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Genetic Location of Genes Encoding Enterobacterial Common Antigen

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A new *rff* mutation (*rff-726*) of *Escherichia coli* is described which affects the biosynthesis of the enterobacterial common antigen. This mutation was detected in an *rfe*-defective strain. A Tn*10* insertion near the *rfe* locus was isolated to facilitate further mapping. Both mutations *rfe* and *rff* were mapped by transduction with bacteriophage P1, giving the gene order *ilv rfe rff uvrD metE*. The F′ factor F14 was able to complement both mutations *rfe* and *rff*, whereas the F′ factor F16 could complement the *rfe* but not the *rff* mutation. The *rff* mutation did not affect the biosynthesis of *N*-acetyl-D-mannosaminuronic acid, as the previously described *rff* mutations in *Salmonella typhimurium* do (H. C. Lew, H. Nikaido, and P. H. Mäkelä, J. Bacteriol. 136:227–233, 1978), and also did not affect the biosynthesis of other enterobacterial common antigen components; however, the biosynthesis of the complete enterobacterial common antigen molecule was blocked.

The enterobacterial common antigen (ECA), discovered by Kunin et al. (10), is a component of the outer membrane of the cell wall of almost all members of the family *Enterobacteriaceae* (8, 16, 23, 28). It is an amphipathic molecule with a hydrophilic amino sugar chain and a hydrophobic (L-glycerophosphatidic acid) moiety (9). The amino sugar chain is built of repeating units each consisting of three amino sugars, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosaminuronic acid (ManNAcA) (20), and 4-acetamido-4,6-dideoxy-D-galactose (4-FucNAc) (14). It is responsible for the serological (ECA) specificity, whereas the lipid moiety anchors ECA in the outer membrane (9).

The investigation of the genetic determination of ECA is hampered by the fact that there is so far no direct way for selecting ECA mutants. Studies on ECA genetics depend therefore on mutants which were either found accidentally or selected indirectly. Such studies have shown that three gene loci are involved in the biosynthesis of ECA: *rfe*, *rff*, and in some *Salmonella* serotypes, the *rfb* gene cluster.

In some O groups of *Salmonella* spp. and *Escherichia coli* the *rfe* genes are required for both ECA synthesis and synthesis of O-antigenic polysaccharide (15). Their mode of action has, despite much effort (19), remained elusive. In contrast, *rff* genes are required exclusively for ECA biosynthesis (12). Several *rff* mutants were detected in *Salmonella typhimurium* and shown to be defective in the biosynthesis of ManNAcA, a component of the sugar part of ECA (12). *E. coli* K-12 also has *rff* genes providing the functions required for the synthesis of ECA (31).

In *S. typhimurium* the genes *rfbA* and *rfbB* are necessary for biosynthesis of dTDP glucose, an intermediate in the synthesis of the ECA component 4-FucNAc and of rhamnose, a component of the *S. typhimurium* O chain (21; H. C. Lew, H. Nikaido, H. Mayer, and P. H. Mäkelä, manuscript in preparation).

The genes *rfe* and *rff* are located close to *ilv* genes at 85 min on the *E. coli* chromosomal map, whereas *rfbA* and *rfbB* are part of the *rfb* operon (1). During mapping experiments with an *rfe*-defective *E. coli* O8 strain, an additional *rff* mutation was found in this strain. This *rff* mutation is so far the only discovered *rff* mutation in *E. coli*. In this study this mutation is mapped and partially characterized.

MATERIALS AND METHODS

Strains and media. Bacterial strains and bacteriophages used in this study are listed in Table 1. Media have been described previously (31). Tetracycline and kanamycin were added at 10 μg/ml and 50 μg/ml, respectively, to select or score for the presence of transposon Tn*10* or Tn*5*.

Genetic procedures. Bacterial conjugation and P1 transduction were performed as described (31). Transposon Tn*10* insertions were isolated after infection of strain C600 with phage λ NK370 (7). About 5,000 colonies were pooled, and P1 was grown on them. To select Tn*10* insertions near the *rfe* gene, strain F1312 was transduced with this P1, and ilv^+ Tc^r recombinants were selected.

ECA determination. ECA antiserum was prepared in New Zealand White rabbits by intravenous immunization with heat-killed *E. coli* O14:K7 (2387) or *E. coli* $O8^-:K27^-$ (F470) (23). The presence or absence of ECA was determined by a passive hemagglutination test (17). A modification of this method was developed to allow a rapid analysis of many bacteria. Bacteria were transferred to small areas (3 to 4 mm in diameter) of an agar plate and incubated for 18 h. The bacteria were scraped from the surface of the plate and resuspended in 10 μl of NaCl (0.9%) in Eppendorf tubes. The tubes were treated for 1 h at 100°C. The bacterial sediment was removed by centrifugation (2 min, 10,000 × *g*), and the clear supernatant was transferred to a second centrifuge tube and mixed with 0.5 ml of erythrocyte suspension (5 ml of NaCl [0.9%] plus 30 μl of packed sheep erythrocytes). The tubes were incubated 1 h at 37°C; after centrifugation (5 s, 10,000 × *g*), the sediment was washed once with 1.5 ml of NaCl (0.9%), and the pellet was resuspended in 0.5 ml of NaCl (0.9%). A total of 50 μl of this suspension and 50 μl of an ECA antiserum dilution (1:40, 1:80, 1:160, or 1:320 in NaCl [0.9%]) were transferred into a Cook microtiter plate

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TABLE 1. Bacterial strains and bacteriophages

E. coli strain or phage	Known markers	Reference or source
Strain		
EH726	O8⁻:K27⁻ *rfe rff-726*	(31)
AB1206	K-12 *thi-1 his-4 proA2 lacY1 galK2 rpsL8 tfr-3 supE44* Δ(*ilvE-argH*)/F14	Received as CGSC 1206 (25, 27)
AB1528	K-12 *proA2 his-4 ilvC7 argE3 thi-1 lacY1* or *lacZ4 galK2 supE44*?/F16	Received as CGSC 1528 (11, 25)
AB3505	K-12 *proA2 trp-4 his-4 ilvD188 metE46 argH1 lacY1* or *lacZ4 galK2 xyl-2 mtl-1 tsx-3 supE44*	Received as CGSC 3505 (32)
CGSC 4230	K-12 *ilv-282 argA42 argR40 argS41 galT23* Hfr PO63	(3)
C600	K-12 *thr-1 leu-6 thi-1 supE46 lacY1 tonA21*	Laboratory stock
CSH47	K-12 *supSu*⁺ Hfr	Cold Spring Harbor Collection (24) via B. Rak, Frb.
GW3703	K-12 *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	(26)
JC5088	K-12 *recA56* Hfr PO45	A. J. Clark via C. Beck
JC10241	K-12 *srl-300*::Tn*10* Hfr PO45	A. J. Clark via C. Beck
F459	O8:K27⁻ *ilv* Hfr45	(30)
F470	O8⁻:K27⁻ *met his pro mtl ara mal xyl rfb* Str^r	Laboratory collection
F1312	O8:K27⁻ *ilv his pro pyr met rha* Str^r	(31)
F1467	O8:K27⁻ as F1312, except *ilv*⁺ *rff-726*	This paper; P1 (EH726) → F1312, *ilv*⁺ selection
F1469	O8⁻:K27⁻ as F1312, except *ilv*⁺ *rfe rff-726*	This paper; P1 (EH726) → F1312, *ilv*⁺ selection
F1470	K-12 × O8 as 20895, except *his*⁺ O8⁺	This paper; F459 × 20895, *his*⁺ selection
2387	O14:K7	Laboratory stock
2537	O14:K7 *ilv his pro lac xyl* Str^r	Laboratory stock
20895	as AB3505, except Str^r	Spontaneous Str^r
21090	as CGSC 4230, except *ilv*⁺ *rff-726*	This paper; P1 (F1467) → CGSC 4230, *ilv*⁺ selection
21259	as F1312, except *ilv*⁺ *zie-2*::Tn*10*	This paper; P1 (C600::Tn*10*) → F1312, *ilv*⁺ Tc^r selection
21260	as F1470, except *ilv*⁺ *rfe rff-726*	This paper; P1 (EH726) → F1470, *ilv*⁺ selection
21261	as 21260, except *recA956*	This paper; P1 (JC5088) → P1 (JC10241), 21260, Tc^r *srl*⁺ selection
21262	as 21260, except *zie-2*::Tn*10*	This paper; P1 (21259) → 21260, Tc^r selection
21265	as 21260/F16 *zie-2*::Tn*10*	This paper; [P1 (21259) → AB1528, Tc^r selection] × 21260, Tc^r selection

Continued

TABLE 1—*Continued*

E. coli strain or phage	Known markers	Reference or source
21297	as F1467, except *zie-2*::Tn*10*	This paper; P1 (21259) → F1467, Tc^r selection
21316	O10:K5 Str^r	Laboratory stock
21318	as CSH47, except *zie-2*::Tn*10* *rff-726*	This paper; P1 (21292) → CSH47, Tc^r selection
21331	as 21261, except /F16 *zie-2*::Tn*10*	This paper; 21265 × 21261, Tc^r selection
21347	as GW3703, except *zie-2*::Tn*10* *rff-726*	This paper; P1 (21297) → GW3703, Tc^r selection
21352	as AB1206, except *zie-2*::Tn*10*	This paper; P1 (21259) → AB1206, Tc^r selection
21363	as 21260, except /F14 *zie-2*::Tn*10*	This paper; 21352 × 21260, Tc^r selection
21375	as 21261, except /F14 *zie-2*::Tn*10*	This paper; 21363 × 21261, Tc^r selection
Bacteriophage		
Ω8		Laboratory stock (5)
P1		Laboratory stock
λ NK370	*b221 cI857 cI171*::Tn*10* *Ouga-261*	N. Kleckner via B. Rak

(V shape). The plate was incubated for 1 h at 37°C. ECA-positive strains showed hemagglutination with these four antiserum dilutions, whereas ECA-negative strains showed no hemagglutination with any of these dilutions.

Determination of O8, O10, and K7 antigens. Phage Ω8 (5) was used to detect O8 chain synthesis as described (31). O10 antigen was determined by agglutination with O10 antiserum (kindly provided by B. Jann, Max-Planck Institut, Freiburg, Federal Republic of Germany) diluted in NaCl (0.2%). Twofold serial dilutions (50 μl) were mixed with equal volumes of bacterial suspensions (treated 1 h at 100°C) and incubated for 1 h at 37°C. Presence of K7 antigen was tested by the method of Kaijser (6) by using a K7-specific antiserum.

Chemical methods. ECA was isolated according to the method of Männel and Mayer (20). Hydrolysis and amino acid analysis were carried out as described previously (9).

RESULTS

***rfe* mutant strain EH726 has an additional *rff* mutation.** P1 transductions were performed with *E. coli* EH726 (*ilv*⁺; O8⁻ ECA⁻) as donor and the *E. coli* O8 strain F1312 (*ilv*; O8⁺ ECA⁺) as recipient. Only two types of *ilv*⁺ transductants were expected: one that would not receive *rfe* from the donor and remained O8⁺ and ECA⁺ and another type that received *rfe* from the donor and was therefore O8⁻ and ECA⁻. However, besides these two types of transductants, recombinants were obtained which were O8⁺ and ECA⁻ (8 of 215 *ilv*⁺ transductants). This phenotype is expected for *rff* mutants. From these data it was concluded that the donor strain EH726 had not only an *rfe* mutation but also an additional *rff* mutation. The *rff* mutation cannot be detected in this strain because the phenotype of an *rfe rff* mutant is similar to that of an *rfe rff*⁺ strain.

TABLE 2. Ordering the *rfe rff* region by three-factor transductional crosses

Transduction	Donor	Recipient	Selected marker scored	No. of transductants	Unselected markers	No. of transductants
a	F1467 *ilv*$^+$ *rff metE*$^+$	F1470 *ilv rff*$^+$ *metE*	*ilv*	42	*rff*$^+$ *metE*$^+$	1
					rff$^+$ *metE*	23
					rff metE$^+$	2
					rff metE	16
			metE	157	*rff*$^+$ *ilv*$^+$	0
					rff$^+$ *ilv*	143
					rff ilv$^+$	7
					rff ilv	7
b	F1273 *ilv*$^+$ *rfe metE*$^+$	F1470 *ilv rfe*$^+$ *metE*	*ilv*	67	*rfe*$^+$ *metE*$^+$	0
					rfe$^+$ *metE*	13
					rfe metE$^+$	20
					rfe metE	34
			metE	94	*rfe*$^+$ *ilv*$^+$	0
					rfe$^+$ *ilv*	88
					rfe ilv$^+$	2
					rfe ilv	4
c	21297 *ilv*$^+$ *zie-2*::Tn*10 rff*	20888 *ilv* Tc^s *rff*$^+$	*ilv*	100	*rff*$^+$ Tc^s	38
					rff$^+$ Tc^r	23
					rff Tc^s	0
					rff Tc^r	39
			Tc^r	100	*rff*$^+$ *ilv*$^+$	22
					rff$^+$ *ilv*	42
					rff ilv$^+$	19
					rff ilv	17
d	21262 *ilv*$^+$ *zie-2*::Tn*10 rfe*	F1470 *ilv* Tc^s *rfe*	*ilv*	99	*rfe*$^+$ Tc^s	33
					rfe$^+$ Tc^r	1
					rfe Tc^s	0
					rfe Tc^r	65
			Tc^r	66	*rfe*$^+$ *ilv*$^+$	4
					rfe$^+$ *ilv*	7
					rfe ilv$^+$	46
					rfe ilv	9
e	21259 *zie-2*::Tn*10 rfe*$^+$ *rff*$^+$	21260 Tc^s *rfe rff*	Tc^r	104	*rfe*$^+$ *rff*$^+$	76
					rfe$^+$ *rff*	19
					rfe rff?	9
f	21262 *zie-2*::Tn*10 rfe rff*	F1470 Tc^s *rfe*$^+$ *rff*$^+$	Tc^r	66	*rfe*$^+$ *rff*$^+$	11
					rfe$^+$ *rff*	0
					rfe rff?	55
g	21347 *rff uvrD*::Tn*5 metE*$^+$	20888 *rff*$^+$ Km^s *metE*	Km^r	119	*rff*$^+$ *metE*$^+$	13
					rff$^+$ *metE*	13
					rff metE$^+$	12
					rff metE	81
			metE	89	*rff*$^+$ Km^s	43
					rff$^+$ Km^r	19
					rff Km^s	3
					rff Km^r	24

***rfe* and *rff* mutations are located between *ilv* and *metE*.** For mapping the *rfe* and *rff* mutations, P1-mediated transductions were performed with the double-mutant EH726 (*rfe rff*) and the single mutant F1467 (*rff*) as donors. The recipient strain F1470 was a derivative of *E. coli* K-12 AB3505. Since *E. coli* K-12, a rough strain, does not produce O side chains, it was not possible to distinguish between *rfe* and *rff* mutants. To overcome this problem, in a cross the *his rfb* region of F459 (*E. coli* O8, Hfr) was introduced into AB3505 selecting for *his*$^+$ Str^r recombinants. The recombinant F1470 produced O8 chains, determined by the O8 *rfb* genes it had obtained. Selections were made for *ilv*$^+$ or *metE*$^+$, and the cotransduction frequencies with *rff* or *rfe* were determined.

rff is located between *ilv* and *metE* and is closer to *ilv* than

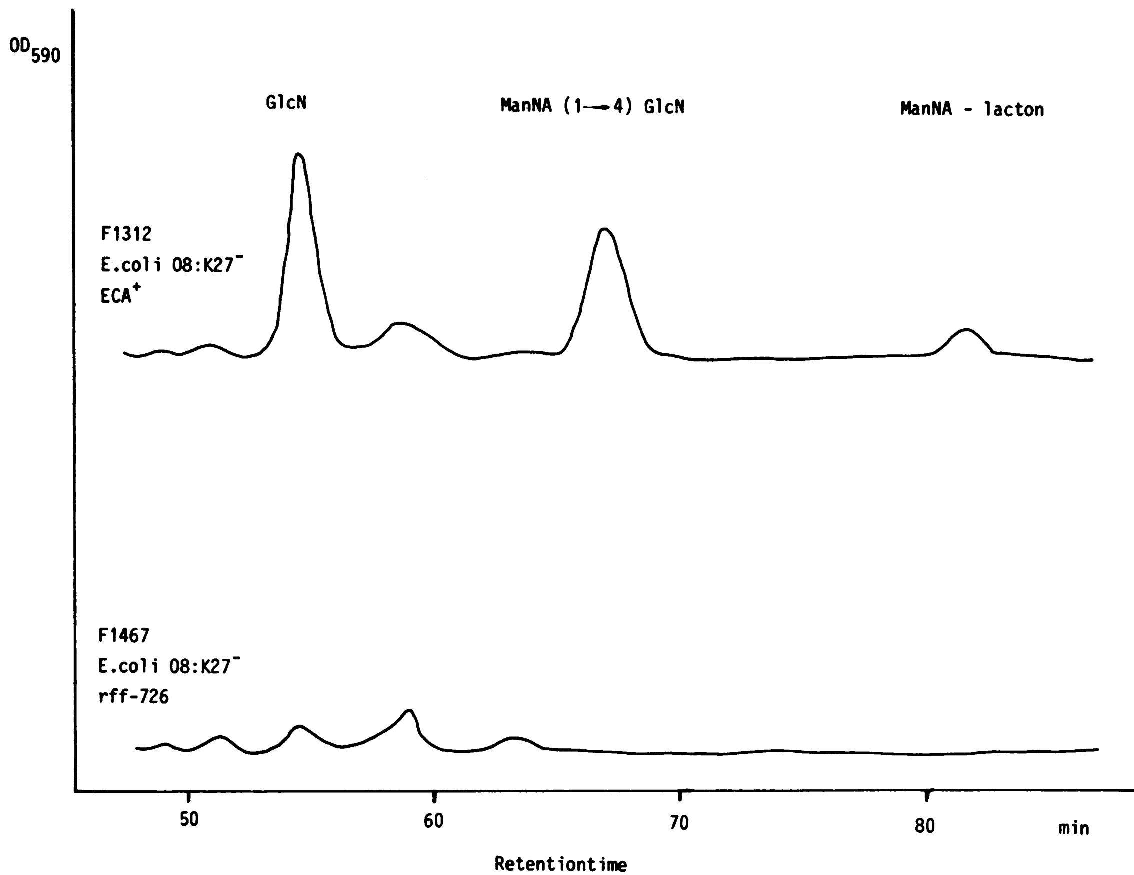

FIG. 1. Elution profile of hydrolysates of the PLL fraction from the ECA-positive strain F1312 and the *rff-726* mutant strain F1467.

to *metE*. The cotransduction frequency of *rff* with *ilv* was 0.43 (18 of 42) and that of *rff* (Table 2, transduction a) with *metE* was 0.09 (14 of 157). This suggests that *rfe* is located between *ilv* and *metE* and closer to *ilv* than to *metE* (Table 2, transduction b). The cotransduction frequency of *rfe* with *ilv* was 0.81 (54 of 67) and that of *rfe* with *metE* was 0.06 (6 of 94).

Insertion of transposon Tn*10* near *rfe* and *rff*. To facilitate mapping and strain construction, the following procedure was used to isolate a Tn*10* element near *rfe* and *rff*. Phage P1 was grown on a population of C600 clones containing Tn*10* elements inserted at random sites of the chromosome. The P1 lysate was used to transduce F1312 (*ilv*); ilv^+ Tc^r transductants were selected. These transductants were examined for cotransduction frequency of *rfe* with Tn*10*, and a transductant with a cotransduction frequency of ca. 0.88

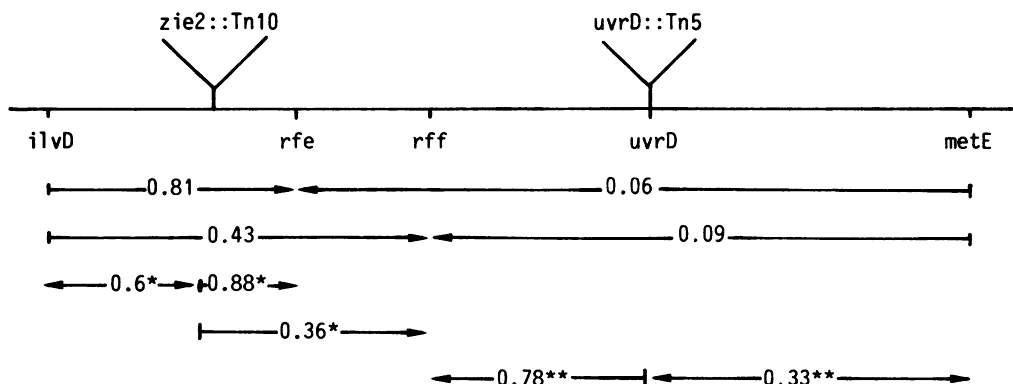

FIG. 2. Genetic map of the *rfe rff* region of *E. coli* showing cotransduction frequencies for markers connected by arrows. The arrows point to the unselected markers in P1 transductions. The order of the genes was determined from the data shown in Table 2. Symbols: *, cotransduction frequency including transduction of Tn*10*; **, cotransduction frequency including transduction of Tn*5*.

(150 of 170) was used for further mapping experiments. The transposon insertion *zie-2*::Tn*10* was transduced into F1467 (*rff*). One Tc^r *rff* transductant (21297) was used as donor in P1-mediated transduction; the recipient was 20888 (*ilv*). Selection was carried out either for ilv^+ or Tc^r, and nonselected markers were scored. From these data (Table 2, transduction c) we conclude that the transposon insertion *zie-2*::Tn*10* is located between *ilv* and *rff*. The cotransduction frequency of *ilv* with *zie-2*::Tn*10* was 0.52 (103 of 200) and that of *rff* with *zie-2*::Tn*10* was 0.36 (36 of 100).

In a similar experiment the transposon insertion *zie-2*::Tn*10* was transduced into 21260 (*rfe*). An *rfe* Tc^r transductant (21262) was used as donor and F1470 (*ilv*) was used as recipient in the following P1-mediated transduction experiment. Selection was carried out for ilv^+ or Tc^r, and nonselected markers were scored (Table 2, transduction d). The transposon insertion *zie-2*::Tn*10* maps between *ilv* and *rfe*.

Position of *rfe* and *rff* in relation to the transposon insertion *zie-2*::Tn*10*. So far it is not possible to detect phenotypically an additional *rff* mutation in an *rfe* strain. Therefore it is difficult to determine the sequential order of *rfe* and *rff*. To overcome this difficulty, two P1-mediated transductions were performed. Selection was carried out each time for the transposon insertion *zie-2*::Tn*10*. In one case an rfe^+ rff^+ strain (21259) was used as donor and an *rfe rff* strain (21260) was used as recipient (Table 2, transduction e), whereas in a second transduction experiment the donor was *rfe rff* (21261) and the recipient was rfe^+ rff^+ (F1470) (Table 2, transduction f).

In the first transduction 95 of the 104 Tc^r transductants had received the donor rfe^+ and 76 of these 95 also had received the donor rff^+. In the second transduction 11 of the 66 Tc^r transductants had retained the recipient's rfe^+ and could be scored for *rff*: none of them had received the donor *rff*. These data make it likely that the order of genes is *zie-2*::Tn*10* *rfe* *rff*.

***uvrD* gene is located between *rff* and *metE*.** We transduced the transposon insertion *uvrD*::Tn*5* to strain 21297 (*rff*) and isolated a Km^r *rff* transductant (21347). This strain was used as donor in a P1 transduction with AB3505 (*metE*) as recipient. Selection was done either for Km^r or $metE^+$. The results indicate (Table 2, transduction g) that the gene order is *rff* *uvrD*::Tn*5* *metE*. The cotransduction frequency of *rff* with *uvrD*::Tn*5* was 0.78 (93 of 119).

Complementation of *rfe* and *rff* mutations with F′ factors. The F′ factor F14 carries the *E. coli* chromosomal region from *ilvE* to *argH* (27). To facilitate further matings, the transposon insertion *zie-2*::Tn*10* was transduced from 21259 (*zie-2*::Tn*10*) to F14 in strain AB1206 [*his* Δ(*ilvE-argH*)/F14] by P1. A Tc^r transductant (21352) was used as donor and 21260 (*rfe rff metE arg trp*) was used as recipient. In suitably supplemented minimal agar, his^+ $metE^+$ recombinants were selected. After single colony isolation, the colonies were tested for Tc^r character and for the presence of ECA and O8 specificity. All recombinants tested were Tc^r and synthesized ECA and O8 antigen, indicating joint transfer of $metE^+$, rfe^+, rff^+, and Tn*10*.

In a second mating one of these recombinants was used as donor and a *recA rfe rff* strain (21261) was used as recipient. All Tc^r recombinants produced ECA and O8 antigen. These matings show that F14 is able to complement both the *rfe* and *rff* mutations.

To determine whether the smaller F′ factor F16, which carries *ilvE* and 28.4 kilobase pairs of adjacent chromosomal DNA (11), also complements the *rfe* and *rff* mutations, the following experiments were carried out. Tn*10* was again introduced into F16 by P1 transduction by using 21259 (*zie-2*::Tn*10*) as donor and AB1528 (*his*/F16) as recipient. Since the transposon insertion can be transduced in this strain either to F16 or to the chromosome, we used for further matings a pool of 10 Tc^r transductants as donor and the strain 21260 (*rfe rff arg trp*) as recipient. his^+ Tc^r recombinants were selected on suitably supplemented minimal agar. All recombinants had the O8 antigen but not ECA. In the following cross one of these recombinants (21265) was used as donor and 21261 (*rfe rff recA*) was used as recipient. arg^+ trp^+ Tc^r recombinants were tested for the presence of ECA and O8 antigen. All recombinants tested synthesized O8 but not ECA. These data suggest that F16 carries a part of the *E. coli* chromosome which complements the *rfe* but not the *rff* mutation. This conclusion is in agreement with the observation that *rfe* is located closer to *ilv* than is *rff*.

***rff* mutant strain F1467 does not synthesize ECA polysaccharide.** Männel and Mayer (20) described the isolation of a fraction (PLL) containing highly enriched ECA. By using the same procedure, the PLL fractions from an ECA-positive strain (F1312) and from its *rff* derivative (F1467) were isolated. After acid hydrolysis the hydrolysis products of the two PLL fractions were compared in an amino acid analyzer. The partial hydrolysate of ECA gives a characteristic pattern (9), based on the ECA constituents GlcNAc, ManNAcA-GlcNAc-disaccharide, and the lactone of ManNAcA. This pattern was obtained with the PLL fraction of the ECA-positive strain F1312, but not with the PLL fraction of the *rff* mutant F1467 (Fig. 1). This indicates that the *rff* strain did not synthesize ECA polysaccharide.

***rff-726* mutation does not affect the biosynthesis of ManNAcA.** The described *rff* mutants of *S. typhimurium* have defects in ManNAcA biosynthesis (12). To investigate whether the *rff* mutation in *E. coli* described here also affects ManNAcA biosynthesis, the *rff* mutation was introduced into *E. coli* serotype O14:K7. In this strain ManNAcA is not only a component of ECA but also of the K7 antigen (22). An *Hfr* cross was performed with 21090 (*E. coli* K-12 [ilv^+ *rff*]) as donor and with 2537 (*E. coli* O14:K7 [*ilv*]) as recipient. ilv^+ recombinants were selected and tested for the presence of ECA and the K7 antigen. Of 96 ilv^+ recombinants, 17 were ECA^- (*rff*) but only 5 of these were also K7 negative. This indicates that the *rff* mutation does not affect the biosynthesis of the K7 antigen and hence not of ManNAcA.

***rff-726* mutation does not affect the biosynthesis of 4-FucNAc.** The O10 antigen is known to contain, like ECA, the rare sugar component 4-FucNAc (4). To test whether the *rff* mutation affects the biosynthesis of 4-FucNAc, a cross with 21319 (*E. coli* K-12 [*Hfr*, *zie-2*:Tn*10* *rff*]) as donor and 21316 (*E. coli* $K5^-$:O10 [Str^r]) as recipient was performed. Tc^r Str^r prototrophic recombinants were selected. Of the 20 recombinants, 19 were ECA negative, but all of them were agglutinated by anti-O10 antiserum. This indicates that the *rff* mutation does not affect the biosynthesis of the O10 antigen and hence not of 4-FucNAc.

DISCUSSION

A genetic map of the *rfe rff* region (Fig. 2) could be constructed from the data of the P1 transduction experiments described. The ECA-determining genes *rfe* and *rff* are both located between *ilv* and *uvrD*; *rfe* is closer to *ilv* than *rff* is.

The *rffD* and *rffE* mutations in *S. typhimurium* have been

found in lipopolysaccharide-defective *rfb* deletion mutants. When *rff* in these strains is replaced by *rff*$^+$, the recombinants again accumulate *rff* mutations (18). The *rfb* deletion strains have a defect in the biosynthesis of 4-FucNAc, a sugar of the ECA repeating units. This will cause the accumulation of an oligosaccharide lipid intermediate, a condition that could be alleviated by the *rff* mutations (Lew et al., manuscript in preparation). The *rff-726* mutation described in this paper has been found in a lipopolysaccharide-defective *E. coli* strain possessing an *rfe* mutation. It may be of interest whether the *rfe* mutation in *E. coli*, like the *rfb* mutations in *S. typhimurium*, favors the appearance of secondary mutations in *rff*.

It is difficult to construct a strain containing *rfe* and *rff*$^+$ genes, since both genes are located very close to each other. Starting with a *rfe rff* double mutant, most *rfe* transductants are expected to be also *rff*, since both genes are cotransduced with high frequency. A further problem is that it is not possible to differentiate phenotypically whether an *rfe* transductant is *rff* or *rff*$^+$.

Another way to examine whether *rfe* mutant strains accumulate *rff* mutations similar to the reported *rfb* deletion strains (18) is complementation studies with the F16 plasmid. As shown in this paper, this plasmid complements *rfe* but not *rff* mutations. *rfe rff* double mutants containing the F16 plasmid synthesize O8 chains but do not produce ECA. On the contrary, *rfe rff*$^+$ strains containing the F16 plasmid should be able to synthesize both O8 chains and ECA. In further experiments we want to examine several independent *rfe* mutants for complementation with F16. An analysis of these recombinants will show whether *rff* mutations accumulate in *rfe* mutant strains.

It could be shown that the *rff-726* mutation did not affect the biosynthesis of two typical ECA components, ManNAcA and 4-FucNAc. Furthermore, the *rff* mutant had normal lipid A (13) and thus must be able to synthesize GlcNAc. The same is true of murein (2) and the lipid anchor of ECA, which is identical to the lipid part of phospholipids. In fact the lethality of the GlcNAc mutation has been shown (29). By exclusion it seems likely that the *rff-726* mutation may affect an ECA transferase activity.

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