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Structure of a polysaccharide from the lipopolysaccharide of *Vibrio vulnificus* clinical isolate YJ016 containing 2-acetimidoylamino-2-deoxy-L-galacturonic acid

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ABSTRACT

A polysaccharide isolated after mild acid degradation of the lipopolysaccharide of *Vibrio vulnificus* clinical isolate YJ016 was found to contain L-Fuc, D-GlcpNAc, 2,4-diacetamido-2,4,6-trideoxy-D-glucose (di-*N*-acetylbacillosamine, D-QuiNAc4NAc), and 2-acetimidoylamino-2-deoxy-L-galacturonic acid (L-GalNAmA). The last sugar derivative was confirmed by correlations for nitrogen-linked protons in 2D TOCSY and ROESY spectra measured in a H_2O–D_2O mixture. The following structure of the polysaccharide was established by ^1H and ^{13}C NMR spectroscopy, including 2D ROESY and ^1H,^{13}C HMBC experiments:

→3)-α-L-Gal*p*NAmA-(1→3)-β-D-Qui*p*NAc4NAc-(1→3)-α-L-Fuc*p*-(1→3)-α-D-Glc*p*NAc-(1→
4
↑
1
β-D-Glc*p*NAc6Ac

where the degree of 6-O-acetylation of the lateral β-GlcNAc residue is ~70%.

1. Introduction

Vibrio vulnificus, a bacterium from the γ-group of Proteobacteria, is an etiologic agent of severe human infections acquired through wounds or contaminated seafood. It shares morphological and biochemical characteristics with other human vibrio pathogens, including *V. cholerae* and *V. parahaemolyticus*. Based on phenotypic characteristics and host range criteria, *V. vulnificus* isolates were primarily grouped into two biotypes:[1] indole$^+$, ornithine decarboxylase$^+$ (ODC$^+$) biotype 1 strains associated with pathogenicity in humans and indole$^-$, ODC$^-$ biotype 2 strains that are pathogenic for eels. Later, this subdivision was questioned as indole$^-$ and ODC$^-$ strains were found to be pathogenic also for humans and indole$^+$ and/or ODC$^+$ strains pathogenic for eels. In addition, a third *V. vulnificus* biotype has been described in Israel;[2] it comprises cellobiose-negative strains isolated from patients who handled Saint Peter's fish (*Tilapia* sp.).

Therefore, all biotypes of *V. vulnificus* are potentially pathogenic for humans but biotype 1 strains are most frequently isolated from clinical specimens.[3] These opportunistic pathogens can attack immunocompromised patients and cause gastroenteritis (inflammation of mucous membranes of stomach and intestine), wound infections, and primary septicemia (spread of the microorganisms through the blood). Biotype 1 strains are the major cause of death from eating raw oysters, especially in people with liver damage. *V. vulnificus* YJ016 is a biotype 1 hospital isolate from Taiwan;[4,5] it contains two large chromosomes and a conjugative plasmid (pYJ016) completely sequenced.[6]

In this work, we characterized chemically a polysaccharide from the lipopolysaccharide (LPS) of *V. vulnificus* YJ016 belonging to biotype 1. Recently, we have elucidated the structure of a polysaccharide from the LPS of a biotype 2 (serotype E) isolate *V. vulnificus* CECT4602.[7]

2. Results and discussion

The lipopolysaccharide was isolated by phenol/water extraction of dried cells of *V. vulnificus* YJ016. A high-molecular-mass polysac-

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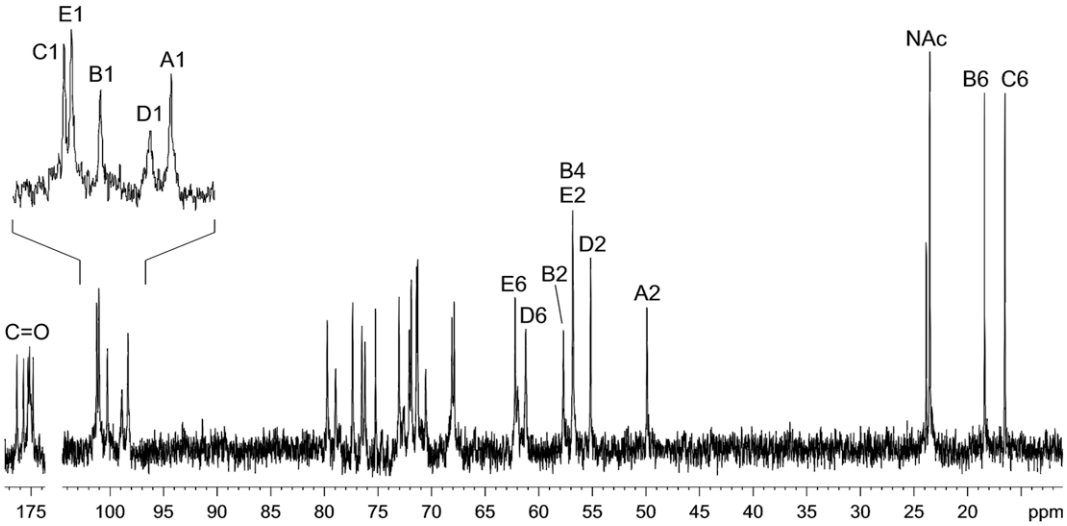

Figure 1. 125-MHz ^{13}C NMR spectrum of the modified polysaccharide (MPS) from *Vibrio vulnificus* YJ016. Arabic numerals refer to carbons in sugar residues denoted by letters as shown in Table 1.

Table 1
^{1}H and ^{13}C NMR data of the modified polysaccharide (MPS) from *Vibrio vulnificus* YJ016

Sugar residue	Atom	δ_C	δ_H	Inter-residue correlations	
				NOE (ROESY)[a]	$^{3}J_{H,C}$ (HMBC)[b]
→3,4)-α-L-Gal*p*NA-(1→ **A**	1	98.3	5.12	**A**1/**B**3	**A**1/**B**3
	2	49.9	4.33		
	3	71.9	3.92		**A**3/**D**1
	4	76.5	4.59		**A**4/**E**1
	5	72.6	4.02		
	6	175.0			
NAc	1	174.8			
	2	23.5	1.95		
→3)-β-D-Qui*p*N4N-(1→ **B**	1	100.2	4.56	**B**1/**C**3	**B**1/**C**3
	2	57.7	3.94		
	3	76.2	3.88		
	4	56.8	3.68		
	5	72.1	3.59		
	6	18.4	1.21		
NAc-2	1	175.7[c]	1.97[d]		
	2	23.5	1.98[d]		
NAc-4	1	175.3[c]			
	2	23.5			
→3)-α-L-Fuc*p*-(1→ **C**	1	101.2	4.99	**C**1/**D**3	**C**1/**D**3
	2	67.9	3.89		
	3	78.9	3.89		**C**3/**B**1
	4	70.5	3.86		
	5	68.1	4.32		
	6	16.5	1.17		
→3)-α-D-Glc*p*N-(1→ **D**	1	98.9	5.00	**D**1/**A**3	**D**1/**A**3
	2	55.2	3.99		
	3	79.7	3.57		**D**3/**C**1
	4	71.4	3.77		
	5	73.0	3.98		
	6	61.2	3.96, 3.88		
NAc	1	175.1	2.03		
	2	23.5			
β-D-Glc*p*N-(1→ **E**	1	101.0	4.68	**E**1/**A**4	**E**1/**A**4
	2	56.8	3.72		
	3	75.2	3.53		
	4	71.3	3.45		
	5	77.4	3.41		
	6	62.2	3.97, 3.77		
NAc	1	176.3	2.09		
	2	23.8			

Arabic numerals refer [a]to protons or [b]before slash to protons and after slash to carbons in sugar residues denoted by letters.
[c,d] Assignment could be interchanged.

charide (PS) was released by mild acid degradation of the lipopolysaccharide with dilute acetic acid and was isolated by GPC on Sephadex G-50.

Sugar analysis of the PS by GLC–MS of the acetylated alditols derived after full acid hydrolysis revealed fucose, GlcN, and a 2,4-diamino-2,4,6-trideoxyhexose. Further studies by NMR spectroscopy showed that the last sugar is bacillosamine (2,4-diamino-2,4,6-trideoxyglucose, QuiN4N) and yet another sugar component is present, namely, 2-amino-2-deoxygalacturonic acid (GalNA), which has not been seen in GLC analysis. Determination of the absolute configurations of the acetylated (*S*)-2-octyl glycosides by GLC indicated that fucose is L and GlcN is D. The absolute configurations of QuiN4N and GalNA were established using known regularities in ^{13}C NMR chemical shifts (see below).

The ^{13}C NMR spectrum of the PS showed a structure heterogeneity, which was suggested to result from non-stoichiometric O-acetylation or/and N-acylation of one of the amino sugars with an acetimidoyl group. Indeed, in addition to signals of *N*-acetyl groups, those of an *N*-acetimidoyl group were present in the spectrum at δ 20.3 (CH_3) and 167.5 (C=N) and of an *O*-acetyl group at δ 21.5 (CH_3) and 174.8 (CO), respectively, but the intensities of the CH_3 groups appeared to be lower than those of any of the *N*-acetyl groups and CH_3 groups of 6-deoxy sugars. The corresponding signals were also present in the ^1H NMR spectrum of the PS at δ 2.23 and 2.15 for the *N*-acetimidoyl and *O*-acetyl groups, respectively, and the assignment of both carbon and proton signals of these groups was confirmed by a $^1H/^{13}C$ HMBC spectrum. Moreover, we observed a decrease in the intensities of the acetimidoyl signals while measuring spectra. Therefore, the PS was treated with aq ammonia to remove the *O*-acetyl group and to convert the *N*-acetimidoyl group into an *N*-acetyl group.

The ^{13}C NMR spectrum of the modified polysaccharide (MPS) (Fig. 1) contained signals for five anomeric carbons at δ 98.3–101.2, two CH_3–C groups (C-6 of Fuc and QuiN4N) at δ 16.5 and 18.4, two $HOCH_2$–C groups (C-6 of GlcN) at δ 61.2 and 62.2, five nitrogen-linked carbons of amino sugars at δ 49.9–57.7, other sugar ring carbons at δ 67.9–79.7, five *N*-acetyl groups at δ 23.5–23.8 (CH_3), and six CO groups (C-6 of GalNA and five NAc groups) at δ 174.8–176.3. The ^1H NMR spectrum of the MPS showed six signals in a low-field region for five anomeric protons and H-4 of GalNA at δ 4.56–5.12, two CH_3–C groups (H-6 of Fuc and QuiN4N) at δ 1.17 and 1.21, other sugar protons at δ 3.40–4.33, and five *N*-acetyl groups at δ 1.95–2.09. Therefore, the MPS has a pentasaccharide repeating unit containing two residues of GlcNAc and one residue each of Fuc, QuiNAc4NAc, and GalNAcA.

Assignment of the ^1H and ^{13}C NMR spectra of the MPS by tracing connectivities in 2D $^1H/^1H$ COSY and TOCSY spectra and by using a $^1H/^{13}C$ HSQC experiment revealed five spin systems for α-GalNAcA (**A**), β-QuiNAc4NAc (**B**), α-Fuc (**C**), α-GlcNAc (**D**), and β-GlcNAc (**E**) (Table 1). The amino sugars were confirmed by correlations of protons at nitrogen-bearing carbons with the corresponding carbons at δ 49.9–57.7. The anomeric configurations of the monosaccharides were inferred by relatively large $^3J_{1,2}$ coupling constants of ~7 Hz for β-linked sugars and by relatively small values of <4 Hz (non-resolved H-1 signals) for α-linked sugars. The absence in the ^{13}C NMR spectrum of non-anomeric carbon signals in a lower field than 80 ppm characteristic for furanosides[8] indicated that all monosaccharides are in the pyranose form.

Linkage and sequence analysis of the MPS was performed using a 2D ROESY experiment (Fig. 2), which revealed the following correlations between anomeric protons and protons at the linkage carbons: **A** H-1,**B** H-3; **B** H-1,**C** H-3; **C** H-1,**D** H-3; **D** H-1,**A** H-3; and **E** H-1,**A** H-4 (Table 1). The **A**-(1→3)-**B**, **B**-(1→3)-**C**, **C**-(1→3)-**D**, **D**-(1→3)-**A,** and **E**-(1→4)-**A** fragments were confirmed by correlation between anomeric protons and linkage carbons and vice versa in the $^1H/^{13}C$ HMBC spectrum (Fig. 3, Table 1). The positions of substitution of the monosaccharides were confirmed by significant downfield displacements, as a result of glycosylation, of the signals for the linkage carbon of units **A** (C-4), **B**, **C,** and **D** (all C-3) to δ 76.2–79.7, that is, by 3.5–8.5 ppm as compared with their positions

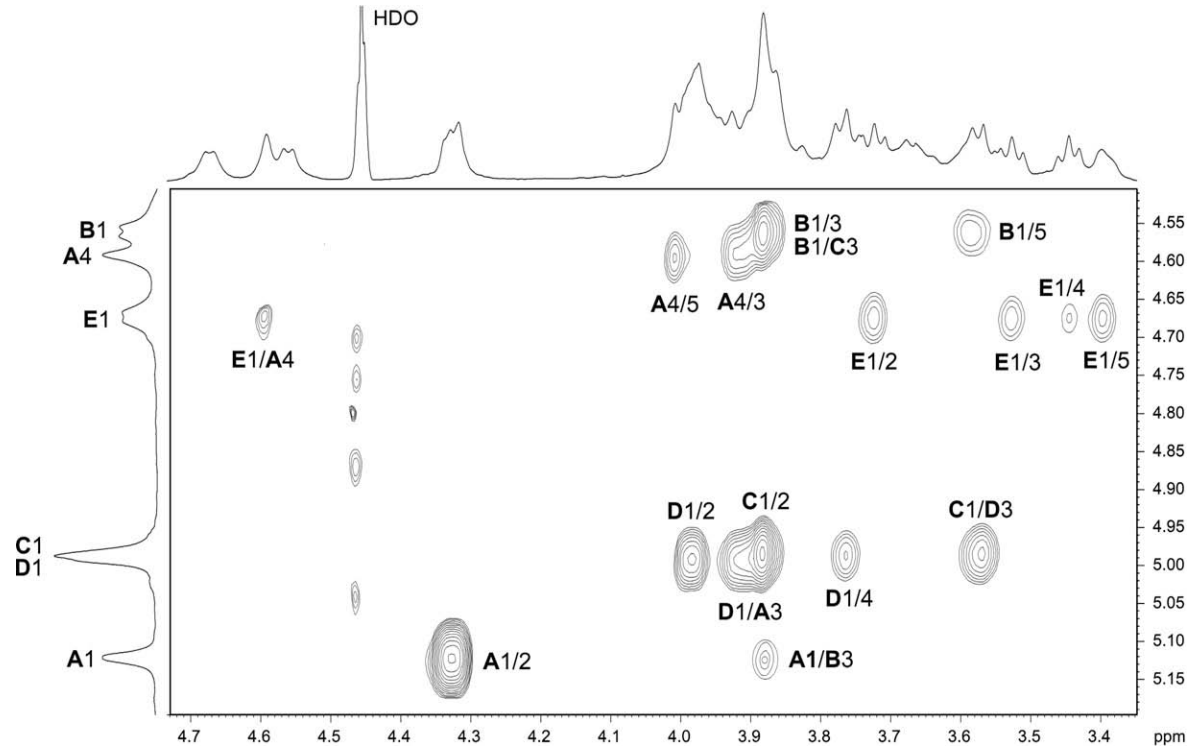

Figure 2. Part of a 2D ROESY spectrum of the modified polysaccharide (MPS) from *Vibrio vulnificus* YJ016. The corresponding parts of the ^1H NMR spectrum are displayed along the axes. Arabic numerals refer to protons in sugar residues denoted by letters as shown in Table 1.

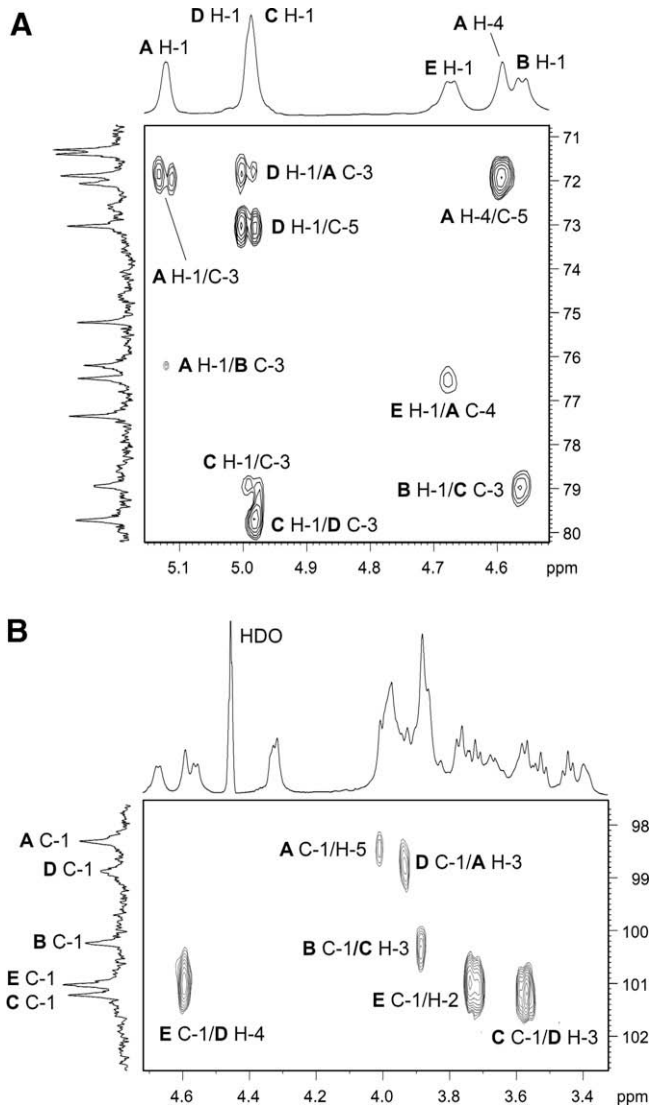

Figure 3. Parts of a $^{1}H/^{13}C$ HMBC spectrum of the modified polysaccharide (MPS) from *Vibrio vulnificus* YJ016 showing correlations for anomeric protons (A) and anomeric carbons (B). The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Letters denote sugar residues as shown in Table 1.

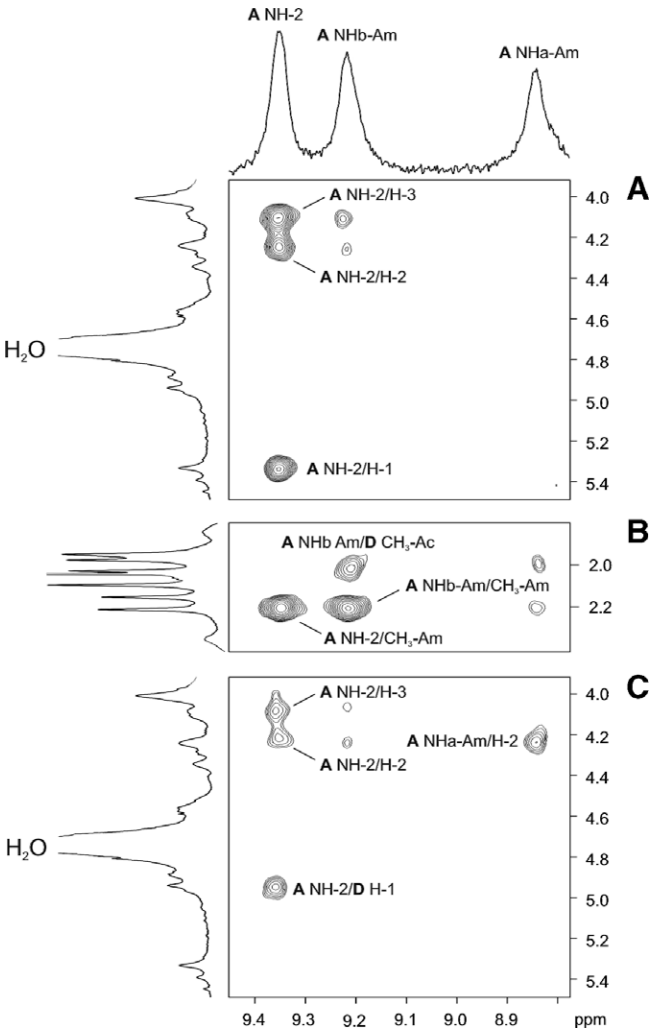

Figure 4. Parts of 2D TOCSY (A) and ROESY (B, C) spectra of the polysaccharide (PS) from *Vibrio vulnificus* YJ016 showing correlations for nitrogen-linked protons of the acetamidino group. The corresponding parts of the ^{1}H NMR spectra are displayed along the axes. The spectra were measured in a 17:3 H_2O–D_2O mixture. Letters denote sugar residues as shown in Table 1.

in the corresponding free monosaccharides at δ 70.4–72.7.[8–10] The signal for **A** C-3 shifted downfield less significantly (δ 71.9 vs 68.7[9]) owing to an opposite effect of glycosylation at C-4 on the C-3 chemical shift. In accordance with a lateral position of unit **E**, its signals for C-2–C-6 were close to those of unsubstituted β-GlcNAc.[10]

A relatively small downfield displacement by 4.5 ppm of the C-1 signal of β-QuiNAc4NAc (δ 100.2 in the ^{13}C NMR spectrum of the MPS vs δ 95.7 in a free di-*N*-acyl β-QuiN4N derivative[9]) indicated[10,11] that the constituent monosaccharides in the β-QuiNAc4NAc-(1→3)-L-Fuc disaccharide fragment have different absolute configurations, that is, that QuiNAc4NAc is D. Furthermore, a relatively large downfield displacement by 6.8 ppm of the C-1 signal of α-GlcNAc (δ 98.9 in the MPS vs δ 92.1 in free α-GlcNAc[10]) indicated[10,11] different absolute configurations of the monosaccharides in the α-D-GlcNAc-(1→3)-GalNAcA disaccharide and, hence, the L configuration of GalNAcA. The last conclusion was confirmed by a close similarity between the chemical shifts of β-QuiNAc4NAc in the MPS and in an O-polysaccharide of *Pseudomonas aeruginosa* NCTC 8505, which includes a similar α-L-GalNAcA-(1→3)-β-D-QuiNAc4NAc fragment.[9]

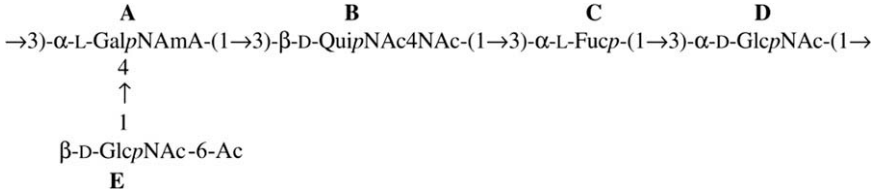

Chart 1. Structure of the polysaccharide from *Vibrio vulnificus* YJ016. The degree of 6-O-acetylation of unit **E** is ~70%.

To determine location of the *N*-acetimidoyl group, NMR spectra of a PS sample were measured in a 17:3 H_2O–D_2O mixture that enabled detection of NH protons (Fig. 4). NH-2 proton of GalNA resonated in a significantly lower field at δ 9.34 in the ^1H NMR spectrum of the PS compared to δ 8.17 in the spectrum of the MPS. This proton showed a strong correlation with CH_3 of the *N*-acetimidoyl group at δ 9.34/2.23 in the ROESY spectrum of the PS.

2-Acetimidoylamino-2-deoxygalacturonic acid (GalNAmA) was also confirmed by a lower field position of its C-2 signal at δ 53.8 in the spectrum of the PS as compared with the C-2 chemical shift δ 49.9 of GalNAcA in the spectrum of the MPS.

Location of the *O*-acetyl group was determined by a comparison of the $^1H/^{13}C$ HSQC spectra of the MPS and PS, which showed a downfield displacement in both dimensions of the major part of **E** H-6a,6b,C-6 cross-peaks from δ 3.97, 3.77/62.2 to 4.86, 4.22/63.4 and an upfield displacement in the ^{13}C dimension of the major part of **E** H-5,C-5 cross-peak from δ 3.41/77.4 to 3.67/74.8, respectively. These shifts are characteristic[12] for O-acetylation of unit **E** at position 6 which is 6-O-acetylated. The position of the O-acetyl group was confirmed directly by observation of correlations for a CO carbon at δ 174.8 with CH_3 protons of OAc at δ 2.15 and H-6b of unit **E** at δ 4.86. The degree of O-acetylation was ~70% as estimated by comparison of the intensities of the signals for O-acetylated and non-acetylated β-GlcNAc.

Therefore, the polysaccharide from *V. vulnificus* YJ016 has the structure shown in Chart 1. It contains two uncommon amino sugar derivatives, di-*N*-acetylbacillosamine and *N*-acetimidoyl-L-galactosaminuronic acid. After its first discovery[13] in a polysaccharide of *Bacillus subtilis* (*Bacillus licheniformis*), di-*N*-acetylbacillosamine and some other *N*-acyl derivatives of bacillosamine have been found in a number of bacterial carbohydrates (see Bacterial Carbohydrate Structure Database at http://www.glyco.ac.ru/bcsdb). *N*-Acetimidoyl-L-galactosaminuronic acid is less common in bacteria; recently, it has been identified[14] in the O-polysaccharide of *Pseudoalteromonas rubra* ATCC 29570^T.

V. vulnificus YJ016 is the first biotype 1 strain with a defined polysaccharide structure and DNA sequence completely determined. The data of this bacterium will be helpful for elucidation of the LPS genetics and biosynthesis and as well as for isolation and studies of LPS mutants to better understand the *V. vulnificus* biotype 1 pathogenesis in humans.

3. Experimental

3.1. Growth conditions and isolation of the lipopolysaccharide and polysaccharide

V. vulnificus YJ016, a biotype 1 representative strain from a hospital in Taiwan, was grown on Tryptone broth (TB) (1% tryptone, 1% NaCl, pH 7.2) at 37 °C for 24 h. Bacterial cells were obtained from 50 L culture as described.[7]

The LPS was isolated from dried cells (5 g) by phenol–water extraction[15] and was purified as described.[7] The yield of the purified LPS was 2.9% of the dried cells weight.

A portion of the LPS (100 mg) was degraded with 2% HOAc for 2 h at 100 °C, a precipitate was removed by centrifugation and the supernatant was fractionated on Sephadex G-50 Superfine as described.[7] A high-molecular-mass polysaccharide (PS) was isolated in a yield of 45% of the lipopolysaccharide weight. A PS sample (10 mg) was modified by treatment with 12% aq ammonia for 6 h at 50 °C, ammonia was flushed out, and the following lyophilization afforded the MPS.

3.2. Sugar analysis

The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2 h), after evaporation the monosaccharides were converted into the alditol acetates and were analyzed by GLC on a Hewlett-Packard 5890 chromatograph equipped with an HP-1ms column using a temperature gradient of 160–290 °C at 10 °C min^{-1}. For determination of the absolute configurations of the monosaccharides,[16] the polysaccharide hydrolyzate was peracetylated, subjected to methanolysis (CH_3OH/2 M HCl, 85 °C, 2 h), peracetylated, treated with (*S*)-2-octanol/1 M HCl (100 °C, 2 h), peracetylated and analyzed by GLC as described above.

3.3. NMR spectroscopy

NMR spectra were obtained on a Bruker DRX-500 spectrometer using standard Bruker software at 40 °C in 99.95% D_2O or a 17:3 H_2O–D_2O mixture. Prior to the measurements in D_2O, samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O. Chemical shifts are referenced to internal TSP (δ_H 0.00) or acetone (δ_C 31.45). A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively. Other NMR parameters were set essentially as described.[17]

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