A New Gene Cluster *rfe* Concerned with the Biosynthesis of Salmonella Lipopolysaccharide

By P. HELENA MÄKELÄ AND M. JAHKOLA

State Serum Institute, Helsinki, Finland

AND O. LÜDERITZ

Max Planck Institut für Immunbiologie, Freiburg, Germany

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SUMMARY

A new class of rough mutants has been found in both *Salmonella montevideo* and *S. minnesota*. The mutants resemble phenotypically *rfb* mutants, having a complete lipopolysaccharide core but no O-specific material in lipopolysaccharide or as free hapten. The site of these *rfe* mutations is near the isoleucine and valine gene *ilv*, and is thus separate from the known lipopolysaccharide gene clusters *rfa* for the core, *rfb* for the O-specific side-chains, and *rfc* for O side-chain polymerization. The function of the *rfe* gene(s) is not known. It is suggested that they might participate in the utilization of the antigen carrier lipid for lipopolysaccharide synthesis, or that they might regulate the activity of *rfb* genes. The gene determining phosphorylation of the heptose in the lipopolysaccharide core of *S. minnesota* was identified as one within the *rfa* cluster.

INTRODUCTION

The O-antigenic lipopolysaccharide of Salmonella is thought to have a central core which is common to all Salmonella species. Attached to it in an unknown way are the O side-chains specific for each O-antigenic type (Fig. 1). Mutants in which the synthesis of lipopolysaccharide is defective are culturally rough (R). Three classes of rough mutants have been described (Subbaiah & Stocker, 1964; Naide *et al.* 1965; Beckmann, Subbaiah & Stocker, 1964). *rfa* mutants have a defective core, and produce a low molecular weight 'hapten' with O specificity. The *rfa* mutations are located in a part of the chromosome near *xyl* (Fig. 2). Later many, although not all, of the *rfa* mutations were shown to be cotransducible with *pyrE* and *cysE* between *xyl* and *ilv* (Kuo & Stocker, 1968). The hapten is thought to consist of polymerized O-specific repeating units as precursors of O side-chains which cannot be attached to the unfinished core (Kent & Osborn, 1968). *rfb* mutants on the other hand have a complete core, but produce no O-specific material. Their mutations map in a tight cluster near *his*. The third class of mutants, *rfc*, are unable to polymerize the O-specific repeating units. Their sites of mutations are located between *trp* and *gal*.

However, some R mutants do not fit into this scheme. These have a complete core with N-acetylglucosamine, and also O-specific hapten (Beckmann, Subbaiah & Stocker, 1964; Gemski & Stocker, 1967), which they apparently cannot attach to the core. Some of these (*rfbT*) have mutations that map in the *rfb* cluster. Other mutations

called *rfaL* resemble *rfa* in being cotransducible with *pyrE* (Kuo & Stocker, 1968). The LPS core in both cases is also complete in the sense that it can accept O side-chains, when incubated with cell-free preparations of *rfb* bacteria for example. It is therefore assumed that both *rfbT* and *rfaL* mutants might lack a component of a ligase or 'translocase' specified by these genes. Cell envelope preparations of *rfaL* mutants have been shown to be unable to catalyse the translocation step (Cynkin & M. J. Osborn, unpublished results quoted by Osborn, 1969).

Fig. 1. Schematic structure of the Salmonella lipopolysaccharide (Lüderitz, Jann & Wheat, 1968; Fuller & Staub, 1968; Osborn, 1969). The innermost part of lipopolysaccharide contains KDO (= ketodeoxyoctonate), ethanolamine, phosphate and lipid A. To this are attached polysaccharide side-chains composed of a core and an O side-chain. The O side-chain is built up of repeating units, which carry the O-specific antigenic determinants characteristic of each Salmonella O group. Examples of the structure of the repeating unit in different Salmonella species, belonging to different O groups, are shown on the right.

Rough mutants have so far been analysed genetically only in *Salmonella typhimurium* strain LT2. While studying rough mutants in two other Salmonella species, *S. montevideo* (O antigens 6, 7) and *S. minnesota* (O antigen 21), we found a new class of R mutants (*rfe*) which produce a complete core, but no O-specific hapten. Their sites of mutation are located close to *ilv* and are clearly separate from *rfa*, *rfb* and *rfe*. The symbol *rfe* is preferred to *rfd*, which would be similar to the *rouD* used earlier to describe leaky *rfa* mutants (Gemski & Stocker, 1967). Typical *rfa* mutations in these species, as in *S. typhimurium*, occur close to *xyl*.

METHODS

Bacterial and phage strains. *Salmonella typhimurium* strain LT2 and various rough (R) mutants of it were obtained from Dr B. A. D. Stocker, Stanford University, California, as were the O- and R-specific phages (Wilkinson & Stocker, 1968)

Fig. 2. Part of the chromosome map of *Salmonella* (after Sanderson, 1967; Mäkelä & Stocker, 1969). Positions not accurately known are indicated by arrows, and loci whose order is not known are bracketed. Genes labelled outside circle affect lipopolysaccharide synthesis. Gene symbols as follows: *gal* = galactose utilization, *trp* = tryptophan biosynthesis, *his* = histidine biosynthesis, *str* = resistance to streptomycin, *xyl* = xylose utilization, *pyrE* = orotidylic acid pyrophosphorylase, *cysE* = cysteine biosynthesis, *ilv* = biosynthesis of isoleucine and valine, *pmi* = phosphomannose isomerase, *rfa*, *rfb*, *rfc*, *rfe* = biosynthesis of the O-antigenic lipopolysaccharide.

Salmonella montevideo was strain no. 129 of Edwards & Bruner (1942), used earlier by Mäkelä (1966); its mutants used are listed in Table 1. *Salmonella minnesota* was strain s99 obtained from Dr F. Kauffmann, State Serum Institute, Copenhagen, Denmark (Lüderitz *et al.* 1965); its R mutants mR3 and mR592 were those described by Lüderitz *et al.* (1966). It turned out that these mutants had more than one R mutation. Single R-mutant derivatives were therefore isolated from them as recombinants in crosses with the smooth parent strain first made F⁺; they are listed in Table 1. Nutritional, fermentation or streptomycin-resistant mutants were selected from these strains by standard procedures (Lederberg, 1950). R mutants were selected as morphologically rough colonies on complete media or as clones resistant to the Felix-O1 phage. Diethylsulphate was the mutagen used (10 mg./ml. added to an overnight broth culture, and incubation continued for 30 min. at 37°, after which the culture was diluted 1/100 in fresh medium and incubated overnight before plating out). The gene symbols are explained in the legend for Fig. 2.

Bacteriological techniques. In the bacterial crosses, F⁺ strains with the F factor derived from *Escherichia coli* K12 (Mäkelä, Lederberg & Lederberg, 1962) were used as donors. The donors were streptomycin-sensitive and streptomycin (1 mg./ml.) was added to the selective medium. All R strains were identified by cultural

Table 1. *Salmonella* strains used

Species	Strain no.	Mating type	Relevant genotype							Source
			<i>rfa</i>	<i>rfb</i>	<i>rfe</i>	<i>his</i>	<i>ilv</i>	<i>xyl</i>	<i>str</i>	
<i>S. typhimurium</i> line LT 2	SL 1027	•	+	+	+	•	•	•	•	From B. A. D. Stocker
	SL 748	•	+	—	+	•	•	•	•	From B. A. D. Stocker
	SL 758	•	—	+	+	•	•	•	•	From B. A. D. Stocker
	SL 1060	•	—	+	+	•	•	•	•	<i>rfaH</i> (Wilkinson & Stocker, 1968)
<i>S. montevideo</i> line 129 (Edwards & Bruner, 1942)	SW 829	F ⁺	+	+	+	+	+	+	+	F from <i>E. coli</i> K 12
	SH 1675	F ⁻	+	+	3623	—	—	—	R	From SH 1622, <i>his ilv xyl str</i> (<i>rfb</i> 3795 is very leaky)
	SH 1676	F ⁻	+	3795	3624	—	—	—	R	
	SH 1679	F ⁻	+	+	3627	—	—	—	R	
	SH 1688	F ⁻	+	+	3636	—	—	—	R	
	SH 1711	F ⁻	3646	3610	+	—	—	—	R	
	SH 1717	F ⁻	3652	3618	+	—	—	—	R	From cross SW 829 × SH 1717*
	SH 1723	F ⁻	3658	3625	3796	—	—	—	R	
	SH 1724	F ⁻	3659	3626	+	—	—	—	R	
	SH 1732	F ⁻	3667	3624	+	—	—	—	R	
	SH 3269	•	3652	+	+	+	—	—	R	
<i>S. mimesota</i> line s 99 (Lüderitz et al. 1965)	SH 309	F ⁺	+	+	+	+	+	+	+	F from <i>E. coli</i> K 12
	mr 3†	F ⁻	3791, 3792	+	3793†	—	—	—	R	Lüderitz et al. 1966
	mr 592†	F ⁻	3789	+	3790†	—	—	—	R	Lüderitz et al. 1966
	SH 1756	•	+	+	3793	—	—	+	R	From cross SH 309 × mr 3
	SH 1757	•	+	+	3790	—	—	+	R	From cross SH 309 × mr 592
	SH 3237	•	3791, 3792	+	+	—	+	—	R	From cross SH 309 × mr 3
	SH 3264	•	3789	+	+	—	+	—	R	From cross SH 309 × mr 592

* Crosses are written as donor × recipient.

† *his*, *ilv*, *xyl* and *str* mutations were selected in the original mr 3 or mr 592, checking the phage sensitivity and agglutination pattern of the strains at each step. However, as new R mutations might have accumulated during this selection, the *rfa* genotype of the original mr 3 and mr 592 was later checked by making them F⁺ and then using them as donors to a smooth *his ilv xyl str* recipient strain; the same *rfa* and *rfe* mutations were recovered as reported in thrfe-3793 may be identical with *rfe*-3790.

characteristics, slide agglutination (4% saline; anti-O sera diluted in 0.2% saline), and sensitivity to O and R-specific phages (Wilkinson & Stocker, 1968). The methods and media were otherwise as described earlier (Mäkelä, 1966).

Chemical methods. Lipopolysaccharides were prepared by the phenol-water procedure followed by centrifugation at 105,000 g. Lipopolysaccharide was obtained as the sediment, while the supernatant represents the L1 fraction and contains the O-specific hapten (Beckmann, Subbaiah & Stocker, 1964). The analyses of these fractions were performed as described previously (Risse *et al.* 1967). The examination of phosphateless (P-) mutants was performed as described by Dröge, Ruschmann, Lüderitz & Westphal, 1968). Haemagglutination inhibition by lipopolysaccharide was examined according to Beckmann, Lüderitz & Westphal (1964).

RESULTS

Characterization of R mutants with bacteriophages

Wilkinson & Stocker (1968) found that the lysis of rough *Salmonella typhimurium* mutants by selected phages correlated well with the chemical composition of the defective lipopolysaccharide. Some of these phages do not attack *S. montevideo* or *S. minnesota* at all, but others produce lytic patterns comparable to those in *S. typhimurium* (Table 2). We found sensitivity or resistance to the Felix-01 phage (called FO in this paper) particularly useful. In *S. typhimurium* sensitivity to this phage has been shown to depend on the presence of *N*-acetylglucosamine—that is, the complete core—in the lipopolysaccharide (Lindberg, 1967). Thus smooth bacteria and R mutants that have a complete core are FO-sensitive, while core mutants are FO-resistant.

Table 2. Phage sensitivity patterns of smooth and rough strains of *Salmonella typhimurium*, *S. montevideo* and *S. minnesota*

Action of phage (clear lysis = +; no lysis = -) was determined by applying the phage dilutions (10^8 plaque-forming units/ml) as drops on nutrient agar plates spread with an overnight broth culture of the bacterial strain and dried for 30 min. (Wilkinson & Stocker, 1968).

				Sensitivity to phages			
		Strain	Mutant	FO (= Felix-01)	Br 60	Ffm	C 21
Smooth		<i>S. typhimurium</i> *	SL 1027	+	-	-	-
		<i>S. montevideo</i>	SW 829	+	-	-	-
		<i>S. minnesota</i>	SH 309	+	-	-	-
Rough							
FO-sensitive	<i>S. typhimurium</i> *	SL 748	<i>rfb</i>	+	+	+	-
	<i>S. montevideo</i>	SH 1675	<i>rfe</i> †	+	+	+	-
	<i>S. minnesota</i>	SH 1756	<i>rfe</i> †	+	+	+	-
FO-resistant	<i>S. typhimurium</i> *	SL 758	<i>rfa</i>	-	+	+	-
	<i>S. montevideo</i>	SH 3269	<i>rfa</i> †	-	+	+	-
	<i>S. minnesota</i>	SH 3237	<i>rfa</i> †	-	+	+	-
	<i>S. typhimurium</i> *	SL 1060	<i>rfaH</i>	-	+	+	+
	<i>S. minnesota</i>	SH 3264	<i>rfa</i> †	-	+	+	+

* According to Wilkinson & Stocker (1968 and personal communication), repeated in this series of experiments.

† Classification according to the data in the present paper.

Table 3. Frequencies of rfe^+ among different classes of recombinants in crosses between a smooth streptomycin-sensitive F^+ donor and streptomycin-resistant $xyl\ ilv\ rfe$ recipients

Crosses were made between exponentially growing broth cultures of donor and recipient, mixed in equal proportions (sw 829 and sh 309 with *S. montevideo* and *S. minnesota* recipients respectively). The mating mixture was kept undisturbed at 37° for 2 hr, then plated for selection of xyl^+str^R , ilv^+str^R or $xyl^+ilv^+str^R$ recombinants, which were recovered at a rate of $c. 10^{-7}$ (= 10 recombinants per plate spread with 0.1 ml. of the mating mixture). The recombinants were streaked on nutrient agar plates, from which single colonies were tested for their nutritional characteristics and LPS character. For the latter purpose colony morphology, agglutinability in 4% saline and in the correct anti-O serum, and phage sensitivity pattern (according to Table 2) were determined.

Recombinant classes	Recipient strains									
	<i>S. montevideo</i>					<i>S. minnesota</i>				
	SH 1675 rfe^{+*}	SH 1676 rfe^+	SH 1679 rfe^+	SH 1688 rfe^+	Total rfe^+		mr 3† rfe^+	mr 592‡ rfe^+	Total rfe^+	% rfe^+
$xyl^- ilv^+$	9/18	4/9	2/5	2/6	17/38			55/121	55/121	45
$xyl^+ ilv^-$	1/18	1/11	0/27	0/36	2/92		1/87	4/78	5/165	3
$xyl^+ ilv^+$	4/5	4/5	7/9	11/11	26/30		104/120	30/50	134/170	78
ilv^+					18/39				61/131	47
xyl^+					11/102				103/340	30
									42/342 = 12%	
									131/340 = 38%	

* rfe strains were culturally rough and agglutinated in 4% saline; rfe^+ were culturally smooth and agglutinated in the corresponding anti-O serum.

† mr 3 is also rfa^- ; rfe^+ could be scored only in recombinants that had become rfa^+ , and the figures given for rfe^+ are only approximate.

‡ mr 592 has an rfa mutation as well as rfe , but the rfa is so leaky that rfe^+ could be scored by agglutination, although the rfa^-rfe^+ recombinants were still resistant to FO.

Genetic analysis of rfe mutants

Most randomly isolated rough mutants of *Salmonella montevideo* were sensitive to FO and rough-specific phages. Most of these mutations mapped near *his*, as does *rfb*, but a number did not. All of these mutants whose mutations did not map in the *rfb* area were isolated from an *xyl ilv* parent strain which was streptomycin-resistant. To locate the site of these mutations each mutant was crossed with an F⁺ streptomycin-sensitive smooth donor strain of *S. montevideo* (sw 829). The frequency of smooth recombinants among *xyl-ilv*⁺, *xyl-ilv*⁻ and *xyl-ilv*⁺ recombinants is given in Table 3. The number of recombinants analysed in each cross is small because of low fertility in F⁺ crosses with this strain. However, the trend was the same in each case: nearly half the *xyl-ilv*⁺ recombinants were smooth, compared with two out of 92 *xyl-ilv*⁻ recombinants. Of the 30 *xyl-ilv*⁺ recombinants 26 were smooth, confirming that the R mutation is in the *xyl-ilv* region of the chromosome.

In these crosses the unselected *xyl*⁺ donor allele appeared in about 3 % of the *ilv*⁺ recombinants, and the unselected *ilv*⁺ donor allele in 10 % of the *xyl*⁺ recombinants, as shown in the lower part of Table 3. The 45 % linkage found between the R mutation and *ilv* suggests that this R mutation is much closer to *ilv* than is *xyl*. We shall call the gene or genes affected in these mutants *rfe*.

A similar R mutation was found in two *Salmonella minnesota* strains. The right half of Table 3 shows some results of mapping this mutation. In both mr 3 and mr 592 the mutation had a similar location to *rfe* in *S. montevideo*, with 45 % linkage to *ilv*. The mutants mr 3 and mr 592 were originally isolated by a complex procedure (J. Schlosshardt, personal communication), and have turned out to contain *rfa* mutations in addition to *rfe*. In mr 592 the *rfa* mutation was so leaky that *rfe*⁺ could be recognized by smooth colony form and smooth-type agglutination in the corresponding anti-O serum, although the recombinants that were still *rfa*⁻ gave a very slight agglutination in 4 % saline, and retained the FO-resistant phage sensitivity pattern of the mr 592 parent. The *rfa* in mr 3 was not leaky—the strain has been demonstrated to have two separate biochemical defects in core synthesis (see p. 101) and hence probably has two independent *rfa* mutations. With this strain the *rfe* genotype could be scored only in recombinants that had become *rfa*⁺. The single *rfe* mutant derivatives obtained in these crosses from mr 3 and mr 592 as *xyl-rfa*⁺ were phenotypically similar to *rfb* mutants, e.g. in sensitivity to FO (Table 2, strain SH 1756). Genetically, however, they were very different, as typical *his*-linked *rfb* mutants could be easily isolated in the *S. minnesota* strain (unpublished observations). Because of the isolation method of mr 3 and mr 592 we do not know whether the *rfe* mutation occurred independently in these two strains or not.

Genetic analysis of rfa mutants

We studied a number of other rough mutants that were resistant to the phage FO. These included five independent mutations of *Salmonella montevideo* and two of *S. minnesota*. As shown in Table 4, all these mutations mapped closer to *xyl* than to *ilv* (34 versus 6 % linkage for the *S. montevideo* mutants, 67 versus 8 % for the *S. minnesota* mutants). The *rfa*⁺ allele was scored in all recombinants as giving rise to an FO-sensitive phage pattern regardless of the possible presence of an *rfe* or *rfb* mutation in the recipients and recombinants.

Table 3. Frequencies of *rfe*⁺ among different classes of recombinants in crosses between a smooth streptomycin-sensitive *F*⁺ donor and streptomycin-resistant *xyl ilv rfe* recipients

Crosses were made between exponentially growing broth cultures of donor and recipient, mixed in equal proportions (sw 829 and SH 309 with *S. montevideo* and *S. minnesota* recipients respectively). The mating mixture was kept undisturbed at 37° for 2 hr, then plated for selection of *xyl*⁺*str*^R, *ilv*⁺*str*^R or *xyl*⁺*ilv*⁺*str*^R recombinants, which were recovered at a rate of *c.* 10⁻⁷ (= 10 recombinants per plate spread with 0.1 ml. of the mating mixture). The recombinants were streaked on nutrient agar plates, from which single colonies were tested for their nutritional characteristics and LPS character. For the latter purpose colony morphology, agglutinability in 4% saline and in the correct anti-O serum, and phage sensitivity pattern (according to Table 2) were determined.

Recombinant classes	Recipient strains									
	<i>S. montevideo</i>						<i>S. minnesota</i>			
	SH 1675 <i>rfe</i> ⁺ *	SH 1676 <i>rfe</i> ⁺	SH 1679 <i>rfe</i> ⁺	SH 1688 <i>rfe</i> ⁺	Total <i>rfe</i> ⁺	% <i>rfe</i> ⁺	mr 3† <i>rfe</i> ⁺	mr 592‡ <i>rfe</i> ⁺	Total <i>rfe</i> ⁺	% <i>rfe</i> ⁺
<i>xyl</i> ⁻ <i>ilv</i> ⁺	9/18	4/9	2/5	2/6	17/38	45		55/121	55/121	45
<i>xyl</i> ⁺ <i>ilv</i> ⁻	1/18	1/11	0/27	0/36	2/92	2	1/87	4/78	5/165	3
<i>xyl</i> ⁺ <i>ilv</i> ⁺	4/5	4/5	7/9	11/11	26/30	87	104/120	30/50	134/170	78
<i>ilv</i> ⁺					18/39	46			61/131	47
<i>xyl</i> ⁺					11/102	11			103/340	30
For comparison, frequency of <i>xyl</i> ⁺ among <i>ilv</i> ⁺					1/39 = 3%		42/342 = 12%			
For comparison, frequency of <i>ilv</i> ⁺ among <i>xyl</i> ⁺					10/102 = 10%		131/340 = 38%			

* *rfe* strains were culturally rough and agglutinated in 4% saline; *rfe*⁺ were culturally smooth and agglutinated in the corresponding anti-O serum.

† mr 3 is also *rfa*⁻; *rfe*⁺ could be scored only in recombinants that had become *rfa*⁺, and the figures given for *rfe*⁺ are only approximate.

‡ mr 592 has an *rfa* mutation as well as *rfe*, but the *rfa* is so leaky that *rfe*⁺ could be scored by agglutination, although the *rfa*⁻*rfe*⁺ recombinants were still resistant to FO.

Relative order of the xyl, rfa, ilv and rfe genes

The relative position of the loci *xyl*, *rfa*, *ilv* and *rfe* could be more closely examined in the two *Salmonella minnesota* mutants MR 3 and MR 592 that have mutations in both *rfa* and *rfe* genes. (It is probable that MR 3 has two separate mutations, in two

Table 5. *Frequencies of different recombinant classes in crosses between a smooth streptomycin-sensitive F⁺ donor and xyl⁻ rfa⁻ ilv⁻ rfe⁻ str^r recipients of S. minnesota*

Crosses were made as in Table 3, *rfe* and *rfa* characterized as in Tables 3 and 4. Numbers of recombinants belonging to each class are listed in columns 5 and 6 (N.D. = not determined). In the last four columns are given numbers of the recombinants whose genotype could be produced only by four or more cross-overs. (The + after some numbers means that there might be a few more recombinants belonging to these groups which were not identified. Their number is certainly small—less than five—and would affect all columns nearly equally.) Different recombinant classes fall into this category when different orders of the four relevant genes are assumed, permitting conclusions of the most likely gene order. This appears to be the first one, *xyl-rfa-ilv-rfe*, which would necessitate quadruple cross-overs in not more than eleven recombinants.

Genetic constitution of recombinants				Recipient strain		Minimum number of quadruple cross-overs required to produce the recombinants observed, assuming gene order			
<i>xyl</i>	<i>rfa</i>	<i>ilv</i>	<i>rfe</i>	MR 3	MR 592	<i>xyl-rfa-ilv-rfe</i>	<i>xyl-rfa-rfe-ilv</i>	<i>rfa-xyl-ilv-rfe</i>	<i>rfa-xyl-rfe-ilv</i>
+	—	—	—	N.D.	23	—	—	—	—
+	+	—	—	86	51	—	—	—	—
+	+	+	—	17	19	—	36	—	36
+	+	+	+	103	28	—	—	—	—
—	+	+	+	12	8	—	—	20	20
—	—	+	+	167	47	—	—	—	—
—	—	+	—		62	—	—	—	—
+	—	+	—	3	1	6	6	—	1+
+	—	+	+		2	—	—	—	—
+	—	—	+	N.D.	2	2+	2+	2+	—
+	+	—	+	1	2	3	—	3	—
—	+	+	—	0	4	—	4	4	4
Totals				389	249	11+	48+	29+	61+
Recombinants with at least two donor markers (excluding <i>ilv⁺rfe⁺</i> with the recipient MR 3)				222	164				

different *rfa* genes. In this genetic analysis they were never separated, and we feel justified in treating them as a single mutation.) These mutants were crossed with SH 309 (smooth, F⁺), and all *xyl*⁺ or *ilv*⁺ recombinant classes analysed (Table 5). Taking into account the fairly close linkage between *ilv* and *rfe*, and between *xyl* and *rfa*, the four loci can be assigned four different orders as shown in the last four columns of Table 5. Assuming the gene order *xyl-rfa-ilv-rfe* we see that most of the recombinant classes can be produced through a double cross-over, integrating the donor alleles in the recipient chromosome as a single block. The four classes *xyl⁺rfa⁻ilv⁻rfe⁻*, *xyl⁺rfa⁻ilv⁺rfe⁺*, *xyl⁺rfa⁻ilv⁻rfe⁺* and *xyl⁺rfa⁺ilv⁻rfe⁺*, however, cannot be produced in this simple way but require at least four cross-overs. Eleven representatives of these classes were found among the 386 recombinants that had at least two donor alleles and therefore could contribute to the analysis. Assuming any of the other three gene orders, a larger number of recombinants (48, 29 and 61

Table 6. *Composition of the lipopolysaccharide of rfa and rfe mutants of S. montevideo and S. minnesota*

Lipopolysaccharide was isolated by phenol-water extraction followed by centrifugation at 105,000 g (Beckmann, Subbaiah & Stocker, 1964). Polysaccharide was prepared from lipopolysaccharide by removing the lipid A (Dröge *et al.* 1968); phosphate and glucosamine were determined from the polysaccharide. The analyses followed previously described methods (Risse *et al.* 1967) and the results were calculated as moles/2 (or 1) moles of heptose. For the expected lipopolysaccharide structure see Fig. 1.

Species	Strain	Relevant genotype		Mole content of the various lipopolysaccharide constituents, with heptose normalized as 2 or 1							
		<i>rfa</i>	<i>rfe</i>	KDO	Heptose	Phosphate in LPS†	Phosphate in PS	Glucose	Galactose	Glucos-amine in PS	Galactos-amine* or mannose
<i>S. montevideo</i>	SH 3269	3652	+	1·3	2		1·8	1·2	0·7	< 0·15	Trace
	SH 1675	+	3623	1·4	2	3·4	2·3	1·2	1·2	0·99	0
	SH 1679	+	3627	1·3	2	3·1	2·4	1·0	1·1	0·78	0
<i>S. minnesota</i>	SH 3237	3791, 3792	+	2·4	1		0·5	0	0	0	0
	mr 3	3791, 3792	3793	1·4	1	2·2	0·7	0	0	0	0
	mr 592	3789	3790	2·3	2	2·3	0·8	1·3	0·6	0·09	0
	SH 1756	+	3793	1·3	2	3	1·5	0·73	1·2	0·29	0
	SH 1757	+	3790	1·7	2	3	2·1	1·4	1·6	1·4	0
Expected of a strain with a complete core (Fig. 1)				2	2	3	2	2	2	1	

* The O-specific sugars are mannose in *S. montevideo* and galactosamine in *S. minnesota* (Fuller & Staub, 1968; Lüderitz *et al.* 1966).

† LPS, lipopolysaccharide; PS, polysaccharide.

respectively) would require quadruple cross-overs, and therefore the first-mentioned order appears the most likely one.

These results thus locate the *rfa* cluster of *Salmonella minnesota* between *xyl* and *ilv*, as it is in *S. typhimurium*. To make its homology with the *rfa* in *S. typhimurium* more certain one would like to test its cotransducibility with, for example, *pyrE*, which is between *xyl* and *ilv* and cotransducible with *rfa* in *S. typhimurium*. The *rfe* locus remains clearly separated.

Chemical composition of the lipopolysaccharide and LI fraction of the mutants

For studying the chemical composition of the mutant lipopolysaccharide we prepared separate *rfa* or *rfe* mutant strains from the original double or triple mutants by crossing these with smooth donors of the same species. Thus SH 3269 (Table 6) is a recombinant from the cross between SW 829 as the smooth donor and SH 1717 as the recipient; it was selected as *his⁺rfb⁺*, and has the *rfa*-3652 mutation of SH 1717. Similarly, a cross between SH 309, the smooth F⁺ *Salmonella minnesota* strain and MR 3 gave the *rfa* mutant SH 3237 as an *ilv⁺rfe⁺* recombinant, and the *rfe* mutant SH 1756 as an *xyl⁺rfa⁺* recombinant. The other single *rfe* mutant SH 1757 was prepared as *xyl⁺rfa⁺* from a similar cross between SH 309 and MR 592.

The monosaccharide composition of the lipopolysaccharide prepared from these mutants was determined, and the results are given in Table 6. *N*-acetylglucosamine was analysed from the polysaccharide (PS) obtained from lipopolysaccharide after removal of lipid A (Dröge *et al.* 1968) because lipid A also contains glucosamine. Phosphate was determined in both lipopolysaccharide and polysaccharide. The molar proportions of these constituents found in strains with the complete lipopolysaccharide core are given as the bottom line of the Table (see also Fig. 1, for the structure of the core).

Let us first examine the *rfe* mutants. Two of these were studied in each species: SH 1675 and SH 1679 of *Salmonella montevideo* and SH 1756 and SH 1757 (which may or may not represent the same *rfe* mutation—see p. 97) of *S. minnesota*. The sensitivity of these mutants to the phage FO strongly suggests that they contain the complete lipopolysaccharide core. All the core constituents were indeed found in the lipopolysaccharide prepared from them, and the polysaccharide contained the expected amount of *N*-acetylglucosamine. SH 1756 was the only exception, with a lower value for *N*-acetylglucosamine.

The serological specificity of the *rfe* lipopolysaccharide was studied by a haemagglutination inhibition technique (Beckmann, Lüderitz & Westphal, 1964). When red blood cells are coated with lipopolysaccharide of various R mutants they become agglutinable by the specific anti-R serum prepared against this sort of R lipopolysaccharide; this agglutination can be specifically inhibited by the same sort of lipopolysaccharide but not by others. In this test the *rfe* lipopolysaccharide could inhibit the haemagglutination by anti-*rfaL* of cells coated with *rfaL* lipopolysaccharide and by anti-*rfb* of cells coated with lipopolysaccharide from several *rfb* mutants. The *rfe* lipopolysaccharide thus resembles that of *rfaL* and *rfb* mutants, both of which are known to contain the complete core (see Introduction).

Two *rfa* mutants SH 3269 and SH 3237 were examined. They had no or very little *N*-acetylglucosamine in the polysaccharide, indicating deficient synthesis of the core. The defect in SH 3269 is apparently leaky, permitting some core stubs to be completed

and then capped by the O-specific side-chains, as indicated by the presence of some *N*-acetylglucosamine and traces of O-specific sugars. This strain behaves like a leaky R mutant also in its reactivity with the specific anti-O serum.

Strain SH 3237 (and its parent MR 3) has a more defective core lacking the second heptose and all other constituents distal to it. Strain MR 3 has been described as a mutant deficient in the transfer of the second heptosyl residue to the first heptose of the core (Risse *et al.* 1967). Later experience (unpublished observations) has shown that it also lacks the ability to phosphorylate lipopolysaccharide heptose. These two defects probably have their basis in two separate mutations, provisionally designated as *rfa*-3791 for the defect in heptosyl transfer, and *rfa*-3792 for the defect in phosphorylation. In the genetic analysis we have so far been unable to separate the two hypothetical mutations, which might indicate that they represent a deletion encompassing at least parts of two genes; however, no deletion mutations have so far been found in the *rfa* cluster (Mäkelä & Stocker, 1969). Strain MR 3 has been shown to possess at least one enzyme participating in core synthesis, namely the transferase for the first glucose residue (Risse *et al.* 1967), which in *Salmonella typhimurium* is determined by a gene *rfaG* in the *rfa* cluster (Wilkinson & Stocker, 1968; Osborn, 1968). The hypothetical deletion could thus not extend to this gene. The presence of either two separate mutations or a deletion would also account for the apparent non-leakiness of the defect in core synthesis.

The *rfa*-3789 of the *Salmonella minnesota* mutant MR 592 was not examined chemically because of its leakiness. *rfa*-3789 *rfe*⁺ derivatives of MR 592 were nearly smooth in cultural behaviour and O-specific agglutination although they retained a rough FO-resistant phage sensitivity pattern (SH 3264 in Table 2). A conspicuous feature of the *rfa rfe* double mutant MR 592 itself is the decreased amount of phosphate in both lipopolysaccharide and polysaccharide, indicating a lack of the phosphate normally attached to the core (Table 6). This type of mutant lipopolysaccharide has been described as P- as opposed to the normal P+ (Dröge *et al.* 1968). The P- lipopolysaccharide is characterized by (1) less phosphate in the lipopolysaccharide and polysaccharide than in P+ strains; (2) no heptosephosphate obtained on hydrolysis of lipopolysaccharide performed according to Slein & Schnell (1953); and (3) a peak of methylated heptoses obtained after methylation and methanolysis, not seen with P+ lipopolysaccharide. The lack of phosphorylation prevents the completion of the core by preventing the transfer of galactose (Mühlradt, Risse, Lüderitz & Westphal, 1968). The defect in phosphorylation in MR 592 is probably very leaky, as high amounts of glucose and galactose and even some *N*-acetylglucosamine are found in the lipopolysaccharide and polysaccharide. The absence of O-specific sugars in the lipopolysaccharide preparation is probably accounted for by the *rfe* mutation in MR 592.

When the *rfa* of either MR 3 or MR 592 was replaced by the *rfa*⁺ allele, the resulting *rfe* recombinants SH 1756 and SH 1757 had, as shown above, gained the ability to synthesize the complete core. The P+ character of their lipopolysaccharide was specifically confirmed by all the three criteria mentioned above. Thus it has been demonstrated that the phosphorylation of lipopolysaccharide heptoses is determined by a gene belonging to the *rfa* cluster.

As *rfa* mutants synthesize an incomplete core, they are unable to attach O-specific side-chains to their lipopolysaccharide. In *Salmonella typhimurium rfa* mutants the

Table 7. Paper chromatographic analysis of the monosaccharide constituents in the L1 (hapten) fraction of the rfa and rfe mutants

The L fraction was obtained as the supernatant after centrifugation (100,000 g) of the phenol-water extract to precipitate the lipopolysaccharide (Beckmann, Subbaiah & Stocker, 1964). This fraction is expected to contain the O-specific hapten.

Species	Strain	Relevant genotype		Monosaccharides in the L 1 fraction					
		rfa	rfe	Heptose	Glucose	Galactose	Glucosamine	Galactosamine*	Mannose*
<i>S. montevideo</i>	SH 3269	3652	+	-†	+	-	+	-	+
	SH 1675	+	3623	-	+	-	-	-	-
	SH 1679	+	3627	-	+	-	-	-	-
<i>S. minnesota</i>	SH 3237	3791, 3792	+	-	-	+	+	+	-
	mr 3	3791, 3792	3793	-	+	-	-	-	-
	mr 592	3789	3790	-	+	-	-	-	-
	SH 1756	+	3793	-	+	-	-	-	-
	SH 1757	+	3790	-	+	-	-	-	-
				-	+	-	-	-	-

* The O-specific sugars are mannose in *S. montevideo*, galactosamine in *S. minnesota* (Fuller & Staub, 1968; Lüderitz *et al.* 1966).

† Stands for a large amount of the sugar detected, - for none; no intermediate amounts were detected.

O-specific material is found as haptenic polysaccharide in the so-called L I fraction after phenol-water extraction and high-speed centrifugation, which removes lipopolysaccharide (Beckmann, Subbaiah & Stocker, 1964). We isolated the L I fraction from our *S. montevideo* and *S. minnesota* mutants in the same way, and analysed its monosaccharide content by paper chromatography (Table 7). The L I fraction of the *S. montevideo* *rfa* mutant SH 3269 contained large amounts of glucose, mannose and glucosamine, which are sugars constituting the O-specific side-chains in this species (Fuller & Staub, 1968). Of these sugars mannose is 'O-specific', occurring only in the O side-chains in the bacterium. Similarly the *rfa* mutant SH 3237 of *S. minnesota* contained in its L I fraction galactose, glucosamine and galactosamine, which are the constituents of its O side-chains (Lüderitz, Jann & Wheat, 1968), and of which galactosamine is O-specific. None of the four *rfe* mutants contained O-specific sugars in the L I fraction. Glucose was found in most of the L I fractions; it probably derives from cell-wall glucans. The original *S. minnesota* mutants mR 3 and mR 592 have no hapten no doubt because of their *rfe* mutation—when the *rfe* was replaced by the *rfe*+ allele (to obtain SH 3237 from mR 3), the strain gained the ability to synthesize hapten as expected of an *rfa* mutant.

DISCUSSION

Mutations at two separate locations in the *xyl*, *ilv* region of *Salmonella montevideo* and *S. minnesota* resulting in defects in the synthesis of lipopolysaccharide were demonstrated. One group corresponds to the *rfa* gene cluster described in *S. typhimurium*, which determines the synthesis of the lipopolysaccharide core, including its phosphorylation. The other group, the *rfe* mutants, has not been detected in *S. typhimurium*, and its function is unknown. The *rfe* mutants resemble phenotypically the *rfb* and *rfaL* mutants of *S. typhimurium*. All of these are sensitive to FO and several rough-specific phages, and they all synthesize a core lipopolysaccharide with *N*-acetylglucosamine. The *rfaL* mutants contain O-specific hapten and the *rfaL* gene is apparently concerned with the translocation of the O side-chain from its lipid carrier to the lipopolysaccharide core (Osborn, 1969). The *rfb* mutants are defective in the synthesis of O-specific sugars or in their assembly into the O side-chains, and therefore have no hapten (Beckmann, Subbaiah & Stocker, 1964; Nikaido, Nikaido, Subbaiah & Stocker, 1964; Nikaido, Levinthal, Nikaido & Nakane, 1967).

The *rfe* mutants of *Salmonella montevideo* and *S. minnesota* synthesize no hapten either (Table 7), and therefore it seems most likely that their mutation interferes with the biosynthesis of O-specific material. The *rfb* cluster is generally known to harbour most of the genes whose products participate in the synthesis of O-specific repeating units (Stocker, Wilkinson & Mäkelä, 1966), both the genes for the synthesis of the O-specific monosaccharides and the genes for the transferases assembling the repeating units. This was specifically shown to be true of the *rfb* cluster of *S. montevideo*. When the *rfb* region of the *S. montevideo* chromosome was introduced, by conjugation, into the cells of *S. typhimurium*, these started synthesizing a lipopolysaccharide of the *S. montevideo* type (Mäkelä, 1966; Nikaido, Nikaido & Mäkelä, 1966). It was concluded that all the information necessary for the synthesis of *S. montevideo*-specific lipopolysaccharide, and not shared by *S. typhimurium*, was located in the *rfb* gene cluster. Thus if the *rfe* gene(s), remote from *rfb*, specify a product necessary for the

synthesis of O side-chains, this function must be common to *S. montevideo* and *S. typhimurium*. What could such a common function be? The only monosaccharide found in the O side-chains of both these species is mannose (Fig. 1). However, the enzymes participating in the synthesis of mannose (as its guanosine-diphosphate nucleotide) are specified by *rfb* genes, or by a gene *pmi* (for phosphomannose isomerase) between *trp* and *gal* (Stocker, Wilkinson & Mäkelä, 1966; Nikaido *et al.* 1967). Furthermore, mannose is not present in the O side-chains of *S. minnesota*, in which *rfe* gene(s) were also identified. The antigen carrier lipid (Wright, Dankert & Robbins, 1965) on which the O side-chains are assembled, is very likely to be common to all *Salmonella* species. The possible role of *rfe* in antigen carrier lipid synthesis now seems to deserve serious investigation. There are, however, immediate objections to this hypothesis: a similar antigen carrier lipid is involved in the synthesis of the cell-wall peptidoglycan (Higashi, Strominger & Sweeley, 1967). Bacteria with an impaired synthesis of peptidoglycan would not be viable under the normal growth conditions used in the isolation and study of all our R mutants. Therefore we might rather think that the antigen carrier lipid will have to be modified in a certain way to mark it for either peptidoglycan or lipopolysaccharide (or some other) synthesis. This modification could be a slight chemical change or perhaps rather a fixed localization of the lipid in association with the enzymes participating in the relevant synthesis. On the other hand, the *rfe* gene(s) might have a regulatory function, e.g. for the *rfb* genes. So far no genetic regulation of the *rfb* genes has been found, and the *rfb*-determined enzymes have shown the same levels of activity whatever the growth conditions of the bacteria (Nikaido, 1968).

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