The Effect of Lipopolysaccharide Modifications – Antigenic Factors 1, 5, 12₂ and 27 – on the Virulence of Salmonella Strains for Mice

By V. V. VALTONEN AND P. HELENA MÄKELÄ

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

(Accepted for publication 13 August 1971)

SUMMARY

The virulence of *Salmonella typhimurium* is known to depend on the presence and on the quality of smooth-type O side-chains in the lipopolysaccharide (LPS). We have studied the effect on virulence of O side-chain modifications – O factors 5, 1, 12₂ and 27 which correspond to acetyl or glucosyl side-branches or a change in the linkage between repeating oligosaccharide units of the LPS (27). The LD₅₀ values of sister strains with and without these factors did not differ significantly, nor could we detect selection during mouse passage for or against forms possessing a variable O factor except perhaps in the case of factor 27. These O side-chain modifications thus appear neutral in respect of virulence.

INTRODUCTION

A complete lipopolysaccharide (LPS) with long, smooth-type O side-chains is necessary for virulence in salmonellas (Roantree, 1967). Recently it was shown that O side-chains of different serological specificities like 4, 5, 12; 9, 12 or 6, 7 confer different degrees of virulence to a *Salmonella typhimurium* strain, to which they were donated by transduction (Valtonen, 1970). It has also been claimed that a minor O antigen factor such as 5 would be an important determinant of virulence in *S. typhimurium* (Jenkin & Rowley, 1965) or in *S. paratyphi-B* (Felix, 1952).

The basic structure of the repeating units of the O side-chains is determined by the *rfb* cluster of genes. The basic structure can be modified, e.g. by the addition of glucosyl side-branches or O-acetyl groups. Many of these modifications depend on antigenic conversion by bacteriophages, while others are determined by bacterial genes. These modifications are not necessary for the synthesis of a complete, smooth-type LPS, and they are probably added as one of the later steps in the synthesis (Mäkelä & Stocker, 1969). Furthermore, many modifications are subject to form variation so that in a culture the corresponding factor is well developed in some bacteria, but absent in others (Kauffmann, 1940).

The basic, *rfb*-determined, O factors are 4 and 12 in group B strains, e.g. *Salmonella typhimurium* (Fig. 1). In addition, the bacteria can have the O antigen factors 1 and 12₂ corresponding to glucosyl side-branches attached to C-6 or C-4 respectively of the galactose of the repeating units (Fig. 1). Phage P22 and related phages cause the appearance of factor 1. Factor 12₂ is dependent on a bacterial gene *oafR*, which maps close to *purE* (Fig. 2) (Mäkelä & Mäkelä, 1966). Both these antigens are form variable. The structure responsible for factor 5 is an O-acetyl group attached to the abequose (Hellerqvist, Lindberg, Svensson, Holme & Lindberg, 1968). Factor 5 is determined by a bacterial gene *oafA*, which maps close to the *rfb* region but is separate from it (Smith & Stocker, 1962). Factor 27 represents a more basic modification: the linkage between repeating units, normally formed under the direction of

the bacterial polymerase (gene *rfc*), is altered (Bagdian, Lüderitz & Staub, 1966). This change is brought about by phage P27, whose prophage attachment site is close to *purE* (Fig. 2) (Bagdian & Mäkelä, 1971).

In this work we have studied the possible effect of LPS modifications on the mouse virulence of a basically virulent *Salmonella typhimurium* strain with the antigenic formula 4, 5, 12. 1 +, 5 -, 12₂ + or 27 + derivatives were prepared from it by means of lysogenic conversion, mutation or genetic recombination.

Fig. 1. A schematic structure of the O side-chain in group B LPS. The basic repeating unit Abe—Man—Rha—Gal is printed in bold face, the modifications with normal. (Abe = abequose, Man = mannose, Rha = rhamnose, Gal = galactose, AcO = O-acetyl).

Fig. 2. A simplified chromosome map of *Salmonella* (according to Sanderson, 1970; Mäkelä & Stocker, 1969). The point of entry and direction of injection of the donor (Hfr or F') chromosomes are indicated by arrows on the circle. Genes participating in LPS synthesis or modification are marked on the outside of the circle. Gene symbols are explained in footnote of Table 1 except *attP 22*, *attP 27* = attachment site of prophage P22, P27.

METHODS

Bacterial and phage strains. The bacterial strains used in this work are listed in Table 1. The basic strains were *Salmonella typhimurium* SH2183 and SH2191, with the O antigens 4, 5, 12 (Valtonen, 1970). Spontaneous factor 5 negative mutants were selected with anti-5 serum (Iseki & Sakai, 1952). The strains are kept in the collection of P. H. Mäkelä, Central Public Health Laboratory, Helsinki.

Phage P27 was received from Dr L. Le Minor, Pasteur Institute, Paris, France. It was originally isolated from *Salmonella schwarzengrund* and then propagated on *S. paratyphi-B*.

Bacteriological techniques. In conjugation equal amounts of exponentially growing donor (Hfr) and recipient (F⁻) broth cultures containing about 5×10^8 bacteria/ml. were mixed and the mixture was incubated for 2 h. without shaking before plating on appropriate selective media (Mäkelä, 1966).

Serological methods followed standard techniques of Kauffmann (1966). O antigens were determined by slide agglutination in 4% saline and in antisera appropriately diluted with 0.2% saline. In case of variable antigens (1, 12₂, 27) 20 sister colonies were tested by agglutination. 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.; single-factor sera were obtained from anti-O sera by absorption with heat-killed strains lacking the particular O factor.

Virulence test. Five tenfold dilutions of an overnight broth culture of bacteria were injected intraperitoneally into groups of ten mice and the LD₅₀ value was calculated from the 10-day survival according to Reed & Muench (1938). Virulence of two classes of bacteria (e.g. with and without a particular antigen factor) were compared by forming a set of 2 × 2 tables from the original data, according to Mantel & Haenszel (1959). The details of our virulence test and the statistical methods have been described before (Valtonen, 1970).

RESULTS

Effect of factor 1 on virulence

The smooth *Salmonella typhimurium* LT2 strain SH2183 (O antigen type 4, 5, 12 and LD₅₀ value about 10⁵) was lysogenized with phage P22. After two subcultures a colony, lysogenic for phage P22, was selected. A marked form variation in regard of factor 1 was noticed (Iseki & Kashiwagi, 1955; Kauffmann, 1940), when 20 separate colonies in a subculture of the P22 lysogenic strain were agglutinated with anti-1 serum. Four colonies were used to prepare the inocula for the virulence test, two of which (SH2284, SH2285) were strongly agglutinated in anti-1 serum while two (SH2283, SH2286) appeared 1-negative.

When the virulence of the strains SH2283 and SH2286, which had a low proportion of 1-positive cells was compared with the virulence of the strains SH2284 and SH2285, which had a higher proportion of 1-positive cells, no statistically significant difference was detected (Table 2). Similarly, the virulence of the 1-negative parent strain SH2183 did not differ significantly from the virulence of its P22-lysogenic derivatives SH2283 to 2286. When the proportion of 1+ cells in the inoculum was compared with the corresponding proportion in the culture recovered from the heart blood of the dead mice no selection tendency for or against factor 1 was apparent.

Table 1. *Salmonella* strains used and their main features

Species	Strain no.	O Antigen	Mating type	Relevant genotype*	Source and/or reference
<i>Salmonella abony</i>	SW 1403	4, 5, 12	HfrH2	<i>met-1151 aro-851 str-501</i>	Mäkelä, 1963
<i>S. enteritidis</i>	SH 673	9, 12 _a	FT59	<i>trp</i>	Mäkelä & Mäkelä, 1966
<i>S. typhimurium</i>	SH 2183	4, 5, 12	F ⁻	<i>his-6116 metA22 trpB2 str flaA66</i>	Valtonen, 1970
<i>S. typhimurium</i>	SH 2191	4, 5, 12	F ⁻	<i>gal-23 his-6116 metA22 trpB2 str flaA66</i>	Valtonen, Sarvas & Mäkelä, 1971
<i>S. typhimurium</i>	SH 2283 to 2286	1, 4, 5, 12	F ⁻	<i>his-6116 metA22 trpB2 str flaA66</i>	P ₃₂ SH 2183 †
<i>S. typhimurium</i>	SH 2287, 2288	4, 5, 12 _a	—	<i>his-6116 metA22 trpB2 str flaA66 oafR⁺stable§</i>	<i>gal</i> ⁺ recombinants from cross SH 673—× SH 2191 ‡
<i>S. typhimurium</i>	SH 2292				P ₂₇ → SH 2183
<i>S. typhimurium</i>	SH 2290	4, 5, 12	—	<i>his-6116 metA22 trpB2 str flaA66 oafR^{variable} </i>	
<i>S. typhimurium</i>	SH 2296, 2297	4, 5, 12, 27	F ⁻	<i>his-6116 metA22 trpB2 str flaA66</i>	
<i>S. typhimurium</i>	SH 2298, 2299				
<i>S. typhimurium</i>	SH 2701	4, 12	F ⁻	<i>his-6116 metA22 trpB2 str flaA66 oafA6</i>	Spontaneous 5-negative mutant of SH 2183
<i>S. typhimurium</i>	SH 2711, 2712	4, 5, 12	—	<i>metA22 trpB2 str flaA66</i>	<i>his</i> ⁺ <i>oafA</i> ⁺ recombinants from cross SW 1403—× SH 2701
<i>S. typhimurium</i>	SH 2713, 2714	4, 12	—	<i>metA22 trpB2 str flaA66 oafA6</i>	<i>his</i> ⁺ recombinants from cross SW 1403—× SH 2701

* Gene symbols as follows: *aro* = phenylalanine + tyrosine biosynthesis, *fla* = flagellar synthesis, *gal* = galactose utilization, *his* = histidine biosynthesis, *met* = methionine biosynthesis, *pro* = proline biosynthesis, *pur* = purine biosynthesis, *str* = resistance to streptomycin, *trp* = tryptophan biosynthesis, *rfb*, *rfc* = biosynthesis of the O-antigenic lipopolysaccharide, *oafA*, *oafR* = determinants of the modification factors 5 and 12_a.

† Antigenic conversions are described as follows: $\xrightarrow{\text{phage}}$ *Salmonella* strain.

‡ Crosses are described as follows: (donor) × (recipient).

§ *gal*⁺ and *oafR*⁺ *stable* from the *S. enteritidis* parent.

|| *gal*⁺ from *S. enteritidis*; *oafR*^{variable} from LT2.

Effect of factor 27 on virulence

The same virulent *Salmonella typhimurium* strain (SH2183) as above was lysogenized with phage P27 which determines factor 27 (Le Minor, Le Minor & Nicolle, 1961). The resulting *S. typhimurium* strain, although stably lysogenic for P27, showed strong form variation of factor 27 and thus we could not obtain even nearly pure 27+ or 27- cultures for the virulence test. Table 3 gives the LD₅₀ values of four strains with different proportions of 27-positive cells. Strains which are predominantly 27-positive were slightly less virulent than the parent strain SH2183, which has no factor 27. There was a difference ($P = 0.02$) in virulence between the parent strain and the group of its four P27-lysogenic derivatives, suggesting that the presence of factor 27 may slightly reduce virulence. Furthermore, there was a slight selection tendency against factor 27 during mouse passage. The most virulent one of the four sister P27-lysogenic strains was SH2296, which had almost lost its 27 specificity during the animal passage.

Table 2. *Effect of factor 1 on virulence*

Strain	O Antigen	LD ₅₀	1 + Bacteria	
			In inoculum	In dead mice
SH2183*	4, 5, 12	$1-5 \times 10^5 \ddagger$	0	0
SH2283†	1, 4, 5, 12	6×10^4	3/20	1/20
SH2284†	1, 4, 5, 12	1×10^5	16/20	17/20
SH2285†	1, 4, 5, 12	2×10^5	9/20	8/20
SH2286†	1, 4, 5, 12	4×10^5	7/20	4/20

* Parent.

† P22-Lysogenic derivatives.

‡ Four independent determinations.

Table 3. *Effect of factor 27 on virulence*

Strain	O Antigen	LD ₅₀	27 + Bacteria	
			In inoculum	In dead mice
SH2183*	4, 5, 12	$1-5 \times 10^5 \ddagger$	0	0
SH2296†	4, 5, 12, 27	2×10^5	13/20	1/20
SH2297†	4, 5, 12, 27	7×10^5	18/20	9/20
SH2298†	4, 5, 12, 27	1×10^6	18/20	12/20
SH2299†	4, 5, 12, 27	1×10^6	10/20	11/20

* Parent.

† P27-Lysogenic derivatives.

‡ Four independent determinations.

Effect of factor 5 on virulence

Several independent factor 5-negative mutants were isolated from the same *Salmonella typhimurium* strain (SH2183) as above using the method of Iseki & Sakai (1952). After five serial passages in anti-5 serum, it was easy to find mutants which agglutinated only slightly or not at all in anti-5 serum. The strain SH2701 was the best 5-negative mutant found. It did not agglutinate at all in any of our three independent anti-5 sera. We could not detect 5-positive revertants after several subcultures or after animal passage. When this 5-negative mutant was crossed with an 4, 5, 12 Hfr donor sw 1403, approximately 80 per cent of the *his*⁺ recombinants were 5+ indicating that the mutated locus was close to *his*; the known gene *oafA* that determines factor 5 is in this region. The LD₅₀ values of this 5-negative mutant and its 5+ parent were about 10^5 (Table 4), indicating that factor 5 is not essential for

virulence. Because this result was unexpected in view of the work of Jenkin & Rowley (1965), we performed the following control. If factor 5 were an important determinant in virulence, its reintroduction into the 5-negative mutant should affect the virulence of the strain. Therefore we determined the LD₅₀ values of two 5-positive and two 5-negative *his*⁺ sister recombinants produced in the above mentioned cross with SW1403 and again there were no differences in virulence between the 5-positive and the 5-negative strains. So it is clear that the presence or absence of factor 5 does not affect the virulence of *S. typhimurium*.

Table 4. *Effect of factor 5 on virulence*

Strain	O Antigen	LD ₅₀
Parent	SH 2183	4, 5, 12
Its 5-negative mutant	SH 2701	4, 12
<i>his</i> ⁺ Recombinants from the cross sw 1403 (donor) × SH 2701 (recipient)	SH 2711	4, 5, 12
	SH 2712	4, 5, 12
	SH 2713	4, 12
	SH 2714	4, 12

Table 5. *Effect of factor 12₂ on virulence*

Strain	O Antigen	LD ₅₀	12 ₂ + Bacteria	
			In inoculum	In dead mice
SH 2191*	4, 5, 12 ₂ variable	3 × 10 ⁵	0/20	0/20
SH 2287†	4, 5, 12 ₂ stable	2 × 10 ⁵	20/20	20/20
SH 2288†	4, 5, 12 ₂ stable	2 × 10 ⁵	20/20	20/20
SH 2292†	4, 5, 12 ₂ stable	7 × 10 ⁴	20/20	20/20
SH 2290†	4, 5, 12 ₂ variable	3 × 10 ⁵	1/20	0/20

* Recipient strain.

† *gal*⁺ Recombinants from cross SH 673 (donor) × SH 2191 (recipient).

Effect of factor 12₂ on virulence

Factor 12₂ is, like factors 1 and 27, subject to form variation. The *Salmonella typhimurium* LT2 line has a variable 12₂, but it is predominantly in the 12₂-negative form: none of 20 colonies tested was 12₂-positive (Table 5). In some other strains factor 12₂ is stable. A gene *oafR* controls the rate of the 12₂ variation. The *Salmonella enteritidis* strain SH673 has an allele *oafR*^{+stable}, which determines the stability of the 12₂ + form (Mäkelä & Mäkelä, 1966). If the *oafR*^{variable} allele in the LT2 strain is replaced with the *oafR*^{+stable} allele from the *S. enteritidis* strain in conjugation, the recombinants are stable 12₂ +. So we made a cross to obtain 12₂-positive and 12₂-negative recombinants for virulence test.

A virulent *gal*⁻ mutant (SH2191) of *Salmonella typhimurium* LT2 was crossed with the stable 12₂-positive *S. enteritidis* donor SH673 and *gal*⁺ recombinants were selected. As expected about 40% of the recombinants were 12₂-positive in a stable fashion during several subcultures and also during animal passage. We tested for virulence three stable 12₂-positive recombinants and one recombinant (SH2290), which had apparently retained the predominantly negative allele of *oafR* of the LT2 strain. There were no significant differences between the LD₅₀ values of the 12₂-positive and 12₂-negative strains (Table 5) indicating that the 12₂ specificity is not an important virulence determinant.

DISCUSSION

We studied the possible effect of LPS modifications on virulence in regard to glucosyl side-branches (factors 1 and 12₂), O-acetylation (factor 5) or linkage between repeating units (factor 27). It has become evident that these modifications do not affect virulence except perhaps factor 27, whose presence may slightly reduce the virulence of a strain.

We have previously found with this same virulence test significant differences in virulence between *Salmonella typhimurium* strains with different smooth-type O side-chains (Valtonen, 1970) and between strains with and without the T1 antigen (Valtonen, Sarvas & Mäkelä, 1971). In the present work our virulence test was even more sensitive, because we had in addition to LD₅₀ values an opportunity to detect any possible selection tendency during mouse passage for or against the form variable specificities. In spite of this greater sensitivity we could not detect differences in the virulence of strains with or without factors 1, 5 or 12₂. The presence of factor 27, however, appeared to reduce virulence. This effect was detectable as a difference in virulence between the 27-positive and negative strains ($P = 0.02$) and as a slight selection against factor 27 during mouse passage.

The *Salmonella typhimurium* strains compared had little genetic differences except for the gene(s) determining the particular O factor. In case of factors 1 and 27 we compared both the parent with its lysogenic derivatives, and predominantly 1+ and 1- or 27+ and 27- variants of a lysogenic culture. Lysogenization itself had no effect on virulence as judged from the first comparison. In case of factor 5 we compared the 5-positive parent strain with its single step 5-negative mutant. Only in case of factor 12₂ the strains to be compared were recombinants with varying and unknown amounts of the donor *S. enteritidis* gene material. As they were all alike in virulence it seems unlikely that any virulence determinants were included in the exchanged material.

One might have expected the LPS modifications now studied to have an effect on virulence as they alter the physicochemical state of the cell surface. The acetyl groups (factor 5) increase and glucosyl side-branches (factor 1 and 12₂) decrease the lipophilic nature of the LPS. However, the results of this work indicate that these modifications do not affect virulence. This is the more surprising when we know that apparently small changes, such as a change from 4, 12 to 9, 12 in the basic structure of the O side-chain, have a marked effect on virulence (Valtonen, 1970).

Spaun (1952) isolated a variant of *Salmonella typhi* without antigen 12₂ after several mouse passages. This variant was more pathogenic than the original strain with factor 12₂. It seems possible that the increase in virulence observed by Spaun was an effect of serial animal passage alone which usually increases virulence, e.g. by selecting virulent mutants. The lack of factor 12₂ in the most virulent strain could have been due to form variation, especially when the stability of factor 12₂ in his strains had not been determined.

Rowley and co-workers have reported that a virulent strain of *Salmonella typhimurium* possesses a labile surface antigen, which they suspected to be factor 5, and that specific antibody against it is crucial for the expression of immunity to *S. typhimurium* infections (Auzins & Rowley, 1963; Jenkin & Rowley, 1965; Jenkin, Karnovsky & Rowley, 1967). They also have reported that there are differences in the quantity of the single O factors between virulent and avirulent *S. typhimurium* strains (Archer & Rowley, 1969). However, the amount of each O-antigenic determinant 4, 5 and 12 in their virulent strain was about twice greater than the corresponding amount in the avirulent strain without any special increase in factor 5. Our present results indicate that factor 5 plays no role in the virulence of *S. typhimurium*.

Felix studied the Vi antigen of *Salmonella paratyphi-B* which is probably identical with factor 5 in the Kauffman-White scheme (Felix, 1952). The presence of Vi was clearly correlated with mouse virulence in *S. paratyphi-B*. However, it was present in smaller amounts in *S. typhimurium*, and did not affect the virulence of this species. As our study only concerned *S. typhimurium* it leaves open the possibility that factor 5 may increase the inherently low mouse virulence of *S. paratyphi-B*. On the other hand, the identity of the Vi of *S. paratyphi-B* with factor 5 has not been finally established on the chemical level, and we do not know the role and composition of the 'slime wall' of *S. paratyphi-B*. A re-examination of the differences between *S. paratyphi-B* and *S. typhimurium* with present chemical and genetic methods should clarify the situation.

Even if factor 5 and the other modification factors did not affect the virulence of *Salmonella typhimurium*, they may be of importance in active immunization as is suggested e.g. by the above mentioned work of Rowley and co-workers (1967). An answer to this question could be achieved by determining the efficiency of these isogenic 5-positive and 5-negative strains in producing protection in mouse typhoid.

We thank Mrs Aila Åkerlund and Mrs Eila Tikkanen for technical assistance. This work was supported by grants from the Sigrid Jusélius Foundation and the Emil Aaltonen Foundation.

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