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EXPERIMENTAL ARTICLES

Effect of Flavonoids on the Composition of Surface Glycopolymers of *Azospirillum lipoferum* Sp59b

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Abstract—Cultivation of the type strain *Azospirillum lipoferum* Sp59b in the presence of flavonoid quercetin induced modification of the structure of the bacterial lipopolysaccharide. Cultivation in the presence of the flavonoid was shown to result in altered serological characteristics of the bacteria, increased heterogeneity of the outer membrane lipopolysaccharide pool, as well as in modified composition and fatty acid ratio of lipid A. The flavonoid was shown to induce the synthesis of the O-specific polysaccharide with the repeating structure represented by a tetrasaccharide consisting of a linear trisaccharide fragment of α-L-Rha*p* residues in the main chain and the terminal β-D-Glc*p* residue. The structure of this O-specific polysaccharide was identical to the previously determined structure of the capsular polysaccharide of these bacteria grown without quercetin. Modifications in the structural composition of the capsular polysaccharide induced by cultivation in the presence of quercetin were revealed.

Keywords: *Azospirillum lipoferum*, lipopolysaccharide, flavonoids, quercetin, bacterial capsular polysaccharides

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Plant flavonoids are known to play an important role in plant–microbial interactions in the rhizosphere. Flavonoid-enriched exudates were shown to activate expression of *Rhizobium* genes involved in the establishment of legume–*Rhizobium* symbiosis [1, 2]. Flavonoids play an important role in the induction of the *GmGin1* gene in fungi during formation of arbuscular–mycorrhizal symbioses: they induce expression of the *vir* gene in the phytopathogens of the genus *Agrobacterium* [3], and they are exuded from plant roots as chemoattractants and growth promoters of many bacterial cultures [4]. For bacteria of the genus *Sinorhizobium*, effect of flavonoids on formation of symbiotic relationships was demonstrated to involve the glycopolymers of the bacterial surface, in which flavonoids induce changes in the composition of carbohydrate surface components, i.e., exopolysaccharides (EPS), capsular polysaccharides (CPS), and lipopolysaccharides (LPS) [5]. Involvement of flavonoids in the regulation of activity of the genes participating in EPS and LPS synthesis has been demonstrated in endophytic plant symbionts, bacteria of the genus *Herbaspirillum* [6]. In the recent past, data on induction of O-specific polysaccharide (OPS) modification in associative symbionts of pseudomonads by flavonoids [7], as well as the data on the effect of wheat exudates on the electrophoretic profile of azospirilla LPS [8], were published. However, the effect of flavonoid compounds on the composition of carbohydrate components of the cell membrane in associative microorganisms has not been studied yet. Bacteria of the genus *Azospirillum* are typical representatives of associative microorganisms. Since CPS and LPS are involved in the interaction of azospirilla with plants [9, 10], the problem of the possible effect of flavonoids on the carbohydrate components of cell membranes is important for the understanding of the role of flavonoid metabolites in formation of plant–microbial associates.

Choice of the strain *A. lipoferum* Sp59b was caused by the fact that earlier, the presence of antigenic and structural differences between the membrane PS (from LPS) and capsular PS (from the lipopolysaccharide–protein complex, LPPC), which is rather rare among azospirilla, was demonstrated for the culture in study, when grown on a selective mineral medium with sodium malate. Moreover, previously [11, 12] we have established the structures of repeated units for the O-specific (I) and capsular (II) polysaccharides, which are presented below.

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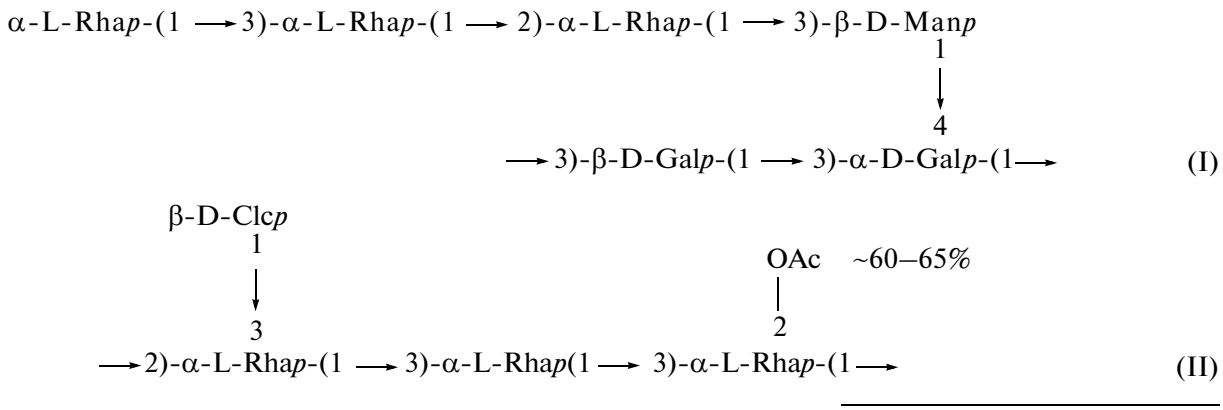

This specific feature of the bacteria provides the opportunity for observation of the changes in the composition of polysaccharides of two types—LPS and CPS—upon the effect of plant secondary metabolites. The choice of the bacteria–plant metabolite model pair was stipulated by the fact that among phenolic secondary metabolites, various flavonoids are present in soil, including quercetin [13–15]. Quercetin, while being a widely spread representative and a starting compound for synthesis of many flavonols, is the most studied one from the point of view of physiological activity toward various organisms [16]. Moreover, at the primary stages of the research, in a comparative experiment, a plant secondary metabolite, more hydrophobic than quercetin, naringenin, and a more hydrophilic one, rutin, were analyzed.

The goal of the present work was to reveal the possible effect of flavonoids on the structural features of the lipid and carbohydrate components of glycopolymers on the surface of *A. lipoferum* Sp59b cells in a model experiment.

MATERIALS AND METHODS

Type strain *A. lipoferum* Sp59b (ATCC 29707) [17] isolated form wheat roots was kindly provided by the collection of microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov, Russia). *A. lipoferum* Sp59b culture was grown in a liquid synthetic malate medium [18] at 30°C and constant stirring on a shaker until the end of the exponential growth phase. Flavonoids in the desired concentration were added at inoculation in the form of solution in dimethylformamide.

Bacteriostatic activity of the solvents and flavonoid solutions was determined by the method of radial diffusion of the preparations in agarized nutrient medium, on which a lawn of azospirilla culture under study was grown. Bacteriostatic activity of the flavonoids was determined from the diameter of growth inhibition zones.

The capsule was removed from the cell surface mechanically by stirring in 0.15 M NaCl solution for 6 days with exchange of the washing solution every 12 h. Capsular material was dialyzed against distilled water during 2 days using a semipermeable membrane with 12 kDa cut-off (Sigma, United States), concentrated on a rotary evaporator, and lyophilized. The cells washed from the capsules were dehydrated by triple centrifugation in acetone and dried in air.

LPS_Q was obtained by extraction of acetone-dried cells grown in the presence of quercetin, with 45% solution of hot phenol without separation of the layers [19]. LPS_Q and CPS_Q preparations were purified from low molecular weight compounds on Sepharose CL-4B and Sephadex G-50 (GE Healthcare, Untied States) columns as described previously [18]. The fractions eluted with void volume of the column and containing carbohydrates were combined, concentrated, and lyophilized. Detection of the separation products in the eluates was performed by an LKB 2142 (LKB, Sweden) differential flow refractometer; the presence of carbohydrate components was assayed by adsorption at $\lambda = 492$ nm upon eluate reaction with phenol and sulfuric acid.

Additional purification of the obtained preparations from quercetin was performed by extraction of colored metabolites from acidified aqueous solutions of glycopolymers with five volumes of ethyl acetate.

For electrophoretic screening of the effect of flavonoids on bacteria, LPS was extracted from cell biomass with EDTA [20]. SDS-PAGE of the LPS and CPS preparations was performed in 15% gel [21]. Components were visualized in gels by silver nitrate-based stain [22].

For immunodot detection, extracts of LPS were applied onto nitrocellulose membranes with pore diameters of 0.2 μm (Sigma, United States). Free binding sites on the membrane were blocked by 1 h incubation in 1% skim milk. Immunodetection was performed by incubation of the dots with polyclonal rabbit antibodies against the LPS and LPPC preparations (Ab_{LPS} and Ab_{LPPC}) [12]. Horseradish peroxidase-conjugated goat anti-rabbit antibodies (Sigma) were used as secondary antibodies and visualized by interaction with the substrate (3,3'-diaminobenzidine).

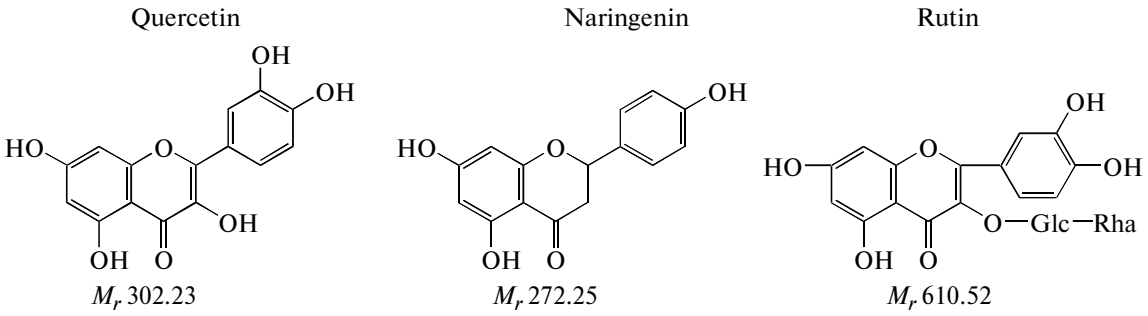

Fig. 1. Structural formulae of the flavonoids used in the study.

Carbohydrates, protein admixtures, and nucleic acids in LPS were determined using common spectrophotometric techniques [18]. Methylation of fatty acids was performed according to the technique described in [23]. Fatty acids were identified by GLC using the standard solutions from Sigma on a GC-2010 chromatograph (Shimadzu, Japan).

Analysis of monosaccharide composition of PS was performed by GLC on a Hewlett Packard 5890 chromatograph upon preliminary hydrolysis of the samples with 2 M CF_3COOH (120°C, 2 h), reduction with $NaBH_4$, and acetylation [24]. Separation was performed on an HP-5 capillary column in a temperature gradient from 160°C (1 min) to 290°C at the heating rate of 7°C/min. Absolute configurations of the sugars were determined by GLC of acetylated glycosides with (R)-2-octanol under similar conditions [25].

PS were methylated with CH_3I in dimethyl sulfoxide in the presence of methylsulfinylmethanide. PS were then hydrolyzed in 2 M CF_3COOH (120°C, 2 h), reduced with $NaBH_4$, and acetylated. Analysis of partially reduced methylated polyol acetates was performed by GLC-MS on a Hewlett Packard 5989A chromatograph equipped with an HP-5ms capillary column in a temperature gradient from 150°C (3 min) to 320°C at the heating rate of 5°C/min.

NMR spectroscopy analyses were performed on a DRX-500 (Bruker, Germany) spectrometer in a 99.96% D_2O solution at 27°C (acetone was used as an internal standard, δ_H 2.225, δ_C 31.45). The samples were preliminarily lyophilized twice from 99.9% D_2O. Two-dimensional spectra were registered using the bundled mathematical software by Bruker the XWINNMR 2.1 software package was used for collection and processing of the data. In TOCSY and NOESY experiments mixing times were 150 and 200 ms, respectively.

RESULTS AND DISCUSSION

Interest in flavonoids—quercetin, rutin, and naringenin—occurs because they belong to various groups of plant phenolic metabolites widely spread in cereal exudates (Fig. 1).

In the development of the *Azospirillum*–flavonoids model system, the choice of acting concentrations of plant secondary metabolites able to induce changes in the composition of bacterial surface glycopolymers was an important stage. While flavonoids are known to express bacteriostatic and even bactericidal properties, they are poorly soluble in water (for example, quercetin, by less than 0.01%); therefore, for the primary dissolution (preparation of stock solutions) of the flavonoids, organic solvents were used in the experiments. In the row of weakly polar solvents (methanol, ethanol, and dimethylformamide), the latter one had the least inherent bactericidal activity. In preliminary experiments, bacteriostatic concentrations of flavonoids for azospirilla were established to be 13.3 and 10.0 mM for naringenin and quercetin, respectively. Rutin, even at concentration of 13 mM, did not express bacteriostatic properties against azospirilla. To reveal their effect on the LPS composition, bacteria were cultured in liquid malate medium with flavonoids at concentrations several times lower than the bacteriostatic ones: 0.3, 0.7, and 1.3 mM for quercetin; 1.1, 1.5, and 1.8 mM for naringenin; and 0.8, 1.6, and 3.2 mM for rutin.

LPS were isolated from the biomass of bacteria, grown under conditions described, by EDTA extraction; changes in macromolecular organization of LPS were evaluated by visualization of the results of electrophoretic separation with a silver nitrate-based dye after periodate oxidation. Experiments demonstrated that upon addition of quercetin to the growth medium, starting from the concentration of 0.7 mM, heterogeneity of the membrane LPS pool increased (Fig. 2). Molecules with O-specific polysaccharides of various length (S forms of LPS molecules), as well as molecules with the carbohydrate part represented by the core oligosaccharide (R forms) were present in the LPS, which was evidenced by the presence of bands in the lower part of electrophoretic track (Fig. 2, track *3*). For bacteria cultivated in the presence of naringenin (1.8 mM), predomination of the molecules with a long O-specific chain (Fig. 2, track *5*) was observed in LPS

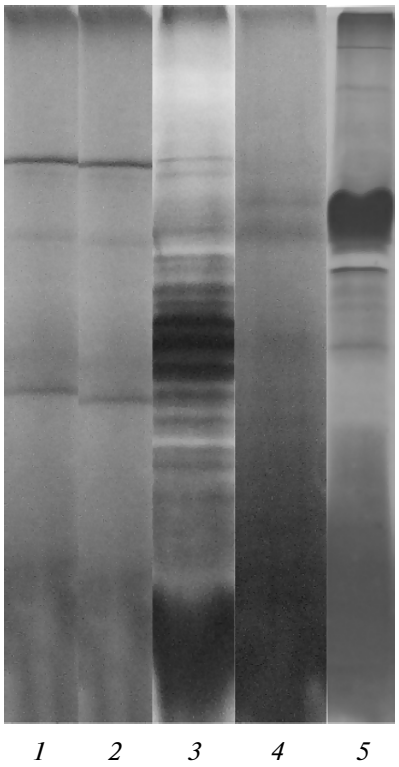

Fig. 2. SDS-PAGE of LPS preparations obtained from *A. lipoferum* Sp59b cultured in a malate medium (control) (*1*), in the presence of 0.3 mM (*2*), 0.7 mM (*3*), and 1.3 mM (*4*) quercetin and 1.8 mM naringerin (*5*).

extracts. Bacterial growth in the presence of rutin at indicated concentrations did not lead to any changes in the electrophoretic profile of LPS (data not shown). The established acting concentrations were comparable with the values reported in the literature on the content of individual flavonoids in the rhizosphere of some plants [26]. Despite the fact that data on flavonoid concentrations in plant tissues, rhizosphere, and soil are contradictory, and are determined by the species-specific features of plants, the fact of changes in LPS heterogeneity according to electrophoresis data evidences the possibility of occurrence of similar modifications of the surface components of azospirilla under in vivo conditions.

Based on these data, quercetin at concentration of 1.3 mM in growth medium was used in further experiments. According to microscopic observations, the presence of quercetin did not affect motility of a living culture of *A. lipoferum* Sp59b, while biomass production decreased by approximately 30%. At early stages of growth in the presence of quercetin, considerable aggregation of the cells was already observed (Fig. 3), which is apparently associated with a significant (by 31%) increase in the relative hydrophobicity of bacterial surface, which had been determined by the method of salt aggregation [27].

Testing of the antigenic properties of *A. lipoferum* Sp59b cultured in the presence of quercetin was performed by immunodot using the polyclonal rabbit antibodies against LPS of the outer membrane and LPPC of the capsule of the initial culture. LPS of the intact *A. lipoferum* Sp59b culture used as a reference preparation interacted only with the homologous antibodies (Fig. 4), which was in agreement with the structural differences between the membrane and capsular glycopolymers of *A. lipoferum* Sp59b [12]. At the same time, Ab_{LPS} exhibited weak affinity to the LPS_Q preparation, in contrast to Ab_{LPPC}, which made it possible to visualize only LPS_Q (Fig. 4). On the basis of these data, we supposed that the structure of bacterial O-antigens within the LPS_Q exposed to the environment changed in response to exposure to quercetin.

For further analysis, LPS_Q was extracted by hot water–phenolic mixture from bacterial cells with the capsular material washed off. Fractionation by gel-permeation chromatography on a Sepharose CL-4B

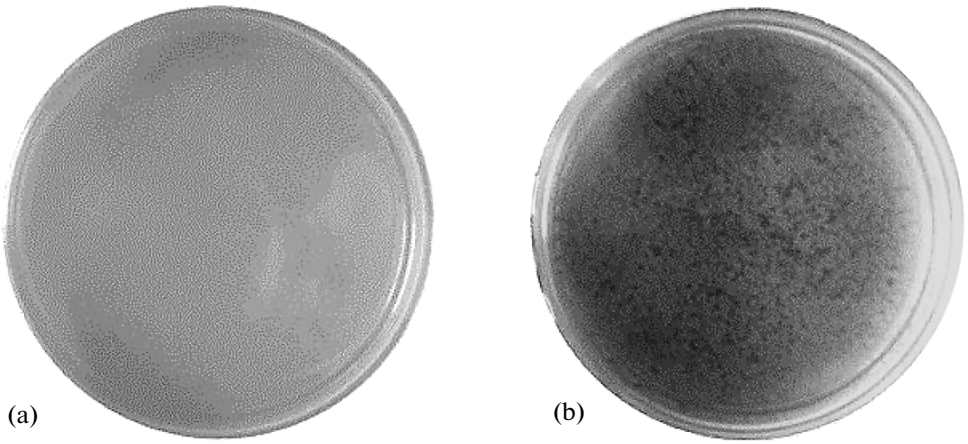

Fig. 3. Formation of cell aggregates upon 18-h cultivation of *A. lipferum* Sp59b in malate medium (a) and malate medium supplemented with quercetin (1.3 mM) (b).

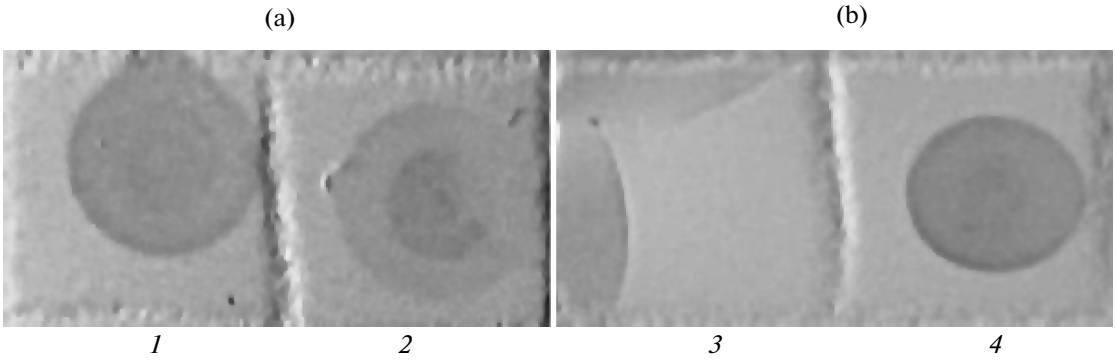

Fig. 4. Results of immunodot analysis of EDTA extracts of LPS from the cells of *A. lipoferum* Sp59b (*1, 3*) and from the same culture grown in the presence of quercetin (*2, 4*). Visualization with antibodies against preparations of LPS (a) and LPPC (b) of the *A. lipoferum* Sp59b.

column allowed for removal of low-molecular weight admixtures from the LPS_Q preparation. To remove residual amounts of quercetin from the LPS_Q preparation, its acidified solution was additionally extracted with ethyl acetate followed by gel-permeation chromatography on a Sephadex G-50 column. Capsular materials (CPS_Q) were isolated from the washings off the cell surface and fractionated under similar conditions.

In the course of comparative analysis of the chemical composition, an increase in the relative carbohydrate content from 38.8% in the control LPS preparation to 53.9% in LPS_Q was established, together with the decrease in the amount of 2-keto-3-deoxyoctulosonic acid (KDO), twice against the control preparation. Decrease in the amount of KDO, as well as the increase in the carbohydrate content in LPS in bacteria grown in the media supplemented with flavonoids correlates well with the data of the work [28] obtained for the capsular polysaccharides of bacteria of the genus *Sinorhizobium* grown in a flavonoid-containing medium.

Study of the lipid components of LPS_Q by GLC revealed significant differences in the composition of fatty acids of lipid A in the experimental sample and in the control LPS (table). Thus, LPS_Q was characterized by the absence of 2-hydroxydodecanoic, 3-hydroxydodecanoic, and dodecanoic acids, which predominated in the reference sample. In the LPS_Q, the major acids (by content) were 3-hydroxytetradecanoic, hexadecanoic, 3-hydroxyhexadecanoic, and octadecenoic acids, with 3-hydroxyalkanoic acids, which belong to mandatory components of the LPS of gram-negative bacteria being responsible for 65% revealed fatty acids.

O-Specific polysaccharide (OPS_Q) was obtained by mild acidic degradation of LPS_Q followed by gel-permeation chromatography on a Sephadex G-50 column. GLC analysis of polyol acetates and (R)-2-octyl glycosides obtained upon complete hydrolysis of OPS_Q demonstrated the presence of L-Rha and D-Glc in the ratio of ~2.7 : 1. GLC-MS analysis of partially methylated polyol acetates allowed for the identification of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucytol, 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol, and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol. Therefore, the repeated unit of OPS_Q is built from a 3-substituted Rha, a 2,3-disubstituted Rha at branching point, and a terminal Glc residue, which differs considerably from the composition of OPS of bacteria of the strain Sp59b grown in the absence of quercetin, but coincides with the CPS composition.

The ^{13}C NMR spectrum of OPS_Q (Fig. 5) contained signals of various intensity, probably due to the non-stoichiometric O-acetylation supported by the presence of the CH_3 signal of *O*-acetyl group at 21.6 ppm. To confirm the supposition, OPS_Q was *O*-deacetylated by treatment with NH_4OH and the obtained polysaccharide was studied by NMR spectroscopy.

Composition of fatty acids in the LPS of *A. lipoferum* Sp59b cultures grown in a synthetic malate medium (LPS) and in the presence of 1.3 mM quercetin (LPS_Q)

Fatty acids	FAME content (% to the total peak area)	
	LPS_Q	LPS*
$C_{12:0}$	–	21.9 ± 0.6
2-OH-$C_{12:0}$	–	8.2 ± 0.2
3-OH-$C_{12:0}$	–	30.7 ± 1.3
3-OH-$C_{14:0}$	52.3 ± 1.3	12.6 ± 0.2
$C_{16:0}$	9.7 ± 0.4	13.0 ± 0.1
3-OH-$C_{16:0}$	13.8 ± 0.6	–
i $C_{17:0}$	2.2 ± 0.1	–
$C_{18:1}$	22.0 ± 0.3	10.1 ± 1.8

* Data from [12].

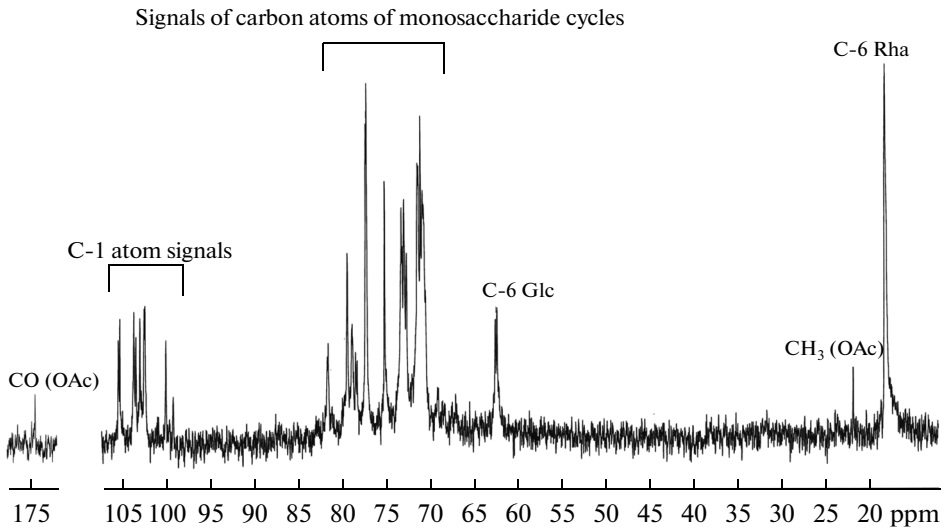

Fig. 5. ^{13}C NMR spectrum of the O-specific polysaccharide of *A. lipoferum* Sp59b cultivated in the presence of quercetin.

The ^{13}C NMR spectrum contained the signals of four anomeric carbon atoms at 102.2–105.2 ppm, 17 carbon atoms of monosaccharide cycles in the region of 62.2–81.3 ppm, together with the three methyl groups (C-6 Rha at 18.0 ppm). The absence of signals in the region of 82–88 ppm characteristic of furanosides evidences that all monosaccharide residues were in the pyranose form. In the ^{1}H NMR spectrum, signals of four anomeric protons were present at 4.67–5.15 ppm, together with the signals of the group of protons of the monosaccharide residues at 3.35–4.49 ppm and three CH_3 groups of rhamnose at 1.27–1.33 ppm. Consequently, ^{13}C and ^{1}H NMR spectra of the *O*-deacetylated OPS_Q were identical to those of *O*-deacetylated capsular polysaccharide of the microorganism [12].

The position of *O*-acetyl group in the initial OPS_Q was established using the $^{1}H/^{1}H$ ROESY and $^{1}H/^{13}C$ HMBC experiments. On the basis of the position of the H-2 proton signal of one of Rha residues in the weak field at 5.35 ppm, and a C-2 signal of the same residue at 73.2 ppm, as well as the presence of a correlation between the H-2 of this rhamnose residue and CH_3 group protons and carbon atoms of the carboxyl group, partial *O*-acetylation of Rha at position 2 was confirmed. The rate of acetylation of Rha residues was determined to be 25–30% by the analysis of integral intensities of proton spectra. Therefore, the repeated units of OPS_Q and of the previously studied capsular polysaccharide of *A. lipoferum* Sp59b possess very similar structures. The difference is merely in the rate of acetylation of one of rhamnose residues, which in CPS is 60–65%.

Analysis of the monosaccharide composition of CPS_Q of *A. lipoferum* Sp59b, in its turn, revealed the presence of a considerable amount of D-Gal, in contrast to the composition of the CPS preparation represented by rhamnoglucan in the ratio of 3 : 1 (Fig. 6).

Thus, for the first time, cultivation of *A. lipoferum* Sp59b in the presence of a plant secondary metabolite quercetin was shown to induce synthesis of OPS_Q, the repeated unit of which is different from the OPS structure of bacteria cultured in a selective sodium malate medium without quercetin. It should be noted that PS with a similar structure were identified previously in CPS of *A. lipoferum* Sp59b and LPS of *A. lipoferum* SR66 [29]. Formation of rhamnans as a factor of adaptation to environmental conditions in the presence of flavonoids was demonstrated for pseudomonads, free-living associative rhizobacteria [7]. Rhamnans in the structure of LPS are typical of many representatives of azospirilla. Thus, the presence of linear D-rhamnose polymers in OPS is a chemical basis for serological relatedness in a number of azospirilla strains related to serogroup I [30], while representatives of serogroup III of azospirilla are grouped by the presence of a trisaccharide fragment →3)-α-L-Rha*p* (1 → 2)-α-L-Rha*p* (1 → 3)-α-L-Rha*p*-(1 → [29].

The effect of the flavonoid on the microorganism was so significant that a synthesis of another capsular polysaccharide, which probably was formed as an element of adaptation to environmental conditions in tight association with the plant (endophytic) was induced on bacterial surface, where the concentration of secondary metabolites was higher than in the rhizosphere. The specific features observed evidence the important role of flavonoids in plant–bacterial communication of azospirilla, realization of which involves bacterial polysaccharides. Taking into account that the chosen flavonoids are representatives of the group of secondary metabolites in cereal plants, and bacterial LPS participate in realization of molec-

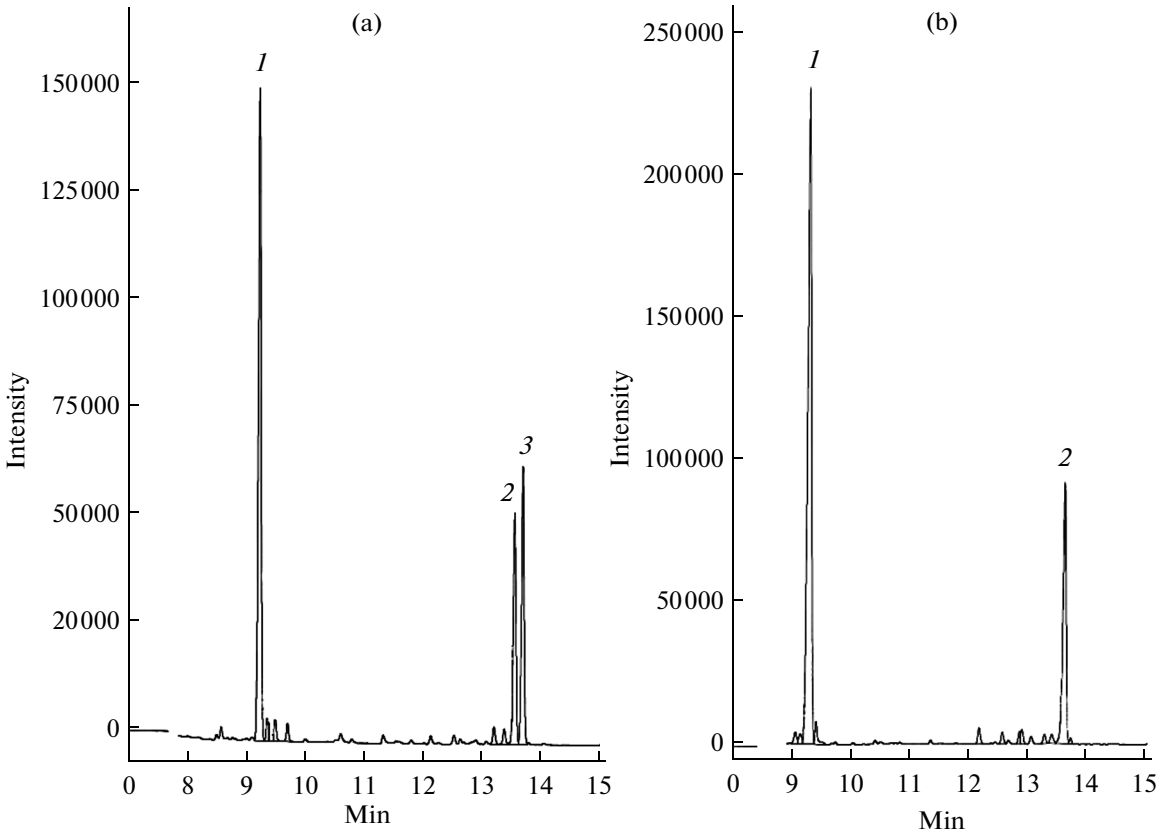

Fig. 6. GLC of polyol acetates of CPS from *A. lipoferum* Sp59b cultured in the presence of a flavonoid quercetin (a) and without it (b): rhamnose (*1*), glucose (*2*), and galactose (*3*).

ular mechanisms of interaction, our results open new perspectives in understanding of the role of flavonoids in formation of associative relationships between azospirilla and cereal plants.

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