Mouse Virulence of Salmonella Strains: The Effect of Different Smooth-type O Side-chains

By V. V. VALTONEN

*Department of Serology and Bacteriology, University of Helsinki and
the Central Public Health Laboratory (State Serum Institute),
Helsinki, Finland*

(Accepted for publication 16 October 1970)

SUMMARY

A loss or reduction in the O side-chain material of the cell-wall lipopolysaccharide is known to abolish or reduce the virulence of Salmonella strains. The present report concerns the effect on virulence of altering the quality of smooth-type O side-chains in a basically virulent *Salmonella typhimurium* line. The original *rfb^B* locus determining the structure of 4, 12-specific repeating units was replaced either in transduction or in conjugation by the wild-type *rfb^D* locus of group D (O antigens 9, 12), or *rfb^C* of group C (O antigens 6, 7). The LD₅₀ values of the 4, 5, 12 recombinants or transductants were about 10⁵ and like those of the 4, 5, 12 parent, whereas the LD₅₀ values of the 9, 12 transductants were about 10⁶, and the LD₅₀ values of the 6, 7 recombinants and transductants were over 10⁷. The reduced virulence of both 9, 12 and 6, 7 recombinants could be restored to the original level by reintroducing the *rfb^B* locus into these strains through conjugation. It seems, therefore, that different kinds of O side-chains confer different degrees of virulence on *S. typhimurium*.

INTRODUCTION

It has long been known that the change in colony morphology from smooth to rough (S → R variation), with accompanying loss of O agglutinating ability, greatly reduces virulence of Salmonella bacteria (Lingelsheim, 1913; Arkwright, 1927). Since the first observation in 1913, further evidence has accumulated for the importance of the structure of the O antigen in virulence of Salmonella strains (Roantree, 1967).

The O antigen – also called endotoxin or lipopolysaccharide (LPS) – forms the outermost layer in the Salmonella cell wall and contains a lipid and a polysaccharide. The latter has a core which is common to all Salmonella species, and to which are attached long O side-chains (Lüderitz, Jann & Wheat, 1968). The O side-chains of at least groups B, D and E are polymers of several repeating units per side-chain. The sugar composition of the repeating units (the O side-chain) determines the O specificity characteristic for each Salmonella group (Fig. 1).

The S → R variation is caused by mutations blocking the synthesis of the polysaccharide. The avirulent R forms thus have an incomplete LPS with no O side-chains; *rfa* mutants are blocked in the synthesis of the core, *rfb* in the synthesis of the repeating unit, and *rfc* in their polymerization (Mäkelä & Stocker, 1969). Both *rfa* and *rfb*

mutants are phenotypically R, but *rfc* mutants have one O-specific repeating unit per side-chain and are SR, intermediate between S and R. Genetic crosses between different O antigen groups (Mäkelä, 1966) have demonstrated that the *rfb* cluster of genes, which is close to the *his* locus on the bacterial chromosome, contains all the information required for the synthesis of O repeating units. If a *Salmonella* has the *rfb^B* (of group B), its LPS has the group B specificity 4, 12, and if the *rfb* is of group D (*rfb^D*) the bacterium has the group D specificity 9, 12, etc.

Fig. 1. Schematic structure of the O side-chains. According to Lüderitz, Jann & Wheat (1968); the structure for the group C side-chain is only tentative (Fuller & Staub, 1968). Abe = abequose, Gal = galactose, Glu = glucose, GNAc = *N*-acetylglucosamine, Man = mannose, Rha = rhamnose, Tyve = tyvelose.

Some recent studies suggest that even in non-R forms possessing O side-chains the quantity of the O side-chain material plays a role in virulence. The mouse virulence of an SR form, which has only one repeating unit per side-chain, is intermediate between the virulence of the S and that of the R form (Nakano & Saito, 1969). A highly virulent smooth *Salmonella typhimurium* was shown to possess about twice as many of the antigenic determinants 4, 5 and 12, measured by an immunological technique, as another smooth but avirulent *S. typhimurium* strain (Archer & Rowley, 1969). The ribose- and galactose-containing TI side-chains appear to confer only a slight degree of virulence on a *rfb* mutant of *S. typhimurium*, much less than do the original 4, 5, 12 side-chains, suggesting that the quality of the side-chain may also affect virulence (Valtonen, 1969).

The purpose of this work was to make a systematic study of the possible effects on virulence of altering the quality of smooth-type O side-chains in a known virulent line of *Salmonella typhimurium*. The alteration was accomplished by genetic replacement of the original *rfb^B* locus (that is *rfb* of group B), which determines the structure of 4, 12-specific repeating units, with the *rfb^D* locus of group D (O antigens 9, 12) or with *rfb^C* locus of group C (O antigens 6, 7). The LD₅₀ value for mice was used as a measure of virulence in this work.

METHODS

Bacterial and phage strains. The bacterial strains with their main properties and origins are listed in Table 1. The basic strain was SL 1027 of *Salmonella typhimurium* line LT2. This strain was originally obtained from Dr B. A. D. Stocker, Stanford University, California, U.S.A. The SH strains are kept in the collection of Professor P. H. Mäkelä, Central Public Health Laboratory, Helsinki, Finland. New mutations

Table 1. *Salmonella* strains used

Species and group	Strain no.	Mating type	LPS characteristic	<i>rfb</i> from group	Genetic markers	Source and reference
<i>S. typhimurium</i> B	SL748	F ⁻	R	B	<i>metA22 trpB2 str flaA66 rfb-430</i>	B. A. D. Stocker; Gemski & Stocker (1967)
<i>S. typhimurium</i> B	SL901	F ⁻	SR (4, 5, 12)	B	<i>metA22 trpB2 str flaA66 rfc-497</i>	B. A. D. Stocker; Gemski & Stocker (1967)
<i>S. typhimurium</i> B	SL1017	F ⁻	S (4, 5, 12)	B	<i>metA22 trpB2 str flaA66</i>	B. A. D. Stocker; Gemski & Stocker (1967)
<i>S. typhimurium</i> B	SL1060	F ⁻	R	B	<i>metA22 trpB2 str flaA66 rfaH487</i>	B. A. D. Stocker; Wilkinson & Stocker (1968)
<i>S. abony</i> B	SW1403	HfrH2	S (4, 5, 12)	B	<i>met-1151 aro-851 str-501</i>	Mäkelä (1963)
<i>S. typhimurium</i> B	SH1	F ⁻	S (4, 5, 12)	B	<i>hisIF135</i>	P. E. Hartman; Loper <i>et al.</i> (1964)
<i>S. typhimurium</i> * B	SH1011	F ⁻	S (6, 7)	C	<i>str purG302</i>	P. H. Mäkelä
<i>S. typhimurium</i> † B	SH1036	F ⁻	S (6, 7)	C	<i>str purG302</i>	P. H. Mäkelä
<i>S. typhimurium</i> B	SH1331, 1333	F ⁻	S (4, 5, 12)	B	—	<i>his</i> ⁺ transductants from SH1011 × SH1†
<i>S. typhimurium</i> B	SH1360, 1361	F ⁻	S (6, 7)	C	—	<i>his</i> ⁺ transductants from SH1011 × SH1
<i>S. montevideo</i> C	SH1605	HfrH14	S (6, 7)	C	<i>leu-1357</i>	P. H. Mäkelä; Mäkelä (1966)
<i>S. typhimurium</i> B	SH2183	F ⁻	S (4, 5, 12)	B	<i>his-6116 metA22 trpB2 str flaA66</i>	SL1027 (des)§
<i>S. typhimurium</i> B	SH2201, 2202, 2203	F ⁻	S (4, 5, 12)	B	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ transductants from SH1036 × SH2183
<i>S. typhimurium</i> B	SH2204, 2205, 2206	F ⁻	S (9, 12)	D	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ transductants from SH1036 × SH2183
<i>S. typhimurium</i> B	SH2233	F ⁻	S (9, 12)	D	<i>his-6118 metA22 trpB2 str flaA66</i>	SH 2206 (des)
<i>S. typhimurium</i> B	SH2241, 2242	F ⁻	S (6, 7)	C	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SH1605 × SH2183
<i>S. typhimurium</i> B	SH2245, 2246	F ⁻	S (4, 5, 12)	B	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SH1605 × SH2183
<i>S. typhimurium</i> B	SH2259	F ⁻	S (6, 7)	C	<i>his-6120 metA22 trpB2 str</i>	SH2241 (des)
<i>S. typhimurium</i> B	SH2266, 2267	F ⁻	S (4, 5, 12)	B	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SW1403 × SH2233
<i>S. typhimurium</i> B	SH2268	F ⁻	S (9, 12)	D	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SW1403 × SH2233
<i>S. typhimurium</i> B	SH2273, 2274	F ⁻	S (4, 5, 12)	B	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SW1403 × SH2259
<i>S. typhimurium</i> B	SH2275	F ⁻	S (6, 7)	C	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ recombinants from SW1403 × SH2259
<i>S. typhimurium</i> B	SH2710	F ⁻	S (4, 5, 12)	B	—	Fresh isolate from a patient

* Strain with the *rfb* but not *his* derived in a conjugational cross from a strain of *S. montevideo* (group C) as donor.

† Strain with the *rfb* but not *his* derived in a conjugational cross from a strain of *S. enteritidis* (group D) as donor.

‡ Transductions (by phage P22) and crosses are described as: (donor) × (recipient).

§ Mutagenesis by diethylsulphate.

|| Gene symbols as follows: *aro* = phenylalanine + tyrosine biosynthesis; *fla* = flagellar synthesis; *gal* = galactose utilization; *his* = histidine biosynthesis; *met* = methionine biosynthesis; *pur* = purine biosynthesis; *rfa*, *rfb*, *rfc* = biosynthesis of the O antigenic lipopolysaccharide; *str* = streptomycin resistance; *trp* = tryptophan biosynthesis; *xyl* = xylose utilization.

were induced by adding diethylsulphate (10 mg./ml.) to an overnight broth culture and incubating for 30 min. at 37°. The culture was then diluted 1:100 in fresh medium and incubated overnight before plating on appropriate suboptimally supplemented media. The genetic donors were Hfr derivatives of *Salmonella abony* (Mäkelä, 1963) and *S. montevideo*. The gene symbols are explained in a footnote to Table 1.

The S- and R-specific phages were originally obtained from Dr R. G. Wilkinson, University of Melbourne, Parkville, Australia. The following phages were used in this work: S-specific P22c2, 9NA, P22h; R-specific P221, Br2, Ffm, Br60, C21, S13, ϕ X174 and 6SR; and FO which attacks both S and R bacteria (Wilkinson, 1966; Wilkinson & Stocker, 1968). Drops of phage lysates containing about 10^8 plaque-forming units were applied to an agar plate previously spread with the bacterial strain to be tested.

Fig. 2. A simplified chromosome map of *Salmonella* (according to Sanderson, 1967; Mäkelä & Stocker, 1969). Positions not accurately known are indicated by symbols in parenthesis. The point of entry and direction of injection of donor chromosomes are indicated by arrows on the circle. For gene symbols, see footnote to Table 1.

Phage P22 was used for transduction experiments.

Media. Broth: Difco antibiotic medium 3 (1.75 g./100 ml.). Nutrient agar: broth solidified with 1.3 g. agar/100 ml. Selective media: Davis minimal medium (Lederberg, 1950) containing agar, 1.2 %; glucose, 0.2 %; required amino acids, 20 μ g./ml.; streptomycin, 1 mg./ml. where indicated. Suboptimally supplemented media: selective media with only 0.2 μ g./ml. of the amino acid for which mutants were selected (Hartman, Loper & Serman, 1960).

Genetic methods. In conjugation experiments, equal amounts of exponentially growing donor (Hfr) and recipient (F^-) broth cultures, containing about 5×10^8 bacteria/ml., were mixed and incubated at 37° for 2 h. without shaking before plating on appropriate selective media (Mäkelä, 1966). In transduction experiments, the phage

(10^9 particles/ml.) was mixed with the exponentially growing recipient (10^9 bacteria/ml. broth), and the mixture left for 10 min. at 37° with gentle shaking and then plated on the selective medium (Hartman *et al.* 1960). The recombinants or the transductants appearing after incubation for 48 h. were restreaked on nutrient agar plates from which single colonies were picked for testing. The Hfr strains used and a schematic map of the *Salmonella* chromosome are given in Fig. 2.

Serological methods. O and H antigens were determined by slide agglutination in 4 % saline and in antisera appropriately diluted with 0.2 % saline. Anti-O sera were prepared by immunizing rabbits with saline suspensions of nutrient agar cultures of smooth strains kept at 100° for 2.5 h.; for anti-H sera the immunogen was a motile broth culture killed with formalin (Kauffmann, 1966).

Virulence test. The LD₅₀ value in mice was used to measure the virulence of the *Salmonella* strains (see Statistical methods). The SAW mice (Swiss Albino Webster strain) used in this work came from two different sources; line I from the State Serum Institute, Helsinki, Finland, and line II from the Department of Serology and Bacteriology, University of Helsinki. There was a small difference in their sensitivity to *Salmonella typhimurium* infection (see Results). Line I was used in only one set of experiments. Test animals of both sexes (aged 8 to 10 weeks, weighing between 22 and 25 g.) were used.

The bacterial strain to be tested was grown in broth for 18 h. (to early stationary phase, about 10^9 bacteria/ml.), tenfold dilutions of the culture were made in 0.9 % saline and the viable count was determined. Five consecutive dilutions (0.5 ml. each) were injected intraperitoneally into five groups of ten mice kept in separate cages. Special care was taken to keep the cages physically separate and to prevent contamination in feeding and handling. The animal room was disinfected after each experiment.

Statistical methods. The LD₅₀ value was calculated from 10 day survival according to Reed & Muench (1938). In the standard assay, virulence of two recombinant classes from one cross (or transduction) possessing different O antigens was compared. The statistical significance was calculated by forming a set of 2×2 tables from original data, according to the method of Mantel & Haenszel (1959). As a rule, three such tables for three different dose levels were made as follows:

Table of the i^{th} dose level

No. of mice	Recombinant class I	Recombinant class II	Total
Surviving	x_{11}	x_{21}	x_1
Dead	$N_{11} - x_{11}$	$N_{21} - x_{21}$	$N_1 - x_1$
Sum	N_{11}	N_{21}	N_1

where

x_{11} and x_{21} = numbers of surviving mice which had received the same dose (a) of recombinant class I bacteria (x_{11}) or recombinant class II bacteria (x_{21}),

N_{11} and N_{21} = total numbers of mice inoculated with the same dose (a) of recombinant class I bacteria (N_{11}) or recombinant class II bacteria (N_{21}),

$x_1 = x_{11} + x_{21}$ (= total number of surviving mice),

$N_1 = N_{11} + N_{21}$ (= total number of infected mice).

If we consider the distribution of, say, x_{11} conditionally on the marginal frequencies N_{11} , N_{21} , x_1 , $N_1 - x_1$, under H_0 (null hypothesis) it has the hypergeometric distribution $\bar{e}$. Under these circumstances χ^2 should be computed as the ratio of the square of a deviation from its expected value to its variance (Mantel & Haenszel, 1959).

Since,

$$E(x_{11}) = x_1 N_{11} / N_1,$$

and

$$\text{var}(x_{11}) = N_{11} N_{21} x_1 (N_1 - x_1) / N_1^2 (N_1 - 1),$$

then

$$\frac{[\sum x_{11} - \sum E(x_{11})]^2}{\sum \text{var}(x_{11})}$$

has a χ^2 distribution with one degree of freedom. The probability that a stated value of χ^2 would be exceeded under H_0 is tabulated as P . The result is considered significant, when $P \leq 0.01$ and highly significant with $P \leq 0.001$.

An example of the calculation from the original data in Table 2 is as follows:

	4, 5, 12	9, 12	Total
Dose level (5×10^4)			
Surviving	22	28	50
Dead	8	2	10
Sum	30	30	60
Dose level (5×10^5)			
Surviving	10	25	35
Dead	20	5	25
Sum	30	30	60
Dose level (5×10^6)			
Surviving	2	9	11
Dead	28	21	49
Sum	30	30	60

$$\chi_{(1)}^2 = \frac{\{22 + 10 + 2 - [50(30)/60 + 35(30)/60 + 11(30)/60]\}^2}{[30^2/60^2(59)] [50(10) + 35(25) + 11(49)]} = 24.2$$

($P < 0.001$)

Table 2. *The lethal effect in mice of smooth 4, 5, 12 or smooth 9, 12 recombinants of Salmonella typhimurium*

The table records the number of deaths in groups of 10 mice infected with tenfold dilutions of overnight broth cultures of S (4, 5, 12) or S (9, 12) recombinants of *S. typhimurium*.

Strain	Dose (bacteria/mouse)				
	5×10^7 *	5×10^6	5×10^5	5×10^4	5×10^3
SH 2201 S (4, 5, 12)	10	10	5	5	0
SH 2202 S (4, 5, 12)	10	8	6	1	0
SH 2203 S (4, 5, 12)	10	10	9	2	0
SH 2204 S (9, 12)	8	7	1	0	0
SH 2205 S (9, 12)	8	5	2	2	0
SH 2206 S (9, 12)	10	9	2	0	0

* For SH 2201 the actual doses were 6×10^7 , 6×10^6 , etc., and for SH 2203 the corresponding doses were 4×10^7 , 4×10^6 , etc.

RESULTS

Reliability of the virulence test

Elimination of cross-infection. Control mice (110 in all) were injected intraperitoneally with 0.5 ml. of 0.9 % NaCl. Most of them were kept in their own cages placed between experimental cages, but a small number (15) were kept in groups of three in the same cages as infected animals. None of the control mice died during the 10 day observation period.

Stability of the strain during virulence test. Bacterial stability was controlled in each experiment by testing 20 colonies for their O antigen serologically and with S- and R-specific phages: 20 colonies were obtained both from the inoculum and from the cultures isolated from heart blood and intraperitoneal fluid of several dead mice. Most isolations were made within 1 h. from the moment of death. No reversions from R to S forms or other changes in the O antigens were found after animal passage.

Table 3. *Time of death in mice after infection with virulent and avirulent strains of Salmonella typhimurium*

Cumulative deaths in groups of ten mice injected intraperitoneally with 0.5 ml./mouse of tenfold dilutions of overnight broth cultures of virulent and avirulent strains of *Salmonella typhimurium*.

Days after injection	Number of mice dead after injection with														
	SH 2183 S (4, 5, 12)					SH 2710 S (4, 5, 12)					SL 748 (R)				
	Dose (bacteria/mouse)					Dose (bacteria/mouse)					Dose (bacteria/mouse)				
	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^5	5×10^4	5×10^3	5×10^2	50	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	9	1	0	0	0	1	0	0	0	0	8	0	0	0	0
2	10	4	1	0	0	2	0	1	1	0	8	2	0	0	0
3	10	9	1	1	0	6	0	1	1	0	9	1	0	0	0
4	10	9	2	1	0	8	3	3	6	0	10	2	0	0	0
5	10	9	3	1	0	10	5	5	7	3	10	3	0	0	0
6	10	10	5	2	0	10	5	5	7	3	10	3	0	0	0
7	10	10	5	2	0	10	9	6	9	3	10	3	0	0	0
8	10	10	5	4	0	10	9	8	9	3	10	5	0	0	0
9	10	10	6	4	0	10	9	10	9	5	10	5	0	0	0
10	10	10	6	5	0	10	9	10	9	5	10	5	0	0	0
30	10	10	7	5	0	10	10	10	9	7	10	5	0	0	0

LD₅₀ = 1×10^5

LD₅₀ < 10^2

LD₅₀ = 5×10^7

The LD₅₀ value calculated from the 10 day survival.

Choice of the observation period. For obvious reasons a relatively short observation period was desirable. Essential information does not seem to be lost by our choice of the 10 day period as compared with the conventional 30 day experiment, because over 90 % of the deaths in 30 day experiments occur during the first 10 days (Table 3). The shorter period may have an advantage in recording deaths due to the original inoculum since it is not likely to include deaths from secondary infection from cage mates.

Choice of the Salmonella strain. We wanted to use *Salmonella typhimurium* LT 2 line as a basic strain, because it is genetically well characterized. The problem was that the

LT 2 line has a fairly low virulence compared with many *S. typhimurium* strains isolated from clinical specimens, e.g. SH2710 in Table 3, LD₅₀ < 10². The LD₅₀ values of SH1 and SL1027 used in this work were about 10⁵, while several other smooth LT 2 lines were even less virulent (LD₅₀ > 10⁶). New auxotrophic mutants isolated from SL1027 after mutagen treatment had often lost their virulence, and for this reason the virulence of each new mutant was checked before use. In further tests some of the new avirulent mutants were still smooth, but their loss of virulence could not be clearly correlated with the known auxotrophic mutations because their prototrophic revertants were still avirulent. The same phenomenon has been described earlier (Herzberg, 1962).

Reproducibility of the LD₅₀ values in the same mouse line was good (Table 4).

Table 4. LD₅₀ values for smooth *Salmonella typhimurium* strain LT 2 and its SR or R mutants

Strain no.	Somatic antigen	Mutant LPS genes	SAW mice line I		SAW mice line II		
			(i)	(ii)	(iii)	(iv)	(v)
SL1027	S (4, 5, 12)	—	4 × 10 ⁴	3 × 10 ⁴	1 × 10 ⁵	5 × 10 ⁵	1 × 10 ⁵
SL901	SR (4, 5, 12)	<i>rfc</i>	1 × 10 ⁶	2 × 10 ⁶	—	—	—
SL748	R	<i>rfb</i>	5 × 10 ⁷	1 × 10 ⁸	1 × 10 ⁸	—	—
SL1060	R	<i>rfaH</i>	2 × 10 ⁸	1 × 10 ⁸	—	—	—

(i) etc. refer to different experiments.

Effect of S → R variation on virulence in the chosen experimental conditions. The LD₅₀ value of the smooth LT 2 line was about 10⁵, while the LD₅₀ values of rough forms (*rfb*, *rfaH*) were about 10⁸ (Table 4). The difference between S and R forms, therefore, was only about a thousandfold as compared to the almost millionfold difference seen between S and R forms when starting from highly virulent *Salmonella* strains (Arkwright, 1927). However, in spite of this narrow scale, the virulence of the semirough mutant (*rfc*) with only one repeating unit per side-chain was clearly intermediate (LD₅₀ ~ 10⁶) between the virulence of the smooth and the R forms. There was no difference between the *rfb* and the *rfaH* mutants, agreeing with previous observations on different R mutants (Nakano & Saito, 1969; Edebo & Normann, 1970). Although both mutants were avirulent, they could be isolated as pure cultures from the heart blood of dead mice, indicating that when injected in large enough amounts they were also capable of causing infections. The endotoxin (LPS) effect seems to be lethal only at very high dose levels (> 10⁸ bacteria/mouse): some deaths were observed on the first day after intraperitoneal injection of about 10⁹ heat-killed bacteria/mouse, but there were no deaths after a dose of about 10⁸.

The effect of 9, 12-specific side-chains

The *rfb* locus of *Salmonella typhimurium* determining 4, 12 specificity was replaced through P22 transduction by the *rfb* locus of *S. enteritidis* determining 9, 12 specificity. Phage P22 grown on the *his*⁺ 9, 12 donor SH1036 was applied to the *his*⁻ 4, 5, 12 virulent (LD₅₀ = 10⁵) recipient SH2183; some of the *his*⁺ transductants had the O antigens 9, 12 indicating that they had inherited the *rfb*^D locus of the donor, while the remaining *his*⁺ transductants were antigenically 4, 5, 12. The virulence of three

transductants from both classes was determined (Tables 2, 5). The LD₅₀ values of three *his*⁺ 4, 5, 12 transductants were about 10⁵, like that of the recipient, while the 9, 12 transductants showed slightly reduced virulence (LD₅₀ ~ 10⁶). The difference between these two recombinant classes was highly significant ($P < 0.001$). As a control, a *his*⁻ mutant was made from one 9, 12 transductant and was used as a recipient in a conjugational cross with an avirulent (LD₅₀ = 10⁸) 4, 5, 12 *S. abony* Hfr strain. It was very unlikely that the avirulence loci in *S. abony* (Krishnapillai & Baron, 1964) would have been transferred in this conjugation, because of the *his*⁺ selection and the direction of chromosome injection by the donor (Fig. 2). The LD₅₀ values of two *his*⁺ 4, 5, 12 recombinants obtained in this cross were about 10⁵, indicating a restoration of the original virulence together with the 4, 5, 12 specificity. The virulence of a *his*⁺ 9, 12 recombinant was unchanged (LD₅₀ > 10⁶). It thus appears that 4, 5, 12-specific side-chains confer a slightly higher degree of mouse virulence on a *S. typhimurium* strain than do 9, 12-specific side-chains.

Table 5. LD₅₀ values of smooth *Salmonella typhimurium* strains with the *O* specificity 4, 5, 12 or 9, 12

Origin of transductants or recombinants		LD ₅₀ values		
Donor	Recipient	Strain no.	<i>O</i> specificity	
			4, 5, 12	9, 12
P22 transduction		Recipient		
SH 1036	SH 2183	SH 2183	1 × 10 ⁵	—
(<i>his</i> ⁺ , 9, 12)	(<i>his</i> ⁻ , 4, 5, 12)	Transductants		
		SH 2201	2 × 10 ⁵	—
		SH 2202	4 × 10 ⁵	—
		SH 2203	1 × 10 ⁵	—
		SH 2204	—	3 × 10 ⁶
		SH 2205	—	3 × 10 ⁶
		SH 2206	—	1 × 10 ⁶
Selection for <i>his</i> ⁺				
Back-cross (conjugation)		Recipient		
SW 1403	SH 2233	SH 2233	—	2 × 10 ⁶
(<i>his</i> ⁺ , 4, 5, 12)	(<i>his</i> ⁻ , 9, 12)	Recombinants		
		SH 2266	1 × 10 ⁵	—
		SH 2267	3 × 10 ⁵	—
		SH 2268	—	4 × 10 ⁶
Selection for <i>his</i> ⁺				

SH 2233 (*his*⁻, 9, 12) was derived from transductant SH 2206.

The effect of 6, 7-specific side-chains

To convert the type 4, 5, 12 *Salmonella typhimurium* to type 6, 7, conjugation was first used instead of transduction, because there were no suitable transducing phages available for such an intergroup system. The results were later confirmed with 6, 7 and 4, 5, 12 transductants, obtained in the round about way described below.

The same virulent *his*⁻ 4, 5, 12 *Salmonella typhimurium* recipient (SH 2183) as before was crossed with an avirulent (LD₅₀ = 10⁸) *S. montevideo* donor (Hfr) strain and *his*⁺ recombinants were selected. As expected (Mäkelä, 1966), about 80 % were serologically 6, 7 and had therefore inherited the *rfb*^c cluster from the *S. montevideo* donor, while the remaining 20 % were 4, 5, 12 and had thus retained the original *rfb*^B locus. Virulence of two *his*⁺ 6, 7 and two *his*⁺ 4, 5, 12 recombinants was determined (Table 6). These recombinants were also *trp*⁻ like the recipient, which put a limit on the amount

of donor material on one side of the *his* marker (see Fig. 2); on the other side the 6, 7 recombinants could have inherited a larger fraction of the donor chromosome. This fraction was probably quite small, however, and the two recombinant classes differed from each other mainly in the *rfb* region, because in intergroup crosses only short chromosome fragments are usually incorporated (Stocker & Mäkelä, 1971). The two recombinants with 4, 5, 12 specificity were as virulent as the recipient (LD 50 $\sim 10^5$), while the 6, 7 recombinants were avirulent (LD 50 = 10^7 to 10^8). The difference between the LD 50 values of these two recombinant classes was highly significant ($P < 0.001$). As a further check it was tested whether the virulence of the 6, 7 recombinant could be restored to the level of the parent, by reintroducing the *rfb^B* locus determining 4, 5, 12 specificity, to a *his⁻* mutant of the 6, 7 recombinant. This 'back-cross' was performed using the same *S. abony* Hfr donor as with the 9, 12 transductants above. The LD 50 values of two *his⁺* 4, 5, 12 recombinants were between 10^5 to 10^6 , while the LD 50 value of a *his⁺* 6, 7 recombinant was unchanged (10^8), strongly indicating that the 4, 5, 12 side-chains had restored virulence to these recombinants (Table 6). The fact that the original LD 50 value of 10^5 was not quite achieved could be due to the manipulations (mutagenesis and crossing), which the strains had experienced before virulence testing.

Table 6. LD 50 values of smooth *Salmonella typhimurium* strains with the O specificity 4, 5, 12 or 6, 7

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
		SH 2282	—
		Recipient	—
		SH 1	4 × 10 ⁵
		Transductants	—
		SH 1331	2 × 10 ⁵
		SH 1333	8 × 10 ⁵
		SH 1360	—
		SH 1361	—

Origin of recombinants or transductants		LD 50 values	
Donor	Recipient	Strain no.	O specificity
Cross (conjugation)			
SH 1605	×	SH 2183	—
(his ⁺ , 6, 7)	(his ⁻ , 4, 5, 12)	Recipient	4, 5, 12
		SH 2183	1 × 10 ⁵
		Recombinants	6, 7
		SH 2245	1 × 10 ⁵
		SH 2246	2 × 10 ⁵
		SH 2241	—
		SH 2242	—
		Recipient	—
		SH 2259	n.d.
		Recombinants	—
		SH 2273	5 × 10 ⁵
		SH 2274	8 × 10 ⁵
	</		

n.d. = not determined.

SH 2259 (*his⁻*, 6, 7) was derived from recombinant SH 2241.

It seems very probable, therefore, that the loss of virulence in the 6, 7 recombinants was due to the presence of the *rfb^C* locus, although possible 'avirulence loci' (Krishnapillai & Baron, 1964) closely linked to *his* and present in the avirulent

Salmonella montevideo donor, could not be excluded. Because of the drastic reduction of virulence it was felt desirable to limit the amount of genetic material transferred along with *his*. This could be achieved in transduction, but the available transducing phage P22 would not attack 6, 7 bacteria. A P22 lysate could, however, be prepared by induction of a strain of *S. typhimurium* which was lysogenic for P22 and which had been given *rfb^C* in a conjugational cross with *S. montevideo* as the Hfr donor. The lysate had a fairly low titre, and transduction did not succeed with SH2183 as a recipient. It did succeed, however, with another *his⁻* *S. typhimurium* strain LT2 (SH1). The LD50 values of two *his⁺* 4, 5, 12 transductants were again 10^5 to 10^6 like those of the recipient, while the corresponding values of *his⁺* 6, 7 transductants were 10^7 to 10^8 (see Table 6).

DISCUSSION

This work has demonstrated that different O side-chains confer different degrees of mouse virulence on a basically virulent *Salmonella typhimurium*. *Salmonella typhimurium* with the original 4, 5, 12-specific side-chains is slightly more virulent than the same strain with 9, 12 specificity, and this is clearly more virulent than the same *S. typhimurium* strain with 6, 7 side-chains. Yet all the strains studied were smooth according to all available criteria: sensitivity to S-specific phages and resistance to R-specific phages; S-type agglutination with specific anti-O sera but no agglutination with 4 % (w/v) saline or with antirough sera.

In each experiment the virulence of several sister recombinants was compared in order to keep the strains as isogenic as possible, the only difference between them being in the O side-chain. When comparing the 9, 12 and 4, 5, 12 strains this purpose appears to have been well achieved. The strains were prepared by transduction, so that the non-identical fraction of the genome of the two transductant classes was less than 1 % (the amount of DNA transferred in transduction is of the order of 1 % of the total chromosome; both transductant classes also had common areas in the histidine locus received from the donor). The 6, 7 strains were prepared by conjugation and by transduction. In both cases the result was the same, low virulence always accompanying the 6, 7 characteristic and high virulence accompanying the 4, 5, 12 specificity. Combining the data from the transduction and the conjugation analyses, four strains of 9, 12 specificity were all less virulent than six sister 4, 5, 12 strains and five strains of 6, 7 specificity were clearly less virulent than seven sister 4, 5, 12 strains. There is strong evidence in these results for the association of virulence with the quality of the O side-chain as determined by the *rfb* locus. Other genes determining virulence and closely linked with the *rfb* locus are not excluded but if they exist their number is limited.

A limitation of the action of foreign (groups C or D) genetic material in the group B genome is also possible. Again the effect, if present, must be limited to *rfb* and a few other closely linked genes. If this impairment occurred, it was not large enough to make the strains detectably non-smooth, although searching criteria for smoothness were applied (Lindberg, Sarvas & Mäkelä, 1970). Pleiotropic effects of the foreign *rfb* genes are also conceivable but improbable.

We do not know how the quality of the O side-chain affects virulence. There are at least two possibilities: (a) the quality of the monosaccharide constituents of the different types of O side-chains is of major importance, or (b) the main factor is the

degree of exposure of the deeper layers of the cell wall including the LPS core, resulting from differences in length and/or arrangement of the O side-chains of different specificities.

The quality of the monosaccharides is very similar in the repeating units of the 4, 12 and 9, 12 side-chains. The only difference lies in the dideoxyhexose which forms an immunodominant side branch in the main chain (Fig. 1), and which is abequose in 4, 12 and tyvelose in 9, 12. It is not easy to understand why the mouse reacts differently to the two sorts of dideoxyhexoses. The 4, 12 strains used in this work also had factor 5, which corresponds to an O-acetyl group on the abequose (Hellerqvist *et al.* 1968), but in another study isogenic *Salmonella typhimurium* strains with and without factor 5 were found to be equally virulent, indicating that this factor does not affect virulence (V. V. Valtonen & P. H. Mäkelä, in preparation). The 6, 7 side-chains on the other hand are very different, lacking both the deoxyhexose rhamnose and the dideoxyhexose.

From the point of view of the host in relation to the observed differences in virulence, two mechanisms can be suggested: resistance could be based either on an immunological mechanism or associated with phagocytosis. The 6, 7-specific side-chains may be more immunogenic than the 4, 5, 12 side-chains, or the mice may have 'natural antibodies' against 6, 7 antigen giving better protection against a *Salmonella* strain with 6, 7 specificity. If these were the main causes of the observed differences in virulence, we have to assume that a 9, 12-specific LPS is also more immunogenic than the 4, (5), 12 LPS, or that the mice possess more 'natural antibodies' against 9, 12 than 4, (5), 12 determinants. We have studied the role of the immune response in infection with the transductant strains used in this work and the results are to be published soon.

There are several reports which have shown that avirulent *Salmonella* strains are killed more rapidly by phagocytic cells than are virulent ones (Furness, 1958; Biozzi *et al.* 1964; Fauve, 1964; Ushiba, 1965). The mechanism by which virulent strains can resist phagocytosis is unknown, but it seems probable that the structure of the LPS is of major importance in this respect. An obvious experimental approach would be to determine whether the 4, 5, 12 LPS is more 'antiphagocytic' than 9, 12 or 6, 7 LPS.

Similar differences in virulence associated with cell-wall polysaccharides have also been found in *Pneumococcus* and *Escherichia coli*. MacLeod & Krauss (1950) found that both the amount and the quality of the capsular polysaccharide have an effect on the virulence of *Pneumococcus*. The capsular polysaccharides are known to be 'antiphagocytic' substances. The capsular polysaccharide of type VII *Pneumococcus* is a particularly efficient antiphagocytic substance; it contains L-rhamnose and other deoxysugars (How, Brimacombe & Stacey, 1964). Medearis and co-workers (1968) have found that the sugar composition of the cell wall strongly affects the virulence of *E. coli*. Apart from a rough mutant they also used an apparently smooth mutant of *E. coli* O-111 strain lacking only a terminal dideoxyhexose colitose in its LPS, and found this to be clearly less virulent than the parent strain. Loss of virulence in this mutant was accompanied by diminished resistance to phagocytosis. The present work with *Salmonella* strains has clear analogies with these observations. The 4, 5, 12 and 9, 12 side-chains, which confer stronger mouse virulence than the 6, 7 side-chains, also contain both L-rhamnose and a dideoxyhexose (abequose or tyvelose respectively), while the 6, 7 side-chain does not contain either. One is tempted to suggest that

these lipophilic deoxy- or dideoxysugars, which are rare in nature, are also important determinants of anti-phagocytic activity and thus of bacterial virulence.

I thank Professor P. Helena Mäkelä for her help in preparing the manuscript and also for help with materials, Professor O. Miettinen for suggesting the statistical significance test applied, Miss Marianne Hovi for preparing some of the strains, and Mrs Aila Åkerlund and Mrs Eila Tikkanen for their technical assistance. This work was supported by grants from the Sigrid Jusélius Foundation to Professor Mäkelä and from the Emil Aaltonen Foundation.

REFERENCES

- ARCHER, J. R. & ROWLEY, D. (1969). A quantitative comparison of the antigenic structure of a virulent and an avirulent strain of *Salmonella typhimurium*. *Immunology* **17**, 551–558.
- ARKWRIGHT, J. A. (1927). The value of different kinds of antigen in prophylactic 'enteric' vaccines. *Journal of Pathology and Bacteriology* **30**, 345–364.
- BIOZZI, G., LE MINOR, L., STIFFEL, C., MOUTON, D. & DAOULAS, F. (1964). Correlation between virulence and phagocytosis of genetic recombinant between *Escherichia coli* and *Salmonella typhimurium*. *Nature, London* **202**, 819–820.
- EDEBO, L. & NORMANN, B. (1970). Virulence and immunogenicity of mutant strains of *Salmonella typhimurium*. *Acta pathologica et microbiologica scandinavica* **78 B**, 75–84.
- FAUVE, M. R. (1964). Résistance cellulaire à l'infection bactérienne. *Annales de l'Institut Pasteur, Paris* **107**, 472–483.
- FULLER, N. A. & STAUB, A. M. (1968). Immunochemical studies on *Salmonella* 13. Chemical changes appearing on the specific polysaccharide of *S. cholerae suis* (6₂, 7) after its conversion by phage 14 (6, 7). *European Journal of Biochemistry* **4**, 286–300.
- FURNSS, G. (1958). Interaction between *Salmonella typhimurium* and phagocytic cells in cell culture. *Journal of Infectious Diseases* **103**, 272–277.
- GEMSKI, P. JUN. & STOCKER, B. A. D. (1967). Transduction by bacteriophage P 22 in non-smooth mutants of *Salmonella typhimurium*. *Journal of Bacteriology* **93**, 1588–1597.
- HARTMAN, P. E., LOPER, J. C. & SERMAN, D. (1960). Fine structure mapping by complete transduction between histidine-requiring *Salmonella* mutants. *Journal of General Microbiology* **22**, 323–353.
- HELLERQVIST, C. G., LINDBERG, B., SVENSSON, S., HOLME, T. & LINDBERG, A. A. (1968). Structural studies on the O-specific side-chains of the cell wall lipopolysaccharide from *Salmonella typhimurium* 395 ms. *Carbohydrate Research* **8**, 43–55.
- HERZBERG, M. (1962). Living organisms as immunizing agents against experimental salmonellosis in mice. I. Virulence of auxotrophic mutants. *Journal of Infectious Diseases* **111**, 192–203.
- HOW, M. J., BRIMACOMBE, J. S. & STACEY, M. (1964). The pneumococcal polysaccharides. *Advances in Carbohydrate Research* **19**, 303–358.
- KAUFFMANN, F. (1966). *The Bacteriology of Enterobacteriaceae*. Copenhagen: Munksgaard.
- KRISHNAPILLAI, V. & BARON, L. S. (1964). Alterations in the mouse virulence of *Salmonella typhimurium* by genetic recombination. *Journal of Bacteriology* **87**, 598–605.
- LEDERBERG, J. (1950). Isolation and characterization of biochemical mutants of bacteria. *Methods in Medical Research* **3**, 5–22.
- LINDBERG, A. A., SARVAS, M. & MÄKELÄ, P. H. (1970). Bacteriophage attachment to the somatic antigen of *Salmonella*: effect of O-specific structures in leaky R mutants and S, T₁ hybrids. *Infection and Immunity* **1**, 88–97.
- LINGELSHEIM, V. (1913). Zur Frage der Variation der Typhusbacillen und verwandter Gruppen. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* (Abteilung I Originale) **68**, 577–582.
- LOPER, J. C., GRABNAR, M., STAHL, R. C., HARTMAN, Z. & HARTMAN, P. E. (1964). Genes and proteins involved in histidine biosynthesis in *Salmonella*. *Brookhaven Symposia in Biology* **17**, 15–52.

- LÜDERITZ, O., JANN, K. & WHEAT, R. (1968). Somatic and capsular antigens of Gram-negative bacteria. In *Comprehensive Biochemistry*, vol. 26 A, pp. 105-228. Edited by M. Florkin and E. H. Stoltz. Amsterdam: Elsevier.
- MACLEOD, C. M. & KRAUSS, M. R. (1950). Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed *in vitro*. *Journal of Experimental Medicine* **92**, 1-9.
- MANTEL, N. & HAENSZEL, W. (1959). Statistical aspects of the analysis of data from retrospective studies of disease. *Journal of The National Cancer Institute* **22**, 719-748.
- MEDEARIS, D. N. JUN., CAMITTA, B. M. & HEATH, E. C. (1968). Cell wall composition and virulence in *Escherichia coli*. *Journal of Experimental Medicine* **128**, 399-414.
- MÄKELÄ, P. H. (1963). Hfr males in *Salmonella abony*. *Genetics* **48**, 423-429.
- MÄKELÄ, P. H. (1966). Genetic determination of the O antigens of *Salmonella* groups B (4, 5, 12) and C₁ (6, 7). *Journal of Bacteriology* **91**, 1115-1125.
- MÄKELÄ, P. H. & STOCKER, B. A. D. (1969). Genetics of polysaccharide biosynthesis. *Annual Review of Genetics* **3**, 291-322.
- NAKANO, M. & SAITO, K. (1969). Chemical components in the cell wall of *Salmonella typhimurium* affecting its virulence and immunogenicity in mice. *Nature, London* **222**, 1085-1086.
- REED, L. G. & MUENCH, H. (1938). A simple method of estimating fifty per cent end-points. *American Journal of Hygiene* **27**, 493-497.
- ROANTREE, R. J. (1967). *Salmonella* O antigens and virulence. *Annual Review of Microbiology* **21**, 443-466.
- SANDERSON, K. E. (1967). Revised linkage map of *Salmonella typhimurium*. *Bacteriological Reviews* **31**, 354-372.
- STOCKER, B. A. D. & MÄKELÄ, P. H. (1971). Genetic aspects of biosynthesis and structure of *Salmonella* lipopolysaccharide. In *Microbial Toxins*, vol. 4. Edited by S. J. Ajl. New York: Academic Press.
- USHIBA, D. (1965). Two types of immunity in experimental typhoid: 'cellular immunity' and 'humoral immunity'. *Keio Journal of Medicine* **14**, 45-61.
- VALTONEN, V. V. (1969). Virulence of *Salmonella* strains with a reduced amount of O antigen. *Journal of General Microbiology* **57**, xxviii.
- WILKINSON, R. G. (1966). *Rough Mutants of Salmonella typhimurium*. Ph.D. Thesis, University of London.
- WILKINSON, R. G. & STOCKER, B. A. D. (1968). Genetics and cultural properties of mutants of *Salmonella typhimurium* lacking glucosyl or galactosyl lipopolysaccharide transferases. *Nature, London* **217**, 955-957.