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Influence of O Side Chains on the Attachment of the Felix O-1 Bacteriophage to Salmonella Bacteria

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The adsorption rate constant (ARC) of the Felix O-1 (FO) bacteriophage to sensitive *Salmonella* strains was used to determine the effect of variations in surface antigens on phage attachment. The *N*-acetylglucosamine of the common-core polysaccharide of the *Salmonella* lipopolysaccharide (LPS) was found to be an essential part of the receptor for the FO phage in conformation with earlier reports. It was found that (i) the ARC was low for strains having O side chains containing two or three non core monosaccharides, (ii) the ARC varied when the O side chain contained no, or only one, noncore monosaccharide, (iii) the ARC was high when the O side chain contained only one repeating unit, and (iv) the ARC was high to mutants of chemotype Ra in which the *N*-acetylglucosamine was the terminal sugar of the LPS. Since a good correlation was found between the ARC of the FO phage and the phage-inactivating capacity of phenol water-extracted LPS, the results suggest that only the structure and composition of the LPS determines the adsorption rate of the FO phage. The phage-inactivating capacity of LPS from the Ra mutants increased in parallel with higher glucosamine contents in the core polysaccharide. In smooth strains having long and numerous O side chains, the access of the FO phage to its receptor is probably blocked by the presence of the side chains, whereas short and numerous side chains or T1 side chains do not interfere with the FO attachment.

Sensitivity of bacteria to bacteriophage infection depends primarily on the presence of specific receptor sites on the surface of the cells. Burnet (2) observed that rough mutants of certain types of *Salmonella* were resistant to some phages lysing the smooth wild-type strain. Sensitivity to these phages was shown to depend on the presence of the specific O antigens which were not formed in the rough mutants. Other phages lysed both smooth and rough forms of the bacteria, indicating that the surface of the rough mutant also may harbor phage receptors. Levine and Frisch (13, 14, 15) and Burnet (3) demonstrated that cell extracts containing the O antigens showed phage-inhibiting activity.

The lipopolysaccharide (LPS) molecule of *Salmonella* consists of a lipid part (lipid A) covalently linked to a polysaccharide. This is composed of a core polysaccharide, to which an O antigenic side chain is linked. The O side chain consists of up to 30 repeating units. When the enzyme which polymerizes the repeating units is missing, only one repeating unit is transferred to the core, resulting in a semirough (SR) strain (18). T1 strains are devoid of O specificity and the side chains contain galactose and ribose (19). By means of sexual recombination, *Salmonella* strains with both O and T1 specificities can be produced. These hybrid strains have an LPS comprising a common-core polysaccharide to which are attached two sorts of side chains, one with O specificity and the other with T1 specificity.

It was shown earlier that the presence of *N*-acetyl glucosamine in the common polysaccharide core of the *Salmonella minnesota* LPS was essential for the attachment and inactivation of the Felix 0-1 (FO) phage (16). This amino sugar forms an endgroup in the core (H. Nikaido, *unpublished data;* C. G. Hellerqvist, *unpublished data*), which means that it is accessible to the phage also in smooth strains (Fig. 1). Preliminary experiments showed that the adsorption rate of the FO phage to its smooth propagating strain, *S. paratyphi* B, and to smooth strains of *S. typhimurium* was exceedingly low. Rough mutants of chemotype Ra, with a terminal *N*-acetylglucosamine (Fig. 1), adsorbed the phage at high rates. Studies on the composition and structure

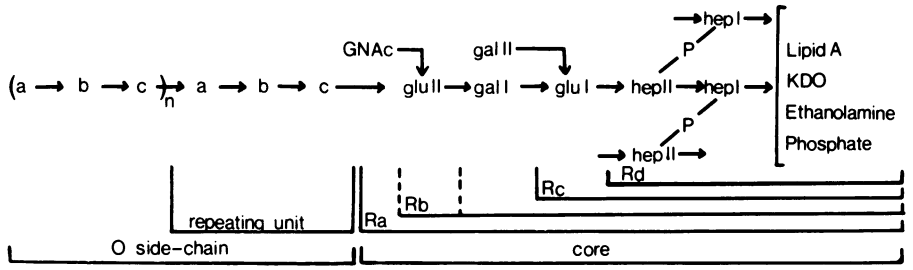

FIG. 1. *Proposed chemical structure for the lipopolysaccharide of Salmonella strains. Abbreviations: a, b, and c, monosaccharides of the repeating unit of the O side chain; glu, glucose; gal, galactose; GNAc, N-acetyl-glucosamine; hep, heptose; KDO, 2-keto-3-deoxyoctonate; P, phosphate. The structure is from Lüderitz, Jann, and Wheat (17) and Nikaido (unpublished data).*

of the O side chains of various smooth *Salmonella* strains have shown that they vary in nature and complexity (17). The object of this investigation was to study the influence of the O side chains on the attachment of the FO phage to whole bacteria and the phage-inactivating capacity of isolated LPS.

MATERIALS AND METHODS

Individual bacterial strains are listed in Table 1. The nonsmooth mutants of *S. typhimurium* 395 MS have been described (9). *S. typhimurium* strain LT2 mutants TV160, TV161, TV163, and TV225 were obtained from B. A. D. Stocker, Department of Microbiology, Stanford University. *S. minnesota* S99 and the mutant strains R60 and R345 were obtained from O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. *S. paratyphi* B T1 strain SH 1308 and the hybrid *S. paratyphi* B T1, 4, 5, and 12 strain SH 1152/3, as well as the SR mutants *S. typhimurium* SH 810 and SH 811, were obtained from M. Sarvas, Department of Bacteriology and Serology, University of Helsinki, Finland. The different *Salmonella* IS strains were obtained from the National Salmonella Center, Statens Bakteriologiska Laboratorium, Stockholm. Strains were tested for their sensitivity to the FO phage [10^8 plaque-forming units (PFU)/ml] by spot test (10).

FO phage (4) was propagated on *S. paratyphi* B76 by means of the soft-agar layer technique (1). Crude lysate, containing 2×10^{11} PFU per ml, was stored at +4 C and used as a stock.

For determination of the adsorption rate constant (ARC), bacteria were grown in nutrient broth (Difco), containing 0.15 M NaCl, at 37 C to a concentration of $10^8 \times$ to 3×10^8 bacteria/ml, determined by viable count. Potassium cyanide was added to a concentration of 0.001 M to arrest bacterial growth. After standing for some minutes, the FO phage, diluted in salt-containing nutrient broth, was added to give multiplicities of infection of about 110^{-3} phage per bacterium. At various times, samples were withdrawn for measurement of unattached phage and the number of infected bacteria.

For measurement of free phage, 1.0-ml samples were centrifuged at 3,000 × *g* for 1 min., 0.1 ml was withdrawn from the supernatant fluid and assayed for unattached phage by means of the soft-agar layer method, with *S. paratyphi* B76 as the indicator strain. Unattached phage was also measured by dilution of the adsorption mixture into nutrient broth containing a few drops of chloroform, which destroys infective centers.

Measurements of phages causing infection were done according to the method of Hershey and Davidson (8). They used antiphage serum to neutralize unadsorbed phage when the fraction of adsorbed phages was small. Anti-FO serum with a K value of 890 was adsorbed with *S. paratyphi* B 76, so that no agglutination was demonstrated when the serum was diluted 1:10. A 0.1-ml amount of the adsorption mixture was diluted into 0.9 ml of anti-FO serum (diluted 1:100) and kept at 37 C for 16 min. The suspension of bacteria was then titrated to determine the number of cells yielding phage. At low multiplicities, this procedure measures the number of adsorbed phages which have caused infection of the bacteria.

Phage adsorption follows the kinetics of a first-order reaction (1, 20). The ARC was determined, up to 90% inactivation, according to the equation $dP/dt = KNP$, where P is the number of unattached phage particles after a time (t), N is the concentration of bacterial cells in the adsorption mixture, and K is the ARC. ARC values given have to be multiplied by 10^{-11} ml/min.

The LPS were extracted from whole cells by the phenol-water method of Westphal, Lüderitz, and Bister (23) and were then purified as described by Lindberg (16).

The phage-inhibiting capacity of the phenol-water-extracted LPS was measured according to the method of Lindberg (16). Activity was expressed as PhI_{50}, the concentration of LPS (micrograms of sugar per milliliter) required to inactivate 50% of the FO phages under experimental conditions.

Hexosamine was estimated according to the method of Gatt and Berman (5) on polysaccharides obtained by acid hydrolysis in acetic acid (22). Glucosamine and galactosamine were identified by paper chromatography according to the method of Hais and Macek by the use of a solvent of *n*-butanol-pyridin-water (6:4:3). The anthrone method (24) was used for the determinations of the carbohydrate content of the LPS preparations, with glucose as the standard.

RESULTS

Role of cations for the attachment of the FO phage. Since the attachment of a bacteriophage to a bacterial strain is dependent on the immediate ionic environment, the slow adsorption of the FO phage to its propagating strains in nutrient broth could be due to an unfavorable medium. Therefore, the rate of adsorption of the FO phage to the rough mutant *S. paratyphi* BR140 was studied by using various concentrations of NaCl and

TABLE 1. *Correlation among FO bacteriophage adsorption rate constants (ARC), phage-inhibiting activity of phenol-water-extracted lipopolysaccharide, and polysaccharide content of N-acetylglucosamine*[a]

Bacterium	Strain	Serogroup	Chemotype or noncore sugars in O side chain	Sensitivity to FO in spot test	ARC (k × 10^{-11} ml/min)	PhI_{50} (μg/ml)	GNAc per cent of polysaccharide
Salmonella typhimurium	395 MR10		Rd	−	<5	>100	1.3
S. typhimurium	395 MR9		Rc	−	<5	>100	0.7
S. typhimurium	395 MR1		Rb	−	<5	1.70	1.4
S. typhimurium	395 MR6		Rb	−	<5	21.0	1.0
S. typhimurium	395 MR8		Rb	−	<5	2.50	0.8
S. typhimurium	TV 160	RI	Rb	−	<5	24.5	1.1
S. typhimurium	TV 161	RI	Rb	−	<5	14.0	0.6
S. minnesota	R 345	RI	Rb	−	<5	>100	
S. minnesota	R 60	RII	Ra	+	411	0.002	7.8
S. typhimurium	395 MR0	RII	Ra	+	213	0.004	16.7
S. typhimurium	395 MR3	RII	Ra	+	290	0.08	4.8
S. typhimurium	TV 163	RII	Ra	+	228	0.13	3.3
S. typhimurium	TV 225	RII	Ra	+	160	0.21	2.7
S. paratyphi B	R 140	RII	Ra	+	220		
S. paratyphi B	T1, SH1308	T1	Rib, Gal	+	457		
S. paratyphi B	SH1152/3	T1/B	Rib, Gal, Abe, Man, Rha	+	69		
S. typhimurium	SH810	SR	Abe, Man, Rha	+	210		
S. typhimurium	SH811	SR	Abe, Man, Rha	+	302		
S. kirkee	IS97	J		+	391		
S. quinhon	IS983	X		+	<5	12	27.2
S. kaolack	IS786	X		+	297		
S. dahlem	IS772	Y		+	20		
S. djakarta	IS694	Y		+	410	0.16	9.3
S. minnesota	S99	L	GalNAc	+	42	0.29	
S. bareilly	IS44	C1	Man	+	<5	3.50	14.1
S. deversoir	IS521	W	Fuc	+	230		
S. weslaco	IS247	T	Rha	+	310	0.01	30.9
S. waycross	IS237	S	Rha	+	65		
S. riogrande	IS231	R	Man, GalNAc	+	<5		
S. poona	IS91	G1	Fuc, GalNAc	+	8		
S. dakar	IS652	M	Rha, GalNAc	+	22		
S. telaviv	IS106	M	Rib, GalNAc	+	11		
S. greenside	IS842	Z	Col, GalNAc	+	25		
S. anatum	IS78	E1	Man, Rha	+	13		
S. vejle	IS119	E1	Man, Rha	+	10		
S. champaign	IS169	Q	Man, Fuc, GalNAc	+	<5		
S. paratyphi B	76	B	Abe, Man, Rha	+	<5	2.50	6.0
S. typhimurium	IS9	B	Abe, Man, Rha	+	<5		
S. typhimurium	395MS	B	Abe, Man, Rha	+	<5	20.8	3.6
S. paratyphi A	IS1	A	Par, Man, Rha	+	30	0.38	10.8
S. paratyphi A	IS256	A	Par, Man, Rha	+	27		
S. enteritidis	IS64	D1	Tyv, Man, Rha	+	10	1.8	4.8
S. sendai	IS71	D1	Tyv, Man, Rha	+	31		

[a] Nomenclature of the serotypes is from Kauffman (11). Abbreviations: GNAc, *N*-acetylglucosamine; Rib, ribose; Gal, galactose; Abe, abequose; Man, mannose; Rha, rhamnose; GalNAc, *N*-acetylgalactosamine; Fuc, fucose; Col, colitose; Par, paratose; Tyv, tyvelose.

$CaCl_2$ in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-buffered solutions (*p*H 7.2) at 37 C. First-order velocity constants were used to characterize the adsorption rates as the ionic composition of the medium was changed (Fig. 2).

A maximal attachment to the rough mutant *S. paratyphi* B R140 was observed at concentrations of approximately 0.1 M for monovalent and 0.05 M for divalent cations in addition to what was present in the 0.01 M Tris-hydrochloride buffer. However, no adsorption (ARC <5) was demonstrable by using only the buffer. The adsorption rate was depressed markedly with higher or lower salt concentrations. With *S. paratyphi* B76, the propagating strain, the value of the ARC was always <5 with any of the salt concentrations tested. Using the technique of Hershey and Davidson (8), in which unadsorbed phages were neutralized by antiphage serum, it could be shown that 4% of the FO phages are adsorbed and produce infection after 16 min. This corresponds to an ARC of 1.5. No difference in the values of the ARC were observed when the multiplicity of infection was varied between 0.001 and 1.

Since the maximal adsorption rate was the same in buffers with optimal concentrations of salt and in nutrient broth with 0.15 M NaCl, no specific cofactors appeared to be required for the adsorption of the FO phage. The following determinations of the adsorption velocities were performed in salt-supplemented nutrient broth.

Attachment of the FO phage to various Salmonella strains. In spot test, all strains except those of chemotypes Rb, Rc, and Rd were lysed by the FO phage. The ARC, determined by measuring free phage in the supernatant fluid, are represented in Table 1 and Fig. 3. Mutants of the chemotypes Rb, Rc, and Rd all had an ARC <5, apparently because they lack the receptor structure for the FO phage. The mutants of chemotype Ra, the T1 and SR strains, all show an ARC above 160. The hybrid strain and strains belonging to chemotype I or having only one noncore monosaccharide in their repeating units display a variation in their ARC between <5 and 410. Strains of chemotype I have smooth colonies but the chemical analysis of the LPS shows no presence of noncore sugars (12). This probably means that the O side chain consists of the core monosaccharide constituents, glucose, galactose, and *N*-acetylglucosamine. Strains having two or three noncore sugars in their repeating units show low ARC; the highest value recorded was 31.

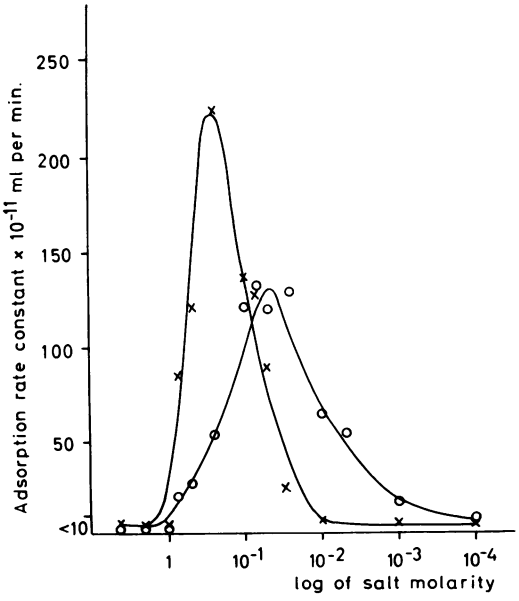

FIG. 2. *ARC of the FO phage to S. paratyphi BR140 in various concentrations of NaCl and $CaCl_2$ in 0.01 M Tris-hydrochloride buffer (pH 7.2) at 37 C. Symbols: ×, NaCl; ○, $CaCl_2$. The scale refers to additional cations beyond what was present in the buffer.*

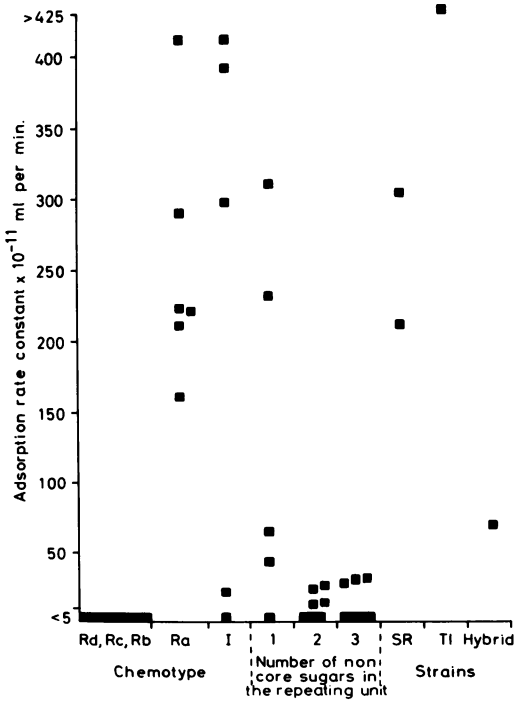

FIG. 3. *FO ARC of the Salmonella strains investigated. Figures represent the mean of two experiments. Figures on the abscissa represent the number of noncore sugars in the O side chain (12).*

The determination of unattached phage remaining in the supernatant fluid after centrifugation of the phage-bacterium mixture is not very sensitive when the fraction of phage which has adsorbed is small. Some attachment, however, must occur to all strains which were lysed in spot test. By counting the number of phages causing infection, it could be shown that the actual ARC for *S. typhimurium* 395 MS and *S. bareilly* IS 44 are 0.6 and 2.5, respectively.

Inhibition of the FO phage by extracted LPS. Of the *Salmonella* strains for which the kinetics of the FO phage attachment have been assayed, 22 were chosen for a further study. The strains represent different chemotypes. The LPS were extracted by the phenol-water method and assayed for FO phage-inactivating capacity in nutrient broth supplemented with 0.15 M NaCl, expressed as PhI_{50}. In Fig. 4, the experimental data for four of the PhI_{50}-determinations are presented. The highest FO phage-inactivating capacity was obtained with LPS isolated from the Ra mutants *S. minnesota* R60 and *S. typhimurium* 395 MRO, with PhI_{50} values of 0.002 and 0.004 μg/ml, respectively. The LPS from other Ra mutants, 395 MR3, TV163, and TV225, had PhI_{50} values between 0.08 and 0.21 μg of LPS/ml. A high phage-inactivating capacity was noted also for LPS extracted from the smooth strains *S. minnesota* S99 (0.29 μg/ml), *S. djakarta* IS694 (0.16 μg/ml), *S. weslaco* IS247 (0.01 μg/ml), and *S. paratyphi* A IS1 (0.38 μg/ml). The concentrations of LPS needed for PhI_{50} for the other strains were >1.7 μg/ml.

Correlations between the FO phage ARC, the LPS-inhibiting activity, and the glucosamine content of the polysaccharides. All strains of chemotype Ra showed high ARC, and the LPS displayed a high phage-inactivating capacity. The glucosamine values (*S. typhimurium* 395 MRO, 395 MR3, TV163, and TV225) were higher than those from chemotype Rb; the concentration varied between 2.7 and 16.7%. There was a good correlation between a high phage-inactivating capacity of the LPS and a high glucosamine content of the polysaccharide. Most of the amino sugar in the lipid-free polysaccharide of *S. minnesota* R60 was shown by paper chromatography to be *N*-acetyl-glucosamine.

Mutants of chemotype Rb, Rc, and Rd, which were not lysed by the FO phage in spot test, did not adsorb the phage (Table 1, Fig. 3). Some of the Rb strains yielded LPS which showed phage inhibition activity, although the concentrations of LPS needed were high. All these mutants, except *S. minnesota* R 345, had very low glucosamine contents in their cell wall polysaccharides. *S. minnesota* R 345 contains 13.8% amino sugar in the polysaccharide, but it could be shown that most of the amino sugar was derived from *N*-acetylgalactosamine, which is a constituent of the repeating unit of the smooth parent strain *S. minnesota* S99 (12).

The two strains of chemotype I behave differently. *S. quinhon* had a low ARC and yielded an LPS with a low phage-inhibiting activity, although the percentage of glucosamine in the polysaccharide was high (27.1%). *S. djakarta*, on the other hand, had a high ARC and yielded an LPS

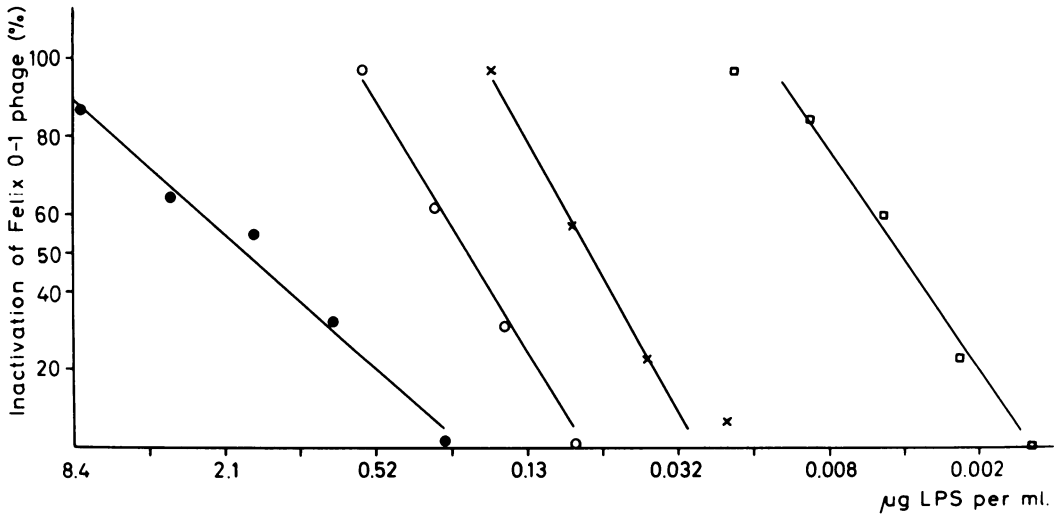

FIG. 4. *FO phage-inactivating capacity of phenol-water-extracted lipopolysaccharides. Symbols:* □, *S. typhimurium 395 MRO;* ×, *S. typhimurium 395 MR3;* ○, *S. typhimurium TV 225;* ●, *S. enteritidis IS 64.*

with a high FO phage-inactivating capacity; glucosamine represents 9.3% of the polysaccharide.

Two of the strains with one noncore monosaccharide in the repeating unit were studied. The polysaccharide of *S. bareilly* contains 14.1% glucosamine, but the ARC and the phage-inactivating capacity of the LPS were low. *S. weslaco* contains a polysaccharide with 30.9% glucosamine and had a high ARC and a high phage-inactivating capacity.

The three strains with a repeating unit containing three noncore monosaccharides had ARC values between <5 and 31. The concentrations of LPS needed for inhibition of the phage varied between 0.38 and 2.5 μg/ml, and the glucosamine content varied from 4.8 to 10.8%.

DISCUSSION

The ARC for the FO phage appears to be directly related to the accessibility of the receptors on the bacterial surface. A comparison of the ARC from the strains studied shows that great differences exist. Rough mutants of chemotype Ra have ARC values >160, whereas the ARC in the 15 smooth *Salmonella* strains with O side chains containing two or three noncore monosaccharides did not exceed 31 (Table 1). The reason for this difference can be looked for in the structural arrangement of the LPS, since it has been shown that the entire FO receptor is located in the LPS (16). In rough mutants of chemotype Ra, the *N*-acetylglucosamine is at the end of the polysaccharide chain (Fig. 1), and no other sugar groups interfere with the attachment of the phage. Two strains, SH 810 and SH 811, in which the core has been capped by only one repeating unit (19), had ARC values of 210 and 302, respectively. Thus, the introduction of one repeating unit outside the receptor area does not interfere with the attachment of the FO phage. Since the semirough strains showed no serological cross-reaction with the mutants of chemotype Ra, it is probable that the core stubs of the semirough strains were more efficiently capped by O side chains than were those of smooth strains in which serological cross-reactivity with Ra mutants was often found (O. Lüderitz, *personal communication*). Thus, interference by direct covering of the FO receptor with the O side-chain seems less probable. On the other hand, the linking of an O side chain with an average of 11 repeating units, as in *S. typhimurium* 395MS (7), greatly reduced the accessibility of FO receptors (ARC 2.5). Although the *N*-acetylglucosamine is terminal also in this strain, it is situated deeply within the LPS molecule. (The O side chain of *S. typhimurium* 395MS has been estimated to extend approximately 15 nm from the complete core.) It is probable that the O side chains interfere with the attachment of the FO phage by steric hindrance. Observations on the attachment process of the phages T2 and T4 to *Escherichia coli* (21) suggested that the phages attached by the distal ends of the long tail fibers. The second major step appeared to be a repositioning of the phage so that the short tail pins of the base plate were brought into close proximity to the cell wall. It seems probable that the presence of the O side chains could interfere with both of these steps.

Of the 15 strains with O side chains containing two or three noncore monosaccharides, 7 have ARC values which are demonstrable by measuring unattached phage. This fact would mean that the steric hindrance should be less pronounced for these strains than for *S. typhimurium* 395MS. Two explanations may account for this reduced steric hindrance: either the O side-chains are short and numerous or they are relatively few and long. Studies on *S. paratyphi* A IS1, which has an ARC of 30, shows the LPS structure to have few and long O side chains (*unpublished data*), leaving more core stubs uncapped and, consequently, making it easier for the FO phage to reach its receptor area. In the T1 strain studied, SH 1308, the only side chains present are of the type T1, containing galactose and ribose. The T1 side chain apparently does not interfere at all with the attachment of the FO phage since the ARC is the same as that for the rough mutants of chemotype Ra.

For most *Salmonella* strains, the structure and length of the O side chain is not known. The subdivision of the smooth strains according to the number of noncore sugars in the O antigenic side chain may be misleading since the core sugars glucose, galactose, and *N*-acetylglucosamine may be constituents of the O repeating unit as well. It appears, however, that the strains with two or three noncore sugars mostly have rather numerous and long side chains. The low ARC displayed by some of the strains with no, or only one, noncore sugar might be explained by the presence of O side chains completely or to a large extent built up by core sugars.

It could be assumed that the hydrophilic character of the O side chain would be a contributory cause to the low adsorption rate of the FO phage to many of the smooth strains. The addition of cations to the adsorption medium did not, however, increase the ARC of the FO phage to its smooth propagating strain *S. paratyphi* B76. This fact makes it more probable that steric hindrance is the major cause for the interference with phage attachment to the smooth strains.

The FO phage-inactivating capacity of phenol-water-extracted LPS showed a good agreement

with the adsorption rate determinations to whole bacteria; strains having high ARC values yielded an LPS with a high phage-inactivating capacity and strains having low ARC values yielded an LPS with a low phage-inactivating capacity. This direct correlation indicates that the phenol-water extraction does not affect the FO receptor structure in the LPS and that the LPS is the only cell surface structure which exerts any significant influence on the FO phage attachment process. As a consequence, determination of the attachment rate to whole bacteria may be used as a measuring criterion for studies on the FO receptor and LPS structure.

Determination of the glucosamine concentration of lipid-free polysaccharide showed that different mutants of chemotype Ra vary considerably in their content of *N*-acetylglucosamine. This variation is in agreement with earlier investigations (22). The fact that a good correlation is found between the content of core *N*-acetylglucosamine and the phage-inactivating capacity in these strains supports the assumption that this amino sugar is an essential part of the receptor for the FO phage. However, the *N*-acetylglucosamine must be present in association with the core for attachment. *S. quinhon* and *S. bareilly* yielded polysaccharides with high contents of glucosamine, but still the ARC values were low as was the phage-inactivating capacity of their LPS. It is probable that the amino sugar is a component also of the O side chain in these strains but inactive as a receptor component in that position.

Rough mutants of the chemotypes Rb, Rc, and Rd were not lysed by the FO phage, and no attachment of the phage to whole bacteria was demonstrated. However, the LPS from some of these strains inactivated the FO phage, although the concentrations needed were high. These mutants contained small amounts of the sugars found in the O side chain of the parent strain (9), suggesting that the mutations leading to roughness probably were incomplete (leaky). These results indicate that few but complete core stubs and O side chains are synthesized, with the concomitant appearance of FO receptors.

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