Lipopolysaccharide Modification Accompanying Antigenic Conversion by Phage P27

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A structural study by methylation analysis was made of the lipopolysaccharides from *Salmonella bredeney* and strains of *Salmonella typhimurium* which had been lysogenized with phage P27 and thereby converted to have the new O-antigen factor 27. The results were compared with previous studies on the non-converted, 27⁻, parental strains. The change from 27⁻ to 27⁺ was accompanied by a change in the linkage between the repeating units of the O side-chain from galactose-(1→2)-mannose to galactose-(1→6)-mannose. In *S. bredeney* (P27), where the expression of antigen 27 is stable, 98 % of the linkages were changed, whereas only 31 to 45 % of the linkages were affected in two strains of *S. typhimurium* (P27). As 71 to 85 % of the cells in the same batches of *S. typhimurium* bacteria were 27⁺ it seems probable that in these strains many bacteria contained both types of linkages in their lipopolysaccharide.

INTRODUCTION

In *Salmonella* serogroups B and D some species isolated from natural sources have the antigen factor 27 and show the O-antigen formula (1), 4, (5), 12, 27 or (1), 9, 12, 27. Variants of the 27⁺ species which lack factor 27 are found both in nature and in the laboratory. The presence of factor 27 has been shown to depend on a temperate phage, P27 (LeMinor *et al.*, 1961), which converts both 27⁻ variants of naturally 27⁺ strains and strains normally 27⁻ into 27⁺ (LeMinor, 1962). Factor 27 is a 'form variable' antigen factor (Kauffman & Rhode, 1961): some strains exhibit this variation so that the same culture contains both 27⁺ and 27⁻ organisms, while other strains are stably 27⁺.

The chemical basis of factor 27 has been investigated in a *Salmonella bredeney* strain, by comparing a 27⁻ variant and its 27⁺ derivative obtained by lysogenization with P27 originally isolated from *Salmonella schwarzengrund* (Bagdian *et al.*, 1966; Staub & Bagdian, 1966). Factor 27, like other O-antigen factors, corresponds to a specific structural feature in the O-specific polysaccharide chain of the wall lipopolysaccharide (LPS) (Fig. 1). These chains are composed of oligosaccharide repeating units, which are polymerized into long chains attached to a carrier lipid before their final transfer to the LPS core (Osborn, 1969). The 27⁺ and 27⁻ strains differed in the linkage between repeating units so that factor 27 represents a galactose-(1→6)-mannose linkage. In the 27⁻ form the corresponding linkage was thought to be galactose-(1→4)-mannose (Bagdian *et al.*, 1966). Later, the structure of the O side-chain of *Salmonella typhimurium*, a naturally 27⁻ strain of group B, was studied by more reliable analytical methods and the linkage between repeating units was shown to be

Table 1. *Strains of Salmonella bredeney (line B15-60) and S. typhimurium (line LT2, subline SD14) used in this study*

Organism	Strain	Prophages		Agglutination* in factor antisera				
		P22	P27	1	4	5	12	27
<i>S. bredeney</i>	SH363	—	—	+	+	—	+	—
<i>S. bredeney</i>	SH363(P27)	—	+	+	+	—	+	+
<i>S. typhimurium</i>	SH2183(P22)	+	—	±	+	+	+	—
<i>S. typhimurium</i>	SH2183(P22,P27)	+	+	—	+	+	+	+
<i>S. typhimurium</i>	SH5110(P27)	—	+	—	+	+	+	+

* +, strong; ±, incomplete; —, no agglutination.

(Hellerqvist *et al.*, 1968). Samples were taken before harvesting and spread on nutrient agar plates (Difco). After overnight incubation, isolated colonies were tested by slide agglutination against antisera to O factors 4, 5 and 27 and against 4 % saline. The supernatant fluid obtained after harvesting the submerged culture was analysed for the content of either phage P27 or phages P27 and P22 using appropriate indicator strains.

Extraction of lipopolysaccharide and separation of polysaccharide. LPS was extracted by hot phenol/water (Westphal *et al.*, 1952) from formaldehyde-killed bacteria (Lindberg & Holme, 1972) and purified by repeated cycles of ultracentrifugation at 105 000 g for 4 h until virtually free from specific absorption at 260 nm. The LPS fractions were then hydrolysed in aqueous acetic acid, pH 3.2, at 100 °C for 60 min to split the acid-labile 3-deoxyoctulosonic linkages in the basal core (Dröge *et al.*, 1968). This resulted in a polysaccharide fraction free from lipid A. The polysaccharide was fractionated on a Sephadex G-50 column with 0.05 M-pyridine/acetate buffer, pH 5.5, as eluant. The fractions were analysed for carbohydrate content using the phenol-sulphuric acid method (Whistler & Wolfrom, 1962). The peak fractions were pooled and subjected to chemical analyses.

Analysis of monosaccharides. The pooled fractionated polysaccharides were hydrolysed to give a mixture of monosaccharides which were reduced with an excess of sodium borohydride at room temperature for 16 h. Excess borohydride was destroyed by addition of Dowex 50 (H⁺) and boric acid was removed as methyl borate by several distillations with methanol under reduced pressure. The syrups were dried by distillation under reduced pressure with benzene, and then fully acetylated with acetic anhydride/pyridine (1:1, v/v) at 100 °C for 1 h. Excess acetic anhydride was removed by co-distillation with toluene. The resulting mixture of fully acetylated alditol acetates was dissolved in chloroform and analysed by gas-liquid chromatography (Sawardeker *et al.*, 1965). The different sugars were identified by their retention times (T values) and mass spectra (Golovkina *et al.*, 1966).

Analysis of polysaccharide structure. The fractionated polysaccharides were methylated, and the methylated sugars were converted to alditol acetates and analysed by gas-liquid chromatography (Björndal *et al.*, 1967a, b). The components were identified by mass spectrometry and corresponding retention times; T values are given relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

Presence of O-acetyl groups. This was investigated by infrared spectrometry. The spectral evidence was corroborated by looking for methyl acetate by gas-liquid chromatography/mass spectrometry after treating the LPS preparations with 4 % (v/v) methanolic HCl (2 mg LPS in 0.5 ml) for 1 h at 100 °C.

Alkali-labile groups. The presence of these groups was investigated in the fractionated polysaccharide according to the method of DeBelder & Norrman (1968). The fully acetylated polysaccharide was then methylated and analysed as described earlier (Hellerqvist *et al.*, 1968).

RESULTS

Serology and stability of the strains

The *S. bredeney* 27[—] strain was completely stable; all its colonies agglutinated in anti-1, anti-4 and anti-12 factor sera (Table 1). The converted *S. bredeney* (P27) was likewise stable; in the batch from which the LPS was extracted 50 out of 50 colonies gave a strong agglutination in anti-27 serum as well as in anti-1, anti-4 and anti-12 factor sera.

Salmonella typhimurium strains, when converted by P27, showed rapid form variation. The batch culture for preparing the LPS was always started from a fresh colony which gave a strong agglutination reaction in anti-27 serum. Nevertheless, a large fraction of the colonies grown from the final batch were 27[—]: 23 % in SH2183(P22,P27), 15 % in SH5110 (Table 1). In

Table 2. *Monosaccharide content (molar proportions of detected sugars) of the five LPS preparations studied*

Component	Derived from*	<i>S. bredeney</i>		<i>S. typhimurium</i>		
		27 ⁻	27 ⁺	27 ⁻	27 ⁺ †	27 ⁺ ‡
Abequose	O-PS	20	18	19	9	12
L-Rhamnose	O-PS	22	18	20	14	18
D-Mannose	O-PS	23	18	19	14	18
D-Galactose	O-PS and core	25	25	30	18	21
D-Glucose	O-PS and core	10	14	12	12	6
Heptose	core	3	6	ND	5	ND

ND, Not detected.

* O-PS, O-polysaccharide; core, core polysaccharide; see Fig. 1.

† Strain SH2183(P22,P27). ‡ Strain SH5110(P27).

strain SH2183 the phage P22 determined the production of antigenic factor 1, which is based on glucose (1→6)- α -linked to galactose in the O-repeating unit (Stocker *et al.*, 1960). This factor is also form variable.

Lipopolysaccharide analysis

The overall compositions of the LPS of the strains examined are given in Table 2 and are as expected. Abequose, mannose and rhamnose derive exclusively from the O-repeating units and should occur in equal amounts when the acid-lability of abequose is accounted for (Hellerqvist *et al.*, 1968). When this was not done, however, lower values for abequose were observed (Table 2). Galactose occurs both as an integral component of the repeating units and in the core, and should therefore be found in slightly higher amounts than the other O-specific sugars. Glucose is an integral component of the core (where it occurs in equimolar amounts with galactose) and is also present as a variable branch substituent of the O-polysaccharide. As expected, it was found in variable amounts and, on the basis of the methylation analyses (see below), corresponded to between 0 and 30 % substitution of the galactose units of the O-polysaccharide.

The antigenic factor 5 is absent in *S. bredeney* but present in *S. typhimurium* where it is based on an alkali-labile group at O-2 in abequose (Kotelko *et al.*, 1961). We found evidence (see Methods) of O-acetyl substituents in all three strains of *S. typhimurium* but not in the two *S. bredeney* strains. Neither could we find any alkali-labile groups (DeBelder & Norrman, 1968) in the *S. bredeney* strains.

The structure of the O-polysaccharides was studied by methylation analysis. The molar percentages of the different methyl ethers of the 27⁺ LPS are given in Table 3, together with those obtained from the 27⁻ LPS. From the mass spectra and T values, most peaks could be unambiguously identified. Peak B revealed the presence of both a 6-deoxy-2,3-di-O-methylhexose and a 2,3,4,6-tetra-O-methylhexose. These components were fully separated and identified on a OS138 (polyphenylether) column as 2,3-di-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-glucose.

The major difference in the analyses of 27⁻ and 27⁺ LPS was the presence of peak M in 27⁺ LPS. The retention time and mass spectra of peak M identified it as a derivative of 2,4-di-O-methyl-D-mannose. In the *S. bredeney* SH363(P27) LPS, this peak corresponded to 90 % of the total mannose, the remainder being divided between 4,6-di-O-methyl-D-mannose (peak H) and 2,4,6-tri-O-methyl-D-mannose (peak E). In analysis of the 27⁻ LPS, no 2,4-di-O-methyl-D-mannose was found; the mannose was here found as 4,6-di-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-mannose. It can be assumed that in the 27⁺ LPS the 2,4-di-O-methyl-D-mannose is derived from mannose in the phage 27-converted galactose-(1→6)-mannose linkage between the repeating units. The 4,6-di-O-methyl-D-mannose represents the non-converted 27⁻ galactose-(1→2)-mannose linkage, and 2,4,6-tri-O-methyl-

Table 3. Molar percentages of methyl ethers* from the hydrolysates of methylated LPS of 27⁻ *S. bredeney* and *S. typhimurium* parent strains and their 27⁺-converted lysogenic derivatives

Component	Derived from†	Peak	T‡	<i>S. bredeney</i>			<i>S. typhimurium</i>		
				27 ⁻ SH363	27 ⁺ SH363(P27)	SH2183(P22)	27 ⁻ SH2183(P22)	27 ⁺ SH2183(P22,P27)	27 ⁺ SH5110(P27)
2,4-Di- <i>O</i> -methylabequose	O-PS	A	0.32	11.9	14.0		22.5	10.0	10.3
2,3-Di- <i>O</i> -methyl-L-rhamnose	O-PS	B	0.98	22.0	22.0		22.0	22.0	22.0
2,3,4,6-Tetra- <i>O</i> -methyl-D-glucose	O-PS	B	1.00	5.0	5.2		5.1	2.4	1.1
2,3,4,6-Tetra- <i>O</i> -methyl-D-galactose	core	C	1.25	3.0	1.7		2.3	3.0	5.1
2,4,6-Tri- <i>O</i> -methyl-D-glucose	core	D	1.98	ND	ND		1.5	ND	ND
2,4,6-Tri- <i>O</i> -methyl-D-mannose	O-PS	E	2.08	1.5	2.0		6.3	3.6	7.5
2,4,6-Tri- <i>O</i> -methyl-D-galactose	O-PS	F	2.29	15.0	19.7		15.7	21.6	22.3
3,4,6-Tri- <i>O</i> -methyl-D-galactose	core	G	2.50	1.1	1.7		6.7	4.4	NE
4,6-Di- <i>O</i> -methyl-D-mannose	O-PS	H	3.30	23.1	0.5		18.1	12.6	6.2
2,6-Di- <i>O</i> -methyl-D-galactose	O-PS	J	3.65	ND	ND		2.3	0.3	ND
3,6-Di- <i>O</i> -methyl-D-glucose	core	K	4.30	1.5	1.6		2.3	2.5	3.1
2,4-Di- <i>O</i> -methyl-D-glucose	core	L	5.10	2.7	1.7		4.2	2.4	4.7
2,4-Di- <i>O</i> -methyl-D-mannose	O-PS	M	5.30	ND	22.0		ND	5.7	5.1
2,4-Di- <i>O</i> -methyl-D-galactose	O-PS	N	6.35	4.6	4.5		5.1	ND	ND

ND, Not detected; NE, not estimated.

* The results are normalized to give the same molar percentages for the L-rhamnose derivatives.

† For definitions, see Table 2.

‡ Retention times, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (approximately 20 min), for the corresponding alditol acetates on ECNSS-M (180 cm) at 170 °C with a carrier gas flow rate of 25 ml min⁻¹.

Table 4. Adsorption rate constants of the P22 and P27 bacteriophages to parent and converted *S. bredeney* and *S. typhimurium* strains

Strain	10 ¹¹ × Adsorption rate constant (ml min ⁻¹)	
	P22	P27
<i>S. bredeney</i> SH363	402	260
<i>S. bredeney</i> SH363(P27)	< 5	< 5
<i>S. typhimurium</i> LT2	297	ND
<i>S. typhimurium</i> SH2183(P22)	41	312
<i>S. typhimurium</i> SH2183(P22,P27)	52	228

ND, Not determined.

D-mannose the methyl ether which originates from the mannose residue which terminates each O side-chain (see Fig. 1). In the 27⁺-converted *S. typhimurium* strains, we likewise found 2,4-di-O-methyl-D-mannose which was absent from the corresponding 27⁻ LPS.

The relative amount of 2,4,6-tri-O-methyl-D-mannose compared with total mannose allowed us to calculate the average number of repeating units per O-polysaccharide chain. These are 16 and 12, respectively, for the 27⁻ and 27⁺ *S. bredeney* strains, 4 for the 27⁻ *S. typhimurium*, and 6 and 3, respectively, for its two 27⁺ derivatives. The distribution of 4,6-di-O-methyl-D-mannose and 2,4-di-O-methyl-D-mannose shows that in the 27⁺ *S. bredeney* the conversion is almost complete: 98 % of the linkages between repeating units are of the 27⁺ type [galactose-(1→6)-mannose]. The situation is quite different in the two 27⁺ *S. typhimurium* strains where only 31 to 45 % of the linkages are of this type.

Some other differences were seen between the LPS preparations. 2,4,6-Tri-O-methyl-D-glucose (peak D) was found only in the 27⁻ *S. typhimurium* strain and may derive from an incomplete core (Hellerqvist & Lindberg, 1971). 2,6-Di-O-methyl-D-galactose (the minor peak J) originates from the galactose residue in the O-repeating unit which carries D-glucose linked to C-4, determining O-12₂ specificity (Tinelli & Staub, 1960). This peak was not found in the two *S. bredeney* strains, consistent with the reported absence of antigen 12₂ in *S. bredeney*. In the 27⁻ *S. typhimurium* it represented 10 % of the repeating unit galactosyl residues, much less in the 27⁺ strain SH2183(P22,P27), and was absent from the other 27⁺ derivative SH5110(P27). Finally peak N was present in all the LPS samples except the two from the P27-converted *S. typhimurium* strains. It corresponds to O-antigen 1, which is thus present on the average on every fourth or fifth repeating unit; antigen 1 was detected by agglutination in the three strains whose LPS yielded peak D.

Effect of phage P27 conversion on phage attachment

The smooth-specific P22 phage lyses only *Salmonella* strains that have O-antigen 12, i.e. serogroups A, B and D (Zinder & Lederberg, 1952). Common to these bacteria is a galactose-(1→2)-α-mannose linkage between the repeating units (Hellerqvist *et al.*, 1971*a, b, c*). Furthermore, the O-chain must consist of more than one repeating unit (Naide *et al.*, 1965). The entire P22 receptor is found in the extracted LPS (Lindberg & Hellerqvist, 1971). Phage P27 also attacks only smooth strains of serogroups A, B and D which have the O-antigen 12. However, its attachment site or receptor has not been studied. The change of linkage between the repeating units in the 27⁺-converted *S. bredeney* strain abolished the attachment of both phages P22 and P27 (Table 4). The *S. typhimurium* SH2183(P22) strain, lysogenic for P22, adsorbs this phage with a much reduced efficiency. The P27 conversion did not affect the P22 attachment rate in a measurable way while the P27 attachment rate was slightly decreased. This result probably reflects the fact that only every third linkage had been converted by phage P27.

DISCUSSION

The earlier observation that the linkage galactose-(1→6)-mannose corresponds to 27⁺ appears to be correct. The physiological repeating unit in biosynthesis is abequose→mannose→rhamnose→galactose (Nikaido, 1965; Nikaido & Nikaido, 1965), and so the action of P27 concerns the linkage between repeating units, which is changed from (1→2) to (1→6). The conversion was the same in both the naturally occurring 27⁺ *S. bredeney* and in 27⁺ *S. typhimurium* which is never found in nature. The P27-converted *S. bredeney* was completely stable. Calculation of the number of converted linkages (2,4-di-*O*-methyl-D-mannose/4,6-di-*O*-methyl-D-mannose) showed that only 2 % of the linkages were galactose-(1→2)-mannose instead of galactose-(1→6)-mannose.

In contrast, P27 lysogenization of *S. typhimurium* never resulted in stable 27⁺ strains. In the two 27⁺ *S. typhimurium* derivatives studied here, only 71 to 85 % of the colonies were 27⁺ although the cultures had been grown from a single 27⁺ colony. This represents form variation of the antigen 27; the P27 phage was still found in the supernatants. In the corresponding LPS preparations the number of converted galactose-(1→6)-mannose linkages was 31 to 45 %, respectively, while the remaining 69 to 55 % were still of the parental galactose-(1→2)-mannose type. Therefore many 27⁺ bacteria probably contained LPS with both types of linkages in their O-chains.

Salmonella bredeney has antigen 1 which is not form variable. In the O-chains of both 27⁺ and 27⁻ strains every fourth or fifth galactose residue was substituted at C-6 by glucose which determines O-antigen 1 specificity (Stocker *et al.*, 1960). Thus phage P27 does not interfere with the glucosylation of the repeating unit suggesting that this type of glucosylation can take place irrespective of the neighbouring galactose-mannose linkage and certainly when it is either (1→2) or (1→6).

On the other hand, the antigen 1 in *S. typhimurium* is determined by phage P22 (Stocker *et al.*, 1960) and is form variable. The 27⁻ strain was lysogenic for P22 and therefore inherently capable of antigen 1 synthesis; correspondingly, in its LPS every fourth galactose of the O-repeating units was substituted by glucose at C-6.

The change of the galactose-(1→2)-mannose linkage to galactose-(1→6)-mannose between the repeating units in the O-polysaccharide chains of the 27⁺ bacteria alters the stereochemistry and probably the biological properties of the LPS. A change is observed in its antigenic properties as appearance of a new antigen factor 27. In strains where the conversion to the new (1→6) linkage is nearly complete, as in *S. bredeney*, it is very probable that some antigenic features associated with the (1→2) linkage are lost. This has, however, never been specifically tested.

The altered LPS structure also alters the reactivity of the LPS as phage receptor. Both P22 and P27 attach to the O-chains of the LPS in serogroups A, B and D, a reaction that can be measured as inactivation of free phage (Lindberg & Hellerqvist, 1971). Thus the conversion of the linkage between the repeating units in *S. bredeney* resulted in abolition of the attachment of both P22 and P27. In 27⁺ *S. typhimurium*, where only every third linkage was converted, the effect was less dramatic and both P22 and P27 were found to attach. Only the adsorption of P27 was reduced compared with the 27⁻ parent strain; the adsorption of P22 was low in both strains due to P22 lysogeny.

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