 Biosynthesis of Enterobacterial Common Antigen in Escherichia coli

BIOCHEMICAL CHARACTERIZATION OF Tn10 INSERTION MUTANTS DEFECTIVE IN ENTEROBACTERIAL COMMON ANTIGEN SYNTHESIS*

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Twelve independent Tn10 insertion mutants of Escherichia coli K12 were isolated that were defective in the synthesis of enterobacterial common antigen (ECA). The mutants were identified by screening a random pool of Tn10 insertion mutants for their ECA phenotype using a colony-immunoblot assay. All 12 of the Tn10 insertion mutants were found to be located in the chromosomal region of the rff-rfe genes. Four of the Tn10 insertions were in rff genes while the remaining eight Tn10 insertions were in rfe genes. All of the rff::Tn10 insertion mutants were defective in the synthesis of GlcNAc-pyrophosphorylunodecaprenol (C20-PP-GlcNAc, lipid I), the first lipid-linked intermediate involved in ECA synthesis. Biochemical characterization of the rff::Tn10 insertion mutants revealed that they were defective in various steps of ECA synthesis subsequent to the synthesis of lipid I. These defects included: (i) the inability to synthesize UDP-ManNAcA due to Tn10 insertions in the structural genes for UDP-GlcNAc-2-epimerase (rffE) and UDP-ManNAcA (N-acetyl-D-mannosaminuronic acid) dehydrogenase (rffD), (ii) defects in the synthesis of Cas-GlcNAc-ManNAcA (lipid II) due to insertion of transposon Tn10 in the structural gene for the UDP-ManNAcA transferase (rffM), (iii) the inability to synthesize TDP-Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose) due to Tn10 insertions in the structural gene for the transaminase that catalyzes the conversion of TDP-4-keto-6-deoxy-D-glucose to TDP-4-amino-4,6-dideoxy-D-galactose (rffA), and (iv) defects in steps subsequent to the synthesis of Cas-GlcNAc-ManNAcA-Fuc4NAc (lipid III). In addition, a re-examination of a mutant possessing the rff-726 lesion revealed that it was defective in the synthesis of lipid III due to a defect in the structural gene for the Fuc4NAc transferase (rffT).

Enterobacterial common antigen (ECA) is an outer membrane glycolipid produced by all members of the family Enterobacteriaceae (7, 14, 20). The carbohydrate portion consists of N-acetyl-d-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic acid (ManNAc), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (12, 17). These amino sugars form linear heteropolysaccharide chains that are comprised of trisaccharide repeat units having the structure: → 3)-α-D-Fuc4NAc(1→ 4)-β-D-ManNAcA(1→ 4)-α-D-GlcNAc(1→ 12). Individual heteropolysaccharide chains are linked to phosphatidic acid molecules through phosphodiester linkage in all ECA-containing strains (8). In addition, heteropolysaccharide chains can also be linked to the core region of lipopolysaccharide (LPS) in certain rough strains (5).

The initial steps involved in ECA biosynthesis are now reasonably well understood (Fig. 1). The first intermediate in ECA biosynthesis, GlcNAc-pyrophosphorylunodecaprenol (lipid I), results from the enzymatically catalyzed transfer of GlcNAc-1-phosphate from the donor, UDP-GlcNAc, to the acceptor, undecaprenyl monophosphate (2, 27). Additional steps involve the transfer of ManNAcA from UDP-ManNAcA to lipid I to yield ManNAcA-GlcNAc-pyrophosphorylunodecaprenol (lipid II) and the subsequent transfer of Fuc4NAc from TDP-Fuc4NAc to lipid II to yield Fuc4NAc-ManNAcA-GlcNAc-pyrophosphorylunodecaprenol (lipid III) (1). Lipid III is utilized for the synthesis of ECA heteropolysaccharide chains (1); however, the mechanism of chain elongation remains to be established.

The genetic determinants of ECA synthesis include the rff and rfe gene clusters located at min 85 of the Escherichia coli chromosome (10, 22). The rffE and rffD genes are required for the synthesis of UDP-ManNAcA, and they code for the enzymes UDP-GlcNAc-2-epimerase and UDP-ManNAcA dehydrogenase, respectively (10). It was initially believed that the rffT gene was the structural gene for the transerase that catalyzes the transfer of ManNAcA from UDP-ManNAcA to lipid I (3). However, data obtained during the present study indicate that the rffT gene codes for the transerase that catalyzes the transfer of ManNAcA from UDP-ManNAcA to lipid I (3).

The role of the rfe locus in ECA and O-side chain synthesis has not yet been determined. In Salmonella typhimurium (group B), genes in the his-linked rfb region are also required for ECA synthesis (9). The rfbA and rfbB genes of S. typhimurium code for TDP-glucose pyrophosphorylase and TDP-glucose oxidoreductase, respectively. These enzymes are necessary for the synthesis of TDP-4-keto-6-deoxy-D-glucose which is a precursor of TDP-Fuc4NAc as well as for TDP-rhamnose, the donor of rhamnose.
ECA-negative Tn10 Insertion Mutants


UDP-GlcNAc

UDP-ManNAcA

TDP-Fuc4NAc

UDP

C_{55}-P

UMP

PP-GlcNAc (Lipid I)

PP-GlcNAc-ManNAcA (Lipid II)

PP-GlcNAc-ManNAcA-Fuc4NAc (Lipid III)

ECA

FIG. 2. Colony-immunoblot detection of ECA-negative Tn10 insertion mutants. The arrow indicates the colony location of an ECA-negative mutant. The + and - symbols indicate the colony locations of ECA-positive and ECA-negative control strains, respectively.

EXPERIMENTAL PROCEDURES

RESULTS

Isolation of ECA-negative Tn10 Insertion Mutants—A pool of 7.3 \times 10^{4} Tn10 insertion mutants was prepared by incubating E. coli K12 strain AB1133 with phage \lambda NK 370 at a multiplicity of infection of 0.36 followed by selection for tetracycline resistance. Tetracycline-resistant mutants were subsequently patched to agar plates (about 200 mutants/plate) and screened for their ability to synthesize ECA by colony-immunoblot assay using mouse anti-ECA monoclonal antibody mAb898 (21, 26) (Fig. 2). ECA-negative Tn10 insertion mutants were observed at a frequency of 1 in 1237 random insertions, and a total of 12 mutants were isolated by the colony-immunoblot assay. The 12 insertion mutants were further screened in a passive hemagglutination assay using mouse anti-ECA polyclonal antisera (22) and all were found to be ECA-negative.

Mapping of ECA-negative Tn10 Insertion Mutants—All of the genes known to be involved in ECA synthesis in E. coli K12 are located between ilv and uuFD on the chromosome (7, 22). Therefore, the P1 co-transduction frequencies of the ECA-negative Tn10 insertions with ilv and uuFD were determined. Phage P1 was grown on each of the 12 ECA-negative insertion mutants, and transductions were carried out using strains AB3605 (ilv) and GW3703 (uurD::Tn6) as recipients. All 12 of the ECA-negative Tn10 insertions were co-transducible with ilv and uuFD (Table II). The P1 co-transduction frequencies of the Tn10 insertions with ilv were between 0.94 and 1.0, and the co-transduction frequencies with uuFD were between 0.1 and 0.4.

Additional transductions were carried out using E. coli 2443 (rfb_{0}) as the recipient. Strain 2443 is an E. coli K12 strain that contains the rfb genes of E. coli 08; therefore, strain 2443 synthesizes a complete LPS molecule possessing 08 side chains. Since synthesis of 08 side chains is an rfe-dependent process (31), insertion of Tn10 into rfe should abolish the synthesis of both ECA and 08 side chains. In contrast, insertion of Tn10 into the rff genes should only abolish ECA synthesis. Transduction of each of the 12 Tn10 insertions into strain 2443 revealed that four of the transductants were defective in the synthesis of both ECA and 08 side chains; therefore, these insertions (rfe-48, rfe-88, rfe-91, and rfe-92) were in rfe (Table II). The remaining eight ECA-negative Tn10 insertions (rff-46, rff-50, rff-66, rff-67, rff-68,
ECA-negative Tn10 Insertion Mutants

In Vitro Synthesis of Lipid I by Membranes of Tn10 Insertion Mutants—Lipid I is the first lipid-linked intermediate in the synthesis of ECA. Accordingly, in vitro assays were conducted to ascertain the ability of each of the ECA-negative Tn10 insertion mutants to utilize endogenous undecaprenyl monophosphate as acceptor for GlcNAc-1-phosphate residues for the synthesis of lipid I. Membrane fractions obtained from each of the mutants, as well as the ECA-positive parental strain AB1133, were incubated with UDP-[3H]GlcNAc and the incorporation of radioactivity into lipid I was determined. The in vivo synthesis of lipid I was also assayed using membranes obtained from mutant strain 21701 which possesses the rff-726 lesion; the rff-726 mutation was previously believed to reside within the structural gene for the ManNAcA transferase (3).

Lipid I synthesis was demonstrated in reaction mixtures containing membranes prepared from each of the eight rff::Tn10 mutants as well as the mutant possessing the rff-726 defect (Table III); the amount of [3H]GlcNAc incorporated into lipid I varied from approximately 50 to 165% of the amount incorporated into the parental strain AB1133. In contrast, lipid I synthesis was not detected in reaction mixtures that contained membranes prepared from the four rfe::Tn10 mutants.

In Vivo Accumulation of Lipid-linked Intermediates in rff::Tn10 Insertion Mutants—The rff::Tn10 insertion mutants, as well as E. coli strain 21731 possessing the rff-726 lesion, were examined for the in vivo accumulation of lipid-linked ECA intermediates as an initial attempt to define the step in ECA synthesis affected by each of the respective mutations. The mutants were incubated with [3H]GlcNAc and the amount of radioactivity incorporated into lipids I, II, and III was determined.

Lipid I is readily separated from lipids II and III by chromatography on silica gel-impregnated (SG81) paper or by thin-layer chromatography on silica gel using a variety of solvent systems. Thus, the amount of radioactive lipid I can be determined directly following chromatography on SG81 paper or silica gel thin layers. In contrast, no solvent system has thus far been identified that affords the separation of lipids II and III from one another when analyzed by either of the above chromatographic methods. Accordingly, the region of SG81 chromatograms corresponding to lipids II and III was eluted and the radioactive lipid-linked intermediates were subjected to mild acid hydrolysis. The amount of radioactivity incorporated into the water-soluble disaccharide and trisaccharide released from lipids II and III, respectively, was then analyzed by gel permeation chromatography using Bio-Gel P-2.

Three phenotypically distinct groups of rff mutants were defined by the radioactive lipid-linked intermediates that they accumulated in vivo (Table IV). Three mutants (strains 21546, 21566, and 21568) accumulated lipid I, but they did not accumulate lipids II or III. Accordingly, these mutants appeared to either possess a defective ManNAcA transferase or they were defective in the synthesis of UDP-ManNAcA. The second group of rff::Tn10-insertion mutants (strains 21550, 21567, and 21598), as well as mutant strain 21731 which possesses the rff-726 lesion, accumulated both lipids I and II; the major amount of radioactivity was accumulated in lipid II. These strains were candidates for mutants that possessed either a defect in the Fuc4NAc transferase that catalyzes the transfer of Fuc4NAc from TDP-Fuc4NAc to lipid II or a defect in one of the enzymes involved in the synthesis of TDP-Fuc4NAc. The third group of mutants (strains 21585 and 21594) accumulated [3H]GlcNAc-labeled lipids II and III, lesser amounts of radioactive lipid I also accumulated. Since these mutants could synthesize the complete ECA trisaccharide repeat unit, they appeared to be defective in some later steps of ECA synthesis involving the elongation of ECA hexasaccharide chains.

In Vitro Synthesis of Lipid II by Membranes of rff::Tn10 Insertion Mutants—Mutant strains belonging to Group I appeared to be defective in the conversion of lipid I to lipid II (Table IV). Thus, the nature of the enzymatic defect in these mutants was examined by measuring the ability of the mutants to perform the de novo synthesis of lipid II as determined by in vitro assay. Membrane fractions obtained from Group I mutants were incubated with UDP-[3H]GlcNAc and UDP-[14C]ManNAcA, and the incorporation of radioactivity into lipid II was determined (Table V). Similar assays were performed using membranes obtained from mutants belonging to Groups II and III and the wild-type parental strain.

The in vitro synthesis of [14C]ManNAcA-labeled lipid II was demonstrated using membranes obtained from two of the Group I mutant strains, strains 21546 (rff::Tn10-46) and 21566 (rff::Tn10-66). These data indicate that the in vivo accumulation of lipid I by strains 21546 and 21566 is due to their inability to synthesize UDP-ManNAcA. In contrast, [14C]ManNAcA-labeled lipid II was not detected in reaction mixtures that contained membranes from mutant strain 21568 (rff::Tn10-68). Thus, the lack of detectable lipid II synthesis using membranes from this mutant suggests that the Tn10 insertion results in a defective ManNAcA transferase.

It is interesting to note that the in vitro synthesis of [14C]ManNAcA containing lipid II by membranes from mutant strains 21546 and 21566 was not accompanied by the parallel incorporation of [3H]GlcNAc (Table V). This may be due to the accumulation of lipid I in the membranes (Table IV) and a corresponding lack of free undecaprenyl monophosphate, the acceptor for [3H]GlcNAc-1-phosphate residues. However, the endogenous lipid I is apparently able to act as an acceptor of [14C]ManNAcA residues. This conclusion is supported by the observation that the amount of [14C]ManNAcA incorporated into lipid II by membranes of strain 21566 did not differ when reactions were carried out either in the presence or absence of exogenously supplied UDP-GlcNAc (data not shown). In addition, the in vitro incorporation of [14C]ManNAcA into lipid II by membranes of wild-type strains of E. coli is dependent on prior synthesis of lipid I (2).
conversion of UDP-GlcNAc to UDP-ManNAcA was not detected in cell-free extracts obtained from mutant strains 21546 (rff::TnlO-46) and 21566 (rff::TnlO-66). Additional in vitro assays revealed that strain 21546 possessed a defective UDP-ManNAc dehydrogenase and strain 21566 was defective in both UDP-GlcNAc-2-epimerase and UDP-ManNAc dehydrogenase activities (Table V1). Thus, the rff::TnlO-46 insertion appears to be in the structural gene for UDP-ManNAc dehydrogenase, whereas the location of the rff::TnlO-66 insertion is unclear. It is possible that the rff::TnlO-66 insertion is located in the structural gene for the epimerase, and the lack of both epimerase and dehydrogenase activity may be due to a polar effect.

In Vitro Synthesis of Lipid III by Membranes from rff::TnlO Insertion Mutants—Group II rff::TnlO insertion mutants were able to synthesize lipid II, but they were defective in the synthesis of lipid III (Table IV). These data suggest that the accumulation of lipid II by these mutants may be due to either their inability to synthesize TDP-Fuc4NAc or to a defective Fuc4NAc transferase. In order to distinguish between these possibilities, membrane fractions of Group II mutants were incubated with TDP-[3H]Fuc4NAc, and the incorporation of radioactivity into lipid III was determined. Similar assays were also conducted using membranes prepared from mutants in Groups I and III. Accordingly, UDP-GlcNAc and UDP-ManNAcA were included in all reaction mixtures.

No incorporation of [3H]Fuc4NAc into lipid III was observed when reaction mixtures contained membranes from strain 21731 (rff-726) (Table VII). Thus, the in vivo accumulation of lipid II by this mutant appears to be due to a defective Fuc4NAc transferase. In contrast, significant incorporation of [3H]Fuc4NAc into lipid III was observed when reaction mixtures contained membranes from Group II mutant strains 21550 (rff::TnlO-50), 21567 (rff::TnlO-67), and 21593 (rff::TnlO-93) (Table VII). These data indicate that the in vivo accumulation of lipid II by these mutants is due to their inability to synthesize TDP-Fuc4NAc rather than to a defective Fuc4NAc transferase.

The in vitro synthesis of [3H]Fuc4NAc-labeled lipid III was also demonstrated in reaction mixtures containing membranes from the Group III mutant strains 21559 (rff::TnlO-85) and 21594 (rff::TnlO-94) (Table VII). These results, together with the observation that these mutants accumulate lipid III in vivo (Table IV), further support the conclusion that the Group III mutants are defective in some later step of ECA synthesis involving the elongation of ECA heteropolysaccharide chains.

The inability of membranes from mutant strain 21568 (rff::TnlO-68, Group I) to function in the synthesis of lipid III is in agreement with the previous conclusion that this mutant possesses a defective ManNAcA transferase. In contrast, marked incorporation of [3H]Fuc4NAc into lipid III was observed when membranes from Group I mutants 21546 and 21566 were added to standard reaction mixtures containing exogenously supplied UDP-ManNAcA and UDP-GlcNAc. These data support the earlier conclusion that these mutants are unable to synthesize lipid II in vivo due to defects in the synthesis of UDP-ManNAcA (Tables IV and V1).

Synthesis of TDP-Fuc4NAc by Mutants Defective in Lipid III Synthesis—The ability of Group II mutants 21550, 21567, and 21593 to synthesize TDP-Fuc4NAc was assayed in vitro in order to determine at which step in TDP-Fuc4NAc synthesis each of the mutants was defective. Cell-free soluble enzyme fractions obtained from the parental strain and each of the mutants were incubated with TDP-[14C]glucose, and the in vitro conversion of the labeled nucleotide sugar to TDP-[14C]Fuc4NAc was assayed as previously described (18). Significant degradation of TDP-[14C]glucose was encountered following its introduction into extracts prepared from the parental strain AB1133. In order to avoid this difficulty, the mutations rff-726, rff::TnlO-50, rff::TnlO-67, rff::TnlO-85, and rff::TnlO-85 were transduced into E. coli Y10 using phage P1 since this strain has been used previously for assay of the enzymes involved in TDP-Fuc4NAc synthesis (18, 23).

Soluble enzymes from strain Y10 catalyzed the conversion of TDP-[14C]glucose to a single labeled product with a chromatographic mobility identical to that of authentic TDP-Fuc4NAc when analyzed by paper chromatography (Table VIII). In addition, mild acid hydrolysis of the product resulted in the release of a labeled compound with mobilities identical to that of Fuc4NAc when analyzed by both paper chromatography and high voltage paper electrophoresis (Table VIII). In contrast, soluble enzyme fractions from transductants possessing transposon insertions rff::TnlO-50 (strain B2111), rff::TnlO-67 (strain B2112), and rff::TnlO-93 (strain B2114) synthesized a single labeled product with a chromatographic mobility of R_PDP-FucNAc = 0.78-0.82 when analyzed by paper chromatography using Solvent A (Table VIII). In addition, the radioactive sugar released from the compound by treatment with mild acid was electrophoretically neutral at pH 4.2 and migrated with an RsecNAc = 1.48 when analyzed by paper chromatography using Solvent B. The above properties of both the intact compound and the free sugar are in agreement with their identification as TDP-4-keto-6-deoxy-D-glucose and 4-keto-6-deoxy-D-glucose, respectively (23). Thus, the TnlO insertions in these mutants appear to be located in the structural gene for the transaminase that catalyzes the conversion of TDP-4-keto-6-deoxy-D-glucose to TDP-4-amino-6-dideoxy-D-glucose (TDP-fucosamine, TDP-FucN), the immediate precursor of TDP-Fuc4NAc. Indeed, additional experiments revealed that the soluble fractions obtained from these mutants were capable of catalyzing the conversion of TDP-[14C]FucN to TDP-[14C]Fuc4NAc (data not shown).

Mutants possessing the rff-726 and rff::TnlO-85 lesions were not found to be defective in the conversion of TDP-glucose to TDP-Fuc4NAc (Table VIII).

Effect of TnlO Insertions on the Synthesis of ECA and K7 Capsule in E. coli 014:K7—One of the amino sugars of the ECA trisaccharide repeating unit, ManNAcA, is also a component of the K7 capsule produced by E. coli 014:K7 (19, 24). In order to test the effects of each of the TnlO insertions on the synthesis of ECA in E. coli 014:K7, recombinants were constructed by mating Hfr strains containing each of the TnlO insertions with E. coli 014:K7 strain 2537. Tetracycline- and streptomycin-resistant recombinants were screened for their ability to synthesize ECA by passive hemagglutination assay.

All of the recombinants possessing the TnlO insertions rff-68, rff-46, rff-88, rff-91, and rff-92 were ECA-negative (Table IX). In contrast, all of the recombinants possessing the TnlO insertions rff-46, rff-66, rff-67, rff-85, and rff-94 were ECA-positive. In addition, both ECA-negative and ECA-positive recombinants were observed among transconjugants possessing the TnlO insertions rff-50 and rff-93. These results suggest that the chromosome of E. coli 014:K7 contains genes outside of the rff-rff region that are able to complement the defects resulting from insertion of transposon TnlO into many of the rff genes.

In order to determine if genes involved in K7 capsule synthesis function to complement these defects, mutants carrying the various TnlO insertions were transformed with cosmids, pGB26, which contains the genes necessary for bio-
synthesis of the K7 capsule (29). All of the transformants expressed the K7 capsule as determined by immunoprecipitation using K7-specific antiserum (Table IX). In addition, ECA synthesis was rescued in two of the transformants possessing Tn10 insertions in genes involved in UDP-ManNAcA synthesis (rff-46 and rff-66). Thus, it seems likely that the region of the E. coli O14:K7 chromosome involved in K7 capsule synthesis includes genes required for UDP-ManNAcA synthesis. However, the location and primary function of genes able to complement the other rff::Tn10 insertions are less well understood. Some of these genes might be closely linked to the rff region; for example, those complementing the rff-50 and rff-93 lesions. In this event, both ECA-positive and ECA-negative recombinants could result depending on whether the recombinants maintained the O14:K7 region containing these genes or lost these genes due to acquisition of the corresponding E. coli K12 region lacking these genes, respectively.

DISCUSSION

Mutations that affect ECA synthesis can be divided into two genetically distinct groups; mutations in rff genes and mutations in rfe genes. Mutations in rfe genes affect not only ECA synthesis, but they also affect the synthesis of lipopolysaccharide O-side chains in certain organisms (7, 24, 31). In contrast, mutations in rff genes affect only ECA synthesis regardless of the organism in which they occur. All 12 of the independently isolated ECA-negative Tn10 insertion mutations described in this report were found to be located in the rff-rfe region at 84-85 min on the E. coli chromosome. Four of the mutants possessed Tn10 insertions in rfe genes. Accordingly, the Tn10 insertions in these mutants also abolished the synthesis of O-side chains when transduced into a derivative of E. coli K12 containing rfeB genes (Table II). The remaining eight mutants possessed Tn10 insertions in rff genes since the insertions in these mutants only affected the synthesis of ECA.

The specific enzymatic steps in ECA synthesis affected by each of the Tn10 insertions were identified, and they are summarized in Fig. 3. Two of the mutants, strains 21546 (rff::Tn10-46) and 21566 (rff::Tn10-66), accumulated lipid I in vivo (Table IV), and they were found to be defective in the synthesis of UDP-ManNAcA (Table VI). The rff::Tn10-46 lesion appears to be in the structural gene for UDP-ManNAc dehydrogenase (rfd), whereas the location of the rff::Tn10-66 insertion remains less clear. The rff::Tn10-66 insertion abolished both UDP-GlcNAc-2-epimerase and UDP-ManNAc dehydrogenase activities. It is possible that this Tn10 insertion is located in the structural gene for the epimerase (rffE), and the lack of both epimerase and dehydrogenase activity may be due to a polar effect.

Mutant strains 21585 (rff::Tn10-85) and 21594 (rff::Tn10-94) were able to synthesize lipid III as determined by both in vivo (Table IV) and in vitro (Table VII) experiments. Accordingly, the lack of detectable ECA synthesis by these mutants suggests that the Tn10 insertions in these strains affect some later step in ECA synthesis that involves the elongation of ECA heteropolysaccharide chains. However, the mechanism of chain elongation remains to be established.

In many instances the amount of in vitro incorporation of radioactive sugars into lipid-linked intermediates by membranes of rff::Tn10 insertion mutants varied considerably from that observed using membranes from the wild-type parental strain. Thus, membranes from mutants defective in the synthesis of UDP-ManNAcA catalyzed the incorporation of approximately 160% more [3H]GlcNAc into lipid I than did membranes isolated from the wild-type strain (Table III). In contrast, Tn10 insertions in the structural genes for the ManNAcA transferase (rffM) and the FucNAc transferase (rffT) did not alter the amount of [3H]GlcNAc incorporated into lipid I in vitro. All other rff::Tn10 insertions, affecting either TDP-FucNAc synthesis or steps in ECA synthesis subsequent to the synthesis of lipid III, resulted in decreased in vitro incorporation of [3H]GlcNAc into lipid I relative to that observed with wild-type membranes. Thus, the amount of [3H]GlcNAc incorporated into lipid I appears to be correlated with the type of mutation. Similar relationships were not observed for either the in vitro incorporation of [3H]GlcNAc and [14C]ManNAcA into lipid II or the incorporation of [3H]FucNAc into lipid III by Tn10 insertion mutants capable of synthesizing these intermediates. Accordingly, the in vitro incorporation of [3H]GlcNAc and [14C]ManNAcA into lipid II was reduced for all rff::Tn10 insertion mutants when compared to the amounts incorporated using membranes from the wild-type strain (Table V). In contrast, the in vitro incorporation of [3H]FucNAc into lipid III was significantly enhanced for all Tn10 insertion mutants able to synthesize this intermediate (Table VII). The underlying basis for the above relationships remains to be established.

The function of genes in the rfe region is not yet understood. All mutant possessing rfe::Tn10 insertions were found to be defective in lipid I synthesis (Table III). This observation suggests the possibility that the rfe gene codes for the transferase which catalyzes the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to undecaprenyl monophosphate. However, this interpretation fails to explain why the synthesis of other polysaccharides, such as the O-side chains of E. coli 08, 09, 020, 0101, S. montevideo, and S. minnesota is not rfe dependent. The repeat units of these polymers either lack GlcNAc or they contain GlcNAc in a position that does not suggest the involvement of lipid I in their assembly. For example, the O-side chains of E. coli 08 and 09 are homopolymers of mannose containing α-(1 → 2)- and α-(1 → 3)-mannosyl linkages; the mannose residues of the 08 and 09 side chains are arranged in trisaccharide and pentasaccharide repeat units, respectively (24). In addition, the O-side chain of F. coli 020 also lacks GlcNAc; the repeat unit of this polymer consists of the disaccharide → 4)-α-D-Gal-(1 → 2)-α-D-Rib-(1 → 24). Furthermore, the O-side chain of S. minnesota is comprised of pentasaccharide repeat units containing GlcNAc, GalNAc, and Gal; however, the GlcNAc occurs as a branch residue (11). Thus, the structures of these polymers suggest that the rfe gene is not the structural gene.
for the GlcNac-1-phosphate transferase involved in lipid I synthesis. Accordingly, it is possible that the rfe gene has a different function but that mutations in this gene nevertheless abolish the synthesis of lipid I in some unknown manner. However, it should be noted that no mutations in the rfe region or other regions of the chromosome have been described that affect GlcNac-1-phosphate transferase activity in the above organisms without affecting O-side chain synthesis. Consequently, it is possible that the rfe gene has a different function but that mutations in this gene nevertheless abolish the synthesis of lipid I in some unknown manner.

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UDP-ManNAcA is the donor of ManNAcA residues for the synthesis of both ECA and the K7 capsule in E. coli 014:K7. In addition, the genes involved in UDP-ManNAcA synthesis appear to be rearranged or duplicated in this organism. Accordingly, genes located in the region of the chromosome concerned with K7 capsule synthesis are able to complement mutations that abolish the function of the rffA and rffE genes (Table IX). E. coli 014-K7 also possesses other genes that are not located in the region concerned with K7 capsule synthesis, and these genes can complement the loss of functions of the rffA gene and the rffE genes involved in the elongation of ECA heteropolysaccharide chains (Table IX). Neither the locations or the primary functional roles of these genes are known. It is possible that these genes originated as a result of the duplication of rff genes and their subsequent translocation to chromosomal regions involved in the synthesis of other polysaccharides. Such a process may have contributed significantly to the pronounced diversity that exists in the structures of O-side chains and other cell-surface polysaccharides among members of the Enterobacteriaceae.

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REFERENCES

ECA-negative Tn10 Insertion Mutants

Relevant phenotypes of transconjugants of strain 2427 possessing Tn10 insertions and colimxillin selection frequencies of Tn10 insertions with Em and MnO

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The occurrence of ECA and 61 side-chains were determined by passive hemagglutination assay and sensitivity to lysis by lysis.

The authors of Tn10 insertions at Em and MnO (mutants) were selected for resistance to spectinomycin and scored secondary for Em and MnO, respectively.
### TABLE V

Effect of Tn10-insertion mutations on the in vivo incorporation of radioactivity from UMP, GDP-Man, and UDP-[14C]ManNAc into lipid II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Radioactivity incorporated into lipid II$^a$</th>
<th>[14C]ManNAc</th>
<th>GDP-Man</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1153</td>
<td>wild type</td>
<td>0.90 (100)</td>
<td>1.00 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Group I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Radioactivity incorporated into lipid II$^a$</th>
<th>[14C]ManNAc</th>
<th>GDP-Man</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Group II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Radioactivity incorporated into lipid II$^a$</th>
<th>[14C]ManNAc</th>
<th>GDP-Man</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>21566</td>
<td>ΔTn10-65</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-66</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the percentage incorporation relative to the wild type strain.

### TABLE VI

Effect of Tn10-insertion mutations on the in vitro synthesis of UMP-ManNAc

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>UMP-ManNAc</th>
<th>GDP-ManNAc</th>
<th>UMP-ManNAc</th>
<th>GDP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1153</td>
<td>wild type</td>
<td>0.46 (46)</td>
<td>0.46 (46)</td>
<td>0.46 (46)</td>
<td>0.46 (46)</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
<td>0.66 (36)</td>
</tr>
</tbody>
</table>

*Units of activity expressed as nanomoles per milligram protein per hour.

### TABLE VII

Effect of Tn10-insertion mutations and the ΔTn10 mutation on the in vivo incorporation of radioactivity from UMP, GDP-ManNAc, and UDP-[14C]ManNAc into lipid II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>[14C]ManNAc incorporated into lipid II$^a$</th>
<th>GDP-ManNAc</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1153</td>
<td>wild type</td>
<td>7.02 (100)</td>
<td>1.00 (100)</td>
<td>1.00 (100)</td>
</tr>
</tbody>
</table>

Group I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>[14C]ManNAc incorporated into lipid II$^a$</th>
<th>GDP-ManNAc</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>1.711 (248)</td>
<td>0.26 (41)</td>
<td>0.26 (41)</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>1.711 (248)</td>
<td>0.26 (41)</td>
<td>0.26 (41)</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-60</td>
<td>1.711 (248)</td>
<td>0.26 (41)</td>
<td>0.26 (41)</td>
</tr>
</tbody>
</table>

Group II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>[14C]ManNAc incorporated into lipid II$^a$</th>
<th>GDP-ManNAc</th>
<th>UMP-ManNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>21566</td>
<td>ΔTn10-66</td>
<td>3.070 (407)</td>
<td>0.42 (65)</td>
<td>0.42 (65)</td>
</tr>
<tr>
<td>21566</td>
<td>ΔTn10-66</td>
<td>3.070 (407)</td>
<td>0.42 (65)</td>
<td>0.42 (65)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the percentage incorporation relative to the wild type strain.

### TABLE VIII

Chromatographic mobilities of radioactive compounds derived from Tn10/GlcNAc by viable strains of Tn10-insertion mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>TDP-sugar</th>
<th>Sugar</th>
<th>Chromatographic mobility of radioactive compound$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y11</td>
<td>wild type</td>
<td>1.90</td>
<td>1.90</td>
<td>1.90</td>
</tr>
<tr>
<td>Y111</td>
<td>ΔTn10-66</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Y112</td>
<td>ΔTn10-62</td>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
</tr>
<tr>
<td>Y114</td>
<td>ΔTn10-64</td>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
</tr>
<tr>
<td>Y113</td>
<td>ΔTn10-65</td>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
</tr>
</tbody>
</table>

*Chromatographic mobilities of the radioactive TDP-sugar and the labeled sugar released from the TDP-sugar by mild and hydrolysis were determined by descending paper chromatography employing solvents A and B, respectively. The values expressed are the chromatographic mobilities of the TDP-sugar and the sugar relative to TDP-Fucitol and Fucitol, respectively.

*Hydrolysis paper chromatography was carried out as described under "Experimental Procedures." The values expressed are the chromatographic mobilities of the sugar relative to Fucitol.

### TABLE IX

Effect of Tn10-insertion mutations on the synthesis of ECA and K1-poly saccharide

<table>
<thead>
<tr>
<th>Mutation (gene locus)</th>
<th>ECA-negative</th>
<th>KT</th>
<th>ECA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔTn10-64 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-66 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-62 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-63 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-65 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-64 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-65 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTn10-66 (ECA)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values represent the number of recombinants unable to synthesize either the K1 capsule polysaccharide or ECA relative to the total number of recombinants examined. The presence of ECA and K1 polysaccharides were determined by passive hemagglutination assay and immunoprecipitation with specific antibodies, respectively.

*The occurrence or absence of polymers is designated by + and - respectively.