

# Structure and Gene Cluster of the K93 Capsular Polysaccharide of *Acinetobacter baumannii* B11911 Containing 5-*N*-Acetyl-7-*N*-[(*R*)-3-hydroxybutanoyl]pseudaminic Acid

A. A. Kasimova<sup>1,2</sup>, M. M. Shneider<sup>3</sup>, N. P. Arbatsky<sup>1</sup>, A. V. Popova<sup>4,5</sup>, A. S. Shashkov<sup>1</sup>,  
K. A. Miroshnikov<sup>3</sup>, Veeraraghavan Balaji<sup>6</sup>, Indranil Biswas<sup>7</sup>, and Yu. A. Knirel<sup>1\*</sup>

<sup>1</sup>Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russia; E-mail: yknirel@gmail.com

<sup>2</sup>Higher Chemical College of the Russian Academy of Sciences,

Dmitry Mendeleev University of Chemical Technology of Russia, 125047 Moscow, Russia

<sup>3</sup>Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences,  
119997 Moscow, Russia; E-mail: mm\_shn@mail.ru

<sup>4</sup>Moscow Institute of Physics and Technology, 141701 Dolgoprudny,  
Moscow Region, Russia; E-mail: popova\_nastya86@mail.ru

<sup>5</sup>State Research Center for Applied Microbiology and Biotechnology, 142279 Obolensk, Moscow Region, Russia

<sup>6</sup>Department of Clinical Microbiology, Christian Medical College, Vellore,  
632004 Tamil Nadu, India; E-mail: vbalaji@cmcvellore.ac.in

<sup>7</sup>Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center,  
Kansas City, KS 66160, USA; E-mail: ibiswas@kumc.edu

Received October 13, 2016

Revision received November 5, 2016

**Abstract**—Capsular polysaccharide (CPS) assigned to the K93 type was isolated from the bacterium *Acinetobacter baumannii* B11911 and studied by sugar analysis along with one- and two-dimensional <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. The CPS was found to contain a derivative of pseudaminic acid, and the structure of the branched tetrasaccharide repeating unit was established. Genes in the KL93 capsule biosynthesis locus were annotated and found to be consistent with the CPS structure established. The K93 CPS has the α-D-Galp-(1→6)-β-D-Galp-(1→3)-D-GalpNAc trisaccharide fragment in common with the K14 CPS of *Acinetobacter nosocomialis* LUH 5541 and *A. baumannii* D46. It also shares the β-D-Galp-(1→3)-D-GalpNAc disaccharide fragment and the corresponding predicted Gal transferase Gtr5, as well as the initiating GalNAc-1-P transferase ItrA2, with a number of *A. baumannii* strains.

DOI: 10.1134/S0006297917040101

**Keywords:** *Acinetobacter baumannii*, capsular polysaccharide structure, capsule biosynthesis locus, pseudaminic acid, glycosyltransferase

Recently, *Acinetobacter baumannii* has become one of the most widespread agents causing health-care associated infections. Their treatment is complicated owing to the ability of the bacteria to acquire and to accumulate various antibiotic resistance mechanisms [1]. *Acineto-*

*baumannii* has a short-chain lipooligosaccharide without any O-polysaccharide chain [2] but possesses a capsular polysaccharide (CPS), which consists of many oligosaccharide repeats (K units). The CPS forms a thick layer around the bacterial cell and protects *A. baumannii* from the action of immune system components and penetration of antimicrobial agents into the cell [3].

*Acinetobacter baumannii* strain B11911 was isolated from a patient with bloodstream infection in 2014 in Vellore, India. Its genome has been sequenced previously [4] (GenBank accession number NZ\_LFYX00000000.1) and was found to contain a novel capsule biosynthesis cluster at the K locus. It was designated KL93, as suggest-

**Abbreviations:** COSY, correlation spectroscopy; CPS, capsular polysaccharide; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; Pse, pseudaminic acid; RHb, (*R*)-3-hydroxybutanoyl; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

\* To whom correspondence should be addressed.

ed by J. J. Kenyon (personal communication), and, accordingly, the CPS of this strain was assigned to the K93 type. In this work, we established the structure of this CPS and assigned putative functions to the genes present in the KL93 locus.

## MATERIALS AND METHODS

### Cultivation of the bacterium and isolation of CPS.

*Acinetobacter baumannii* B11911 was cultivated in 2× TY medium for 24 h. Cells were collected by centrifugation at 10,000g per 20 min, washed with a 7 : 3 (v/v) acetone–water mixture, and dried.

CPS was isolated by phenol–water extraction [5] of bacterial mass (450 mg), and then the crude extract was dialyzed without layer separation and freed from insoluble contaminations by centrifugation. Proteins and nucleic acids were removed by precipitation with aqueous 50%  $\text{CCl}_3\text{CO}_2\text{H}$  at 0°C; after centrifugation, the supernatant was dialyzed against distilled water and lyophilized.

CPS preparation (85 mg) was heated with aqueous 2% HOAc (100°C, 3 h), lipid precipitate was removed by centrifugation (13,000g per 20 min), and the purified CPS sample (33 mg) was isolated from the supernatant by gel-permeation chromatography on a column (56 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 0.05 M pyridinium acetate buffer (pH 4.5) monitored using a differential refractometer (Knauer, Germany).

**Composition analysis.** A CPS sample (0.5 mg) was hydrolyzed with 2 M  $\text{CF}_3\text{CO}_2\text{H}$  (120°C, 2 h). Monosaccharides were analyzed by GLC of the alditol acetates on a Maestro (Agilent 7820) chromatograph (Interlab, Russia) equipped with a HP-5 column (0.32 mm × 30 m) using a temperature program of 160°C (1 min) to 290°C at 7°C/min. The absolute configurations of monosaccharides and 3-hydroxybutanoic acid were determined by GLC of the acetylated (*S*)-2-octyl glycosides [6] and trifluoroacetylated (*S*)-2-octyl ester as described [7].

**NMR spectroscopy.** Samples were deuterium-exchanged by lyophilization from 99.9%  $\text{D}_2\text{O}$  and then examined as solutions in 99.95%  $\text{D}_2\text{O}$  at 65°C using internal sodium 3-trimethylsilylpropanoate-2,2,3,3- $\text{d}_4$  ( $\delta_{\text{H}}$  0.0,  $\delta_{\text{C}}$  –1.6) as a reference for calibration. The  $^{13}\text{C}$  NMR spectra were recorded on a Bruker DRX-500 instrument, and two-dimensional NMR spectra were run on a Bruker Avance II 600 MHz spectrometer (Germany) using standard Bruker software. Relaxation delay was set to 3 s in the  $^{13}\text{C}$  NMR experiment. A spin-lock time of 60 ms and a mixing time of 150 ms were used in TOCSY and ROESY experiments, respectively. A 60-ms delay was applied for evolution of long-range coupling to optimize the  $^1\text{H}$ ,  $^{13}\text{C}$  HMBC experiment. Other NMR parameters were set as previously described [8]. The BrukerTopSpin 2.1 program was used to acquire and process the NMR data.

**Bioinformatics.** The K locus sequence for *A. baumannii* B11911 was obtained from GenBank (accession number NZ\_LFYX00000000.1, base position range: 3339095 to 3366570). The sequence was annotated using the established nomenclature system [2], and novel genes were assigned names by J. J. Kenyon (personal communication). Protein functions were predicted using the BLASTp bioinformatics tool [9].

## RESULTS AND DISCUSSION

**Structure elucidation of the CPS.** A crude CPS preparation was isolated from cells of *A. baumannii* B11911 by phenol–water extraction and freed from contaminations by heating under mild acidic conditions followed by gel-permeation chromatography on Sephadex G-50. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of the purified CPS sample compared to those of the crude preparation demonstrated that the acid treatment introduced no structural modification.

Sugar analysis of the CPS using GLC of the alditol acetates derived after full acid hydrolysis revealed Gal and GalN in the ratio 1 : 0.25 (detector response). Further studies showed that the CPS also contained a derivative of pseudaminic acid (Pse), namely 5-acetamido-3,5,7,9-tetra-deoxy-7-(3-hydroxybutanoylamino)-*L*-glycero-*L*-manno-non-2-ulosonic acid (Pse5Ac7Hb). GLC analysis of the acetylated (*S*)-2-octyl glycosides indicated that Gal and GalN had the *D*-configuration. 3-Hydroxybutanoic acid was released by acid hydrolysis and identified as the *R* isomer by GLC of the trifluoroacetylated (*S*)-2-octyl ester.

The  $^1\text{H}$  NMR spectrum showed signals for three anomeric protons at  $\delta$  4.48–4.97 (table), two C- $\text{CH}_2$ -C groups at  $\delta$  1.57, 2.50 (Pse H-3ax, H-3eq) and  $\delta$  2.35 (2 H, NHb H-2), two  $\text{CH}_3$ -C groups at  $\delta$  1.22 (Pse H-9 and NHb H-4), and two NAc groups at  $\delta$  2.02 and 2.03. The  $^{13}\text{C}$  NMR spectrum of the CPS (Fig. 1) contained signals for four anomeric carbons at  $\delta$  100.1–106.0, two C- $\text{CH}_2$ -C groups at  $\delta$  37.1 (Pse C-3) and  $\delta$  46.4 (NHb C-2), four  $\text{CH}_3$ -C groups at  $\delta$  19.2, 23.6 (2 C), and 23.8 (Pse C-9, NHb C-4, and 2 NAc C-2), three C- $\text{CH}_2\text{O}$  groups at  $\delta$  62.5, 65.1, and 67.9 (C-6 of GalN and 2 Gal, data of the attached proton test), one C-CHOH-C group at  $\delta$  66.3 (NHb C-3), three CH-NH groups at  $\delta$  49.9, 54.9, and 53.0 (Pse C-5 and C-7, GalN C-2), four CO groups at  $\delta$  174.1–176.2 (C-1 of Pse, NHb, and 2 NAc), and other sugar carbons at  $\delta$  68.1–82.7 (table).

These data together indicated that the CPS is composed of tetrasaccharide K units containing two *D*-Gal residues and one residue each of *D*-GalN and Pse. Two amino groups of the amino sugars are *N*-acetylated, and one is *N*-acylated with a (*R*)-3-hydroxybutanoyl group.

The  $^1\text{H}$  NMR spectrum of the CPS was assigned using two-dimensional shift-correlated  $^1\text{H}$ ,  $^1\text{H}$  COSY,

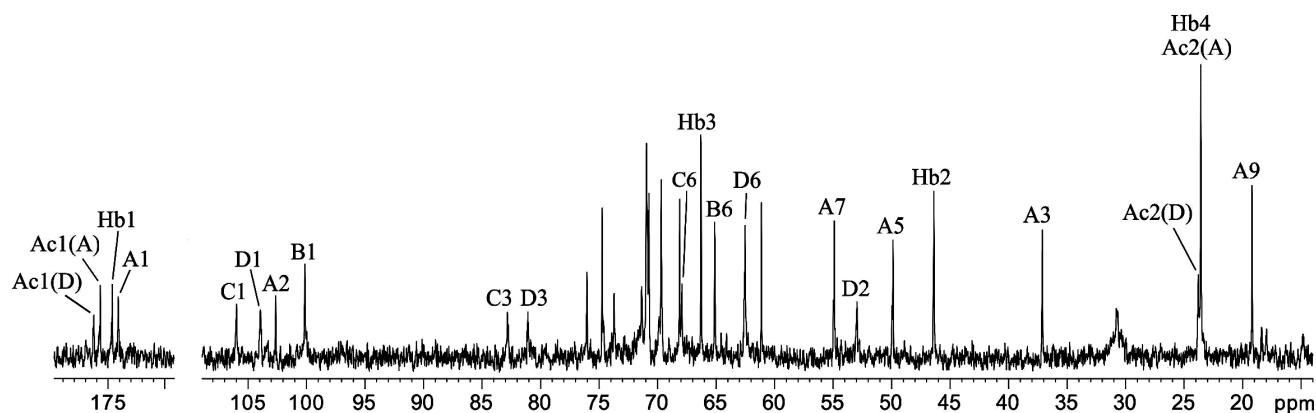
$^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts ( $\delta$ , ppm) of the K93 CPS of *A. baumannii* B11911

Residue	H-1	H-2	H-3 (3ax,3eq)	H-4	H-5	H-6 (6a,6b)	H-7	H-8	H-9
	<i>C-1</i>	<i>C-2</i>	<i>C-3</i>	<i>C-4</i>	<i>C-5</i>	<i>C-6</i>	<i>C-7</i>	<i>C-8</i>	<i>C-9</i>
$\beta$ -Psep5Ac7RHb-(2 $\rightarrow$			1.57, 2.50	3.87	4.18	3.96	4.00	4.07	1.22
<b>A</b>	<i>174.1</i>	<i>102.6</i>	<i>37.1</i>	<i>68.1</i>	<i>49.9</i>	<i>74.7</i>	<i>54.9</i>	<i>70.9</i>	<i>19.2</i>
$\rightarrow$ 6)- $\alpha$ -D-Galp-(1 $\rightarrow$	4.97	3.83	3.80	3.95	4.05	3.56, 3.95			
<b>B</b>	<i>100.1</i>	<i>69.7</i>	<i>70.7</i>	<i>70.7</i>	<i>70.8</i>	<i>65.1</i>			
$\rightarrow$ 3,6)- $\beta$ -D-Galp-(1 $\rightarrow$	4.48	3.63	3.72	4.17	3.87	3.69, 3.69			
<b>C</b>	<i>106.0</i>	<i>71.4</i>	<i>82.7</i>	<i>69.9</i>	<i>73.7</i>	<i>67.9</i>			
$\rightarrow$ 3)- $\beta$ -D-GalpNAc-(1 $\rightarrow$	4.74	4.04	3.88	4.11	3.66	3.75, 3.75			
<b>D</b>	<i>103.9</i>	<i>53.0</i>	<i>81.1</i>	<i>69.7</i>	<i>76.0</i>	<i>62.5</i>			
RNHb		2.35	4.16	1.22					
	<i>174.6</i>	<i>46.4</i>	<i>66.3</i>	<i>23.6</i>					

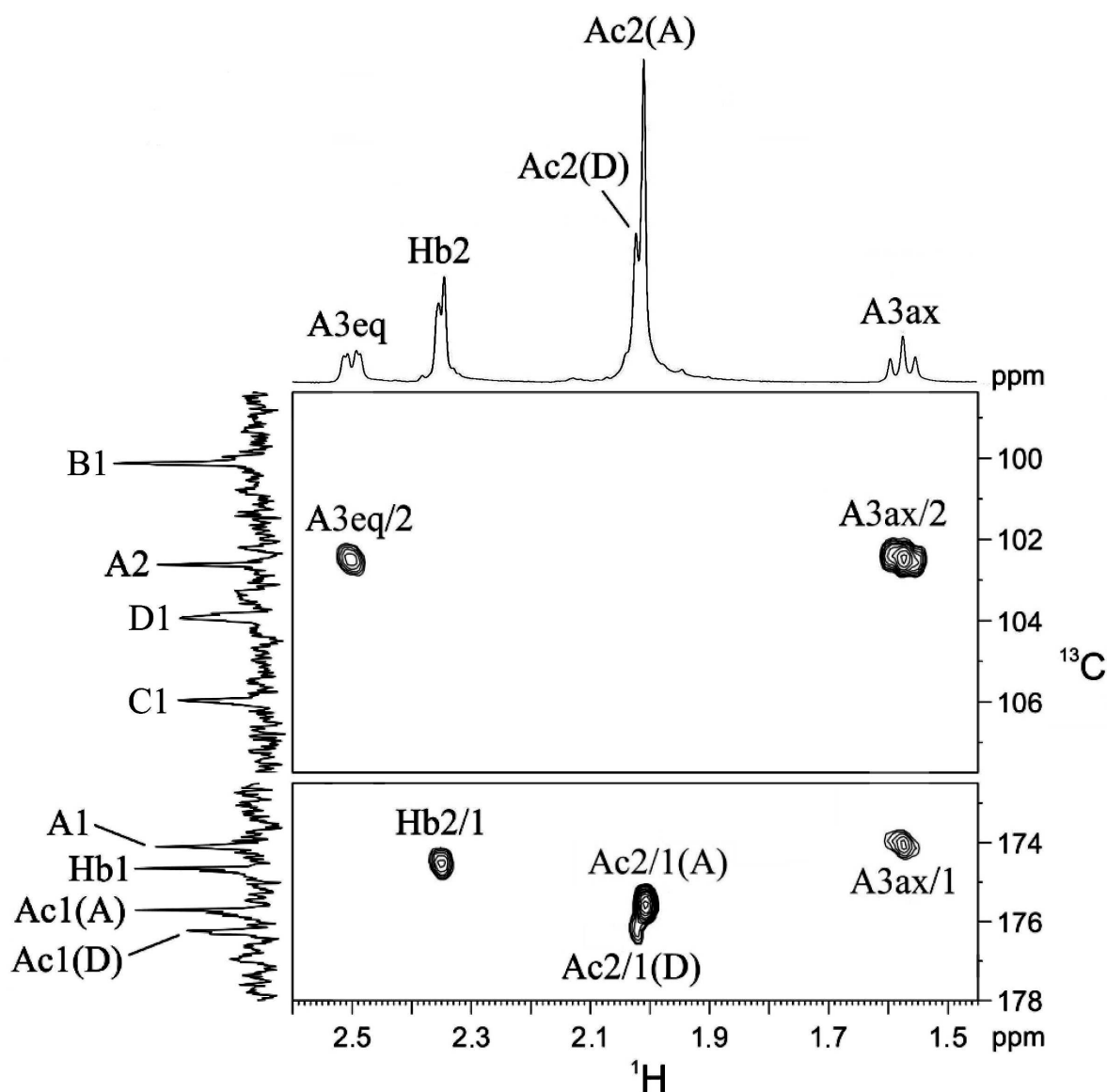
Note:  $^{13}\text{C}$  NMR chemical shifts are italicized. Chemical shifts for the N-acetyl groups are  $\delta_{\text{H}}$  2.02 and 2.03,  $\delta_{\text{C}}$  23.6 and 23.8 (both Me), 175.7 and 176.2 (both CO).

$^1\text{H}$ ,  $^1\text{H}$  TOCSY, and  $^1\text{H}$ ,  $^1\text{H}$  ROESY experiments. Spin-systems for four sugar residues were revealed, which were designated as units **A** ( $\beta$ -Psep), **B** ( $\alpha$ -Galp), **C** ( $\beta$ -Galp), and **D** ( $\beta$ -GalpN), all being in the pyranose form (table). Particularly, the monosaccharides were identified by the following cross-peaks in the TOCSY spectrum: H-3ax/H-4,5, H-3eq/H-4,5, and H-9/H-8,7,6 for unit **A**, H-1/H-2,3,4 for units **B**, **C**, and **D**, combined with correlations between neighboring protons within each monosaccharide residues that were demonstrated by the COSY spectrum. H-1/H-5 correlations for units **C** and **D** in the  $^1\text{H}$ ,  $^1\text{H}$  ROESY spectrum (figure; see Supplement to this paper on the site of the journal ([\[bio.msu.ru/biokhimiya\]\(http://protein.bio.msu.ru/biokhimiya\)\) and Springer site \(Link.springer.com\)\) enabled assignment of the H-5 signals and defined the  \$\beta\$  configuration of these units. The  \$\alpha\$  configuration of unit \*\*B\*\* was inferred by a relatively low  \$^3J\_{1,2}\$  coupling constant of <3 Hz. With the  \$^1\text{H}\$  NMR spectrum assigned, the  \$^{13}\text{C}\$  NMR spectrum was assigned using two-dimensional  \$^1\text{H}\$ ,  \$^{13}\text{C}\$  HSQC and  \$^1\text{H}\$ ,  \$^{13}\text{C}\$  HMBC experiments \(table\).](http://protein.</a></p>
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That unit **A** was a 3-deoxynonulosonic acid was confirmed by H-3ax/C-1, H-3ax/C-2, and H-3eq/C-2 correlations at  $\delta$  1.57/174.1, 1.57/102.6, and 2.50/102.6, respectively, in the  $^1\text{H}$ ,  $^{13}\text{C}$  HMBC spectrum (Fig. 2). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts of unit **A** and  $^3J_{\text{H,H}}$  coupling constants estimated from the two-dimensional



**Fig. 1.**  $^{13}\text{C}$  NMR spectrum of the K93 CPS of *A. baumannii* B11911. Numbers refer to carbons in sugar residues denoted by letters as shown in the table.



**Fig. 2.** Part of a two-dimensional  $^1\text{H}$ ,  $^{13}\text{C}$  HMBC spectrum of the K93 CPS of *A. baumannii* B11911. The corresponding parts of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are shown along the horizontal and vertical axes, respectively. Numbers before and after oblique stroke refer to protons and carbons, respectively, in sugar residues denoted by letters as shown in the table.

NMR spectra were consistent with a non-substituted 5,7-diamino-3,5,7,9-tetraoxy-*L*-glycero- $\beta$ -*L*-manno-non-2-ulopyranosonic (pseudaminic) acid and differed from the spectral parameters of the other known stereoisomers [10-13]. Particularly, relatively large  $J_{3\text{ax},4}$  ( $\sim 12.5$  Hz) and small  $J_{4,5}$  and  $J_{5,6}$  ( $< 5$  Hz) coupling constants indicated the axial orientation of H-4 and the equatorial orientation of H-5. Combined with the  $J_{6,7}$  coupling constant of  $\sim 10$  Hz, these values suggested the *L*-glycero-*L*-manno configuration of unit A, which is thus a derivative of Pse. A relatively large difference between the H-3ax and H-3eq chemical shifts (0.93 ppm) indicated the axial orientation of the carboxyl group, i.e. the  $\beta$  configuration of Pse [10-13]. The absolute configuration of Pse was con-

firmed by analysis of the gene cluster for the CPS biosynthesis (see below).

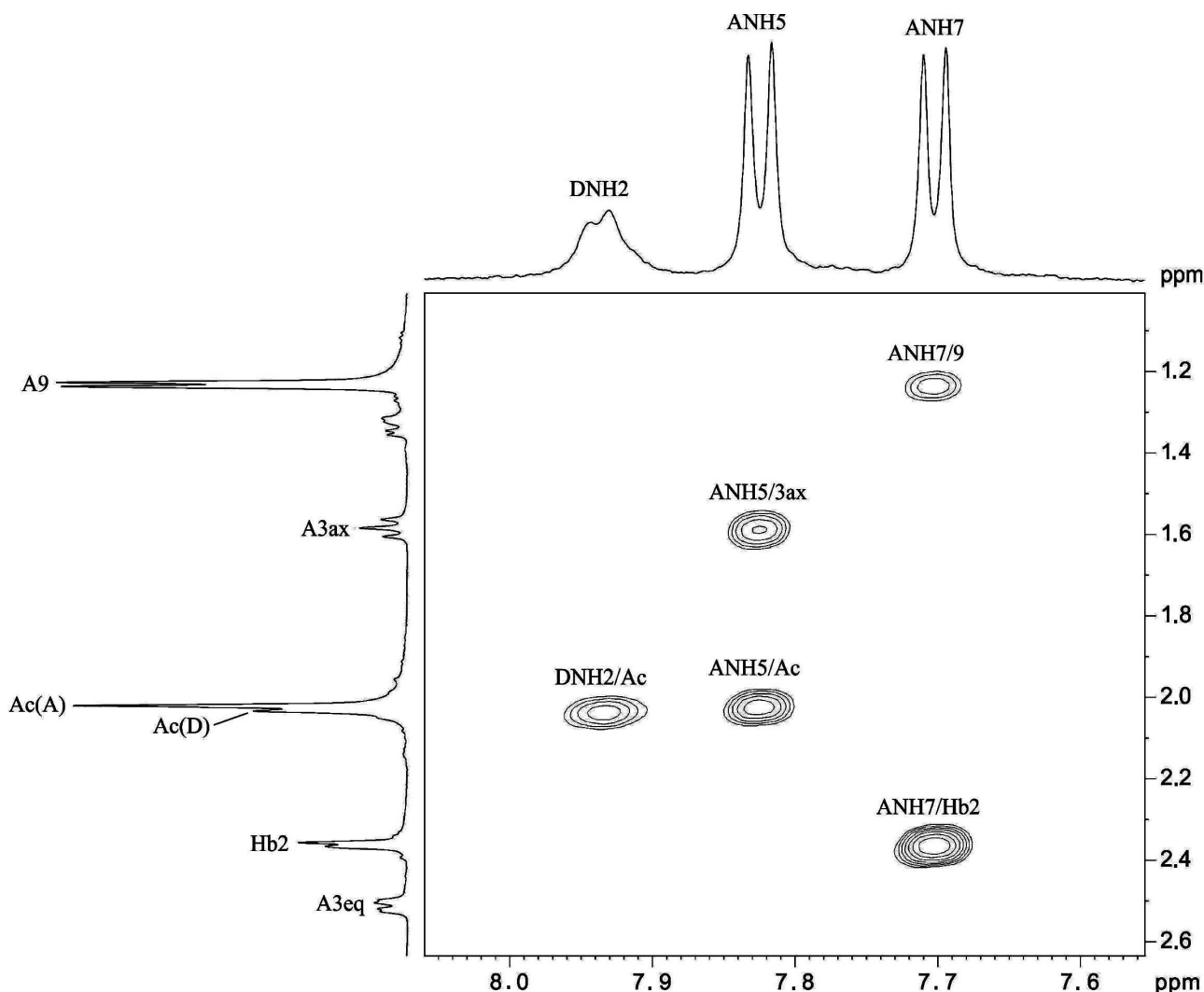
To determine positions of the N-acyl substituents, the NMR spectra of the CPS were measured in a 9 : 1  $\text{H}_2\text{O}$ - $\text{D}_2\text{O}$  mixture, which enabled detection of the nitrogen-linked protons. Signals for three NH protons were observed at  $\delta$  7.70, 7.82, and 7.93 and were assigned to Pse NH-7 and NH-5 and GalN NH-2, respectively, by correlations with CH protons of Pse in the  $^1\text{H}$ ,  $^1\text{H}$  TOCSY spectrum: NH-5/H-3ax,3eq,4,5 and NH-7/H-6,7,8,9. The  $^1\text{H}$ ,  $^1\text{H}$  ROESY spectrum (Fig. 3) showed correlations of GalN NH-2 and Pse NH-5 with the NAc groups at  $\delta$  7.93/2.03 and 7.82/2.02, respectively, and Pse NH-7 with H-2 of the NHb group at  $\delta$  7.70/2.35. The position of the

NHb group was confirmed by a correlation between Pse H-7 and NHb C-1 (CO group) at  $\delta$  4.00/174.6 in the  $^1\text{H}$ ,  $^{13}\text{C}$  HMBC spectrum. Therefore, Pse5Ac7RHb was present in the CPS.

Low-field positions of the signals for C-3 of unit **D**, C-3 and C-6 of unit **C**, and C-6 of unit **B** at  $\delta$  81.1, 82.7, 67.9, and 65.1, respectively, compared with their positions in the corresponding non-substituted monosaccharides at  $\delta$  72.4, 74.1 (both C-3), 62.4 and 62.2 (both C-6), respectively [14], showed that the CPS is branched and defined the glycosylation pattern in the K unit. The  $^1\text{H}$ ,  $^1\text{H}$  ROESY experiment (figure in Supplement) showed **C** H-1/**D** H-3, **D** H-1/**C** H-2,3,4, and **B** H-1/**C** H-6 inter-residue correlations. The  $^1\text{H}$ ,  $^{13}\text{C}$  HMBC spectrum showed a correlation between C-2 of unit **A** (Pse) and H-6b of unit **B** at  $\delta$  102.6/3.95. These data were consistent with the  $^{13}\text{C}$  NMR chemical shift data of the linkage car-

bons and defined the monosaccharide sequence in the CPS.

Based on these data, we concluded that the K93 CPS of *A. baumannii* B11911 has the structure shown in Fig. 4. The CPS contains  $\beta$ -Pse5Ac7RHb that belongs to a class of higher acidic monosaccharides that are rather common in *A. baumannii* strains. Particularly,  $\alpha$ -Pse5Ac7RHb has been found in K42 [15],  $\alpha$ -Pse5Ac7Ac in K2 [16, 17] and K33 [18], and  $\beta$ -Pse5Ac7Ac in K6 [19]. The presence of genes for synthesis of a nucleotide precursor of Pse in the K locus of some other *A. baumannii* strains [17] suggests that there are more CPS K types that include a Pse derivative. Although, to our knowledge, the K93 CPS has a unique structure among known bacterial polysaccharides, it shares the  $\alpha$ -D-Galp-(1 $\rightarrow$ 6)- $\beta$ -D-Galp-(1 $\rightarrow$ 3)-D-GalpNAc trisaccharide fragment with K14 of *A. nosocomialis* LUH 5541 and *A. baumannii* D46 [20, 21]. CPS of



**Fig. 3.** Part of a two-dimensional  $^1\text{H}$ ,  $^1\text{H}$  ROESY spectrum of the K93 CPS of *A. baumannii* B11911 measured in 9 : 1  $\text{H}_2\text{O}$ - $\text{D}_2\text{O}$  mixture. The corresponding parts of the  $^1\text{H}$  NMR spectrum are shown along the axes. Numbers refer to protons in sugar residues denoted by letters as shown in the table.

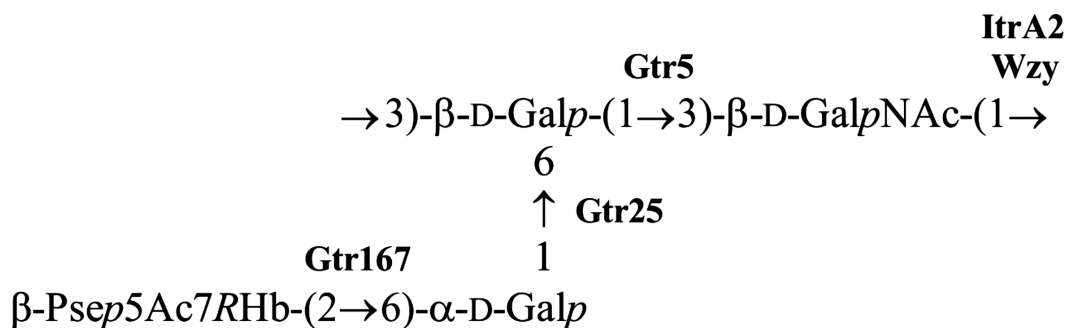


Fig. 4. Structure of the K93 CPS of *A. baumannii* B11911. Glycosyltransferases are shown next to the linkage each is assigned to.

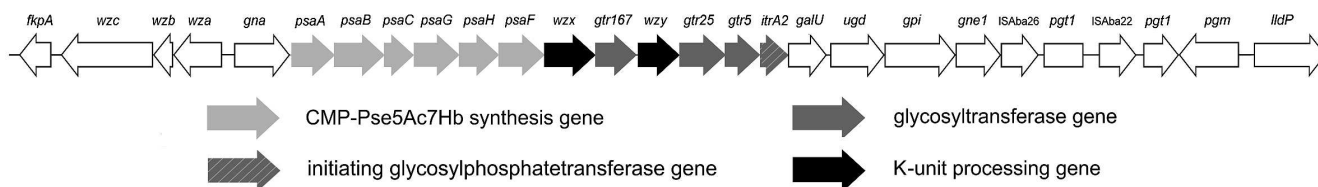


Fig. 5. Organization and content of the KL93 locus of *A. baumannii* B11911. The figure is drawn to scale from GenBank accession number NZ\_LFYX000000001 (base position range: 3339095 to 3366570).

a number of other *A. baumannii* strains, including K2 [16, 17], K6 [19], K27 [22], K33 [18], and K44 [22], have the  $\beta\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-D-GalpNAc}$  disaccharide fragment in common with K93.

#### Characterization of the CPS biosynthesis gene locus.

The gene locus for synthesis and export of the K93 CPS, KL93, has the common genetic organization for *A. baumannii*, with a central region containing genes for synthesis of a specific sugar component (Pse5Ac7RHb), glycosyltransferases for the K-unit assembly, Wzx translocase, and Wzy polymerase (Fig. 5). This CPS-specific portion is flanked by a block of *wz* genes for capsule export on one side and a block of genes for synthesis of common sugars on the other side [2]. In KL93, the latter is distinguished by the occurrence of an insertion sequence (IS) immediately adjacent to the *pgf1* gene for phosphoglycerol transferase, which is interrupted by another IS (Fig. 5).

In KL93, there is a set of six *psa* genes that are close homologs of the genes involved with the synthesis of Pse5Ac7RHb, which are present in KL42 [15]. The same pseudaminic acid derivative is present in the K93 CPS, and hence the *psa* genes were assigned to its synthesis. Other monosaccharides found in K93 (Gal and GalNAc) are common sugars in *A. baumannii* and, accordingly, no other monosaccharide synthesis module is present in KL93.

KL93 encodes an initiating glycosylphosphate transferase having 99 and 97% sequence identity with ItrA2 of KL2 [17] and KL14 [21], respectively. This enzyme initiates the K-unit synthesis by transfer of D-GalpNAc-1-P from UDP-D-GalpNAc to the undecaprenol phosphate

carrier in *A. baumannii* ATCC 17978 (KL3) [23], which is consistent with the presence of GalNAc in K93 CPS and shows that this monosaccharide is the first in the K unit. The Wzy polymerase would then catalyze the formation of the  $\beta\text{-D-GalpNAc}\text{-}(1\rightarrow 3)\text{-D-Galp}$  linkage between the K93 units.

In accordance with the presence of three internal linkages in the K93 unit, KL93 includes three glycosyltransferases. Two of them are 98 and 90% identical to the Gtr5 and Gtr25 predicted glycosyltransferases, which have been assigned to the  $\beta\text{-D-Galp}\text{-}(1\rightarrow 3)\text{-D-GalpNAc}$  and  $\alpha\text{-D-Galp}\text{-}(1\rightarrow 6)\text{-D-Galp}$  linkages in the K14 CPS [21], respectively. Both linkages are present in the K93 unit, and the two Gtr proteins were named accordingly (Fig. 5). The remaining glycosyltransferase gene is evidently responsible for formation of the  $\beta\text{-Psep5Ac7RHb}\text{-}(2\rightarrow 6)\text{-D-Galp}$  linkage. It was named *gtr167*, as suggested by J. J. Kenyon (personal communication). The predicted Gtr167 protein is 42% identical and 62% similar to the Gtr16 inverting glycosyltransferase of *A. baumannii* RBH4 (KL6), which was assigned to the  $\beta\text{-Psep5Ac7Ac}\text{-}(2\rightarrow 4)\text{-D-Galp}$  linkage [19].

Therefore, the genes in the KL93 capsule biosynthesis locus of *A. baumannii* B11911 are consistent with the K93 CPS structure established.

#### Acknowledgements

We thank J. J. Kenyon for her help and critical reading of the manuscript.

This work was supported by the Russian Foundation for Basic Research (grant No. 14-04-00657).

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