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# *Salmonella* Bacteriophage Glycanases: Endorhamnosidase Activity of Bacteriophages P27, 9NA, and KB1

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Four bacteriophages, P22, P27, 9NA, and KB1, active on smooth *Salmonella* strains belonging to serogroups A, B, and D1 were investigated for endoglycosidase activity and specificity in enzyme hydrolysis assays. Purified phage was incubated with phenol-water-extracted lipopolysaccharide preparations which had been partially delipidated. Dialyzable oligosaccharides, released by phage glycosidase activity, were analyzed by sugar and methylation analyses. Phages P27, 9NA, and KB1, as well as P22 assayed earlier (U. Eriksson et al., J. Gen. Virol. **43:** 503-511, 1979; S. Iwashita and S. Kanegasaki, Biochem. Biophys. Res. Commun. **55:**403-409, 1973), were all found to have phage-associated endorhamnosidase activity hydrolyzing the O-polysaccharide chain common to bacteria of serogroups A, B, and D1

$$\rightarrow 2\ \text{D-mannose}\ 1 \underset{\alpha}{\rightarrow} 4\ \text{L-rhamnose}\ 1 \underset{\alpha}{\rightarrow} 3\ \text{D-galactose}\ 1 \underset{\alpha}{\rightarrow}$$

(R attached at position 3 of D-mannose)

between the L-rhamnose and D-galactose residues. The nature of the R monosaccharide, abequose, tyvelose, or paratose, had no effect on the activity or specificity of the endorhamnosidase, whereas a change of the D-galactose → D-mannose linkage from α1,2 to α1,6 made the O-polysaccharide chain resistant to the endorhamnosidases. Modification of the O chain by glucosylation of the D-galactose residue at *O*-4 or *O*-6 revealed two glycosidase specificities: the phage P22 and P27 enzymes hydrolyzed O chains glucosylated at *O*-4 but not *O*-6, whereas the phage 9NA and KB1 enzymes hydrolyzed chains glucosylated at *O*-6 but not *O*-4. Phage KB1, like P22 and P27, had a short, noncontractile tail containing a base plate with tail spikes (morphologically Bradley group C), whereas 9NA had a long, flexible tail ending with a base plate-like appendage (Bradley group B), which suggests that the endorhamnosidase activity can be associated with different tail structures.

Phages infecting encapsulated or smooth enterobacteria like *Salmonella, Escherichia coli, Klebsiella,* and *Shigella* often have glycanase activity associated with their tail structures (for a review, see reference 17). The glycanases, besides being responsible for recognition of the bacterial phage receptors, also assist in the penetration of the cell envelope. Thus, the search for a postulated membrane receptor, where triggering of nucleic acid ejection occurs, is facilitated, and the likelihood of a productive infection is increased.

Several phages have endorhamnosidase activity hydrolyzing the rhamnosyl bonds in the polysaccharide chain of lipopolysaccharides (LPS) from *Salmonella* and *Shigella* (16, 20). In a study of 12 different phages isolated by Lilleengen (15) and active on smooth *Salmonella typhimurium* strains, all were found to have enzymatic activity hydrolyzing the L-rhamnose 1 $\underset{\alpha}{\rightarrow}$ 3 D-galactose linkage in the polysaccharide chain (Fig. 1; 28). The LPS of *Salmonella* serogroups A, B, and D can be modified by two sorts of glucosylation. The glucose is in each case attached to the D-galactose residue of the polysaccharide chain, linked to either *O*-4 or *O*-6, giving rise to O-antigen $12_2$ ($O12_2$) and O1 specificity, respectively (23, 27, 29).

Phage P22, which hydrolyzes the L-rhamnose 1 $\underset{\alpha}{\rightarrow}$ 3 D-galactose linkage, is a converting phage and causes O1 antigen specificity by glucosylation at *O*-6 of D-galactose in the LPS (27, 34). A strain which is P22 lysogenic is immune to P22

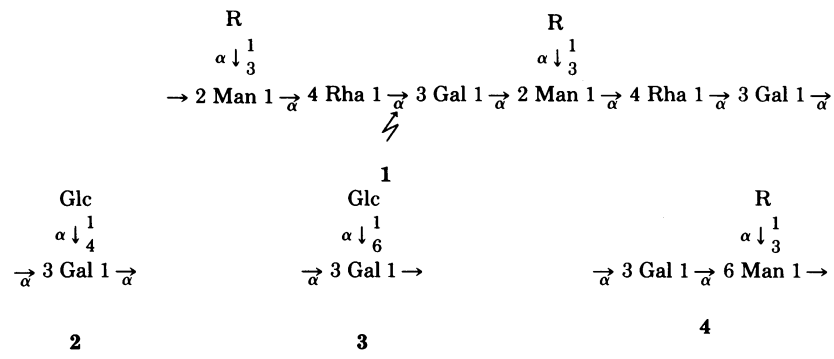



FIG. 1. *Structure of the O-polysaccharide chain of Salmonella serogroup A, B, and D bacteria (1). R is paratose in serogroup A, abequose in B, and tyvelose in D. Structure shown in 2 gives the bacteria O12₂ specificity; the structure in 3 gives O1 specificity; and the structure in 4 gives O27 specificity. Abbreviations: Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Glc, D-glucose. Arrow indicates linkage hydrolyzed by the phage enzymes.*

(8, 9). However, phages such as KB1 (6) act on P22 lysogenic as well as P22 nonlysogenic strains. Another *Salmonella*-specific phage, 9NA, is active on O12₂-negative strains but not on O12₂-positive strains (22). A third *Salmonella*-specific phage, P27, is converting and modifies the linkage between successive repeating units in the O-polysaccharide chain (1, 14, 18). Phage P27 lysogenic strains are resistant to phage P22.

This study was undertaken to investigate whether phages like KB1, 9NA, and P27 have endorhamnosidase activity and whether their enzymes are active on O-polysaccharide chains with *O*-4 or *O*-6 glucosylated D-galactose residues or on otherwise modified O-polysaccharide chains. (The glycanase will, for simplicity, be called endorhamnosidase, although it is not known whether it hydrolyzes other types of glycosidic linkages.)

## MATERIALS AND METHODS

**Bacterial strains.** *Salmonella* strain SH 1262 is a stable O12₂-negative recombinant derived from a cross of an *S. montevideo* Hfr donor to an *S. enteritidis* recipient and has O antigens 9 and 12 (23). *S. typhimurium* strain SH 4305 is a stable O12₂-positive derivative of *S. typhimurium* strain LT2, owing its stable O12₂-positive character to introduction of a gene *oaf*R⁺ from an O12₂-stable strain of *S. enteritidis* subsp. Chaco (22). *S. typhimurium* strain SL 3622 is a P22 lysogenic derivative of *S. typhimurium* LT2 subline SL 1027; as such, it is expected to have O1, which, however, is subject to form variation so that single-colony isolates may give either a strong or a weak reaction in O-factor 1 antiserum. *S. typhimurium* SL 696 is an auxotrophic subline of *S. typhimurium* strain LT2 with an unaltered O-antigen character, i.e., O antigens 4, 5, and 12; it is correspondingly sensitive to phage P22 and lacks O1. *S. wilhelmsburg* strain S 2932/78, O4, 27, is a strain with well-developed O27 so that single-colony isolates all react strongly on O-factor 27 antiserum (and, as expected, negatively in O-factor 1 and 12₂ antisera). *S. paratyphi* A strain 5/68, O2, 12, was selected because it did not agglutinate in O-factor 1 and 12₂ antisera (thus corresponding to *S. paratyphi* A subsp. Durazzo according to Kauffmann [12]).

Before and after cultivation for extraction of LPS, each strain was reisolated from single colonies and shown to behave as expected when tested for nutritional characteristics, O-antigenic specificity, and phage sensitivity. Table 1 summarizes these reactions; Table 2 summarizes the efficiency of plating (EOP; by the soft agar layer method) for some phage-strain pairs.

**Bacteriophages.** Phages P27 and 9NA were obtained from P. H. Mäkelä, Central Public Health Laboratory (State Serum Institute), Helsinki, Finland, and phage KB1 was obtained from B. A. D. Stocker, Department of Microbiology, Stanford University, Stanford, Calif. Phage P22*c*2, a clear-plaque mutant of P22, was from the collection at the National Bacteriological Laboratory, Stockholm, Sweden.

**Preparation of phage stocks.** All phages were propagated in submerged culture on *S. typhimurium* SL 696. Log-phase cells (approximately $2 \times 10^8$ cells/ml), grown at 37°C in tryptone-yeast medium with 1% glucose, were infected at a multiplicity of infection of 5 and incubated for 4 to 5 h with constant heavy aeration. Complete lysis of cells was then imposed by the addition of a few milliliters of chloroform. Cell debris was sedimented by low-speed centrifugation at $2,000 \times g$ for 20 min at 4°C. The titer of raw lysates varied between $8 \times 10^8$ and $4 \times 10^{10}$ PFU per ml. Phages were precipitated by polyethylene glycol according to Yamamoto and Alberts (33). The pellet was suspended in 0.005 M ammonium carbonate buffer, pH 7.0, to about 1/100 of the original volume, and polyethylene glycol was removed by repeated precipitation with chloroform and low-speed centrifugation. After extensive dialysis against 0.005 M ammonium carbonate buffer, pH 7.0, at 4°C, the phages were spun down at $37,000 \times g$ for 60 min at 4°C on a cesium

TABLE 1. *Bacterial strains*

| Strain | Relevant characters | Source and/or reference |
|---|---|---|
| *S. enteritidis* SH 1262 | $O9,12_2^-$ | Mäkelä and Mäkelä (23) |
| *S. typhimurium* SH 4305 | $O4,5,12_2^+$ | Mäkelä (22) |
| *S. typhimurium* SL 3622 | O1,4,5,12 (P22-lysogenized SL $1027^+$) | Stocker strain collection |
| *S. paratyphi* A 5/68 | $O2,12_2^-$ | SBL[a] strain collection |
| *S. wilhelmsburg* S:2932/78 | O4,27 | SBL strain collection |
| *S. typhimurium* SL 696 | Propagating strain O4,5,12 | Wilkinson et al. (31) |

[a] SBL, National Bacteriological Laboratory.

TABLE 2. *EOP for and extent of hydrolysis of Salmonella O polysaccharides by phages P22, P27, 9NA, and KB1*

| Bacterial strain | P22c2 | | P27 | | 9NA | | KB1 | |
|---|---|---|---|---|---|---|---|---|
| | EOP[a] | % Release[b] | EOP | % Release | EOP | % Release | EOP | % Release |
| *S. typhimurium* SL 696 | 1.0 | ND[c] | 1.0 | ND | 1.0 | ND | 1.0 | ND |
| *S. enteritidis* SH 1262 | $9 \times 10^{-4}$ | 88 | $1 \times 10^{-3}$ | 79 | | 69 | $5 \times 10^{-3}$ | 74 |
| *S. typhimurium* SH 4305 | 0.5 | 75 | | 67 | | 3 | | 4 |
| *S. typhimurium* SL 3622 | | 61 | | 62 | 0.4 | 69 | 0.6 | 63 |
| *S. paratyphi* A 5/68 | $4 \times 10^{-3}$ | 41 | | 53 | $9 \times 10^{-2}$ | 27 | | 50 |
| *S. wilhelmsburg* S:2932/78 | | <1 | | <1 | | <1 | | <1 |

[a] Determined in soft agar layer in relation to *S. typhimurium* SL 696. Entries left blank indicate an EOP of $<10^{-5}$.

[b] Amount of dialyzable oligosaccharides released, estimated by the phenol-sulfuric acid method, upon incubation of phage and Alk-PS preparation from bacterial LPS. The percentage represents the amount of phenol-sulfuric acid-positive material released, with the color reaction of the non-phage-treated Alk-PS preparation set as 100%.

[c] ND, Not determined.

chloride pad (1.65 g/ml) and collected. The titer of phage stocks varied from $1 \times 10^{12}$ to $8 \times 10^{12}$ PFU/ml.

**Preparation of LPS.** Each strain was grown in submerged culture in tryptone-yeast-glucose medium to late logarithmic phase, harvested, and washed, and the LPS was extracted from formaldehyde-killed bacteria by the phenol-water method (19). Partially delipidated LPS, referred to as Alk-PS, was prepared from LPS by treatment with 0.15 M sodium hydroxide at 100°C for 60 min. Cleaved lipids were extracted by partition with chloroform. The Alk-PS were extensively dialyzed against water and lyophilized.

**Phage hydrolysis.** In this procedure, 50 mg of Alk-PS and phage ($10^{10}$ PFU per mg of Alk-PS) in 25 ml of 0.005 M ammonium carbonate buffer, pH 7.0, was dialyzed against $4 \times 250$ ml of the same buffer for 2 days at 37°C in the presence of chloroform. The pooled dialysates were lyophilized and subjected to chemical analyses.

**Chemical analyses.** Total carbohydrate content was estimated by the phenol-sulfuric acid method (24) with the Alk-PS from each strain as a standard. The structure of Alk-PS and hydrolyzed material was determined by methylation analysis (4). Sugar derivatives obtained on methylation analysis were separated and analyzed by gas-liquid chromatography. Separations were performed with a Hewlett-Packard model 5830-A instrument equipped with a well-coated open tubular (WCOT) glass capillary column (25 m by 0.25 mm) coated with SP-1,000. For gas-liquid chromatography-mass spectrometry, a Varian MAT 311 gas-liquid chromatograph-mass spectrometer, fitted with an OV-225 or ECNSS-M column, was used.

**Electron microscopy.** Phage 9NA and KB1 preparations were stained with uranyl acetate by the following procedure. A droplet of the preparation to be examined was placed on a grid (200-mesh copper with carbon-shadowed Parlodian film), and excess fluid was aspirated with filter paper. A drop of freshly prepared 1% (wt/vol) uranyl acetate was then applied to the grid. After a few minutes, excess fluid was again removed by touching the grid to the corner of an absorbent filter paper. The preparations were examined in a Philips EM 200 electron microscope at a magnification of about 114,000.

## RESULTS

**Phage morphology.** Phages P22 and P27 belong to group C in Bradley's morphological classification (5), with a hexagonal head symmetry and a short, noncontractile tail terminating in a base plate (30). Electron microscopy revealed that phage KB1 (Fig. 2A) had a similar morphology: the hexagonal head had a diameter of about 60 nm; the tail was approximately 15 nm long and terminated in a base plate approximately 25 nm wide. Thus, the KB1 phage also belongs to group C according to Bradley (5). Phage 9NA, however, had a different morphology (Fig. 2B). To the symmetrical head, which was about 60 nm long and about 60 nm wide, was attached an approximately 150-nm-long thin, noncontractile tail. A 30-nm-wide base

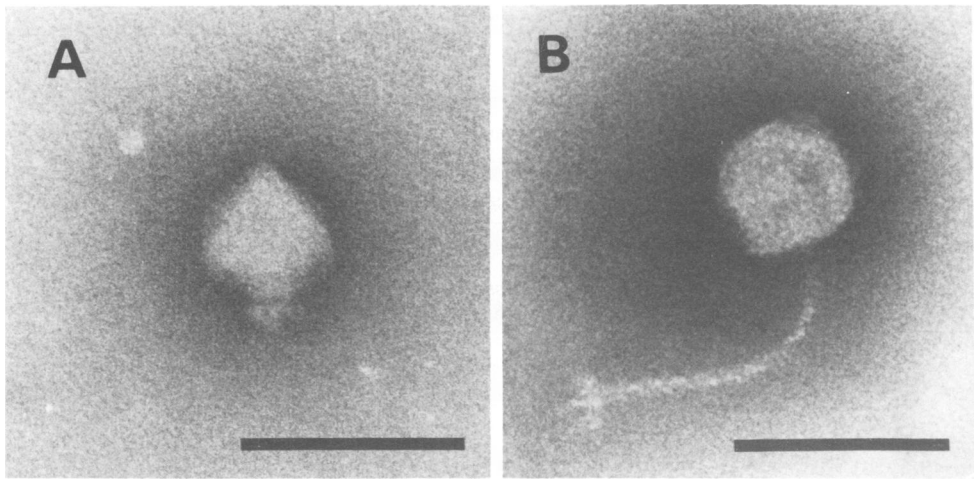



FIG. 2. *Electron micrographs of (A) phage KB1 and (B) phage 9NA. Bars represent 100 nm.*

plate-like structure was attached to the distal end of the tail. Phage 9NA thus belongs to group B of Bradley.

**Phage glycanase activity.** The EOP data for phages P22, P27, 9NA, and KB1 gave no information about the glycanase activity of the phages (Table 2). A low or undetectable EOP and yet digestion of LPS indicated restriction in some cases (e.g., phage P22 and *S. enteritidis* SH 1262) and lysogenic immunity in others (e.g., P22 and *S. typhimurium* SL 3622). A low EOP in spite of Alk-PS degradation may also reflect a sensitivity of the phage enzyme to *O*-acetyl groups present in the native (i.e., not alkali-treated) LPS. Glycanase activity was instead shown in experiments where phage was incubated with extracted, nondialyzable (Alk-PS) preparations which had been partially delipidated to prevent irreversible binding of the phage to its receptor. After incubation with phage, $\sim 10^{10}$ PFU per mg of Alk-PS, dialyzable oligosaccharides was split off (Table 2). Phages P22 and P27 were found to release oligosaccharides (41 to 88% of added Alk-PS carbohydrates) from all Alk-PS preparations tested except *S. wilhelmsburg*, the only O27-positive and O12-negative LPS tested. Phages 9NA and KB1 were active on the same Alk-PS, but only small amounts, 3 and 4%, respectively, were released from the Alk-PS preparation of *S. typhimurium* SH 4305, the LPS of which was glucosylated at *O*-4 of the D-galactose residues in the polysaccharide chain (Fig. 1, **1 + 2**).

**Structural studies of released oligosaccharides.** The Alk-PS preparations were subjected to sugar and methylation analyses. The qualitative and quantitative monosaccharide compositions obtained on sugar analysis were as expected (Fig. 1). Also, the methyl ethers from the five Alk-PS preparations (Table 3) were in accordance with the established structures (Fig. 1). In *S. typhimurium* SH 4305 (a stable $O12_2$-positive strain), calculation of the relative amounts of the 2,6-di-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose ethers showed that 75% of the D-galactosyl residues in the O chain were substituted with D-glucose at *O*-4, to give the expected $O12_2$ antigenic specificity. In the *S. typhimurium* SL 3622 strain (P22 lysogenic and O1 positive, but expected to be subject to form variation), calculation of the 2,4-di-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose ethers showed that in the preparation studied 33% of the D-galactosyl residues were substituted at *O*-6, giving the O1 antigenic specificity.

Oligosaccharides obtained from the dialysates were subjected to methylation analysis to determine which linkage in the O-polysaccharide chain had been cleaved. The amount and nature of the different methyl ethers in the phage-liberated oligosaccharide preparations were next compared with those obtained from the untreated Alk-PS preparations (Table 3).

**Phage P22.** Oligosaccharides liberated from *S. enteritidis* SH 1262 (stable $O12_2$ negative), *S. typhimurium* SL 3622 (P22 lysogenic, O1 positive), and *S. paratyphi* A strain 5/68 (O1 and $O12_2$ negative) on phage P22 hydrolysis yielded a 2,3,4,6-tetra-*O*-methyl-D-galactose ether not present in the methylated Alk-PS (not exposed to phage P22). In the oligosaccharides released from *S. typhimurium* SH 4305 ($O12_2$ positive), a 2,3,6-tri-*O*-methyl-D-galactose ether was found which was not present in methylated Alk-PS not phage exposed. These data are compatible with

TABLE 3. *Methylation analysis of Alk-PS and oligosaccharide fractions obtained by phage-mediated hydrolysis of Salmonella Alk-PS preparation*

| Methylated sugar[a] | Retention time[b] | *S. enteritidis* SH 1262 (structure **1**, Fig. 1) | | | | | *S. typhimurium* SH 4305 (structure **1** + **2**, Fig. 1) | | | | | *S. typhimurium* SL 3622 (structure **1** + **3**, Fig. 1) | | | | | *S. paratyphi* A 5/68 (structure **1**, Fig. 1) | | | | *S. wilhelmsburg* S: 2932/78 (structure **4**, Fig. 1), Alk-PS[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Alk-PS[d] | P22 | P27 | 9NA | KB1 | Alk-PS | P22 | P27 | 9NA | KB1 | Alk-PS | P22 | P27 | 9NA | KB1 | Alk-PS | P22 | P27 | 9NA | |
| 2,4-DDH | | 19 | 15 | 18 | 15 | 21 | 12 | 23 | 16 | 24 | 18 | 19 | 22 | 21 | 16 | 26 | 15 | 21 | 18 | 18 | 11 |
| 2,3-Rha | 0.93 | 30 | 23 | 25 | 25 | 25 | 25 | 23 | 18 | 20 | 14 | 26 | 24 | 26 | 21 | 21 | 27 | 23 | 25 | 23 | 23 |
| 2,3,4,6-Glc | 1.00 | —[e] | — | — | — | — | 19 | 23 | 18 | 14 | 17 | 10 | 2 | 2 | 10 | 7 | — | — | — | — | |
| 2,3,4,6-Gal | 1.14 | — | 8 | 9 | 8 | 11 | — | — | 1 | 1 | 2 | — | 9 | 9 | 4 | 4 | — | 6 | 6 | 4 | |
| 2,3,6-Gal | 1.86 | — | — | — | — | — | — | 8 | 9 | 2 | 2 | — | — | — | — | — | — | — | — | — | |
| 2,4,6-Man | 1.87 | 2 | 1 | 1 | 1 | 1 | 2 | — | — | — | — | 3 | 2 | 2 | 2 | 1 | 8 | 3 | 4 | 5 | 10 |
| 2,4,6-Gal | 1.94 | 27 | 20 | 16 | 20 | 17 | 6 | 3 | 1 | 14 | 19 | 14 | 14 | 15 | 10 | 10 | 28 | 19 | 18 | 22 | 33 |
| 2,3,4-Gal | 2.42 | — | — | — | — | — | — | — | — | — | — | — | — | — | 3 | 2 | — | — | — | — | |
| 4,6-Man | 2.65 | 21 | 33 | 30 | 31 | 25 | 20 | 28 | 22 | 14 | 20 | 20 | 25 | 26 | 27 | 22 | 22 | 28 | 29 | 27 | |
| 2,4-Man | 2.77 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 23 |
| 2,6-Gal | 3.80 | — | — | — | — | — | 18 | 19 | 16 | 11 | 9 | — | — | — | — | — | — | — | — | — | |
| 2,4-Gal | 4.19 | — | — | — | — | — | — | — | — | — | — | 7 | 1 | — | 7 | 6 | — | — | — | — | |

[a] DDH, 3,6-Dideoxy-D-xylohexose (abequose) in *S. typhimurium* and *S. wilhelmsburg*, 3,6-dideoxy-D-ribohexose (paratose) in *S. paratyphi* A, and 3,6-dideoxy-D-arabinohexose (tyvelose) in *S. enteritidis*; Rha, L-rhamnose; Glc, D-glucose; Gal, D-galactose; Man, D-mannose; 2,3,4,6-Glc, 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.

[b] Retention time of the corresponding alditol acetates relative to 2,3,4,6-Glc set as 1.00.

[c] There was no release of oligosaccharide with phages P22, P27, 9NA, and KB1.

[d] Methyl ethers deriving from the core of the Alk-PS are not presented. Data represent peak area as percentage of total peak areas as detected by flame ionization.

[e] —, Not detected.

hydrolysis of the L-rhamnosyl $1\xrightarrow{\alpha}3$ D-galactosyl linkages in the O-polysaccharide chains by the endorhamnosidase activity of phage P22 (Fig. 1).

**Phage P27.** The cleavage pattern for phage P27 was similar to that observed for phage P22 (Table 3). All methylated oligosaccharide preparations contained 2,3,4,6-tetra-*O*-methyl-D-galactose ethers (although the amount in the *S. typhimurium* SH 4305 oligosaccharides was considerably less than that of the 2,3,6-tri-*O*-methyl-D-galactose ether). The data are compatible with the presence of endorhamnosidase activity also in phage P27.

**Phage 9NA.** The oligosaccharides released by phage 9NA were likewise shown by methylation analysis to have galactose methyl ethers not present in the untreated Alk-PS preparation (Table 3). As for P27, all four oligosaccharide preparations contained the 2,3,4,6-tetra-*O*-methyl-D-galactose ether. In the oligosaccharide from *S. typhimurium* SL 3622, a 2,3,4-tri-*O*-methyl-D-galactose ether was also found in amounts comparable to those found for the tetra-*O*-methyl ether. The hydrolase associated with phage 9NA is thus an endorhamnosidase. Although the phenol-sulfuric acid assay indicated that phages 9NA and KB1 released 27 and 50%, respectively, of material from the Alk-PS of the *S. paratyphi* A strain, chemical analyses by gas-liquid chromatography showed that only minor amounts of oligosaccharides unsuitable for analysis of the KB1-released material had been released.

**Phage KB1.** The de novo-formed galactose methyl ethers found in the KB1 hydrolysates were the same as for phage 9NA (Table 3). The amount of oligosaccharide released from *S. paratyphi* A strain 5/68 was, however, too small for analysis (see above). Phage KB1, like the other phages studied, also displayed endorhamnosidase activity.

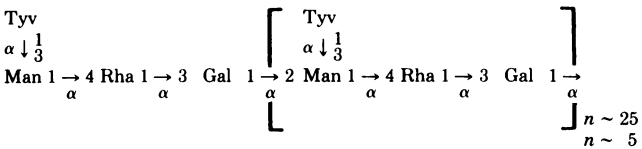
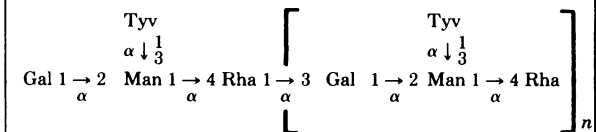
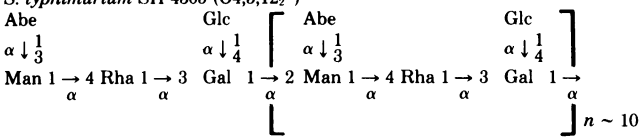
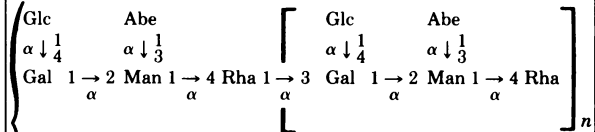
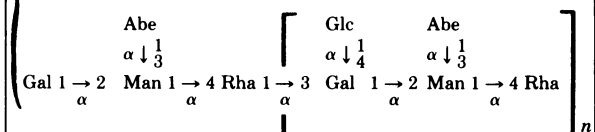
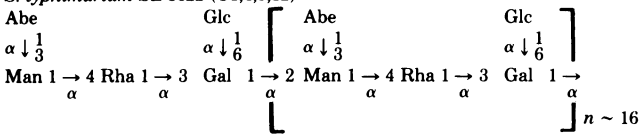
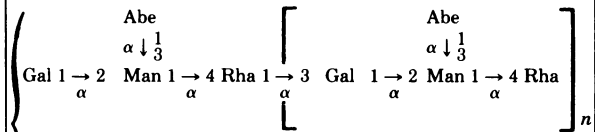
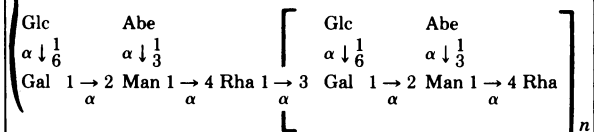
## DISCUSSION

Phages P27, 9NA, and KB1, like P22, all had endorhamnosidase activity hydrolyzing the L-rhamnose $1\xrightarrow{\alpha}3$ D-galactose linkage within the repeating unit of the O-polysaccharide chain of *Salmonella* serogroups A, B, and D (Tables 2 and 3; Fig. 1). Based on the enzymatic activity against Alk-PS prepared from *S. enteritidis* SH 1262, *S. typhimurium* SH 4305, and *S. typhimurium* SL 3622, two patterns were discernible (Table 4).

Phages P22 and P27 both hydrolyzed *S. enteritidis* SH 1262 (O1 negative, $O12_2$ negative), where the D-galactosyl residue was unsubstituted, and *S. typhimurium* SH 4305 (O1 negative, $O12_2$ positive), where the D-galactosyl residue was substituted with D-glucose at *O*-4 (Tables 2 and 4). Hydrolysis of Alk-PS from *S. typhimurium* SL 3622, where the D-galactosyl was substituted with D-glucose at *O*-6, was also seen and was 61 and 62% for P22 and P27, respectively; this is not in accordance with what was previously reported for phage P22 (9). Oligosaccharides released from *S. typhimurium* SL 3622 (O1 positive, $O12_2$ negative) by P22 and P27 yielded only 2,3,4,6-tetra-*O*-methyl-D-galactose and no 2,3,4-tri-*O*-methyl-D-galactose ethers. This shows that hydrolysis took place only at rhamnosyl-galactosyl linkages where no glucosylation at *O*-6 of D-galactose was present. The nearly complete absence of glucose in the oligosaccharides (only 2% detected in the P22-released oligosaccharides) attests that hydrolysis took place only in O-polysaccharide chains which were nonglucosylated. Such chains perhaps resulted from O1-negative clones by form variation or, less probably, from the appearance of non-P22 lysogenic revertants in the batch culture of strain SL 3622. No 2,3,4,6-tetra-*O*-methyl-D-galactose ether was found in the methylation analysis of oligosaccharides released by phage P22 from *S. typhimurium* SH 4305 (O1 negative, $O12_2$ positive), and only a small amount was found in the oligosaccharides released by phage P27 (Table 3). Since about one-quarter of the D-galactosyl residues are unsubstituted in the Alk-PS preparation, this indicates that both the P22 and P27 endorhamnosidases preferentially hydrolyze rhamnosyl-galactosyl linkages were the galactosyl is glucosylated at *O*-4. Within the released oligosaccharides the galactosyl residues were glucosylated 90 and 96%, respectively, which is higher, by approximately 3:1 preference, than was seen in the Alk-PS preparation. These data show that if the D-galactose residue is glucosylated at *O*-6, no hydrolysis of the rhamnosyl $1\xrightarrow{\alpha}3$ galactosyl linkage takes place, which is in accordance with earlier data (8), whereas glucosylation of the D-galactose at *O*-4 has no such effect but, in contrast, seems to facilitate hydrolysis by these enzymes.

Phages 9NA and KB1 both readily hydrolyzed *S. enteritidis* SH 1262 (O1 negative, $O12_2$ negative) and *S. typhimurium* SL 3622 (O1 positive, $O12_2$ negative), but only small amounts of oligosaccharides were released from *S. typhimurium* SH 4305 (O1 negative, $O12_2$ positive) (Table 2). The SH 1262 and SH 4305 oligosaccharides gave exclusively 2,3,4,6-tetra-*O*-methyl-D-galactose as the ether formed de novo. From the oligosaccharide fraction of *S. typhimurium* SL

TABLE 4. *Structure of Salmonella O-polysaccharide chain and oligosaccharides released by phage-mediated hydrolysis*

| Structure of O chain | Released oligosaccharide(s) | Mean size of dialyzable oligosaccharide released | | |
|---|---|---|---|---|
| | | | *S. enteritidis* SH 1262 | *S. paratyphi* A 5/68 |
| *S. enteritidis* SH 1262 (O9,12$_2^-$) and *S. paratyphi* A 5/68 (O2,12) | | | | |
|  |  | P22<br>P27<br>9NA<br>KB1 | *n*~2.4<br>*n*~1.8<br>*n*~2.3<br>*n*~1.3 | *n*~2.1<br>*n*~1.9<br>*n*~2.2<br>Not determined |
| *S. typhimurium* SH 4305 (O4,5,12$_2^+$) | | | | |
|  |  | P22<br>P27 | *n*~2.6<br>*n*~1.5 | |
| |  | 9NA<br>KB1 | *n*~6.0<br>*n*~5.5 | |
| *S. typhimurium* SL 3622 (O1,4,5,12) | | | | |
|  |  | P22<br>P27 | *n*~1.3<br>*n*~1.4 | |
| |  | 9NA<br>KB1 | *n*~1.9<br>*n*~2.2 | |

[a] In *S. paratyphi* A, the tyvelose residue is replaced by paratose.

3622, the 2,3,4-tri-*O*-methyl- and tetra-*O*-methyl-D-galactose ethers were found in approximately equal amounts (Table 3). The virtual absence of the 2,3,6-tri-*O*-methyl-D-galactose ether in the SH 4305 oligosaccharide fractions demonstrates that the phage 9NA and KB1 endorhamnosidases cannot hydrolyze the rhamnosyl $1 \xrightarrow[\alpha]{} 3$ galactosyl linkage when the D-galactose residue is substituted with a D-glucose residue at *O*-4 (Table 4). In the oligosaccharides released from the SL 3622 (O1 positive, $O12_2$ negative) Alk-PS, the almost equal amounts of 2,3,4,6-tetra-*O*-methyl- and 2,3,4-tri-*O*-methyl-D-galactose suggest that the endorhamnosidase of 9NA and KB1 hydrolyzes the rhamnosyl $1 \xrightarrow[\alpha]{} 3$ galactosyl linkage equally well irrespective of the $\alpha$1,6-glucosylation of the D-galactose residue.

All phages hydrolyzed one or another Alk-PS from the *S. paratyphi* A, *S. typhimurium*, and *S. enteritidis* strains (Table 2). The dideoxyhexosyls $\alpha$1,3-linked to D-mannose in these species are paratose, abequose, and tyvelose, respectively (21). Thus, the nature of the dideoxyhexosyl substituent did not influence the substrate specificity of the phage enzymes. This is in accordance with what has been found for other endorhamnosidase-containing phages (8).

The Alk-PS from *S. wilhelmsburg* S:2932/78 (O4, 27) was not hydrolyzed by any of the phages (Table 2) (Fig. 1, **1** + **4**). The repeating unit of this O-polysaccharide chain is identical to that of *S. typhimurium* (Fig. 1, **1**), but the repeating units are linked $\alpha$1,6 instead of $\alpha$1,2. It is likely that this change, which depends on the presence of the temperate P27 phage (1), is sufficient to make the substrate resistant to the endorhamnosidases. Attachment of P22 and P27 was abolished in an *S. bredeney* strain converted by phage P27 (18).

The average size of the oligosaccharides released in the dialysate was determined by calculating the ratio of the D-galactose ether(s) derived from the terminal nonreducing ends produced upon phage hydrolysis to the total amount of D-galactose ethers and of the D-mannose ether derived from the nonreducing end of the O chain to the total amount of D-mannose. The average size was found to vary from two to seven repeating units, with the majority being approximately three repeating units. It is likely that the dialysate contained a mixture of oligosaccharides, since earlier studies of the products of phage $\epsilon^{15}$ and P22 hydrolysis have been found to consist of a series of oligosaccharides of different sizes (2, 8).

The O chains of *Salmonella* groups A, B, and D are modified by two sorts of glucosylation: the $\alpha$1,4-glucosylation of D-galactose, as in strain SH 4305, and the $\alpha$1,6-glucosylation of D-galactose, as in strain SL 3622. These correspond to $O12_2$ and O1, respectively. In many strains which have $O12_2$, there is a rapid change to an $O12_2$-negative form and back again. This is known as form variation (11). It is, however, not known in form-variable strains how the O chains are glucosylated. Four models are possible: (i) some bacteria have their LPS glucosylated and others do not; (ii) in each bacterium some chains are completely glucosylated and others are not; (iii) there are clusters of glucosylated repeating units within chains; and (iv) there is random distribution of glucosylation within chains. Phages P22 and P27 released oligosaccharides from *S. typhimurium* SH 4305 which were almost completely glucosylated when the substrate was only about 75% glucosylated and released from *S. typhimurium* SL 3622 oligosaccharides which were almost completely nonglucosylated when the substrate was about 33% glucosylated. This suggests that the first or second alternative, that some O chains are completely glucosylated and others are nonglucosylated, is the more likely one. Such a distribution would also be biosynthetically more plausible, since stepwise addition of D-glucose units to the growing O polysaccharide has been a favored model (32).

That endorhamnosidase activity is found associated with phages P27 and KB1 is not surprising, since hydrolytic activity seems to be a common quality of phages belonging to group C of the morphological classification of Bradley (13, 17, 26). Among the best-characterized phage-associated hydrolyses are the endorhamnosidase in P22 (8; this communication), the endoglucosidase in $\phi$29 (3), and the *O*-acetylases in Vi phages (13). Of particular interest is that glycanase activity has been found in phage 9NA, which belongs to the Bradley group B phages, and that this enzyme has a specificity very similar to that of the phage KB1 endorhamnosidase. A depolymerase in a group B phage has previously been described only for a *Pseudomonas aeruginosa* phage (17) and a coliphage (25). Although not proven in this investigation, it is likely that the endorhamnosidase activities of phages P27, 9NA, and KB1 reside in tail appendages (tail spikes or tail fiber), as has been demonstrated for P22 (7), $\phi$29 (3), and $\epsilon^{15}$ (2). The *O*-deacetylase activity of Vi phage III is also localized in the tail (13). The bacteriophage tail has been considered an organ which is clearly in the process of evolution, since it exhibits a more or less complete gradation in morphology and functional complexity (10). Our studies attest to the hypothesis. Endorhamnosidase activity has now been found in all 16

phages active on smooth *Salmonella* bacteria of serogroups A, B, and D (28; this communication). The endorhamnosidase is found in phages with a long flexible tail, like 9NA, and a short tail, like P22. The endorhamnosidases have also evolved in specificity as judged by their ability to hydrolyze linkages where the D-galactose residue is substituted at *O*-4, as in *S. enteritidis* SH 4305, or *O*-6, as in *S. typhimurium* SL 3622. It would be interesting to investigate whether these differences in enzyme specificity are the result of minor (e.g., single amino acid) or major (e.g., oligopeptide) differences in the primary structure of the tail protein.

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