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Role of the *rfe* Gene in the Biosynthesis of the *Escherichia coli* O7-Specific Lipopolysaccharide and Other O-Specific Polysaccharides Containing *N*-Acetylglucosamine

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We report that *rfe* mutants of wild-type strains of *Escherichia coli* O7, O18, O75, and O111 did not express O-specific polysaccharide unless the *rfe* mutation was complemented by a cloned *rfe* gene supplied in a plasmid. The O polysaccharides in these strains are known to have *N*-acetylglucosamine (GlcNAc) in their O repeats. In addition, in vitro transferase assays with bacterial membranes from either the O7 wild-type strain or its isogenic *rfe* mutant showed that GlcNAc is the first carbohydrate added onto the lipid acceptor in the assembly of the O7 repeat and that this function is inhibited by tunicamycin. Our results indicate that the *rfe* gene product is a general requirement for the synthesis of O polysaccharides containing GlcNAc.

Lipopolysaccharide (LPS) is a surface glycolipid molecule found on the outer leaflet of the outer membrane in gram-negative bacteria (7, 33). It consists of lipid A, a core oligosaccharide, and the O-specific side-chain or O antigen made of repeating oligosaccharide units (see reference 33 for a review). The character of a specific O antigen is determined by the composition and mode of linkage of the monosaccharides in the O repeat (7, 9, 33). Our laboratory is using the O7-specific antigen of *Escherichia coli* as a model system to study the molecular details of the biosynthesis of O antigens (18, 19, 30). The structure of the O7 repeating unit has been elucidated (15) and found to consist of galactose (Gal), mannose, rhamnose, 4-acetamido-4,6-dideoxyglucose, and *N*-acetylglucosamine (GlcNAc) (Fig. 1).

Early studies by several investigators (reviewed in reference 7) have shown that the biosynthesis of the O repeat is carried out by the sequential addition of each of its sugar components, delivered as nucleotide sugar precursors, onto the undecaprenol lipid carrier. Most of the metabolic enzymes for the formation of nucleotide sugar precursors as well as specific glycosyltransferases necessary for the biosynthesis of the O-specific LPS are encoded by the *rfb* cluster located at 44 min of the *E. coli* chromosomal map between the histidine operon and the genes for the formation of colanic acid (8, 27). Studies in *Salmonella enterica* have shown that the biosynthesis of the O repeat begins with the addition of Gal onto the undecaprenol phosphate lipid carrier (24). This function is mediated by *rfbP*, a gene encoding a polypeptide with features of an integral membrane protein (8). The other specific glycosyltransferases for the addition of the remaining sugar components of the O repeat do not appear to be integral membrane proteins; possibly they are loosely associated with the membrane by ionic interactions (13). Despite the fact that Gal is a component of the *E. coli* O7 repeat (Fig. 1), the *E. coli* O7 *rfb* (rfb_{EcO7}) gene cluster does not have any gene or gene product similar to RfbP and contains gene candidates for only four of the five glycosyltransferases needed for the assembly of the O7 repeat (17).

The *rfe* gene is involved in the biosynthesis of certain O polysaccharides and in the synthesis of a glycolipid shared by all members of the family *Enterobacteriaceae* which is known as enterobacterial common antigen (11, 12). Recent work by other investigators (20) suggests that *rfe* is the structural gene of the UDP-GlcNAc:undecaprenyl phosphate GlcNAc-1-phosphate transferase which catalyzes the transfer of GlcNAc onto undecaprenol phosphate, the first step in enterobacterial common antigen biosynthesis (21, 25). The DNA sequence of the *rfe* gene has been determined (20, 22), and the analysis of its protein product predicts multiple membrane-spanning domains, suggesting a protein structure similar to that of RfbP in spite of the lack of significant amino acid sequence identity (10).

Since the O7 repeat also contains GlcNAc, we investigated whether *rfe* plays a role in its biosynthesis. In this study, we demonstrate that *rfe* is essential for the first step in the biosynthesis of the O7 repeat by mediating the incorporation of GlcNAc onto undecaprenol phosphate. Also, we show that *rfe* is essential for the formation of other O types with GlcNAc in the O repeat, such as O18, O75, and O111, suggesting a general role for this gene in the biosynthesis of O-specific polysaccharides.

Role of *rfe* in the expression of O7 polysaccharide. To investigate the possible participation of *rfe* in the biosynthesis of the O7 repeat, the *E. coli* K-12 strains 21548(*rfe*::Tn*10*) and the isogenic rfe^+ parent strain AB1133 (Table 1) were transformed with pJHCV32::Tn*3*HoHo1-151, a plasmid containing the cloned rfb_{EcO7} region (19). pJHCV32::Tn*3*HoHo1-151 also has the transposon insertion 151, which maps outside the rfb_{EcO7} region, providing an antibiotic resistance marker for the selection of transformants (19). *E. coli* 21548(pJHCV32::Tn*3*HoHo1-151) transformants were not agglutinated with the O7-specific polyclonal antiserum, whereas AB1133(pJHCV32::Tn*3*HoHo1-151) control cells were slide agglutination positive (Table 2). To confirm that pJHCV32::Tn*3*HoHo1-151 was functionally intact in *E. coli* 21548, the isolated plasmid from this strain was transformed back into AB1133; the resulting transformants displayed a positive slide agglutination with the O7 antiserum (Table 2).

E. coli 21548(pJHCV32::Tn*3*HoHo1-151) was transformed with pMAV11 (Table 1) carrying the intact *rfe* gene. Transformants exhibited an O7-positive slide agglutination, suggesting

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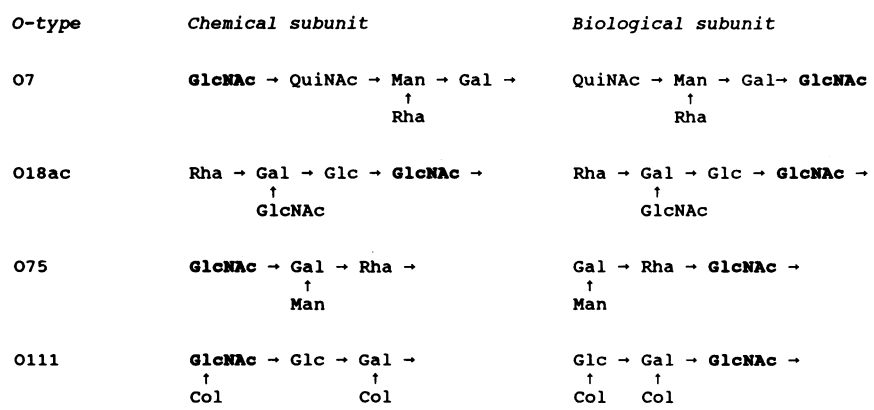

FIG. 1. Structure of the O repeating units of O7, O18, O75, and O111 polysaccharides. Under the heading chemical subunits are listed the structures of the O repeats drawn as elucidated by chemical methods (5, 6, 9, 15). Under the heading biological subunits are listed the biological orders of the O repeats, with GlcNAc the first sugar added onto the lipid carrier according to data presented in this study. Abbreviations: QuiNAc, 4-acetamido-4,6-dideoxyglucose; Man, mannose; Rha, rhamnose; Glc, glucose; Col, colitose (3,6-dideoxy-L-galactose).

that the defect in the expression of O7 LPS by this strain is associated solely with the mutation in the *rfe* gene. Since *rfe* is the first gene of the *rfe-rff* gene cluster (20, 22), this also shows that the lack of O7 LPS expression in *E. coli* 21548 is not caused by a polar effect of Tn*10* on genes located downstream from the insertion site on *rfe*. Purified LPS from *E. coli* 21548(pJHCV32::Tn*3*HoHo1-151) did not have O7-specific bands, as determined by Western blot (immunoblot) analysis, whereas LPS from the strains AB1133(pJHCV32::Tn*3*HoHo1-151) and 21548(pJHCV32::Tn*3*HoHo1-151, pMAV11) expressed O7-specific polysaccharides (Table 2).

To determine whether the results obtained with the *rfe* mutant in *E. coli* K-12 are applicable to the wild-type *E. coli* O7, we constructed an *rfe* mutant of this strain using P1 transduction; Tet^r transductants were screened by slide agglutination and by sensitivity to rough-specific bacteriophages BR2 and FfM (30). P1 transductions were carried out according to standard protocols (4), except that prior to transduction, VW187 cells were grown in the presence of 50 μg of diphenylamine per ml. This treatment decreases considerably the formation of O polysaccharide (28) and facilitates the adsorption of the P1 bacteriophage onto wild-type *E. coli* cells (1). In contrast to the wild-type strain, no Tet^r transductants examined agglutinated with O7 antiserum and were lysed by the phages BR2 and FfM. One of these isolates, designated MV501, was utilized for further studies. LPS from MV501 and VW187 was prepared and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. Figure 2a, lane B, shows that MV501 does not display O polysaccharides, suggesting a defect in the biosynthesis of O7 LPS. This defect was associated with the *rfe* mutation, since the formation of the O7 LPS was restored upon transformation of pMAV11 into the strain MV501 (Fig. 2a, lane C). The lipid A core band of MV501 showed an electrophoretic profile similar to that of the core in the wild-type VW187 strain (Fig. 2b, lanes A and B), suggesting that *rfe* is involved in the formation of O7 polysaccharide but has no apparent effect on the biosynthesis of the lipid A core in this strain.

In vitro incorporation of radiolabelled UDP-Gal onto decaprenol phosphate depends on the presence of UDP-GlcNAc. To investigate whether GlcNAc is the first sugar attaching to the bactoprenol lipid carrier in the biosynthesis of the O7 repeat, membranes were prepared from strains VW187 and MV501 and

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

Strain or plasmid	Relevant properties	Source or reference
Strains		
AB1133	K-12; *thr-1 leuB6*(Δ*gpt-proA*)*66 hisG4 argE3 thi-1 rfbD1 lacY ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 SupE44*	B. Bachmann
21548	AB1133; *rfe*::Tn*10* Tc^r	P. Rick 25
VW187	O7:K1; clinical isolate	29
MV501	21548 × VW187; like VW187 but *rfe*::Tn*10* Tc^r	This study
RS172	O18ac:K1; clinical isolate	32
MV502	RS172; *rfe*::pINT*rfe* Km^r Ap^r	This study
RS501	O75:K1; clinical isolate	R. Silver
MV504	RS501; *rfe*::pINT*rfe* Km^r Ap^r	This study
EC88-624	O111; clinical isolate	H. Lior
MV503	EC88-624; *rfe*::pINT*rfe* Km^r Ap^r	This study
Plasmids		
pJHCV32::Tn*3*HoHo1-151	pJHCV32 carrying Tn*3*HoHo1 insertion 151, O7$^+$ Tc^r Ap^r	19
pACYC184	Cloning vector, P15 replicon, Tc^r Cm^r	2
pRL100	1.46-kb *Cla*I-*Xma*III fragment containing *rfe* cloned in pBR322, Ap^r	20
pMAV11	1.4-kb *Cla*I-*Ava*I fragment from pRL100 cloned in pACYC184, *rfe*$^+$ Cm^r	This study
pINT*rfe*	Integration plasmid; Ori$_{pSC101}^{ts}$, 0.34-kb *Hin*dIII-*Bam*HI fragment of *rfe*, Km^r	22

TABLE 2. Expression of O7 polysaccharide in *rfe* and *rfe*$^+$ *E. coli* K-12 strains

Strain	Plasmid	O7 agglutination[a]	Western blot[b]
AB1133	None	−	−
21548	None	−	−
AB1133	pJHCV32::Tn*3*HoHo1-151	+	+
21548	pJHCV32::Tn*3*HoHo1-151	−	−
AB1133	pJHCV32::Tn*3*HoHo1-151[c]	+	+
21548	pJHCV32::Tn*3*HoHo1-151, pMAV11	+	+

[a] Assayed by slide agglutination with an O7-specific rabbit polyclonal antiserum prepared as described in reference 31.

[b] LPS was extracted, separated in SDS-polyacrylamide gels, and electrotransferred to nitrocellulose membranes according to standard procedures (19). Membranes were reacted with O7-specific antiserum as previously described (19, 31). −, lack of O7-reactive polysaccharide bands; +, presence of O7-reactive O polysaccharides.

[c] This plasmid was isolated from *E. coli* 21548 and transformed back into strain AB1133.

used in an in vitro transferase assay in which [^{14}C]UDP-Gal and cold UDP-GlcNAc were added to the mixture. If GlcNAc is the first sugar attached to the bactoprenol lipid carrier, the incorporation of labelled galactose into a lipid-associated form would require the formation of a GlcNAc-bactoprenyl intermediate that would serve as an acceptor for galactose (Fig. 1).

Membranes were isolated as described by Osborn et al. (23), and transferase assays were conducted by previously described methods (13, 23) with some modifications. The reaction mixture contained the membrane fraction (200 μg of protein [14]), 15 μg of exogenous decaprenol phosphate carrier lipid, 7 μg of UDP-GlcNAc (98% purity; Sigma Chemical Co., St. Louis, Mo.), 0.05 μCi of [^{14}C]UDP-Gal (Amersham Canada, Oakville, Ontario, Canada), 50 mM Tris-acetate (pH 8.5), 1 mM EDTA, and 10 mM $MgCl_2$ in a total volume of 250 μl. The exogenous decaprenol phosphate was omitted in some experiments, since it did not affect the results (see below). After 30 min of incubation at 37°C, the lipid-associated material was extracted twice with 250 μl of 1-butanol. The combined 1-butanol extracts were washed once with 500 μl of distilled water. The radioactive counts of the original aqueous fraction, which contains the excess of sugar nucleotides, membranes, and water soluble products, and the counts of the 1-butanol fraction and aqueous butanol wash were determined with a Beckman liquid scintillation counter.

In the presence of the VW187 membrane fraction and UDP-GlcNAc, 3.5% of total radioactive counts (corresponding to 29 pmol of Gal/mg of protein) were detected in the butanol extract, indicating that it was lipid associated, presumably bactoprenyl-GlcNAc-Gal intermediates (Table 3). In contrast, over 150-fold less incorporation of radioactive Gal was detected in the butanol extracts of assays with VW187 membranes in the absence of UDP-GlcNAc (Table 3). The MV501 membrane preparation did not support the incorporation of significant amounts of labelled Gal irrespective of the presence of UDP-GlcNAc (Table 3). However, an experiment using a mixture of membranes from MV501 and from *E. coli* K-12 W1485 (*rfe*$^+$) showed an increased incorporation of radiolabelled Gal (corresponding to 7.2 pmol of Gal/mg of protein) in the butanol extract in the presence of UDP-GlcNAc (Table 3), whereas W1485 membranes alone incorporated much smaller amounts of label in the butanol phase. Therefore, we conclude that MV501 membranes were functional and could support the continuation of the biosynthesis of the O7 repeat if the *rfe*-mediated function was supplied by membranes of an *rfe*$^+$ strain, indicating a biochemical complementation of the *rfe* defect. W1485 membranes showed a higher background of incorporation of Gal than membranes prepared from strains VW187 and MV501. However, this higher incorporation of Gal was not associated with the presence of GlcNAc (Table 3 and data not shown) and was not inhibited by tunicamycin (see below). Since this strain cannot form O-specific LPS (26, 34), the increased incorporation of Gal reflects the formation of bactoprenyl-sugar intermediates of a distinct surface polymer.

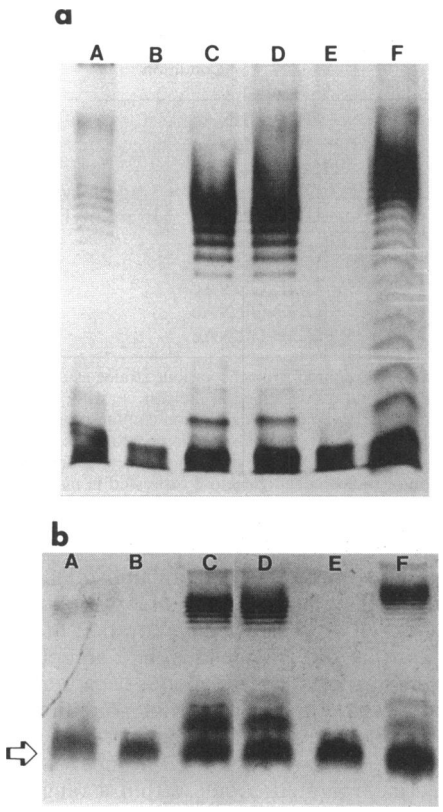

FIG. 2. (a) Silver-stained 14% polyacrylamide gel showing the LPS banding pattern of the various wild-type and *rfe* mutant strains. (b) Silver-stained 20% polyacrylamide gel to visualize core-lipid A bands (indicated by the arrow). Lanes: A, VW187 (O7:K1); B, MV501 (O7:K1 *rfe*::Tn*10*); C, MV501(pMAV11); D, RS172 (O18ac:K1); E, MV502 (O18ac:K1 *rfe*::pINT*rfe*); F, MV502(pMAV11).

Tunicamycin is an antibiotic that specifically prevents the transfer of GlcNAc-1-phosphate from UDP-GlcNac to polyprenyl monophosphate acceptors (16), and it has also been found to inhibit the function of Rfe (20, 25). Consequently, the effect of tunicamycin on O7 LPS synthesis was investigated to gain further insight into the role of *rfe* in the formation of O7. No incorporation of radioactive Gal was detected in the butanol extracts when the transferase assays were carried out with VW187 membranes in the presence of 3 μg and 10 μg of tunicamycin per ml (Table 3). Since tunicamycin does not inhibit incorporation of Gal into polyprenyl lipid acceptors (16), the lack of radioactivity in the butanol extracts has to be the result of an inhibition in the transfer of GlcNAc. We then conclude that UDP-GlcNAc must be the first sugar attached to the lipid acceptor in our experimental system.

To characterize further the in vitro assay system, we carried out an experiment adding increasing amounts of decaprenol phosphate (Fig. 3a). Surprisingly, the percent incorporation of

TABLE 3. Incorporation of [^{14}C]UDP-Gal

Membrane fraction[a]	Condition[b]	Aqueous fraction (cpm)[c]	1-Butanol fraction (cpm)[d]	Incorporated counts (%)[e]	Incorporated galactose (pmol/mg)[f]
VW187	+UDP-GlcNAc	49,471 ± 6	1,741 ± 2	3.5	29
VW187	−UDP-GlcNAc	52,300 ± 8	10 ± 17	0.02	0.16
VW187	Heat treated	20,505 ± 3.5	9 ± 21	0.04	0.38
VW187	+UDP-GlcNAc, +3 μg of Ty/ml	20,393 ± 1	19 ± 14	0.09	0.8
VW187	+UDP-GlcNAc, +10 μg of Ty/ml	82,393 ± 1	15 ± 15	0.01	0.15
MV501	+UDP-GlcNAc	40,668 ± 2.5	38 ± 10	0.09	0.8
MV501	−UDP-GlcNAc	28,422 ± 5	11 ± 19	0.04	0.33
MV501 + W1485	+UDP-GlcNAc	53,028 ± 1	441 ± 3	0.83	7.2
MV501 + W1485	−UDP-GlcNAc	56,538 ± 1	108 ± 6	0.19	1.6
W1485	+UDP-GlcNAc	56,905 ± 1	176 ± 4	0.31	2.6

[a] Membranes were prepared from the various strains as described in the text. See Table 1 for strain descriptions.
[b] Assay was carried out as described in the text. +, with; −, without; Ty, tunicamycin. Membranes were heat inactivated by boiling for 5 min.
[c] Means ± standard deviations. This fraction contains unincorporated [^{14}C]UDP-Gal.
[d] Means ± standard deviations. This fraction contains lipid-associated [^{14}C]Gal.
[e] Percent counts in the 1-butanol extract of the total counts used in the experiment.
[f] Incorporation of radiolabelled galactose expressed in picomoles of galactose per mg of membrane proteins.

radioactive counts in the butanol fraction remained more or less constant even in the absence of exogenous decaprenol phosphate, suggesting that the incorporation of GlcNAc and Gal takes place on the endogenous lipid acceptor present in the membrane fractions, and exogenous decaprenol phosphate is not used under our experimental conditions. This was confirmed by an experiment adding increasing amounts of membranes (Fig. 3b), which showed a linear increase in the percent lipid-incorporated counts within a range from 50 to 600 μg of membrane proteins. The reduction in incorporation with increasing membrane concentrations above 700 μg of protein (Fig. 3b) may be due to substrate saturation or to the presence of an inhibitor interfering with the reaction. Other investigators have recently reported that the GlcNAc phosphate transferase cannot utilize decaprenol phosphate as an acceptor (26). Thus, we conclude from these experiments that the endogenous acceptor, probably undecaprenol phosphate, present in the membrane preparation is the substrate for the *rfe*-mediated transfer of GlcNAc.

Role of *rfe* in the formation of O polysaccharides in other enteric bacteria. Many O-specific polysaccharides in *E. coli* possess GlcNAc as a component of the O repeat (7, 9). Therefore, we investigated whether *rfe* is also necessary for the synthesis of other O-specific polysaccharides using O18, O75, and O111 as examples (Fig. 1), and for this purpose, we sought to construct *rfe* mutants in *E. coli* serotypes O18, O75, and O111. Several attempts to obtain *rfe*::Tn*10* mutants in these strains using P1 transduction failed. A Southern blot hybridization experiment using chromosomal DNA from these strains digested with various restriction endonucleases and a 0.34-kb *Hin*dIII-*Bam*HI *rfe*-specific probe fragment isolated from pINT*rfe* (Table 1) confirmed that the *rfe* gene is present in these strains (1). Thus, the failure to obtain *rfe* mutants by P1 transduction was probably due to the inability of this phage to infect the *E. coli* O18, O75, and O111 strains. A different mutagenesis approach was followed as described by Ohta et al. with the plasmid pINT*rfe* (22). At 42°C, pINT*rfe* is unable to replicate, facilitating homologous recombination and the subsequent integration of the plasmid in the *E. coli* chromosome and effectively disrupting the *rfe* gene (22). *rfe* mutants of the *E. coli* O18 strain RS172, the *E. coli* O75 strain RS501, and the *E. coli* O111 strain EC88-624, designated MV502, MV504, and MV503, respectively, were obtained by this procedure, and in contrast to the wild-type strains they were susceptible to lysis by bacteriophages BR2 and FfM and also failed to display

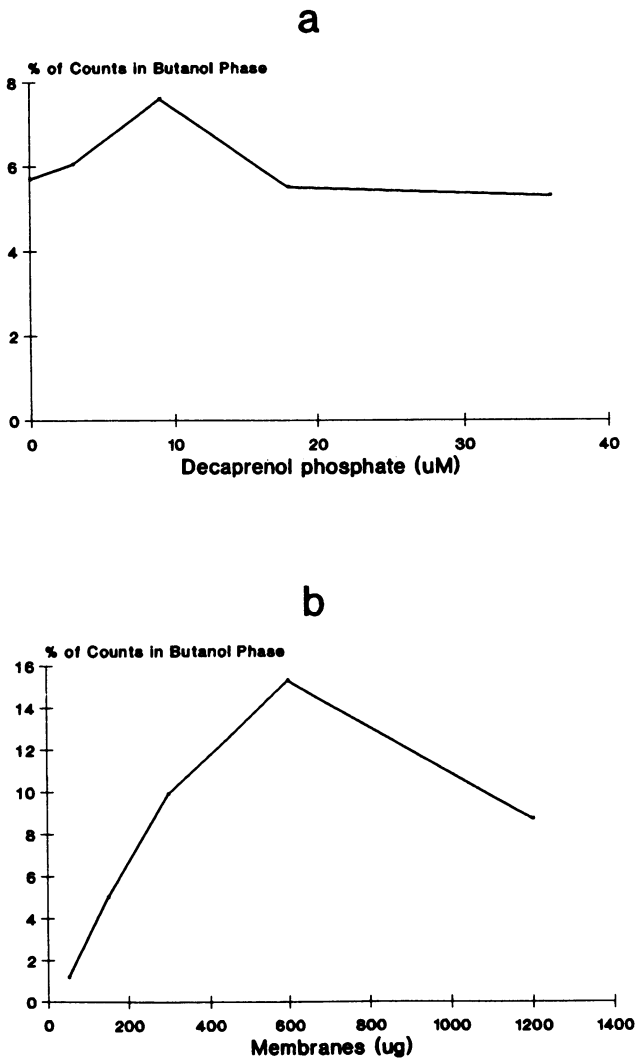

FIG. 3. Analysis of the incorporation of radioactive Gal in the butanol-extractable fraction as a function of the concentration of exogenous decaprenol phosphate (a) and the amount of membranes in the assay (b). In vitro transferase assays were carried out as described in the text.

O-specific polysaccharide as determined by SDS-PAGE and silver staining (Fig. 2a, lane E, and data not shown). Transformation of MV502, MV503, and MV504 with pMAV11 restored their wild-type phenotypes (Fig. 2a, lane F, and data not shown). These experiments demonstrate that the *rfe* gene is also required for biosynthesis of the O-specific polysaccharide in *E. coli* O18, O75, and O111.

Concluding remarks. In the present study, we demonstrate that *rfe* is involved in the first step of the biosynthesis of the O7 repeat, mediating the addition of GlcNAc onto a lipid acceptor. This is supported by three lines of evidence: (i) *rfe* mutants abolish the expression of O7-specific polysaccharide but do not appear to affect the synthesis of the core lipid A, suggesting a specific defect in the formation of the O7 repeat; (ii) membranes of the wild-type strain VW187 mediate the in vitro incorporation of radioactive Gal into a butanol-extractable fraction only in the presence of UDP-GlcNAc, suggesting the formation of a Gal-GlcNAc-pyrophosphoryl-undecaprenol intermediate involved in the synthesis of the O7 repeat; (iii) in vitro incorporation of radiolabelled Gal is inhibited by tunicamycin, a drug known to inhibit the formation of GlcNAc-pyrophosphorylundecaprenol (16).

The chemical structures of the O repeating units are obtained from the analysis of partial hydrolysis products of the O polysaccharide chain. Since most of the chemical methods employed for structural elucidation cleave the polysaccharide chain at acid-labile linkages, the resulting oligosaccharide units may or may not represent the biological order of synthesis. In view of the biochemical information provided in this study, we propose that the structure of the O7 repeat be redrawn as shown in Fig. 1 to reflect the biological order in the sequential addition of precursors, beginning with GlcNAc at the nonreducing end of the oligosaccharide.

We provide additional evidence that *rfe* is involved in the formation of three other O polysaccharides: O18, O75, and O111. The chemical structures of the O18, O75, and O111 repeats have also been established (5, 6, 9), and both have in common the presence of GlcNAc (Fig. 1). We predict that *rfe* also mediates the incorporation of GlcNAc as the first sugar attached to the bactoprenol lipid carrier in these cases, and accordingly, the proposed biological structures of the O18, O75, and O111 repeats are also shown in Fig. 1.

In addition of its role in the synthesis of enterobacterial common antigen, *rfe* has been shown to be involved in the biosynthesis of O8 and O9 in *E. coli* (7) and O1 in *Klebsiella pneumoniae* (3). In these cases, GlcNAc is not found as a structural component of the O repeating units, which contain mannose in the case of O8 and O9 (7) and Gal in the case of *K. pneumoniae* O1 (3). Therefore, the role of *rfe* in these cases is not clearly understood. Studies on the biosynthesis of O8 and O9 have identified a glucosyl-lipid acceptor (7), and a regulatory function for *rfe* has been proposed (7, 33).

On the basis of genetic complementation studies, it has recently been suggested that in *Shigella dysenteriae* type 1 (9) and in *Shigella flexneri* (34) GlcNAc is the first sugar in the biological O repeating unit. Also, Stevenson et al. have shown by an in vitro transferase assay that GlcNAc is the first sugar added in the biosynthesis of the K-12/O16 repeat in an *rfe*-dependent manner (26). This work confirms and extends these observations by adding O7, O18, O75, and O111 to the list of O polysaccharides requiring *rfe* for the biosynthesis of O repeats. On the basis of all these observations, we propose that *rfe* plays a general role in the biosynthesis of O polysaccharides containing GlcNAc.

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