ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 332 (2001) 279–284

CARBOHYDRATE RESEARCH

Structure of the O-specific polysaccharide of *Vibrio cholerae* O9 containing 2-acetamido-2-deoxy-D-galacturonic acid

Nina A. Kocharova,[a,b] Yuriy A. Knirel,[a,b] Per-Erik Jansson,[a,*] Andrej Weintraub[c]

[a]*Karolinska Institute, Clinical Research Centre, Huddinge University Hospital, Novum, 141 86 Huddinge, Sweden*
[b]*N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia*
[c]*Department of Microbiology, Pathology and Immunology, Division of Clinical Bacteriology, Karolinska Institute, Huddinge University Hospital, 141 86 Huddinge, Sweden*

Received 27 November 2000; received in revised form 9 February 2001; accepted 2 March 2001

Abstract

The O-specific polysaccharide (OPS) was isolated by mild-acid degradation of the lipopolysaccharide of *Vibrio cholerae* O9 and studied by carboxyl reduction, sugar and methylation analyses, Smith degradation, and two-dimensional NMR spectroscopy, including COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ HMQC experiments. The following structure of the pentasaccharide-repeating unit of the OPS was established:

α-D-Glc*p*
↓
4
→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*NAcA-(1→3)-α-D-Gal*p*NAcA-(1→3)-α-D-Glc*p*NAc-(1→

Keywords: *Vibrio cholerae*; Lipopolysaccharide; O-Specific polysaccharide structures; 2-Acetamido-2-deoxy-D-galacturonic acid

1. Introduction

Until 1992, O1 was considered as the only *Vibrio cholerae* serogroup associated with epidemic cholera.[1] The emergence then of an epidemic strain from another *V. cholerae* serogroup, O139, prompted monitoring of diarrhoea in endemic areas also for other non-O1 serogroups. Some of the non-O1, non-O139 serogroups were found to be increasingly associated with cholera-like diseases.[2–7] In particular, *V. cholerae* O9 was found in several cases of diarrhoea in a prospective study in India between 1993 and 1995.[6] The virulence mechanisms of this serogroup of bacteria are not known, and we have therefore undertaken structural studies of the lipopolysaccharide.

Serogrouping of *V. cholerae* is based on the reactivity of specific antibodies with antigens that reside in the O-specific polysaccharide chain (OPS) of the lipopolysaccharides. Structures of the OPS of *V. cholerae* vary significantly from serogroup to serogroup.

* Corresponding author. Tel.: +46-8-58583821; fax: +46-8-58583820.
E-mail address: pererik.jansson@kfcmail.hs.sll.se (P.-E. Jansson).

0008-6215/01/$ - see front matter © 2001 Elsevier Science Ltd. All rights reserved.
PII: S0008-6215(01)00069-6

Structural studies have been performed on the OPS of serogroups O1–O3, O5, O8,[8] O10, O21, O22, O76, O139, O144, and O155, and a number of unique sugars and non-carbohydrate groups have been identified as OPS components (for review see Refs. 9 and 10). Now we report on the structure of the OPS of *V. cholerae* O9 containing 2-acetamido-2-deoxy-D-galacturonic acid.

2. Results and discussion

The O-specific polysaccharide (OPS) was prepared by mild-acid degradation of the lipopolysaccharide of *V. cholerae* O9 followed by gel-permeation chromatography on Sephadex G-50. Acid hydrolysis of the OPS revealed glucose and 2-amino-2-deoxyglucose, which were identified by GLC of the alditol acetates. After carboxyl reduction, in addition to these sugars, 2-amino-2-deoxygalactose was identified, which was evidently derived from 2-amino-2-deoxygalacturonic acid (GalNA). The GlcN:GalN ratio was 1:0.8, which, taking into account the presence in the repeating unit of the OPS of one GlcN and two GalNA residues (see below), indicated reduction of 40% GalNA residues. Determination of the absolute configuration of the monosaccharides in the carboxyl-reduced OPS by GLC of the acetylated (+)-2-octyl glycosides showed that they all have the D configuration.

Methylation analysis of the OPS revealed similar amounts of terminal and 4-substituted glucose residues, but only a trace amount of an amino sugar derivative. When the carboxyl reduced OPS was analysed, derivatives from the 3-substituted GlcN and 4-substituted and 3,4-disubstituted GalN residues were identified in the ratios 1:0.5:0.2, respectively. The absence of the corresponding 3-substituted GlcN derivative in methylation analysis of the initial OPS may be accounted for by the slow hydrolysis of the glycosidic linkage of GalNA attached to GlcN (see below).

The ^{13}C NMR spectrum of the OPS (Fig. 1) contained signals for five anomeric carbons at δ 98.5–102.3, three carbons bearing nitrogen at δ 50.4–54.3, three $HOCH_2$–C groups at δ 62.1–62.3, 17 other carbons bearing oxygen at δ 69.1–81.7, three *N*-acetyl groups at δ 23.4–23.6 (CH_3) and 175.6–176.1 (CO), and carboxyl groups at δ 173.6. The absence of signals above δ 82 indicated that all sugars were pyranoid. Accordingly, the ^1H NMR spectrum of the OPS contained signals for five anomeric protons at δ 5.00–5.28, three *N*-acetyl groups at δ 1.97–2.05, and other protons in the region δ 3.48–4.56. These data showed that the OPS has a pentasaccharide repeating unit, containing one residue of D-GlcNAc and two residues each of D-Glc and D-GalNAcA.

The ^1H and ^{13}C NMR spectra of the OPS were assigned using two-dimensional COSY, TOCSY, and H-detected ^1H,^{13}C HMQC ex-

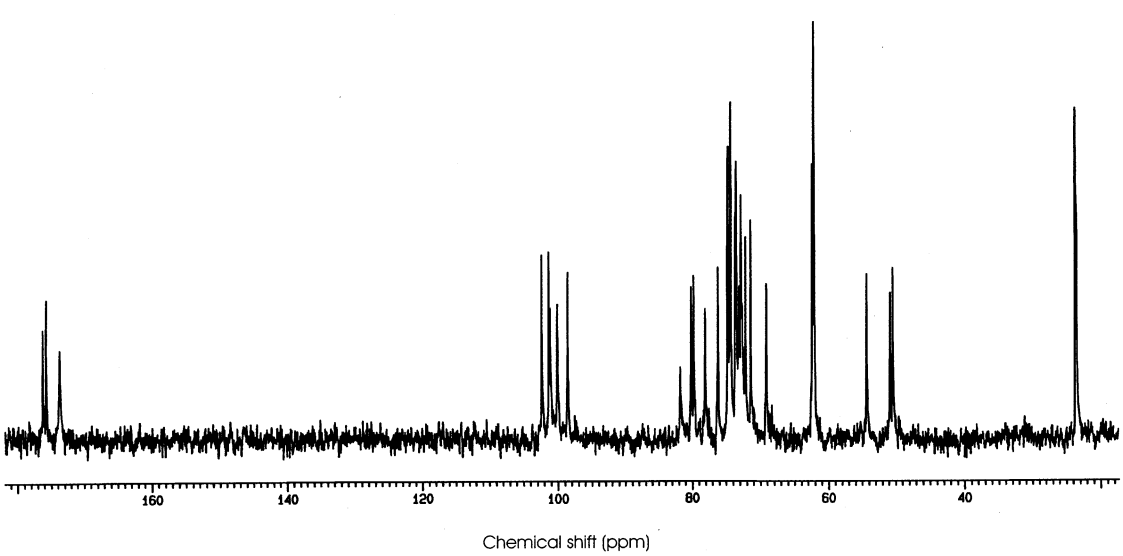

Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide of *V. cholerae* O9.

Table 1
^{1}H NMR chemical shifts (δ, ppm) [a]

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6
OPS ***1***							
→4)-α-D-Glc*p*-(1→	(**A**)	5.00	3.48	4.00	3.66	4.12	3.81
→4)-α-D-Gal*p*NAcA-(1→	(**B**)	5.21	4.42	3.92	4.45	4.40	
→3,4)-α-D-Gal*p*NAcA-(1→	(**C**)	5.28	4.52	4.07	4.56	4.45	
→3)-α-D-Glc*p*NAc-(1→	(**D**)	5.28	4.03	3.89	3.72	3.80	3.86
α-D-Glc*p*-(1→	(**E**)	5.04	3.52	3.79	3.51	3.96	3.86
Oligosaccharide ***2***							
α-D-Gal*p*NAcA-(1→	(**B**)	5.13	4.20	3.89	4.32	4.36	
→3)-α-D-Gal*p*NAcA-(1→	(**C**)	5.25	4.34	3.97	4.39	4.34	
→3)-α-D-Glc*p*NAc-(1→	(**D**)	5.05	4.01	3.86	3.70	3.81	3.85

[a] Additional chemical shifts for NAc are δ 1.97, 2.00, and 2.05 in the OPS; δ 1.97, 2.00, and 2.04 in the oligosaccharides.

Table 2
^{13}C NMR chemical shifts (δ, ppm) [a]

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
OPS ***1***							
→4)-α-D-Glc*p*-(1→	(**A**)	101.1	73.6	74.7	78.0	72.6	62.1
→4)-α-D-Gal*p*NAcA-(1→	(**B**)	98.5	50.8	69.1	80.1	72.8	173.6
→3,4)-α-D-Gal*p*NAcA-(1→	(**C**)	101.3	50.4	76.1	79.7	73.0	173.6
→3)-α-D-Glc*p*NAc-(1→	(**D**)	100.0	54.3	81.7	72.1	74.3	62.3
α-D-Glc*p*-(1→	(**E**)	102.3	73.4	74.3	71.3	74.6	62.1
Oligosaccharide ***2***							
α-D-Gal*p*NAcA-(1→	(**B**)	97.7	50.9	68.6	72.0	73.1	174.9 [b]
→3)-α-D-Gal*p*NAcA-(1→	(**C**)	100.8	49.6	80.3	71.0	73.1	174.5 [b]
→3)-α-D-Glc*p*NAc-(1→	(**D**)	98.4	54.0	81.0	72.6	74.0	61.8
→2)-Erythritol		61.1 [c]	76.3 [c]	68.7 [c]	64.0 [c]		

[a] Additional chemical shifts for NAc are δ 23.4 and 23.6 (2C) (CH_3), 175.6 (2C) and 176.1 (CO) in the OPS; 23.5, 23.6 (2C) (CH_3), 175.9, 176.0, and 176.3 (CO) in the oligosaccharide. Assignment of signals with a chemical shift difference $\leq$0.2 ppm could be interchanged.
[b] Assignment could be interchanged.
[c] Tentative assignment.

periments (Tables 1 and 2). The spin systems of the amino sugar residues were identified by correlation of protons at carbons bearing nitrogen to the corresponding carbons. The GalNAcA spin systems were distinguished from that of GlcNAc by characteristic low $J_{3,4}$ and $J_{4,5}$ coupling constant values (not resolved, <3 Hz) and relatively high δ-values of the signals for H-2, H-4, and H-5 (δ 4.40–4.56). Chemical shifts of the anomeric protons ($\delta >$ 5.00) and small $J_{1,2}$ coupling constant values (<4 Hz) indicated that all monosaccharides are α-linked.

Sugar residues in the repeating unit of the OPS were designated as shown in Fig. 2 and Tables 1 and 2. Their sequence was determined by the following interresidue correlations between the transglycosidic protons revealed by a NOESY experiment on the OPS: **A** H-1/**B** H-4, **C** H-1/**D** H-3, **D** H-1/**A** H-4, and **E** H-1/**C** H-4 at δ 5.00/4.45, 5.28/3.89, 5.28/3.66, and 5.04/4.56, respectively. **B** H-1 at δ 5.21 gave two interresidue cross-peaks, with H-3 and H-4 of unit **C** at δ 4.07 and 4.56, respectively, but since the **E**-(1→4)-**C** sequence followed from the **E** H-1/**C** H-4 correlation, unit **B** is attached to unit **C** at position 3. The glycosylation pattern in the OPS was confirmed by low-field displacements to δ 76.1–81.7 of the signals for C-4 of units **A** and

B, C-3 of unit **D**, and C-3 and C-4 of unit **C** in the ^{13}C NMR spectrum of the OPS (compare chemical shifts δ 69.1–73.5 for C-3 and C-4 in the corresponding nonsubstituted sugars.[11,12]) The C-2–C-6 chemical shifts of unit **E** were close to those in α-Glc.[11] These data together show that the repeating unit of the O-specific polysaccharide of *V. cholerae* O9 has structure **1** shown in Fig. 2.

Smith degradation of the OPS resulted in oxidation of both glucose residues to give a mixture of two oligosaccharides, each containing all three amino sugars present in the OPS. The negative ion mode ESI mass spectrum of the mixture showed two intense singly charged $[M-H]^-$ pseudomolecular ions at m/z 758.4 and 800.2. The former, major peak corresponded to the target Smith degradation product **2** (Fig. 2) containing erythritol as aglycon (calculated monoisotopic molecular mass 759.3 Da). The other, minor peak belonged to an oligosaccharide having a 42 Da higher molecular mass, which corresponded to the presence of an additional C_2H_2O fragment. Most likely, this fragment was a 2-hydroxyethylidene group derived from the C-1–C-2 fragment of the oxidised 4-substituted glucose residue, which formed a stable cyclic acetal with erythritol rather than a hemiacetal with water with subsequent elimination by mild-acid hydrolysis (**3**, Fig. 2). Smith degradation of (1→4)-linked glucans and some bacterial polysaccharides containing a 4-substituted hexose residue has been reported to give similar by-products (Ref. 13 and refs. cited therein).

The structures of the oligosaccharides were established using the ^1H and ^{13}C NMR data (Tables 1 and 2). In particular, a low field position of the C-3 signal of unit **C** compared to that of terminal unit **B** (δ 80.3 vs. 68.6) indicated substitution of the former at posi-

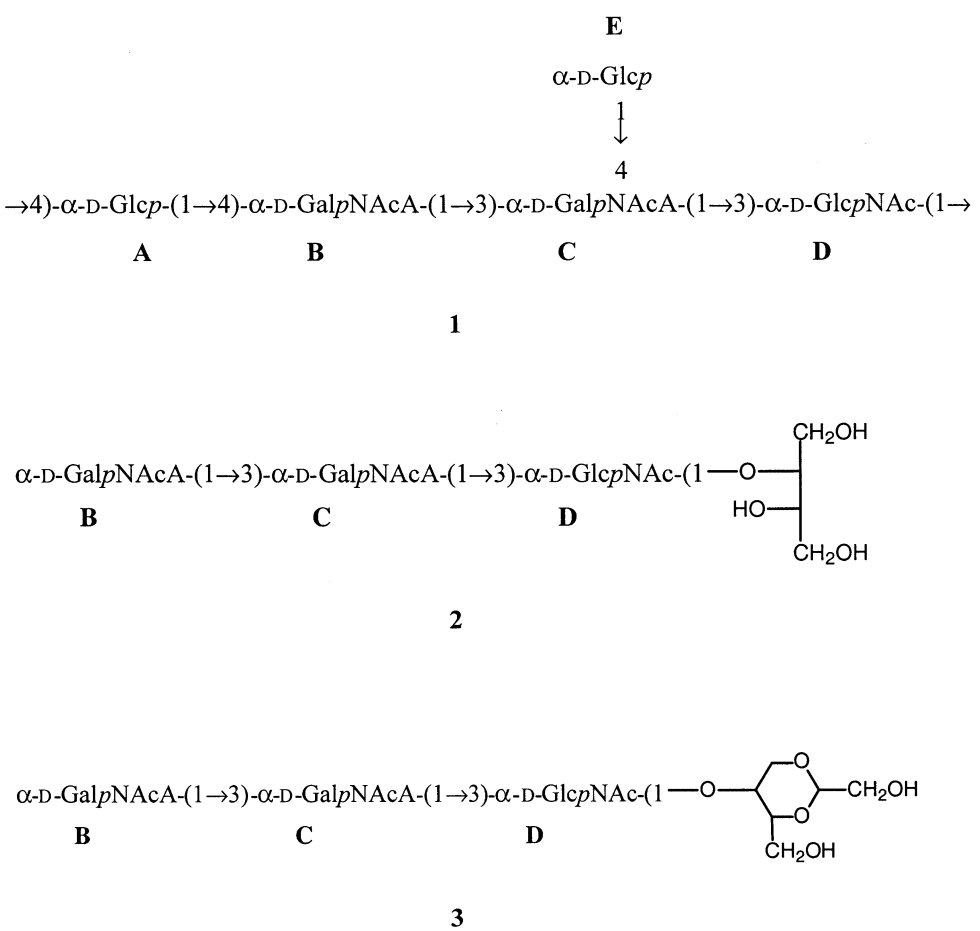

Fig. 2. Structures of the repeating unit of the O-specific polysaccharide of *V. cholerae* O9 (**1**) and oligosaccharides derived by Smith degradation of the OPS (**2** and **3**). GalNAcA stands for 2-acetamido-2-deoxygalacturonic acid.

tion 3 in the oligosaccharide and thus confirmed the attachment of unit **E** to unit **C** at position 4 in the OPS. A signal at δ_H 5.00 (t, J 3 Hz, O–CH–O), giving correlations with signals at δ_H 3.67 (C–CH_2OH) in the COSY spectrum and at δ_C 104.5 in the ^{1}H,^{13}C HMQC spectrum, demonstrated the presence of an acetal group and a 1,3-dioxane ring in the minor oligosaccharide **3** (compare published data[13]). Structures **2** and **3** of the Smith degradation products confirmed structure **1** of the O-specific polysaccharide of *V. cholerae* O9 (Fig. 2).

Noteworthy, the polysaccharide studied possesses inter alia a feature in common with a few other polysaccharides, characterised by the presence of a GalNA→GalNA disaccharide element (1→3)-linked to an *N*-acetylamino sugar having the D-*gluco* configuration (D-GlcNAc in *V. cholerae* O9 and *Shigella dysenteriae*;[14] 2-acetamido-2,6-dideoxy-D-glucose in *Pseudomonas aeruginosa*,[15] *Francisella tularensis*,[16] and *Vibrio anguillarum*;[17] or 2,4-diacetamido-2,4,6-trideoxy-D-glucose in *Acinetobacter haemolyticus*[18]).

3. Experimental

Growth of the bacterium, isolation and degradation of the lipopolysaccharide.—*V. cholerae* O9, strain CO987, from Professor G.B. Nair (Department of Microbiology, National Institute of Cholera and Enteric Diseases, Calcutta, India) was grown in a rich tryptone-yeast extract as described earlier.[19] Pelleted bacterial cells were suspended in water and extracted with hot aq phenol[20] to give a crude lipopolysaccharide, which was purified by treatment with DNAse, RNAse and Proteinase K.[21] The purified lipopolysaccharide was degraded with 2% AcOH for 10 h at 100 °C; the precipitate was removed by centrifugation, and the OPS was isolated by gel-permeation chromatography on Sephadex G-50 (S) (Pharmacia, Sweden) using 0.05 M pyridinium acetate pH 4.5 as eluent; monitoring was performed using a Bischoff differential refractometer (USA).

NMR spectroscopy and MS.—An OPS sample was exchanged twice with D_2O (99.9%), lyophilised, and dissolved in D_2O (99.96%); pD of the solution was 6.5. The ^{13}C NMR spectrum of the OPS was run on a Bruker AM-360 instrument (Germany) at 80 °C for the polysaccharide and that of the oligosaccharide on a JEOL EX-270 instrument (Japan) at 25 °C, with acetone (δ 31.45) as the internal reference. The ^{1}H NMR and two-dimensional NMR spectra were recorded on a JEOL JNM ECP500 instrument (Japan) at the same temperature, using sodium 3-trimethylsilylpropanoate-d_4 (δ 0.0) as the internal reference. A mixing time of 100 ms was used in the TOCSY and NOESY experiments.

Electrospray ionisation (ESI) MS was run in the negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, UK) with aq 50% MeCN containing 1 mM ammonia as the mobile phase at a flow rate of 10 μL/min. The sample was dissolved in aq 50% MeCN at a concentration of ~50 pmol/μL, and 10 μL was injected via a syringe pump into the electrospray source.

Sugar analysis.—Sugar analysis was carried out before and after carboxyl reduction, which was performed according to the published method.[22] Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), and monosaccharides were identified by GLC as the alditol acetate derivatives[23] on a DB-5 fused-silica capillary column (25 m × 0.25 mm), using a Hewlett–Packard 5880 instrument (USA) and a temperature gradient from 160 °C (1 min) to 250 °C at 3 °C/min. For determination of the absolute configuration of monosaccharides,[24] the hydrolysate of the carboxyl-reduced OPS was N-acetylated with Ac_2O in an aq satd solution of $NaHCO_3$ (20 °C, 30 min), heated with (+)-2-octanol (0.15 mL) in the presence of anhyd CF_3CO_2H (0.03 mL) at 100 °C for 16 h, acetylated with Ac_2O in Py (100 °C, 1 h), and analysed by GLC as above.

Methylation analysis.—Methylation of the OPS and the carboxyl reduced OPS was performed using methyl iodide in dimethyl sulphoxide in the presence of sodium methylsulphinylmethanide.[25] Hydrolysis was performed as in sugar analysis, partially methylated monosaccharides derived were reduced with $NaBH_4$, converted to alditol acetates, and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph (USA)

equipped with a NERMAG R10-10L mass spectrometer (France) under the same chromatographic conditions as in GLC.

Smith degradation.—The OPS (18 mg) was oxidised with of 0.1 M $NaIO_4$ at 20 °C for 48 h in the dark. Ethylene glycol was added to destroy the excess oxidant; the product was reduced with an excess of $NaBH_4$ at 20 °C for 2 h, acidified with HOAc, desalted on Biogel P-2 (Bio-Rad) in 0.05 M pyridinium acetate pH 4.5, hydrolysed with 2% AcOH (2 h, 100 °C), and the resulting oligosaccharide mixture (7.7 mg) was separated by gel-permeation chromatography on Biogel P-2.

Acknowledgements

We thank Professor G.B. Nair for the *V. cholerae* O9 strain. This work was supported by a grant from the Swedish Natural Science Research Council (to P.E.J.). N.A.K. and Y.A.K. thank the Royal Swedish Academy of Sciences and Karolinska Institute for fellowships.

References

1. Albert, M. J.; Ansaruzzaman, M.; Bardhan, P. K.; Faruque, A. S. G.; Faruque, S. M.; Islam, M. S.; Mahalanabis, D.; Sack, R. B.; Salam, M. A.; Siddique, A. K.; Yunus, M. D.; Zaman, K. *Lancet* **1993**, *342*, 387–390.
2. Bagchi, K.; Echeverria, P.; Arthur, J. D.; Sethabutr, O.; Serichantalergs, O.; Hoge, C. W. *J. Clin. Microbiol.* **1993**, *31*, 1315–1317.
3. Dalsgaard, A.; Albert, M. J.; Taylor, D. N.; Shimada, T.; Meza, R.; Serichantalergs, O.; Echeverria, P. *J. Clin. Microbiol.* **1995**, *33*, 2715–2722.
4. Dalsgaard, A.; Forslund, A.; Bodhidatta, L.; Serichantalergs, O.; Pitarang, C.; Pang, L.; Shimada, T.; Echeverria, P. *Epidemiol. Infect.* **1999**, *122*, 217–226.
5. Finch, M. J.; Valdespino, J. L.; Wells, J. G.; Perez-Perez, G.; Arjona, F.; Sepulveda, A.; Bessudo, D.; Blake, P. A. *Am. J. Trop. Med. Hyg.* **1987**, *36*, 393–397.
6. Mukhopadhyay, A. K.; Garg, S.; Mitra, R.; Basu, A.; Rajendran, K.; Dutta, D.; Bhattacharya, S. K.; Shimada, T.; Takeda, T.; Takeda, Y.; Nair, G. B. *J. Clin. Microbiol.* **1996**, *34*, 2537–2543.
7. Sharma, C.; Thungapathra, M.; Ghosh, A.; Mukhopadhyay, A. K.; Basu, A.; Mitra, R.; Basu, I.; Bhattacharya, S. K.; Shimada, T.; Ramamurthy, T.; Takeda, T.; Yamasaki, S.; Takeda, Y.; Nair, G. B. *J. Clin. Microbiol.* **1998**, *36*, 756–763.
8. Kocharova, N. A.; Perepelov, A. V.; Zatonsky, G. V.; Shashkov, A. S.; Knirel, Y. A.; Jansson, P.-E.; Weintraub, A. *Carbohydr. Res.* **2001**, *330*, 83–92.
9. Jansson, P. E. In *Endotoxin in Health and Disease*; Brade, H.; Opal, S. M.; Vogel, S. N.; Morrison, D. C., Eds.; Marcel Dekker: New York, 1999; pp. 155–178.
10. Knirel, Y. A.; Kochetkov, N. K. *Biochemistry (Moscow)* **1994**, *59*, 1325–1383.
11. Jansson, P. E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169–191.
12. Knirel, Y. A.; Kocharova, N. A.; Shashkov, A. S.; Kochetkov, N. K.; Kholodkova, E. V.; Stanislavsky, E. S. *Eur. J. Biochem.* **1987**, *166*, 189–197.
13. L'vov, V. L.; Shashkov, A. S.; Dmitriev, B. A.; Kochetkov, N. K.; Jann, B.; Jann, K. *Carbohydr. Res.* **1984**, *126*, 249–259.
14. Knirel, Y. A.; Dashunin, V. M.; Shashkov, A. S.; Kochetkov, N. K.; Dmitriev, B. A.; Hofman, I. L. *Carbohydr. Res.* **1988**, *179*, 51–60.
15. Knirel, Y. A.; Skvortsov, I. M.; Shashkov, A. S.; Dmitriev, B. A.; Kochetkov, N. K.; Stanislavsky, E. S.; Mashilova, G. M. *Eur. J. Biochem.* **1985**, *150*, 551–557.
16. Vinogradov, E. V.; Shashkov, A. S.; Knirel, Y. A.; Kochetkov, N. K.; Tochtamysheva, N. V.; Averin, S. P.; Goncharova, O. V.; Khlebnikov, V. S. *Carbohydr. Res.* **1991**, *214*, 289–297.
17. Eguchi, H.; Kaya, S.; Araki, Y.; Kojima, N.; Yokota, S. *Carbohydr. Res.* **1992**, *231*, 159–169.
18. Haseley, S. R.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1997**, *244*, 761–766.
19. Knirel, Y. A.; Paredes, L.; Jansson, P. E.; Weintraub, A.; Widmalm, G.; Albert, M. J. *Eur. J. Biochem.* **1995**, *232*, 391–396.
20. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
21. Lambden, P. R.; Heckels, J. E. *J. Immunol. Methods* **1982**, *48*, 233–240.
22. Taylor, R. L.; Shively, J. E.; Conrad, H. E. *Methods Carbohydr. Chem.* **1976**, *7*, 149–151.
23. Sawardeker, J. S.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1603.
24. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
25. Hakomori, S. I. *J. Biochem.* **1964**, *55*, 205–208.