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Escherichia coli Capsule Bacteriophages

VII. Bacteriophage 29-Host Capsular Polysaccharide Interactions

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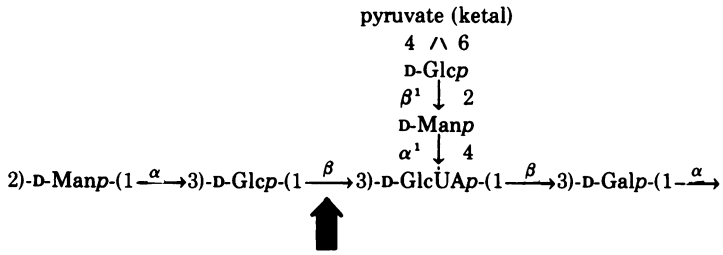
Different interactions between particles of *Escherichia coli* capsule bacteriophage 29 and its receptor, the *E. coli* serotype 29 capsular polysaccharide have been studied. The *inactivation* of phage 29 (8×10^3 PFU/ml) by isolated host capsular glycan was found to be physiologically insignificant (50% inactivation dose = 100 μg after 1 h at 37 C). No adsorption ($< 2 \times 10^4$ PFU/μg) of the viruses to K29 polysaccharide-coated erythrocytes (at 0 or 37 C) was observed either. The phage particles were, however, found to catalyze the hydrolysis of β-D-glucosido-(1→3)-D-glucuronic acid bonds (arrow) in the receptor polymer, leading, ultimately, to the formation of a mixture of K29 hexasaccharide (one repeating unit), dodecasaccharide, and octadecasaccharide:



Testing derivatives of K29 polysaccharide, as well as 82 heterologous bacterial (mainly *Enteriobacteriaceae*) capsular glycans, the viral glycanase was found to be highly specific; in accordance with the host range of phage 29, only one enzymatic cross-reaction (with the *Klebsiella* K31 polysaccharide) was observed. These and previous results, as well as the electron optical findings of M. E. Bayer and H. Thurow (submitted for publication), are discussed in terms of a unifying mechanism of phage 29-host capsule interaction. We propose that the viruses penetrate the capsules by means of their spike-associated glycanase activity, which leads them along capsular polysaccharide strands to membrane-cell wall adhesions where ejection of the viral genomes occurs.

Bacteriophages generally infect a restricted range of related host bacteria only. This specificity is largely, but by no means exclusively, due to the fact that for initiation of infection the viruses demand different chemical structures in the outer regions of the host cell surface—the bacteriophage receptors (29).

A variety of experimental techniques have been used for the distinction of such "receptive areas" (54) from the rest of the host surface constituents.

(i) Phage-inactivating agents. The now classical method is the extraction, purification, and analysis of phage-inactivating agents (PIA) from the host bacteria (e.g., 6, 11, 24, 55), estimating the activity of the fractions obtained in terms of the amount needed to inactivate 50% of a given phage sample (ID_{50} [6]), or, alternatively, in terms of the inactivation velocity constant (55). Although reports to the contrary have been published (9, 26), it has often been observed (e.g., 24, 28, 58) that rather large "lumps" of the bacterial surface area needed to cause ejection of the viral genome (24), i.e., that PIA loses activity upon separation into subfractions. In the case of complicated bacterial viruses with several morphologically distinct types of receptor-complementary organelles, one explanation for these findings is offered by the assumption that each type of organelle (e.g.,

in the case of coliphage T4, the long [4] and the short [25] tail fibers, as well as the spikes [41] needs its own distinct receptor; it also appears that the receptors (in this case presumably identical) for several organelles of the same type may have to exhibit a certain sterical arrangement (30).

(ii) Host mutants. Another widely used technique (e.g., 13, 20, 21, 28, 44, 46, 58) is the structural comparison of surface constituents from wild-type and adsorption-resistant host mutant cells. In contrast to the analysis of PIA, this approach often led to the identification of very small surface areas, the presence of which is necessary for the attachment of a given bacterial virus. Examples are the findings that *Salmonella* bacteriophage "Felix 01" (28) and *Escherichia coli* phages T3 and T4 (P. Prehm, Ph.D. thesis, Freiburg University, 1974; P. Prehm et al., submitted for publication) will not adsorb to host mutants which lack the distal 2-acetamido-2-deoxy-D-glucopyranoside or the subterminal D-glucopyranoside residue, respectively, in the core region of host cell wall lipopolysaccharide.

(iii) Electron microscopy. Host surface constituents that are clearly distinguishable by electron microscopy can also be identified as phage receptors by visualization of the virus-cell complexes. This method has, consequently, met with special success in the case of pilus-dependent (e.g. 12, 16) and flagellotropic (e.g., 38) bacteriophages.

(iv) Adsorption to carriers coated with isolated host surface constituents. The ability of isolated host surface components to absorb homologous phage has been tested by incubation of a virus suspension with suitably coated carrier particles, notably erythrocytes (43, 52). For instance, about 10^{11} PFU of Vi phage II were, in this manner, found to absorb to 1 μg of Vi polysaccharide at 0 C (52).

(v) Host surface constituents as substrates of bacteriophage-borne enzymes. A rapidly increasing number of examples for a totally different type of bacteriophage-receptor relationship—analogous to the long-known animal virus neuraminidases (e.g., 17)—is being reported in recent years, viz., the degradation of host surface components by virus-associated enzymes. Among these are the deacetylation of Vi polysaccharide by Vi phages (27, 47, 50) and the hydrolysis of glycosidic linkages in *Enterobacteriaceae* cell wall lipopolysaccharides (22, 36, 49), as well as capsular polysaccharides (5, 37, 53). Incubation of host surface constitutents with bacterial viruses and subsequent analysis of the former for chemical alterations thus constitutes another experimental approach to receptor identification.

Further techniques for this purpose are, (vi) the chemical modification of the host cell envelope (by extraction [e.g., 10, 15], enzymatic digestion [e.g., 15, 58], or with specific reagents [e.g., 40, 56]) and evaluation of its influence upon phage attachment, and (vii) the inhibition of PIA-blocking antibodies by host surface haptens (31). Finally, the techniques for identifying receptors of single types of phage organelles shall be mentioned, viz., (viii) the testing of host surface constituents for enzymatic digestion (e.g., 5, 22, 37) or (ix) of PIA for blocking (57) by isolated phage tail parts.

Since in the same phage-host system the different methods often involve different host surface areas (for instance, phage "Felix 01," which adsorbs only to *Salmonella* strains carrying the terminal 2-acetamido-2-deoxy-D-glucose of the cell wall lipopolysaccharide core, is not inactivated by isolated complete lipopolysaccharide after it has been sonically treated [28], and flagellotropic phage is not inactivated by isolated flagella [38]), bacteriophage receptors appear to be rather ill-defined entities.

Aiming at a unifying molecular interpretation of the results of these different biological techniques in one suitable system, we have applied several methods of receptor analysis to *E. coli* capsule bacteriophage 29 (46) and the homologous capsular polysaccharide (14). Our work was substantially complemented by the simultaneous electron optical investigations of M. E. Bayer and H. Thurow (submitted for publication).

(This paper was taken in part from a thesis submitted by F. F. in partial fulfillment of the requirements for the Dr.rer.nat. degree from Freiburg University.)

MATERIALS AND METHODS

Many of the materials and methods used have been described or cited previously: (i) the media and general techniques (44, 46); (ii) the growth and purification of *E. coli* capsule bacteriophage 29 particles (37); (iii) the isolation of host capsular polysaccharide by the phenol-water-Cetavlon procedure (14); (iv) the determination of capsule depolymerase activity and the definition of the depolymerase (glycanase, enzyme) unit (37); (v) the constituent and methylation analysis of K29 oligosaccharides (14); and (vi) the labeling and identification of reducing end sugars in capsular oligosaccharides (45).

Bacteriophage. *E. coli* capsule bacteriophage 29 (37, 46) was used exclusively.

Bacteria. Besides the host strain, *E. coli* Bi 161/42 [09:K29(A):H^-] and the other serological test strains for different *E. coli* K(A) antigens previously listed

(46), *E. coli* E75 [09:*K34*(A):H⁻], *E. coli* F9095/41M (059:K?:H19:M) (which produces colanic acid [M antigen] [35]), and 72 serological test strains for *Klebsiella* K antigens (34) were also employed. All strains were kindly supplied by F. Ørskov and I. Ørskov, World Health Organization International Escherichia Center, Statens Seruminstitut, Copenhagen, Denmark.

Inactivation. Phage 29 (8×10^3 PFU) in 1 ml of Merck standard I broth containing between 0.1 and 200 μg of host capsular polysaccharide was incubated for 1 h at 37 C and then titrated against a control.

Adsorption. For the control of polysaccharide fixation to erythrocytes, 2 mg of $NaBH_4/NaB^3H_4$ (125 mCi/mmol) was added to 13 mg of K29 glycan in 1.3 ml of 0.01 N aqueous NaOH. After storage at room temperature overnight, excess $NaBH_4/NaB^3H_4$ was destroyed with dilute acetic acid, and the mixture was dialyzed extensively against distilled water (removal of borate checked by flame coloring) and lyophilized (radioactivity of the product, 9×10^5 counts/min per mg). Samples (10 and 100 μg) of the labeled polymer were added to 0.05 ml of packed formalinized sheep erythrocytes in 1 ml of phosphate-buffered saline, (PBS), and the mixture was agitated at 37 C for 1 h and then stored at 4 C overnight. The modified erythrocytes were sedimented and washed three times with 1 ml of PBS. The radioactivity of the first supernatant and of the washings was determined using a Packard Tri-Carb model 2450 liquid scintillation spectrometer.

For the absorption of phage 29 to polysaccharide-coated erythrocytes (compare references 43 and 52), 1-ml portions of P medium (46) at 0 or 37 C containing 10^5 (or 7×10^6) PFU of phage 29 were added to 0.05-ml samples of packed erythrocytes which had previously been modified with 20 μg of cold (or 10 μg of hot) K29 polysaccharide as described above. The mixtures were agitated for 2, 4, 6, 8, or 10 min (one series at 0 and one at 37 C) and centrifuged (2 min at $700 \times g$), and the supernatants were titrated in duplicate against a control (unmodified red cells). In the case of the hot samples, the radioactivity of all supernatants and washings was again determined.

Conditions of depolymerization. (i) Temperature. Samples of 0.15 M sodium phosphate buffer, pH 7, containing 3×10^{10} PFU of purified phage 29 particles and 2 mg of K29 polysaccharide per ml were incubated in Ostwald viscosimeters at temperatures between 15 and 70 C, and the efflux times were determined at intervals. The results were expressed as the time required for a 50% reduction of the viscosity number ($\eta - \eta_\infty/\eta_0 xc$). The temperature stability of the viral enzyme (in PBS) was estimated by storage at different temperatures and determination of the residual depolymerase activity as described previously (37).

(ii) pH. Analogous experimental conditions were used for determining the pH dependence of the depolymerization reaction (37 C; in 0.15 M sodium phosphate buffers of pH 5.5 to 8, or 0.1 M citric acid-sodium phosphate buffers of pH 3.5 to 5.5) and the pH stability of the enzyme.

(iii) Phage concentration. Solutions containing 2 mg of polysaccharide and between 10^3 and 5×10^{10} PFU per ml of PBS (compare Fig. 2c) were incubated for 18 h at 37 C in Ostwald viscosimeters, and the efflux time was then determined. After 18 and 42 h of incubation, the 3×10^{10}-PFU reaction sample was also analyzed for reducing sugars using the Park-Johnson technique with D-glucose as a standard (see 37).

Isolation of depolymerization products. Portions (50 mg) of lyophilized K29 polysaccharide and about 8×10^{11} PFU of purified phage 20 particles in 25 ml of PBS were incubated for 18 h at 37 C. The mixture was then placed on a Sephadex G10 column (length, 80 cm; area, 7 cm^2) and eluted (20 ml/h) with a volatile buffer (pyridine-glacial acetic acid-water, 10/4/1,000, vol/vol/vol, pH 4.5), analyzing the fractions for carbohydrates with the phenol-sulfuric acid reagent (19). The K29 oligosaccharides appeared in the void volume; they were pooled and lyophilized. The desalted mixture of depolymerization products was taken up in 2 ml of a 0.05 M Tris-hydrochloride buffer, pH 7, containing 0.2 M NaCl, and absorbed to a DEAE-Sephadex column (length, 50 cm; area, 7 cm^2; flow rate, 5.4 ml/h). After washing with the same buffer, the oligosaccharides were eluted with a 0.2 to 0.4 M linear NaCl gradient. The peak fractions (compare Fig. 3) were pooled, desalted by passage over Sephadex G10 as above, and lyophilized (yield: oligosaccharide P1, 15 to 17.5 mg [30 to 35%, wt/wt, from the polymer {Both the lyophilized polysaccharide and the oligosaccharides obtained from it generally contained 10 to 15%, wt/wt, of water as determined by drying for 18 h at 40 C in vacuo over phosphorus pentoxide}]; P2, 10 to 12.5 mg [20 to 25%]; P3, 15 to 17.5 mg [30 to 35%]). The products were checked for homogeneity by paper electrophoresis (in pyridine/glacial acetic acid/water, 5/2/43, vol/vol/vol; pH 5.2, mobility relative to D-glucuronic acid [GlcUA] = 0.59, P1; 0.66, P2; 0.73, P3), and subjected to constituent analysis by methods previously described (14).

Identification of reducing and nonreducing end sugars in the depolymerization products. Using a procedure described previously (45), the reducing end sugar in oligosaccharides P1, P2, and P3 was identified by reduction with $NaBH_4/NaB^3H_4$, hydrolysis, and paper electrophoresis of the radioactive hexitol in an arsenite buffer. The nonreducing end sugar was identified (as well as quantitatively determined) as follows: 2 ml of a 40 mM aqueous solution of sodium metaperiodate was added to 2 ml of 1% (wt/vol) solutions of the K29 oligosaccharides, and the mixtures were stored at 4 C in the dark for 24 h. Upon addition of a 0.1 volume of ethylene glycol and after 60 min at room temperature, the solutions were brought to pH 8 with dilute aqueous NaOH, and 10-mg portions of $NaBH_4$ were admixed. After storage at 4 C for another 18 h, excess borohydride was destroyed with dilute acetic acid. The products were desalted by passage over Sephadex G10 with a volatile buffer (see above), lyophilized, and analyzed for GlcUA by the carbazole-sulfuric acid method (18).

Size determinations of the depolymerization products. The size of oligosaccharides P1, P2, and P3 was determined by the method of Moyer and Isbell

(32), as well as by comparative glucose analysis before and after reduction with $NaBH_4$.

(i) Labeling with $Na^{14}CN$. A 75-μl portion of a 100 mM solution of $NaCN/Na^{14}CN$ (60 μCi/mmol) in 0.1 M sodium carbonate buffer, pH 9.2, was added to 75 μl each of between 1 and 7 mM solutions (in the same buffer) of oligosaccharides P1, P2, and P3, of a 2 mM solution of D-glucose (standard), and of buffer alone (control). Toluene (5 μl) was then added, and the reaction vessels were closed tightly and stored at room temperature for 10 days. After the addition of 3 drops of 10% aqueous formic acid, the mixtures were evaporated to dryness at room temperature. This procedure was repeated four times. The final dry residues were taken up in 100 μl of water, and the radioactivity was determined as above.

(ii) Determination of reducible glucose. About 6 μmol of $NaBH_4$ in 0.01 N NaOH was added to 200 μl of 2.5 mM aqueous solutions of the K29 oligosaccharides, and the mixtures were stored overnight at 4 C in the dark. After decomposition of excess reducing agent with dilute acetic acid, decationization with dry Amberlite IR/H^+, and chasing of the boric acid with methanol, the products were brought to dryness. Reduced and not reduced aliquots of each oligosaccharide were then hydrolyzed with 2 N HCl (18 h at 100 C), and the acid was removed by evaporation. D-Glucose was determined in the hydrolyzates using fungal glucose oxidase (EC 1.1.3.4; Boehringer, no. 15982) as described by Schlubach and Repenning (39).

Methylation analysis of oligosaccharide P1. About 5 mg of P1 was reduced with NaB^2H_4 in deuterium oxide (labeling of the reducing end for mass spectrometry) as described above. The reduced oligosaccharide was permethylated and passed over a Merckogel PVA 500 column (length, 30 cm; area, 0.7 cm^2; eluant, methanol; flow rate, 5 ml/h) for purification. The product was hydrolyzed, and the methyl sugars obtained were analyzed by gas-liquid chromatography-mass spectrometry of the alditol acetates as cited (e.g., 7) and detailed previously (14).

Substrate specificity of the phage 29 glycanase. (i) Effect of phage 29 particles on derivatives of K29 polysaccharide. Mildly alkali-treated, carboxyl-reduced, periodate-oxidized and borohydride-reduced, as well as depyruvylated, K29 polysaccharide was prepared as described previously (14). The materials (2 mg/ml of PBS) were incubated (18 h at 37 C) with 3×10^{10} PFU of phage 29 particles per ml. The reducing power of all derivatives—before and after incubation with phage—was determined by the Park-Johnson technique using glucose as a standard (compare 37).

(ii) Effect of phage 29 particles on heterologous bacterial capsular polysaccharides. *E. coli* serotypes 26, 27, 28, 30, 31, 34, 38, and 42, as well as the *Klebsiella* serotype 11 capsular polysaccharides were all isolated by the phenol-water-Cetavlon procedure (23, 33, 35) and checked for homogeneity by analytical ultracentrifugation (after mild alkali treatment), as well as by Ouchterlony agar gel double diffusion against a rabbit OK serum (compare 14). Solutions of all polymers (2 mg/ml of PBS) (the pneumococcus type III capsular polysaccharide was a gift from M. Heidelberger, New York University College of Medicine) were incubated in Ostwald viscosimeters with phage 29 particles (3×10^{10} PFU/ml) as above, and the viscosity numbers (at 37 C) were determined before and after incubation.

(iii) Extended host range. Portions (50 μl) of a phage 29 suspension containing 2×10^{11} PFU/ml were placed on freshly seeded lawns of 72 *Klebsiella* K antigen test strains (see 34) on broth agar plates and incubated overnight. As a control, the same amount of phage was also incubated on an outgrown (18 h at 37 C) and then heat-killed (90 min at 60 C) lawn of the host. The relative efficiency of plating (EOP) on *E. coli* K29-*Klebsiella* K31 was accurately titrated, and simulation of this cross-reaction by lysogeny of *E. coli* Bi161/42 was excluded by testing a culture filtrate of this strain.

RESULTS

Inactivation and adsorption of E. coli capsule bacteriophage 29 by isolated host capsular polysaccharide. After 1 h at 37 C in broth, roughly 100 μg (ID_{50}) of K29 glycan per ml was found to cause half-inactivation of 8×10^3 PFU/ml.

Following the procedure of Taylor and Taylor (52), formalinized sheep erythrocytes were modified with K29 polysaccharide (also with labeled polymer to determine the extent of modification) and then used to absorb phage from suspension. Under the conditions employed (see above), and independent of subsequent contact with phage, about 40% (viz., 4 or 8 μg) of the glycan used was found to adhere to the red cells (0.05 ml), but no measurable adsorption of phage (from 7×10^6 or 10^5 PFU in 1 ml) to the modified erythrocytes was detected after between 2 and 10 min of incubation at 0 or 37 C.

Depolymerization of isolated K29 polysaccharide as catalyzed by phage 29 particles. Conditions of depolymerization. Originally, the depolymerization reactions were carried out in "P buffer," i.e., growth medium (46) without glucose and amino acids. Since, however, the addition of NH_4Cl_4, $MgSO_4$ and $CaCl_2$, was then found to be unnecessary (the depolymerization was not inhibited by EDTA either), phosphate buffers were used as a medium for all further experiments.

The temperature and pH stability range of the viral enzyme after 48 h of storage were found to extend to about 40 C (at pH 7), and from about pH 6.5 to 9 (at room temperature) (compare 37). The temperature and pH optima of the depolymerization reaction, about 60 C and pH 4.2, were determined as shown in Fig. 1. Because of the limited stability of the enzyme, all further reactions were carried out at 37 C in PBS of pH 7.

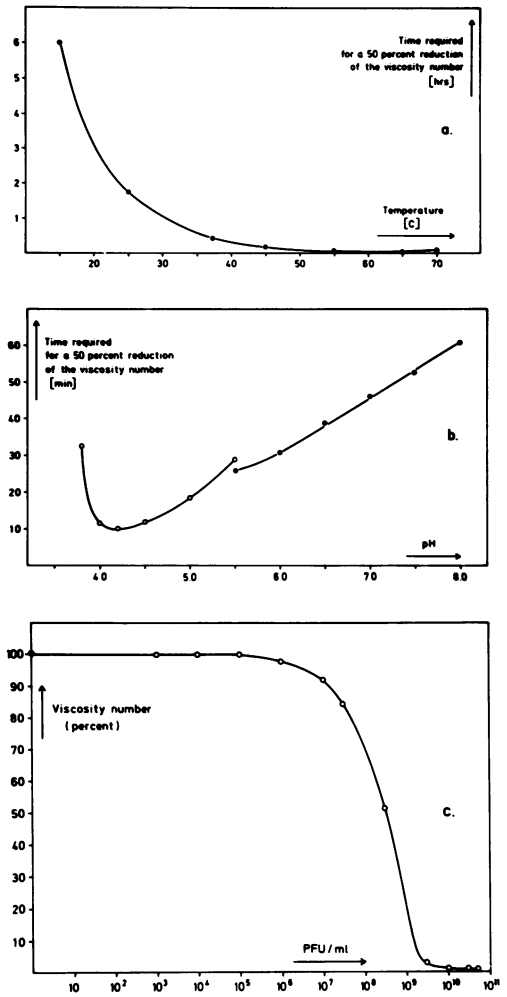


FIG. 1. *Conditions of depolymerization of E. coli K29 polysaccharide by phage 29 particles. (a) Temperature dependence. Solutions containing 2 mg of polysaccharide and 3 × 10¹⁰ PFU per ml of 0.15 M sodium phosphate buffer, pH 7, were incubated in Ostwald viscosimeters at temperatures between 15 and 70 C. The efflux times were determined at intervals, and the results were expressed as the time required for a 50% reduction of the viscosity number. (b) pH dependence. The same experimental procedure was used, but the reactions were carried out at 37 C throughout using 0.1 M citric acid-sodium phosphate buffers for pH 3.5 to 5.5 (○) and 0.15 M sodium phosphate buffers for pH 5.5 to 8 (●). (c) Phage concentration. Solutions containing increasing concentrations of phage and 0.2% (wt/vol) polysaccharide in PBS were incubated for 18 h at 37 C and the viscosity numbers were then determined.*

Figure 1 also shows the results of an experiment designed to determine the concentration of phage necessary for maximal depolymerization of a polysaccharide solution within a reasonable period of time. It can be seen that, after 18 h of incubation, 10^{10} PFU/ml or more had caused a complete loss of viscosity in a 2 mg/ml solution of the polymer. A 15-μg amount of the products obtained by the action of 3×10^{10} PFU/ml was found to exhibit a reducing power equivalent to 1 μg of glucose, and this value was not affected by further incubation (up to 42 h).

The conditions ("standard conditions") thus established—viz., incubation of a 0.2% (wt/vol) solution of polysaccharide in PBS with about 3×10^{10} PFU/ml for 18 h at 37 C—were employed for all subsequent depolymerization reactions.

Depolymerization products. Larger samples of K29 polysaccharide were incubated with phage 29 particles under standard conditions, and the depolymerization products were separated by ion-exchange chromatography (see Fig. 2) and checked for homogeneity by paper electrophoresis.

All three K29 oligosaccharides—which were obtained with a yield of 30 to 35% (P1), 20 to 25% (P2), and 30 to 35% (P3) (wt/wt, polysaccharide)—were found to consist of Glc:Man:GlcUA:Gal:pyruvate in a molar ratio approaching 2:2:1:1:1 as the polymer (14).

For an identification of the reducing and the nonreducing end sugars, the isolated oligosaccharides were reduced with $NaBH_4$-NaB^3H_4

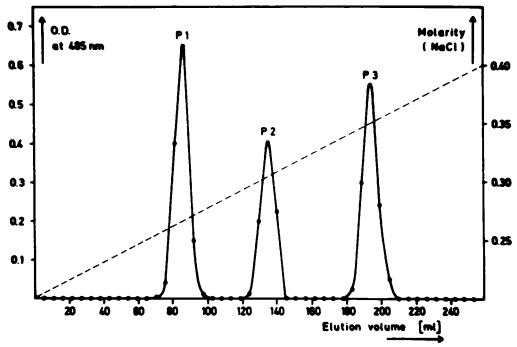


FIG. 2. *Separation of K29 polysaccharide degradation products by ion-exchange chromatography. The mixture of oligosaccharides, produced by extensive phage action upon 50 mg of glycan, was desalted and absorbed to a DEAE-Sephadex A25 column (length, 50 cm; area, 7 cm²) from a 0.05 M Tris-hydrochloride buffer, pH 7, containing 0.2 M NaCl. After washing, oligosaccharides P1, P2, and P3 were eluted (5.4 ml/h) with a linear 0.2 to 0.4 M NaCl gradient in the same buffer. The fractions (2.7 ml) were analyzed for carbohydrates with the phenol-sulfuric acid reagent (19), reading the extinction at 485 nm. Yields (after desalting and lyophilization): P1, 15 to 17.5 mg (30 to 35%); P2, 10 to 12.5 mg (20 to 25%); P3, 15 to 17.5 mg (30 to 35% wt/wt, from the polymer). For further experimental details see Materials and Methods.*

and hydrolyzed, and the hydrolyzates were analyzed for radioactive hexitols (Fig. 3), or oxidized with periodate, reduced with $NaBH_4$, and analyzed for uronic acid (Table 1). It can be seen that all depolymerization products were found to end in reducing glucose, and that periodate destroyed all, one-half, or one-third of the glucuronic acid, which is periodate stable in the polymer (14, 33) in oligosaccharides P1, P2, or P3, respectively.

Besides identifying GlcUA as the nonreducing end sugar, the results of periodate oxidation also indicated that the size of the depolymerization products corresponded to one (P1), two (P2), and three (P3) hexasaccharide repeating units of K29 polysaccharide (see 14). This was confirmed by comparative glucose analysis before and after reduction with $NaBH_4$, as well as by direct molecular weight determination according to Moyer and Isbell (32). The results are included in Table 1.

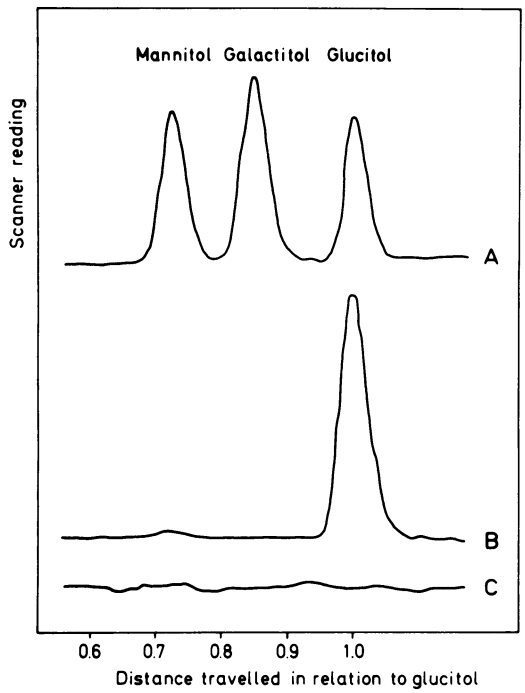


FIG. 3. *Reducing end sugar identification. K29 oligosaccharide P1 was reduced with $NaBH_4$/NaB^3H_4 and hydrolyzed, and the hydrolyzate was analyzed for radioactive hexitols by paper electrophoresis in arsenite buffer; the electropherogram was analyzed with a Packard radiogram scanner. (A) Radioactive standards, (B) sample, (C) K29 polysaccharide, reduced and hydrolyzed in the same manner (control). Oligosaccharides P2 and P3 gave the same results. Under the electrophoresis conditions used, L-gulonic acid shows a mobility of 1.86 relative to glucitol; the samples did not yield any radioactivity in this region of the electropherograms. The experimental details have been described previously (45).*

TABLE 1. *Quantitative reducing (D-glucose) and nonreducing (D-glucuronic acid) end sugar analysis and molecular weight determination of K29 oligosaccharides P1, P2, and P3*

Oligosaccharides[a]	% D-Glucose reduced by sodium borohydride[b]	% D-Glucuronic acid oxidized by periodate[c]	Mol wt[d]
P1	52	92	1,065
P2	23	46	2,045
P3	17	27	3,070

[a] Compare Fig. 3.

[b] Glucose was determined with fungal glucose oxidase in hydrolyzates of the oligosaccharide before (= 26.9 ± 1.7%, wt/wt, anhydro Glc) and after reduction with sodium borohydride.

[c] Using the carbazole-sulfuric acid method (18), the oligosaccharides were analyzed for uronic acid before (= 16.6 ± 0.6%, wt/wt, anhydro GlcUA) and after oxidation with periodate and subsequent reduction with sodium borohydride.

[d] Weighed samples of the dry oligosaccharides were labeled with $Na^{14}CN$, and the radioactivity was determined relative to a glucose standard treated in the same manner (32). A molecular weight of 1,056 is calculated for the hexasaccharide repeating unit of K29 polysaccharide (see Discussion).

Oligosaccharide P1 was also reduced with sodium borodeuteride in deuterium oxide, permethylated, and hydrolyzed, and the methyl sugars obtained were analyzed by gas-liquid chromatography-mass spectrometry as the alditol acetates (7). The results are given in Table 2. It can be seen that the following compounds, in a molar ratio approaching 1:2:1:1, were identified: 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-hexitol, monodeuterated in position 1; 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-mannitol; 1.3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol; and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-glucitol.

Substrate specificity. Some derivatives of K29 polysaccharide, prepared as described in the accompanying paper (14), were each incubated with phage 29 particles under standard conditions (see above), and the materials were analyzed for reducing power before and after phage action. The results are given in Table 3. It can be seen that carboxyl reduction, periodate oxidation followed by borohydride reduction, as well as depyruvylation all led to protection of K29 polysaccharide against action of the phage enzyme.

Under the same experimental conditions, ten heterologous bacterial capsular polysaccharides

TABLE 2. *Gas-liquid chromatography-mass spectrometry of the O-acetyl-O-methyl-alditols obtained from deuterated-reduced and permethylated K29 oligosaccharide P1*[a]

Alditol derivative[a, b]	T[c]		Primary fragments found (m/e)										Ratio of peak areas[a, d]
	Literature	found	45	89	117	133	161	189	205	233	249	261	
1.2.4.5.6-Glc[e]		0.38	+[f] (46)	+[f] (90)		+			(206)[g]		+[f] (250)		1.0
3.4.6-Man[h]	1.95	1.98	+				+	+	+	+			2.2
2.4.6-Gal	2.28	2.31	+		+		+			+			0.9
2.3-Glc[h]	5.39	5.39			+		+					+	1.0

[a] The methylated derivative of GlcUA is not obtained under the conditions used.

[b] 1.2.4.5.6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol, etc.

[c] Retention time relative to 2.3.4.6-Glc (T = 1.00) and 2.3-Glc (T = 5.39) on an ECNSS-M column at 170 C (7).

[d] Height times breadth at half height.

[e] Identified as a Glc derivative by the deuterium label (compare Fig. 4).

[f] Both normal and monodeuterated fragment found.

[g] Only monodeuterated fragment found.

[h] For identification compare also reference 14.

TABLE 3. *Action of E. coli K phage 29 particles on derivatives of K29 polysaccharide*

Substrate[a]	Material (μg) equivalent to 1 μg of glucose in reducing power	
	Before phage 29 action[b]	After phage 29 action[b]
E. coli K29 capsular polysaccharide		
Native	1,600	15
Alkali treated[c]	1,600	23
Periodate oxidized and reduced with $NaBH_4$	406	417
Carboxyl reduced[d]	889	752
Depyruvylated[e]	179	171

[a] The preparation of all substrates is described in the accompanying paper (14).

[b] The polymers (2 mg/ml of PBS) were incubated (18 h at 37 C) with purified phage 29 particles (3×10^{10} PFU/ml), and the resulting mixture—as well as the same materials before phage treatment—was analyzed for reducing power by the Park-Johnson method with glucose as a standard (for further details see 37).

[c] After mild alkali treatment, the polysaccharide exhibits an altered sedimentation behavior (14) and is seen, by electron microscopy, to have decayed into filamentous subunits (M. E. Bayer and H. Thurow, submitted for publication).

[d] Carboxyl reduction was achieved by esterification with ethylene oxide and subsequent reduction with sodium borohydrode. As determined by the carbazole-sulfuric acid technique (18), 80 to 85% of the glucuronic acid residues were reduced in this manner.

[e] Hydrolysis with 0.125% aqueous oxalic acid (2 h at 100 C) removed 95% of the pyruvate ketal residues (but caused some hydrolysis of the polysaccharide chain also).

(*E. coli* serotype 26, 27, 28, 30, 31, 34, 38, 42, *Klebsiella* serotype II, and pneumococcus serotype III), as well as colanic acid (M antigen), were incubated with phage 29 virions, and the solutions were tested for loss of viscosity. No significant drop of the viscosity number (i.e., below 90% or the value before phage action) was observed in any of these cases.

An extension of the phage 29-host range experiments (compare 46) using 72 *Klebsiella* K antigen test strains (34) was also carried out. With the exception of *Klebsiella* 6258 (K31), which was lysed by phage 29 with a relative EOP (on *E. coli* K29-*Klebsiella* K31) of 7 to 8, all strains were found to be resistant (relative EOPs on *E. coli* K29-Klebsiella Kn > 10^{10}). Since the amount of phage applied (10^{10} PFU) was amply sufficient to cause (under the same conditions of incubation) visible decapsulation and O agglutinability of an outgrown and then heat-killed lawn of the host bacteria, the experiment allowed conclusions not only on the host range of phage 29 but also on the substrate specificity of the glycanase associated with it.

DISCUSSION

E. coli capsule bacteriophage 29 (46) is a small virus consisting of an isometric head (about 45 nm in diameter) that carries a six-pointed base plate (14 nm in diameter) with six (or possibly, a multiple thereof) roughly cylindrical spikes (5 by 15 nm) linked to it (37). Only nine different polypeptides were detected in the virions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two of which (about 29,500 and 57,000 daltons) occur in the spikes

(37) which obviously establishes first contact with the host surface.

The cells of *E. coli* Bi161/42 (09:K29(A):H$^-$), the host of phage 29, are surrounded by thick (300 to 400 nm as seen in India ink preparations) heteropolysaccharide capsules (46; M. E. Bayer and H. Thurow, submitted for publication). The primary structure of this polysaccharide (the *E. coli* serotype 29 capsular glycan) has been elucidated (14), its strands (both after extraction as well as in situ, where they extend radially from the cell surface) have been visualized by electron microscopy (M. E. Bayer and H. Thurow, submitted for publication), and its conformation is tractable by present day methods. Structural details of the cell wall components underneath the capsule of *E. coli* Bi161/42 have also been established and are under further investigation (see Discussion in accompanying paper [14]).

The comparatively simple structures both of the phage 29 virions (especially also of their receptor complementary organelles) and of the outer cell surface component of its host, as well as the rather advanced chemical analyses of both "reactants", make this system a suitable model for attempts to elucidate the molecular basis of bacteriophage-host surface interactions, notably of the various phage-receptor relationships enumerated above.

(i) Inactivation. The results of inactivation of phage 29 by isolated host capsular polysaccharide suggest that the viruses do not eject their nucleic acid on contact with the host capsule. This follows from the finding (U. Feige, unpublished data) that one colony-forming *E. coli* Bi161/42 cell has a dry weight of about 7.5×10^{-3} g: even if half of this weight is assumed to be capsular polysaccharide (actually, 7.5% [wt/wt] was obtained by the phenol-water-Cetavlon procedure [14]), the ID_{50} shows that the glycan from a very large number of bacteria is needed to inactivate one phage within 1 h—almost three latent periods (46). Our experiments do not exclude one alternative explanation, viz., that the capsular polysaccharide in situ, in its natural conformation on the bacterial surface, does induce phage 29 to eject its genome, but that this conformation is destroyed during the extraction procedure (compare 24, 28, 58). This possibility is, however, excluded by the electron optical findings of M. E. Bayer and H. Thurow (submitted for publication). One explanation for the small extent of inactivation obtained is the assumption that the K29 polysaccharide preparations were contaminated with minute amounts of other surface constituents, possibly the "knob-like" elements seen by M. E. Bayer and H. Thurow (submitted for publication).

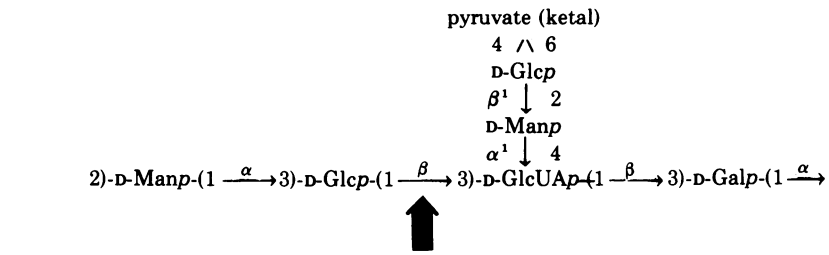
(ii) Host mutants. As previously reported (46), phage 29 does not absorb to and infect acapsular host mutants (relative EOP on K$^+$/K$^-$ $> 10^{10}$). This may be taken to indicate that the viruses are altered in some way by the polysaccharide capsule: contact with the host cell wall seems to lead to infection only, if the phages had contact with the capsule first.

(iii) Electron microscopy. As stated above, our work was substantially complemented by the excellent electron optical investigations carried out in parallel by M. E. Bayer and H. Thurow (submitted for publication). Among other results, these authors showed that phage 29 penetrates the host capsule without ejection of its nucleic acid, leaving, at 0 C (but not at 37 C), a visible "tunnel" behind it, that ejection of the genome does occur upon contact with the cell wall underneath, and there, generally, at specific sites where cell wall and cytoplasmic membrane adhere in plasmolyzed bacteria (1-3), and that upon temperature shift of host mutants exhibiting temperature sensitivity of capsule formation, newly synthesized K29 polysaccharide threads appear to emerge from these same sites of adhesion.

(iv) Adsorption to carriers coated with K29 polysaccharide. Erythrocytes, coated with host capsular polysaccharide, do not absorb significant amounts of phage 29 (i.e., $< 2 \times 10^4$ PFU/μg; compare 27, 43, 47, 52), either at 0 C (where depolymerization is very slow; see Fig. 1a) or at 37 C (although the K29 oligosaccharides formed remain attached to the red cells). By electron microscopy, in contrast, phage 29 particles were seen to adhere to the polymer (in situ, or isolated) at 0 C, whereas, at 37 C the attachment also remained doubtful (M. E. Bayer and H. Thurow, submitted for publication). These results, which are corroborated by reports on a similarly labile phage-receptor attachment in related systems (at 37 C, Vi phages II and III do not absorb to Vi. polysaccharide-coated erythrocytes either [47, 52], and the empty capsids of these bacteriophages fall off the infected host cells again after ejection of the viral genome [48, 51]) are very difficult to interpret. One explanation is offered by the (totally unproven) assumption that a comparatively firm attachment is achieved during the infective process (and only then) by contact of several spikes with properly positioned glycan molecules (see 30).

(v) K29 polysaccharide as a substrate of phage 29 depolymerase. In a previous note (45) we have shown that there is a depolymerase

activity associated with particles of phage 29 which liberates reducing glucose in host capsular glycan. The active center of this enzyme is



part of the virus spikes, and, within these organelles, part of subunits containing polypeptides of 57,000 daltons only (37).

In conjunction with the primary structure of K29 polysaccharide (14), the results presented justify the following conclusions about the viral enzyme. (i) Phage 29 depolymerase catalysis is independent of bivalent cations and shows a pH optimum around 4.2 (Fig. 1); the enzyme is not stable above 40 C, and beyond about pH 6.5 to 9 (compare 37). (ii) Catalysis by phage 29 particles ultimately leads to a quantitative depolymerization of K29 glycan to oligosaccharide fragments P1, P2, and P3 (Fig. 2). (iii) β-D-Glucosido-(1 → 3)-D-glucuronic acid bonds (arrow) are split exclusively, and P1, P2, and P3 thus all end in reducing glucose and nonreducing glucuronic acid (Fig. 3; Table 1 and 2); they have the size of one, two, and three K29 hexasaccharide repeating units (Table 1). (iv) The enzyme catalyzes a hydrolysis, and not a β-elimination, as judged from the fact that the nonreducing end glucuronic acid residues in the oligosaccharides give a positive carbazole-sulfuric acid reaction before, but not after, periodate oxidation and sodium borohydride reduction (Table 1). The finding (F. Fehmel, Ph.D. thesis, Freiburg University, 1972) that phage 29 action on a K29 polysaccharide solution results in a fast loss of viscosity concomitant with a comparatively slow increase in reducing power further seems to indicate that the enzyme acts by an endo mechanism. (v) Phage 29 glycanase is highly specific. It does, for instance, not tolerate the removal of the pyruvate ketal, or the reduction of the GlcUA residues in the substrate (Table 3), and depolymerizes but one—the *Klebsiella* serotype 31 capsular polysaccharide—of 82 heterologous bacterial glycans tested, among these the pneumococcus type III and the *Klebsiella* type 11 capsular polysaccharides which also contain the β-D-glucosido-(1→3)-D-glucuronic acid disaccharide (Table 4). Unfortunately, the structure of the *Klebsiella* K31 polysaccharide is not known; it is, however, interesting to note that it contains the same sugar monomers as the *E. coli* K29 glycan (34).

The results presented also verify some conclusions on the structure of the K29 polysaccharide discussed in the accompanying paper (14). The finding that the glycan is depolymerized by cleavage of Glc→GlcUA bonds shows that they are part of the chain (and not of the branch), and the periodate lability of nonreducing GlcUA in P1, P2, and P3 (Table 1) justifies the conclusion that Glc is linked to position 3 and Man to position 4 of the GlcUA (and not vice versa).

The following hypothetical model, which is similar to those of other authors (e.g., 3, 51; compare also M. E. Bayer and H. Thurow, submitted for publication), would account for

TABLE 4. *Structure of partially homologous bacterial capsular polysaccharides not depolymerized by phage 29 particles*

Polysaccharide	Structure
Pneumococcus serotype III	4)-D-Glcp-(1—β→3)-D-GlcUAp-(1—β→
Klebsiella serotype 11[a]	Pyruvate(ketal) 4 /\ 6 D-Galp α¹↓4 3)-D-Glcp-(1—β→3)-D-GlcUAp-(1—β→3)-D-Galp-(1—α→

[a] See H. Thurow et al. (Carbohydr. Res., in press).

most of the findings on phage 29-host capsule interaction. After contact of several phage 29 spike tips with the ends or sides of K29 polysaccharide molecules, the virus glucanase subunits (possibly capable of conformational alterations) move along the polymer strands, the cleavage of glycosidic bonds opening a path for the phage head and providing the energy for the movement. Such a mechanism might lead both to a vertical (through the capsule) as well as to a lateral (to the sites of cell wall-membrane adhesion where the capsule strands originate) movement of the virions relative to the host surface. Although nothing is known about the molecular events after this first stage, the finding that phage 29 ejects its nucleic acid upon contact with the host cell wall (and outside the cytoplasmic membrane) seems to imply the existence of a second receptor, as well as a mechanism akin to transfection for the final uptake of the viral genome by the host cell. Also, in the case of the *E. coli* K29 system, the "bacteriophage receptor" appears to resolve into several "substrates and receptors of viral tail organelles," a view which may generally be helpful for a better understanding of discrepancies in phage receptor identification (see above).

Although this model obviously has to be substantiated by much further experimentation and actually fails to explain some of the available facts (notably the finding [M. E. Bayer and H. Thurow, submitted for publication] that phage 29 can penetrate the host capsule at 0 C where glycanase action is exceedingly slow, which may be taken to indicate an involvement of preformed capsular pores [compare 42]), some traits of it may well be of more general validity, e.g., also for the interaction of most Vi phages (27, 47, 48, 50, 51), P22 (22), ϵ^{15} (49), and Ω8 (36) with the respective host surface polysaccharides, and possibly for many other, especially Bradley group B and C virions (8) infecting host cells with capsules or thick cell walls. It is also worth noting that this model has some elements in common with virus-host cell (or cell organelle) surface interactions observed with animal viruses, notably the host cell surface carbohydrate degradation by *Myxovirus* neuraminidases and *Adenovirus* movement to and interaction with nuclear pores (see 17).

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