

Mapping of Genes Determining Penicillin-resistance and Serum-sensitivity in *Salmonella enteritidis*

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Two presumptive single-step mutants, resistant to penicillin and sensitive to the bactericidal effect of normal human serum, were isolated on penicillin gradient plates from a smooth, penicillin-sensitive, serum-resistant strain of *Salmonella enteritidis* (O9,12; gal hisEI cys). The chemical composition of their lipopolysaccharides, their phage-sensitivity patterns, and their serological and cultural properties showed one to be 'part-rough' and the other smooth. Hfr strains with O antigens O4,5,12 or O1,4,5,12 (two *Salmonella typhimurium* and one *S. abony*) and one *S. enteritidis* F', O9,12, were crossed with the *S. enteritidis* O9,12 mutants. The results with the part-rough mutant indicate that its penicillin-resistance, serum-sensitivity and rough phage pattern result from a single mutation between *hisEI* and the part of the *rfb* gene cluster determining O specificity, 4 (abequose) or 9 (tyvelose). Transduction experiments confirmed that the mutation is closely linked to the *his* operon. This mutation is inferred to cause an incomplete defect in a transferase for galactose, mannose or rhamnose, the smooth sugars common to O4,5,12 and O9,12. Results from similar crosses to the smooth, serum-sensitive, penicillin-resistant *S. enteritidis* mutant indicate that its serum-sensitivity is not linked to *his*. The occasional independent segregation of penicillin-resistance and serum-sensitivity suggests that other loci modify penicillin-resistance.

INTRODUCTION

Michael & Braun (1958) reported that passage of strains of *Shigella dysenteriae* or *Escherichia coli* on a series of gradient plates, each with a higher concentration of penicillin than the preceding one, yielded penicillin-resistant mutants which were more easily killed by exposure to normal serum (antibody plus complement) than were the parent strains, but which retained the O-antigenic specificities of the parent strains. Roantree & Steward (1965) used this method to derive serum-sensitive mutants from serum-resistant, smooth *Salmonella enteritidis* and *Salmonella typhimurium* strains, to test whether becoming serum-sensitive reduced virulence. They observed that in a series of mutants, derived stepwise from a given parent strain, only one or a few of the mutations causing increased penicillin-resistance were associated with increased sensitivity to serum. Some serum-sensitive strains were apparently smooth, others were rough, as indicated by colonial morphology and growth with deposit in broth. Nelson & Roantree (1967) characterized lipopolysaccharides (LPS) from these mutants and from single-step penicillin- or cephalothin-resistant mutants of the same two parent strains. The mutants fell into five groups: (a) hapten-negative rough mutants lacking detectable O-specific material in the LPS and L1 fraction [the supernatant after ultra-

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centrifugation in the phenol-water procedure (Westphal *et al.*, 1952) for the extraction of LPS]; (b) a hapten-positive rough mutant with O-specific material in the L1 fraction only; (c) part-rough mutants, with phenotypes intermediate between smooth and rough; (d) mutants which were serum-sensitive but unaltered in smooth character; (e) penicillin-resistant mutants not altered in resistance to serum, in antigenic character or LPS sugar composition, but less virulent (for mice) than the parent strain.

We report here genetic and other investigations of two serum-sensitive mutants of a genetically marked smooth *S. enteritidis* strain, obtained at a single step by selection for resistance to penicillin. One mutant, R146, fell into the part-rough category *c* described above. The other, R147, possessed the characteristics of category *d*, i.e. serum-sensitive but apparently smooth. Tests of the activity of extracts of R146 (by Dr H. Nikaido) showed that it was not defective in any of the enzymes needed for synthesis of the nucleotide precursors of the components of the repeat unit of group B or D LPS.

METHODS

Media. The media used were Difco nutrient broth and nutrient agar and, as defined medium, that of Davis & Mingioli (1950), solidified with purified agar (Difco Noble agar) and with glucose (2 g l⁻¹) as energy source; its citrate component was omitted for tests on sugar utilization, etc.

Bacterial strains and phages. The bacterial strains used are listed in Table 1. *Salmonella enteritidis* strain R134, from which penicillin-resistant mutants were isolated, is a *his-1461 cys gal* derivative, obtained by three successive mutations, each after exposure to ethyl methanesulphonate, from a 'wild' mouse-virulent *S. enteritidis* strain, I203 (Roantree & Steward, 1965), of typical antigenic composition, i.e. with O antigen O9,12 and phase-1 H antigen *g,m* and no phase-2 flagellar antigen. Mutation *his-1461* of R134 falls within the *hisE11659* deletion (P. E. Hartman, personal communication). The cystine requirement of R134 is satisfied by sulphide or thiosulphate but not by sulphite, which indicates its *cys* mutation is either at *cysC* or *cysD* within the *cysCDHIJ* cluster at 60 units, or at *cysG* at 72 units (Sanderson & Hartman, 1978). The *gal* mutation of R134 did not alter its smooth character, which shows that *galE* function was not affected. Strain R134 is as penicillin-sensitive and as serum-resistant as its 'wild' ancestor, I203, but, for unknown reasons, of reduced mouse-virulence (intraperitoneal route). The strains used as conjugational donors of O-group B or D (Table 1) were all smooth, serum-resistant and about as penicillin-sensitive as strain R134. Phages P22 and P22h (Wilkinson *et al.*, 1972) were used for transduction. For the phages used to test LPS character ('phage pattern'), see Wilkinson *et al.* (1972).

Phage and genetic methods. Phage-sensitivity patterns were determined as described by Wilkinson *et al.* (1972), except that the medium used was Difco nutrient agar, and the stock of smooth-specific phage 9NA had been propagated on a derivative of *S. enteritidis* I203 instead of an *S. typhimurium* LT2 subline. Recombinants were tested for sensitivities to phages P22.c2, 9NA, 6SR and Br2. For transduction, phage and broth culture were mixed, to give phage:bacterium ratios of about 1:1, 5:1 or 10:1, and then centrifuged; after incubation at 37 °C for 10 min (for smooth strains) or 3 h (for the part-rough strain, R146) the mixture was centrifuged again and samples of washed cells were plated on selective medium. For conjugational crosses, equal volumes of young broth cultures of donor and recipient were mixed, centrifuged, incubated at 37 °C for 2 h and then centrifuged again; samples of washed-cell suspensions were plated on selective media. Transconjugants and transductants were purified by single-colony reisolation on selective medium before testing their phenotype (where appropriate by prong replication from a nutrient agar master plate). Penicillin-sensitivity was determined by streaking loopful amounts of broth cultures (about 10⁶ cells) on plates of nutrient agar with penicillin G (5, 10, 15 or 20 units ml⁻¹). For tests of sensitivity to the bactericidal effect of normal serum, inocula of about 0.001 ml of overnight broth cultures were mixed with 0.2 ml of fresh normal human serum (not inactivated), and samples (about 0.001 ml) of this mixture were streaked on nutrient agar immediately after mixing and again after 2 h incubation at 37 °C.

To score the H (flagellar antigen) alleles of recombinants from crosses of diphasic donors (*S. typhimurium* or *S. abony*), they were first prong-replicated to plates of semi-solid medium containing anti-*g,m* serum (final concentration 1/50) and then recombinants giving spreading growth (i.e. able to express an H antigen other than that specified by the *H1* allele of the recipient) were further tested by slide agglutination. The O-antigen character of recombinants was determined by a slide agglutination test, using O4,5,12 and O9,12 sera at a final dilution of 1/20 in normal saline. Phage patterns of recombinants were inferred from their sensitivity or resistance to two smooth-specific phages, P22.c2 and 9NA, and two rough-specific phages, 6SR and Br2.

Table 1. *Principal Salmonella strains used*

Strain no.	Description*	Origin/reference
I203	<i>S. enteritidis</i> (O9,12): wild-type (prototrophic, smooth, serum-res, pen-sen)	Roantree & Steward (1965)
R134	<i>S. enteritidis</i> : gal-707 his-1461 cys-1159 (smooth, serum-res, pen-sen)	From I203 by EMS†-induced mutations
R146	<i>S. enteritidis</i> : gal-707 his-1461 cys-1159 rfb-968 (part-rough, serum-sen, pen-res)	Spontaneous mutant of R134, penicillin selection
R147	<i>S. enteritidis</i> : gal-707 his-1461 cys-1159 (smooth, serum-sen, pen-res)	Spontaneous mutant of R134, penicillin selection
PG189	<i>S. typhimurium</i> LT2 HfrB2 (O1,4,5,12): proA26(P22) clb ⁺ tre ⁺ (smooth, serum-res, pen-sen)	SU418; Sanderson <i>et al.</i> (1972)
SR305	<i>S. typhimurium</i> LT2 HfrA (O4,5,12): hisD23 gal-50 (smooth, serum-res, pen-sen)	Zinder (1960)
SW1444	<i>S. abony</i> HfrH1 (O1,4,5,12): aroD851 metA1151 rpsL501 (smooth, serum-res, pen-sen)	SW1391; Mäkelä (1963)
SL987	<i>S. enteritidis</i> M178 trp (F'59 pyrB): (smooth, serum-res, pen-sen)	Mäkelä & Ziegler (1966)

* For definitions of serum-resistance and sensitivity (serum-res and serum-sen), and penicillin-resistance and sensitivity (pen-res and pen-sen), see Methods; smooth indicates smooth phage pattern; clb⁺ and tre⁺ indicate increased ability to utilize cellobiose and trehalose, respectively.

† EMS, Ethyl methanesulphonate.

RESULTS

Isolation and general characterization of penicillin-resistant, serum-sensitive mutants

Mutant R146 was the only serum-sensitive strain of 35 strains picked from penicillin-resistant colonies appearing on gradient plates (20 units ml⁻¹) seeded with about 10⁸ bacteria of the parent strain R134. Mutant R147 was the one serum-sensitive strain of 15 tested from a similar selection. As may be seen in Table 2, R146 behaves like a part-rough strain being weakly agglutinated in anti-O9 serum and giving an R-sens phage pattern (not affected by smooth-specific phages but lysed by FO phage and the rough-specific phages 6SR and Br2) which in *S. typhimurium* is characteristic of mutants making complete core LPS without O chains (Wilkinson *et al.*, 1972). Characteristics of a *S. typhimurium* rfaL transductant with the same phage pattern are included in Table 2. Mutant R147 is like its parent except for its penicillin-resistance and serum-sensitivity.

Analysis by thin-layer chromatography and spectrophotometric estimation of the products of the Dische reaction of LPS extracted from mutant R146 by the hot phenol method (by Dr Bruce Cohn) revealed much reduced amounts of the smooth-specific sugars tyvelose, mannose and rhamnose, compared with the parent strain. O-specific sugars were nearly absent in the supernatant (L1 fraction) after ultracentrifugation, indicating that R146 is not a mutant of the rfa or rfbT class (see Stocker & Mäkelä, 1971). Its ability to ferment mannose indicates it is not a pmi mutant.

The rfb gene cluster of *S. typhimurium* includes genes for various enzymes concerned in the synthesis of the immediate precursors of the O-specific sugars of the oligosaccharide repeating unit, i.e. TDP-rhamnose, CDP-bequose and GDP-mannose (Nikaido *et al.*, 1967). Table 3 records the activity of these enzymes in extracts of the smooth, serum-resistant *S. enteritidis* parent and of its part-rough mutant R146, as determined by Dr Nikaido. The activities of the extracts of mutant R146 did not differ significantly from those of the smooth parent. Thus, the reduced content of O-specific material in the LPS of strain R146 does not result from inability to synthesize any of the precursors of its O-specific sugar components.

Table 2. *Comparison of pertinent phenotypic characteristics of S. enteritidis and S. typhimurium smooth parent strains and their penicillin-resistant derivatives*

Strain	Serum-sensitivity*	Penicillin m.i.c.† (units ml ⁻¹)	Deposit in broth	Agglutination (O9)	Phage pattern
<i>S. enteritidis</i>					
R134	Res	8	0	+++	Smooth
R146	Sen	20	++	+	R-sens
R147	Sen	16	0	+++	Smooth
<i>S. typhimurium</i> ‡					
SL1515	Res	6	0	+++	Smooth
SL3749(rfaL)	Sen	8	++	0	R-sens

* Res, Resistant; Sen, sensitive.

† m.i.c., minimum inhibitory concentration.

‡ Data from Roantree *et al.* (1977).Table 3. *Activities of enzymes involved in nucleotide-sugar synthesis; data of Dr H. Nikaido*

Enzymes were assayed as described by Nikaido *et al.* (1967). The results are expressed as percentages of the specific activities found in *S. typhimurium* LT2.

		Enzyme specific activity*											
Strain	O4/9	Phage pattern†	UDPG PPase (galU)	Rha			Abeq			Man		GNAc	Man
				2	1	3	1	2	3	3	2	4	1 (pmi)
R143 HfrA	O4	Smooth	98	108	124	74	70	23	64a‡	105	119	93	97
R134	O9	Smooth	128	45	90	51	116	22	123t	70	115	100	64
R146	O9	Part-rough	218	37	84	57	135	31	82t	72	133	107	75

* Abbreviations for the names of enzymes: UDPG PPase, UDPglucose pyrophosphorylase; Rha-1, TDPglucose pyrophosphorylase; Rha-2, TDPglucose oxidoreductase; Rha-3, TDPphamnose synthetase; Abeq-1, glucose-1-phosphate cytidyltransferase; Abeq-2, CDPglucose oxidoreductase; Abeq-3, CDPabequose synthetase; Man-1, phosphoglucomutase; Man-2, phosphomannomutase; Man-3, mannose-1-phosphate guanylyltransferase; GNAC, UDP-*N*-acetylglucosamine pyrophosphorylase.

† See Table 1.

‡ In Abeq-3, *a* and *t* indicate the synthesis of CDPabequose and CDPtyvelose, respectively.

Genetic analysis of part-rough mutant R146

Mutant R146 was used as recipient in crosses with two *S. typhimurium* Hfr strains (PG189 or SR305) or the *S. abony* Hfr strain SW1444. In the cross recorded in Table 4 the donor was strain PG189, an HfrB2 derivative of *S. typhimurium* LT2 (Sanderson *et al.*, 1972), which transfers its chromosome clockwise from about 37 units on the recalibrated map (Fig. 1). Of 48 recombinants selected for possession of the donor *hisEI*⁺ and recipient *pro*⁺ (Table 4*a*), 37 resembled the smooth donor parent in their level of penicillin-sensitivity, resistance to killing by serum, and phage patterns. The 11 other *his*⁺*pro*⁺ recombinants resembled the recipient parent, R146, in penicillin-resistance, serum-sensitivity, and phage-sensitivity pattern. The co-inheritance of either the three mutant phenotypic traits of R146 or of the corresponding wild-type characters indicates that all three traits resulted from a single mutation. Three of the 37 *his*⁺*pro*⁺ recombinants which had acquired donor smooth character were strongly agglutinated by the anti-O4 serum and not by anti-O9 serum; the other 45 recombinants were agglutinated only by anti-O9 serum like the recipient parent, R146. No other unselected donor 'marker' was represented in more than one of the 48 recombinants tested. In the same cross (Table 4*b*), two of 49 recombinants selected for possession of *cys*⁺ of the donor and *pro*⁺ of R146 were donor-like in penicillin-sensitivity, serum-resistance and phage pattern. The rest were recipient-like in those traits.

It appears from these results that the mutation causing the penicillin-resistance, serum-

Table 4. Frequency of recombinant classes in crosses of *S. typhimurium* PG189 HfrB2 with *S. enteritidis* R146

The donor was *S. typhimurium* PG189 HfrB2 [LT2 *proA*(P22) *clb*⁺ *tre*⁺ *H1*(i) *H2*(1,2), O1,4,5,12, serum-resistant, penicillin-sensitive, smooth phage pattern]. The recipient was *S. enteritidis* R146 [*gal hisEI cys H1*(g,m), O9,12, serum-sensitive, penicillin-resistant, rough phage pattern]. There were negligible numbers of *hisEI*⁺ or *cys*⁺ revertants in the R146 controls and of *proA*⁺ revertants in the PG189 HfrB2 controls.

No.	Loci, in order transferred:							Serum-sensitivity*	Penicillin-sensitivity*	Phage pattern†
	<i>H1</i>	<i>hisEI</i>	<i>O4/9</i>	<i>H2</i>	<i>cys</i>	<i>proA</i>	<i>gal</i>			
(a) Selected as <i>his</i> ⁺ <i>pro</i> ⁺ , i.e. for donor <i>hisEI</i> ⁺ and recipient <i>proA</i> ⁺										
34	g,m	+	O9	—	—	+	—	Res	Sen	Smooth
11	g,m	+	O9	—	—	+	—	Sen	Res	Rough
2	g,m	+	O4	—	—	+	—	Res	Sen	Smooth
1	i	+	O4	1,2	—	+	—	Res	Sen	Smooth
Total with donor allele	1	48	3	1	0	0	0	37	37	37
(b) Selected as <i>cys</i> ⁺ <i>pro</i> ⁺ , i.e. for donor <i>cys</i> ⁺ and recipient <i>proA</i> ⁺										
47	g,m	—	O9	—	+	+	—	Sen	Res	Rough
1	g,m	—	O9	—	+	+	—	Res	Sen	Smooth
1	g,m	—	O4	—	+	+	—	Res	Sen	Smooth
Total with donor allele	0	0	1	0	49	0	0	2	2	2
(c) Selected as <i>his</i> ⁺ <i>cys</i> ⁺ <i>pro</i> ⁺ , i.e. for donor <i>hisEI</i> ⁺ and <i>cys</i> ⁺ and recipient <i>proA</i> ⁺										
8	g,m	+	O9	—	+	+	—	Sen	Res	Rough
7	g,m	+	O4	—	+	+	—	Res	Sen	Smooth
4	g,m	+	O9	—	+	+	—	Res	Sen	Smooth
2	g,m	+	O4	—	+	+	—	Sen	Res	Rough
1	i	+	O4	—	+	+	—	Sen	Res	Rough
1	i	+	O4	—	+	+	+	Res	Sen	Smooth
Total with donor allele	2	23	11	0	23	0	1	12	12	12

* Res, Resistant; Sen, sensitive.

† Smooth phage pattern indicates sensitivity to phages P22.c2 and 9NA and resistance to phages 6SR and Br2 (the donor, HfrB2, was resistant to P22.c2 because of lysogeny by phage P22). Rough phage pattern indicates sensitivity to phages 6SR and Br2 and resistance to phages P22.c2 and 9NA like the recipient.

sensitivity and rough phage-sensitivity of R146 is closely linked to *hisEI*, and closer to it than is the part of the *rfb* gene cluster which determines O4 or O9 serological character. Both its phenotype and this result suggest that the mutation of R146 is within the *rfb* gene cluster (and we therefore assign it mutation number *rfb*-968). On this interpretation the gene order is *his-rfb-968-O4/9*, where O4/9 indicates the part(s) of the *rfb* gene cluster which determine the serological character of the O-repeat unit, O4 or O9.

Mutant R146 was crossed with two other smooth group B Hfr strains: SW1444, which is *S. abony* HfrH1, and SR305, which is an HfrA derivative of *S. typhimurium* strain LT2 (Table 1). The results of selection for donor *hisEI*⁺ were generally similar to those in the cross with the HfrB2 donor. Table 5 summarizes the distribution of part-rough versus smooth character and of O-antigenic character in recombinants selected as *hisEI*⁺ (and also includes a summary of the results in the HfrB2 cross). Most recombinants resembled R146 in penicillin-resistance, serum-sensitivity and phage pattern, or instead resembled the smooth donor in all three respects. In both the HfrH1 and the HfrA crosses, the relative frequency of *hisEI*⁺ recombinants with the donor smooth character or with both the smooth character and O4 character of the donor indicated the gene order: *his-rfb-968-rfb*(4/9). However, the closeness of linkage of *rfb*(4/9) with *his* was different in the *S. abony* HfrH1

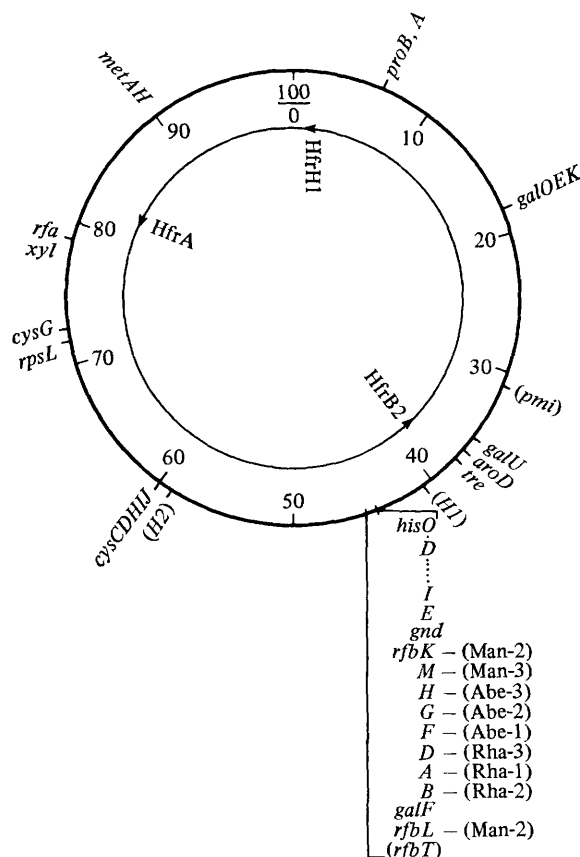


Fig. 1. The linkage map of *Salmonella typhimurium*. Map positions of standard loci, expressed in units according to Sanderson & Hartman (1978). Points of origin of Hfr strains are indicated as are the map positions of loci concerned with the biosynthesis of LPS. The relative order of loci shown within parentheses is unknown.

Table 5. *O* antigen and serum-sensitivity (and correlated characters) of *his*⁺ recombinants from conjugational crosses with *S. enteritidis* R146 as recipient

The donor strains were *S. typhimurium* HfrA and HfrB2 and *S. abony* HfrH1 [O4,5,12 or O1,4,5,12, serum-resistant, penicillin-sensitive, smooth phage pattern]. The recipient was *S. enteritidis* R146 [*gal hisEI cys rfb-968 HI*(g,m), O9,12, serum-sensitive, penicillin-resistant, rough phage pattern].

Donor			No. of <i>his</i> ⁺ recombinants in class				
Strain no.	Contra-selected gene	Selected gene	Total	O9, serum-resistant	O9, serum-sensitive	O4, serum-resistant	O4, serum-sensitive
PG189	<i>proA</i>	<i>hisEI</i> ⁺	48	34	11	3	0
HfrB2	<i>proA</i>	<i>hisEI</i> ⁺ <i>cysC</i> ⁺	23	4	8	8	3
SW1444	<i>metA</i>	<i>hisEI</i> ⁺	25	4	6	15	0
HfrH1	<i>aroD</i>	<i>hisEI</i> ⁺	25	5	5	14	1
SR305 HfrA (2 crosses)	<i>hisD</i>	<i>hisEI</i> ⁺	136*	54	68	4	1

* These 136 included nine exceptional recombinants. Four were inagglutinable in O4 or O9 antiserum (three were recipient-like in serum-sensitivity, penicillin-resistance and phage pattern; the other was recipient-like except for a smooth phage pattern). Five had new recombinations of the three correlated characters. Three (two O4, one O9) were recipient-like in serum-sensitivity and phage pattern, but were not as penicillin-resistant as the recipient. Two others (one O4, the other O9) were penicillin-sensitive and resistant to rough phages, like the donor, but were serum-sensitive.

cross (30 of 50 *his*⁺ recombinants with donor O4 character) from that in the two *S. typhimurium* Hfr crosses (14 of 71 and 8 of 136 *his*⁺ with donor O4 character). The LT2 HfrA strain used (Zinder, 1960; Sanderson *et al.*, 1972) requires histidine because of a mutation at *hisD*; recombinants obtained on medium without histidine were therefore selected for retention of *hisD*⁺ of the recipient, as well as for acquisition of *hisEI*⁺ of the donor. But as the order of the relevant genes (Fig. 1) is known to be *hisD*-*hisIE*-*rfb*, selection against donor *hisD* is not expected to affect the representation of *rfb* genes amongst recombinants acquiring donor *hisEI*⁺. Two *his*⁺ recombinants with O antigen O4 but retaining the penicillin-resistance, serum-sensitivity and part-rough character of R146 were encountered amongst 186 *his*⁺ recombinants in the crosses with the HfrH1 and HfrA donors; these recombinants and three similar ones in the HfrB2 cross (Table 5) we attribute to quadruple crossover events. The *his*⁺ recombinants from the HfrA cross included four which were not agglutinable in O4 or O9 serum; three of them were recipient-like in serum-sensitivity, penicillin-resistance and sensitivity to rough phages, the other was recipient-like except for a smooth phage pattern. Another five *his*⁺ recombinants from this cross appeared to have new recombinations of the three correlated characters. Three recombinants (two O4⁺, the other O9⁺) were recipient-like in serum-sensitivity and sensitivity to rough phages but were not as penicillin-resistant as the recipient. Two others (one O4⁺, the other O9⁺) were penicillin-sensitive and resistant to rough phages, like the donor, but were serum-sensitive. We did not further investigate these recombinants, for instance to determine if the O-inagglutinable strains made complete core or incomplete core LPS. The representation of unselected loci gave no indication as to their origin, perhaps by unequal crossing over within the partly non-homologous *rfb* clusters, or by coincidental mutation at *rfb* or some other locus affecting LPS character or serum-sensitivity or penicillin-sensitivity or (improbably, we think) by crossing over between two closely linked mutant loci in R146, for instance one affecting penicillin-sensitivity and the other causing part-rough character, with sensitivity to rough phages and to serum. Completely rough recombinants from O4 × O9 crosses have been observed but rarely in previous reports (Mäkelä, 1965; Johnson *et al.*, 1965; Nikaido *et al.*, 1966). Part-rough recombinants would probably have been overlooked in those studies.

The whole of the *rfb* gene cluster of *S. typhimurium* can be cotransduced with the *his* operon by phage P22, which, however, is smooth-specific and inactive on R146. The agglutinability of mutant R146 by anti-O9 serum and the presence of small amounts of O-specific sugars in its LPS showed that it synthesizes some 'smooth' LPS side-chains and suggested that it might be susceptible to P22-mediated transduction, despite its resistance to lysis by this phage (cf. Gemski & Stocker, 1967). Phage P22 propagated on strain I203, the *S. enteritidis* wild-type (*his*⁺, penicillin-sensitive, serum-resistant, smooth phage pattern) ancestor of R146, evoked *his*⁺ transductants, at a frequency of about 1 per 10⁸ plaque-forming units, from strain R134, the smooth *his* immediate parent of mutant R146. The same lysate also evoked *his*⁺ transductants from R146, but the yield per plaque-forming unit was about fivefold lower. A total of 40 *his*⁺ transductants evoked from R146 by either this P22 lysate or by a P22h lysate of the wild-type strain I203 were tested; 32 resembled the recipient, R146, in their penicillin-resistance, serum-sensitivity and rough phage pattern but eight were donor-like in all these three characters (except that they were unaffected by phage P22 . c2, probably because they had become P22-lysogenic, since they were sensitive to 9NA, the other O-specific phage used).

Genetic analysis of smooth, penicillin-resistant mutant R147

This mutant differed from its smooth parent, R134, only in being penicillin-resistant and serum-sensitive (Table 2). Thin-layer chromatography of a hydrolysate of its LPS showed about the same amount of O-specific sugars as the corresponding materials from the parent strain (B. Cohn, personal communication).

Several Hfr or F' crosses were made with the object of mapping the mutation causing serum-sensitivity and penicillin-resistance (without loss of smooth character) in mutant R147 (details of crosses not shown). In crosses with *S. abony* HfrH1 as donor, 36 of 100 recombinants selected for possession of donor *hisEI*⁺ and recipient *aroD*⁺ of *metA*⁺ had antigen O4, like the donor; 33 of these 36 were recipient-like in serum-sensitivity and penicillin-resistance. This strongly indicates that the relevant mutation of R147, unlike the part-rough mutation of R146, is not located between *rfb*(O4/9) and *hisEI*. Only two of the 100 *hisE*⁺ recombinants from these selections were donor-like (wild-type) in both their resistance to serum and their sensitivity to penicillin and these two were amongst the only four recombinants which had the donor *gal*⁺ allele. The HfrH1 donor was crossed to R147 and selection was made for donor *gal*⁺ and recipient *metA*⁺; only one of 100 *gal*⁺*metA*⁺ recombinants was donor-like in penicillin-sensitivity and serum-resistance, which makes it unlikely that the mutation of R147 is closely linked to *gal*. The only conclusions that could be drawn from the crosses of R147 with the HfrH1 donor were that the mutation of R147 was not between *his* and *rfb*, and not closely linked to *hisEI*, *gal* or *cys*, the loci used as selected donor markers.

In crosses of a *xyl* mutant of R147 (the *xyl* mutation was obtained following ethyl methanesulphonate mutagenesis) with the *S. enteritidis* F' strain, SL987, 39 of 51 recombinants selected for possession of the donor *cys*⁺ or *hisEI*⁺ possessed all three of the donor characters, (*hisEI*⁺, *cys*⁺ and *xyl*⁺) and 35 of these 39 recombinants were donor-like in serum and penicillin character. However, no close linkage of the serum/penicillin character with any of these loci was apparent in this or the other crosses made. This suggests that the mutation of R147 is probably located in the *his-cys-xyl* segment, i.e. between unit 44 and unit 78 in the current version of the *S. typhimurium* linkage map (Sanderson & Hartman, 1978), but gives no indication as to its position within that segment.

DISCUSSION

As mutant R146 was isolated by a single cycle of selection for penicillin-resistance and its three characteristic properties, penicillin-resistance, serum-sensitivity and part-rough character, almost always segregated together in conjugational crosses, it is reasonable to infer that all three properties result from a single mutation. The five recombinants with non-parental combinations of the three correlated characters encountered amongst 136 *his*⁺ recombinants in the HfrA × R146 cross (Table 5) casts some doubt on the hypothesis that the three altered characters of R146 all result from a single mutation. Fuller examination of these five recombinants might have shown whether their exceptional combinations resulted from coincidental mutations affecting LPS character, serum-sensitivity or penicillin-sensitivity, or from crossing over between closely linked mutated genes in R146, for instance one causing part-rough character (sensitivity to rough phages and to serum) and the other causing penicillin resistance – but we think this last an unlikely hypothesis. The results of conjugational crosses of 'smooth' donors of group B (antigen O4, abequose as the dideoxyhexose of the O-repeat unit) to R146 as recipient (Table 5) and P22-mediated transductional crosses with the wild-type *S. enteritidis* ancestor of R146 as donor and R146 as recipient clearly show that the mutation of R146 is closely linked to *hisEI* and to *rfb*, or, more precisely, to the part(s) of the *rfb* gene cluster which determine O4 or O9 serological character, that is abequose or tyvelose as the dideoxyhexose branch of the O-repeat unit. On this interpretation the five recombinants which had antigen O4 from the donor but retained recipient penicillin-resistance, etc. resulted from quadruple crossover events, a class expected to be rare. The phenotype of these O4⁺ part-rough recombinants shows that the *rfb-968* mutation of R146 causes part-rough phenotype even when the O-specific material made contains abequose instead of tyvelose; it is therefore unlikely that the R146 mutation affects the gene for an enzyme which is specific for tyvelose, such as the (postulated) enzyme for

transfer of this dideoxyhexose from CDPtyvelose to the trisaccharide backbone of the newly formed repeat unit.

In *S. typhimurium* the enzyme which converts CDP-4-keto-6-deoxyglucose to CDP-abequose is determined by gene *rfbH*, whose position in relation to other mapped genes within the *rfb* cluster (Table 3, Fig. 1) is known from tests of enzymic activity of strains with deletions extending into the cluster from the side nearest *his* (Nikaido *et al.*, 1967). It seems reasonable to assume that the enzyme which in *S. enteritidis* converts CDP-4-keto-6-deoxyglucose to CDPtyvelose (CDPtyvelose synthetase) is specified by a corresponding '*rfbH*' gene. If so, the segregation of O-antigen character and of 'part-rough' character in recombinants selected for possession of donor *his*⁺ indicates that the *rfb*-968 mutation of R146 lies between *his* and *rfbH* (Fig. 1) specifying the dideoxyhexose of the repeat units. Since other possible mutations have been eliminated (Table 3), we infer that the *rfb*-968 mutation of R146 affects a glycosyl-transferring enzyme needed for the assembly of the repeating unit. Since the part-rough character was present in O4 as well as O9 recombinants it is unlikely that *rfb*-968 affects the tyvelose-transferase gene. Thus, one of the other three transferases (galactosyl-, mannosyl- or rhamnosyl-transferases) involved in the assembly of the O-repeat units of group B and D *Salmonella* must be affected. These are known to be part of the *rfb* gene cluster but their position in relation to the mapped genes for biosynthetic enzymes is not known, except for some unpublished evidence as to the location of the gene for the galactosyl-transferring enzyme (Nikaido *et al.*, 1967; H. Nikaido, personal communication). The results shown in Table 4 indicate that at least one of these three transferase genes is on the *his* side of gene *rfbH*, the enzyme catalysing the last step in the synthesis of CDPabequose (in *S. typhimurium*) and, we assume, a corresponding gene for the enzyme synthesizing CDPtyvelose in *S. enteritidis*.

In *Salmonella* and related genera, rough mutants, of all types tested, are readily killed by exposure to complement-containing normal serum (Nelson & Roantree, 1967), and selection for penicillin-resistance yields mutants some of which are 'rough', as discussed in the Introduction. Thus, the salient features of the phenotype of R146 are all explicable by the postulated partial defect of a transferase function needed for assembly of the O-repeat unit. Why the absence of O chains, by itself or in consequence of the absence of one or more of the outer units of the LPS core chain (Roantree *et al.*, 1977), should cause somewhat reduced sensitivity to penicillin remains to be discovered. Mutation of *rfb*-968 of mutant R146 should result in production of complete rough core LPS with only a few O chains, thus resembling that of *rfaL* strains. Mutant R146 shows increased resistance to novobiocin and cephalothin as well as to penicillin, similar to that previously shown in *rfaL* transductants (Roantree *et al.*, 1977). The *rfa* mutations in *Salmonella* conferring increased resistance to penicillin are presumably analogous to the *lpc* (*lps*) mutations conferring ampicillin-resistance in *Escherichia coli* K12 (Eriksson-Grennberg *et al.*, 1971). In *E. coli* K12 increased penicillin-resistance caused by mutation at the *his*-linked region corresponding to *rfb* of *S. typhimurium* would not have been detected since strain K12 is already rough, but probably would be detected if rough mutants of smooth *E. coli* were tested.

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REFERENCES

- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**, 17-28.
- ERIKSSON-GRENNBERG, K. G., NORDSTRÖM, K. & ENGLUND, P. (1971). Resistance of *Escherichia coli* to penicillins. IX. Genetics and physiology of class II ampicillin-resistant mutants that are galactose negative or are sensitive to bacteriophage C21 or both. *Journal of Bacteriology* **108**, 1210-1223.
- GEMSKI, P., JR & STOCKER, B. A. D. (1967). Transduction by bacteriophage P22 in nonsmooth mutants of *Salmonella typhimurium*. *Journal of Bacteriology* **93**, 1588-1597.
- JOHNSON, E. M., KRAUSKOPF, B. & BARON, L. S. (1965). Genetic mapping of Vi and somatic antigenic determinants in *Salmonella*. *Journal of Bacteriology* **90**, 302-308.
- MÄKELÄ, P. H. (1963). Hfr males in *Salmonella abony*. *Genetics* **48**, 423-429.
- MÄKELÄ, P. H. (1965). Inheritance of the O antigens of *Salmonella* groups B and D. *Journal of General Microbiology* **41**, 57-65.
- MÄKELÄ, P. H. & ZIEGLER, L. (1966). An F' episome of *Salmonella abony*. *Acta pathologica et microbiologica scandinavica* **67**, 547-554.
- MICHAEL, J. G. & BRAUN, W. (1958). Relationships between bacterial resistance to serum and penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **97**, 104-107.
- NELSON, B. W. & ROANTREE, R. J. (1967). Analyses of lipopolysaccharides extracted from penicillin-resistant, serum-sensitive *Salmonella* mutants. *Journal of General Microbiology* **48**, 179-188.
- NIKAIDO, H., NIKAIDO, K. & MÄKELÄ, P. H. (1966). Genetic determination of enzymes synthesizing O-specific sugars of *Salmonella* lipopolysaccharides. *Journal of Bacteriology* **91**, 1126-1135.
- NIKAIDO, H., LEVINTHAL, M., NIKAIDO, K. & NIKANE, K. (1967). Extended deletions in the histidine-rough B region of the *Salmonella* chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 1825-1832.
- ROANTREE, R. J. & STEWARD, J. P. (1965). Mutations to penicillin-resistance in the *Enterobacteriaceae* that affect sensitivity to serum and virulence for the mouse. *Journal of Bacteriology* **89**, 630-639.
- ROANTREE, R. J., KUO, T.-T. & MACPHEE, D. G. (1977). The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. *Journal of General Microbiology* **103**, 223-234.
- SANDERSON, K. E. & HARTMAN, P. E. (1978). Linkagemap of *Salmonella typhimurium*, edition V. *Microbiological Reviews* **42**, 471-519.
- SANDERSON, K. E., ROSS, H., ZIEGLER, L. & MÄKELÄ, P. H. (1972). F⁺, Hfr, and F' strains of *Salmonella typhimurium* and *Salmonella abony*. *Bacteriological Reviews* **36**, 608-637.
- STOCKER, B. A. D. & MÄKELÄ, P. H. (1971). Genetic aspects of biosynthesis and structure of *Salmonella* lipopolysaccharide. In *Microbial Toxins*, vol. 4, pp. 369-438. Edited by G. Weinbaum, S. Kadis & S. J. Ajl. London: Academic Press.
- WESTPHAL, O., LÜDERITZ, O. & BISTER, F. (1952). Über die Extraktion von Bakterien mit Phenol/Wasser. *Zeitschrift für Naturforschung* **7b**, 148-155.
- WILKINSON, R. G., GEMSKI, P., JR & STOCKER, B. A. D. (1972). Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. *Journal of General Microbiology* **70**, 527-554.
- ZINDER, N. D. (1960). Sexuality and mating in *Salmonella*. *Science* **131**, 924-926.