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Preliminary Structure Determination of the Capsular Polysaccharide of *Vibrio cholerae* O139 Bengal Al1837

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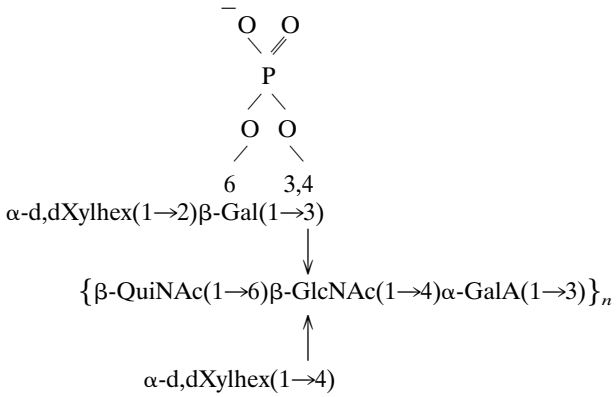
***Vibrio cholerae* O139 Bengal has recently been identified as a cause of epidemic cholera in Asia. In contrast to *V. cholerae* O1, *V. cholerae* O139 Bengal has a polysaccharide capsule. As determined by high-performance anion-exchange chromatography and ^{1}H nuclear magnetic resonance analysis, the capsular polysaccharide of *V. cholerae* O139 Bengal strain Al1837 has six residues in the repeating subunit; this includes one residue each of *N*-acetylglucosamine, *N*-acetylquinovosamine (QuiNAc), galacturonic acid (GalA), and galactose and two residues of 3,6-dideoxyxylohexose (Xylhex). The proposed structure is**



The disease cholera has traditionally been attributed to cholera toxin-producing strains of *Vibrio cholerae* within O group 1 (*V. cholerae* O1) (14). In October 1992, cases of cholera associated with a *V. cholerae* strain which did not agglutinate with O1 antisera were first noted in Madras, India (22). During the next several months, this strain (designated *V. cholerae* O group 139, synonym Bengal) spread in epidemic form across India and Bangladesh and has now been introduced into much of the rest of Asia (5, 8, 11, 18, 20). Molecular epidemiologic studies have demonstrated that *V. cholerae* O139 Bengal strains are virtually identical to *V. cholerae* O1 El Tor, the bioserogroup responsible for the seventh cholera pandemic, ongoing since 1961 (7, 18, 21). However, in contrast to *V. cholerae* O1 (but in common with other non-O1 *V. cholerae* strains [12]), *V. cholerae* O139 Bengal is encapsulated (13). We report here our preliminary determination of the structure of the *V. cholerae* O139 Bengal capsular polysaccharide.

These studies utilized *V. cholerae* O139 Bengal strain Al1837; this strain was isolated from a cholera patient in Bangladesh and has been shown to cause cholera in healthy North American volunteers (19). Methods for extraction of the capsular polysaccharide follow those previously described for non-O1 *V. cholerae* and other *Vibrio* species (12, 23). The final preparation had <0.01% lipopolysaccharide (LPS), as determined by a standard *Limulus* amoebocyte lysate assay (Sigma).

Approximately 200 μg of *V. cholerae* O139 polysaccharide was taken in a screw-cap Teflon tube (13 by 100 mm), and 200 μl of aqueous trifluoroacetic acid was added. After hydrolysis in a heating block, the tube was cooled and the acid was removed by evaporation with nitrogen gas. The residue was dissolved in 200 μl of water, and 10 μl was used for each high-performance liquid chromatography (HPLC) injection. The system used for high-performance anion-exchange chromatography consisted of a BioLC gradient pump (Dionex Corp., Sunnyvale, Calif.) with a pulsed amperometric detector. A Carbopac PA1 pellicular anion-exchange column (4 by 250 mm; Dionex Corp.) equipped with Carbopac guard column was used with a flow rate of 1 ml/min at room temperature. In these experiments, eluant 1 was 16 mM NaOH (suitable for neutral and amino sugars) and eluant 2 was 100 mM NaOH plus 150 mM sodium acetate (suitable for acidic sugars). Detection was done with a pulsed amperometric detector, using a gold working electrode (9).

Nuclear magnetic resonance (NMR) spectra were recorded at a ^{1}H frequency of 500 MHz on a General Electric GN500 spectrometer at 60°C with a reverse polarization transfer probe

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for ^{1}H-detected ^{13}C and ^{31}P experiments. Polysaccharide samples of approximately 15 mg were D_2O exchanged and dissolved in 0.5 ml of 99.996% D_2O (Merck Sharp & Dohme Co., St. Louis, Mo.). All chemical shifts are reported relative to internal 4,4-dimethyl 4-silapentane sulfonate, using acetone as a secondary internal standard. Two-dimensional NMR data (correlation spectroscopy and nuclear Overhauser effect spectroscopy and ^{1}H-detected ^{13}C heteronuclear multiple quantum coherence [HMQC]) were recorded with standard pulse sequences. Nuclear Overhouser effect spectroscopy mixing time was 100 ms, and total correlation spectroscopy mixing was with a MLEV-16 sequence for 20 or 93 ms. Long-range ^{1}H-^{13}C correlation experiments were done with the heteronuclear multiple bond coherence (HMBC) sequence (3) with a delay time of 42 or 50 ms. A carbonyl-selective HMBC experiment with a delay of 50 ms was done with selective low-power ^{13}C 90° pulses of 400 μs. ^{1}H-detected ^{31}P spin echo experiments were done with delay periods ranging from 20 to 100 ms. All NMR data were processed on Silicon Graphics workstations running the FELIX program (Biosym Corp., San Diego, Calif.).

After acid hydrolysis with 0.05 M trifluoroacetic acid at 60°C for 3 h, carbohydrate analysis showed release of 3,6-dideoxyxylohexose which cochromatographed with an authentic sample of abequose in solvent 1. Hydrolysis for 10 h in 1 M trifluoroacetic acid at 100°C yielded peaks corresponding to quinovosamine, glucosamine, galactose, and a reduced amount of 3,6-dideoxyxylohexose, using elution conditions (solvent 1) which are suitable for neutral sugars (23). Using HPLC conditions suitable for acidic sugars (solvent 2), a peak whose retention time corresponds to that of galacturonic acid (GalA) was detected, but the yield was less than stoichiometric apparently because of difficulty of cleaving the glycosidic linkages of uronic acids under conditions which do not degrade them.

The polysaccharide appears to be stable to degradation with a mild base. Treatment for 12 h with aqueous NH_4OH (pH 11) at 4°C did not modify the NMR spectrum of the polysaccharide.

^{1}H NMR spectra of the polymer at room temperature show fairly broad lines with poor resolution, but at 60°C the spectra show reasonably narrow lines. The HMQC spectrum (Fig. 1) shows six resonances typical of anomeric C-H pairs between 98 and 103 ppm in the ^{13}C dimension and between 4.4 and 5.4 ppm in the ^{1}H dimension. The hypothesis that there are six residues in the repeating subunit was tested by tracing the connectivity of each pyranoside ring spin system from the anomeric ^{1}H signal, using established methods which allow identification of the sugar residue from coupling patterns (1). A residue of β-*N*-acetylglucosamine (residue B) was identified by large coupling constants (7 to 11 Hz) for H1-H2, H2-H3, H3-H4, and H4-H5 along with a characteristic downfield chemical shift of the C2 resonance. (See Table 1 for resonance assignments.) A residue of β-*N*-acetylquinovosamine (β-QuiNAc; residue A) was identified by large H-H coupling constants, a downfield shift for C2, and chemical shifts characteristic of a methyl group at C6. Two residues of α-3,6-dideoxyxylohexose (α-3,6-dd-Xylhex; residues D and F) were similarly identified by homonuclear coupling constants, characteristic chemical shifts for the C3 and H3 methylene group and the C6 methyl group. A residue of β-galactose (β-Gal; residue E) was identified by large coupling constants between H1-H2 and H2-H3 with small (1- to 3-Hz) couplings between H3-H4 and H4-H5. Large nuclear Overhauser effect cross-peaks were observed between H4 and H3 and H5 as well as between H1 and H3 and H5. The residue of α-galacturonic acid (residue C) was identified by a large coupling between H2-H3 and a small coupling between H1-H2 and between H3-H4 and H4-H5. Large nuclear Overhauser effect cross-peaks were observed between H1-H2 and between H4-H3 and H4-H5. A large HMBC cross-peak was observed between H1 and C3 and C5, as is characteristic of residues with the α-configuration (1, 17). The anomeric configurations of all the residues were confirmed by values of $^{1}J_{C\text{-}H}$ as measured by coupled HMQC spectra (Table 1).

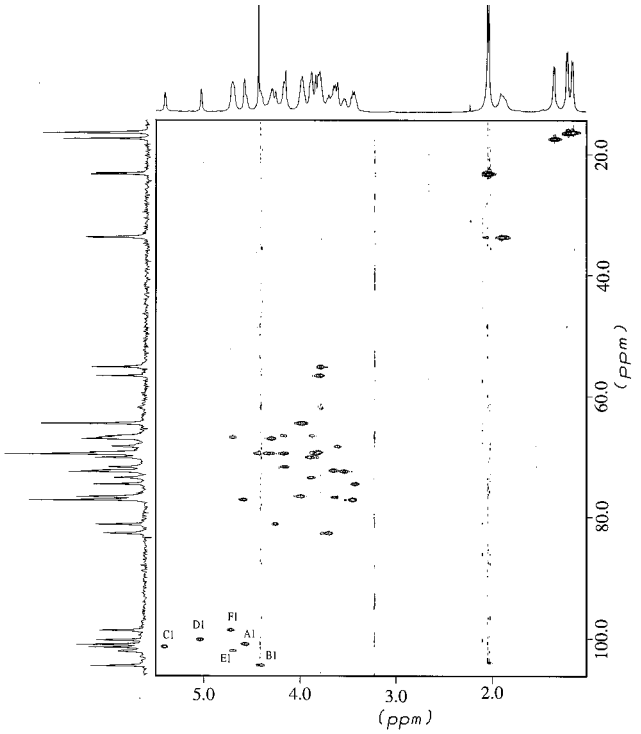


FIG. 1. Two-dimensional ^{1}H-detected ^{13}C NMR spectrum (HMQC) at 500 MHz of the capsular polysaccharide of *V. cholerae* O139 Bengal strain Al1837 at 60°C. A total of 256 pairs of t_1 data of 1,024 points each were collected with a sweep width in the ^{1}H dimension of 2,400 Hz and in the ^{13}C dimension of 12,500 Hz.

An HMBC experiment with ^{13}C long low-power pulses selective for carbonyl carbon resonances was used to assign the three carbonyl carbon resonances observed in the direct ^{13}C spectrum. The data (not shown) indicate that two of the carbonyl carbon resonances are coupled to methyl protons typical of *N*-acetyl groups, showing that both amino sugars are N acetylated. The third carbonyl resonance is coupled to the narrow multiplet assigned to H5 of the α-GalA residue, confirming the identity of this residue.

Phosphate analysis showed 1 mol of phosphate per repeating unit which was not susceptible to digestion with alkaline phosphatase.

The ^{31}P NMR spectrum showed a single resonance at a chemical shift typical of phosphate. ^{1}H-detected ^{31}P spin echo difference spectra were used to show that ^{31}P is coupled ($^{3}J_{PH}$ = 20 Hz) to the resonance assigned to H6 of β-galactose with smaller coupling values to H3, H4, and H5 of β-galactose. These data suggest a cyclic phosphate at C6 also connected to C3 or C4 of galactose. The complete assignment of all the ^{1}H and ^{13}C signals is shown in Table 1.

Long-range ^{13}C-^{1}H correlation data (not shown) were used to identify all the positions of the glycosidic linkages indicated in the structure of the scheme. Cross-peaks between H1 and Cx were observed for the linkage between GalA (residue C) and

TABLE 1. NMR chemical shifts of the *V. cholerae* O139 native polysaccharide in D_2O at 60°C

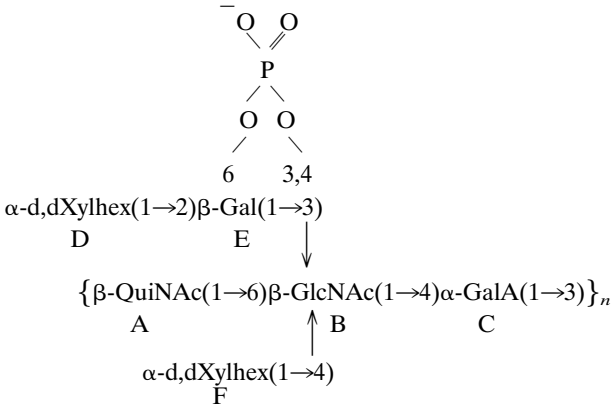
Assignment	Chemical shift (ppm) of the following residue[a]:					
	A (β-QuiNAc)	B (β-GlcNAc)	C (α-GalA)	D (α-3,6-d,d-Xylhex)	E (β-Gal-6PO_4)	F (α-3,6-d,d-Xylhex)
^1H						
H1	4.565	4.412	5.407	5.035	4.695	4.720
H2	3.785	3.798	3.808	3.989	3.633	3.975
H3	3.704	3.995	3.886	1.901	3.885	2.050
H3′				1.862		1.864
H4	3.451	3.657	4.258	3.830	4.584	4.169
H5	3.540	3.425	4.155	4.294	3.608	4.699
H6	1.351	4.173			4.434	
H6′		3.879			4.320	
CH_3	2.026	2.045		1.214		1.162
^{13}C						
C1	100.89	104.35	101.32	100.15	101.97	98.59
C2	55.20	56.63	69.28	64.49	76.72	64.49
C3	82.66	76.59	70.11	33.78	73.48	33.82
C4	77.18	72.33	81.14	69.54	77.17	69.50
C5	72.47	74.55	71.72	67.04	68.32	66.81
C6	17.46	66.56	173.93	16.49	69.47	16.36
CH_3	23.27	23.45				
C═O	175.40	174.85				

[a] See the text and the scheme for further discussions of the residues. The residues had the following $^1J_{C1H1}$ coupling constants (in hertz): A, 159.5; B, 159.7; C, 172.2; D, 170; E, 158.5; F, 169.2.

QuiNAc (residue A), between QuiNAc and GlcNAc (residue B), and between dideoxyxylohexose (residue D) and β-Gal. Cross-peaks between C1 and Hx were observed for the linkages between dideoxyxylohexose (residue D) and β-Gal, for dideoxyxylohexose (residue F) and GlcNAc (residue B), for GlcNAc and GalA, and for β-Gal and GlcNAc. Confirming evidence for the assignment of the linkage positions was observed in the NOESY spectrum, which showed cross-peaks between GalA H1 and QuiNAc H3, between dideoxyxylohexose (residue D) H1 and Gal H2, between dideoxyxylohexose (residue F) H1 and GlcNAc H4, and between Gal H1 and GlcNAc H3. The linkage between QuiNAc and GlcNAc was confirmed by the characteristic downfield chemical shift of GlcNAc C6.

The chemical and NMR data indicate that the capsular polysaccharide of *V. cholerae* O139 has six pyranoside residues in the repeating subunit with the following linkage configuration:

At this point the absolute configurations of the monosaccharides have not been unambiguously determined. Therefore, the residues of 3,6-dideoxyxylohexose could be designated either as colitose (the L isomer) or as abequose (the D isomer).



V. cholerae O139 Bengal has a semirough LPS (10, 16). Comstock et al. and others (6, 25) have shown that a single transposon insertion can result in loss of both encapsulation and the ability to produce LPS beyond the core oligosaccharide, suggesting that the same genes are responsible for expression of the capsule and the addition of the short O antigen. Hisatsune and colleagues (10) have reported that the LPS (core and O antigen) of O139 Bengal strains contains colitose and fructose as terminal sugars, together with glucose, L-glycero-D-manno-heptose, glucosamine, and quinovosamine. Further studies will be necessary to fully elucidate the genetic and biochemical relationship between LPS and capsule in these strains.

To our knowledge, this is the first *V. cholerae* strain for which the structure of the capsular polysaccharide has been determined. In prior work, we have found that quinovosamine, glucosamine, and galactose are not uncommon constituents of capsular polysaccharides among strains of *Vibrio vulnificus* (9, 23, 24). The 3,6-dideoxyxylohexose, in contrast, appears to be unusual among the members of the family *Vibrionaceae*, although abequose (and, to a lesser extent, colitose) is a recognized constituent of LPS O antigen side chains among *Salmonella* and *Yersinia* species (2, 4, 15). Genes associated with capsule expression have now been cloned from *V. cholerae* O139 Bengal, including one 11-kb region which is not present in *V. cholerae* O1 (6). Portions of this region hybridize with other encapsulated strains of non-O1 *V. cholerae* and *V. vulnificus*, as well as strains in other *Vibrio* species. By knowing the structure of the O139 Bengal capsule (and with our growing knowledge of other *Vibrio* capsular polysaccharides and the genetic elements which control their expression), we can begin to define the genetic elements required for O139 Bengal capsule biosynthesis.

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