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Interaction Between Bacteriophage Sf6 and *Shigella flexneri*

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The *Shigella flexneri* phage Sf6 has an isometric head with hexagonal symmetry 53 nm in diameter. The noncontractile tail is 16 nm long and terminates with a base plate containing six spikes. Sf6 is typical of the C phages in the morphological classification of Bradley. Phage Sf6 posesses α-1,3-endorhamnosidase activity as demonstrated by methylation and reducing end group sugar analyses of the products obtained on interaction with the O-polysaccharide chain of *S. flexneri* strains which have the O-group 3,4 antigen. The major end product was an octasaccharide with the following structure: Rha III-GlcNAc-Rha I-Rha II-Rha III-GlcNAc-Rha I-Rha II. Acetylation of 0-2 of rhamnose III of the O-polysaccharide chain, either brought about by Sf6 lysogenization or found in wild-type *S. flexneri* (*3b*) strains, prevented enzymatic hydrolysis. *O*-deacetylation of the polysaccharide chain again made it susceptible to the Sf6 endorhamnosidase.

It has long been recognized that the O-antigen polysaccharide chain of the lipopolysaccharide (LPS) layer of the outer cell membrane of gram-negative bacteria functions as a receptor site for specific adsorption of some bacteriophages (9). More recently, it has been revealed that such phage adsorption is concomitant with an enzymatic hydrolysis of the polysaccharide chain. Tail components of *Salmonella* phages ϵ^{15} and P22 (for review, see 9) have been shown to express endorhamnosidase activities, which cause the hydrolysis of Rha $1\underset{\alpha}{\rightarrow}3$ Gal linkages of the O-polysaccharide receptor. Likewise, coliphage Ω8, which uses *Escherichia coli* polysaccharide chain with O-antigen 8 specificity as receptor, possesses an endorhamnosidase responsible for hydrolysis of the Man $1\underset{\alpha}{\rightarrow}3$ Man linkage between successive repeat units of the the O8 polysaccharide chain (13).

Many phages which require such O-antigen in polysaccharide chains as receptors for adsorption and infection of the host bacterium also are able to cause O-antigen conversion. This event usually means a glycosylation, acetylation, or other structural modification of the O-polysaccharide chain of the host, which leads to the expression of a new O-antigenic specificity (9). Recently, *Shigella* phage Sf6 has been shown to possess the ability to convert the *S. flexneri* group 3,4 antigen to the group 6 antigen, presumably by means of a phage-directed *O*-acetylation of the polysaccharide chain (5, 10). Genetic and adsorption studies also revealed that the O-polysaccharide chain with group 3,4 antigen of *S. flexneri* functioned as the receptor in the adsorption step of Sf6 infection. Moreover, modification of the O-polysaccharide chain affected the efficiency with which Sf6 adsorbs to host cells (5). The structure of the repeating unit of the O-polysaccharide chain of *S. flexneri* varient Y (-: 3,4) has recently been determined (1) (7):

-3)-β-D-GlcNAc*p*-(1-2)-α-L-Rha*p*-(1-2)-
α-L-Rha*p*-(1-3)-α-L-Rha*p*-(1- (1)

The three L-rhamnose residues will be called Rha I, Rha II, and Rha III herein. The structure(s) in the O-polysaccharide chain responsible for O-group 3,4-antigenic specificity is, however, not known. The structural, and thus O-antigenic, variation is provided by adding α-D-glycopyranosyl groups (type antigens I, II, IV, and V and group antigen 7,8) and *O*-acetyl groups (type antigen 6) to various positions of the basic structure 1.

In the present study we show that phage Sf6 causes hydrolysis of the Rha II $1\underset{\alpha}{\rightarrow}3$ Rha III linkage in the *S. flexneri* O-polysaccharide chain with group 3,4-antigenic specificity, and that the presence of an acetyl group of 0-2 of Rha III prevents the enzymatic hydrolysis.

MATERIALS AND METHODS

Bacterial strains. The characteristics of *S. flexneri* serotypes pertinent to this study are summarized in Table 1. *S. flexneri* strain F3, used as the host for phage Sf6, is a natural Y variant which produces only the group 3,4 somatic antigen complex (5). *S. flexneri*

TABLE 1. *Host range and adsorption of phage Sf6*

S. flexneri strain	O-antigen		Lytic sensitivity	Adsorption rate,[a] K (ml/min)	Structure of O-polysaccharide chain	Reference
	Group	Type				
F3 (variant Y)	3,4		+	1.1×10^{-10}	→3 GlcNAc 1→2 Rha I 1→2 Rha II 1→3 Rha III 1→ (α; β; α; α)	8
F3 (Sf6)	3,4,6		−	2.5×10^{-11}	OAc (2) on 3 Rha III 1→	10
17-14-18 (*2a*)	3,4	II	−	2.1×10^{-11}	Glc (α 1,4) on 3 Rha III 1→	B. Lindberg (personal communication)
G127-1-2 hybrid of (17-14-18)	3,4		+	1.2×10^{-10}	As strain F3	
YG127-1-2 (Sf6)	3,4,6		−	2.0×10^{-11}	As strain F3 (Sf6)	
FH-4 (*3a*)	6,7,8	III	−	3.9×10^{-11}	Glc (α 1,3) on 2 Rha I→	B. Lindberg (personal communication)
FH-5 (*4a*)	3,4	IV	−	7.1×10^{-11}	Glc (α 1,6) on →3 GlcNAc 1→	B. Lindberg (personal communication)
FH-7 (*5b*)	7,8	V	−	2.8×10^{-11}	Glc (1,3) on 2 Rha II 1→	7

[a] Adsorption of Sf6. Approximately 2.5×10^8 PFU were added to 2.5×10^9 bacterial cells in Penassay broth at 37°C. Periodically samples were diluted into chilled Penassay broth containing $CHCl_3$, thoroughly mixed, and subsequently assayed in soft agar for unabsorbed phages with *S. flexneri* as the indicator host (5).

F3 (Sf6), a lysogenic, phage-converted derivative of F3, expresses both the O-group 3,4 and O-group 6 antigens of *S. flexneri* (5). In addition, for host range studies we used *S. flexneri* strains which express both the type-specific and the group 3,4 antigens. *S. flexneri* strain Sc 596 (*3b*) was from the strain collection at the National Bacteriological Laboratory, Stockholm. The presence of *S. flexneri* somatic antigens was scored by slide agglutination tests using O-group factor 6, O-group factor, 3,4, and various O-type-specific antisera.

Media. Penassay broth (Difco), brain heart infusion broth (Difco), and Trypticase soy agar (BBL) were used for cultivation of bacteria, as previously described (5).

Phage methods. Procedures for routine propagation and titration of phage Sf6 as well as for measurements of Sf6 adsorption have been described previously (5). Sf6 was purified for electron microscopic examination and for the hydrolysis experiments by means of CsCl density gradient centrifugation. To prepare such gradients, 10 g of CsCl was added to 11 ml of phage lysate. The final density of the suspension was adjusted to 1.50 g/cm^3. The suspensions were centrifuged for 40 h at 44,000 rpm in a Beckman Spinco Ti60 rotor. The gradients were harvested by suction through a needle puncturing the bottom of the tubes and assayed for Sf6 PFU, using *S. flexneri* F3 as the host. Fractions with phage were dialyzed against 0.1 M ammonium acetate (pH 7.0) before preparation for electron microscope examination and against 0.005 M ammonium carbonate buffer before hydrolysis experiments.

LPS preparation. The bacteria were grown overnight in brain heart infusin broth under aeration. After the bacteria were washed twice with distilled water, the cell paste was extracted for LPS, using the phenol-water method (4, 14), and the aqueous phase was lyophilized. LPS was subjected to weak acid hydrolysis in acetic acid (pH 3.1, 100°C for 90 min) (4). Precipitated lipid A was removed by centrifugation at 10,000 × *g* at 4°C for 20 min.

Chemical analysis of poly- and oligosaccharides. Total carbohydrate content was estimated by the phenol-sulfuric acid method (11), with rhamnose as a standard.

The nature of native polysaccharide and hydrolyzed oligomeric material was determined by methylation analysis (1) on borodeuteride-reduced material.

Terminal reducing sugar in oligomeric material was determined by a procedure including reduction of the oligosaccharide with sodium borodeuteride for 16 h. The mixture was brought to pH 5 with 50% aqueous acetic acid, concentrated, and codistilled with methanol (3 × 3 ml) at reduced pressure to remove formed boric acid. The product was hydrolyzed with 0.25 M sulfuric acid (2 ml) for 16 h on boiling water, neutralized with barium carbonate, filtered, and concentrated to dryness. The hydrolysate was heated with hydroxylamine in methanol (2 ml) for 30 min at 60°C, concentrated to dryness, and acetylated with acetic anhydride (0.2 ml) and pyridine (0.2 ml) for 30 min in boiling water. This procedure yields a product in which the terminal reducing sugar in the oligosaccharide becomes an alditol acetate, and the other sugar residues are transformed into aldononitriles.

Sugar derivatives obtained on methylation analysis and terminal reducing-sugar determination were separated and analyzed by gas-liquid chromatography. Separations were performed with a Perkin-Elmer model 990 instrument, equipped with a glass column (200 by 0.3 cm) containing 3% OV-225 on GasChrom Q (100/120 mesh). Peak areas were measured with a Hewlett-Packard 3370-B electronic integrator. For gas-liquid chromatography–mass spectrometry a Perkin-Elmer 270 gas chromatograph-mass spectrometer fitted with an OV-225 SCOT column was used.

Electron microscopy. The phage Sf6 preparation, suspended in 0.1 M ammonium acetate (pH 7.0), was examined soon after preparation in a JEOL 100-C electron microscope. For staining, a small droplet of the phage preparation was placed on the grid (200-mesh copper with carbon-shadowed Parlodion film) and then mixed with a droplet of freshly prepared and filtered 1% phosphotungstic acid. After 10 min, the staining solution was removed by touching the grid to the corner of an absorbent filter paper. The preparations were examined at a magnification of about 50,000. The exact magnification was determined by using a calibration grid made from a carbon grating. The calculation of the phage dimension was made by comparing measurements of the phage particles to measurements of the calibration grid taken at the same magnification.

RESULTS

Host range and adsorption characteristics of phage Sf6. Previous studies have revealed that the O-polysaccharide chain with group 3,4-antigenic specificity in *S. flexneri* functioned, at least in part, as the cell surface receptor site for Sf6 adsorption (5). This conclusion is supported by the findings summarized in Table 1 on the lytic spectrum and adsorption characteristics of Sf6. Of the various serotypes examined, only *S. flexneri* Y strains (with the 3,4 group antigen) appear to be lytically sensitive to Sf6. Adsorption of Sf6 to strains with an O-antigenic specificity additive to 3,4 was also greatly reduced (Table 1).

The additional O-antigenic specificity in strains listed in Table 1 is a consequence of the presence of a substituent on the O-polysaccharide chain. For example, *S. flexneri* serotype *4a* (group antigen 3,4, type antigen IV) is the result of addition of D-glucose α-1,6-linked to *N*-acetyl-D-glucosamine. The finding of a low adsorption rate to strains with additional O-antigenic specificities makes it likely that for adsorption of Sf6 the basic structure 1 of the *S. flexneri* Y strain (group antigen 3,4) is required.

Morphology of Sf6. Electron micrographic examination of the Sf6 phage preparation revealed typical phage particles, consisting of a head portion and short tail, which are uniform in size. Sf6 phage has an isometric head with hexagonal symmetry 53 mm in diameter (Fig. 1). This kind of image indicates that the true shape of the head is probably icosahedral (3). The tail

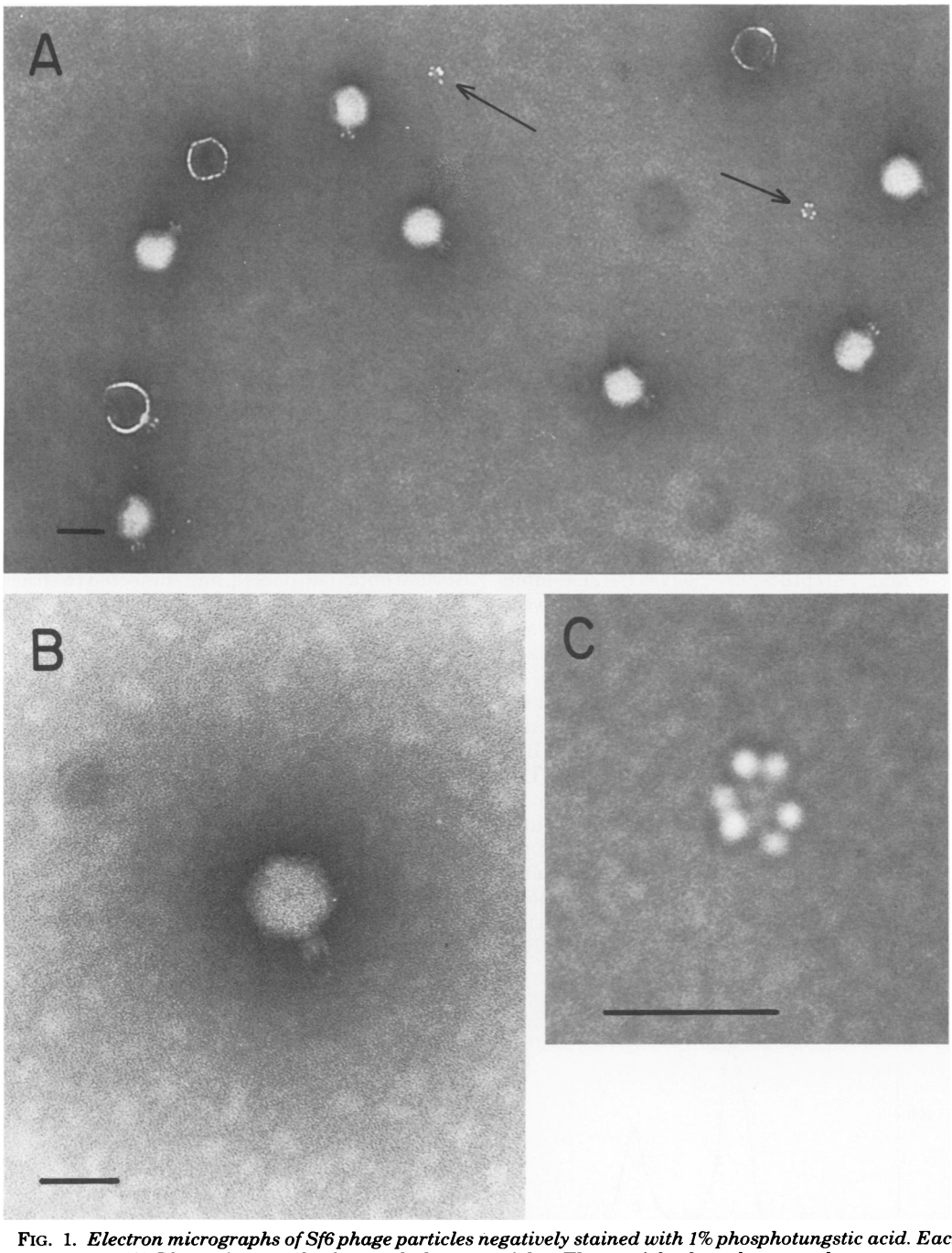

FIG. 1. *Electron micrographs of Sf6 phage particles negatively stained with 1% phosphotungstic acid. Each bar = 50 nm. (A) Photomicrograph of several phage particles. The particles have hexagonal symmetry and short tails composed of tail spikes. The tail spikes are apparently attached to a base plate or tail plate. The arrows point to two tail plates that have broken from the phage tail and are viewed end-on. The tail spikes when viewed end-on appear as clusters of circles. Tail plates observed in this way contain up to six tail spikes. Also observed were some particles that have apparently lost their DNA so that with the negative stain the empty heads appear only as a bright outline of head. (B) A 2.5 × 10^5-fold magnification of an Sf6 phage particle, which clearly shows the hexagonal symmetry. The diameter of the phage is 53 nm. The complete tail, including base plate and tail spikes, is 16 nm long. (C) An enlargement, 6 × 10^5-fold, of the end-on view of one of the base plates observed in (A). Six tail spikes are evident. All the base plates observed had six or fewer tail spikes.*

of the phage, about 16 nm long, terminates with a base plate containing six spikes. No contractile sheath was observed. The appearance of Sf6 phage resembles *Salmonella* phage P22 and is typical of type C phages in the morphological classification of Bradley (2).

Preparation of *S. flexneri* Y F3 O-polysaccharide. Preliminary experiments showed that upon incubation of potassium cyanide-poisoned *S. flexneri* F3 bacteria with CsCl-purified bacteriophage Sf6 (ratio, 10 phages per cell), a dialyzable, carbohydrate-rich product was released. Because it was likely that this material came from the O-polysaccharide chain of the outer cell membrane, further studies were done with LPS extracted from *S. flexneri* F3. Previous experience indicated that phages hydrolyzing the O-polysaccharide chain in the LPS are irreversibly inactivated by the micellar complex of LPS found in aqueous solutions but not by the lipid A-free polysaccharides (6, 9). Therefore, *S. flexneri* F3 LPS was subjected to mild acid hydrolysis in 1% acetic acid (pH 3.1) at 100°C for 90 min. This procedure cleaves the acid-labile 2-keto-3-deoxyoctulosonic acid linkages between the heptose I residue in the core of the polysaccharide chain and the glucosamine residue of lipid A (12). The precipitated lipid A was removed by centrifugation, and the supernatant, after extensive dialysis against distilled water, was lyophilized. Approximately 70% of the LPS was recovered as a polysaccharide fraction. This material (109 mg) was next subjected to gel chromatography on a Sephadex G100 column (Fig. 2). Two peaks were detected using the phenol-sulfuric acid assay for carbohydrates. The fractions under peaks I and II were pooled and contained 35 and 40 mg (dry weight) of carbohydrate, respectively. Sugar analysis showed that peak I contained rhamnose and glucosamine. In peak II glucose, galactose, and heptose were the predominant sugars. Thus, peak I contained sugars which are expected in the O-polysaccharide chain of *S. flexneri* Y strains, whereas peak II contained almost exclusively sugars representative of the core (P. E. Jansson, B. Lindberg, A. A. Lindberg, and R. Wollin, Carbohydr. Res., in press). Only the material in peak I was subjected to further analysis.

Phage Sf6 hydrolysis of *S. flexneri* O-polysaccharides. Purified phage Sf6 and peak I polysaccharide from *S. flexneri* strain Y were incubated at 37°C in 0.005 M carbonate buffer, pH 7.0, at a ratio of 10^8 PFU of Sf6 per mg of polysaccharide. The reaction mixture was contained in a dialysis bag which allowed escape of released oligosaccharides into the dialysis fluid (ammonium carbonate buffer). The dialysis fluid was changed daily for 3 days. The pooled dialysates were concentrated to dryness and fractionated on Bio-Gel P2 (Fig. 3). Two peaks (A and B) were obtained, pooled, and subjected to structural analysis. Since the structure of the *S. flexneri* O-polysaccharide chain is known (7), analysis of *N*-acetyl-D-glucosamine was not necessary.

Methylation analysis of borodeuteride-reduced pool B material yielded (Table 2) 1,3,4,5-tetra-*O*-methyl-rhamnitol, 2,3,4-tri-*O*-methyl-rhamnitol, 3,4-di-*O*-methyl-rhamnitol, and 2,4-di-*O*-methyl-rhamnitol in the proportions 9:14:59:18. (Part of the volatile tetra- and trimethyl-methyl-rhamnitol derivatives are lost during concentration.) The amount of 2,3-di-*O*-methyl-rhamnitol in the pool B fraction was only half of what was found in the native O-polysaccharide. Based upon the known structure

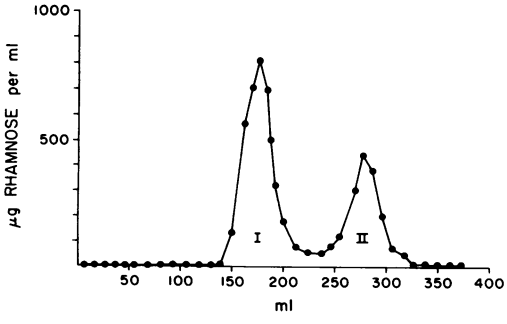

FIG. 2. *Gel chromatography of S. flexneri F3 polysaccharide on Sephadex G100 (column size, 2.5 by 100 cm; bed volume, 350 ml). The column was irrigated with pyridine-acetic acid-buffer (4:10:1,000 ml), pH 5.5, at a flow rate of 48 ml/h. Fraction size, 3.1 ml. Fractions were assayed for carbohydrate content by the phenol-sulfuric acid method.*

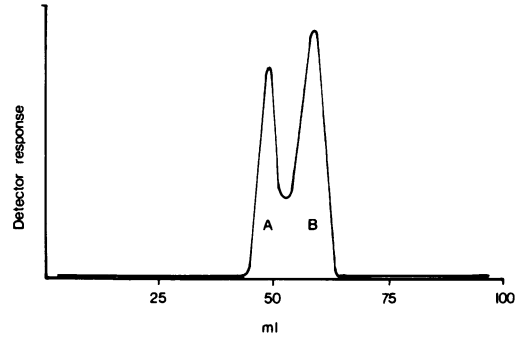

FIG. 3. *Gel chromatography of phage Sf6-hydrolyzed S. flexneri O-polysaccharide on a Bio-Gel P2 (200 to 400 mesh) column (1.6 by 90 cm) eluted with water (10.0 ml/h). Fractions were assayed for refractive index with a differential refractometer R403 (Waters Associates, Milford, Mass.).*

of the *S. flexneri* O-polysaccharide (1), the methylethers of column B in Table 2 indicate that the pool B fraction contains an octasaccharide with the following structure (2):

$$\begin{array}{c}\text{Rha II } 1\xrightarrow{\alpha}3 \text{ GlcNAc } 1\xrightarrow{\beta}2 \text{ Rha I } 1\xrightarrow{\alpha}2\\ \text{Rha II } 1\xrightarrow{\alpha}3 \text{ Rha III } 1\xrightarrow{\alpha}3 \text{ Glc NAc } 1\xrightarrow{\beta}2 \quad (2)\\ \text{Rha I } 1\xrightarrow{\alpha}2 \text{ Rha II}\end{array}$$

That the oligosaccharide contained six L-rhamnose residues, as required for an octasaccharide, was also demonstrated by the sugar analyses, which gave rhamnitol and the aldononitrile of rhamnose in the ratio 21:79. The expected ratio for an octasaccharide is 17:83.

Methylation analysis of borodeuteride-reduced pool A material gave the same methylethers as were found in pool B. The amounts indicated the presence of oligosaccharides larger than the octasaccharide, e.g., dodecasaccharides and higher.

Lysogenization of *S. flexneri* F3 leads to the appearance of the O-group antigen 6; at the same time the lysogenized bacteria become Sf6 resistant, and the adsorption rate is decreased almost 10-fold (Table 1). This infers that the presence of an *O*-acetyl on C2 of the Rha III residue makes the O-polysaccharide less fit as an Sf6 receptor. The hypothesis was tested with *S. flexneri 3b* strain Sc 596, which has the O-group antigens 3,4:6 and type antigen III and thus is serologically indistinguishable from an Sf6-lysogenized *S. flexneri* Y strain. Strain Sc 596 is resistant to phage Sf6. When polysaccharide from strain Sc 596 (prepared as the *S. flexneri* Y polysaccharide) was treated with Sf6, no degradation was seen. After the polysaccharide had been *O*-deacetylated (completely as judged by nuclear magnetic resonance spectroscopy) with 0.25 N NaOH at 25°C for 18 h before the addition of Sf6, degradation was detectable. Methylation analysis and terminal reducing-sugar determination also revealed that an octasaccharide identical to that obtained from strain F3 had been released by phage Sf6 hydrolysis (Table 2, column C).

TABLE 2. *Methylation analysis of original polysaccharide and oligosaccharides derived by hydrolysis with Sf6*[a]

Sugar	T[b]	Analysis of pools		
		A	B	C
1,3,4,5-Rhamnitol[c]	0.30		9	8
2,3,4-Rhamnitol	0.50	3	14	15
3,4-Rhamnitol	0.87	60	59	53
2,4-Rhamnitol	0.94	37	18	24

[a] (A) Original *S. flexneri* Y polysaccharide; (B) obtained on Sf6 hydrolysis of *S. flexneri* Y polysaccharide (peak B); and (C) obtained on Sf6 hydrolysis of deacetylated *S. flexneri 3b* polysaccharide.

[b] Relative retention of the derived alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol.

[c] 1,3,4,5-Rhamnitol = 1,3,4,5-tetra-*O*-methyl-rhamnitol; 2,3,4-rhamnitol = 2,3,4-tri-*O*-methyl-rhamnitol, etc.

DISCUSSION

Phage Sf6 has a narrow lytic spectrum (Table 1). It adsorbs to and forms plaques on *S. flexneri*, which have an O-polysaccharide chain with the group 3,4 antigen (Y variant). What chemical structure is responsible for the 3,4-antigenic specificity is not known—only that it is represented in the tetrasaccharide repeating unit of the O-chain shown in 1. Addition of α-D-glycopyranosyl and *O*-acetyl groups to the repeating unit decreases or eliminates the adsorption (Table 1). Our results show that upon adsorption of Sf6 either to whole bacteria or a lipid-free O-polysaccharide (from phenol-water-extracted LPS) oligosaccharides are released. Methylation analysis of the main oligosaccharide and sugar analysis of the reduced oligosaccharide showed that it is the Rha II $1\xrightarrow{\alpha}3$ Rha III linkage in 1 that has been hydrolyzed (Table 2, Fig. 3), and that this product is an octasaccharide. Larger oligosaccharides, presumedly dodeca- and hexadecasaccharides, were also isolated.

We therefore conclude that the component of Sf6 which functions in adsorption has endorhamnosidase activity. Phage Sf6, which belongs to morphological group C according to Bradley (2), has an icosahedral-shaped head with a short, noncontractile tail (Fig. 1). The tail appears to contain a base plate with six spikes extending from it. *Salmonella* phages ϵ^{15} and P22 and several of the *E. coli* and *Klebsiella* phages with endoglycosidase activity also belong to group C of Bradley. In these phages the enzymatic activity has been located in the tail. It seems reasonable to assume that the endorhamnosidase activity of Sf6 is also located in the tail.

Lysogenization of *S. flexneri* Y strains with Sf6 results in the appearance of group antigen 6 specificity, associated with an acetyl group of 0–2 in rhamnose III (11). Sf6-lysogenized *S. flexneri* (Table 1) and wild-type *S. flexneri 3b* strains (O-group antigens 3,4:6 and type antigen III) are Sf6 resistant (Table 1). The adsorption rate of Sf6 is also low, demonstrating that the acetyl on 0–2 of rhamnose III prevents adsorption. When the O-polysaccharide of *S. flexneri 3b* was deacetylated, however, Sf6 endorhamnosidase activity was observed. It is known that the O-polysaccharide of *S. flexneri* Y strains

may contain *O*-acetyl groups, although their location has not been determined (10). Thus, the presence of *O*-acetyl groups per se is not incompatible with phage Sf6 adsorption, but their position appears to be important.

Phage Sf6 also adsorbs poorly to, and does not lyse, strains with specificities other than the group 3,4 antigen (*S. flexneri 2a* and *4a*, Table 1). Both strains have α-D-glucopyranosyl groups in the oligosaccharide repeating unit of the O-antigen (Table 1). It seems probable that these glucose residues, like the acetyl on 0-2 of the rhamnose III residue, prevent the Sf6 endorhamnosidase activity.

Based on our findings, one can surmise that the narrow host range of phage Sf6 and the specificity of its endorhamnosidase are interrelated. Phages ϵ^{15} and P22 also have α-1,3-endorhamnosidase activity, but the Sf6 phage does not adsorb to any of the ϵ^{15}- or P22-susceptible *Salmonella* strains, nor does it hydrolyze the O-antigenic polysaccharide in these bacteria.

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