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Structure of the K82 Capsular Polysaccharide from *Acinetobacter baumannii* LUH5534 Containing a D-Galactose 4,6-Pyruvic Acid Acetal

STRUCTURE OF K82 CAPSULAR POLYSACCHARIDE FROM *Acinetobacter baumannii* LUH5534

A. A. Kasimova[1,2]*, J. J. Kenyon[3,4], N. P. Arbatsky[1], A. S. Shashkov[1], A. V. Popova[5,6], Y. A. Knirel[1], and R. M. Hall[3,7]

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[1]Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russia; E-mail: yknirel@gmail.com

[2]Higher Chemical College of the Russian Academy of Sciences, D. I. Mendeleev University of Chemical Technology of Russia, 125047 Moscow, Russia; E-mail: nastia-kasimova979797@mail.ru

[3]School of Molecular Bioscience, The University of Sydney, Sydney, NSW 2006, Australia

[4]Institute of Health and Biomedical Innovation, School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Brisbane, QLD 4059, Australia; E-mail: johanna.kenyon@qut.edu.au

[5]Moscow Institute of Physics and Technology, 141700 Dolgoprudny, Moscow Region, Russia; E-mail: popova_nastya86@mail.ru

[6]State Research Center for Applied Microbiology and Biotechnology, 142279 Obolensk, Moscow Region, Russia

[7]School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia; E-mail: ruth.hall@sydney.edu.au

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* To whom correspondence should be addressed.

Abbreviations: COSY, correlation spectroscopy; CPS, capsular polysaccharide; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; Und-P, undecaprenyl phosphate.

Abstract—Type K82 capsular polysaccharide (CPS) was isolated from *Acinetobacter baumannii* LUH5534. The structure of a linear tetrasaccharide repeating unit of the CPS was established by sugar analysis along with one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy. Proteins encoded by the KL82 capsule gene cluster in the genome of LUH5534 were assigned to roles in the synthesis of the K82 CPS. In particular, functions were assigned to two new glycosyltransferases (Gtr152 and Gtr153) and a novel pyruvyltransferase, Ptr5, responsible for the synthesis of D-galactose 4,6-(*R*)-pyruvic acid acetal (D-Gal4,6*R*Pyr).

Keywords: *Acinetobacter baumannii*, capsular polysaccharide structure, pyruvic acid acetal, K locus, genetics of capsule biosynthesis

Acinetobacter baumannii has become one of the most widespread agents causing nosocomial infections. Currently, the majority of *A. baumannii* isolates display resistance to almost all therapeutically suitable antibiotics (World Health Organization (2017) Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics at http://www.who.int/medicines/publications/global-priority-list-antibioticresistant-bacteria/en/).

A thick polysaccharide capsule (CPS, K antigen) surrounds the *A. baumannii* cell and protects the bacterium from the action of immune system components, as well as disinfectants, desiccation, and some antimicrobial compounds. The CPS is composed of many oligosaccharide repeats (K units) and is characterized by high structural diversity mainly due to variability of the gene content at the chromosomal K locus (KL) driving the CPS biosynthesis. To date, more than 120 various gene clusters at the K locus have been recognized ([1, 2]; J. J. Kenyon, unpublished data). The chemical structures for about 40 different *A. baumannii* CPSs have been established ([3-7] and references cited in [3]) and form the basis for classification of strains of these bacteria by K antigens. Most of these structures are consistent with putative functions of CPS synthesis genes located at the K locus.

In this work, we report on the structure and gene cluster of the CPS that is specific for *A. baumannii* LUH5534. The CPS gene cluster originally designated PSgc3 has been sequenced and genes have been annotated by comparison with sequences in available databases [2]. Here, we re-specified this gene cluster using the established nomenclature system for *A. baumannii* CPS [1] and rename it KL82. Accordingly, the CPS of *A. baumannii* LUH5534 was assigned to the K82 type.

MATERIALS AND METHODS

Cultivation of bacteria. *Acinetobacter baumannii* strain LUH5534 was cultivated in 2× TY media for 24 h. Cells were harvested by centrifugation (10,000*g*, 20 min), washed with phosphate-buffered saline (pH 7.4), suspended in a 7 : 3 acetone/water mixture (v/v), precipitated, and dried.

Isolation of capsular polysaccharide. CPS was isolated by phenol–water extraction [8] of bacterial cells (1.12 g). The extract was dialyzed without layer separation, freed from insoluble contaminations by centrifugation, and proteins and nucleic acids were precipitated by aqueous 50% CCl_3CO_2H at 4°C. After dialysis

against distilled water and centrifugation, the supernatant was fractionated by gel-permeation chromatography on a column (60 × 3.5 cm) of Sephadex G-50 in 0.1% CH_3CO_2H, and a high-molecular-mass fraction was freeze-dried to give a CPS preparation (65 mg).

Deacetylation. A CPS sample (16 mg) was treated with 4% CH_3CO_2H (1.5 ml, 100°C, 7 h), the products were fractionated by gel-permeation chromatography on a column (110 × 2 cm) of Sephadex G-25 in water, and a high-molecular-mass fraction (6 mg) was subjected to anion-exchange chromatography on a column (8 × 1 cm) of DEAE-Toyopearl TSK 650M in water to give a neutral modified polysaccharide (MPS) (1.5 mg).

Monosaccharide analysis. A CPS sample (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h). Monosaccharides were analyzed by GLC as the alditol acetates on a Maestro (Agilent 7820) chromatograph (Interlab, Russia) equipped with an HP-5 column (0.32 mm × 30 m) using a temperature program of 160°C (1 min) to 290°C at 7°C/min.

NMR spectroscopy. Samples were deuterium-exchanged by freeze-drying from 99.9% D_2O and then examined as solutions in 99.95% D_2O. Sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (δ_H 0, δ_C −1.6) was used as internal reference for calibration. NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer (Germany) at 60°C using standard Bruker software and Bruker TopSpin 2.1 program to acquire and process the NMR data. A 60-ms MLEV-17 spin-lock time and a 150-ms mixing time were used in TOCSY and ROESY experiments, respectively. A 60-ms delay was used for evolution of long-range couplings to optimize $^{1}H,^{13}C$ HMBC experiments for the coupling constant of $J_{H,C}$ 8 Hz.

Bioinformatics. The BLASTp database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and the Pfam database (http://pfam.xfam.org/) were used to assign encoded proteins to CPS biosynthesis roles.

RESULTS AND DISCUSSION

A crude CPS preparation was isolated from *A. baumannii* LUH5534 by phenol–water extraction. Sugar analysis of the CPS by GLC of the acetylated alditols revealed

the presence of D-Gal, D-GlcNAc, and D-GalNAc in the ratio ~0.6 : 1 : 0.5, respectively. The CPS was studied by NMR spectroscopy, including one-dimensional ^{1}H NMR and ^{13}C NMR (Fig. 1) spectra and two-dimensional ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and HMBC experiments. Four sugar spin systems were identified, including those for β-Gal*p*NAc (unit **A**), α-Glc*p*NAc (unit **C**), and two β-Gal*p* residues (units **B** and **D**), all monosaccharides being in the pyranose form. The assigned ^{1}H and ^{13}C NMR chemical shifts of the CPSs are tabulated in Table 1. Therefore, the CPS has a tetrasaccharide K unit.

Relatively large $J_{1,2}$ coupling constants of 7-8 Hz indicated that the Gal*p* and Gal*p*NAc residues were β-linked, whereas an α-linked Glc*p*NAc was characterized by a smaller coupling constant ($J_{1,2}$ < 4 Hz).

The NMR spectra also showed signals for a pyruvic acid acetal (Pyr). They were identified by C/H correlations in the ^{1}H,^{13}C HSQC spectrum (Table 1) and C1/H3 and C2/H3 correlations at δ 175.0/1.51 and 101.2/1.51, respectively, in the ^{1}H,^{13}C HMBC spectrum.

Low-field positions at δ 82.0, 78.7, 80.3 and 79.2 of the signals for C3 of units **A**, **B**, and **C**, and C2 of unit **D**, respectively, showed that the CPS is linear, the first three monosaccharide residues in the repeating unit being 3-substituted and residue **D** 2-substituted. The signals for C4 and C6 of β-Gal*p* were shifted downfield to δ 72.7 and 66.2, respectively, and the C5 signal was shifted upfield to δ 67.1, as compared with their positions in non-substituted β-Gal*p* [9], showed that Pyr is attached at positions 4 and 6 of this monosaccharide. The chemical shift of δ 26.3 for C3 of Pyr indicated that the acetal has the (*R*)-configuration [10].

The sequence of the monosaccharides in the repeating unit was determined by the ^{1}H,^{1}H ROESY and ^{1}H,^{13}C HMBC experiments, which showed correlations of the anomeric protons and carbons with atoms of the neighboring sugar residues (Table 2). These data also confirmed the substitution pattern in the K unit.

The K82 CPS structure was corroborated by cleavage of Pyr by mild acid hydrolysis followed by structure elucidation of the resulting modified polysaccharide (MPS) by NMR spectroscopy as described above for the CPS (the assigned ^{1}H and ^{13}C NMR chemical shifts are tabulated in Table 1).

Based on these data, it was concluded that the K82 CPS from *A. baumannii* LUH5534 has the structure shown in Fig. 2. A peculiar feature of this CPS is the presence of (*R*)-configured D-galactose 4,6-pyruvic acid acetal. Earlier, a similar acetal

but on a D-GalNAc residue was reported in the K4 CPS from *A. baumannii* D78 [11].

The KL82 gene cluster in *A. baumannii* LUH5534 (GenBank accession number KC526908) between conserved genes *gna* and *galU* contains genes involved specifically with the synthesis of the K82 CPS (Fig. 3). The *itrA2* gene in the KL82 locus encodes an initiating transferase, ItrA2 (WeeH in GenPept, accession number AHB32565.1), which is 99% identical to ItrA2 of *A. baumannii* KL2 (AGK44809.1 in GenPept) shown to use D-GalNAc as the initiating sugar in the K-unit assembly [12, 13]. ItrA2 in KL82 is 99% identical to ItrA2 of *A. baumannii* ATCC 17978 (KL3). Hence, D-GalNAc is the first sugar of the K82 unit, and the Wzy polymerase (AHB32563.1 in GenPept) would catalyze formation of the β-D-Gal*p*NAc-(1→3)-D-Ga*lp* linkage between the K units (Fig. 2).

Glycosyltransferase Gtr5 encoded by KL82 (GenPept accession number AHM95430.1) is 95% identical to Gtr5a from *A. baumannii* KL2 (GenPept accession number AHM95430.1), which is responsible for formation of the β-D-Ga*lp*-(1→3)-D-Gal*p*NAc linkage in the K2 CPS [14]. This linkage is the first in the K82 unit, and Gtr5 was assigned accordingly.

Two further glycosyltransferase genes, *gtr152* and *gtr153*, were also identified in KL82 (Fig. 3) and are evidently responsible for adding the next two sugar residues to complete the assembly of the tetrasaccharide K unit (Fig. 2). Gtr153 (WafK in GenPept, accession number AHB32562.1) is 54% identical to Gtr58 from *A. baumannii* KL27 (ALL34866.1 in GenPept) that was previously predicted to form an α-D-Glc*p*NAc-(1→3)-D-Gal*p* linkage [15]. Accordingly, Gtr153 would catalyze this linkage, which also is present in the K82 CPS, and Gtr152 (WafJ in GenPept, accession number AHB32561.1) was assigned to the last β-D-Gal*p*-(1→3)-D-Glc*p*NAc linkage (Fig. 2).

The product of the gene located immediately downstream of the *wzx* gene was identified as a pyruvyl transferase belonging to protein family (Pfam) PF04230 and was named Ptr5 (WafI in GenPept, accession number AHB32560.1). Ptr5 is not significantly related to Ptr1 from *A. baumannii* KL4 (GenPept accession number JN409449.3) putatively involved with formation of a D-GalNAc4,6Pyr [15], though it would be responsible for the similar attachment of pyruvic acid to the Gal residue (**D**) in the K82 CPS.

The generally conserved *galU-ugd-gpi-gne1-pgm* arrangement in *A. baumannii* is interrupted in KL82 by a partial copy of ISAba13 (138-1016 of 1039 bp) and a gene encoding a putative acyltransferase (GenPept accession number AHB32571.1

previously annotated as CgmA) that belongs to Pfam PF01757. The first 142 amino acids of the acyltransferase (201 a.a. totally) is 82% identical to Atr5 (142 a.a.) from *A. baumannii* KL4 (GenPept accession number ACJ39541.2), and neither appears to modify the K4 [11] or K82 CPS. An additional gene cluster KL124 differs from KL82 only in this region (Fig. 3).

Finally, the occurrence in KL82 of genes for flippase Wzx and polymerase Wzy indicated that, as for all other known CPSs of *A. baumannii*, the K82 CPS is synthesized by the Wzx/Wzy-dependent pathway. Therefore, the content of the KL82 gene cluster and predicted gene functions are consistent with the K82 CPS structure established in this work.

Acknowledgments

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Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) K82 CPS from *A. baumannii* LUH55534

Residue	C1 *H1*	C2 *H2*	C3 *H3*	C4 *H4*	C5 *H5*	C6 *H6 (6a,6b)*
CPS						
→3)-β-D-Gal*p*NAc-(1→	103.4	52.6	82.0	69.5	76.1	61.7
A	*4.76*	*4.03*	*3.82*	*4.14*	*3.65*	*3.82*
→3)-β-D-Gal*p*-(1→	106.0	70.5	78.7	66.4	76.1	62.4
B	*4.47*	*3.63*	*3.66*	*4.04*	*3.59*	*3.71, 3.76*
→3)-α-D-Glc*p*NAc-(1→	95.7	53.0	80.3	68.8	72.8	62.5
C	*5.01*	*4.13*	*4.06*	*3.70*	*4.01*	*3.75, 3.83*
→2,4,6)-β-D-Gal*p*-(1→	100.5	79.2	73.6	72.7	67.1	66.2
D	*4.64*	*3.75*	*3.75*	*4.13*	*3.55*	*3.90, 4.02*
Pyr	175.2	101.2	26.3			
			1.50			
MPS						
→3)-β-D-Gal*p*NAc-(1→	103.1	52.6	82.2	69.6	76.1	61.6
A	*4.77*	*4.03*	*3.82*	*4.15*	*3.64*	*3.82*
→3)-β-D-Gal*p*-(1→	106.1	70.4	78.6	66.3	76.1	62.3
B	*4.47*	*3.62*	*3.67*	*4.06*	*3.60*	*3.75*
→3)-α-D-Glc*p*NAc-(1→	95.5	53.6	80.1	69.2	69.9	62.4
C	*5.02*	*4.11*	*4.08*	*3.66*	*4.00*	*3.73, 3.82*
→2)-β-D-Gal*p*-(1→	101.8	79.5	75.0	70.1	76.4	62.4
D	*4.59*	*3.66*	*3.69*	*3.86*	*3.69*	3.73

Notes: ^{1}H NMR chemical shifts are italicized. Chemical shifts for N-acetyl groups are: δ_C 23.4-23.6 (Me) and 175.2-176.0 (CO), δ_H 2.01-2.13.

Table 2. Correlations for H1 and C1 in the two-dimensional ^{1}H,^{1}H ROESY and ^{1}H,^{13}C HMBC spectra of the K82 CPS from *A. baumannii* LUH55534

Atom in sugar residue (δ)	Correlations to atoms in sugar residues (δ)	
	^{1}H,^{1}H ROESY	^{1}H,^{13}C HMBC
A H1 (4.76)	**D** H2 (3.75), **A** H3 (3.82), **A** H5 (3.65)	**D** C-2 (79.2)
A C1 (103.4)		**D** H2 (3.75), **A** H2 (4.03), **A** H5 (3.65)
B H1 (4.47)	**A** H3 (3.82), **B** H2 (3.63), **B** H3 (3.66), **B** H5 (3.59)	**A** C3 (82.0)
B C1 (106.0)		**A** H3 (3.82), **B** H2 (3.63), **B** H5 (3.59)
C H1 (5.01)	**B** H2 (3.63), **B** H3 (3.66), **B** H4 (4.04)	**B** C3 (78.7), **C** C3 (80.3), **C** C5 (72.8)
C C1 (95.7)		**B** H3 (3.66)
D H1 (4.64	**C** H2 (4.13, w), **C** H3 (4.06), **C** H4 (3.70, w), **D** H3 (3.75), **D** H5 (3.55)	**C** C3 (80.3)
D C1 (100.5)		**C** H3 (4.06), **D** H2 (3.75), **D** H5 (3.55)

Note: w, weak.

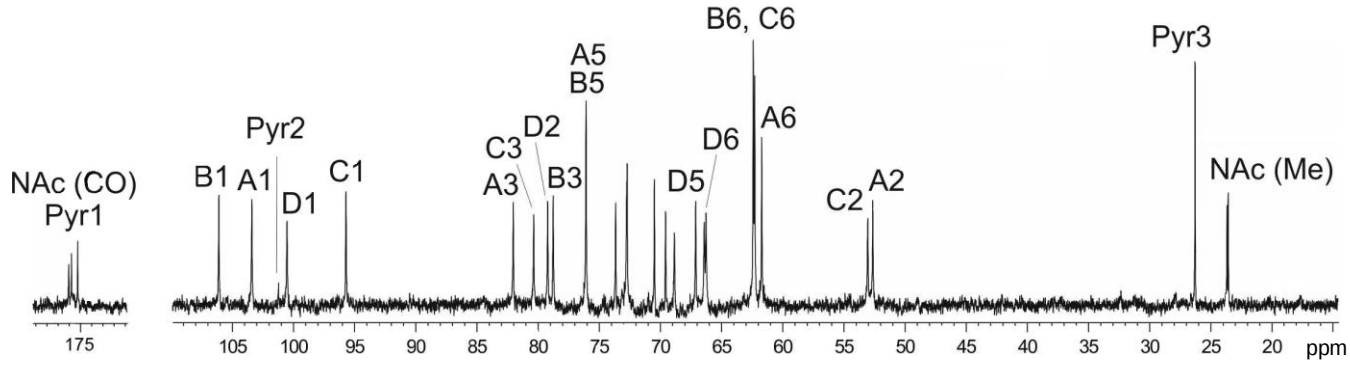

Fig. 1. ^{13}C NMR spectrum of the K82 CPS from *A. baumannii* LUH5534. Numbers refer to carbons in Pyr and sugar residues denoted by letters as shown in Table 1 and Fig. 2.

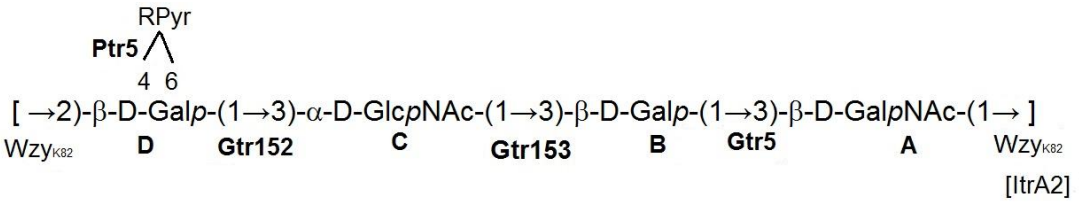

Fig. 2. Structure of the K82 CPS from *A. baumannii* LUH5534. *R*Pyr indicates (*R*)-1-carboxyethylidene (pyruvic acid acetal). Transferases are shown near the linkage they are predicted to catalyze the formation of.

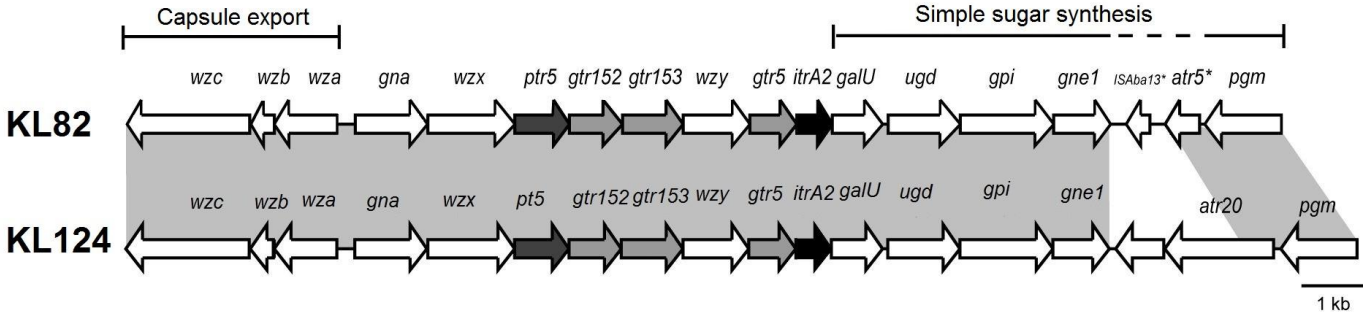

Fig. 3. Comparison of the *A. baumannii* KL82 and KL124 capsule biosynthesis gene clusters. Shading between gene clusters indicates shared regions of >95% nucleotide sequence identity.