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Characterization of an *Escherichia coli rff* Mutant Defective in Transfer of *N*-Acetylmannosaminuronic Acid (ManNAcA) from UDP-ManNAcA to a Lipid-Linked Intermediate Involved in Enterobacterial Common Antigen Synthesis

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The *rff* genes of *Salmonella typhimurium* include structural genes for enzymes involved in the conversion of UDP *N*-acetyl-D-glucosamine (UDP-GlcNAc) to UDP *N*-acetyl-D-mannosaminuronic acid (UDP-ManNAcA), the donor of ManNAcA residues in enterobacterial common antigen (ECA) synthesis. An *rff* mutation (*rff-726*) of *Escherichia coli* has been described (U. Meier and H. Mayer, J. Bacteriol. 163:756–762, 1985) that abolished ECA synthesis but which did not affect the synthesis of UDP-ManNAcA or any other components of ECA. The nature of the enzymatic defect resulting from the *rff-726* lesion was investigated in the present study. The in vitro synthesis of GlcNAc-pyrophosphorylundecaprenol (lipid I), an early intermediate in ECA synthesis, was demonstrated by using membranes prepared from a mutant of *E. coli* possessing the *rff-726* lesion. However, in vitro synthesis of the next lipid-linked intermediate in the biosynthetic sequence, ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II), was severely impaired. Transduction of wild-type *rff* genes into the mutant restored the ability to synthesize both lipid II and ECA as determined by in vitro assay and Western blot (immunoblot) analyses done with anti-ECA monoclonal antibody, respectively. Our results are consistent with the conclusion that the *rff-726* mutation is located in the structural gene for the transferase that catalyzes the transfer of ManNAcA from UDP-ManNAcA to lipid I.

Enterobacterial common antigen (ECA) is an acidic cell surface glycolipid synthesized by essentially all bacteria belonging to the family *Enterobacteriaceae* (12, 13, 17). The carbohydrate portion of ECA consists of a heteropolysaccharide comprised of *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (8). These sugars are linked to one another to form linear chains of trisaccharide repeat units having the sequence →4)-β-D-Man_pNAcA-(1→4)-α-D-Glc_pNAc-(1→3)-D-Fuc_p4NAc-(1→ (8). Individual ECA polysaccharide chains are linked to a phosphatidic acid moiety by glycosidic linkage of a reducing terminal GlcNAc residue to the phosphate to form a phosphodiester (2). The lipid aglycone presumably serves to anchor the molecule in the outer membrane.

Several genetic loci are involved in the biosynthesis of ECA. These include the *ilv*-linked *rfe* and *rff* gene clusters located at min 83 and 85 of the *Salmonella* and *E. coli* chromosomes, respectively (10, 11, 15, 22). In addition, group B salmonellae require genes located at the *rfb* locus (6, 11, 14). Our current understanding of the roles of these various genes in ECA synthesis has been recently summarized (6).

Of particular importance for the present study are the functions of the *rff* genes. Mutants of *Salmonella typhimurium* possessing defective *rff* genes have been described that are unable to carry out the conversion of UDP-GlcNAc to UDP-ManNAcA, the donor of ManNAcA residues in ECA synthesis (7). Indeed, all *rff* genes characterized thus far have been found to be involved in the synthesis of ManNAcA. More recently, a new *rff* mutation (*rff-726*) was described in *E. coli* O8 (15). The *rff-726* mutation abolished the synthesis of ECA but did not affect the biosynthesis of UDP-ManNAcA or any other ECA components. Thus, it has been suggested that the *rff-726* lesion might reside within the structural gene for the UDP-ManNAcA transferase involved in ECA synthesis. However, until recently an examination of this possibility was precluded by a lack of information regarding the mechanism of ECA biosynthesis.

Previous studies demonstrated that GlcNAc-pyrophosphorylundecaprenol is an early intermediate in ECA biosynthesis (18). More recently, we have developed an in vitro system to define the individual enzymatic steps involved in ECA biosynthesis (1). These studies revealed that membranes of *E. coli* possess enzymes that catalyze the following sequence of reactions (P-lipid designates undecaprenyl monophosphate):

$$\text{UDP-GlcNAc} + \text{P-lipid} \xrightleftharpoons{Mg^{2+}} \text{GlcNAc-PP-lipid} + \text{UMP} \quad (1)$$

$$\text{UDP-ManNAcA} + \text{GlcNAc-PP-lipid} \rightleftharpoons \text{ManNAcA-GlcNAc-PP-lipid} + \text{UDP} \quad (2)$$

In this study we demonstrate that membranes obtained from *rff-726* mutants of *E. coli* O8 possess the enzyme activity that catalyzes the synthesis of GlcNAc-pyrophosphorylundecaprenol (lipid I) according to reaction 1 above. However, these mutants are unable to carry out the enzymatic conversion of this intermediate to ManNAcA-GlcNAc-PP-undecaprenol (lipid II). The results presented here support the conclusion that the *rff-726* lesion resides within the structural

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gene for the transferase that catalyzes reaction 2 described above.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Experiments were done with *E. coli* F1467 (O8:K27$^-$ *rff-726 his pro pyr met rha* Str^r) (15), *E. coli* 21259 (O8:K27$^-$ *zie-2*::Tn*10 his pro pyr met rha* Str^r) (15), and *S. typhimurium* PR122 (*hisF1009 trpB2 metA22 xyl-1 rpsL galE nagB* F$^-$) (20). Cultures were grown with vigorous aeration in L medium containing 0.2% glucose (4) or Proteose Peptone-beef extract (PPBE) medium (21) as indicated.

Radiochemicals, chemicals, and reagents. UDP-*N*-acetyl-D-[6-^{3}H(N)]glucosamine (20.4 Ci/mmol) and UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (283 mCi/mmol) were purchased from New England Nuclear Corp. UDP-*N*-acetyl-D-[U-^{14}C]mannosaminuronic acid (283 mCi/mmol) and unlabeled UDP-ManNAcA were enzymatically synthesized from UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (283 mCi/mmol) and UDP-GlcNAc, respectively (1). Tunicamycin (mixture of isomers A, B, C, and D) was purchased from Sigma Chemical Co. Bacterial alkaline phosphatase was purchased from Cooper Biomedical, Inc. Mouse anti-ECA monoclonal antibody mAb898 (IgG2a) was kindly provided by D. Bitter-Suermann, Johannes-Gutenberg-University, Mainz, Federal Republic of Germany (16).

Genetic methods. Transductions were done with bacteriophage P1 *vir* as described by Silhavy et al. (23). Tetracycline-resistant transductants were selected on PPBE plates containing tetracycline at a final concentration of 25 μg/ml.

Assays for the synthesis of GlcNAc-PP-lipid and ManNAcA-GlcNAc-PP-lipid. Cell envelopes were prepared as previously described (1) with the following modifications. Cultures were grown in L medium (25 ml) containing 0.2% glucose at 37°C to an A_{600} of 0.5 to 0.6. The cells were washed, disrupted by sonication, and centrifuged at 160,000 × *g* for 60 min at 4°C. Cell envelopes were then suspended in 60 μl of cold Tris hydrochloride (15 mM, pH 8.0) and used immediately in the assays.

Reaction mixtures for the synthesis of GlcNAc-PP-lipid and ManNAcA-GlcNAc-PP-lipid contained the following in a final volume of 52 μl: 50 mM Tris hydrochloride (pH 8.2), 30 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 5 μM UDP-[^{3}H]GlcNAc (8 × 10^5 cpm/nmol), 5 μM UDP-ManNAcA, 0.16 U of bacterial alkaline phosphatase, and cell envelopes (200 to 250 μg of protein). UDP-[^{14}C]ManNAcA (282 mCi/mmol) and tunicamycin were added to reaction mixtures, where indicated, to give a final concentration of 0.26 μM and 10 μg/ml, respectively. Incubations were done at 37°C for 30 min. Reactions were terminated by the addition of 1.0 ml of $CHCl_3$-CH_3OH (3:2 [vol/vol]) and processed as described previously (1). Chloroform-soluble products were analyzed by ascending chromatography on EDTA-treated SG-81 paper by using $CHCl_3$-CH_3OH-H_2O-NH_4OH (88:48:10:1, by volume) as the solvent. After chromatography, lanes of the chromatogram were cut into 1-cm sections and soaked in 0.5 ml of 1.25% sodium dodecyl sulfate for 6 to 8 h at 42°C. The sections were then analyzed for radioactivity by liquid scintillation counting by using toluene-Liquiflor-Bio-Solv (19).

Incorporation of [^{14}C]ManNAcA into exogenously supplied [^{3}H]GlcNAc-pyrophosphorylundecaprenol. [^{3}H]GlcNAc-PP-lipid was synthesized in vitro as described above by using membranes prepared from either strain F1467 or 21259 as indicated. The radioactive intermediate was purified by ascending chromatography on EDTA-treated SG-81 paper by using $CHCl_3$-CH_3OH-H_2O (65:25:4, by volume) as the solvent. [^{3}H]GlcNAc-PP-lipid was eluted from the chromatogram with $CHCl_3$-CH_3OH-H_2O (10:10:3, by volume), and the solvent was removed at room temperature under a stream of nitrogen. The dried lipid was stored until use in a nitrogen atmosphere at −20°C.

Reactions making use of exogenously supplied [^{3}H]GlcNAc-PP-lipid as an acceptor of [^{14}C]ManNAcA residues were done as follows. [^{3}H]GlcNAc-PP-lipid (11,200 dpm) was dried in the presence of Mg^{2+}-EDTA according to the method of Behrens and Tabora (3). The residue was solubilized by brief bath sonication after the addition of Tris hydrochloride (50 mM, pH 8.2), $MgCl_2$ (30 mM), 2-mercaptoethanol (5 mM), and Triton X-100 (0.01%). After sonication, membranes from strain 21259 (250 to 300 μg of protein) were added to reaction mixtures either in the presence or absence of UDP-[^{14}C]ManNAcA. Reaction mixtures were incubated at 37°C for 30 min and then terminated by the addition of 1 ml of $CHCl_3$-CH_3OH (3:2 [vol/vol]). Radioactive components extracted into the organic phase were subsequently analyzed by ascending chromatography on EDTA-treated SG-81 paper by using $CHCl_3$-CH_3OH-H_2O-NH_4OH (88:48:10:1 by volume) as the solvent. The location of radioactivity on the chromatogram was determined as described above.

Western blot analysis. Western blot (immunoblot) analyses were done as previously described (18) except that after electrophoretic transfer, the nitrocellulose papers were incubated in buffer A (10 mM K_2HPO_4-KH_2PO_4 [pH 7.5], 15 mM NaCl) containing 2% gelatin for 18 h before incubation with antibody.

Chromatographic procedures. Paper chromatography was done with silica gel-impregnated papers (Whatman, Inc.; SG-81) which were pretreated by being dipped in EDTA (0.68 M, pH 7.7) and dried at room temperature. The papers were heated at 110°C for 10 min immediately before use.

RESULTS

In vitro synthesis of ECA intermediates by membranes obtained from wild-type (*rff*$^+$) and mutant (*rff-726*) strains. The ability of *E. coli* 21259 (*rff*$^+$) and F1467 (*rff-726*) to synthesize lipid I and lipid II was investigated by using previously developed in vitro assays (1). Incubation of membranes from strain 21259 with UDP-[^{3}H]GlcNAc and UDP-[^{14}C]ManNAcA resulted in the incorporation of radioactivity into both lipid I and lipid II (Fig. 1A). Under the conditions of the assay, the incorporation of [^{3}H]GlcNAc into both intermediates occurred to the same extent. In addition, [^{14}C]ManNAcA was incorporated only into lipid II, in agreement with the earlier characterization of this component as ManNAcA-GlcNAc-PP-lipid (1). Parallel experiments with membranes prepared from strain F1467 revealed the incorporation of [^{3}H]GlcNAc into lipid I to a similar extent (Fig. 1B). However, in contrast to the results observed with membranes obtained from strain 21259, almost no transfer of [^{14}C]ManNAcA to [^{3}H]GlcNAc-labeled lipid I was observed.

The possibility that membrane preparations obtained from strain F1467 contained an inhibitor of the ManNAcA transferase that catalyzes the transfer of ManNAcA from UDP-ManNAcA to lipid I was eliminated by mixed membrane experiments. Thus, the addition of membranes from strain F1467 to reaction mixtures containing membranes from strain 21259 did not inhibit the synthesis of lipid I (data not shown).

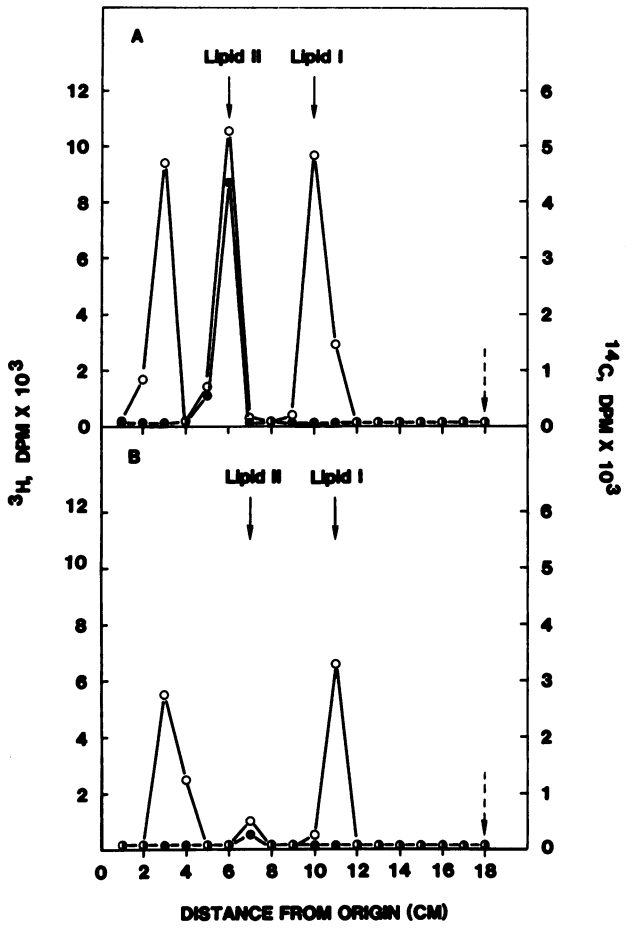

FIG. 1. In vitro incorporation of radioactivity from UDP-[^{3}H]GlcNAc and UDP-[^{14}C]ManNAcA into lipid I and lipid II. Cell envelope membranes (200 to 250 μg of protein) were incubated with UDP-[^{3}H]GlcNAc and UDP-[^{14}C]ManNAcA in the standard reaction mixture (50 μl) at 37°C for 30 min. Reactions were terminated by the addition of chloroform-methanol (3:2 [vol/vol]), and radioactive components extracted into the organic phase were analyzed by ascending chromatography on EDTA-treated SG-81 paper. The broken arrows designate the position of the solvent front. Additional details are provided under Materials and Methods. Symbols: ●, carbon-14; ○, tritium. (A) Membranes from strain 21259 (*rff*$^+$); (B) membranes from strain F1467 (*rff-726*).

The results described above clearly demonstrate that the synthesis of lipid II is impaired in strain F1467. Moreover, since the synthesis of UDP-ManNAcA is not impaired in strain F1467 (15), these data also suggest that the defect in lipid II synthesis is due to a defect in ManNAcA transferase activity.

Relationship between the *rff-726* lesion and the inability to synthesize lipid II. The chromosome of *E. coli* 21259 carries the transposon insertion *zie-2*::Tn*10*, and the gene order *ilv zie-2*::Tn*10 rfe rff uvrD* has been established (15). In addition, the cotransduction frequency of *zie-2*::Tn*10* with *rff* has been reported to be 0.36 to 0.73. Phage P1-mediated transductions were done by using strain 21259 (*rff*$^+$) as donor and strain F1467 (*rff-726*) as recipient to determine the relationship between the *rff-726* mutation and the defective ManNAcA transferase activity in strain F1467. Tetracycline-resistant transductants were selected and subsequently screened for ManNAcA transferase activity by in vitro assay as well as for their ability to synthesize ECA in vivo.

The screening of the transductants revealed that 68% (17 of 25) of the transductants were able to synthesize ECA as determined by Western blot analysis by using mouse anti-ECA monoclonal antibody (Fig. 2). In addition, the ability of transductants to synthesize ECA correlated well with their observed levels of ManNAcA transferase activity (Table 1). Accordingly, the level of ManNAcA transferase activity possessed by the ECA-negative transductants was similar to that of strain F1467, and this level was approximately sixfold lower than the level of activity observed in the ECA-positive transductants. These findings were supported by the results of double-label experiments in which the incorporation of radioactivity into lipid I and lipid II was measured after the incubation of membranes with UDP-[^{3}H]GlcNAc and UDP-[^{14}C]ManNAcA (Table 2). The extent of [^{3}H]GlcNAc and [^{14}C]ManNAcA incorporation into lipid II was essentially the same with membranes prepared from either the donor strain 21259 or randomly selected transductants identified in the initial screening as possessing wild-type ManNAcA transferase activity. The addition of tunicamycin to reaction mixtures totally inhibited the synthesis of ^{3}H-labeled lipid I, and as a consequence, the synthesis of ^{3}H- and ^{14}C-labeled lipid II was also abolished. Tunicamycin specifically inhibits transferases that catalyze the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenyl monophosphate acceptors (9, 24, 25). Accordingly, earlier studies demonstrated that the in vitro synthesis of lipid I (reaction 1) is completely inhibited by tunicamycin (1). Tunicamycin does not inhibit the ManNAcA transferase; the effect of tunicamycin on lipid II synthesis is a consequence of the action of the drug on lipid I synthesis, and it reflects the precursor-product relationship between lipid I and lipid II (1).

The synthesis of lipid II was found to be markedly decreased when similar analyses were conducted by using membranes prepared from transductants initially characterized as possessing defective ManNAcA transferase activity (Table 2). Although the ability of these transductants to synthesize [^{3}H]GlcNAc-PP-lipid was unimpaired, their ability to transfer [^{14}C]ManNAcA to [^{3}H]GlcNAc-PP-lipid was significantly reduced.

In vitro synthesis of lipid II by using membranes from strain 21259 (*rff*$^+$) and exogenously supplied lipid I obtained from strain F1467. Experiments were conducted to examine the possibility that the inability of strain F1467 to synthesize lipid II is not due to defective ManNAcA transferase activity, but rather that the lipid I synthesized by the mutant might be structurally altered such that it is unable to function as an acceptor of ManNAcA residues. Accordingly, exogenously supplied lipid I synthesized by an *rff*$^+$ strain would be

TABLE 1. Relationship between ManNAcA transferase activity and ECA synthesis in tetracycline-resistant transductants of strain F1467

Strain	Relevant genotype or phenotype[a]	ManNAcA transferase activity[b]
21259	*rff*$^+$	3.50
F1467	*rff-726*	0.35
8 transductants	ECA$^-$	0.88 (± 0.38)
17 transductants	ECA$^+$	5.16 (± 1.18)

[a] ECA synthesis was determined by Western blot analysis as described under Materials and Methods.

[b] ManNAcA transferase activity is expressed as picomoles of lipid II synthesized 30 min^{-1} 250 μg of protein^{-1}. Enzyme activity was assayed by determining the amount of [^{3}H]GlcNAc incorporated into lipid II in vitro when reactions were done in the presence of unlabeled UDP-ManNAcA. Details are described under Materials and Methods.

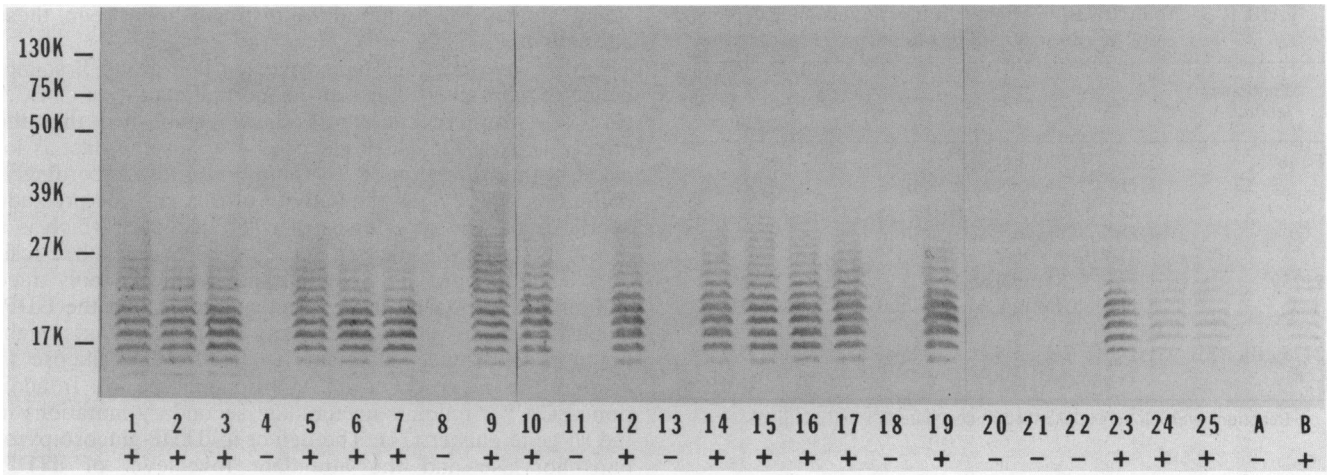

FIG. 2. The relationship between the ManNAcA transferase activity of tetracycline-resistant transductants and their ability to synthesize ECA. The presence or absence of ECA in the soluble fraction of sodium dodecyl sulfate extracts of 25 independent tetracycline-resistant transductants of strain F1467 was determined by Western blot analysis by using monoclonal anti-ECA antibody. The lane numbers in the figure also correspond to the identifying numbers of each transductant. The ManNAcA transferase activity of each transductant was assayed in vitro by measuring the amount of radioactivity incorporated into lipid II after the addition of UDP-[^{3}H]GlcNAc and unlabeled UDP-ManNAcA to reaction mixtures. A + or − under each lane indicates the detection of wild-type transferase activity or severely reduced transferase activity, respectively. Lane A, Recipient strain F1467 (*rff-726*); lane B, donor strain 21259 (*zie-2*::Tn*10* *rff*$^+$).

expected to function as an in vitro acceptor of ManNAcA residues by using membranes from strain F1467 as a source of the transferase. Thus, lipid I was synthesized in vitro by using membranes from strain 21259 (*rff*$^+$). The lipid-linked intermediate was then partially purified and used as an exogenously supplied acceptor of [^{14}C]ManNAcA residues in reaction mixtures containing membranes prepared from strain F1467. No incorporation of [^{14}C]ManNAcA into lipid II was observed when lipid I obtained from either strain 21259 or strain F1467 was used as an exogenously supplied acceptor (Table 3). In contrast, the incorporation of [^{14}C]ManNAcA into lipid II was markedly stimulated after the addition of F1467 lipid I to reaction mixtures containing membranes prepared from strain 21259. The low level of lipid II synthesis observed in the absence of exogenously supplied F1467 lipid I most likely reflects the presence of endogenous lipid I acceptor in the membrane preparations. These data indicate that lipid I synthesized by strain F1467 is indeed able to function as an acceptor of ManNAcA residues. In addition, these data further support the conclusion that the defect in lipid II synthesis in mutants possessing the *rff-726* lesion is due to a defect in ManNAcA transferase activity.

DISCUSSION

Recent studies have established that GlcNAc-pyrophosphorylundecaprenol (lipid I) and ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II) are intermediates in the biosynthesis of ECA (18). Lipid I serves as a precursor of lipid II in a reaction that involves the transfer of ManNAcA from UDP-ManNAcA to lipid I catalyzed by a specific ManNAcA transferase (reaction 2). The synthesis of UDP-ManNAcA occurs by the following sequence of reactions in both *E. coli* (5) and *Salmonella* sp. (7):

$$\text{UDP-GlcNAc} \rightleftharpoons \text{UDP-ManNAc} \quad (3)$$

$$\text{UDP-ManNAc} + \text{NAD}^+ \rightleftharpoons \text{UDP-ManNAcA} + \text{NADH} + \text{H}^+ \quad (4)$$

Reactions 3 and 4 are catalyzed by the enzymes UDP-GlcNAc 2-epimerase and UDP-ManNAc dehydrogenase, respectively. An investigation of *S. typhimurium rff* mutants

TABLE 2. Effect of the *rff-726* lesion on in vitro incorporation of radioactivity from UDP-[^{3}H]GlcNAc and UDP-[^{14}C]ManNAcA into lipid I and lipid II[a]

Strain	Relevant genotype or phenotype[b]	Addition of tunicamycin	Radioactivity (dpm) incorporated[c]		
			^{3}H-labeled lipid I	^{3}H-labeled lipid II	^{14}C-labeled lipid II
21259	*rff*$^+$	−	4,469	4,326	2,585
F1467	*rff-726*	−	3,236	530	206
Transductant 23	Transferase$^+$	−	2,924	4,704	2,759
		+	184	79	61
Transductant 24	Transferase$^+$	−	3,217	6,162	3,644
		+	45	40	46
Transductant 4	Transferase$^-$	−	2,580	487	323
Transductant 21	Transferase$^-$	−	4,532	647	177

[a] Reactions were done as described under Materials and Methods.
[b] Phenotypic designations are based on the results of initial screening assays that determined the amount of radioactivity from UDP-[^{3}H]GlcNAc incorporated into lipid II in vitro when reactions were done in the presence of unlabeled UDP-ManNAcA.
[c] Values shown represent the averages of the results obtained from two assays.

TABLE 3. Ability of F1467 lipid I to function as an acceptor of ManNAcA residues in vitro

Membrane source	Addition(s)	[^{14}C]ManNAcA incorporated into lipid II (dpm)[a]
F1467	UDP-[^{14}C]ManNAcA	0
	UDP-[^{14}C]ManNAcA + lipid I (21259)	35
	UDP-[^{14}C]ManNAcA + lipid I (F1467)	24
21259	UDP-[^{14}C]ManNAcA	134
	UDP-[^{14}C]ManNAcA + lipid I (F1467)	487
21259 (boiled)	UDP-[^{14}C]ManNAcA + lipid I (F1467)	35

[a] Reactions were done as described under Materials and Methods.

defective in the synthesis of UDP-ManNAcA has identified the structural genes for the 2-epimerase (*rffE*) and the dehydrogenase (*rffD*) (7). Although *E. coli* mutants defective in the synthesis of UDP-ManNAcA have not yet been reported, it is assumed that the structural genes for the 2-epimerase and the dehydrogenase are also located in the *ilv*-linked *rff* region of the *E. coli* chromosome. However, the location of the structural gene encoding the ManNAcA transferase, as well as the structural genes for other sugar transferases involved in ECA synthesis, has not been established in either *E. coli* or *Salmonella* sp.

Earlier investigations established that the *rff-726* mutation does not affect the biosynthesis of UDP-ManNAcA, dTDP-Fuc4NAc, or UDP-GlcNAc, all of which are required for ECA synthesis (15). Thus, it was proposed that the *rff-726* mutation might affect the activity of a sugar transferase involved in this process. The results of the present study clearly support the conclusion that the *rff-726* mutation results in defective ManNAcA transferase activity. Accordingly, examination of the ManNAcA transferase activity in membrane preparations obtained from strain F1467 (*rff-726*) and from strains possessing the wild-type allele revealed that the activity of the enzyme in the mutant was significantly impaired. Transduction of wild-type *rff* genes into strain F1467 restored the activity of the ManNAcA transferase to the wild-type level as well as the ability of the transferase-positive transductants to synthesize ECA. It is interesting that the activity of the ManNAcA transferase was not totally abolished by the *rff-726* lesion. However, the residual activity (approximately 16% that of wild-type strains) was apparently insufficient to support the synthesis of ECA in amounts that can be detected either chemically (15) or by Western blot analysis as described in the present study.

The ability of strain F1467 membrane preparations to catalyze the in vitro synthesis of GlcNAc-pyrophosphoryl-undecaprenol (lipid I) was unimpaired. In addition, the lipid I synthesized by the mutant strain appeared to be functionally equivalent to lipid I synthesized by *rff*$^+$ strains, as indicated by its ability to serve as an in vitro acceptor of ManNAcA residues. Thus, the *rff-726* lesion has no apparent affect on the synthesis of either undecaprenylphosphate or the UDP-GlcNAc:undecaprenylphosphate GlcNAc-1-phosphate transferase responsible for the synthesis of lipid I. It seems likely that the *rff-726* mutation resides within the structural gene for the ManNAcA transferase, which we propose to call *rffT*. However, it is also possible that the decreased level of transferase activity is due to a defect in a gene involved in the regulation of enzyme synthesis. Additional studies are required to distinguish between these alternatives.

Earlier investigations have revealed that group B salmonellae require an *rfb* function for normal synthesis of ECA (11, 14). More recent studies have established that the structural gene for dTDP-glucose pyrophosphorylase is located in the *rfb* region of *S. typhimurium* (6). Accordingly, mutations that abolish the activity of this enzyme preclude synthesis of wild-type levels of dTDP-glucose, which is a precursor of dTDP-Fuc4NAc, the donor of Fuc4NAc residues in ECA synthesis. Such mutants synthesize only trace amounts of ECA, and it has been suggested that the UDP-glucose pyrophosphorylase activity of these mutants might function to provide sufficient levels of dTDP-glucose to account for the so-called ECA^{trace} phenotype (6). In addition, ECA^{trace} mutants accumulate secondary mutations in the *rff* gene cluster (14). The defect in dTDP-glucose pyrophosphorylase and the attendant low level of dTDP-Fuc4NAc synthesis presumably leads to an accumulation of lipid II which is deleterious to the cell, and secondary *rff* mutations alleviate this condition (6). However, all secondary *rff* mutations examined thus far have been found to affect the synthesis of UDP-ManNAcA (7). It seems likely that a closer examination of such secondary *rff* mutants might also yield a class possessing a defective ManNAcA transferase as well as secondary mutants defective in the synthesis of lipid I.

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