (Fig. 1). The ORFs potentially encoded 29.7-, 47.8-, 81.6-, 91.5-, 43.9-, and 31.1-kDa polypeptides, and they were termed, according to the number of amino acids encoded by each gene, ORF261, ORF431, ORF708, ORF815, ORF381, and ORF274, respectively. With the exception of ORF274, the products of these genes could be demonstrated by minicell experiments (51). They were located downstream of *rfbK* and were transcribed in the same direction as *rfbK* and *rfbM*. An inverted repeat 9 bp downstream of the stop codon of ORF274, with a free energy of −26.5 kcal (ca. −110.9 kJ)/mol, could act as a transcriptional terminator. The only relatively long (300 bp) intercistronic region was between ORF381 and ORF274.

We also determined a sequence that extended the region downstream of ORF274 and found two ORFs which transcribed in counter direction to *rfb*. One of them showed 98% identity to 3′ end of *E. coli* K-12 *hisF*, and the other showed 92% identity to *E. coli* K-12 *hisIE* (7). There was a gap of 38 bp between the stop codons of ORF274 and *hisIE*. The inverted repeat mentioned above was located within this gap. This result indicated that the *rfb* gene cluster was located directly downstream of *his* operon and that there was no gene between *his* and *rfb* in *E. coli* O9.

G+C content. The G+C content plot and G+C content values of the individual genes in the *E. coli* O9 *rfb* region are shown in Fig. 2. Whereas the *rfbM* and *rfbK* genes had a higher G+C content (0.62) (51), that of the other six genes was close to the usual value for *E. coli* (0.48 to 0.56).

Analysis of ORF431 and ORF261. ORF431 encoded a predicted polypeptide of 47.8 kDa. Its sequence was 39.5% identical with BexA of *Haemophilus influenzae* (27) and 36.5% identical with KpsT of *E. coli* K1 and K5 (27, 50). The molecular mass was larger than those of BexA and KpsT (47.8 versus 25 to 27 kDa). It contained two sequences, GVNGAGKST and ILIVD, homologous to corresponding sites in BexA and KpsT. These motifs correspond to the A and B sites of an ATP-binding motif (9).

ORF261 encoded a predicted polypeptide of 29.7 kDa. Its hydrophobicity profile is shown in Fig. 3 in comparison with that of BexB. The profile indicated of a highly hydrophobic

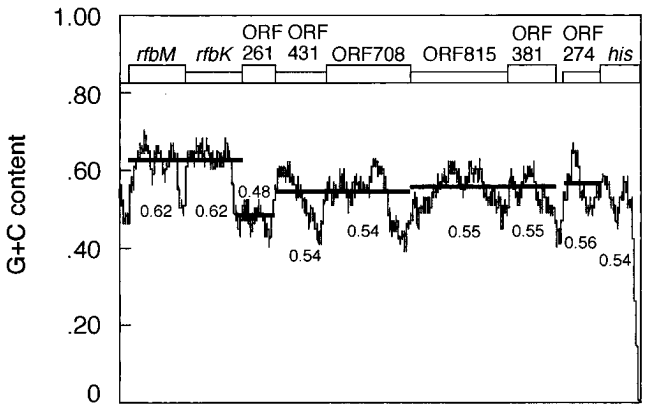

FIG. 2. G+C plot and G+C content of each gene. The G+C content plot is given below the map (each point gives an average of over 199 bases). The G+C content of each gene is shown by a bar above the plot of the *rfb* region.

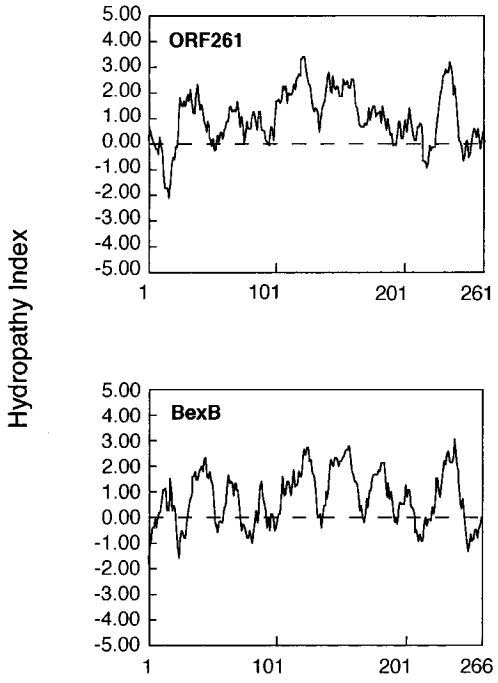

FIG. 3. Hydropathy plots of the predicted ORF261 and BexB proteins generated by the method of Kyte and Doolittle (28) with a window length of 11.

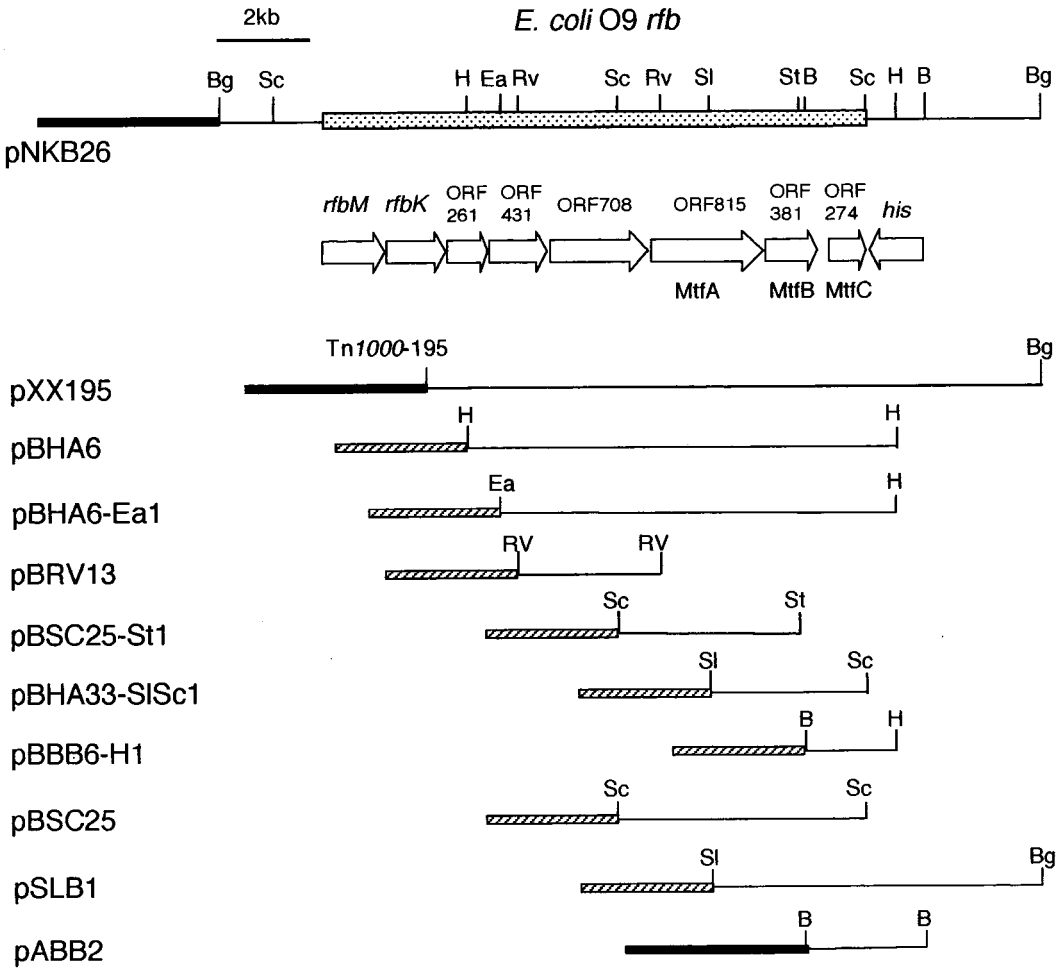

FIG. 4. Restriction enzyme maps of pNKB26 and physical map of subclones used in this study. Solid bars represent the cloning vector pACYC184; hatched bars represent the cloning vector pBluescript II SK(+). Box arrows indicate ORFs deduced from the DNA sequence, and names of the genes determined are also indicated.

protein with six putative membrane-spanning domains. The profile of ORF261 is similar to those of KpsM, BexB, CtrC, VexB, and Y-RfbD of *E. coli* (39, 50), *H. influenzae* (27), *Neisseria meningitidis* (10), *Salmonella typhi* (14), and *Yersinia enterocolitica* (56), which are considered as the integral membrane component of an ABC transport system (9, 15, 42).

Identification of the mannosyl transferase genes. To characterize the O9-specific mannosyl transferase genes, the O9 *rfb* genes were cloned individually (Fig. 4). Membrane preparations of recombinant *E. coli* harboring the complete *rfb* region and of those with single *rfb* genes were incubated with GDP-[^{14}C]mannose, and the mannose incorporation was determined by counting in a Beckman liquid scintillation counter. The results (Table 2) show that membranes from HU1190 harboring all *E. coli* O9 *rfb* genes (on plasmid pNKB26) and some deleted derivatives of pXX195, pBHA6, and pBHA6-Ea1 incorporated mannose actively. Difference in copy number of vectors for pXX195 and pBHA6 seemed to have no effect on the mannose incorporation activities. Deletion of ORF431 resulted in a lower activity of *E. coli* HU1190(pBHA6-Ea1) membranes, although TSK-gel chromatography of the product revealed that the O9 polysaccharide was synthesized (data not shown). If ORF431 is part of a O9 polysaccharide transporter, its deletion may cause a feed back inhibition of the O9 polysaccharide formation, similar to that demonstrated in the ex-

TABLE 2. Membrane activity to incorporate mannose from GDP-mannose

Plasmid (ORFs on plasmid)[a]	Mannose incorporation (pmol/mg of protein)
pNKB26 (all)[b]	404.7
pXX195 (deleted of *rfbKM* and ORF261, -431, -708, -815, -381, and -274)[b]	508.3
pBHA6 (ORF431, -708, -815, -381, and -274)[c]	483.8
pBHA6-Ea1 (ORF708, -815, -381, and -274)[c]	215.5
pBRV13 (ORF708)[c]	0
pBSC25-St1 (ORF815)[c]	0
pBHA33-SlSc1 (ORF381)[c]	0
pBBB6-H1 (ORF274)[c]	65.6
pBSC25 (ORF815 and -381)[c]	0.1
pBSC25 (ORF815 and -381)[c] + pABB2 (ORF274)[b]	77.0
pBSC25-St1 (ORF815)[c] + pABB2 (ORF274)[b]	14.2
pSLB1 (ORF381 and -274)[c]	108.1
pABB2 (ORF274)[b]	9.6

[a] For characterization, see Fig. 1.
[b] In pACYC184.
[c] In pBluescript II SK(+).

pression of the *E. coli* K5 capsular polysaccharide (6). The products of the *rfbK* and *rfbM* genes were not necessary for the membranes to incorporate mannose from GDP-mannose in vitro (pXX195; Table 2). The activity of membranes from HU1190(pBHA6) and from HU1190(pBHA6-Ea1) indicated that ORF261 and ORF431 are not needed for mannose incorporation in vitro. That left ORF708, ORF815, ORF381, and ORF274 as candidates for mannosyl transferase genes. Of all clones with a single *rfb* gene, only membranes from HU1190 (pBBB6-H1) carrying ORF274 incorporated mannose. The low incorporation could be explained by the transfer of only one or few mannose units.

In another series of experiments, membranes from recombinant strains with different combinations of *rfb* genes (Fig. 4) were used for the incubation with GDP-[^{14}C]mannose. The results are shown in Table 2. To generate the gene combinations used, ORF274 was subcloned into pACYC184, which can coexist with pBluescript II SK(+) derivatives in *E. coli*. The low activity of HU1190(pABB2) membranes compared with that of HU1190(pBBB6-H1) membranes may be due to a lower copy number of the cloning vector pACYC184 than that of pBluescript II SK(+).

The results shown in Table 2 suggest that ORF381 may transfer mannose(s) to the synthesized oligosaccharide product of ORF274 and that ORF815 may subsequently transfer mannose(s) to the oligosaccharide product of ORF381. In such a reaction sequence, ORF815 could not complement ORF274 because of the absence of ORF381, which provides the necessary precursor. This would explain the comparable activities given in the combination of pBSC25-St1 plus pABB2. The relatively low activity presented in the combination of pBSC25 plus pABB2 is difficult to explain. However, O9 polysaccharides were synthesized in the membrane (see below). It may be due to a low copy number of pABB2 and an insufficient synthesis of the mannose acceptor.

Characterization of the reaction products. The products obtained by incubation with GDP-[^{14}C]mannose of membranes from strains HU1190(pBBB6-H1) with only ORF274, HU1190(pSLB1) with ORF274 and ORF381, and HU1190(pBSC25 + pABB2) with ORF274, ORF381, and ORF815 were analyzed with respect to molecular size and linkage sequence.

For size determinations, the incubation products were liberated from the membranes by mild acid hydrolysis and subjected to gel permeation chromatography. The results (Fig. 5) revealed that the membrane-derived products are a disaccharide (directed by ORF274), a tetrasaccharide (directed by ORF274 and ORF381), and a polymer (directed by ORF274, ORF381, and ORF815). The polymer from HU1190(pBSC25 + pABB2) with the three ORFs showed the same chromatographic properties as that of authentic O9 polysaccharide. This is an indication that ORF274 directs the first mannosyl transferase, that ORF381 directs the second mannosyl transferase, and that ORF815 directs the third mannosyl transferase. We designate these genes *mtfA*, *-B*, and *-C*, starting from the 5′ end.

To analyze the linkage sequence, the polymeric and the tetrasaccharide incubation products were subjected to periodate oxidation-sodium borohydride reduction followed by mild acid hydrolysis (Smith degradation) (12). This reaction, which cleaves sugar rings by oxidation between vicinal hydroxyl groups, was used to differentiate between 2- and 3-linked mannoses: 2-linked residues were cleaved (between C-3 and C-4), and 3-linked residues were not cleaved (no vicinal hydroxyl groups). Mild acid hydrolysis then fragmented the polysaccharide at the sites of oxidation. The reaction products were sep-

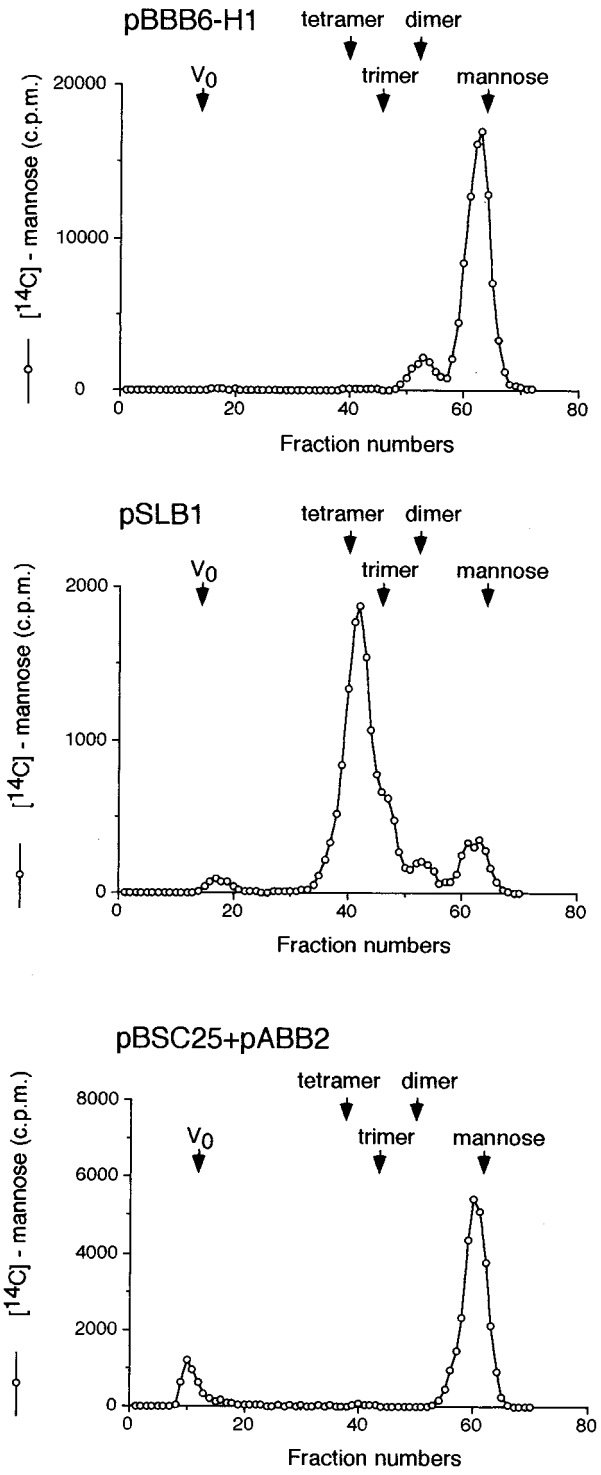

FIG. 5. Profiles of TSK-gel column chromatography of oligo- and polysaccharide fractions synthesized in vitro. The authentic *E. coli* O9 polysaccharides prepared from O9 LPS elute at void volume (V_0). Positions of oligosaccharide eluted are determined by using stachyose (tetramer), raffinose (trimer), cellobiose (dimer), and mannose. Fractions (1 ml) were counted in Beckman scintillation counter. GDP-mannose was converted to mannose in the procedure and therefore was present in the column eluates. This was especially the case when membrane activity was low (top and bottom panels).

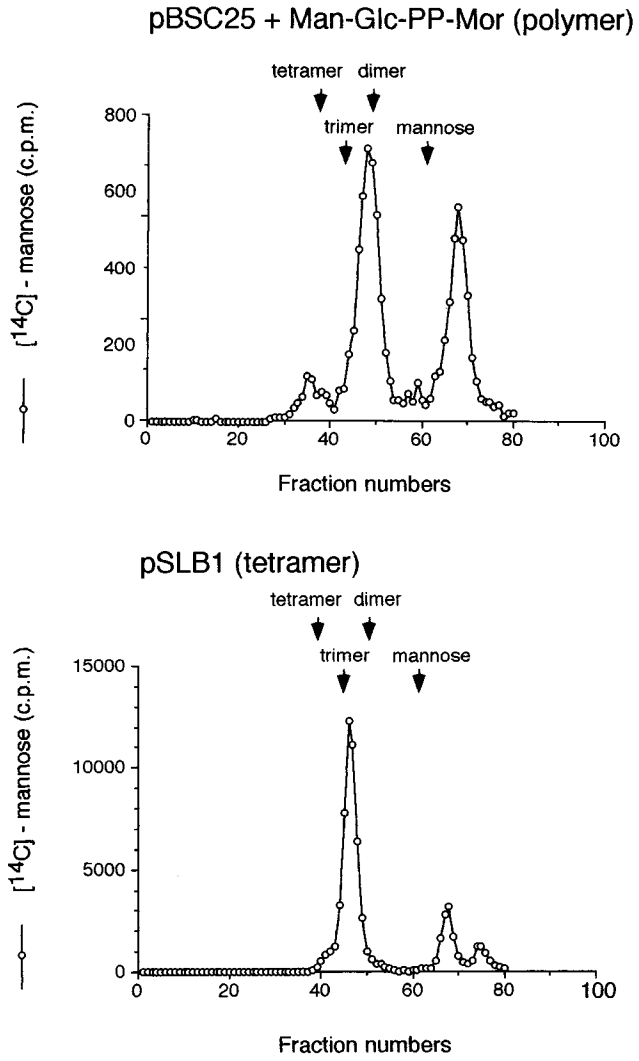

FIG. 6. Linkage sequence analysis of membrane products. Polysaccharide and tetrasaccharide fractions were subjected to the Smith degradation procedure. The resultant oligosaccharides were separated by TSK-gel chromatography. Fractions (1 ml) were counted in Beckman scintillation counter. Mor, moraprenol.

arated by gel permeation chromatography. The results are shown in Fig. 6. Smith degradation of the polymer, obtained by incubation of membranes from HU1190(pBSC25) with synthetic Man-Glc-pyrophosphorylmoraprenol and GDP-[^{14}C] mannose, resulted in two products, one about the size of a disaccharide and the other one smaller than a monosaccharide. They represent the compounds Man-(1→3)-Man-(1→2)-glycerol and glycerol, the glycerol residues arising from the oxidized (2-linked) mannose. This agrees with the reported structure of the O9 polysaccharide as [→2)-Man-(1→2)-Man-(1→2)-Man-(1→3)-Man-(1→3)-Man-1→]$_n$ (18). Smith degradation of the tetrasaccharide from membranes of *E. coli* HU1190(pSLB1) yielded a trisaccharide and glycerol. This result showed that the tetrasaccharide must have the structure Man-(1→3)-Man-(1→3)-Man-(1→3)-Glc.

Membrane reconstitutions. If the sequence of reactions of mannosyl transferases proposed above is correct, the product of MtfC must be a substrate for MtfB, and the product of MtfB must be a substrate for MtfA. We had found previously that

TABLE 3. Specificity analysis of mannosyl transferases

Membrane of HU1190 carrying:	Substrate[a]	Mannose incorporation (pmol/mg of protein)
pBSC25	Man-Glc-PP-Mor	93.3
pBSC25-St1	Endogenous[b]	0
	Man-Glc-PP-Mor	0.7
	Man-Man-Man-Glc-PP-Und[c]	39.1

[a] Mor, moraprenol; Und, undecaprenol.
[b] Butanol extract of HU1190(pSLB1) membrane.
[c] Butanol extract of HU1190(pSLB1) membrane incubated with 4 μM cold GDP-mannose.

Glc-pyrophosphorylundecaprenol is an endogenous mannose acceptor in *E. coli* O9 and that the first product of mannosyl transfer is Man-(1→3)-Glc-pyrophosphorylundecaprenol (17, 53). To verify this in the system presented here, we incubated membranes from recombinant strains with GDP-[^{14}C]mannose and appropriate lipid-linked oligosaccharides as acceptors. We used synthetic Man-Glc-pyrophosphorylmoraprenol and Man-Man-Man-Glc-pyrophosphorylundecaprenol as acceptors for mannosyl transferases. The latter was obtained by incubation of membranes from HU1190(pSLB-1, with *mtfB* and *mtfC*) with nonradioactive GDP-mannose and extraction of the incubated membranes with butanol. The results of the incubation experiments using these acceptors are shown in Table 3. Man-Glc-pyrophosphorylmoraprenol stimulated mannose incorporation in membranes from strain HU1190 (pBSC25) with MtfA and MtfB but not in membranes from HU1190(pBSC25-St1) with only MtfA. The latter were, however, stimulated by Man-Man-Man-Glc-pyrophosphorylundecaprenol. These results showed that only MtfA, -B, and -C are required for the synthesis of the O9 polysaccharide. They indicated that *mtfC* encoded the first, *mtfB* encoded the second, and *mtfA* encoded the third mannosyl transferase. Gel permeation and Smith degradation analyses of the reaction products of HU1190(pBSC25) membranes incubated with Man-Glc-pyrophosphorylmoraprenol showed them to be identical with the O9 polysaccharide (Fig. 6). The results showed that MtfA and MtfB suffice for chain elongation.

***rfe* dependence and acceptor requirement of the first mannosyl transferase (MtfC).** It has been demonstrated that synthesis of the *E. coli* O9 polysaccharide requires the *rfe* gene (19, 52). This observation was based mainly on the fact that an *rfe*-deficient *E. coli* O9 strain did not produce the O9 polysaccharide and that membranes from this strain were inactive but could be reconstituted with Glc-pyrophosphorylmoraprenol. Since this compound is the substrate for the first mannosyl transferase MtfC (see above), an *rfe* dependence of this enzyme should be expected. To demonstrate such an *rfe* dependence, we introduced plasmid pBBB6-H1 (carrying *mtfC*) into the *E. coli rfe*-negative strain 21548. Whereas membranes from the *rfe*-positive strain HU1190(pBBB6-H1) incorporated mannose from GDP-mannose, membranes from the *rfe*-negative strain 21548(pBBB6-H1) did not. A butanol extract from the *rfe*-positive host strain HU1190 restored mannose-incorporating activity in the membranes of 21548(pBBB6-H1) in a dose-dependent way (Table 4). To show that the *rfe* dependence of MtfC was critical for the O9 polysaccharide synthesis, the experiment was repeated with plasmid pNKB26, harboring all *E. coli* O9 *rfb* genes. Also in this case, membranes from *E. coli* 21548(pNKB26) did not incorporate mannose but did so after reconstitution with a butanol extract from *E. coli* HU1190 (Fig.

TABLE 4. *rfe* dependence of the mannosyl transferase activity in *rfe*-negative *E. coli* 21548(pBBB6-H1) membranes

BuOH extract from HU1190 (μl)	Mannose incorporation (pmol/mg of protein)
0	0
5	13.1
10	16.3
20	20.3
30	25.9

7). The product of this incubation was shown to be identical with the O9 polysaccharide.

To analyze the acceptor requirement for MtfC, a number of glycosyl derivatives of pyrophosphorylmoraprenol were used in incubation of membranes from the *E. coli rfe*-negative strain 21548 harboring plasmids pBBB6-H1 and pNKB26 separately. Table 5 shows that the Glc- and GlcNAc-pyrophosphorylmoraprenols are both mannose acceptors.

Mannosyl transferase genes. The *mtf* genes did not show significant homology with other mannosyl transferase genes over the whole length of DNA sequence. The amino acid sequences of MtfA, -B, and -C predicted from the DNA sequence were compared with sequences of the mannosyl transferases identified in the *rfb* clusters of *Salmonella enterica*

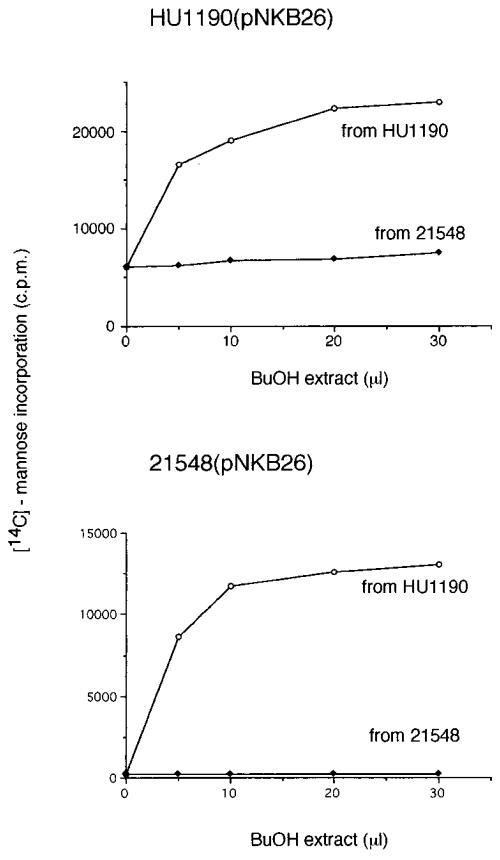

FIG. 7. Membrane reconstitution with butanol extracts from *rfe*-positive (HU1190) and -negative (21548) strains. Membranes from HU1190(pNKB26) and 21548(pNKB26) were incubated with GDP-[^{14}C]mannose in the presence of butanol extracts from HU1190 and 21548. Membranes were collected on a cellulose acetate filter after incubation and counted in Beckman scintillation counter.

TABLE 5. Analysis of substrate specificities for the mannosyl transferase, using the synthetic precursors

Membrane of *rfe*-negative *E. coli* 21548 carrying:	Acceptor	Mannose incorporation (pmol/mg of protein/ mmol of acceptor)
pBBB6-H1	P-Mor[a]	0
	Glc(α)-PP-Mor	136.4
	Glc(β)-PP-Mor	0.6
	GlcNAc-PP-Mor	225.8
	Gal-PP-Mor	0
	GlcUA-PP-Mor	0
pNKB26	P-Mor	0
	Glc(α)-PP-Mor	4,565.9
	Glc(β)-PP-Mor	388.3
	GlcNAc-PP-Mor	5,001.7
	Gal-PP-Mor	117.0
	GlcUA-PP-Mor	21.7

[a] Mor, moraprenol.

groups B, C2, and E1 (30) (Table 6). MtfB had significant similarity to RfbW, a mannosyl transferase of *S. enterica* group C2. The C-terminal sequences showed 53% identity over 85 amino acids (Fig. 8). MtfA is approximately twice as long as MtfB and RfbW. It has two segments with the same pattern as the homology plot between MtfB and RfbW which is not present in other mannosyl transferases. The conserved sequence S--EGFGLP--E was observed in these four segments of MtfA, MtfB, and RfbW. MtfA, MtfB, and RfbW are mannosyl transferases which are thought to transfer mannose to the 2 or 3 position of a mannose residue, whereas the other mannosyl transferases shown in Table 6 transfer mannose to GlcNAc, Gal, or Rha (30).

DISCUSSION

In continuation of previous studies on the biosynthesis of the *E. coli* O9 polysaccharide (11, 17, 19, 20, 23, 26, 53, 54), the *rfb* gene cluster was cloned into *E. coli* K-12. Sequence analysis revealed the presence of eight genes within the *rfb* region. The O9 *rfb* genes were found to be directly adjacent to the *his* operon, and their transcriptional direction was found to be opposite that of the *his* genes. This is in contrast to the gene organization *cps-galF-rfb-gnd-rol-his*, found in *E. coli* O7 (34) and in several *Salmonella* serovars (in which *rol* was termed *cld*) (41). Recently, it was reported that in *E. coli* O9 (strain E69), *gnd* is located upstream of the *rfb* gene (22). Although we have not determined the sequence upstream of *rfb* in the DNA from the O9 strain F719, we suggest that in *E. coli* O9, the *rfb* cluster was inserted between *gnd* and *his*. Therefore, the gene order in *E. coli* O9 probably is *cps-gnd-rfb-his*. The G+C content of the *rfb* region was lower than that from other regions of the chromosome. Such a fact had been described for the *rfb* genes of *Salmonella* strains, of *S. dysenteriae* 1, and of *E. coli* O7 (25, 34, 41, 48). This finding indicated that these *rfb* genes had arisen by horizontal gene transfer from bacteria with a lower G+C content. From the different G+C values of the *rfb* genes from *Salmonella* serovars, *S. dysenteriae*, *E. coli* O7, and *E. coli* O9, one can conclude that *E. coli* O9 *rfb* gene cluster must have arisen from a different source than the others.

Two of the *rfb* genes had been characterized previously as *rfbM* and *rfbK*, encoding the enzymes for mannose activation PMM and GMP (51). They are common to *E. coli* O8 and O9 and to *Klebsiella* O3 and O5 (51), all producing O-specific

TABLE 6. Comparison of amino acid identities of deduced sequences from mannosyl transferase genes in various *rfb* clusters[a]

Protein	% Identity						
	MtfA αM[b](1→2)M	MtfB αM(1→3)M	MtfC αM(1→3)GlcNAc	RfbO βM(1→4)Rha	RfbW αM(1→2)M	RfbZ αM(1→3)Gal	RfbU αM(1→4)Rha
MtfB	14.1		17.6	14.3	24.8	18.7	19.7
MtfC	11.8			16.0	17.8	19.0	17.9
RfbO	13.3				17.4	16.2	15.8
RfbW	13.1					20.0	19.3
RfbZ	14.6						16.9
RfbU	14.3						

[a] Sequences were compared by using a maximum matching program of GENETYX with a gap weight 3 and a length weight 1.
[b] M, mannose.

mannans (17, 55). All remaining genes of the *E. coli* O9 *rfb* cluster except ORF708 were characterized in this study.

The three genes at the 3′ end of the *rfb* cluster were found to direct mannosyl transferases, and they were termed, starting at 5′ of the *rfb* region, *mtfA*, *mtfB*, and *mtfC* (Fig. 1). We analyzed their functions in in vitro incubation studies using membranes of recombinant *E. coli* harboring plasmid-borne mannosyl transferase genes in various combinations and by chemical analysis of the products thus obtained. The results, in combination with previous findings (17, 20, 53), prompt us to suggest the pathway for the O9 mannan shown in Fig. 9. According to this scheme, MtfC as the first enzyme transfers Man-1-phosphate from GDP-Man to Glc (or GlcNAc)-pyrophosphoundecaprenol. This reaction furnishes the acceptor for subsequent chain elongation by the sequential activities of MtfB, transferring two mannosyl units into the 3 position, and of MtfA, transferring three mannosyl units into the 2 positions of the previous mannoses. The mechanism shown in Fig. 9 warrants some comments. (i) The *rfe* dependence of MtfC corroborated previous studies on the overall reactions (17, 20). However, the acceptor property of Glc-pyrophosphorylundecaprenol reported in these studies are in disagreement with a more recent report implying the participation of the GlcNAc- rather than the Glc-pyrophosphorylundecaprenol in the *E. coli* O8-specific polysaccharide synthesis in *E. coli* K-12 (44). Also, the *rfe* gene was found to direct the formation of GlcNAc-pyrophosphorylundecaprenol, a substrate for the synthesis of the enterobacterial common antigen ECA (2, 32, 35, 36). We now found that membranes from an *rfe*$^+$ strain can transfer Glc-1-phosphate or (even better) GlcNAc-1-phosphate from the UDP-activated forms to undecaprenol monophosphate, indicating that the *rfe* gene can direct both transfers. Our data show that MtfC seems to prefer the GlcNac derivative. However, it cannot be ruled out that the structure of the LPS core (terminal GlcNAc in *E. coli* K-12 and terminal Glc in *E. coli* O9 [16, 26, 47, 55]) to which the mannan will be translocated may influence which sugar-1-phosphate will be transferred. (ii) There are fewer transferase genes than sugars in the repeating unit. Since the DNA region containing *mtfC* is common among *rfb* gene clusters of *E. coli* O8 and O9 and *Klebsiella* O3 and O5 (51), the MtfC is probably not strictly O9 specific but also operative in the other strains. This is in accord with its function to mannosylate the endogenous acceptor. All other linkages of the O9 mannan are formed under the actions of the two enzymes MtfA and MtfB. The major acceptor requirement for both enzymes seems to be a terminal α-mannose, irrespective of its linkage. MtfB even transfers mannose to mannose which may be linked either to position 3 of glucose (first MtfB reaction) or to position 2 of mannose (all other transfer reactions). It would be interesting to know whether these enzymes have separate domains for the formation of the different linkages. (iii) At first glance, the postulated mechanism of chain growth is difficult to reconcile with the observed ladder-like pattern in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the O9 LPS. As an explanation, we propose that the growth of the chain must be discontinuous with respect to transfer kinetics. If one of the transfers is slower than the others, the probability for ligation to core-lipid A would be greater at that juncture. The banding pattern of SDS-PAGE indicates that such a probability is realized only above a finite length of the growing chain. It remains to be shown if MtfA and/or MtfB contain domains that are responsible for such a transfer regulation. The conserved sequence S--EGFGLP--E, the function of which is unclear, was found in MtfB and MtfA. It seems possible that this motif is engaged in determining the speed of mannosyl transfer.

Two genes (ORF261 and ORF431), just downstream of *rfbK*, had distinct sequence similarity to several known genes that had been implicated in the translocation of capsular polysaccharides in *H. influenzae*, *E. coli* K1 and K5, and *N. meningitidis* (10, 27, 39, 50) and of the O-specific polysaccharides from *Y. enterocolitica* O3 and *Klebsiella pneumoniae* O1 (5, 56) across the cytoplasmic membrane. We could not find similar genes in the *rfb* clusters of *Salmonella* strains and *S. dysenteriae*. Like the above-mentioned genes, ORF431 and ORF261 seemed to constitute the ATP-binding (energizing) and the transmembrane component of an ABC transporter (9, 15, 42), respectively. The ATP-binding component of the *E. coli* O9 *rfb* had about double the size of the corresponding proteins of the other systems, a fact that we cannot yet interpret.

A comparison of the polymerization mechanisms for the O polysaccharides of *Salmonella* strains and *E. coli* O9 with the respective translocation mechanisms is interesting. It appears that in *Salmonella* strains, the polysaccharide is assembled from ACL-linked oligosaccharide intermediates at the peri-

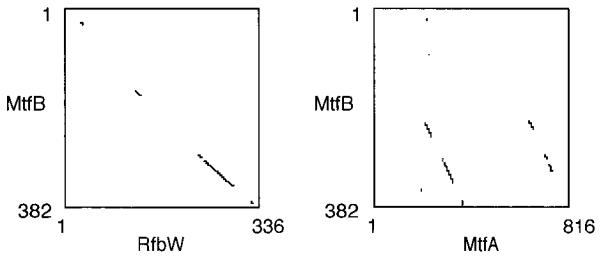

FIG. 8. Homology between MtfB and two mannosyl transferases, RfbW and MtfA. Dot matrix homology alignments of MtfB versus RfbW and MtfA are shown. The point which has more than seven identical amino acids over 15 residues was plotted.

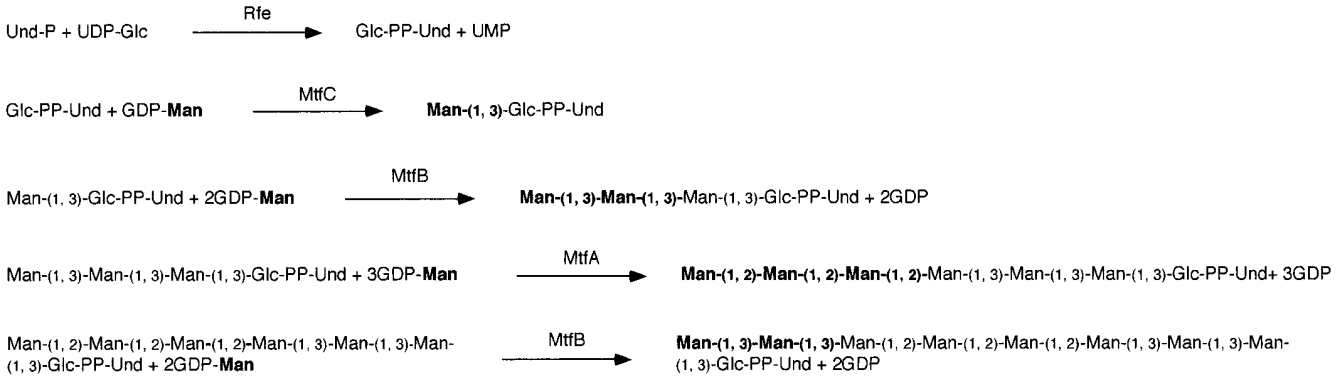

FIG. 9. Proposed sequential actions of mannosyl transferases in the biosynthesis of the O9 polysaccharide of *E. coli*. Und, undecaprenol (moraprenol in the in vitro experiments); Glc, glucose; Man, mannose: MtfABC, mannosyl transferases, signifying the respective mannosyl transferases encoded in the *E. coli* O9 *rfb* gene cluster. The scheme is based on the information given in this report and in references 17, 20, and 53.

plasmic side of the inner membrane, after their translocation across the membrane (38, 43), whereas in *E. coli* O9, the polysaccharide is completely assembled at the cytoplasmic face of the inner membrane (17, 18, 20) and then translocated in toto across the membrane with an ABC transport system. Thus, polymerization and translocation mechanisms seem to be intimately coupled.

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