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EXPERIMENTAL ARTICLES

Characterization of the Lipipolysaccharides of *Pseudomonas chlororaphis*

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Abstract—Lipopolysaccharides (LPS) from two strains of *Pseudomonas chlororaphis* subsp. *aureofaciens*, UCM B-111 and UCM B-306, were isolated and characterized. The LPS preparations exhibited low toxicity, high pyrogenicity, and high antiviral activity. Mild acid hydrolysis was used to obtain the O-specific polysaccharides. Their structures were established by monosaccharide analysis and determination of absolute configurations, as well as by 1D and 2D NMR spectroscopy. The O-polysaccharides were shown to contain the linear tri- or tetrasaccharide repeating units. Both O-polysaccharides were structurally heterogeneous:
P. chlororaphis subsp. *aureofaciens* UCM B-111
→ 4)-α-D-Gal*p*NAc*6Ac*-(1 → 3)-β-D-Qui*p*NAc-(1 → 6)-α-D-Glc*p*NAc-(1→
β-D-Glc*p*NAc-(1 → 3)-];
degree of the non-stoichiometric 6-O-acetylation of GalNAc ~60%;
P. chlororaphis subsp. *aureofaciens* UCM B-306
→ 3)-α-D-Rha*p*-(1 → 4)-α-D-Gal*p*NAcA*N*-(1 → 3)-α-D-Qui*p*NAc4NAc-(1 → ,
where GalNAcAN is 2-acetamido-2-deoxy-D-galacturonamide, the degree of non-stoichiometric amidation of the GalNAcA residue ~60%.

Keywords: Pseudomonas chlororaphis subsp. *aureofaciens*, lipopolysaccharide, O-specific polysaccharide, structure, biological activity

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Lipopolysaccharides (LPS) of various *Pseudomonas* species have been studied in different ways; most publications are concerned with investigation of carbohydrate-containing biopolymers of such species as *Pseudomonas aeruginosa* (Bystrova et al., 2003, 2004; Knirel et al., 2006), which play an important role in human pathology, and of a plant pathogenic species *P. syringae* (Zdorovenko et al., 2001; Zatonsky et al. 2002). Little is known about the LPS of saprophytic species, e.g., *Pseudomonas chlororaphis*, a biologically active representative of pseudomonads. At the beginning of our study, there was only little information on the isolation of LPS from *P. chlororaphis*, as well as about their structure, biological activity, and possible role as taxonomic markers. At the same time, it was known that antibiotics produced by *P. chlororaphis* strains had an important role in biocontrol; the mechanisms of bacterial colonization of plant roots and pathogen suppression, as well as the genes involved in these processes, were studied (Weller, 2007; *Pseudomonas...*, 2008).

Preparations containing strains of *P. chlororaphis* (e.g., Pseudobacterin-2) have been already used against plant mycoses, and the studies on testing and application of novel active strains are in progress (Raio, 2011). In the Institute of Microbiology and Virology, NAS of Ukraine, the complex biopreparation Gaupsin based on two strains of *P. chlororaphis* subsp. *aureofaciens* was elaborated and patented (Ukraine Patents no. 73682, 2005); it suppressed growth of phytopathogenic bacteria and fungi and, apart from antimicrobial action, exhibited considerable entomopathogenic activity. Gaupsin contains strains *P. chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306, which were selected from a large collection of pseudomonads in the course of wide-ranging studies of their antagonistic activities against phytopathogenic fungi and bacteria, as well as against some harmful insects, in particular, apple seedworm and potato beetle. Gaupsin is successfully applied for protection of vegetables, grain crops, fruit trees, vineyards, and woodlands from fungal and bacterial diseases, as well as from insect pests. However, the substances responsible for the biological activity of the preparation were characterized insufficiently or

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were not studied at all. In 2013, from the culture liquids of strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306, we isolated exopolymers, which exhibited high activity against tobacco mosaic virus (TMV) (Kiprianova et al., 2013). These substances decreased the TMV development by 76–96% at a concentration of 10 mg/mL, and by 40–62 and 14–27% at concentrations of 1.0 and 0.1 mg/mL, respectively.

The studied exopolymers contained up to 7.6% of carbohydrates represented by such neutral monosaccharides as fucose, mannose, galactose, and glucose. Since LPS of some *Pseudomonas* strains were known (Shibuya et al., 2007) to activate and initiate expression of defense mechanisms in plants in response to fungal and bacterial infections, we isolated LPS from strains *P. chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306 and studied their activity against TMV (Kiprianova et al., 2013). As a result, it was found that the LPS of these strains were also highly active antiviral substances.

Since characteristics of these LPS were unknown, the goal of the present work was to isolate and identify the LPS from two strains of *P. chlororaphis* subsp. *aureofaciens* and to study their functional and biological activities.

MATERIALS AND METHODS

Research subjects were two strains *Pseudomonas chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306 isolated from soil and cabbage rhizosphere, respectively, and deposited in the Collection of the Institute of Microbiology and Virology, NAS of Ukraine under the numbers IMV B-7097 and IMV B-7096, respectively.

Cultivation of the strains was carried out in 750-mL Erlenmeyer flasks with 150 mL of the semidefined King A medium containing the following (g/L): peptone, 20; K_2SO_4, 20; glycerol, 20; and $MgCl_2$, 7.0 on a shaker (220 rpm) for 24 h at 28°C.

LPS isolation. Lipopolysaccharides were extracted from the cells, which were preliminarily dehydrated with acetone and diethyl ether, by using 45% aqueous phenol solution at 65–68°C. The obtained aqueous fractions were dialyzed sequentially against tap and distilled water for phenol removal. Nucleic acids were removed by precipitation with trichloroacetic acid and subsequent ultracentrifugation of the supernatant (104 000 *g*, 4 h); the purified LPS was lyophilized (Varbanets et al., 2006).

To isolate the individual structural components, the LPS was hydrolyzed with 3% acetic acid (100°C, 6 h); the precipitate of lipid A was collected by ultracentrifugation (25000 *g*, 40 min); the supernatant was concentrated to 10 mL and fractionated on a column (70 × 3 cm) with Sephadex G-50 using 0.025 M pyridine–acetate buffer (pH 4.5) as the eluting agent. As a result, fractions of the O-specific polysaccharide (OPS) and the core oligosaccharide were obtained.

Assays of carbohydrates, nucleic acids, and protein. The amounts of the following components were determined: carbohydrates by reaction with phenol and sulfuric acid (Varbanets et al., 2006); nucleic acids by the method of Spirin (Varbanets et al., 2006); protein by the Lowry method (Varbanets et al., 2006); 2-keto-3-deoxyoctulosonic acid (KDO) by reaction with thiobarbituric acid (Caroff et al., 1987).

Identification of neutral monosaccharides was carried out after hydrolysis of the preparations in 2 M CF_3COOH (2 h, 120°C). Monosaccharides were analyzed in the form of polyol acetates (Albershein et al., 1976) on a Maestro 7820 GC gas chromatograph (Interlab, Russia) with an HP-5ms column at a programmed temperature range from 160°C (1 min) to 290°C scanned at 7°C/min. Monosaccharides were identified by comparing the retention times of polyol acetates in the experimental and standard samples. The quantitative ratio of individual monosaccharides was expressed as the percentage of the sum of peak areas.

The absolute configurations of monosaccharides were determined by GLC of acetylated glycosides with the use of (S)-2-octanol on a Maestro 7820 GC gas chromatograph (Interlab, Russia) with an HP-5ms column at a programmed temperature range from 160°C (1 min) to 290°C scanned at 7°C/min (Varbanets et al., 2006).

NMR spectroscopy. The samples were twice lyophilized from D_2O and analyzed in 99.95% D_2O. The ^1H- and ^{13}C-NMR spectra of the preparations were recorded on a Bruker Avance II 600 MHz spectrometer (Germany) at 30°C. The chemical shifts were determined with the use of sodium-3-trimethylsilyl propanoate-d^4 (δH 0.00) and acetone (δC 31.45) as internal standards. The 2D experiments were carried out with the use of the Bruker and Bruker TopSpin 2.1 software packages. The mixing time in TOCSY and ROESY experiments was 100 ms; the delay time in HMBC was 60 ms.

Fatty acid composition was determined after the sample hydrolysis in 1.5% acetyl chloride solution in methanol (100°C, 4 h); the fatty acid methyl esters were analyzed on an Agilent 6890N/5973 inert chromato–mass spectrometric system with an HP-5MS column (30 m × 0.25 mm × 0.25 μm) at the temperature mode of 150–250°C and temperature gradient, 4°C, with helium as the carrier gas at the flow rate of 1.2 mL/min. The evaporator temperature was 250°C; flow distribution was 1 : 100. Fatty acids were identified using the PC database and the standard mixture of the fatty acid methyl esters. The quantitative ratio of individual fatty acids was expressed as the percentage of the total peak areas.

Immunological studies. To obtain the O-antiserum, heated cells of strains *P. chlororaphis* subsp. *aureofa-*

ciens UCM B-111 and UCM B-306 were used (2.5 h, boiling water bath); the cell concentration was 2×10^9/mL. The rabbits were immunized intravenously five times with 4-day intervals (from 0.1 to 1 mL).

The antigenic activity of LPS was studied by the method of double immunodiffusion in agar according to Ouchterlony (Ouchterlony, 1962).

The LPS pyrogenicity was determined in rabbits weighing 2.0–3.5 kg (Varbanets et al., 2006). Thermometry was performed with an electronic thermometer (Omron Matsusaka Co. Ltd., Japan), which was inserted into the rectum to a depth of 5–7 cm (depending on the weight of a rabbit). All rabbits were pretested for immunoreactivity by intravenous injection of 10 mL/kg of 0.9% sterile apyrogenic sodium chloride solution. The tested LPS preparations were dissolved in a sterile apyrogenic isotonic solution, incubated for 10 min at 37°C before the injection, and introduced intravenously (1 mL/kg of animal weight). The minimal pyrogenic dose of the LPS preparations was determined in a series of dilutions from 0.5 to 0.01 mg/mL. Each series of the solutions was tested in 3 rabbits of similar weight (at a difference not exceeding 0.5 kg).

Prior to injection of the LPS solution, the temperature of the rabbits was measured twice with a 30-min interval. Since the difference between temperature values should not exceed 0.2°C, the animals not satisfying this criterion were not used for investigation. The result of the last measurement was taken as the initial temperature. The LPS solution was injected not later than 15–20 min after the last temperature measurement. After the injection, the measurements were made three times with 1-h intervals. The tested LPS solution was considered apyrogenic if the total temperature increase during 3 h did not exceed 1.4°C.

Determination of LPS toxicity was carried out in healthy white non-pedigreed mice of both sexes weighing 19–21 g, not used previously for any experiments and sensibilized with galactosamine. For this purpose, 0.5 mL of 3.2% D-galactosamine hydrochloride solution in apyrogenic sterile 0.9% NaCl solution was injected intraperitoneally into all mice. Immediately after that, 0.2 mL of LPS in isotonic sterile apyrogenic saline solution heated to 37°C was injected intraperitoneally at a rate of 0.1 mL/s. In the series of LPS dilutions, the preparation dose causing death of 50% tested animals (LD_{50}) was determined and its value was used for assessment of LPS toxicity. The control and experimental groups consisted of 10 mice each. The control mice were injected with 0.2 mL of sterile 0.9% NaCl solution together with D-galactosamine hydrochloride. The animals were observed for 48 h (Varbanets et al., 2006).

The lethal dose LD_{50} was also calculated by the Nowotny method (Nowotny, 1979):

$$\log LD_{50} = \log X_{100} - (\log Fd/n)\ (\Sigma t - n/2),$$

where

$\log X_{100}$ is the logarithm of the largest dose tested;

$\log Fd$ is multiplicity of dilutions of the tested preparations;

Σt is the total number of died animals;

n is the number of animals per dose.

Antiviral effect of LPS was determined as described earlier (Shcherbinskaya et al., 2001). The effective dose (ED_{50}), a minimal amount of the LPS preparation which suppressed development of the virus-specific cytopathogenic dose (CPD) by 50%, was determined as follows. The test virus in a dose of 100 TCD_{50}/ 0.1 mL was added into the culture of the baby hamster kidney (BHK) cells and incubated at 37°C for 1 h. After adsorption of the virus on the cells, its remainder was removed, and the cells were washed with the RPMI-1640 medium; the studied LPS preparations were added into the maintaining medium (the RPMI-1640 medium supplemented with 2% of fetal serum). The value of ED_{50} of the LPS preparation was determined from the absence of CPD in the experimental variant and its presence in the control, as well as from a decrease in the infectious titer in the experimental variant by 2.0 and more of log ID_{50} as compared with control.

RESULTS AND DISCUSSION

The yield of the LPS isolated from strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 by the phenol–water method reached 11.3–12.9% which is higher than the mean values typical of the family *Enterobacteriaceae* (5%), but lower than that in some *Pseudomonas* strains (up to 32%).

Since the LPS preparations were characterized by high content of nucleic acids (up to 30%), which was probably due to the method of isolation applied, they were purified by ultracentrifugation. The purified preparations of the LPS of strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 contained large amounts of carbohydrates (30–35%), insignificant content of nucleic acids (3.1 and 4.3%, respectively), and traces of protein (0.1 and 0.4%, respectively).

The typical LPS components of gram-negative bacteria, heptose and KDO, in strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 made up 3.3–6.2 and 0.7–0.5%, respectively.

Thus, the LPS isolated for the first time from *P. chlororaphis* subsp. *aureofaciens* strains B-111 and B-306 contained all the components typical of these biopolymers.

Analysis of the fatty acid composition of lipid A of the LPS from the studied strains (Table 1) showed the presence of fatty acids with 12 to 16 carbon atoms in their chains, as well as an unidentified component which comprised 13.5–13.7% in both strains. Lipids A of strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 contained 2-hydroxydodecanoic acid as

Table 1. Fatty acid composition of lipid A of the LPS from strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306

LPS of strains	Fatty acids, % of the total peak areas						
	C12:0	2-OH-C12:0	3-OH-C12:0	3-OH-C14:0	C16:0	C16:1	X
B-111	2.3	63.5	8.3	3.1	6.5	2.8	13.5
B-306	4.1	61.9	8.4	4.2	7.7	–	13.7

Table 2. Determination of acute toxicity of LPS

LPS of strains	The number of dead (numerator) and the total number (denominator) of animals after injection of different doses (d, μg/mouse) of the studied LPS					LD_{50}	
	$d = 0.02$	$d = 0.2$	$d = 2.0$	$d = 20$	$d = 200$	μg/mouse	mg/kg of the mouse weight
B-306	0/4	0/4	2/4	3/4	4/4	7.0	0.355

the predominant component (61.9 and 63.5%, respectively) as well as dodecanoic (2.3 and 4.1%, respectively), 3-hydroxydodecanoic (8.3 and 8.4%, respectively), 3-hydroxytetradecanoic (3.1 and 4.2%, respectively), and hexadecanoic (6.5 and 7.7%, respectively) fatty acids. Lipid A of *P. chlororaphis* subsp. *aureofaciens* B-111 also contained an insignificant amount of hexadecenoic acid (2.8%). According to the literature data, unsaturated fatty acids are not common components of lipid A, although they have been revealed in few cases.

It is known that LPS are efficient immunomodulators (Varbanets and Vinarskaya, 2002), which makes them promising objects for elaboration of vaccines and novel methods of immunochemical diagnostics of infections caused by gram-negative bacteria. However, the application of LPS is hindered by their toxicity and pyrogenicity. Lipid A is known to be the endotoxic center of the LPS molecule, which determines such LPS properties as lethal toxicity, pyrogenicity, the Schwartzman reaction, the ability to induce the tumor necrosis factor, and mitogenic activity. Toxicity of the LPS from *P. chlororaphis* subsp. *aureofaciens* B-306 was compared with that of *Escherichia coli* O55:B5 LPS, which is known as a typical endotoxin and is usually applied as a control in the studies of toxic properties of LPS from other microbial strains. Experiments carried out with the D-galactosamine-sensitized mice revealed that lethal toxicity (LD_{50}) of the LPS from the studied strain (7 μg/mouse) was 50-fold less than that of the LPS from *E. coli* (14 μg/mouse) (Table 2). Therefore, LPS of the studied strain can be considered low-toxic substances.

Comparative studies showed that the minimal pyrogenic dose of the LPS preparation was 7.5×10^{-3} μg/mL of apyrogenic isotonic solution. Injection of the LPS solutions from both studied strains caused an increase in the temperature of experimental animals by more than 0.45°C, which is beyond the physiological standard of healthy animals. As seen from Fig. 1, the LPS preparations from *P. chlororaphis* subsp. *aureofaciens* strains B-111 and B-306 showed higher pyrogenic activity than the pharmaceutical preparation Pyrogenal containing LPS of *Shigella typhi*.

The generally known and the best-studied role of LPS is its functioning as the main thermostable antigen of microbial cells. The LPS introduction into organisms of higher animals and humans results in production of complementary antibodies directed against specific structural sites of the LPS molecule, which was used for immunization (*Bacterial Lipopolysaccharides*, 2011). The working titer of O-antisera to the heated cultures of the studied strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 determined by the ring precipitation reaction was 1 : 80000 and 1 : 40000, respectively.

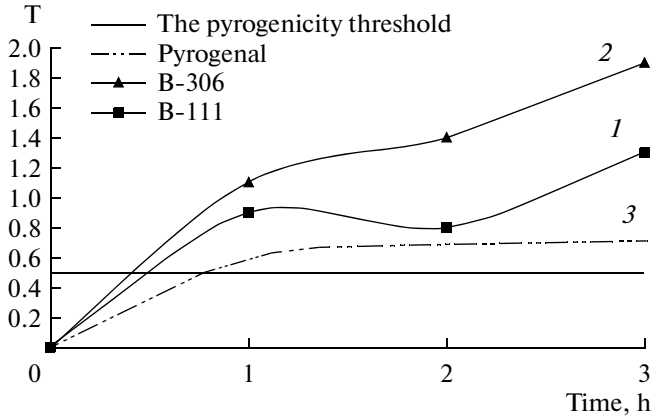

Fig. 1. Pyrogenic activity of LPS from strains *P. chlororaphis* subsp. *aureofaciens* B-111 (*1*) and B-306 (*2*); the pyrogenicity threshold (*3*).

During the double immunodiffusion in agar by Ouchterlony, LPS of the studied strains in a homologous system exhibited an antigenic activity (Fig. 2). In the cross-reactions, LPS of the tested strains did not interact with the antisera.

Since O-specific polysaccharide is the molecular basis of serological affinity, its structure was investigated.

The study of the monosaccharide composition of OPS from *P. chlororaphis* subsp. *aureofaciens* UCM B-306 revealed the presence of glucose, rhamnose, and GlcNAc. Further studies of the OPS showed the presence of residues of 2-amino-2-deoxygalacturonic acid (GalNAcA) and 2,4-diamino-2,3,6-trideoxyglucose (QuiNAc4NAc), which were not revealed by the monosaccharide analysis. Assay of absolute configuration of the rhamnose residues by GLC with the use of (S)-2-octylglycosides showed that rhamnose had the D configuration. The absolute configuration of GalNAcA and QuiNAc4NAc residues was also determined as the D configuration based on the effects of glycosylation in the ^{13}C-NMR spectra of OPS (Shashkov et al., 1988).

The ^{1}H-NMR spectrum of O-polysaccharide contained the signals of six anomeric protons at 5.03–5.27 ppm, signals of CH_3–CH groups (H-6) of Rha and QiuN4N residues at 1.18 and 1.24 ppm, signals of the other protons of the monosaccharide cycle at 3.48–4.42 ppm, and signals of N-acetyl groups in the region of 1.97–2.01 ppm (Table 3). The ^{13}C-NMR spectrum recorded in D_2O at pD 8 displayed the signals of anomeric carbon atoms at 98.4–101.9 ppm, carbon atoms connected with nitrogen atom at 50.3–56.5 ppm, the other carbon atoms of the monosaccharide cycle at 68.1–79.8 ppm, signals of CH_3–CH

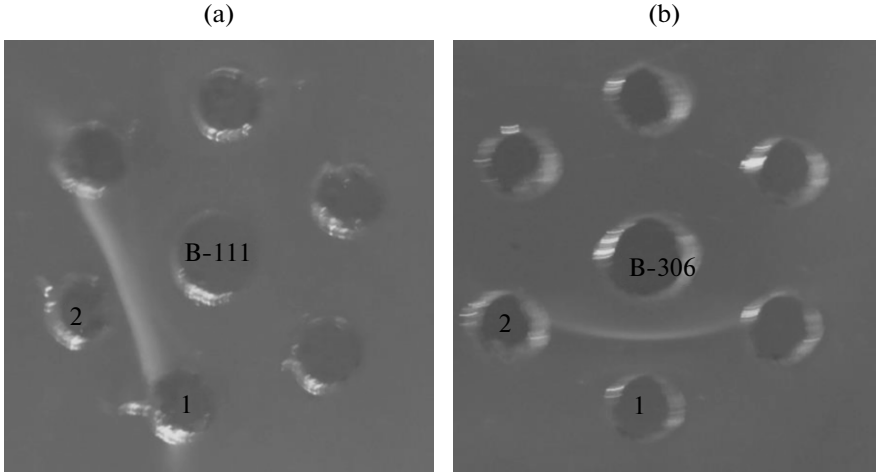

Fig. 2. Reaction of double immunodiffusion in agar according to Ouchterlony of LPS from *P. chlororaphis* subsp. *aureofaciens* B-111 (*1*) and *P. chlororaphis* subsp. *aureofaciens* B-306 (2) with O-antiserum to B-111 (a) and B-306 (b).

Table 3. Data of ^{1}H- and ^{13}C-NMR chemical shifts (ppm) of O-polysaccharide from *P. chlororaphis* subsp. *aureofaciens* UCN B-306 in D_2O at pD 8

Monosaccharide residue		H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
→ 3)-α-D-Qui*p*NAc4NAc-(1 →	A	5.03 100.5	4.12 55.8	4.02 74.3	3.72 56.5	3.99 68.3	1.18 17.7
→ 3)-α-D-Qui*p*NAc4NAc-(1 →	A'	5.01 100.5	4.13 55.8	4.05 74.7	3.72 56.5	4.02 68.1	1.19 17.7
→ 4)-α-D-Gal*p*NAcA-(→	B	5.19 98.4	4.25 50.3	3.94 69.9	4.41 76.9	4.08 72.9	 175.2
→ 4)-α-D-Gal*p*NAcAN-(1 →	B'	5.27 98.5	4.27 50.3	3.98 69.1	4.37 76.1	4.21 72.0	 174.0
→ 3)-α-D-Rha*p*-(1 →	C	5.15 101.5	4.10 71.7	3.84 79.8	3.49 72.8	3.81 70.3	1.24 18.0
→ 3)-α-D-Rha*p*-(1 →	C'	5.13 101.9	4.10 71.7	3.72 79.7	3.56 72.5	3.68 70.9	1.23 18.0

GalNAcAN is 2-acetamide-2-deoxy-D-galacturonamide. Chemical shifts of N-acetyl groups: δ_H, 1.97–2.01; δ_C, 23.2–23.5 (CH_3) and 174.6–175.5 (CO)

groups (C-6) of Rha and QiuN4N at 17.7–18.0 ppm, signals of CH_3 N-acetyl groups at 23.2–23.5 ppm, and signals of CO N-acetyl groups and a carboxyl group (C-6 GalNAcA) at 174.6–175.5 ppm (Table 3). The absence of signals in the region of 82–88 ppm in the ^{13}C-NMR spectrum indicated that all monosaccharide residues were present in the pyranose form (Bock and Pedersen, 1983).

Based on results of ^{13}C-NMR chemical shifts and characteristics of the spin–spin interaction ($^{3}J_{H,H}$), six main spin systems were indicated with the use of 2D ^{1}H, ^{1}H COSY, TOCSY, ^{1}H, ^{13}C HSQC, and HMBC experiments including two systems for each monosaccharide residue: A and A' for QuiNAc4NAc, B and B' for GalNAcA, and C and C' for Rha (Table 3).

Thus, it was established (Zdorovenko et al., 2015) that the O-specific polysaccharide of *P. chlororaphis* subsp. *aureofaciens* UCM B-306 was built up from two types of repeating units of the following structure:

-[→ 3)-α-D-Rha*p*-(1 → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui*p*NAc4NAc-(1-]$_n$ →

-[→ 3)-α-D-Rha*p*-(1 → 4)-α-D-Gal*p*NAcAN-(1 → 3)-α-D-Qui*p*NAc4NAc-(1-]$_m$ →

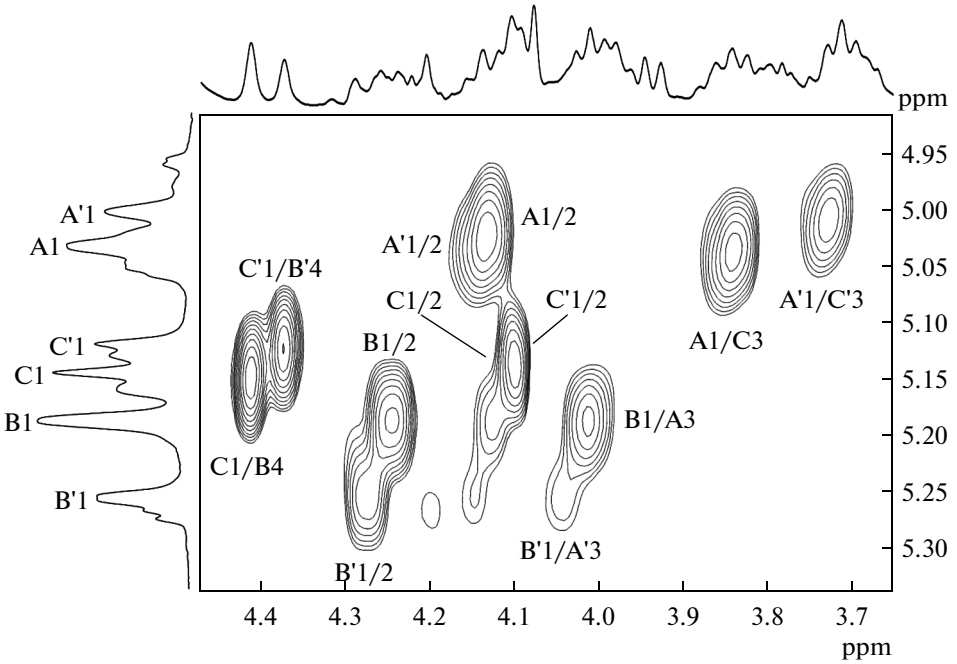

Fig. 3. Fragment of the ROESY spectrum of O-polysaccharide from *P. chlororaphis* subsp. *aureofaciens* UCM B-306 in D_2O at pD 8. Corresponding regions of monomeric ^{1}H-NMR spectrum are shown along vertical and horizontal axes.

where GalNAcAN is 2-acetamide-2-deoxy-D-galacturonamide; the n : m ratio is equal to 3 : 2.

The O-polysaccharide of *P. chlororaphis* subsp. *aureofaciens* UCM B-111 was obtained by mild acid hydrolysis of the LPS with subsequent gel filtration on a column with Sephadex G-50. After complete acid hydrolysis of the O-polysaccharide, monosaccharides were analyzed in the form of polyol acetates; the presence of Glc, 2-acetamide-2,6-dideoxyglucose (N-acetylquinovosamine, QuiNAc), GlcNAc, and GalNAc in a ratio of 1 : 1 : 2 : 1, respectively, was revealed. Subsequent studies showed that Glc residues were probably components of the LPS core oligosaccharide rather than components of a repeating unit (Bystrova et al., 2006; Silipo et al., 2004). Determination of the absolute configurations of monosaccharides by GLC of acetylated glycosides with the use of (S)-2-octanol made it possible to conclude that GlcNAc had the D configuration. Residues of GalNAc and QuiNAc also had D configuration that was established on the basis of the known effects of glycosilation from the data of the ^{13}C-NMR chemical shifts (Shashkov et al., 1988).

The ^{1}H-NMR spectrum of the O-polysaccharide from *P. chlororaphis* subsp. *aureofaciens* UCM B-111 contained signals of different intensity, indicating structural heterogeneity which was most likely associated with non-stoichiometric O-acetylation of the polysaccharide (the spectrum contained a signal of O-acetyl group at 2.15 ppm). The ^{1}H-NMR spectrum of the polysaccharide contained signals of anomeric protons in the range of 4.49–5.50 ppm, one CH_3-C group (H-6 QuiNAc) at 1.33 ppm, and four N-acetyl groups at 2.00, 2.08, and 2.10 ppm (signal of double intensity). The ^{13}C-NMR spectrum of the O-polysaccharide displayed the signals of anomeric carbon atoms in the range of 98.1–104.7 ppm, one CH_3-C group (C-6 QuiNAc) at 17.9 ppm, $HOCH_2$-C groups (C-6 GlcNAc and GalNAc) in the range of 60.7–69.1 ppm, four carbon atoms connected with nitrogen (C-2 aminosugars) in the range of 49.9–56.5 ppm, the other carbon atoms of the monosaccharide cycle in the range of 71.0–78.5 ppm, and four N-acetyl groups in the range of 23.1–23.6 ppm (Me) and 174.9–175.6 ppm (CO).

Comparison of the obtained spectra with that of the O-polysaccharide isolated from strain *P. chlororaphis* 449 (planktonic form) (Zdorovenko et al., 2015) showed their structural identity. As a result, four spin systems were identified, and all the signals were described with the aid of 2D ^{1}H, ^{1}H COSY, TOCSY, ROESY, ^{1}H, ^{13}C HSQC (Fig. 4), and HMBC experiments for residues GalNAc (A and A'), QuiNAc (B), and GlcNAc (C and C', D and D') (Table 4). Thus, the O-specific polysaccharide obtained from the LPS of *P. chlororaphis* subsp. *aureofaciens* UCM B-111 had the following structure:

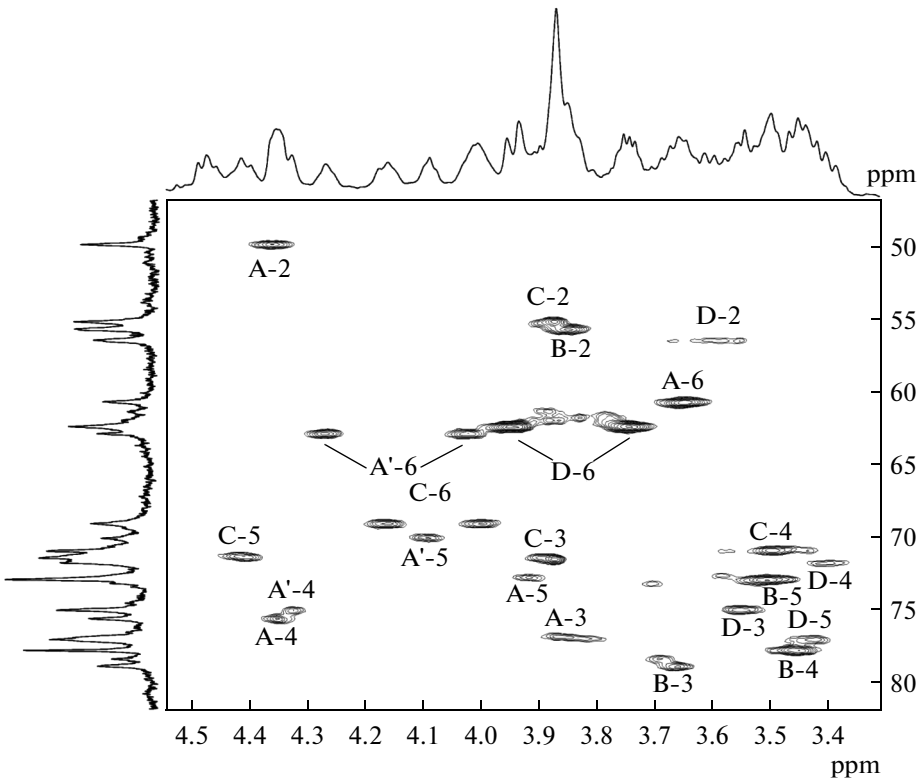

Fig. 4. Fragment of ^{1}H, ^{13}C HSQC spectrum of O-polysaccharide from *P. chlororaphis* subsp. *aureofaciens* UCM B-111 in D_2O. Corresponding regions of monomeric ^{1}H- and ^{13}C-NMR spectra are shown along vertical and horizontal axes.

-[→ 4)-α-D-Gal*p*NAc6Ac-(1 → 3)-β-D-Qui*p*NAc-(1 → 6)-α-D-Glc*p*NAc-(1 →]-$_n$
β-D-Glc*p*NAc-(1 → 3)⌋
-[→ 4)-α-D-Gal*p*NAc-(1 → 3)-β-D-Qui*p*NAc-(1 → 6)-α-D-Glc*p*NAc-(1 →]-$_m$
β-D-Glc*p*NAc-(1 → 3)⌋

where the n : m ratio is equal to 3 : 2.

We have shown earlier (Kiprianova et al., 2013) that the lipopolysaccharides of strains *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306 were highly active antiviral agents, and in experiments carried out in 2010–2012, they invariably demonstrated efficiency against TMV in the case of plants of the family *Solanaceae*. LPS in concentration of 1–10, 0.1, and 0.01 mg/mL suppressed the virus infectivity by 98–100, 57–69, and 43–44%, respectively. LPS in concentration of 1 μg/mL decreased the virus infectivity for various indicator plants, e.g., Jimson weed (*Datura stramonium* L.) and tobacco (*Nicotiana sanderae* H., *Nicotiana tabacum* L.) by 10.2 to 46%. Activities of both LPS were approximately similar. Of interest is the fact that the lipopolysaccharides obtained from microorganisms belonging to other genera and species (*Rahnella aquatilis* and *Ralstonia solanacearum*) showed no activity against TMV and sometimes even stimulated the necrosis formation.

P. chlororaphis subsp. *aureofaciens* B-111 and B-306 on human viruses, in particular, those of the herpes 2 type, were carried out with the use of the culture of the hamster kidney tissues infected with this virus. It was found that the LPS showed no cytotoxic effect on the tissue culture in concentrations from 3.125 to 100 μg/mL; at the same time, their effective dose, that is the minimal concentration of preparation which suppressed development of the virus-specific CPD by 50 % and inhibited reproduction of the herpes virus by 2.0 and more of the log ID_{50} value, was 0.75 μg/mL (Table 5). Index of selectivity (IS) of the studied LPS was 1333; therefore, they can be considered as active antiviral preparations.

Thus, the studied LPS not only showed high activity towards tobacco mosaic virus and activity against

Table 4. The ^{1}H- and ^{13}C-NMR chemical shifts (δ, ppm) of O-polysaccharide from *P. chlororaphis* subsp. *aureofaciens* UCM B-111

Monosaccharide residue		H-1 *C-1*	H-2 *C-2*	H-3 *C-3*	H-4 *C-4*	H- *C-5*	H-6 (6a, 6b) *C-6*
→ 3,4)-α-D-Gal*p*NAc-(1 →	A	5.50 *98.3*	4.36 *49.9*	3.82 *77.1*	4.32 *75.1*	3.92 *72.9*	3.66 *60.7*
→ 3,4)-α-D-Gal*p*NAc-(1 →	A'	5.48 *98.3*	4.36 *49.9*	3.87 *77.0*	4.35 *75.9*	4.09 *70.1*	4.00/4.27 *62.9*
→ 3)-β-D-Qui*p*NAc-(1 →	B	4.59 *102.3*	3.85 *55.7*	3.69 *78.5*	3.45 *77.9*	3.51 *73.0*	1.33 *17.9*
→ 6)-α-D-Glc*p*NAc-(1 →	C	4.93 *98.1*	3.87 *55.3*	3.87 *71.7*	3.50 *71.0*	4.42 *71.3*	4.00, 4.16 *69.1*
→ 6)-α-D-Glc*p*NAc-(1 →	C'	4.85 *98.7*	3.87 *55.3*	3.87 *71.7*	3.50 *71.0*	4.42 *71.3*	4.00, 4.16 *69.1*
β-D-Glc*p*NAc-(1 →	D	4.49 *104.7*	3.57 *56.5*	3.55 *75.0*	3.40 *71.8*	3.42 *77.1*	3.75, 3.95 *62.4*
β-D-Glc*p*NAc-(1 →	D'	4.50 *104.7*	3.57 *56.5*	3.55 *75.0*	3.40 *71.8*	3.42 *77.1*	3.75, 3.95 *62.4*

A', C', and D' are monosaccharide residues of the repeating unit containing 6-O-acetylated residue of GalNAc.

Table 5. The ED_{50} of LPS from *P. chlororaphis* subsp. *aureofaciens* B-111 and B-306

LPS concentration, μg/mL	Virus titer, log TCD_{50}	
	LPS of strains	
	B-111	B-306
6.25	0	2.5
3.125	0	2.0
1.55	2.0	3.0
0.75	3.0	2.0
Control	5.0	4.0

some human and animal viruses, but also inhibited the reproduction of the herpes virus.

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