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Genetic Determination of Enzymes Synthesizing O-Specific Sugars of *Salmonella* Lipopolysaccharides

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ABSTRACT

NIKAIDO, HIROSHI (Massachusetts General Hospital, Boston, Mass.), KISHIKO NIKAIDO, AND P. HELENA MÄKELÄ. Genetic determination of enzymes synthesizing O-specific sugars of *Salmonella* lipopolysaccharides. J. Bacteriol. **91**:1126–1135. 1966.—Levels of enzymes involved in the biosynthesis of various nucleotide sugars were examined in parental strains and recombinants obtained in crosses between *Salmonella* of groups B, C_2, and C_1 with the O antigen specificities 4, 5, 12; 6, 8; and 6,7, respectively. The results showed that smooth strains of groups B and C_2 possessed the enzymes for the synthesis of guanosine diphosphate mannose, cytidine diphosphate abequose, and thymidine diphosphate rhamnose; these sugars are constituents of their lipopolysaccharides. Group C_1 lipopolysaccharide is devoid of both abequose and rhamnose, and the corresponding enzymes for cytidine diphosphate abequose synthesis, as well as the enzyme(s) catalyzing the last step(s) of thymidine diphosphate rhamnose synthesis, were undetectable in *S. montevideo* of this group. Two other enzymes also involved in the biosynthesis of thymidine diphosphate rhamnose were present at a low level of activity; their function in this strain is not known. The analysis of enzyme levels in recombinants indicated that genes determining at least eight of the enzymes involved in the biosynthesis of nucleotide-bound mannose, rhamnose, and abequose were located in the *O* locus known to determine the specificity of the O antigen. In three rough recombinant strains, enzyme levels indicated that crossing-over had presumably occurred within the *O* locus. The results also suggested a high degree of nonhomology in this region of the chromosome between groups B and C_1.

In genetic crosses between *Salmonella* species of different antigenic groups, recombinants were obtained among which the parental and some new types of O antigens segregated (7, 11, 12). A major gene locus controlling O antigen specificity showed close linkage to *his*, the locus for histidine biosynthesis, and was mapped between *his* and *metG*. (Symbols are defined in Table 1.) The *rouB* class of rough mutants, which cannot synthesize any O-specific material, also map at this *O* locus (27).

It is known that the O antigenic specificities in *Salmonella* are determined by the structure of the "O-specific side chain" (or S-specific side chain) portion of the cell wall lipopolysaccharide. Thus the *O* locus should contain information for the structure of these side chains, and, more specifically, it should determine several transferases involved in the orderly incorporation of component sugars into these side chains. These side chains are believed to be composed of many repeating units, each containing several monosaccharide units (22, 25), some of which are known to be present only in the O-specific side chains and are conveniently referred to as the O-specific sugars. Various *Salmonella* serotypes contain widely different O-specific sugars (8). Thus, the O-specific sugars in both groups B and C_2 are D-mannose, L-rhamnose, and abequose. These two groups show no serological cross-reactions in rabbit antisera, in spite of the similar composition of their lipopolysaccharides; linkages between these sugars, therefore, must be completely different (25). In group D, the O-specific sugars are D-mannose, L-rhamnose, and tyvelose; and in group C_1, D-mannose. These monosaccharides are used for the O antigen

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synthesis in their activated form, i.e., as nucleotide sugars, which are synthesized as shown in Fig. 1. In this paper, the three enzymes catalyzing the three successive steps leading from α-D-glucose-1-phosphate (G-1-P) to thymidine diphosphorhamnose will, for the sake of brevity, be designated as rha-1, rha-2, and rha-3; the three enzymes effecting the synthesis of cytidine diphosphoabequose as abe-1, abe-2, and abe-3; and the three enzymes catalyzing the synthesis of guanosine diphosphomannose as man-1, man-2, and man-3 (Fig. 1). Reactions rha-3 and abe-3 undoubtedly involve more than one enzyme each, but for the purpose of this study this point can temporarily be disregarded.

Many of the enzymes concerned with the synthesis of O-specific sugars have no other known functions. Their activity can therefore be expected to differ extensively in various serological groups. One such difference has been found between groups B and D, the former possessing the enzyme converting cytidine diphosphate-4-keto-6-deoxyglucose into CDP abequose, whereas the latter converts the same precursor to CDP tyvelose (4, 14; Nikaido and Nikaido, *in press*).

Furthermore, since in *Salmonella* many genes concerned with related functions are known to be clustered (2), the genes determining most of these enzymes—whose sole function is the biosynthesis of O-specific side chains—might be expected to reside also in the *O* gene cluster, in addition to the genes for the transferases already mentioned. In a rough mutant which maps in the *rouB* or *O* locus, a defect in the enzyme rha-3 has been found, indicating that the gene determining this enzyme is indeed a part of the *O* locus (20).

The present paper is an analysis of the level of these nucleotide sugar-synthesizing enzymes in *Salmonella* strains of groups B, C_2, and C_1, and their hybrids. It will be shown that these groups differ in their content of these enzymes, and that most of the enzymes concerned with the synthesis of the O-specific sugars are genetically determined at the *O* locus.

MATERIALS AND METHODS

Bacterial strains. Recombinants were obtained by crossing the smooth parent strains of different O groups (12). Most of them were selected so as to have inherited the donor *his*$^+$ allele with none, parts, or all of the nearby *O* locus. One recombinant, SH 912, however, was selected for the donor *try*$^+$ marker. In addition to the parent strains and recombinants, several rough mutants of group C_1 were also used for analysis. The main characteristics of the strains are given in Table 1.

Chemicals. Uridine diphosphogalactose was isolated from mutants lacking uridine diphosphogalactose-4-epimerase (16). α-D-Mannose-1-phosphate was a kind gift of G. Ashwell. P^{32}-pyrophosphate was synthesized according to Bergmann (1). Other chemicals were obtained from commercial sources: G-1-P, D-mannose-6-phosphate, and D-fructose-6-phosphate from Sigma Chemical Co., St. Louis, Mo.; uridine triphosphate, thymidine triphosphate, guanosine triphosphate, cytidine triphosphate, cytidine diphos-

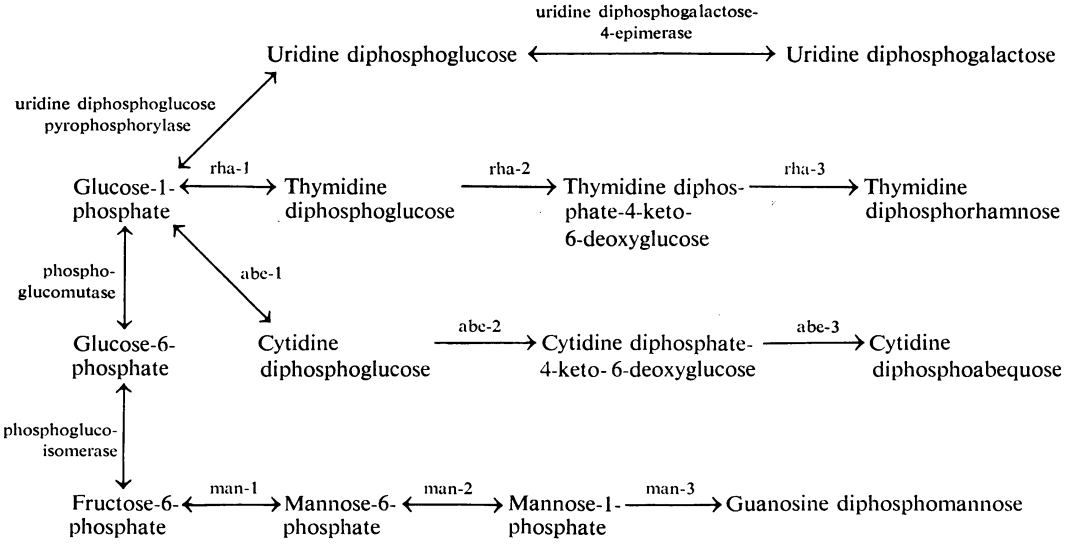

FIG. 1. *Pathways of biosynthesis of nucleotide-sugars in Salmonella* (5). *Abbreviations for the names of enzymes: rha-1, thymidine diphosphoglucose pyrophosphorylase; rha-2, thymidine diphosphoglucose oxidoreductase; rha-3, thymidine diphosphorhamnose synthetase; abe-1, cytidine diphosphoglucose pyrophosphorylase; abe-2, cytidine diphosphoglucose oxidoreductase; abe-3, cytidine diphosphoabequose synthetase; man-1, phosphomannoisomerase; man-2, phosphomannomutase; man-3, guanosine diphosphomannose pyrophosphorylase.*

TABLE 1. *Salmonella strains studied*

Group	Species	Strain	O serotype*	Source
B	*S. abony*	SW 1444†	S 4,5,12	Originally no. 103 of Edwards and Bruner (3)
	S. typhimurium	LT2†	S 4,5,12	Stocker, Zinder, and Lederberg (26)
C_2	*S. takoradi*	SH 685†	S 6_1, 8	Originally no. 239 from Communicable Disease Center, Atlanta, Ga.
C_1	*S. montevideo*	SH 541†	S 6_1, 7	Originally no. 129 of Edwards and Bruner (3)
		SH 692	R	Mutant of SH 541
		SH 705	R	Mutant of SH 541
		SH 721	R	Mutant of SH 541
		SH 727	R	Mutant of SH 541

Recombinants from cross:	Strain	O serotype	Genetic composition (loci in clockwise map order)‡							
			rouC	*try*	*H1*	*his*	*0-4/7*	*0-5*	*metG*	*aroD* or *athA*	*H2*
B × C_2 (*S. abony* donors to *S. takoradi* recipient)	SH 835	SR (4,5,12)	r		r	d	d	d		r	r
	SH 834	SR (4,12)	r		r	d	d	r		r	r
	SH 830	S 4,12	d		r	d	d	r		r	r
B × C_1 (*S. abony* donors to *S. montevideo* recipients)	SL 958	S 6_1,7			r	d	r			r	r
	SH 806	SR (4,5,12)	r			d	d	d		r	
	SL 954	SR (4,5,12)	r		d	d	d	d		d	r
	SH 805	SR (4,12)	r			d	d	r		r	
	SH 817	SR (4,12)	r			d	d	r		r	
	SL 956	SR (4,12)	r		r	d	d	r		r	r
	SL 953	S 4,12	d		r	d	d	r		r	r
	SL 957	R			r	d	?			r	r
	SH 912	R		d		r	?			r	r
C_1 × B (*S. montevideo* donors to *S. typhimurium* recipients)	SH 1049	S 6_2,7			d	r	d		d	r	
	SH 1050	S 6_2,7		d	d		d				r
	SH 1101	S 6_1,7				d	d		d	d	
	SH 1070	R				d	?		d	r	
	SH 1104	R				d	?				
	SH 1115	R				d	?				
	SH 1086	R				d	?		r	r	

* S, smooth; R, rough; SR, semirough (17).

† Strains actually used in crosses were frequently derivatives of these strains. Since the derivatives were presumably isogenic with the parent strains as regards the genes determining nucleotide-sugar synthesis, enzyme levels in the original strains, rather than those of the derivatives, were measured and tabulated in Tables 2–4.

‡ Symbols: r, recipient allele; d, donor allele. The genes outside the segment *rouC-H2* were recipient-like in all cases tested (*metC*, *str*, *ile*, *metA*, *leu*). The symbols for gene loci: *rouC*, elongation of O-specific side-chain; *try*, tryptophan biosynthesis; *H1*, phase 1 flagellar protein; *his*, histidine biosynthesis; *0-4/7*, O-antigen specificities 4,12/6,7/6,8/9,12; *0-5*, O-antigen specificity 5; *metG* (*metC* and *metA*), methionine biosynthesis; *aroD*, biosynthesis of tyrosine and phenylalanine; *athA*, purine biosynthesis; *str*, streptomycin resistance; *ile*, isoleucine biosynthesis; *leu*, leucine biosynthesis. For the genetic map of *Salmonella*, see Sanderson and Demerec (23), Smith and Stocker (24), Mäkelä (10–12).

phoglucose, thymidine diphosphoglucose, D-glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), phosphoglucomutase, and G-6-P dehydrogenase from Calbiochem.

Preparation of cell-free extracts and assay of enzymes. The cells were grown in nutrient broth (Difco), and sonic extracts were prepared as described previously (19). The reactions catalyzed by the enzymes are shown in Fig. 1. It should be noted that all assays were carried out with tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (*p*H 7.5), and that for some of the enzymes this *p*H was not optimal for their activity. Some enzymes were assayed at 37 C (pyrophosphorylases, uridine diphosphogalactose-4-epimerase, rha-2, rha-3, abe-2, and abe-3); the others were assayed at 25 C. Furthermore, owing to the presence of interfering enzymes in the crude extracts, activities of some enzymes were overestimated, and those of some were underestimated. The absolute values obtained, therefore, have little significance.

However, the results do permit the comparison of the relative levels of each enzyme among the strains examined.

Nucleoside diphosphate hexose pyrophosphorylases. These enzymes (rha-1, abe-1, man-3, and uridine diphosphoglucose pyrophosphorylase) were assayed by measuring the hexose-1-phosphate-dependent exchange of labeled pyrophosphate into nucleoside triphosphates, according to the principle established by Neufeld et al. (18). Incubation mixtures contained the following in a final volume of 1.0 ml: Tris-HCl buffer (*p*H 7.5), 100 μmoles; $MgSO_4$, 10 μmoles; and KF, 5 μmoles. In addition, the incubation mixture for uridine diphosphoglucose pyrophosphorylase contained uridine triphosphate, 1.0 μmole; P^{32}-pyrophosphate, 2.0 μmoles; and G-1-P, 1.0 μmole. The mixture for rha-1 contained thymidine triphosphate, 1.0 μmole; P^{32}-pyrophosphate, 1.0 μmole; and G-1-P, 1.0 μmole. The mixture for abe-1 contained cytidine triphosphate, 1.0 μmole; P^{32}-pyrophosphate, 1.0 μmole; and G-1-P, 1.0 μmole. The mixture for man-3 contained guanosine triphosphate, 1.0 μmole; P^{32}-pyrophosphate, 5 μmoles; and α-D-mannose-1-phosphate, 2.0 μmoles. After 10 min of incubation at 37 C, the reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. The tubes were transferred into an ice bath, and 0.2 ml of charcoal suspension [Darco G-60, 15% (w/v) in water] was added. After 3 min, the tubes were centrifuged in the cold, and the charcoal sediment was washed three times by resuspension and centrifugation, each time with 2.5 ml of ice-cold water. The sediment, containing the adsorbed nucleoside triphosphates, was suspended in 3.0 ml of 50% ethyl alcohol, which was 0.3 N in respect to NH_4OH, and samples were plated and counted with a Nuclear-Chicago gas flow counter equipped with a Micromil window. For each determination, controls without hexose-1-phosphate were always run, and the counting rate of the controls was subtracted. Incorporation in these control tubes was very small (about 3% of experimental, group B parent) in the case of rha-1, but was considerably higher in the assay of abe-1.

Thymidine diphosphoglucose oxidoreductase (rha-2) and "thymidine diphosphorhamnose synthetase" (rha-3). These reactions were measured as described by Okazaki et al. (21). For the assay of the latter reaction, thymidine diphosphoglucose was used as substrate, and an excess of thymidine diphosphoglucose oxidoreductase (crude extracts of an *S. typhimurium* mutant strain, TV 208, which is defective in rha-3) was added.

Cytidine diphosphoglucose oxidoreductase (abe-2) and "cytidine diphosphoabequose synthetase" (abe-3). These enzymes were assayed as described elsewhere (Nikaido and Nikaido, *in press*), except that, for the assay of abe-3, cytidine diphosphate-4-keto-6-deoxyglucose-C^{14} (0.12 μmole) was used as substrate and 0.05 μmole of flavin mononucleotide was also added to the reaction mixture.

Phosphomannomutase (man-2). This enzyme was assayed spectrophotometrically by following the reduction of NADP by G-6-P dehydrogenase and G-6-P which was derived from mannose-1-phosphate through reactions man-2, man-1, and phosphoglucoisomerase (Fig. 1). Since the overall reaction was dependent upon the endogenous man-1, phosphoglucoisomerase, and mannose-1,6-diphosphate or glucose-1,6-diphosphate, or both [cofactors of man-2 (6)], contained in the crude extracts, the values obtained for this enzyme represent minima only. Enzyme man-1, however, was found to be present in all cases (Tables 2–4). Phosphoglucoisomerase, whose activity was usually about 100 times higher than that of man-1 (20), was also assayed in cases where the activity of man-2 was found to be low; phosphoglucoisomerase was found to be normal in all of these cases. The reaction mixture for man-2 contained the following in a total volume of 1.0 ml: Tris-HCl buffer (*p*H 7.5), 50 μmoles; $MgCl_2$, 2 μmoles; cysteine, 1 μmole; α-D-mannose-1-phosphate, 1.0 μmole; NADP, 0.4 μmole; G-6-P dehydrogenase, 1 μg; and crude extract. The reduction of NADP was followed, as in all the other assays described below, at 340 mμ with a Cary model 15 recording spectrophotometer. After a long lag (usually about 30 min), the reaction attained a constant rate.

Phosphomannoisomerase (man-1). This enzyme was assayed spectrophotometrically in a reaction mixture similar to that for the assay of man-2, except that mannose-6-phosphate (0.4 μmole) replaced mannose-1-phosphate as substrate and that cysteine was omitted. The reaction was started by the addition of sonic extract. Owing to the contamination of the substrate with small amounts of G-6-P, there was an initial burst of NADP reduction, but after 3 to 5 min the reaction attained a constant rate.

Phosphoglucomutase and phosphoglucoisomerase. These enzymes were also assayed spectrophotometrically. The reaction mixture contained, in a final volume of 1.0 ml: Tris-HCl buffer (*p*H 7.5), 50 μmoles; $MgCl_2$, 5 μmoles; NADP, 0.5 μmole; G-6-P dehydrogenase, 1 μg; and crude extract. In addition, the mixture for phosphoglucomutase contained cysteine, 5 μmoles; G-1-P, 1.0 μmole; and glucose-1,6-diphosphate, 2 mμmoles. The reaction mixture for phosphoglucoisomerase contained fructose-6-phosphate, 1.0 μmole.

Uridine diphosphogalactose-4-epimerase. This enzyme was assayed according to the two-step procedure of Maxwell, Kurahashi, and Kalckar (15).

RESULTS

Enzyme levels in the smooth parent strains. Results of enzyme assays on the smooth parent strains and the rough mutants are given in Table 2. The three enzymes of thymidine diphosphorhamnose synthesis, and the three of cytidine diphosphoabequose synthesis, were present with approximately the same levels of activity in group B (4,5,12) and group C_2 (6,8) organisms; this is consistent with the fact that the organisms of both groups contain rhamnose and abequose in their lipopolysaccharides. In contrast, the group C_1 (6,7) strain, whose lipopolysaccharide is

TABLE 2. *Enzyme levels in smooth Salmonella strains of groups B, C_1, and C_2, and in some rough mutants of group C_1**

Species	O serotype	Strain	Specific activity of enzyme†								
			rha-1	rha-2	rha-3	abe-1	abe-2	abe-3	man-1	man-3	man-3
S. abony	Group B 4,5,12	SW 1444	480	770	24	142	ND‡	ND	670	110	29
			340	360	76	109	180	2.0	620	140	30
S. typhimurium	Group B 4,5,12	LT2	940	450	84	125	140	5.8	390	80	80
S. takoradi	Group C_2 6,8	SH 685	260	500	48	96	ND	4.2	1,150	370	65
S. montevideo	Group C_1 6,7	SH 541	40	40	<2	<10	<10	ND	550	190	250
			30	60	<2	<10	<10	<0.2	560	450	210
S. montevideo	Group C_1 rough	SH 692	120	120	ND	<10	ND	ND	780	180	150
		SH 705	60	60	<2	<10	ND	ND	400	430	320
		SH 721	60	70	<2	<10	ND	ND	510	230	100
		SH 727	40	30	ND	<10	ND	ND	680	70	210

* Abbreviations for the names of enzymes are given in Fig. 1. For SW 1444 and SH 541, values obtained in experiments performed several months apart are shown, to indicate the degree of reproducibility of the assays. The values for these strains shown in Tables 3 and 4 are the average of the two sets of results.

† Expressed as millimicromoles per milligram of protein per hour.

‡ Not determined.

devoid of both these sugars, had undetectable activity in the synthesis of cytidine diphosphoabequose, undetectable activity of rha-3, and only low levels of activity of the other two rhamnose enzymes. These "low" levels were about 10% of those in group B and C_2, but were nevertheless at least five times higher than the lowest detectable levels of these enzymes.

Mannose is an O-specific sugar common to all the groups studied; all the strains had distinct activity of the three enzymes of guanosine diphosphomannose synthesis. The group C_1 organisms had higher values for the enzyme man-3, whereas the group C_2 strain tended to have slightly higher activity of man-1.

The enzymes phosphoglucomutase, phosphoglucoisomerase, uridine diphosphoglucose pyrophosphorylase, and uridine diphosphogalactose-4-epimerase were each found to have essentially identical activities in extracts of the parent strains (not shown in Table 2). The levels of activity were similar to the values already described for the derivatives of *S. typhimurium* LT2 (20).

The rough mutants of group C_1 had approximately the same activity of various enzymes as had the smooth strain from which they were derived. This result is similar to what was found in rough mutants of *S. typhimurium*, where only 1 of 12 analyzed had a defect in one of these enzymes (20).

Enzyme levels in the recombinants. Enzyme assays were made on three recombinants (SH 835, SH 834, and SH 830 of Table 1) with the O antigenic specificity of group B-type from a cross between a group B donor and a group C_2 recipient strain. The activities were very similar in both parents and in all the recombinants (not shown in table). Results of enzyme assays on recombinants of group B × group C_1 crosses are presented in Tables 3 and 4. The values for the parent strains (the same as in Table 2) are given on the first two lines of Tables 3 and 4, to facilitate comparison.

The recombinants in Table 3 are the products of crosses of group B (4,5,12) donors with group C_1 (6,7) recipients. Four main classes of recombinants are represented: smooth, with the recipient O antigen 6,7; semirough (17), with the donor O antigens 4,5,12 or 4,12; smooth, with the donor antigens 4,12; and rough. The values of the three rhamnose enzymes, of the three abequose enzymes, and of one of the mannose enzymes (man-3) differed significantly between the parent strains, and characterized the "group B-like enzyme pattern" and the "group C_1-like enzyme pattern." The recombinant with the group C_1 antigen 6,7 had a group C_1-like enzyme pattern quite similar to that of the recipient group C_1 parent. All the recombinants, both semirough and smooth, with the donor antigens 4,12 (with or without 5), had a group B-like enzyme pattern indistinguishable from that of the group B donor strain.

The two rough recombinants of Table 3 represent very rare types, each encountered only once in the 201 recombinants tested (12). Here the pattern of enzyme levels was essentially a mixture of those of both parents, but with additional en-

TABLE 3. *Enzyme levels in parents and recombinants of crosses between group B (4,5,12) donors and group C_1 (6,7) recipients*

O serotype	Strain	Specific activity of enzyme†									Enzyme pattern
		rha-1	rha-2	rha-3	abe-1	abe-2	abe-3	man-1	man-2	man-3	
S 4,5,12	SW 1444	410	570	50	126	180	2.0	640	130	29	Group B-like
S 6,7	SH 541	40	50	<2	<10	<10	<0.2	560	320	230	Group C_1-like
S 6,7	SL 958	40	50	<2	<10	<10	<0.2	460	180	160	Group C_1-like
SR (4,5,12)	SH 806	380	650	14	37	130	1.4	320	80	49	Group B-like
SR (4,5,12)	SL 954	610	460	23	120	130	2.6	500	130	32	
SR (4,12)	SH 805	450	390	60	84	130	0.8	720	150	36	
SR (4,12)	SH 817	490	470	14	49	240	2.9	400	170	55	
SR (4,12)	SL 956	530	690	44	90	130	1.9	750	280	15	
S 4,12	SL 953	590	410	21	131	80	1.1	370	70	24	
R	SL 957	270	30	<2	<10	<10	<0.2	360	<5	<2	Mixed
R	SH 912	280	<5	<2	<10	<10	<0.2	560	<5	<2	

* SW 1444 and its derivatives were the donor; SH 541 and its derivatives, the recipients; the other strains represent recombinants.

† Expressed as millimicromoles per milligram of protein per hour.

zyme defects. In both strains the abequose enzymes were undetectable, as in the recipient group C_1 parent; of the rhamnose enzymes, rha-1 was donor group B-like in both strains, but rha-2 was group C_1-like in the strain SL 957, and undetectable in the other strain, SH 912; rha-3 was undetectable in both. The mannose enzymes man-2 and man-3 were undetectable in contrast to their presence in both parent strains, whereas man-1 was present at the same level of activity as in both parent strains. (In contrast to man-2 of SH 1086, only traces of man-2 activity were observed in the extracts of SH 912 and SL 957, even when glucose-1,6-diphosphate was added.)

Table 4 gives the results for recombinants from crosses where the donor strain was of group C_1 (6,7), and the recipient of group B (4,5,12), that is, the reverse of the crosses shown in Table 3. All recombinants with donor group C_1 antigens 6_1,7 or 6_2,7 had a group C_1-like enzyme pattern. Four rough recombinants were found among 1,554 recombinants studied (Mäkelä and Hovi, *unpublished data*). One of them had a completely group C_1-like enzyme pattern, two had a group B-like pattern, and one, SH 1086, had a complex pattern. It had donor group C_1-like levels for the three abequose enzymes and the three rhamnose enzymes; of the mannose enzymes, man-3, present in both parents, was undetectable in this strain, but man-1 was present at the same level as in both parents. Its man-2 showed a peculiar behavior: the activity was extremely low (0.01 μmole per mg of protein per hr) when assayed by the regular method, but was normal (0.24 μmole) when the cofactor glucose-1,6-diphosphate (10 mμmoles) was added to the reaction mixture. An assay of endogenous glucose-1,6-diphosphate in a crude extract of this strain revealed that its content was about 50% of that in a crude extract of *S. typhimurium* LT2. It is still not clear, however, whether the peculiar behavior of man-2 is due to a decreased content of endogenous glucose-1,6-diphosphate or to an alteration in the structure of the enzyme (man-2) itself.

DISCUSSION

Inheritance of O antigens in intergroup crosses of Salmonella. It has been shown that the O specificities 4 and 12 of group B, 9 of group D, 6 and 7 of group C_1, and probably 6 and 8 of group C_2 are determined at a locus, *O*, which is closely linked to *his* (7, 11; Stocker, Wilkinson, and Mäkelä, *in press*). The antigen factor 5, which is found only in group B, is determined at a locus close to the *O* locus (11, 12, 24).

Thus, in crosses between strains of group B and C_1 or C_2, the majority of recombinants which have inherited the *his* allele from the donor parent have also inherited the closely linked *O* allele of the donor and are serologically donorlike. There are three complications, however. (i) When the donor parent was of group C_1 with the antigenic formula 6_1,7, most recombinants were found to be 6_2,7 (*see* SH 1049 and SH 1050 of Table 1). The difference is due to a prophage carried by the 6_1,7 strains; the 6_2,7 recombinants can be converted to 6_1,7 by the action of this phage (Mäkelä and Hovi, *to be published*). (ii) When the donor was of group B (4,5,12), most *his*$^+$

TABLE 4. *Enzyme levels in the parents and recombinants of crosses between a group C_1 donor and group B recipients*

O serotype	Strain[a]	Specific activity of enzymes[b]									Enzyme pattern
		rha-1	rha-2	rha-3	abe-1	abe-2	abe-3	man-1	man-2	man-3	
S 6₁,7	SH 541	40	50	<2	<10	<10	<0.2	560	320	230	Group C_1-like
S 4,5,12	LT2	940	450	84	125	140	5.8	390	80	80	Group B-like
S 6₂,7	SH 1049	20	80	<2	<10	<10	<0.2	190	150	210	Group C_1-like
S 6₂,7	SH 1050	20	230	<2	<10	<10	<0.2	290	370	300	
S 6₁,7	SH 1101	50	80	<2	<10	<10	<0.2	650	350	360	
R	SH 1070	30	160	<2	<10	<10	<0.2	290	290	380	
R	SH 1104	1,590	430	73	128	250	1.1	530	50	60	Group B-like
R	SH 1115	1,590	500	80	77	ND[c]	ND	620	70	80	
R	SH 1086	30	150	<2	<10	<10	<0.2	330	10†	<2	Mixed?

[a] A derivative of SH 541 was the donor; derivatives of LT2, the recipients; the other strains represent recombinants.

[b] Expressed as millimicromoles per milligram of protein per hour.

[c] Not determined.

[d] Much higher activity was demonstrated when glucose-1,6-diphosphate was added.

recombinants had these specificities but were not smooth like the donor parent. Instead, they were semirough (SR): either SR (4,12), with the antigens 4 and 12 (*see* SH 805, SH 817, and SL 956 of Table 1), or SR (4,5,12), with antigen 5 as well as 4 and 12 (*see* SH 806 and SL 954 of Table 1). The SR form is believed to contain only one repeating unit per O-specific side chain, presumably owing to defects in a transferase which transfers the second and subsequent repeat units to the incomplete lipopolysaccharide (17). This defect maps far away from *O* and *his*, at a locus provisionally called *SR* or *rouC* and located between *gal* and *try*. (iii) The gene *O-5*, which determines the factor antigen 5, that is, the acetylation of the galactose located in the repeat unit of group B (9), is apparently absent in group C_1 and group C_2. Because this locus is not very closely linked to the main *O* locus, crossovers between these two loci produced 4,12 recombinants when the group B parent was 4,5,12 (12).

Enzyme levels and the composition of lipopolysaccharides. In the three groups, B, C_1, and C_2, the content of enzymes concerned with the synthesis of sugar-nucleotides varied according to the content of the sugar components in their lipopolysaccharides. Enzymes synthesizing uridine diphosphogalactose, uridine diphosphoglucose, and guanosine diphosphomannose were found in all the strains whose lipopolysaccharides are known to contain these sugars. Differences, however, were found in the levels of two of the mannose enzymes. The three abequose enzymes and rha-3 were present at approximately the same levels in groups B and C_2, whose lipopolysaccharides contain both abequose and rhamnose; these enzymes were undetectable in group C_1, whose lipopolysaccharide is devoid of these sugars. The other two rhamnose enzymes, rha-1 and rha-2, were much less active in group C_1 than in the other two groups, but a low level of activity was definitely demonstrated. This is interesting in view of the fact that these two enzymes also participate in the biosynthesis of thymidine diphosphate-4-acetylamino sugars (13), one of which has been found to be a component of the lipopolysaccharide of *Chromobacterium violaceum* (28). Although such sugars have not been isolated from acid hydrolysates of O antigens of enteric bacteria, some workers suspect that the sugars may be present but are broken down during acid hydrolysis (13).

Genes determining the enzymes of nucleotide-sugar synthesis. Our data provide information on the genetic determination of eight of the nine enzymes studied. Strains of groups B and C_1 differed significantly in the activity levels of seven enzymes (abe-1,2,3; rha-1,2,3; and man-3). In all recombinants, the enzyme pattern corresponded to their O antigens: those with the group C_1 specificities 6₁,7 or 6₂,7 had a group C_1-like enzyme pattern, no matter whether the recipient or the donor parent had been of group C_1; and those with the group B antigens 4,12 or 4,5,12, either smooth or semirough, all had a group B-

like enzyme pattern. As the O antigen specificity in these groups is inherited as if determined by one genetic locus, *O*, closely linked to *his* (12), the activity levels of the seven enzymes must also be determined at the same *O* locus. (As a rule the recombinants in *Salmonella* crosses receive only short segments of chromosome from the donor, F^+, Hfr, or F'^+ bacteria. If these enzymes were determined by genes located outside the *O* locus, segregation between the genes and the genes of the *O* locus would have been observed.)

This could either mean that the structural genes for all these enzymes reside in the *O* locus, or that this locus merely controls the activity levels of the enzymes by some kind of repression-derepression mechanism. The former alternative is supported by the enzyme patterns of the rough recombinants from crosses between group B donors and group C_1 recipients (Table 3). The levels of enzymes rha-1 and rha-2, which catalyze the two successive steps of thymidine diphosphorhamnose synthesis and which were both high in the donor and both low in the recipient parent, showed a mixed pattern in the rough recombinants. In SL 957, rha-1 was high and rha-2 low; in SH 912, rha-1 was high and rha-2 was undetectable. These various patterns, which are difficult to explain by repression-derepression, can be easily understood as a result of crossing-over (see below), if the different activity levels are assumed to be the reflection of different structural genes.

The assay for man-2 was only semiquantitative. Thus, it was not possible to map the gene for man-2 in the same way as the genes already discussed. Its activity, however, disappeared in some rough recombinants (SL 957 and SH 912) which are believed to have had crossovers within the *O* locus. This result could be best understood if the structural gene for man-2 is in the *O* locus (see below).

Phosphomannoisomerase (man-1) did not differ significantly in activity among various groups; therefore, its determinant gene could not be mapped from our results alone. However, B. A. D. Stocker and M. J. Osborn (*personal communication*) investigated mutants defective in this gene and found that it mapped between the loci *try* and *gal*, that is, outside the *O* locus.

The presence or absence of antigen 5 in the recombinants with group B specificity was not reflected in their content of the enzymes of nucleotide-sugar synthesis; this is what one would expect if gene *O–5* determines the acetylation of a sugar unit already present in the lipopolysaccharide. Nor were differences found between semirough and smooth forms of 4,12 or 4,5,12 recombinants, also as expected if the semirough form is a result of a defect in one of the transferases (none of which was measured in this study).

Rough recombinants. Of the very few rough recombinants encountered (Tables 3 and 4), two had a recipientlike and one a donorlike enzyme pattern. Their rough character could have resulted from a mutation in the recipient or the donor, or from a crossover within the *O* locus which caused the loss of a transferase or transferases without affecting the enzymes of nucleotide-sugar synthesis. From our data it is not possible to decide between these two alternatives.

In addition to these recombinants, there were three other rough recombinants with a mixed pattern of enzymes. It seems unlikely that they were derived from rough mutants of either parent, because two of them showed defects involving more than one enzyme in each case; the 4 rough mutants of *S. montevideo* studied in this paper (Table 2) and the 12 rough mutants of *S. typhimurium* studied by Nikaido et al. (20) had no defect in these enzymes, with the exception of a single strain with only one defect.

In these three rough recombinants, rha-1 was always donorlike, showing that they all had received at least a part of the donor *O* locus. This finding is significant for the interpretation of SH 912, a recombinant selected for having the donor allele of *try*, which is located quite far from the *O* locus. It is known to have the recipient his^- allele, and thus might be thought unlikely to have the donor *O* allele. Nevertheless, in view of its donorlike level of rha-1, and in view of the fact that its rough character was shown to be genetically closely linked to *his* (Mäkelä, *unpublished data*), it seems to have received a part of the donor *O* allele and to represent a case with quadruple crossovers, one of which should be within the *O* locus.

Rha-2 in the recombinant SH 912, man-2 in in SH 912 and SL 957, and man-3 in all three recombinants were undetectable, but all of these enzymes were present in both parents. We assume that these enzymes were lost as a result of crossovers involving the nonhomologous *O* segments of group B and group C_1 chromosomes; the existence of such nonhomology is indeed suggested also by various other considerations (see below).

The enzymes rha-3 and abe-1,2,3 were undetectable in these three rough recombinants. As they were also undetectable in one of the parents, it is not possible to say whether they were affected by the crossing-over or not.

Structure of O locus. The *O* locus in group B thus probably contains the structural genes for the three enzymes of thymidine diphosphorham-

nose synthesis, for the three of cytidine diphosphoabequose synthesis, and for two mannose enzymes, man-2 and man-3. In group C_1, abequose enzymes and the enzyme rha-3, and thus probably the corresponding genes, are missing; the enzyme man-3 is present, and its determinant gene appears to reside in the *O* locus. Thus, the *O* loci of the two groups must have large structural differences. In addition, even the genes for functionally similar enzymes may not be homologous. Transferases must also be quite different among groups B, C_1, and C_2, since the structure of the end products—O-specific sidechains—seems to be very different, as judged by their distinct immunological specificities. These transferases are apparently also determined genetically at the *O* locus, adding to its nonhomologous character.

It seems possible that an analysis of this kind would allow a mapping of the genes for the various synthetic and transferring enzymes within the *O* locus. Unfortunately, crossovers within the *O* locus seem to be very rare, as one would expect between two parental chromosome segments with little homology and thus little opportunity for effective pairing. It is probable that most of such crossovers would yield serologically rough forms, as a failure in the synthesis of a proper repeat unit would in most cases result in the production of a lipopolysaccharide without any O-specific side chains, that is, a rough antigen. Rough recombinants (as well as possible but apparently exceedingly rare types with new smooth antigens) should therefore be looked for and investigated. An analysis of the specific transferases would be as important as the study of the enzymes of nucleotide-sugar synthesis; we are now planning to attempt such an analysis.

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