JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1141-1149

Vol. 127, No. 3
Printed in U.S.A.

Enterobacterial Common Antigen in *rfb* Deletion Mutants of *Salmonella typhimurium*

P. HELENA MÄKELÄ,* G. SCHMIDT, H. MAYER, H. NIKAIDO, H. Y. WHANG, AND E. NETER

Central Public Health Laboratory, Mannerheimintie 166, SF-00280 Helsinki 28, Finland; Max-Planck-Institut für Immunbiologie, Stübeweg 51, 78 Freiburg-Zähringen, Germany; Department of Bacteriology and Immunology, University of California, Berkeley, California 94720; and Departments of Pediatrics and Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14222*

Received for publication 9 March 1976

The *his-rfb* deletion series of *Salmonella typhimurium* mutants characterized previously by Nikaido et al. was examined for the presence of the enterobacterial common antigen (ECA). All deletions not extending further to the left than the genes for cytidine diphosphoabequose synthesis were ECA positive, whereas longer deletions (extending to the genes for thymidine diphosphorhamnose synthesis or further) were ECA negative. When these long-*his-rfb* deletion strains were studied further, it became clear that they (four out of four studied) had accumulated a second mutation, called *rff*, close to *ilv*, which prevented the synthesis of ECA. When *rff*$^-$ was replaced by *rff*$^+$, the recombinants, now having the *his-rfb* deletion only, produced traces of ECA, showed reduced viability, increased sensitivity to sodium dodecyl sulfate (SDS) and, to a lesser extent, to other anionic detergents, and accumulated secondary "suppressor" mutations upon storage. Such suppressor-containing mutants could be isolated by selecting for resistance to 1% SDS. Thirty of 46 SDS-resistant mutants studied had a second mutation, which alone prevented the synthesis of ECA, close to *ilv*. This *ilv*-linked mutation was similar to the *rff* mutation of the strains studied originally. The new *rff* mutation was similar to previously described *rfe* mutations in its close linkage to *ilv* and association with an ECA-negative phenotype. It differed from *rfe*, however, by not affecting the synthesis of the O antigens (O-6,7) of group C_1. In *Salmonella* group C_1, all ECA genes identified thus far are linked to *ilv* (*rfe* and/or *rff*) and none is linked to *rfb*.

The molecular basis for the enterobacterial common antigen (ECA) described by Kunin et al. in 1962 (10) being a typical component of enteric bacteria has been very elusive, and several conflicting reports have dealt with it (7-9). Its properties are most consistent with glycolipid structure, and this is supported by recent findings in the laboratory of H. Mayer (to be published). This view is in accord with the finding that genes participating in the determination of the cell wall lipopolysaccharide (LPS) are also required for the production of ECA (14, 15).

A study of the well-known series of *Salmonella minnesota* rough (R) mutants (11) showed that the *rfe* gene(s), which is required for the synthesis of the O-antigenic polysaccharide (whose structure is determined by the *rfb* cluster of genes), is also needed for ECA (15). Therefore, *rfe*$^-$ mutants are rough and ECA negative.

The same is true of *S. montevideo* of O group C_1 (O antigens 6,7): the function of *rfe* genes is needed for the synthesis of the O-antigenic polysaccharide chain and of ECA (13, 14). On the other hand, in *S. typhimurium* (O group B, with O antigens 4,12) *rfe* genes are not needed for O antigen synthesis. It has been suggested on the basis of these results that the synthesis of the O-antigenic polysaccharide of groups C_1 and B differs in some basic way, e.g., in their use of a lipid carrier (14). Hybrid strains between groups B and C_1 showed that even in group B there are *rfe* genes (designated B-*rfe*) that participate in the synthesis of ECA. The situation is apparently somewhat more complicated, however: the B-*rfe*$^+$ genes in combination with the *rfb*$^+$ genes of group C_1 could support the synthesis of LPS with the C-*rfb*$^+$-determined O antigens 6,7 but of only minimal amounts of ECA. Even R strains with nonfunctional (mutated) C-*rfb* genes or a likewise nonfunctional hybrid C/B-*rfb* region were of this ECA^{trace} (or ECA^{tr}) phenotype, suggesting that

TABLE 1. *ECA phenotype as determined by rfe and rfb genes*[a]

Genetic constitution		ECA
rfe	*rfb*	
C^+	Any form	+
C^-	Any form	−
B^+	B^+	+
B^+	C^+	tr
B^+	C^-	tr
B^+	C/B	tr
B^-	Any form	−

[a] Reference 14 and unpublished data of P. H. Mäkelä and H. Mayer. The origin of the *rfe* and *rfb* genes from either group C or group B is marked with C or B, respectively; +, functional; −, mutated genes; tr, trace.

the *rfb* region of group B somehow participates in the production of ECA. A summary of these findings is presented in Table 1.

This hypothesis could be tested further since a large series of deletion mutants involving the *rfb* region of group B was available (16). In these strains the deletion extends from the *his* genes clockwise into the *rfb* region (also covering the non-essential genes in between); the extent of the *rfb* region deleted was deduced from the missing function of its product enzymes. These enzymes are those participating in the synthesis of the nucleotides of the sugars abequose, mannose, rhamnose, and galactose, which are part of the O-antigenic polysaccharide chain of group B and are determined by B-*rfb* genes (17). In addition, the B-*rfb* region very probably contains genes determining the transferases required to put together the O-specific repeating units (17). Many of these transferases have not been assayed, and the position of their genes is therefore unknown, although a gene, *rfbP*, required for the production of functional uridine 5'-diphosphate galactose:undecaprenol phosphate galactose 1-phosphate transferase has been mapped (M. Levinthal and H. Nikaido, unpublished data). The presence of still other types of genes in the *rfb* region is probable; e.g., "*X*" and "*Y*" were "identified" on the basis of their effects on levels of certain enzymes (16).

MATERIALS AND METHODS

Bacteria. Most strains are derivatives of *S. typhimurium* LT2; all strains are described in Table 2. Genetic material derived from *Salmonella* group C_1 is marked with the prefix C; otherwise the strains are of group B origin. For clarity, functional genes may be marked with a superscript $^+$: e.g., C-*rfb*$^+$ = functional (nonmutated, wild-type) *rfb* gene cluster from group C_1. Several strains were received from B. A. D. Stocker, Stanford University, Stanford, Calif., and from K. E. Sanderson, Salmonella Genetic Stock Center, University of Calgary, Alberta, Canada.

Culture methods. As a rule, the stock cultures were kept in tubes of nutrient agar (only 0.6% agar) at room temperature. Genetic donor strains were preserved by subculturing at ca. 2-month intervals, at which times they were tested to make certain that they had retained their original properties (this was necessary because of the relative instability of *Salmonella* donor strains [20]). The unstable derivatives prepared in the course of this work were tested within a few months after isolation, and their properties were confirmed each time.

The sensitivity of the bacteria to various antimicrobial agents was tested on nutrient agar plates (20) containing either no addition or sodium dodecyl sulfate (SDS), sodium cholate, deoxycholate, polymyxin E sulfate, erythromycin, or gentian violet at the concentrations indicated in Table 4. A suspension was made from one colony on nutrient agar in 1 ml of sterile water; a glass rod was dipped into this suspension and used to inoculate one section of a testing plate at a time. Growth was recorded by comparing the number and size of colonies with those of colonies on plain nutrient agar. As a supplement, we used the disk sensitivity test (4) on Mueller-Hinton agar with impregnated paper disks from AB Biodisk (11458; Stockholm, Sweden).

LPS characteristics. LPS characteristics of the bacteria were tested by slide agglutination from growth on nutrient agar in 4% saline and in suitable dilutions of O-4,12 and O-6,7 rabbit antisera as described previously (12). In most cases, these results were confirmed by testing the sensitivity of the bacteria to a set of phages selected to characterize various smooth (S) and rough (R) forms (26).

ECA. ECA was determined by the ability of saline extracts (1 h at 100°C) of the bacteria to sensitize sheep or human erythrocytes to agglutination by anti-ECA sera as described previously (15). The anti-ECA sera used were from rabbits immunized with a *Shigella boydii* R mutant (type 3^-, strain 3140) or with *Escherichia coli* O14. All sera gave similar results; most of the strains were tested in two or three of the laboratories with identical results. Usually the results were clearly positive or negative, but with some strains "trace" reactions were seen (14, 15) (designated ECA^{tr}). Both the negative and trace reactions were confirmed by testing the ability of the same extracts to inhibit the hemagglutination between these antisera and cells sensitized by extracts of the ECA-positive *S. typhimurium*.

Genetic methods. Mutants were selected after treatment of the parent culture with diethyl sulfate (13). Bacterial conjugation (20) was the standard method of genetic analysis. All mutants and recombinants were used only after single-colony isolation on nutrient agar.

RESULTS

***rfb* region.** ECA determination of *his-rfb* deletion mutants suggested a pattern (Fig. 1):

TABLE 2. *Salmonella strains used*[a]

Strain no.	Relevant genotype	Derivation, reference, and/or remarks
Extended deletions in the *his-rfb* region and their derivatives		
his-386, *his-399*, *his-658*, *his-809*, and others shown in Fig. 1		(16)
SH5148	*his-386 rff-4272 ilv-1188*	*ilv* mutant selected from *his-386*
SH5149	*his-399 rff-4271 ilv-1189*	*ilv* mutant selected from *his-399*
SH5150	*his-809 rff-4270 ilv-1190*	*ilv* mutant selected from *his-809*
SH4480	*his-658 rff-4273 ilv-1180*	*ilv* mutant selected from *his-658*
SH5185 and others	*his-386* + +	ilv^+ rff^+ recombinants from cross HfrK1-2 × SH5148
SH5187 and others	*his-399* + +	ilv^+ rff^+ recombinants from cross HfrK1-2 × SH5149
SH5177 and others	*his-809* + +	ilv^+ rff^+ recombinants from cross HfrK1-2 × SH5150
SH4499 and others	*his-658* + +	ilv^+ rff^+ recombinants from cross SH3361 × SH4480
SH5838 and others	+ *rff-4271 ilv-1189*	his^+ recombinants from cross SW1403 × SH5159
SH5840 and others	+ *rff-4270 ilv-1190*	his^+ recombinants from cross SW1403 × SH5150
SH5283	*his-386 rff-4135*	SDS-resistant mutants of SH5185 and others
SH5284 and others	*his-386 rff-4136* etc.	
SH5279 and others	*his-399 rff-4131* etc.	SDS-resistant mutants of SH5187 and others
SH5273	*his-809 rff-4125*	SDS-resistant mutants of SH5177 and others
SH5274 and others	*his-809 rff-4126* etc.	
Reference strains		
SL1715	*act-201 rfa-942*	B. A. D. Stocker, personal communication
SL1761	*act-201*	
SH4420	*pmrA5*	Line SD14 (26; P. H. Mäkelä et al., manuscript in preparation
SH5357	*pmrA8 rfa-4041*	
SL1102	*rfaE543*	Line SD14 (26)
TV119, TV149, TV157, TV205, TV208	*rfb* point mutants	Line SD14 (25)
Donor strains		
SL696 (F^+)	*metA22 trpB2 strA120*/F	(20, 26)
SH344 (FS59)	*proAB47 purE66*/F $pyrB^+$	(3, 20)
SA841 (FS400)	*metE338 ilvA401 his-5406 str*/F rfb^+ gnd^+ his^+	(20)
SU418 HfrB2	*proA26*	CW, 0-53-57 (20)
SA464 HfrK1-2	*serA13 rfa-3058*	CW, 0-117 (20)
SA722 HfrK10	*serA15 pur-268*	CCW, 0-123 (20)
SH3357 HfrH1	*purE66 aro-851 met-1151 str-501*	*S. abony* (20)
SW1403 HfrH2	*aro-851 met-1151 str-501*	*S. abony* (20)
SH5152 HfrH2	C-rfb^+ *aro-851 met-1151 str-501*	his^+ rfb^+ recombinant from cross between HfrH14 and a *his* derivative of SW1403
SH471 HfrH14	*pur-258*	*S. montevideo* mutant of SH672 (12)

[a] Standard genetic symbols except as follows: *act*, actinomycin D resistance; *pmr*, polymyxin resistance; and *rff*, gene for synthesis of enterobacterial common antigen as described in this paper. For Hfr strains, the point of origin is shown in minutes as 0- and the direction of chromosome transfer is shown as CW (clockwise) or CCW (counterclockwise).

the strains with a short deletion were ECA positive; those with a long deletion were ECA negative. Mutant *his-520* (not shown in Fig. 1) appeared to be an exception; it extended to the area of the "long" deletions but was ECA^+. Upon further analysis, however, it turned out that this strain had, in fact, two independent mutations near the two ends of the *rfb* cluster (M. Levinthal, personal communication).

The division point between ECA-positive and ECA-negative mutants is between *his-801* and

his-399. *his-801*, the last ECA-positive mutant, has all of the enzymes required for rhamnose synthesis but lacks enzymes of cytidine diphosphoabequose (CDP-abequose) and guanosine diphosphomannose (GDP-mannose) synthesis. Many *rfb* point mutants were also tested for ECA. They were all ECA negative, including TV208, which lacks the enzyme RHA-3 (thymidine diphosphorhamnose [TDP-rhamnose] synthetase [16]). These findings indicated the presence of an "ECA gene" near the *rfbA* gene, which determines TDP-glucose pyrophosphorylase. To confirm this, genetic crosses were made to replace the deleted *his-rfb* region with the functional *his*$^+$-*rfb*$^+$ genes of smooth donor strains. Many different donors of *S. typhimurium* (SL696 [F], SH344 [FS59], SA841 [FS400], and SA722), *S. abony* (SH3357, SW1403, and SH5152), and *S. montevideo* (SH471) were used with the deletion mutants *his-399*, *his-809*, and *his-658* as recipients. All *his*$^+$ recombinants selected were smooth (O-4,12 or O-6,7, depending on the donor), indicating that the entire deleted *his-rfb* area had been replaced by genetic material from the donor. To our surprise, all of these recombinants were still ECA negative.

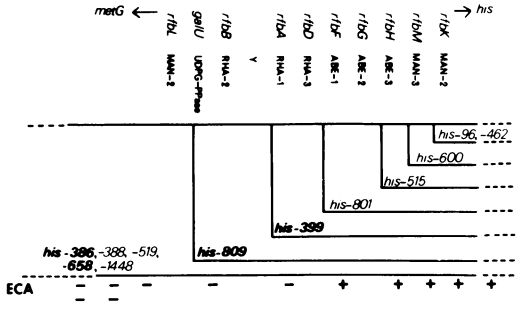

FIG. 1. *Partial schematic map of the rfb region of the S. typhimurium chromosome and the extent of deletions into rfb of the his deletion mutants shown (16). Bottom line: Presence (+) or absence (−) of ECA in these mutants. MAN-2, MAN-3, etc. refer to enzymes determined by the rfb genes (MAN-2, phosphomannomutase; MAN-3, GDP-mannose pyrophosphorylase; ABE-3, "CDP-abequose synthetase"; ABE-2, CDP-glucose oxidoreductase; ABE-1, CDP-glucose pyrophosphorylase; RHA-3, "TDP-rhamnose synthetase"; RHA-1, TDP-glucose pyrophosphorylase; RHA-2, TDP-glucose oxidoreductase; UDPG-PPase, UDP-glucose pyrophosphorylase; Y, unidentified function [deletion of Y results in derepressed levels of the product of rfbL]).*

An *rfe* mutation? The inevitable conclusion from these results was that a mutation, which was located not very close to *his*, was responsible for the ECA-negative phenotype of the strains containing long *his-rfb* deletions. Since the *rfe* genes had already been implicated in ECA synthesis, the next step was obvious: to look for a possible *rfe* mutation in these strains. Operationally, *rfe* mutation would be close to *ilv* at min 122 (13). We therefore isolated *ilv* mutants from four ECA-negative deletion strains and used them as recipients in crosses with *ilv*$^+$ ECA$^+$ donors (HfrK-10, HfrK1-2, and HfrH1) (first class of recombinants in Table 3). No ECA-positive recombinants were found in the *ilv*$^+$ class. Upon closer examination, most of these recombinants differed, however, from their female parent by having trace amounts of ECA. It was considered possible that these *ilv*$^+$ recombinants had received donor *rfe*$^+$ alleles, which changed the ECA phenotype of the *his*-deletion strains from ECA$^-$ to ECA^{tr}. This suggested that the original ECA-negative deletion strains contained two mutations: the *rfb* deletion, which reduced ECA production to a trace level, and an *rfe*-like mutation, which completely abolished the production of ECA. Whether the "*rfe*-like" mutation of these

TABLE 3. *Behavior of LPS and ECA in crosses between ECA-negative ilv*$^-$ *mutants of four long-his-rfb deletion strains as recipients and ECA-positive donors of Salmonella group B*[a]

Recipient			Recombinant class								
			his$^-$ *ilv*$^+$			*his*$^+$ *ilv*$^-$			*his*$^+$ *ilv*$^{+b}$		
Genotype	LPS	ECA	LPS	ECA	Frequency[c]	LPS	ECA	Frequency	LPS	ECA	Frequency
his-386 ilv-1188	R	−	R	tr	25/28	NO	NO	NO	S	+	26/26
his-399 ilv-1189	R	−	R	tr	23/26	S	−	10/10	S	+	30/30
his-809 ilv-1190	R	−	R	tr	13/14	S	−	38/38	S	+	14/14
his-658 ilv-1180[d]	R	−	R	tr	4/12	S	−	38/38	S	+	14/14

[a] R, Rough; S, smooth (O-4,12); tr, trace; NO, none obtained.

[b] This class was obtained by isolating *his*$^+$ recombinants from crosses in which the first class of recombinant (*ilv*$^+$ ECA^{tr}) was used as recipients.

[c] Number of recombinants with this LPS and ECA type/number of recombinants of this class tested.

[d] This *ilv* mutation is very revertable and most *ilv*$^+$ "recombinants" isolated were probably *ilv*$^+$ reversions instead.

strains is identical to *rfe* or not is discussed below. When an ECA^{tr} *ilv*$^+$ recombinant was crossed again as recipient with a smooth *S. typhimurium* donor strain, the *his*$^+$ recombinants isolated were smooth and ECA positive, confirming this hypothesis (last class of recombinants in Table 3). The second class of recombinants in Table 3 shows, for comparison, the phenotype of *his*$^+$ recombinants isolated from similar crosses with the original ECA$^-$ deletion strains: they are all smooth but ECA negative.

Why should this be so? Why do so many (probably all) long-*his-rfb* deletion strains have a second *rfe*-like mutation? The possibility was first considered that, by chance, the long deletions were isolated from a common parent that happened to be ECA negative. This was not true, however. The parental strain for some ECA-positive and ECA-negative *his-rfb* deletion mutants was one strain, and the parent of other ECA$^+$ and ECA$^-$ strains was another LT2 derivative. A second possibility would be selection of a *rfe*-like mutation, e.g., during storage. This would be more likely if the phenotype of the long *his-rfb* deletions could be shown to favor such a selection. Some proof for this was obtained in the following way.

Strains containing only the *his-rfb* deletions. From the crosses shown in Table 3, we could pick *ilv*$^+$ ECA^{tr} recombinants that had only the original *his-rfb* deletion mutation. When such recombinants were examined, they were rough by agglutination and phage sensitivity, as expected from their *rfb* genotype, but looked "sick." Their colonies were smaller and there was more variation in their size and shape on nutrient agar, when compared with wild-type strains.

When tested on detergent-containing media, they appeared to be much more sensitive than wild-type strains or *rfb* point mutants (compare strains of type 2 with types 4, 8, and 9 in Table 4). Their doubly mutant parents (strain type 1) were much closer to normal, although even they were slightly sensitive to sodium cholate and deoxycholate. Of other detergent-sensitive mutants known, the polymyxin-resistant *pmrA* mutants (Mäkelä et al., manuscript in preparation) and the actinomycin D-sensitive *act* mutants (B. A. D. Stocker, personal communication) (types 5 and 6 in Table 4) were not sensitive to SDS. In fact, only the "heptoseless," deep R mutants, which are known to be very sensitive to many toxic agents (19, 21, 22) and have extensive disorganization of their outer membrane structure (1, 23), showed sensitivity to SDS (strain type 7 of Table 4).

In addition, these deletion mutants were slightly more sensitive to polymyxin than most other strains. Their sensitivity to gentian violet and erythromycin was not changed measurably (Table 4), and in this they differed drastically from the very sensitive deep R mutants. No change was observed in their sensitivity to ampicillin, penicillin G, cephalosporins, neomycin, kanamycin, gentamicin, chloramphenicol, tetracycline, trimethoprim, or sulfonamides when tested by the less-accurate paper disk method.

SDS-resistant double mutants. The appar-

TABLE 4. *ECA and growth[a] of various strain types on nutrient agar containing sodium dodecyl sulfate (SDS), sodium cholate (SC), deoxycholate (DOC), polymyxin (PM), gentian violet (GV), or erythromycin (Er)*

Strain type	Relevant genotype	Derivation	LPS	ECA	Growth on medium containing:								
					SDS, 1.0%	SDS, 0.3%	SDS, 0.1%	SC, 3.0%	DOC, 0.3%	PM, 1 IU/ml	PM, 0.3 IU/ml	GV, 10 μg/ml	Er, 4.5 μg/ml
1	*his*$^-$ *ilv*$^-$	*his-386, his-399, his-809*	R	−	+	++	++	+−	+−	+	++	++	++
2	*his*$^-$ *ilv*$^+$	*ilv*$^+$ recombinants of 1	R	tr	−[b]	−[b]	−[b]	−	−	−	++	++	++
3	*his*$^+$ *ilv*$^-$	*his*$^+$ recombinants of 1	S	−	+	++	++	++	++	++	++	++	++
4	*his*$^+$ *ilv*$^+$	*his*$^+$ recombinants of 2	S	+	++	++	++	++	++	++	++	++	++
For comparison													
5	*act*	SL1715, SL1767	R or S	+	+	++	++	++	−	−	++	−	−
6	*pmrA*	SH4420, SH5357	R or S	+	+	++	++	+	+−	++	++	++	++
7	*rfaE*	SL1102	R[c]	+	−	−	−	−	−	−	+	−	−
8	*rfb*	Point mutants	R	+	+	++	++	++	++	++	++	++	++
9	Wild type		S	+	+	++	++	++	++	++	++	++	++

[a] Graded as follows: ++, normal growth; −, no growth; + small colonies; +−, some strains +, some −.
[b] Many resistant colonies.
[c] "Deep rough," chemotype Re (11, 26).

ent permeability defects of the single *his-rfb* deletion mutants possibly accounted for the frequent accumulation of secondary mutations in these strains. If the double mutants grew better and were less sensitive to toxic agents, this would provide a very efficient selection in their favor.

We used the sensitivity to 1% SDS of the single *his-rfb* deletion strains as a method for selecting secondary mutants. Since we hoped that many such secondary mutations would represent the *rfe*-like type described above and therefore be *ilv*-linked, we had to select new *ilv* mutants of the just-isolated *his-rfb* deletion strains (= the *ilv*$^+$ recombinants of Table 3 and type 2 of Table 4). These were then spread on nutrient agar plates containing 1% SDS; a large number of colonies grew on these plates. After single-colony isolation on nutrient agar, such SDS-resistant mutants (all independent isolates) were tested. They all were SDS resistant but varied in their sensitivity to deoxycholate and sodium cholate. In fact, this behavior was very similar to that of the deletion mutants (double mutants) tested originally (strain type 1, Table 4).

To identify the new mutations, we made the following crosses. First, the SDS-resistant mutants were used as recipients in crosses in which *his*$^+$ (also *rfb*$^+$) recombinants were selected. If these recombinants remained ECA negative, the second mutation selected in the SDS resistance selection might be the cause of it, as was the case with the *his*$^+$ recombinants listed in Table 3. These recombinants were then used as recipients in other crosses, selecting *ilv*$^+$ recombinants. If the SDS resistance mutation had occurred near *ilv* and were *rfe*-like, many of the *ilv*$^+$ recombinants should have become ECA positive. Table 5 shows the results of such crosses involving 46 SDS-resistant mutants derived from three separate *his-rfb* deletion strains.

All crosses between these mutants as recipients and HfrB as donor produced *his*$^+$ recombinants (Table 5, part I), which were smooth (O-4,12), as they should be when the recipient's deleted *his-rfb* region is, as a whole, replaced by the donor's *his*$^+$-*rfb*$^+$ region. Thirty of the SDS-resistant mutants gave rise to ECA-negative *his*$^+$ recombinants only: these SDS-resistant mutants had apparently acquired another mutation, not close to *his*, affecting the production of ECA. Seven of the ECA-negative *his*$^+$ recombinants (derived from seven separate SDS-resistant mutants) were used as recipients in second crosses with HfrK1-2 as the donor, with selection for recombinants that received the donor *ilv*$^+$ genes (Table 5, part II). In all seven, the mutation responsible for the ECA-negative phenotype turned out to be close to *ilv*.

The ECA-negative phenotype of the *his*$^+$ recombinants also demonstrated that the *ilv*-linked, *rfe*-like mutation alone, without the *rfb* deletion, was sufficient to block the synthesis of ECA.

In the remaining 16 SDS-resistant mutants (whose *his*$^+$ recombinants were ECA$^+$), the mutation leading to SDS resistance was apparently of a different nature. It either did not affect the synthesis of ECA or was in the *his-rfb* region so that it was replaced by its wild-type allele in the *his*$^+$ recombinants. These mutations were not studied further.

In several cases, we also happened to see the "natural" selection of double mutants from single *his-rfb* deletion strains taking place during storage in agar stabs. On retesting after 6 to 18 months, usually SDS-resistant colonies only were found. On four occasions these were tested by crosses with Hfr donors: the *his*$^+$ recombinants were still ECA negative, confirming the presence of a second mutation.

Further characterization of the *rfe*-like mutation (*rff*?). The *rfe*-like mutation described here has two of the characteristics of a mutation in a *rfe* gene: the gene is close to *ilv* and the mutation affects the synthesis of ECA. A third property of *rfe* mutations is the one by which they were discovered: they prevent the synthesis of the O antigen of *Salmonella* group C_1 (O-6,7). We therefore tested the *rfe*-like mutations in this respect. Table 6 shows the outcome of crosses in which the *his-rfb* deletion was replaced by the intact *his-rfb* region originating

TABLE 5. *Behavior of ECA in crosses between ECA-positive donor strains and 46 independent SDS-resistant mutant derivatives of three his-rfb deletion strains*

SDS-resistant mutants derived from:	(I) Cross: HfrB × SDS-resistant mutants			(II) Cross: HfrK1-2 × *his*$^+$ recombinants from I		
	Mutants tested (ECA$^-$ or ECA^{tr})	*his*$^+$ recombinants[a]		*his*$^+$ strains tested[b] (ECA$^-$)	*ilv*$^+$ recombinants[c]	
		ECA$^+$	ECA$^-$		ECA$^+$	ECA$^-$
his-386	21	11	10	3	3	0
his-399	17	5	12	4	4	0
his-809	8	0	8	0		

[a] In each cross, 10 *his*$^+$ recombinants were tested and behaved identically; the data given are numbers of crosses giving this type of *his*$^+$ recombinant.

[b] Each derived from a separate cross of type I with separate SDS-resistant mutants.

[c] In each cross, 10 *ilv*$^+$ recombinants were tested; 4 to 10 of them were ECA$^+$ in each cross.

TABLE 6. *LPS and ECA characteristics of his⁺ ilv⁻ and his⁻ ilv⁺ recombinants isolated in crosses between the long-his-rfb deletion strains (as found in collection or as new SDS-resistant derivatives) as recipients and O-6,7 donor strains HfrH14 (group C) or SH5152 (C-rfb⁺ derivative of HfrH2 of group B)*

Recipient		Recombinants isolated in crosses with donor					
		SH5152 *his⁺ ilv⁻*			HfrH14 *his⁻ ilv⁺*		
Genotype	Derivation	LPS	ECA	Frequency[a]	LPS	ECA	Frequency[a]
his-386 rff-4272 ilv-1188	Collection	6,7	−	30/30	R	+	36/38
his-399 rff-4271 ilv-1189	Collection	6,7	−	30/30	R	+	15/18
his-809 rff-4270 ilv-1190	Collection	6,7	−	50/50	ND[b]	ND	ND
his-658 rff-4273 ilv-1180	Collection	6,7	−	10/10	ND	ND	ND
his-386 rff-4135	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND
his-386 rff-4136	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND
his-399 rff-4131	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND
his-399 rff-4132	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND
his-809 rff-4125	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND
his-809 rff-4126	SDS-resistant mutant	6,7	−	10/10	ND	ND	ND

[a] Number of recombinants with this LPS and ECA type/number of recombinants of this class tested.
[b] ND, Not done.

in *S. montevideo* of group C_1. For all of the four *his-rfb* deletion strains and the six SDS-resistant mutants derived from them, all of the *his⁺* recombinants were smooth (O-6,7).

It is clear that the *rfe*-like mutation did not prevent the O-6,7 polysaccharide synthesis. It is doubtful, therefore, whether the gene(s) defined by these mutations should be called *rfe* or even *rf*. (which refers to the impaired LPS synthesis of rough mutants [24]) at all. However, until we know more about the function of these genes and that of *rfe*, we would like to propose the mnemonic *rff* for the *rfe*-like mutation here described. If proven to be functionally closely related to *rfe*, *rff* could be changed to *rfe* without much inconvenience. In Tables 2 and 6 we have used the gene designation *rff* with the mutant numbers assigned to each independent mutation.

From Table 6 (*his⁻ ilv⁺* recombinants) it can be seen that the *rfe⁺* genes of group C_1 were sufficient to support the synthesis of ECA even in strains harboring the *his-rfb* deletion. This is different from the behavior of corresponding *his⁻ ilv⁺* recombinants listed in Table 3, in which the *rff⁺* (and *rfe⁺*) genes came from group B. This kind of difference between *rfe-rff* genes of groups B and C has been described before in slightly different experiments (14). As a new feature, however, it should be noted that the C-*rff⁺* recombinants still harboring the *his-rfb* deletion were detergent resistant in contrast to the SDS-sensitive B-*rff⁺* recombinants.

***rfb* deletions of group C_1.** Even if the data accumulated thus far (above and reference 14) have indicated that the *rfe* region (comprised of *rfe* and *rff*) of group C_1 is sufficient for the production of ECA, without requiring the function of any *rfb* genes, we thought it prudent to check this with a series of *rfb* deletion mutants of *S. montevideo* group C_1. These deletions had been selected (P. H. Mäkelä) in the same way as the *S. typhimurium his* deletions (16). Nine independent isolates had been tested for *rfb*-determined enzymes, and all were found (H. Nikaido, personal communication) to be lacking the enzymes MAN-2 and MAN-3 (phosphomannomutase and GDP-mannose pyrophosphorylase) of the GDP-mannose pathway and to have at least nearly normal levels of the enzymes for *N*-acetylglucosamine synthesis (the O polysaccharide of group C_1 contains only mannose, glucose, and *N*-acetylglucosamine [6]). These strains were tested for their ECA content and all were found to be ECA positive. Although we have no proof (because of lack of suitable "marker" enzymes) that any of the deletions comprised a large fraction of the *rfb* region of group C_1, this result tends to support the contention that the *rfb* region of group C_1 does not contain genes participating in the production of ECA.

DISCUSSION

The data presented here lead to the following conclusions. (i) The *rfb* region of group B salmonellae is somehow connected with the production of ECA. This function is localized toward the left end of the *rfb* region, the cut-off point being between the genes for CDP-abequose synthesis (not required for ECA) and those for TDP-rhamnose synthesis (deletion resulting in absence of ECA). This does not necessarily imply, however, that any of the enzymes of the TDP-rhamnose pathway are necessary for the synthesis of ECA. It is quite possible that there

are unidentified genes in this area that might be the ones implicated in ECA synthesis.

It is slightly disconcerting that no *rfb* point mutants have been found which are impaired in the production of ECA. This could, of course, be due to chance, but it is also possible that such a mutant would not be rough (R) at all and would not have been found during the search for *rfb* mutants.

Trace amounts of ECA are regularly found in strains missing large parts of the *rfb* region of group B salmonellae: both deletion mutants, as described here, and hybrids between groups B and C_1 (14). This observation may provide a clue to the function of the *rfb* gene(s) with regard to ECA. However, it will be necessary, as a first step, to be sure of the cause of the trace reaction: above all, whether it is caused by small amounts of the same molecular species as is ECA^+ in other strains.

(ii) The *his-rfb* deletion causing an ECA^{tr} phenotype seems to impair the viability of the bacteria, as evidenced by the very marked tendency for accumulation of second ("suppressor") mutations. Whether or not the small amount of ECA is involved directly is hard to say. At any rate, completely ECA-negative double mutants or single *rfe* or *rff* mutants have better viability (Table 4). Reduced viability is connected with increased sensitivity (= increased permeability?) especially to SDS but also to other anionic detergents; permeability to many other agents is affected much less or not at all. The detergent sensitivity suggests disturbance of membrane integrity, which would not be too surprising—after all, ECA is most likely a component of the outer membrane of the bacteria (2).

At any rate, the detergent sensitivity (and reduced viability) can be alleviated by a second mutation, which itself prevents the synthesis of ECA (not even trace amounts of ECA detected). This sort of suppression of some effects of the *rfb* deletion mutation suggests that the reduced viability may have been caused, for example, through accumulation of a toxic or otherwise deleterious intermediate. An example of this kind of effect would be the *galE* mutation, which prevents the interconversion of UDP-galactose and UDP-glucose and results, under certain conditions, in the accumulation of toxic amounts of some metabolite of galactose (5). That the ECA pathway is rather intimately involved is shown by the fact that the mutations that suppress the SDS sensitivity and reduced viability occur in a gene(s) necessary for ECA synthesis. This contention is supported further by hybrids, in which the *rfe-rff* region comes from group C_1: these hybrids are ECA positive and SDS resistant.

(iii) The SDS sensitivity of certain *his-rfb* deletions has provided a second means of selecting mutants in genes participating in ECA synthesis. The only method available previously was to select R mutants in bacteria with group C_1 (or group L) *rfb* genes and check these for ECA (and/or linkage of the mutation to *ilv*) (14). This R selection yielded one type of mutation, termed *rfe*, affecting synthesis of ECA and the O-6,7 polysaccharide at the same time. All of the *rfe* mutations occurred in a gene(s) close to *ilv*. The new selection has provided a new type of mutation, termed *rff*, affecting the synthesis of ECA but not that of LPS (neither O-6,7 nor O-4,12). Also, these mutations are clustered close to *ilv*. Whether *rfe* and *rff* represent one or more genes each is not known yet. Neither is it known how closely related functionally they might be. The answer to this question may make it necessary to group them together, but, so far, calling them by separate names may prevent some confusion. A mutation corresponding to the description of *rff* has also been accidentally found in a stock of *S. minnesota* (15).

In the work described here, 38 independent *rff* mutations, in combination with 4 separate *his-rfb* deletions, were studied. They all are remarkably alike. Finding more different types of mutations of the ECA pathway might help to clarify both its structure and function. A new method for selecting ECA mutants may be needed: both of those available are indirect and may select an atypical set of mutants only. Unfortunately, at this time a method for the direct selection for the ECA-negative phenotype is not available.

ACKNOWLEDGMENTS

This study was supported by grants from the Finnish Medical Research Council and the Sigrid Jusélius Foundation (to P.H.M.), by the Deutsche Forschungsgemeinschaft (to H.M.), by Public Health Service research grant AI-09644 from the National Institute of Allergy and Infectious Diseases and grant BC-20 from the American Cancer Society (to H.N.), and by Public Health Service research grant AI-00658 from the National Institute of Allergy and Infectious Diseases (to E.N.).

For excellent assistance we thank Marjukka Brandt, Marianne Hovi, Sirkku Waarala, and Sirkka Vene in Helsinki, and Brigitte Straub and Christiane Widemann in Freiburg.

LITERATURE CITED

1. Ames, G. D.-L., E. N. Spudich, and H. Nikaido. 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. 117:406–416.
2. Aoki, S., M. Merkel, and W. R. McCabe. 1966. Immunofluorescent demonstration of the common entero-

bacterial antigen. Proc. Soc. Exp. Biol. Med. 121:230–234.

3. **Bagdian, G., and P. H. Mäkelä.** 1971. Antigenic conversion by phage P27. I. Mapping of the prophage attachment site on the *Salmonella* chromosome. Virology 43:403–411.
4. **Ericsson, H., C. Högman, and K. Wickman.** 1954. A paper disk method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents. Scand. J. Clin. Lab. Invest. 6(Suppl.):11.
5. **Fukasawa, T., and H. Nikaido.** 1961. Galactose-sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. Biochim. Biophys. Acta 48:470–483.
6. **Fuller, N. A., and A. M. Staub.** 1968. Immunochemical studies on *Salmonella*. 13. Chemical changes appearing on the specific polysaccharide of *S. cholerae suis* (6_2,7) after its conversion by phage 14(6,7). Eur. J. Biochem. 4:286–300.
7. **Hammarström, S., H. E. Carlsson, P. Perlmann, and S. Svensson.** 1971. Immunochemistry of the common antigen of *Enterobacteriaceae* (Kunin). J. Exp. Med. 134:565–576.
8. **Johns, M. A., R. E. Whiteside, E. E. Baker, and W. R. McCabe.** 1973. Common enterobacterial antigen. I. Isolation and purification from *Salmonella typhosa* O:901. J. Immunol. 110:781–790.
9. **Kunin, C. M.** 1963. Separation, characterization, and biological significance of a common antigen in *Enterobacteriaceae*. J. Exp. Med. 118:565–586.
10. **Kunin, C. M., M. V. Beard, and N. E. Halmagyi.** 1962. Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other *Enterobacteriaceae*. Proc. Soc. Exp. Biol. Med. 111:160–166.
11. **Lüderitz, O., C. Galasso, H. J. Risse, E. Ruschmann, S. Schlecht, G. Schmidt, H. Schulte-Holtehausen, R. Wheat, O. Westphal, and J. Schlosshard.** 1966. Structural relationship of *Salmonella* O and R antigen. Ann. N.Y. Acad. Sci. 133:349–374.
12. **Mäkelä, P. H.** 1966. Genetic determination of the O antigen of *Salmonella* groups B (4,5,12) and C_1 (6,7). J. Bacteriol. 91:1115–1125.
13. **Mäkelä, P. H., M. Jahkola, and O. Lüderitz.** 1970. A new gene cluster *rfe* concerned with the biosynthesis of Salmonella lipopolysaccharide. J. Gen. Microbiol. 60:91–106.
14. **Mäkelä, P. H., and H. Mayer.** 1974. Participation of lipopolysaccharide of the enterobacterial common antigen: analysis in *Salmonella* groups B and C. J. Bacteriol. 119:765–770.
15. **Mäkelä, P. H., H. Mayer, H. Y. Whang, and E. Neter.** 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis of R mutants of *Salmonella minnesota*. J. Bacteriol. 119:760–764.
16. **Nikaido, H., M. Levinthal, K. Nikaido, and K. Nakane.** 1967. Extended deletions in the histidine-rough-B region of the Salmonella chromosome. Proc. Natl. Acad. Sci. U.S.A 57:1825–1832.
17. **Nikaido, H., K. Nikaido, and P. H. Mäkelä.** 1966. Genetic determination of enzymes synthesizing O-specific sugars of *Salmonella* lipopolysaccharides. J. Bacteriol. 91:1126–1135.
18. **Nikaido, H., K. Nikaido, T. V. Subbaiah, and B. A. D. Stocker.** 1964. Rough mutants of *Salmonella typhimurium*. 3. Enzymatic synthesis of nucleotide-sugar compounds. Nature (London) 201:1301–1302.
19. **Sanderson, K. E., T. MacAlister, J. W. Costerton, and K.-J. Cheng.** 1974. Permeability of lipopolysaccharide-deficient (rough) mutants of *Salmonella typhimurium* to antibiotics, lysozyme, and other agents. Can. J. Microbiol. 20:1135–1145.
20. **Sanderson, K. E., H. Ross, L. Ziegler, and P. H. Mäkelä.** 1970. F^+, Hfr, and F′ strains of *Salmonella typhimurium* and *Salmonella abony*. Bacteriol. Rev. 36:608–637.
21. **Schlecht, S., and O. Westphal.** 1970. Untersuchungen zur Typisierung von Salmonella-R-Formen. 4. Typisierung von *S. minnesota*-R-Mutanten mittels Antibiotica. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 213:365–381.
22. **Schmidt, G., S. Schlecht, and O. Westphal.** 1970. Untersuchungen zur Typisierung von Salmonella-R-Formen. 3. Typisierung von *S. minnesota*-Mutanten mittels chemischer Agenzien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 212:88–96.
23. **Smit, J., Y. Kamio, and H. Nikaido.** 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. 124:942–958.
24. **Stocker, B. A. D., and P. H. Mäkelä.** 1971. Genetic aspects of biosynthesis of *Salmonella* lipopolysaccharide, p. 369–438. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press Inc., New York.
25. **Subbaiah, T. V., and B. A. D. Stocker.** 1974. Rough mutants of *Salmonella typhimurium*. I. Genetics. Nature (London) 201:1298–1299.
26. **Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker.** 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. 70:527–554.