Structure of the *N*-acetylpseudaminic acid-containing capsular polysaccharide of *Acinetobacter baumannii* NIPH67

*N. P. Arbatsky,[a] M. M. Shneider,[b] A. S. Shashkov,[a] A. V. Popova,[c,d] K. A. Miroshnikov,[b] N. V. Volozhantsev,[d] and Y. A. Knirel[a]**

[a]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47 Leninsky prosp., 119991 Moscow, Russian Federation. Fax: +7 (499) 135 5328. E-mail: yknirel@gmail.com*
[b]*M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 16/10 ul. Miklukho-Maklaya, 117997 Moscow, Russian Federation. E-mail: mm_shn@mail.ru*
[c]*Moscow Institute of Physics and Technology, 9 Institutsky per., 141700 Dolgoprudnyi, Moscow Region, Russian Federation. E-mail: popova_nastya86@mail.ru*
[d]*State Research Center for Applied Microbiology and Biotechnology, 142279 Obolensk, Moscow Region, Russian Federation. E-mail: nikvol@obolensk.org*

Capsular polysaccharide (CPS) was isolated from a nosocomial pathogen *Acinetobacter baumannii* (*A. baumannii*) NIPH67 and studied by sugar analysis, Smith degradation, and ^{1}H and ^{13}C NMR spectroscopy. The CPS was found to contain 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse5Ac7Ac), and the structure of the linear trisaccharide repeating unit of the CPS was established as →4)-α-Pse*p*5Ac7Ac-(2→6)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→. The genetic content of the capsule biosynthesis cluster of *A. baumannii* NIPH67, designated KL33, is consistent with the established CPS structure, and thus the capsule of the investigated strain was assigned to K33 group. Functions of proteins including two glycosyltransferases encoded by the genes of the K33 locus were assigned based on the structure of CPS and by the comparison with related proteins of other capsular types of *A. baumannii*.

Key words: *Acinetobacter baumannii*, structure of capsular polysaccharide, KL33 gene cluster, glycosyltransferase, K locus, pseudaminic acid.

In recent years, *Acinetobacter baumannii* (*A. baumannii*) attracts attention as a nosocomial pathogen with a multiple antibiotic resistance. The capsule of the bacterial cell is essential for survival and infectivity of *A. baumannii*.[1] It consists of a capsular polysaccharide (CPS), which is composed of a repeating oligosaccharide unit (K-unit). Gene sequences of the cluster involved in the biosynthesis of CPS (K locus) and the corresponding structures of CPS are highly variable[2,3] allowing classification of strains of *A. baumannii* on the basis of K groups. More than 90 types of capsular loci (KL-groups) have been identified.*

In the present paper, the structure of a CPS from *A. baumannii* NIPH67 was elucidated, which contains 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse5Ac7Ac), and the functions of the transferase-encoding genes of the K locus (designated as K33) of this strain were suggested.

The polysaccharide was isolated from the bacterial cells by the water-phenol procedure. The ^{13}C NMR spectrum of the polysaccharide did not contain signals of fatty acids common to lipopolysaccharides and hence was identified as a CPS. Lipopolysaccharides cannot be extracted from the cells of *A. baumannii* by water-phenol treatment previously suggested[4] for extraction of this type of biopolymers, because in these bacteria lipopolysaccharide is present exclusively in the R-form, which is devoid of the polysaccharide chain,[2,5] and the R-form is isolated using other extraction procedures.[6]

GLC analysis of alditol acetates obtained after complete acid hydrolysis of the CPS revealed the presence of Glc, Gal, and GalNAc in the ratio of ~4 : 2 : 1 (based on the detector response). The further study showed that glucose derived from an associated glucan and this monosac-

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charide was not included into the CPS. ^{1}H and ^{13}C NMR spectra of the isolated CPS were poorly resolved. The absence of signals of O-acetyl groups allowed us to treat the CPS with NH_4OH with a view to enhance resolution.

The NMR spectra of thus processed CPS contained signals of three major and one minor monosaccharide residues. By application of 2D sequences ^{1}H,^{1}H COSY, TOCSY, ROESY and ^{1}H,^{13}C HSQC, the major signals were assigned to β-Gal*p*, α-Gal*p*NAc and 5,7-diacetamido-3,5,7,9-tetradeoxynon-2-ulosonic acid, which was subsequently identified as pseudaminic acid (Table 1). Minor signals were attributed to α-Glc*p*. A correlation between protons H(1) and H(6a) of Glc at 4.97/3.77 ppm in the ROESY spectrum evidenced the presence of α1→6-glucan. This structure was confirmed by the downfield shift of C(6) signal and the upfield shift of C(5) signal to 66.8 and 71.6 ppm compared to corresponding published data[7], namely, 61.9 and 72.7 ppm for unsubstituted α-Glc*p* (α- and β-effects of glycosylation, respectively).

The ROESY spectrum showed H(1) Gal/H(3) GalNAc and H(1) GalNAc/H(4) Pse5Ac7Ac correlations in the major series of signals at 4.48/3.82 ppm and 5.03/4.17 ppm, respectively. Therefore, GalNAc is substituted at position 3, and Pse5Ac7Ac is glycosylated at position 4. The presence of Gal-(1→3)-GalNAc linkage was confirmed by a low-field displacement of C(3) signal to 78.7 ppm (compare published data[7] 68.7 ppm for C(3) of unsubstituted α-Gal*p*NAc). Comparison of ^{1}H and ^{13}C NMR chemical shifts of signals of nonusolonic acid with published data[8] permitted its identification as L-*glycero*-L-*manno*-isomer (Pse5Ac7Ac), and also revealed its pyranose form and α-configuration. The presence of Pse5Ac7Ac in the CPS is in agreement with the presence of the gene module for the synthesis of its nucleotide precursor in the K locus of the strain being studied (*vide infra*).

In the ROESY spectrum, the cross-peaks indicating the position of Pse5Ac7Ac attachment were not detected. However, signals of H(6a,6b) of Gal were substantially shifted upfield from 3.64 and 3.72[9] ppm in unsubstituted β-Gal*p* to 3.45 and 3.55 ppm in the CPS, and the signal of C(5) was shifted from 76.3[7] ppm in β-Gal*p* to 73.5 ppm in CPS (β-effect of glycosylation). These shifts indicated that Gal is substituted at position 6. A small effect of α-glycosylation (0.4 ppm) observed for C(6) of Gal is common for the substitution with a ketose, which in our case is Pse5Ac7Ac. The obtained data show that CPS from *A. baumannii* NIPH67 has structure **1**.

→4)-α-Pse*p*5Ac7Ac-(2→6)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→

1

This structure was substantiated by Smith degradation analysis. First, the CPS was oxidized with $NaIO_4$, then reduced with $NaBH_4$, and the cleavage product was subjected to mild acid hydrolysis. As we expected, the degradation resulted in production of oligosaccharide **2** with glycerol as an aglycone, which was formed by destruction of 6-substituted Gal. Besides, a minor oligosaccharide **3** was isolated as a by-product, which arose from unwanted hydrolysis of the acid-labile linkage of Pse5Ac7Ac. The

Table 1. NMR ^{1}H and ^{13}C data

Residue	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)	H(1)[a]	H(2)	H(3)[b]	H(4)	H(5)	H(6)[c]	H(7)	H(8)	H(9)
CPS **1**																		
→3)-α-D-Gal*p*NAc-(1→	96.2	49.9	78.7	69.5	72.0	62.9	—	—	—	5.03	4.24	3.82	4.16	4.17	3.76	—	—	—
→6)-β-D-Gal*p*-(1→	105.8	71.9	73.9	69.2	73.5	62.6	—	—	—	4.48	3.52	3.63	3.99	3.79	3.45 3.55	—	—	—
→4)-α-Pse*p*5Ac7Ac-(2→	—	101.8	33.7	71.6	48.9	72.3	55.0	68.5	1.16	—	—	1.62 2.16	4.17	4.32	3.90	4.16	4.18	1.16
Glucan																		
→6)-α-Glc*p*-(1→	99.0	72.7	74.6	70.8	71.6	66.8	—	—	—	4.97	3.57	3.72	3.51	3.90	3.77 3.97	—	—	—
Oligosaccharide **2**																		
α-D-Gal*p*NAc-(1→	96.3	51.2	68.6	69.9	72.5	62.9	—	—	—	5.06	4.09	3.77	3.95	4.21	3.79	—	—	—
→4)-α-Pse*p*5Ac7Ac-(2→	—	101.7	33.9	71.5	49.0	72.0	54.9	68.3	17.0	—	—	1.61 2.16	4.23	4.31	3.88	4.20	4.21	1.15
→1)-Gro	65.7	71.6	63.8	—	—	—	—	—	—	3.37 3.46	3.89	3.60 3.67	—	—	—	—	—	—
Oligosaccharide **3**																		
α-D-Gal*p*NAc-(1→	96.4	51.2	68.6	69.9	72.5	62.9	—	—	—	5.04	4.09	3.77	3.96	4.21	3.79	—	—	—
→4)-α-Pse*p*5Ac7Ac	—	97.7	33.5	71.9	49.2	71.5	54.2	68.2	16.7	—	—	1.81 1.95	4.15	4.32	4.06	4.14	4.11	1.10

Note. Chemical shifts of *N*-acetyl groups: δ_H 1.97—2.02; δ_C 23.2—23.4 (Me), 175.0—176.0 (CO). [a] 1a, 1b. [b] 3ax, 3eq or 3a, 3b. [c] 6a, 6b.

structures of **2** and **3** were elucidated by 2D NMR spectroscopy, including $^{1}H,^{1}H$ ROESY and $^{1}H,^{13}C$ HSQC experiments. Assignments of ^{1}H and ^{13}C chemical shifts are shown in Table 1.

α-D-Gal*p*NAc-(1→4)-α-Pse*p*5Ac7Ac-(2→1)-Gro

2

α-D-Gal*p*NAc-(1→4)-Pse5Ac7Ac

3

A distinctive feature of the investigated CPS is the presence of α-linked di-*N*-acetylpseudaminic acid, which was earlier detected in the CPS of *A. baumannii* having K2[10,11] and K42[12] capsule biosynthesis loci. In the case of K42, α-Pse5Ac7Ac is present only in a number of K-units, and the others contain 5-*N*-acetyl-7-*N*-[(*R*)-3-hydroxybutanoyl] derivative (α-Pse5Ac7Hb).[12] β-Linked Pse5Ac7Ac is a component of CPS of the K6 group.[13] Detection of genes encoding the synthesis of the nucleotide precursor of Pse[11] in a number of K loci shows that derivatives of Pse are also present in CPS of *A. baumannii* of other K groups.

K locus of the sequenced earlier genome of *A. baumannii* NIPH67 (GenBank accession number APRA00000000)[14] contains a novel gene cluster that enables CPS biosynthesis, designated as KL33.[11] Respectively, the CPS from *A. baumannii* NIPH67 belongs to the K33 group. KL33 cluster includes two modules. One of them contains capsule export genes (*wza*, *wzb,* and *wzc*), and the other one contains monosaccharides synthesis genes (*galU, ugd, gpi, gne1,* and *pgm*). They flank the central variable region which includes the *gna* gene encoding UDP-D-Glc*p*NAc- or UDP-D-Gal*p*NAc-dehydrogenase together with the K33 capsule polysaccharide gene cluster.

Next to *gna* gene, six genes *pse*[2,11] are located, which encode proteins PseA—PseF involved in the synthesis of nucleotide-activated precursor CMP-Pse5Ac7Ac. They were found to be nearly identical (more than 95% at the amino acid level) to the related genes of the *A. baumannii* KL2 cluster.[11]

Glycosylphosphatetransferase ItrA2 encoded in the KL33 cluster, is 99% identical to ItrA2 of a strain of *A. baumannii* ATCC 17978 (GenPept accession number ABO10556.2, also known as PglC), which initiates synthesis of the K-unit *via* the transfer of D-Gal*p*NAc-P from UDP-D-Gal*p*NAc to undecaprenyl phosphate carrier incorporated in the inner membrane.[15] Hence, D-GalNAc is the first sugar of the K33 unit.

KL33 cluster contains genes designated as *gtr5* and *kpsS2*,[11] which encode two glycosyltransferases. Gtr5 included in KL33 is 84—90% identical to proteins Gtr5 found in KL2,[11] KL6,[13] and KL52/PSgc18 (also known as WafH)[3] clusters from *A. baumannii* which are believed to catalyse formation of the β-D-Gal*p*-(1→3)-D-Gal*p*NAc linkage. As this linkage was detected in the K33 group CPS being studied we concluded that *gtr5* plays the same role in KL33 cluster of *A. baumannii*.

KpsS2 glycosyltransferase can be assigned to the remaining α-Pse5Ac7Ac-(2→6)-D-Gal*p* linkage in accordance with its identity to the enzyme family able to transfer aldulosonic acids by retaining mechanism.[16] Two families of retaining glycosyltransferases from *A. baumannii,* namely KpsS1 and KpsS2 have been described.[11,17] KpsS2 ($KpsS2_{K33}$) glycosyltransferase encoded in KL33 locus is 59% identical to KpsS1 ($KpsS1_{K2}$) encoded in KL2 locus, which was earlier assigned to a similar α-Pse5Ac7Ac-(2→6)-D-Glc*p* linkage in the K2 unit.[11] The variation in the amino acid sequence may be interlinked with the difference in acceptor substrates of these two KpsS enzymes. Meanwhile, $KpsS2_{K33}$ is 95% identical to $KpsS2_{K42}$, which is related to the α-Pse5Ac7Acyl-(2→4)-D-Rib*p* linkage in CPS of K42 group.[12] It can be assumed that KpsS2 proteins recognize binding domains in different monosaccharide acceptors (Rib*p* and Gal*p*).

Experimental

Bacterial strain, cultivation and isolation of CPS. *A. baumannii* NIPH67[18] was provided by Dr. A. Nemec (National Institute of Public Health, Prague, Czech Republic). Bacteria were cultivated in 2TY medium (Bacto triptone, 16 g, Bacto Yeast extract, 10 g, NaCl, 5 g, distilled water, 1 L) for 16 h; the cells were harvested by centrifugation (10000 g, 20 min), washed with phosphate-buffered saline, suspended in aq. acetone (7 : 3 v/v), precipitated by centrifugation and dried in the air.

CPS was isolated by phenol-water extraction[4] of bacterial cells (1.7 g), the extract was dialyzed without layer separation and the insoluble contaminants were removed by centrifugation. The supernatant was treated with cold (4 °C) aq. 50% CCl_3CO_2H to pH 2, and the residue was removed by centrifugation. The supernatant was neutralized with aq. 25%-NH_4OH and CPS was isolated by gel chromatography on a column (56×3.5 cm) with Sephadex G-50 in aq. 0.1% AcOH, monitored with a UV detector «Uvicord» (LKB, Sweden) at 206 nm. Then CPS was treated with aq. 12.5% NH_4OH (37 °C, 16 h), the ammonia was removed with a stream of nitrogen and the polysaccharide was isolated as described above.

Composition analysis. A sample of CPS (1 mg) was hydrolyzed with 2*M* CF_3CO_2H (120 °C, 2 h). Monosaccharides were identified by GLC of the alditol acetates on a Maestro (Agilent 7820) gas chromatograph (Interlab, Russia), equipped with HP-5 column (0.32 mm×30 m), using a temperature program of 160 (1 min) to 290 °C at 7 °C min^{-1}. The absolute configuration of monosaccharides was determined using the glycosylation effects in the ^{13}C NMR spectra of CPS.[19]

Smith degradation. A sample of CPS (10 mg) was oxidized with aq. 1% $NaIO_4$ (2 mL) at 20 °C (72 h in the dark), reduced with $NaBH_4$ (60 mg) at 20 °C for 16 h, the excess of $NaBH_4$ was decomposed with AcOH, the solution was evaporated, metha-

nol was added to the residue and the solution was evaporated (the procedure was repeated 5 times), the residue was dissolved in water (0.3 mL) and desalted by gel chromatography on a column (108×1.2 cm) with Sephadex G-25. The product was eluted with water, hydrolyzed with aq. 2% AcOH (100 °C, 1.5 h) and a mixture of oligosaccharides **2** and **3** (1.6 mg) was isolated by gel chromatography on Sephadex G-25, as described above.

NMR spectroscopy. A CPS sample was freeze-dried twice from 99.9% D_2O and dissolved in 99.95% D_2O («Deutero GmbH», Germany). The NMR spectra were recorded on a Bruker Avance II instrument (Germany; 600 MHz) at 20 °C, using internal sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (Sigma-Aldrich; $\delta_H = 0$ ppm, $\delta_C = -1.6$ ppm) as a reference for calibration. The 2D NMR spectra were recorded using standard Bruker software, and the Bruker TopSpin 2.1 program was used to acquire and process the NMR data. In the 2D TOCSY and ROESY experiments, the mixing time was 150 ms. Other parameters were set as described earlier.[20]

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