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Depolymerization of the capsular polysaccharide from *Vibrio cholerae* O139 by a lyase associated with the bacteriophage JA1

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Abstract

We have studied the interaction between the *Vibrio cholerae* O139 specific phage JA1, belonging to the *Podoviridae* family, and the capsular polysaccharide (CPS) of the parent strain from which the phage was isolated. Upon incubation of the JA1 phage with the CPS, oligosaccharides were isolated and purified. The oligosaccharides derived from one (shown below) and two repeating units of the CPS were characterized using NMR spectroscopy, mass spectrometry and sugar analysis.

α-Col*p*-(1→2)-4,6-*P*-β-D-Gal*p*
1
↓
3
Sug-(1→3)-β-D-Qui*p*NAc-(1→6)-D-Glc*p*NAc
4
↑
1
α-Col*p*

The cleavage was found to occur by β-elimination at the 4-substituted α-linked galacturonic acid, which results in a 4-deoxy-β-L-*threo*-hex-4-enopyranosyl uronic acid group (Sug). The enzyme associated with the JA1 phage responsible for the depolymerization of the *V. cholerae* O139 CPS is thus a lyase.

Keywords: *Vibrio cholerae*; Bacteriophage; Lyase; NMR spectroscopy; Mass spectrometry

1. Introduction

Vibrio cholerae O139 Bengal emerged as a second aetiologic agent of cholera in the Indian subcontinent late in 1992.[1] Genetic studies indicated that this strain arose probably as a result of recombinatorial events in which the chromosomal region encoding the somatic antigen in *V. cholerae* O1 El Tor was replaced

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by novel genes that encode for the O139 specificity.[2] In spite of the striking similarities between *V. cholerae* O139 and *V. cholerae* O1, the former differs from the latter in the possession of a polysaccharide capsule.[3,4] The capsular polysaccharide (CPS) is a polymer of the O-side chain of the lipopolysaccharide (LPS) antigen and the two antigens have been shown to cross-react.[5,6] Structural studies of the CPS showed that it is made up of galactose with a cyclic phosphate, *N*-acetylglucosamine, *N*-acetylquinovosamine, galacturonic acid and two residues of colitose.[7] Since the O-antigen and the CPS are cross-reactive, it is of interest to depolymerize the latter to generate oligosaccharides in order to prepare CPS-based antigens for immunodiagnostics and immunizations. A chemical approach to oligosaccharide preparation is difficult as the polymer contains acid labile groups, i.e., the colitose residues. However, we have isolated a phage (JA1) belonging to the *Podoviridae* family that specifically lyses encapsulated strains of *V. cholerae* O139, whereas non-capsulated variants are resistant to lysis.[8] Since the phage attachment and lysis of a bacterium often involves cleavage of a specific linkage in the cell wall antigen,[9] we have studied the chemical nature of the interaction between JA1 phage and the CPS from *V. cholerae* O139.

2. Results

The oligosaccharides obtained after incubation of the phage with the CPS were subjected to gel-permeation chromatography (GPC). The elution profile showed that different degrees of depolymerization had taken place. This was also confirmed by ^{1}H NMR spectroscopy which showed a relative increase of signals attributed to the sugar residues that were modified upon cleavage of the polysaccharide (vide infra). Material that eluted on the GPC column in the region for oligosaccharides was further analyzed.

The third fraction collected from the GPC column was analyzed with FABMS in the positive mode. The spectrum showed a peak at m/z 1051 attributed to $[M + H]^+$. This is consistent with an oligosaccharide with a molecular mass of 1050 amu arising from one repeating unit of the polysaccharide in which a formal loss of water has taken place.

The ^{1}H NMR spectrum of this hexasaccharide shows, inter alia, six signals for anomeric protons, at δ_H 5.70, 5.04, 5.01, 4.76, 4.70 and 4.60, signals for methyl protons of two colitose (3,6-dideoxy-L-*xylo*hexose) groups at δ_H 1.16 and 1.19, and a signal for one methyl group of a 2-acetamido-2,6-dideoxyglucosamine residue at δ_H 1.30. Two signals for *N*-acetyl groups are visible at δ_H 1.93 and 2.04 and a signal for an olefin proton appears at δ_H 5.90. A weaker anomeric signal is visible at δ_H 4.58, attributed to the small proportion of reducing end appearing in the β anomeric configuration. By employment of a ^{1}H,^{1}H COSY experiment, the two low field signals could be connected into a common spin system, viz., δ_H 5.70, 3.75, 4.32 and 5.90, which arise from H-1 to H-4 in a modified sugar residue. The appearance of this sugar residue with a four-proton spin system, including an unsaturation, indicates that the phage hydrolysis takes place between a GalA residue and a GlcNAc residue in the polysaccharide. Thus, the GalA residue is upon phage hydrolysis transformed into a 4-deoxy-β-L-*threo*-hex-4-enopyranosyl uronic acid group.[10] This is in agreement with FABMS data and as a further confirmation, the ^{1}H and ^{13}C NMR signals of the hexasaccharide were assigned (Table 1, Fig. 1(A)).

The second fraction from the GPC column, in the region of somewhat larger oligosaccharides, was analyzed with MALDI MS (Kratos) in the positive mode. The spectrum showed a pseudo molecular ion at m/z 2167.8 attributed to $[M + 3Na + H]^+$. This is consistent with an oligosaccharide with a molecular mass of 2100 amu arising from two repeating units of the polysaccharide with a formal loss of water (18 amu). In the ^{1}H NMR spectrum of this dodecasaccharide (Fig. 2) signals were observed, inter alia, at δ_H 5.90 ($J_{H,H}$ 2.6 and 0.8 Hz), δ_H 5.70 ($J_{H,H}$ 2.9 Hz), δ_H 5.38 ($J_{H,H}$ 4.1 Hz). The latter signal, which was not present in the spectrum of the hexasaccharide, arises from H-1 in the galacturonic acid residue. Furthermore, signals for two different

Table 1
Chemical shifts (ppm) of the signals in the ^1H and ^{13}C NMR spectra of the oligosaccharide (one repeating unit) from *V. cholerae* 139 CPS treated with phage JA1 [a]

Sugar residue	$^1H/^{13}C$					
	1	2	3	4	5	6
Sug-(1→	5.70 [3]	3.75	4.32	5.90		
	100.0	71.0	66.2	110.5	142.3	166.7
→3,4,6)-α-D-Glc*p*NAc	5.04 [4]	3.94	4.19	3.69	4.01	3.93, 4.06
	91.6	55.3	74.1	73.0	70.8	67.2
α-Col*p*-(1→2)	5.01[4]	3.97	1.81, 1.89	3.75	4.31/4.25	1.18/1.17
	100.3	64.5	33.7	69.6	67.1/67.0	16.4
α-Col*p*-(1→4)	4.77 [4]	3.97	1.86, 2.05	4.17	4.73	1.16
	98.6	64.5	33.7	69.5	67.0	16.4
→2)-4,6-*P*-β-D-Gal*p*-(1→	4.70 [8]	3.62	3.88	4.58	3.61	4.34, 4.42
	101.8	77.0	73.4	77.2	68.3	69.5
→3)-β-D-Qui*p*NAc-(1→	4.60 [8]	3.72	3.79	3.47	3.48	1.30
	100.5	54.9	79.3	77.0	72.6	17.1
→3,4,6)-β-D-Glc*p*NAc	4.58 [8]	3.78	4.02	3.67	3.57	3.88, 4.15
	96.5	57.7	76.4	73.0	75.2	67.4

[a] $J_{H1,H2}$ values are given in Hz in square brackets.

methyl groups of 2-acetamido-2,6-dideoxyglucosamine residues at δ_H 1.34, $J_{H,H}$ 5.3 Hz (3 H) and δ_H 1.29, $J_{H,H}$ 5.3 Hz (3 H), as well as methyl protons of four colitose groups, δ_H 1.1–1.2 (12 H), were also observed. In a $^1H,^1H$ COSY spectrum signals from H-1 and H-5 protons in the QuiNAc residues were observed at δ_H 4.58/4.55 and 3.48/3.51, respectively. In comparison, the NMR spectra of the larger oligosaccharide (Fig. 1(B)) showed good agreement to those of the CPS previously assigned.[11] In the $^1H,^{13}C$ HMBC spectrum of the dodecasaccharide trans-glycosidic heteronuclear correlations were observed, inter alia, from H-1 and C-1 of the non-reducing *N*-acetylglucosamine residue to C-4 and H-4 of the galacturonic acid, respectively, in agreement with the linkage in the CPS. The occurrence of two different QuiNAc residues, only one anomeric proton from a galacturonic acid residue and some heterogeneity in the signals of the two colitose residues, further corroborate that the site of cleavage is between the *N*-acetylglucosamine residue and the galacturonic acid (Fig. 3).

The site of cleavage was further confirmed by gas chromatography–mass spectrometry (GC–MS). The most protruding fragments appearing in the electron impact mass spectrometry (EIMS) spectrum of 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-glucitol, used as a reference compound, are *m*/*z* 144 and 84, resulting from cleavage between C-2 and C-3 and further elimination of acetic acid (60

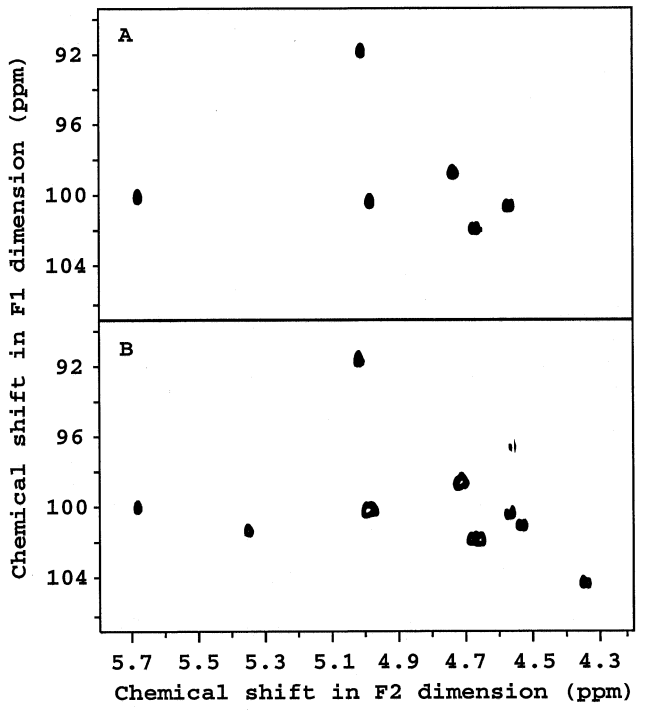

Fig. 1. Anomeric region of the $^1H,^{13}C$ HSQC spectrum at 600 MHz of: (A) the hexasaccharide and (B) the dodecasaccharide originating from one and two repeating units of the polysaccharide, respectively.

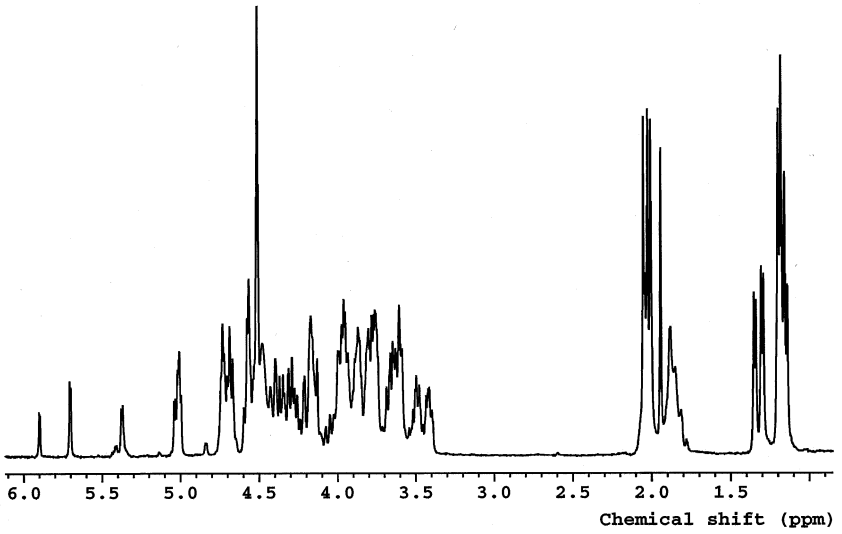

Fig. 2. ^{1}H NMR spectrum at 600 MHz of the oligosaccharide originating from two repeating units of the polysaccharide.

amu). Minor amounts of the fragments at m/z 145 and 85 were also observed which originate from cleavage between C-4 and C-5. The dodecasaccharide and the hexasaccharide samples were analyzed by GC–MS after reduction with $NaBD_4$, hydrolysis, reduction with $NaBH_4$ and acetylation. In the EIMS spectrum of the 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-glucitol derivative from the dodecasaccharide sample, a relative increase of the fragments m/z 145 and 85 could be observed. For the hexasaccharide, only minor amounts of the fragments at m/z 144 and 84 were found. These results indicate that the reducing end of the oligosaccharides, i.e., a GlcNAc residue, had been successfully labeled by a deuterium at C-1. These observations are in complete agreement with a phage cleavage between GlcNAc and GalA residues in the polysaccharide.

An additional MALDI MS spectrum (Bruker) of the dodecasaccharide, acquired in the positive mode, showed a pseudo molecular ion at m/z 2165.7. This molecular mass is 2 amu less than above, indicating an oxidation. Likewise, in the FABMS spectra (JEOL) of the same oligosaccharide peaks were observed at m/z 2165.6 in the positive mode and at m/z 2097.7 in the negative mode, i.e., again a deviation of 2 amu. It is known that underivatized carbohydrates in FABMS partly exhibit a 2 amu lower molecular mass due to oxidation.[12] Furthermore, chemical reactions such as oxidations can be accelerated in FABMS.[13] The terminal residue in the dodecasaccharide is a 4-deoxy-β-L-*threo*-hex-4-enopyranosyl uronic acid with a conjugated 4,5-unsaturation. Thus, the hydroxyl group at C-3 is a secondary allylic alcohol. It is well known that such activated alcohols are more easily oxidized.[14] It is reasonable that an oxidation of the allylic alcohol takes place under the experimental conditions. Applying the post source decay technique,[15] a daughter ion was observed in the MALDI MS spectrum at m/z 2009.2 from the parent ion at m/z 2165.6, which should result from a B-type cleavage[12] of the oxidized terminal group.

ESIMS showed in the region for doubly charged ions $[M-2H]^{2-}$ a peak at m/z 1049.3, as well as two peaks at slightly lower molecular mass. The former peak corresponds to a molecular mass of 2100.6 amu, in complete agreement with the molecular structure proposed in Fig. 3.

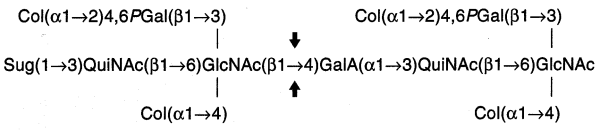

Fig. 3. The dodecasaccharide with a molecular mass of 2100 amu, originating from two repeating units of the capsular polysaccharide from *V. cholerae* O139. Sug = 4-deoxy-β-L-*threo*-hex-4-enopyranosyl uronic acid. Bold vertical arrows indicate the linkage where lyase cleavage occurs.

3. Discussion

We have studied the interaction between the *V. cholerae* O139 specific phage JA1 and the CPS of the parent strain from which the phage was isolated. Upon incubation of the JA1 phage and the purified CPS, it was possible to fractionate and isolate oligosaccharides. The major product was found to be a dodecasaccharide with an *N*-acetyl-D-glucosamine residue at the reducing end, and a 4-deoxy-β-L-*threo*-hex-4-enopyranosyl uronic acid group at the non-reducing end. Hence, the enzyme associated with the JA1 phage responsible for the depolymerization of the *V. cholerae* O139 CPS is a lyase, since cleavage occurs by β-elimination of a 4-substituted uronic acid residue. The *V. cholerae* O139 capsular polysaccharide possesses sugars that are sensitive to acid and phage JA1 can therefore be useful for the generation of oligosaccharides without destruction of the epitopes that may be important for eliciting protective immunity. Since large bacterial polysaccharide polymers are usually poor immunogens as well as T-cell independent antigens,[16] the use of a defined oligosaccharide conjugated to a carrier protein may circumvent the drawbacks of the native CPS. Several bacteriophage associated enzymes which degrade bacterial cell wall associated polysaccharides have been identified.[17–19] Most of them are glycanases which recognize a specific carbohydrate and hydrolyze its linkage at the anomeric carbon. If the polysaccharide is a polymer of repeating units, the resulting oligosaccharides are usually homologues and well defined. A recent MALDI-TOF MS study investigated cartilage degradation by a lyase.[20] However, few of the phage-associated enzymes that have been described are lyases.[21]

The phage JA1 lyses only encapsulated strains of *V. cholerae* O139 suggesting that the active site of the lyase recognizes an epitope unique for the CPS. The structures of the *V. cholerae* O139 CPS,[7] as well as the LPS[5] have been determined. It was shown that the CPS is a polymer of hexasaccharide repeating units. The same hexasaccharide structure was found in the LPS as well. However, the LPS contains only one unit of this repeat attached to the core oligosaccharide. It is therefore surmised that the phage-associated lyase requires at least two repeats to bind and to cleave the linkage between the *N*-acetyl-D-glucosamine and the D-galacturonic acid in the *V. cholerae* O139 CPS.

Recent studies on phage P22 associated *endo*-rhamnosidase showed that this enzyme possesses two active sites.[22] One is specific for binding to a defined epitope whereas the other active site possesses the enzymatic activity. Further studies on the *V. cholerae* O139 specific JA1 phage are necessary to find out if the mechanism is similar to that of the P22-associated *endo*-rhamnosidase.

4. Experimental

Origin, isolation and purification.—The clinical *V. cholerae* O139 Bengal, strain AI-1838, was isolated at the Microbiology Laboratory of the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh. The bacteria were grown in a 30 L fermentor (Belach AB) in a rich tryptone-yeast extract as described earlier.[4] Pelleted bacterial cells were suspended in water and extracted with hot aq phenol.[23] The crude extract was dissolved in Na–acetate buffer (0.1 M, pH 4.2) and heated for 4 h at 100 °C. The precipitate was removed by centrifugation, and the CPS isolated by GPC on Sephadex G-50. The isolation of phage JA1 has been described previously.[8]

Depolymerization.—The depolymerization of the CPS with the phage was performed essentially as described by Svenson et al.[24] Briefly, the JA1 phage was mixed at a ratio of 10^9 PFU/mg CPS in ammonium carbonate buffer (5 mM, pH 7.0) and incubated at 37 °C in a dialysis bag immersed in 10 times the volume of the same buffer. After 72 h of incubation, the surrounding dialysis fluid was concentrated to dryness and heated to 50 °C under diminished pressure in order to remove the remaining ammonium carbonate. The presence of carbohydrates in the dialysate was indicated by the phenol–H_2SO_4 assay.[25]

Oligosaccharide purification.—The oligosaccharides found in the outer dialysate were desalted and purified using GPC on Bio-Gel

P2 and P4 (Bio-Rad) columns (2.5 × 100 cm) irrigated with water containing 1,1,1-trichloro-2-methyl-2-propanol (0.05% w/v) as a preservative. Column effluents were monitored using a differential refractometer (model R403, Waters). All fractions were screened for the presence of carbohydrate using the phenol–H_2SO_4 assay. Fractions containing carbohydrates were pooled and further analyzed.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 50 or 70 °C on JEOL GSX-270 MHz, Varian Unity 400 MHz or Varian Inova 600 MHz spectrometers. Homo- and heteronuclear two-dimensional NMR experiments were used to assign signals. The two-dimensional experiments were $^1H,^1H$ correlated spectroscopy (COSY), $^1H,^1H$ total correlation spectroscopy (TOCSY) with a spin-lock time of 90 ms and gradient versions of a heteronuclear $^1H,^{13}C$ single quantum coherence experiment (gHSQC)[26] and two-dimensional $^1H,^{13}C$ HSQC-TOCSY experiments[27,28] with spin-lock times of 30 and 60 ms. For the evolution of long range connectivities, $^1H,^{13}C$ heteronuclear multiple bond correlation (HMBC) experiments with delays of 50 and 70 ms were used.[29] Chemical shifts are reported in ppm using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) or acetone-d_6 (δ_C 31.00) as internal reference. Data processing was performed using standard JEOL software or VNMR (Varian).

Sugar analysis.—Sugar analysis was performed according to standard procedures.[5,30] The reduction of the oligosaccharides (0.5 mg) with $NaBD_4$, prior to derivatization, was performed in aq NH_4OH (1 N, 0.5 mL) at 80 °C for 2 h.

Mass spectrometry.—Fast-atom bombardment mass spectrometry (FABMS) was recorded on a JEOL SX-102 instrument at a resolution of 5000. Fast Xenon atoms were produced using Xenon ions accelerated by 6 keV. The matrices used were glycerol with addition of triethyl amine or triethyl amine and tetramethyl urea for negative FAB and a mixture of thioglycerol and glycerol for positive FAB.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) was run on a Kratos Kompact Discovery MALDI TOF mass spectrometer in the linear mode with pulsed extraction. Spectra in the positive mode were acquired with dihydroxybenzoic acid (DHB) as a matrix and for internal calibration angiotensin II (1047.2 Da) and insulin (5734.5 Da) were used. MALDI spectra were also recorded in the positive mode on a Bruker Reflex III using DHB as a matrix and polypropylene glycol (2 kDa) for internal calibration.

Electrospray ionization mass spectrometry (ESIMS) was performed on a Finnigan LCQ ion-trap mass spectrometer in the negative mode with a resolution of 3400. The mobile phase was (1:1) water–MeOH.

Gas chromatography–mass spectrometry (GC–MS) using electron impact (EI) was performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused silica column (0.20 mm × 25 m). A temperature program of 190 °C for 3 min followed by 3 °C/min to 250 °C was used with helium as carrier gas.

Acknowledgements

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