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HOMOLOGOUS AND HETEROLOGOUS REACTIONS OF BACTERIOPHAGES ϕ41 AND ϕ12 ON THE CAPSULAR POLYSACCHARIDES FROM *Klebsiella* K41 AND K12

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ABSTRACT

The structures of the capsular polysaccharides from *Klebsiella* K41 and K12 are very similar and differ only in the lateral, terminal group of their respective repeating units. The bacteriophages ϕ41 and ϕ12 are shown to hydrolyze the same α-galactopyranosyl bond in each of the polysaccharides, giving rise to an oligosaccharide characteristic of the starting polysaccharide, irrespective of the phage employed.

The presence of the uronic acid function is essential for the phages to be active, but the carboxyl group of the pyruvic acetal in K12 does not appear to play a role in the recognition process.

INTRODUCTION

In a systematic survey of the bacteriophages exhibiting endoglycanase activity on the capsular polysaccharides of *Klebsiella,* Rieger-Hug and Stirm[1] showed that a high degree of specificity is exhibited by these bacterial viruses and, at the same time, recorded certain cross-reactions, including those involving ϕ41 and ϕ12. Prior to the publication of this survey, we had noted the close similarity in structure of the capsular polysaccharides from the serotypes *Klebsiella* K41 (ref. 2) and K12 (ref. 3) and were interested in possible cross-reactions of the respective bacteriophages.

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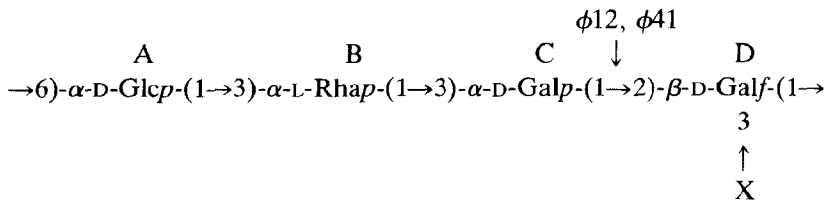


X = β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1 K41 (ref. 2)
G F E

= 4,6-*O*-(1-carboxyethylidene)-β-D-Gal*p*-(1→4)-β-D-Glc*p*A-(1 K12 (ref. 3)
G F E

= 5,6-*O*-(1-carboxyethylidene)-β-D-Gal*f*-(1→4)-β-D-Glc*p*A-(1 K12 (ref. 4)
G F E

The structure for K12 originally published[3] has an *O*-(1-carboxyethylidene) residue linked to positions 4 and 6 of a D-galactopyranose residue. It has now been shown[4] that the acetal is linked to positions 5 and 6 of a D-galactofuranose residue. It should be noted, in particular, that the gentiobiose unit (GF) in the K41 structure is replaced in K12 by an *O*-(1-carboxyethylidene)-D-galactose residue (GF)*, and that in both structures a D-galactofuranose residue constitutes the branch point (D).

The present paper reports the action of the bacteriophages φ41 and φ12 on their homologous substrates K41 and K12 (and modified polysaccharides obtained therefrom), together with a study of the action of φ41 on K12 and, to a lesser extent, of φ12 on K41. While φ41 and φ12 were both known[1] to exhibit galactosidase activity, the precise site of enzymatic hydrolysis remained to be defined. This was the objective of the present investigation.

The isolation of oligosaccharides corresponding to the repeating units of K41 and K12 made possible a detailed examination of their n.m.r. spectra which led to a revision of the K12 structure. The spectral data are discussed in the accompanying paper[4].

RESULTS AND DISCUSSION

Propagation of bacteriophages. — Bacteriophages were isolated from sewage and propagated on their host strains following standard procedures. Bacteriophage φ12 could at best be prepared in concentrations of 10^9 plaque forming units (p.f.u.)/mL, while φ41 had a low burst rate and titres greater than 10^8 p.f.u./mL could not

*In this and the accompanying paper, in any discussion of the K12 oligo- or poly-saccharides, the letter G is used to refer to the pyruvate acetal. Although the pyruvate group is not a monosaccharide residue, this usage emphasizes the similarities between the structures of K12 and K41 and simplifies discussion. All sugars are pyranoid unless otherwise indicated.

TABLE I

GEL PERMEATION CHROMATOGRAPHY OF OLIGOMERS OBTAINED BY ACTION OF ϕ41 ON K41 POLYSACCHARIDE

Oligomer	K_d[a]	*d.p.*	*Repeating units (n)*	*M.W.*[b]
K41-P1	0.49	7	1	1150
K41-P2	0.28	14	2	2282
K41-P3	0.16	21	3	3414
K41-P4	0.09	28	4	4546
K41-P5	0.07	35	5	5678

[a]Partition coefficient on Bio-Gel P6 with 50mM $NaNO_3$ as eluent. [b]Free acid (H^+) form; M.W. = 1132n + 18.

be obtained. Depolymerization had, therefore, to be conducted with 10^{10} p.f.u./g of polysaccharide rather than with the more usual application of 10^{13} p.f.u./g. Furthermore, suspensions of ϕ41 lost much of their titre over a period of weeks. The titre of ϕ12 remained essentially stable.

Action of bacteriophage ϕ41 on Klebsiella *K41 polysaccharide.* — The action of ϕ41 on K41 yielded five oligosaccharides, which were separated by gel filtration and designated K41-P1 through K41-P5 (Table I); P1 is the heptasaccharide (d.p. 7) corresponding to one repeating unit and P2–P5 are simple multiples thereof (M.W. 2282–5678). When log K_d was plotted against d.p., a straight line was obtained for K41-P1 to K41-P4, but the point for K41-P5 lay above this line, perhaps because of a change in conformation[5].

The time for the viscosity of the solution to fall to 50% of its initial value (t_{50}) was, for ϕ41/K41, 42 min, and after 2 h the viscosity remained essentially constant up to 15 d, although the composition of the mixture of oligosaccharides changed (Fig. 1). This figure shows that K41-P2 is the main product, and the fact it cannot be cleaved by ϕ41 implies that K41-P3 is the smallest unit accomodated by the viral

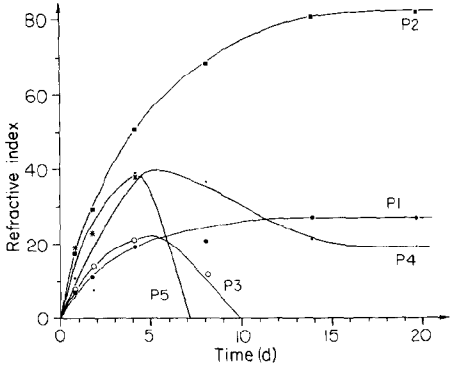


Fig. 1. Distribution of oligomers produced from K41 CPS by action of phage ϕ41. Depolymerization conditions: 4.15mM K41 (90 mg at 4.7 g/L; ϕ41, 4×10^9 p.f.u./g of K41).

TABLE II

GEL PERMEATION CHROMATOGRAPHY OF OLIGOMERS OBTAINED BY ACTION OF ϕ41 ON NATIVE AND MODIFIED K12 POLYSACCHARIDE

Oligomer	K_d[a]	*d.p.*	*Repeating units (n)*	*M.W.*[b]
K12-P1	0.40	6	1	1058
K12-P2	0.19	12	2	2098
K12-P3	0.10	18	3	3138
K12 – GF-P1	0.56	5	1	826
K12 – GF-P2	0.35	10	2	1634
K12 – GF-P3	0.22	15	3	2442
K12 – GF-P5	0.09	25	5	4058

[a]See Table I. [b]Form H^+; K12, M.W. = 1040n + 18; K12 – GF, M.W. = 808n + 18.

enzyme[5]. After ~14 d the composition of the mixture of oligomers remained constant, and P4 was not degraded further, probably because of an inhibition of the enzyme action by the products of the reaction. Furthermore, a residual amount of polysaccharide was still present at this stage.

When a sample of K41 polysaccharide was carboxyl-reduced[6] and the product (K41-CR) was treated with ϕ41, an initial, small fall in viscosity was observed (η_{sp} from 0.68 to 0.64 in 10 min), but thereafter only a slight reduction was noted, and at the end of 8 d no oligosaccharides could be obtained by gel filtration on Bio-Gel P2 or P6. The initial fall is no doubt due to a small amount of native polysaccharide which escaped reduction, and the results indicate that ϕ41 is essentially inactive on the neutral polysaccharide (K41-CR).

Action of bacteriophage ϕ41 on Klebsiella *K12 polysaccharide.* — Gel filtration of the oligosaccharides obtained by phage action yielded K12-P1 (18%), K12-P2 (19%) and some K12-P3 which was incompletely separated from higher oligomers (Table II). A plot of log K_d vs d.p. gave a straight line, with greater slope than in the case of K41 oligosaccharides due to the presence in the repeating unit of two anionic groups, uronic acid and pyruvic acid acetal. For rather similar molecular weights, therefore, the oligomers of K12 exhibit dramatically greater

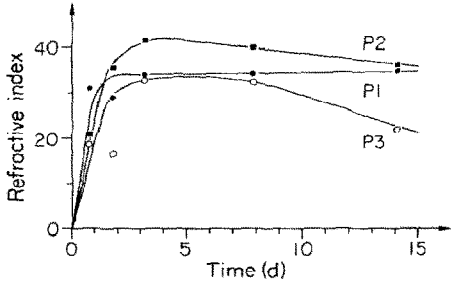


Fig. 2. Distribution of oligomers produced from K12 CPS by action of ϕ41. Depolymerization conditions: 4.52mM K12 (90 mg at 4.7 g/L; ϕ41, 4×10^9 p.f.u./g of K12).

hydrodynamic volumes than those of K41 and, accordingly, lower values of K_d.

An aqueous solution of K12 polysaccharide had an initial, high viscosity of η_{sp} 5.54 which was reduced by the phage to a minimum value of η_{sp} 0.20 in ~20 h. It was interesting to note that t_{50} was 3 min (η_{sp} 2.77) and on this basis one may assess the action of ϕ41 on K12 as 14 times more effective than the homologous reaction of ϕ41 on K41.

The rates of production of K12-P1, -P2, and -P3 are shown in Fig. 2, where it may be seen that, unlike the case of ϕ41, P1 is formed slightly faster than P2, and that P2 seems to be slowly cleaved to smaller units (P1), as can be shown by its steadily decreasing concentration.

Action of bacteriophage ϕ41 on modified K12 polysaccharides. — Carboxyl-reduced (K12-CR) polysaccharide[6] showed an initial small fall in viscosity from η_{sp} 1.15 to 1.03 in 10 min, remaining at η_{sp} 0.62 after 8 d, but no oligosaccharides could be detected by gel filtration. In view of the apparent requirement of the phage for a negative charge on the substrate, it was then of interest to investigate the action of ϕ41 on K12 polysaccharide with the pyruvate acetal removed (K12 minus G, K12 − G)[4]. Pyruvate-acetal groups may be removed by autohydrolysis, but ^{1}H- and ^{13}C-n.m.r. spectroscopy showed that complete removal of the pyruvate was accompanied by extensive depolymerization and loss of the galactose residue carrying the acetal substituent; the product is, therefore, designated K12 − GF (ref. 4). Viscometry could not be used to follow the reaction of ϕ41 on K12 − GF due to this depolymerization, but gel filtration of the products obtained demonstrated the formation of K12 − GF-P1, -P2, -P3, and -P5 (Table II). A plot of log K_d vs d.p. has a smaller slope than in the case of the native K12 oligomers, the K12 − GF oligosaccharides bearing now only one anionic charge per repeating unit. Their hydrodynamic volumes, therefore, are smaller and more similar to those observed for the K41 oligomers (*cf.* Table I).

The kinetic data (Fig. 3) show that K12 − GF-P2 is produced most quickly, and in greatest amount, whereas K12 − GF-P1 is formed only in small amounts. It may be deduced that the phage is incapable of cleaving K12 − GF-P2 into K12 − GF-P1 (*cf.* K12), and that the smallest oligosaccharide recognized and cleaved by ϕ41 is K12 − GF-P3, as for K41-P3, the concentration of which is reduced to zero after ~14 d.

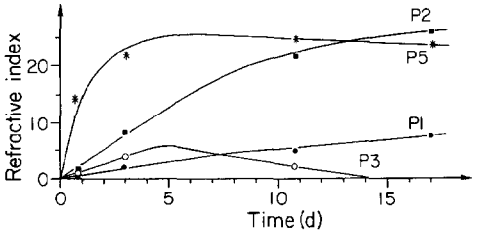


Fig. 3. Distribution of oligomers produced from K12 − GF by action of phage ϕ41. Depolymerization conditions: 3.71mM K12 − GF (45 mg at 3 g/L; ϕ41, 4 × 10^9 p.f.u./g of K12 − GF).

The high M.W. oligomer was designated K12 – GF-P5 on the basis of its K_d value, assuming no conformational changes at higher d.p. The fact that K12 – GF-P5 is formed fastest and in a large amount is probably due to a lesser ability of ϕ41 to attack this modified polymer. Here also a residual amount of polysaccharide survived.

The modification of the side chain of K12 polysaccharide has a significant effect. Thus, the dimeric repetitive unit (K12 – GF-P2) is not susceptible to cleavage as has also been observed[5] in a study on *Klebsiella* K19/ϕ19. This suggests that the action of a phage is closely linked to the conformation of the different substrates (polysaccharides and corresponding oligosaccharides) which, in turn, is dependent on the structure of the side chain. In the case of K12 – GF it is difficult to distinguish the effect of the length of the side chain from that of the absence of the pyruvate acetal. Since the viral enzyme is capable of cleaving K12 – GF it may be assumed that the charge carried by the pyruvate plays a minor, or even zero, role compared to the charge on the glucuronic acid. It should also be noted that the uronic acid is located closer to the linkage cleaved, which could account for its more dominant role.

The fact that K12 – GF-P3 is cleaved, but not K12 – GF-P2, may be explained in the following manner. The former oligosaccharide has three negative charges (uronic acids) of which two are single-unit side-chains in fixed positions, while the third is effectively at the end of a long carbohydrate chain and, therefore, has greater mobility. In contrast, the latter oligosaccharide lacks two negative sites in fixed orientations to each other. If it is assumed that the active site of the phage enzyme must recognize a structure where two negative charges are situated at fixed and ideal distance apart before hydrolysis can occur, the lack of reactivity of K12 – GF-P2 may be rationalized. The oligosaccharide K12 – GF-P3 is, therefore, the smallest oligomer which can resemble, in location of its negative charges, the original polysaccharide.

Action of bacteriophage ϕ12 on Klebsiella *K12 and K41 polysaccharides.* — Depolymerizations were carried out on a small scale and in each case the viscosity decreased rapidly. From K12 the oligosaccharide corresponding to K12-P1 was obtained, while the major product obtained from K41 was K41-P2.

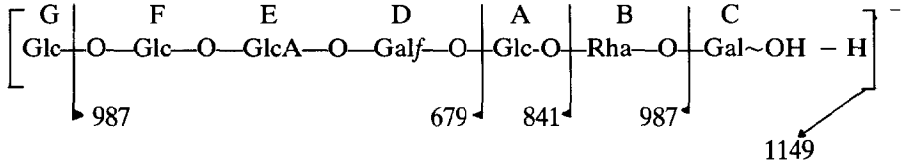
The size of the oligosaccharides could be ascertained from their n.m.r. spectra and confirmed, together with the nature of the reducing terminus (galactose), by the method of Morrison[7]. The oligosaccharide K12-P1 obtained by the homologous reaction was examined by ^{1}H-n.m.r. only, and the spectrum was shown to be identical to that of K12-P1 obtained by the heterologous reaction using ϕ41. In like manner the two samples of K41-P2, produced respectively by ϕ12 and ϕ41, were compared by ^{1}H- and ^{13}C-n.m.r.

The spectra of the compounds obtained by the action of bacteriophage ϕ12 are not given here, as they have been reported elsewhere[8]. They were found to be identical to the spectra of the corresponding oligosaccharides produced by ϕ41.

F.a.b.-m.s. of K41 and K12 oligosaccharides. — Oligosaccharides produced

from bacterial polysaccharides by viral endoglycanases are suitable substrates for f.a.b. mass spectrometry. Accordingly, K41-P1, K41-P2, an K12-P1 were examined by this technique in the negative- and positive-ion modes.

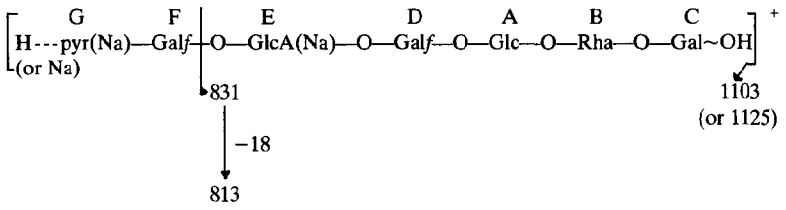
The negative-ion fragmentation of K41-P1 gave a pseudomolecular ion of *m/z* 1149 $(M - H)^-$, confirming the M.W. of K41-P1 as 1150. The ion of *m/z* 987 may arise by loss of either terminal hexose (G or C), while the ion of *m/z* 841 is



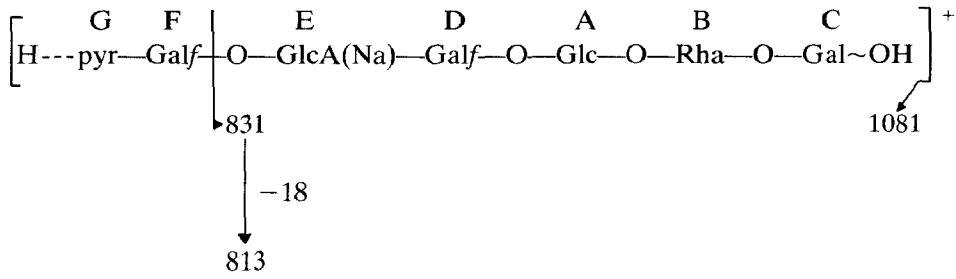
necessarily formed by the loss of two residues (B and C) at the reducing end. The ion of *m/z* 679 may, likewise, be formed by loss of three residues (A, B, and C) from the reducing end, or by a secondary fragmentation of the ion of *m/z* 841 by loss of the terminal, nonreducing unit (G).

The M.W. of K41-P1 having the uronic acid present as the sodium salt is 1172. The positive-ion spectrum thus exhibits the expected ions of *m/z* 1195 $(M + Na)^+$, and 1173 $(M + H)^+$ together with fragments of *m/z* 1033 and 1011 formed by the loss of the terminal glucose unit (G). No fragmenation was observed for K41-P2 (M.W. as Na salt 2326) and the only ions detected were those of *m/z* 2327 $(M + H)^+$ and 2349 $(M + Na)^+$, thus confirming the M.W. of this oligosaccharide.

The positive-ion spectrum of K12-P1 (M.W. 1102 as the disodium salt) showed strong signals for ions of *m/z* 1103 $(M + H)^+$ and 1081 $(M - Na + 2H)^+$ together with a weaker one of *m/z* 1125 $(M + Na)^+$. The only fragment observed



was that of *m/z* 831 (which may dehydrate to give 813), corresponding to the loss of G(Na)-F(H) or G(Na)-F(Na), M.W. 272 or 294, respectively, from the pseudomolecular ions of *m/z* 1103 or 1125. The formation of the ion of *m/z* 831 implies that the fragment eliminated is accompanied by either the proton or sodium of ionization. If this is not the case one should observe an ion of *m/z* 853 due to the loss of G(Na)-F(H), M.W. 272, from the ion of *m/z* 1125 $(M + Na)^+$. If the ion of *m/z* 1081 corresponds to the replacement of the sodium counter-ion of the pyruvate by a proton in the ion $(M + H)^+$ then one would expect the ion of *m/z* 831 by the loss of the units G-F(H) of M.W. 250.



CONCLUSION

These results indicate clearly that the bacteriophages ϕ41 and ϕ12 are capable of depolymerizing the polysaccharides of both their host and related strains. Taken in conjunction with the spectral evidence (see accompanying paper) the results show that both endoglycanases exhibit an α-D-galactopyranosidase activity, and that in both cases bond C–D in the substrate polysaccharides is cleaved, producing similar oligosaccharides. The absence of enzymatic activity *vs.* the carboxyl-reduced polysaccharides (K41-CR and K12-CR) confirms the importance of the uronic acid function in the recognition process, while the reactivity of the modified K12 polysaccharide, lacking the *O*-(1-carboxyethylidene)-D-galactose unit, indicates that this acidic function has little significance for the phage enzyme.

For each of the three oligosaccharides examined by f.a.b.-m.s. the molecular weights observed are in complete accord with the structures proposed, and this provides independent evidence for the d.p. and composition of these oligomers. The application of f.a.b.-m.s. to the study of bacterial polysaccharides of high molecular weight is made possible by their facile depolymerization by viral endoglycanases into oligosaccharides of low enough M.W. to be amenable to this mass spectrometric technique. This is a further example of the utility of bacteriophages in structural investigations, and it may be recalled that where the individual monosaccharide residues in an oligomer have distinguishable formula weights the sequence of the repeating unit may be established directly[9].

EXPERIMENTAL

General methods have been described previously[5,10,11]. Samples of sewage were incubated separately with cultures of *Klebsiella* K41 and K12 and aliquots were plated out on lawns of the host bacteria. Bacteriophages were detected by the appearance of plaques surrounded by haloes. Phages were purified by repicking several times from single plaques[1]. Depolymerizations were carried out in aqueous solution (pH ~7) in the presence of chloroform or NaN_3 to prevent bacterial growth. Oligosaccharides were recovered by dialysis (M.W. cutoff 3,500) against

distilled water or, after sterilization (15 min in a water batch at 100°), by gel filtration on Bio-Gel P6 (eluent, 50mM $NaNO_3$), and purified by passage through Amberlite IR-120 (H^+). Viscosity measurements were made (in 0.1M NaCl) continuously in an automatic apparatus (MS-Fica) using an Ubbelhode viscometer (capillary 0.58 mm) thermostatted at 37°.

K41-P1 had $[\alpha]_D^{23}$ −4.8° (*c* 1.21, water) and K41-P2 had $[\alpha]_D^{23}$ +15.5° (*c* 2.04, water).

Gas chromatography of peracetylated aldononitrile (PAAN) derivatives[7] to measure d.p. was performed using a column of 3% OV-17 on Gas Chrom Q (100–120 mesh), with temperature held at 180° for 4 min and then increased at 2°/min to 220°.

Phages were propagated on their host strains in nutrient broth[5], then the lysates were dialyzed against running water and concentrated to a small volume.

Action of ϕ12 on polysaccharides K41 and K12. — Purified polysaccharide was dissolved in a broth suspension (20 mL) of ϕ12 (~10^9 p.f.u./mL) and, after 48 h at 37°, the mixture was transferred to a dialysis tube (M.W. cutoff 3,500). Dialysis was performed against distilled water (3 × 200 mL; 3 × 24 h) and the combined dialyzates were concentrated to dryness. Oligosaccharides were purified by preparative paper chromatography using 2:1:1 1-butanol–acetic acid–water to yield K12-P1 (10 mg) and K41-P2 (20 mg).

Action of ϕ41 on polysaccharides K41 and K12. — These experiments were conducted as described[5] for ϕ19 and using the same columns.

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