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CHARACTERIZATION OF *AZOSPIRILLUM BRASILENSE* LIPOPOLYSACCHARIDES

Azospirillum brasilense is a gram-negative, nitrogen-fixing bacterium that colonizes the rhizosphere of various types of grasses and cereals. Lipopolysaccharides (LPS) are a class of complex glycolipids present in the cell membrane of gram-negative bacteria and mediate plant-bacteria interactions. Although the effects of LPS of pathogenic plant bacteria on the induction of plant defense mechanisms have been characterized, the role of LPS of beneficial rhizobacteria on plant growth is less clear. Therefore, a very important point is the study of the chemical, biological, and functional activities of A. brasilense LPS, which was the ***aim*** *of this work.* ***Methods.*** *A. brasilense LPSs were isolated from dry bacterial mass by the phenol-water method. The carbohydrates were analyzed by the Dubois method, nucleic acids — by Spirin, protein content — by Lowry and 2-keto-3-deoxyoctonic acid (KDO) — by Osborn. Pyrogenicity of LPS was tested observing the rules of bioethics in rabbits. Serological studies were performed by the Ouchterlony method. The identification of monosaccharides and fatty acids in LPS preparations was carried out on an Agilent 6890N/5973 inert chromatomass spectrometry system. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAAG electrophoresis) was performed according to Laemmli.* ***Results.*** *LPS of 3 strains of A. brasilense were isolated from dry bacterial mass and purified from nucleic acids by ultracentrifugation. The purified LPSs were characterized by different relative yields from 2.44% to 4.75%, which is slightly higher than other strains of the A. brasilense (1—3%). The studied preparations were characterized by a rather high content of carbohydrates from 50.1% to 72.1%. All LPS contained up to 0.17% KDO, which is a specific component of the LPS of gram-negative bacteria. Analysis of the monosaccharide composition indicates that the LPSs of the studied A. brasilense strains turned out to be heterogeneous. At the same time, such monosaccharides as mannose, galactose, glucose, and heptose were recorded in the LPS of all tested strains. The study of the fatty acid composition of LPS showed the presence of fatty acids containing from 14 to 18 carbon atoms. Hydroxylated, saturated, and monounsaturated acids and their cis isomers were found. In the investigated LPS, the dominant fatty acids were 16:0, 18:1, 14:0(3-OH), and 16:0(3-OH), which coincides with the literature data. The research of the pyrogenic effect of LPS of A. brasilense studied strains showed that LPS solutions are apyrogenic. The double immunodiffusion reaction in Ouchterlon agar showed that all tested LPS in homologous systems exhibited an-*

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tigenic activity. Serological cross-reactions can be used as an approach in classifying different bacteria. Thus, we found that antisera to A. brasilense 18-2 and 61 react with all LPSs of the studied strains, which may indicate the presence of common antigenic determinants in them and that these strains belong to the same serogroup. The electrophoretic distribution data indicate that A. brasilense produces S-forms of LPS which differ in the length of O-specific polysaccharide chains. ***Conclusions.*** *For the first time, LPS were isolated from cells of A. brasilense 10/1, 18-2 and 61. A characteristic feature of these LPS is their heterogeneity in monosaccharide and fatty acid composition, all of them were apyrogenic. The results obtained during biological-functional studies of three strains of A. brasilense LPS contribute to the biological characteristics of this species.*

Keywords: *Azospirillum brasilense, lipopolysaccharides, monosaccharide and fatty acid composition, serological and pyrogenic activity.*

Nitrogen-fixing bacteria of the genus *Azospirillum*, due to their potentially high nitrogen-fixing activity and ability to produce phytohormones and other physiologically active substances, are classified as growth-stimulating rhizobacteria. They can be used as a convenient model for identifying the mechanism of associative symbiosis formation, as well as an effective component of biofertilizers used for many crops [1]. A well-studied species of this genus is *Azospirillum brasilense,* whose representatives are closely related to many important agricultural crops and have a beneficial effect on plant growth and productivity [2—4]. Despite intensive research into the physiology and molecular biology of this genus of rhizobacteria, the exact mechanism of the action of *Azospirillum* on plants remains unclear.

In the associative interactions of *Azospirillum* with plants, an important role is played by the surface structures of microorganisms that recognize the host plant and interact with their cells. The initial and most important stage in the formation of an association is the attachment of bacteria to roots involving both nonspecific sorption, which is determined by the charge and hydrophobicity of the bacterial surface, and specific interactions, which at different stages are mediated by surface localized proteins and carbohydrate-containing polymers, in particular, lipopolysaccharides (LPS), one of the main components of the outer membrane of gram-negative bacteria. The data available in the literature allow us to consider LPS as an active component of the *Azospirillum* cell surface, which not only determines bacterial contact interactions with plant roots but also participates in the induction of plant response to these interactions. Fedonenko et al. [5], studying the *Azospirillum* LPSs, found a correlation between the induced mutational changes in their structure and bacterial activity against the roots of a partner plant. A number of studies have shown that LPS can act as an elicitor of plant innate immunity and, depending on the genesis of these molecules and host plant species, can also induce plant cell reactions such as oxidative burst, NO synthesis, entry of calcium ions into cells, and induction or inhibition of the hypersensitivity reaction [6—8]. This fact determines the relevance of further studies of the functional role of LPS as a promising trend for elucidating the molecular mechanisms that promote plant growth and development.

Therefore, the **aim** of the work was to study the properties of LPS of a number of *Azospirillum brasilense* strains.

Materials and methods. The objects of the study were strains *A. brasilense* 10/1 (isolated from the washed roots of the spring triticale variety "Oberig Kharkovsky"), 18-2 (isolated from the rhizosphere of buckwheat), and 61 (isolated from the nodules on mulberry roots), obtained from the cultures collection of the laboratory of plant-microbial interactions of the Institute of Agricultural Microbiology and Agro-Industrial Production of NAAS of Ukraine. Bacteria were grown on a potato agar with the addition of succinic acid for 48 hours at 28—30 ^{0}C. After culti-

vation, the cells were collected by centrifugation (20 min, 5000 g), washed with saline, and dried by treatment with acetone (2 times) and ether (1 time).

LPSs were extracted from dried cells with 45% aqueous phenol solution at 65—68 ^{0}C. The resulting aqueous fractions were dialyzed against the tap and then washed with distilled water to remove phenol [9]. LPSs were purified from nucleic acids by ultracentrifugation (104.000 g, 4 h).

The quantity of neutral carbohydrates was determined by the Dubois method [10]. The results were evaluated by the color change during the reaction of phenol with sulfuric acid on a spectrophotometer SF-26 at 490 nm. The carbohydrate content was determined according to standard calibration curves pre-built for glucose. The content of nucleic acids was assessed by the Spirin method [11], proteins — by Lowry using Folin's reagent [12], 2-keto-3-deoxyoctonic acid (KDO) — by reaction with thiobarbituric acid [13].

Neutral monosaccharides were identified after hydrolysis of preparations in 2 M $C_2HF_3O_2$ (6 h, 100 °C). Monosaccharides were analyzed as polyol acetates [14] on the Agilent 6890N/5973 inert chromatography-mass-spectrometry system equipped with a DB-225mS column (30 m × × 0.25 mm × 0.25 μm); the carrier gas was helium at a flow rate of 1 mL/min. Monosaccharides were identified by comparing the retention time of polyol acetates of the studied samples with standards, as well as using the ChemStation computer database. Quantitative ratios of individual monosaccharides were expressed as % to the total sum of peak areas.

The fatty acid composition was determined after sample hydrolysis in 1.5% acetyl chloride solution in methanol (100 °C, 4 h), and fatty acid methyl esters were analyzed using the above system; the carrier gas was the same. Fatty acids were identified using a personal computer database and a standard mixture of fatty acid methyl esters. The quantitative ratios of individual fatty acids were expressed as % to the total peak areas [15].

The results of the study of monosaccharide and fatty acid composition were obtained using the equipment of the Center for Collective Use at the D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine.

Pyrogenicity was studied on rabbits (weighing up to 3.5 kg) by intravenous administration of the minimum pyrogenic dose of $7.5 \cdot 10^{-3}$ μg/mL, which was established in a series of LPS dilutions, followed by animal thermometry for 3 hours. LPS was considered non-pyrogenic when the sum of the temperature increase in three rabbits was less than or equal to 1.4 °C; when it exceeded 2.2 °C, LPS was considered pyrogenic [16]. The work was conducted in accordance with the "General Ethical Principles of Animal Experiments".

O-antiserum was obtained against heated (2.5 h, boiling water bath) *A. brasilense* cells. Rabbits were immunized intravenously five times, with an interval of 4 days; the cell concentration was $2 \cdot 10^9$CFU/mL (from 0.1 to 1 mL). The antigenic activity of LPS was studied by double immunodiffusion in agar according to Ouchterlony [17].

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAAG electrophoresis) was performed according to Laemmli [18] (4% concentrating and 12% separating gel, current 30 mA). The load on the gel lane was 30, 40, and 50 μg. To visualize LPS, the gels were stained with silver salts according to Tsai's recommendations [19] modified by Kulikov [20].

Statistical analysis of the data obtained was carried out using statistical methods, as well as the Excel 2000 computer program.

Results. Chemical identification of the purified LPS preparations showed (Table 1) that they were characterized by a high enough content of carbohydrates from 50.1% to 72.1%, an insignificant amount of nucleic acids (3.25—4.34%) and protein (3—9.39%). In the composition of all

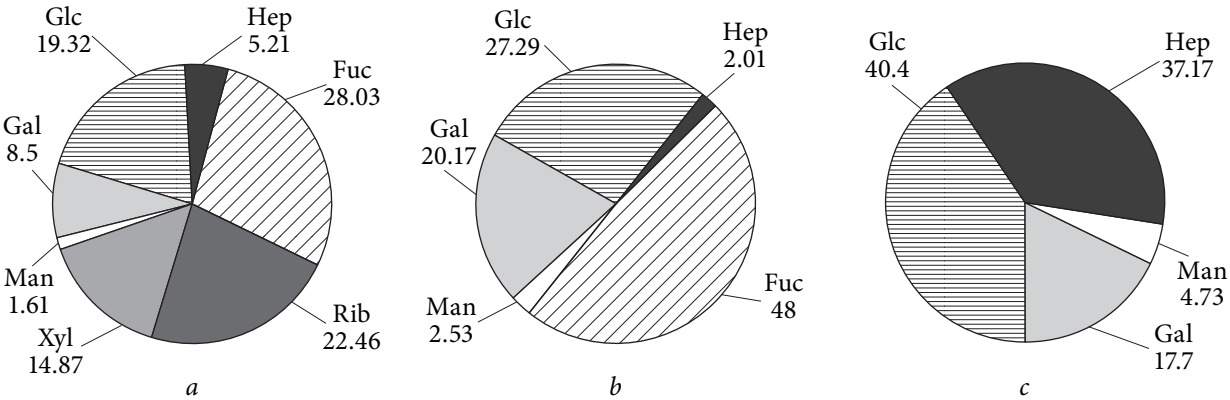


Fig. 1. Monosaccharide composition (% to the total sum of peak areas) of *A. brasilense: a* — 10/1 LPS; *b* — 18-2 LPS; *c* — 61 LPS

LPS, up to 0.17% of KDO was found, which is a specific component of the LPS of gram-negative bacteria. It was also found that the purified LPSs were characterized by different relative yields from 2.44% to 4.75%, which is slightly higher compared to other strains of the *A. brasilense* species (1—3 %).

Analysis of the monosaccharide composition indicates that the LPS of the studied *A. brasilense* strains were quite heterogeneous (Fig. 1). In addition to mannose, galactose, glucose, and heptose, LPS of all studied strains also contained other monosaccharides: fucose (10/1 and 18-2), xylose (10/1), and ribose (10/1).

The LPS of *A. brasilense* 10/1 and 18-2 were similar in their monosaccharide composition, and the dominant monosaccharide for them was fucose (28.03% and 48.0%, respectively), while in the LPS of *A. brasilense* 61 it was absent (Fig. 1). In addition, these LPSs were characterized by a lower mannose content (1.61% and 2.53%, respectively). Ribose (22.46%) and xylose (14.87%) were also identified in *A. brasilense* 10/1 LPS, while they were not found in *A. brasilense* 18-2 and 61 LPS. Depending on the strain, the content of heptose varied from 1.61 to 4.73%.

Table 1. **Chemical composition of *Azospirillum brasilense* LPS**

Strains	Yield of LPS to dry mass of cells,%	Content (% to dry mass of LPS)			
		Carbo-hydrates	Protein	NA*	KDO**
10/1	4.75	72.1	3	3.52	0.13
18-2	4.33	50.1	9.39	3.25	0.17
61	2.44	55.6	6.73	4.34	0.05

Note: * NA — nucleic acids; ** KDO — 2-keto-3-deoxyoctonic acid.

The study of the fatty acid composition of LPS showed (Fig. 2) the presence of fatty acids containing from 14 to 18 carbon atoms. Saturated, monounsaturated, and hydroxy acids and their cis-isomers have been found. The dominant fatty acids in the studied strains were hexadecanoic (16:0), cis-9-octadecenoic (18:1), 3-hydroxy-tetradecanoic (14:0(3-OH)), and 3-hydroxy-hexadecanoic (16:0(3-OH)) acids, which coincides with the literature data [21], since (14:0(3-OH)), (16:0) and (16:0(3-OH)) are characteristic of the LPS of most *A. brasilense* strains.

The presence of 3-hydroxy acids in the fatty acid composition of lipid A is used as one of the additional chemotaxonomic criteria for species differentiation. Lipid A of *A. brasilense* is characterized by the presence of two hydroxy acids (3-hydroxytetradecanoic and 3-hydroxyhexadecanoic), which acylate both amino and hy-

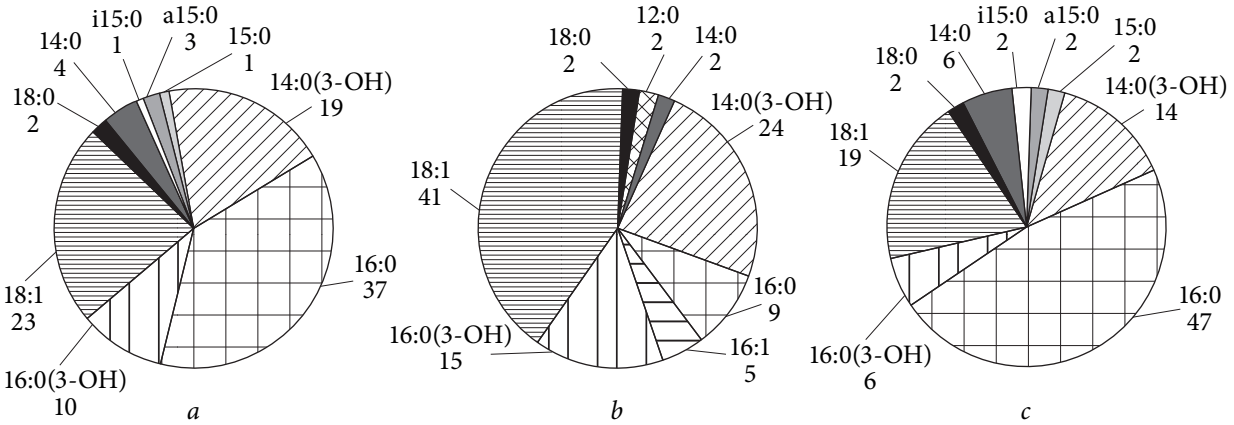


Fig. 2. Fatty acid composition (% to the total sum of peak areas) of *A. brasilense: a* — 10/1 LPS; *b* — 18-2 LPS; *c* — 61 LPS

droxyl groups of glucosamine. Thus, the presence of these acids is characteristic of representatives of the species *A. brasilense*.

It is known that bacterial LPSs exhibit such biological activities as toxicity, pyrogenicity, endotoxin tolerance, leukopenia, adjuvant, mitogenic stimulation, component activation, etc. Since lipid A, the endotoxic center of the LPS molecule, is responsible for all those types of physiological and pathophysiological reactions, we conducted pyrogenicity studies. For a comparative assessment of pyrogenic characteristics, the minimum pyrogenic dose of LPS was established to be $7.5 \cdot 10^{-3}$ μg/mL of pyrogen-free isotonic solution. The results of thermometry showed (Fig. 3) that with the introduction of LPS solutions, no increase in temperature in experimental animals by over 0.5 °C was observed, which is a limit of the physiological norm of healthy animals. Thus, it was established that LPS solutions of the studied strains are not pyrogenic.

As known, the components of the structure and composition of LPS determine its serological specificity as the main antigen. The immunochemical properties of LPS were studied by using polyclonal O-antisera, which were obtained by immunizing rabbits with heated cultures of the studied strains of *A. brasilense*. LPS of 3 strains of *A. brasilense* were used as antigens.

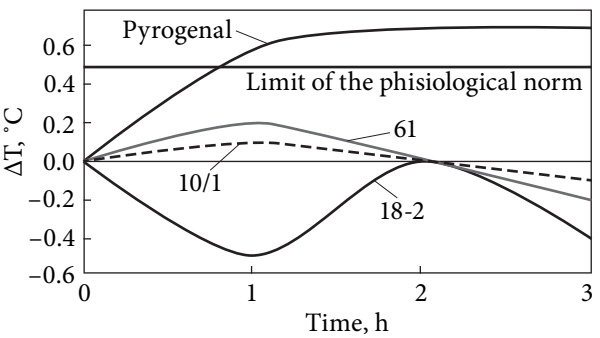
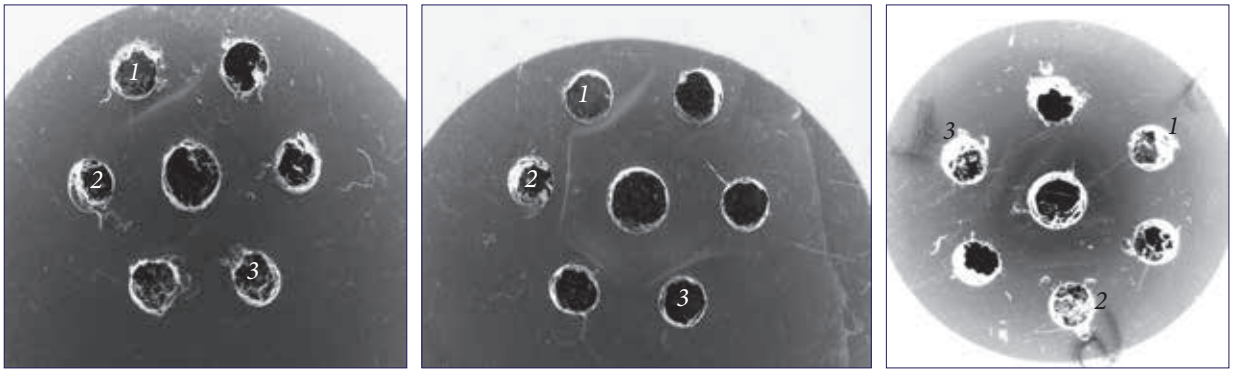


Fig. 3. Pyrogenicity of *A. brasilense* 10/1, 18-2, and 61 LPS

The double immunodiffusion reaction in agar according to Ouchterlony showed (Fig. 4) that all studied LPSs in homologous systems showed antigen activity. As one of the approaches in the classification of different bacteria, cross-serological reactions can be used. Thus, we found that antisera to strains 18-2 and 61 react with all LPS of the studied strains. And the antiserum obtained against *A. brasilense* 10/1 cells reacts only in a homologous system and does not give cross-reactivity with *A. brasilense* 18-2 and 61 LPS. The revealed by us discrepancies in the serological activity of LPS may indicate that the activity is due to the presence in O-specific polysaccharide (OPS) of minor components of a carbohydrate or a not carbohydrate nature, which can be lost



a *b* *c*

Fig. 4. The reaction of double immunodiffusion in agar by Ouchterlony with O-antiserum against *A. brasilense* 10/1, 18-2, and 61 (central holes) and LPS from *A. brasilense: a* — 10/1; *b* — 18-2; *c* — 61. The white bars between the holes indicate the presence of antigen-antibody complexes

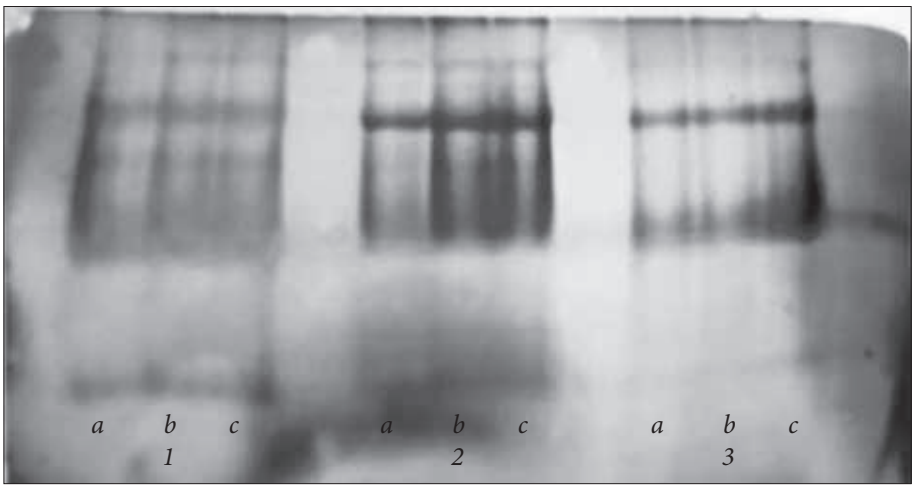


Fig. 5. Electrophoretic distribution in the SDS-PAAG system of *A. brasilense: 1* — 10/1 LPS; *2* — 18-2 LPS; *3* — 61 LPS; *a* — 30 μg, *b* — 40 μg; *c* — 50 μg

during LPS degradation. Further studies related to the determination of the OPS structure by NMR spectroscopy are required to confirm or refute this assumption.

It is known that the LPS molecule is heterogeneous and includes three structural components: OPS, core oligosaccharide (OG-core), and lipid A. PAAG electrophoresis (Fig. 5) showed that *A. brasilense* synthesizes the S-type LPS, presented in the form of a large number of bands characterized by different molecular masses and represent core oligosaccharide (the most mobile band) and core oligosaccharide, substituted by O-specific chains of different lengths (slowly moving zones). Apparently, LPS of *A. brasilense* 10/1 and 18-2 differ in electrophoretic profiles from LPS of *A. brasilense* 61. Thus, additional bands were found in *A. brasilense* 10/1 and 18-2.

The heterogeneity of slowly moving components can be explained by the presence in the molecule of several side chains that differ in molecular mass.. This feature causes the formation of a classical profile in the form of a stepladder on electrophoregrams.

Discussion. *A. brasilense* is one of the well-studied plant-growth-promoting bacteria. Despite the efforts of researchers to unravel the issues of the mechanisms of plant-microbial interactions, there is no knowledge about cellular structures that provide effective interaction. LPSs are **c**onsidered to be factors that play an important role in establishing the symbiotic relationship between bacteria and plants. It has been shown that the LPS cell envelope and its associated glucan located in the periplasmic space perform their important symbiotic functions as intracellular components of bacteria and determine the specificity and efficiency of the *Azospirillum*/plant association.

In this context, the study of the structural features and biological activity of *A. brasilense* LPS associated with plant roots can contribute to understanding the mutual influence of "partners" at the stage of association formation.

LPS preparations of *A. brasilense* 10/1, 18-2, and 61 bacteria were isolated, purified, and characterized. When studying the biopolymer composition, it was shown that the amount of carbohydrates in the studied LPS was higher than in other strains of *A. brasilense* [22]. In the composition of all isolated LPS preparations, KDO was identified, which is the only structural element that is invariably present in LPS. It is known that phosphate groups attach to KDO shield the acid-labile bond, hindering the hydrolysis of LPS and, accordingly, the determination of KDO. This may be the reason for the low KDO content previously reported for LPS of some strains of *A. brasilense* and *A. lipoferum* [21].

An analysis of the monosaccharide composition indicates that the LPS of the studied strains of *A. brasilense* were quite heterogeneous, and all three strains had differences. The presence of such monosaccharides as mannose, galactose, glucose, and heptose was recorded in LPS of all studied strains. The predominant monosaccharide for *A. brasilense* 10/1 and 18-2 LPS was fucose, while for *A. brasilense* 61, it was glucose. This fact is not consistent with the literature data that the predominant monosaccharide for most *A. brasilense* strains is rhamnose [23].

The peculiarities of the fatty acid composition of lipid A are often used as additional chemotaxonomic criteria in the taxonomy of Gram-negative bacteria. Our results for the presence of saturated, monounsaturated, and hydroxylated acids with a chain length of C14—C18 in the composition of LPS of the studied *A. brasilense* strains with the dominance of 3-hydroxytetradecanoic, hexadecanoic, 3-hydroxyhexadecanoic, and octadecenoic acids, as well as minor amounts of tetradecanoic, pentadecanoic, and octadecanoic acids, are consistent with data on the fatty acid composition of LPS of another *Azospirillum* [21, 24].

On the basis of serological studies, strains of *A. brasilense* can be divided into two subgroups. Such division based on thermostable antigens corresponds to the ability of bacteria to form associations with different host plants. The chemical heterogeneity of *A. brasilense* LPS is in good agreement with the serological data. A small number of studied strains of *A. brasilense* makes it difficult to unambiguously divide them into subgroups. Our results show that *Azospirillum* strains belonging to different host plant groups may differ in the LPS composition. This fact can have a significant impact on the interaction of *Azospirillum* with the plant.

The electrophoretic profile of LPS, along with chemical composition, is a generally accepted chemotaxonomic criterion that is often used to establish relationships between large bacterial taxa, as well as between species and strains within individual genera. It is known that LPS can be represented by S- and R-molecular forms. The S-form molecules include lipid A, core oligosaccharide, and OPS, while the R-form lacks OPS and is represented only by lipid A and core oligosaccharide. Although the wide variability of OPS in terms of the number of repeating units and component composition leads to significant

differences in LPS migration during SDS-PAGE electrophoresis, the electrophoretic profile of LPS is characteristic of individual species and even strains [25]. It showed heterogeneity typical for other strains of *A. brasilense*. All studied LPSs are represented by a wide range of molecules of different sizes. SDS-PAGE followed by visualization of the glycopolymer with silver nitrate showed that the investigated LPSs consist of molecules of various masses and contain a large number of S-form molecules. This result is consistent with a fairly high content of carbohydrates in LPS (50.1—72.1%), which is consistent with the previously studied LPS of other strains of azospirilla [26].

A. brasilense lipopolysaccharides 10/1, 18-2, and 61 were purified and chemically characterized. The studied LPS were heterogeneous in both monosaccharide and fatty acid composition. The study of the pyrogenic effect of LPS on *A. brasilense* strains showed that LPS solutions are not pyrogenic. Antisera to *A. brasilense* 18-2 and 61 react with LPS of all studied strains, which may indicate that they have common antigenic determinants and that these strains belong to the same serogroup. The results obtained in the course of biological and functional studies of three *A. brasilense* LPS strains contribute to the study of the biological characteristics of this species.

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ХАРАКТЕРИСТИКА ЛІПОПОЛІСАХАРИДІВ *AZOSPIRILLUM BRASILENSE*

Azospirillum brasilense — це грамнегативна азотфіксуюча бактерія, яка колонізує ризосферу різних трав і злаків. Ліпополісахариди (ЛПС) — це клас складних гліколіпідів, присутніх у клітинній мембрані грамнегативних бактерій і які опосередковують взаємодію рослин-бактерій. Хоча охарактеризовано вплив ЛПС патогенних рослинних бактерій на індукцію захисних механізмів рослин, роль ЛПС корисних ризобактерій на ріст рослин є менш зрозумілою. Тому дуже важливим моментом є вивчення хімічних характеристик, біологічної та функціональної активності ЛПС *A. brasilense,* що і було **метою** даної роботи. **Методи**. ЛПС *A. brasilense* виділяли із сухої бактеріальної маси водно-фенольним методом. Вуглеводи аналізували методом Дюбуа, нуклеїнові кислоти — за Спіріним, вміст білка — за Лоурі та 2-кето-3-дезоксиоктонову кислоту (КДО) — методом Осборна. Пірогенність досліджуваних ЛПС перевіряли з дотриманням правил біоетики на кролях. Серологічні дослідження здійснювали методом подвійної імунодифузії за Оухтерлоні. Ідентифікацію моносахаридів та жирних кислот у препаратах ЛПС проводили хромато-мас-спектрометрією в системі Agilent 6890N/5973. Електрофорез у поліакриламідному гелі в присутності додецилсульфату натрію (електрофорез SDS-PAAG) — за Леммлі. **Результати**. ЛПС 3-х штамів *A. brasilense* виділяли із сухої бактеріальної маси та очищали від нуклеїнових кислот ультрацентрифугуванням. Очищені ЛПС характеризувались різним відносним виходом від 2,44% до 4,75%, що дещо вище, ніж у інших штамів *A. brasilense* (1—3%). Досліджувані препарати характеризувались досить високим вмістом вуглеводів від 50,1% до 72,1%. Усі ЛПС містили до 0,17% КДО, який є специфічним компонентом ЛПС грамнегативних бактерій. Аналіз моносахаридного складу вказує на те, що ЛПС досліджуваних штамів *A. brasilense* виявилися гетерогенними. У той же час у ЛПС усіх досліджуваних штамів реєстрували такі моносахариди, як маноза, галактоза, глюкоза та гептоза. Дослідження жирнокислотного складу ЛПС показало наявність жирних кислот, що містять від 14 до 18 атомів вуглецю. Виявлено гідрокси, насичені, мононенасичені кислоти та їх цис-ізомери. У всіх ЛПС домінуючими жирними кислотами були 16:0, 18:1, 14:0(3-OH), 16:0(3-OH), що

узгоджується з даними літератури. Вивчення пірогенної дії ЛПС штамів *A. brasilense* 10/1, 18-2 і 61 показало, що розчини ЛПС не є пірогенними. Реакцією подвійної імунодифузії в агарі за Оухтерлоні показано, що всі досліджувані ЛПС у гомологічних системах виявляли активність антигену. Серологічні перехресні реакції можна використовувати як один із підходів у класифікації різних бактерій. Таким чином, ми виявили, що антисироватки до *A. brasilense* 18-2 і 61 реагують з усіма ЛПС досліджуваних штамів, що може свідчити про наявність у них загальних антигенних детермінант і приналежність цих штамів до однієї серогрупи. Дані електрофоретичного розподілу вказують на те, що *A. brasilense* продукує S-форми ЛПС, які відрізняються довжиною О-специфічних полісахаридних ланцюгів. **Висновки**. Уперше ЛПС виділено з клітин *A. brasilense* 10/1, 18-2 і 61. За моносахаридним і жирнокислотним складом вони виявилися гетерогенними. Досліджувані ЛПС не проявляли пірогенність, що дає можливість використовувати їх як антагоністів токсичних ЛПС. Одержані результати щодо властивостей ЛПС трьох штамів *A. brasilense* роблять свій внесок у біологічну характеристику виду.

Ключові слова: *Azospirillum brasilense, ліпополісахариди, моносахаридний і жирнокислотний склад, пірогенна та серологічна активність.*