R E V I E W

Pantoea agglomerans LIPOPOLYSACCHARIDES: STRUCTURE, FUNCTIONAL AND BIOLOGICAL ACTIVITY

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This review analyzed literature data, as well as our own research on lipopolysaccharides (LPS) of gram-negative bacteria, focusing mainly on Pantoea agglomerans, a member of the Enterobacteriaceae family. The unique structures of O-specific polysaccharide chains of LPS from Pantoea agglomerans represented by both linear and branched tetra- and pentasaccharide repeating units were described for the first time. The heterogeneity of the LPS molecule itself and the presence of several LPS in the bacterial cell, which differ in the structure of lipids A, O-specific polysaccharide chains, serological activity, as well as endotoxic properties, such as toxicity and pyrogenicity, were shown. Such heterogeneity represents one of the mechanisms of LPS multifunctionality. Based on the antigenicity of LPS, serotyping of P. agglomerans strains and their assignment to 10 serogroups were carried out for the first time. The high immunomodulatory activity of P. agglomerans LPS suggests the possibility to use their oligosaccharide fragments in the development of conjugated vaccines against diseases caused by gram-negative bacteria.

K e y w o r d s: Pantoea agglomerans, O-specific polysaccharide, lipid A, serological activity, TLR 4 receptor, biological activity.

The representatives of the *Enterobacteriaceae* family, an essential part of the biosphere and widely distributed in the environment, were among the first microorganisms that became a subject of research. Their activity affects the various spheres of human life significantly. Although *Enterobacteriaceae* family is the most studied (phenotypically and genotypically) family, its classification is still changing and improving. It happens due to the considerable biological diversity of enterobacteria that complicates the classification of the family members. The greatest difficulties arise when identifying newly discovered and poorly studied species.

However, in the recent years, the improvement to traditional and the development of new methods in systematic have resulted in the identification of 48 *Enterobacteriaceae* genera, among them is *Pan-toea*, which is related (for 25%) to the typical genus *Escherichia*. This level of relationship is fairly high compared to other genera, such as *Budvicia* (1%), *Pragia* (5%) and *Rahnella* (15%) [1].

P. agglomerans is considered one of the most abundant microorganisms, at least in areas inhabited by humans. The ability of these bacteria to compete and survive in various environments makes them especially useful for biocontrol, bioremediation, and biodegradation. *P. agglomerans* representatives have been classed over the years with various bacteria genera and types: *Enterobacter agglomerans*, *Erwinia herbicola*, *Erwinia milletiae*. Only in 1989, the new genus *Pantoea*, which includes the *P. agglomerans* species having a diverse range of properties, was applied to the *Enterobacteriaceae* family [2]. Initially, representatives of this species were found

in plants [1, 3], later they have been determined in water, soil, dust, air, plant and animal products, in the body of arthropods and other animals, and sometimes in humans (in wounds, blood, internal organs) [1, 4, 5]. This bacterium in humans can have an ambiguous effect, both pathogenic and beneficial. On the one hand, it can cause diseases in people who inhale organic dust [6], on the other hand, it can stimulate the production of substances that are effective in the treatment of cancer and other diseases [7].

Recently, Nicoletti et al. suggested that *P. agglomerans* along with other 8 bacteria species (*Aeromonas hydrophila*, *Brevundimonas vesicularis*, *Chromobacterium violaceum*, *Citrobacter youngae*, *Empedobacter brevis*, *Pseudomonas putida*, *Pseudomonas stutzeri* and *Streptococcus mitis*) found in the Comano spring water can contribute to its regenerative and wound-healing properties [8].

The publications of the recent years have considered the promising potential of *P. agglomerans* strains in fighting malaria. Bacteria of this species were detected in the midgut of *Anopheles* mosquitoes infected with *Plasmodium* [9-11]. About 300-500 million cases of malaria resulting in 1.2 million deaths are registered annually [11]. The symbiotic existence of *Pantoea* with malaria mosquitoes enables the development of a strategy called paratransgenesis, in which, bacteria express antiplasmodic effector proteins in the mosquito's midgut. Researchers demonstrated that the introduction of *P. agglomerans* strains inhibited (98%) the development of the human malaria parasite *Plasmodium falciparum* and the rodent *Plasmodium berghei* [11]. These results provide new approaches to combating this lethal disease.

Lipopolysaccharides (LPS), the main components of the outer membrane of gram-negative bacteria, play an important role in host-pathogen interactions in animals and plants [12]. The outer membrane of gram-negative bacteria has a unique asymmetric phospholipid bilayer structure: its inner leaflet consists of glycerophospholipids, whereas the outer one is rich in lipopolysaccharide covering about 75% of the cell surface. Integral membrane proteins, porins, are also embedded in the outer membrane, and together with LPS provide a barrier of permeability for different types of molecules, such as detergents, antibiotics, metals. The barrier properties of the outer membrane are attributed to low fluidity and a highly ordered structure of the LPS monolayer [1, 13, 14].

Although lipopolysaccharides (endotoxins) were discovered more than 100 years ago, these complex macromolecules still draw attention not only of biochemists, but also chemists, physicists, molecular biologists who study their structure and biological activity.

One of the mechanisms that determine the lipopolysaccharide polyfunctionality could be the heterogeneity of its molecule, consisting of carbohydrate and lipid parts, and the presence in the bacterial cell of several lipopolysaccharides, which differ in composition, structure and functions. Studies of lipopolysaccharides facilitate the solving such fundamental problems as: the bacteria classification, determining the correlation between the structure and function of lipopolysaccharides, the mechanisms of the immune response, the development of serological classification schemes, based on structural variations in LPS O-specific chains. Thus, the structural features of LPS have been successfully used in chemotaxonomy of some gram-negative bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*. However, in *P. agglomerans* taxonomy, there are still many unsolved problems. At the same time, the published data on isolation and characteristics of the lipopolysaccharides from *P. agglomerans* representatives remain extremely limited. Since *P. agglomerans* constantly enter into the gastrointestinal tract of humans and farm animals with vegetables and other plant foods, study the functional and biological properties of LPS, isolated from representatives of this species, in particular, toxicity, pyrogenicity, antigenic properties, are vitally important. These properties are known to depend, first of all, on the composition and structure of LPS molecules [15, 16].

Structure and peculiarity of the structural arrangement of *P. agglomerans* LPS. The structure of LPS from *P. agglomerans*, like the structure of LPS from other gram-negative bacteria, include three components, namely O-specific polysaccharide (known as O-antigen) (OPS), core oligosaccharide (COS) and lipid A (Fig. 1).

The LPS molecule is anchored into the outer membrane of gram-negative bacteria via hydrophobic interaction between lipid A fatty acids and phospholipids.

The complete LPS molecule, which comprises all three components, is called the smooth (S)-form LPS. The LPS, which lacks the O-specific polysaccharide and/or part of the core oligosaccharide, is called the rough (R)-form LPS (Fig. 2).
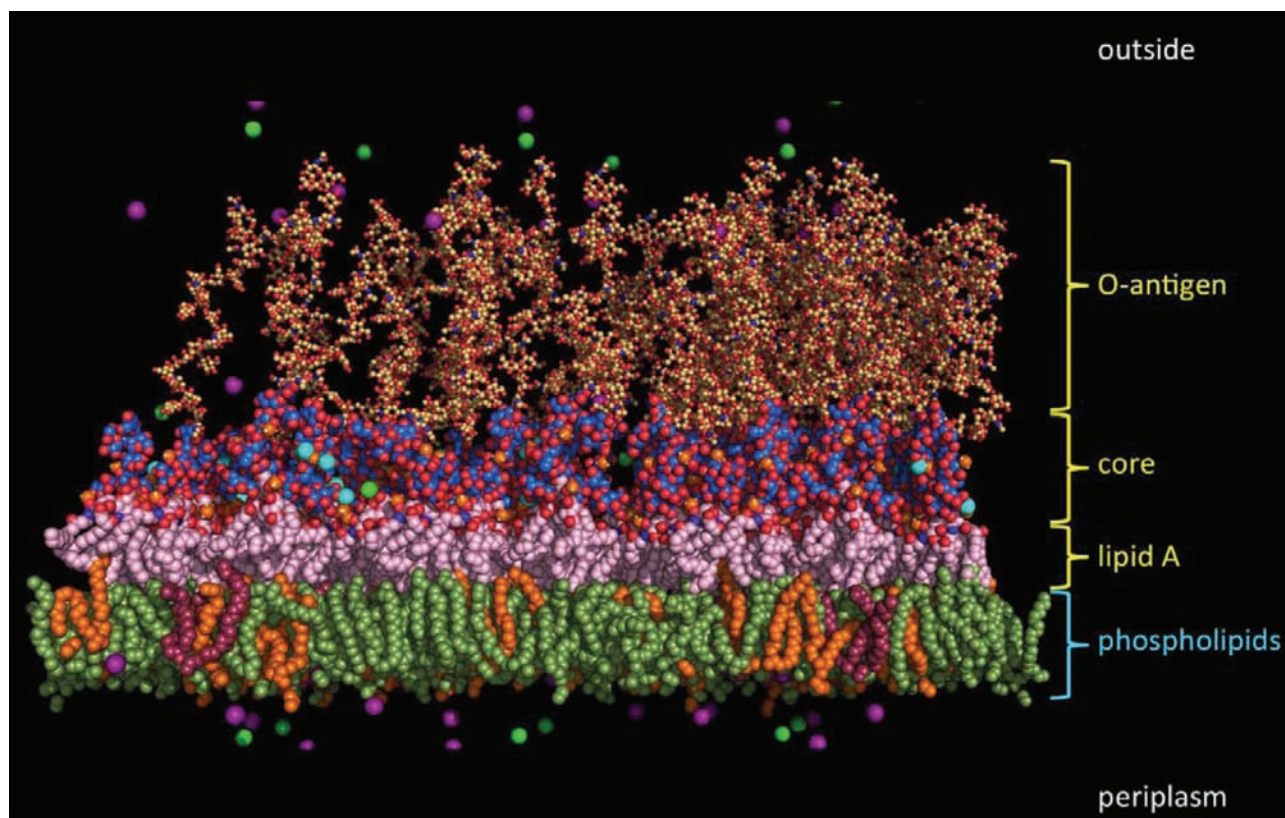
Fig. 1. Typical E. coli LPS (O-antigen polysaccharide, core oligosaccharide, and lipid A) arrangement in outer membrane [15]

The absence of OPS in LPS of some bacteria occurs as a result of either the absence or inactivation of the O-antigen gene cluster. When bacteria are not able to polymerize the O-chain, they synthesize LPS consisting of the only one O-unit attached to COS. Several different forms of LPS can coexist in the same strain. The OPS chain varies considerably in length: from one to more than 50 O-units. The distribution of the chain length is modal (except for bacteria that have S-LPS) and specific for different bacteria strains.

The lipid A structure is the most conservative component of the LPS molecule, whereas the O-polysaccharide chains are the most variable one. Each component performs specific functions: OPS are responsible for the serological activity of bacterial cells and serve as receptors for bacteriophages and bacteriocins; the core oligosaccharide
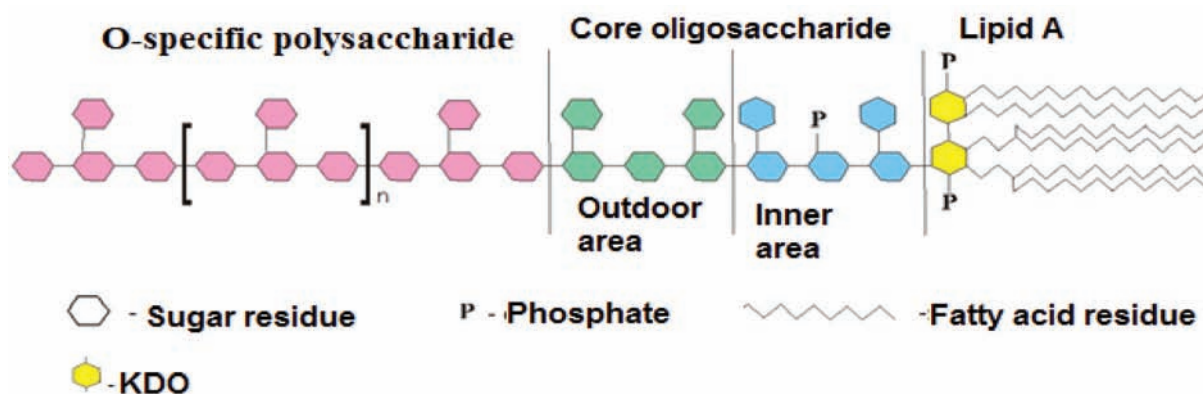

Fig. 2. The common structure of the LPS molecule [26]

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is involved in maintaining the integrity of the outer membrane, and lipid A is the LPS anchor to embed in the bacterial outer membrane via electrostatic and hydrophobic interactions with phospholipids and is responsible for the endotoxic properties of gram-negative bacteria [16].

The carbohydrate part (O-specific polysaccha-ride and core oligosaccharide) is attached to lipid A by a glycosidic bond through the residue of 2-keto-3-deoxyoctonic acid (KDO) (Fig. 3) [16, 17]. This labile acid bond can be selectively cleaved under mild acid hydrolysis conditions into carbohydrate and lipid parts.

Lipid A consists of two β (1→6)-linked D-glucosamine residues, which carry fatty acids and phosphate groups attached via amide and ester bonds at the C-1 and C-4′ positions [18-20] (Fig. 4). Fatty acids can be saturated (in rare cases, unsatu-

rated), as well as hydroxylated. 3-OH-hydroxy acids are differentiating for certain types of bacteria. Therefore, the composition of fatty acids is used as one of the chemotaxonomic criteria in the bacterial systematics.

In lipid A of the studied *P. agglomerans* [21] strains, the following fatty acids were found to pre-dominate: 3-OH-C14:0 (31.7-60.6%), C14:0 (12.9-30.8%), C12:0 (8.2-30.4%), C16:1 (4.2-27.4%), C16:0 (3.3-17.3%). LPS of the two studied strains also contained 2-OH-C14:0 acid. Only one strain lacked C14:0 acid and three strains contained C18:0 acids. In some cases (3 strains), insignificant amounts of C18:0, as well as cis- and trans-C18:1 acids were de-tected; aic C15:0 acid was found in only one strain. The fatty acid composition of lipid A from studied early *P. agglomerans* strain was shown to distin-guish by the absence of C16:1, C18:1 and C18:0 acids
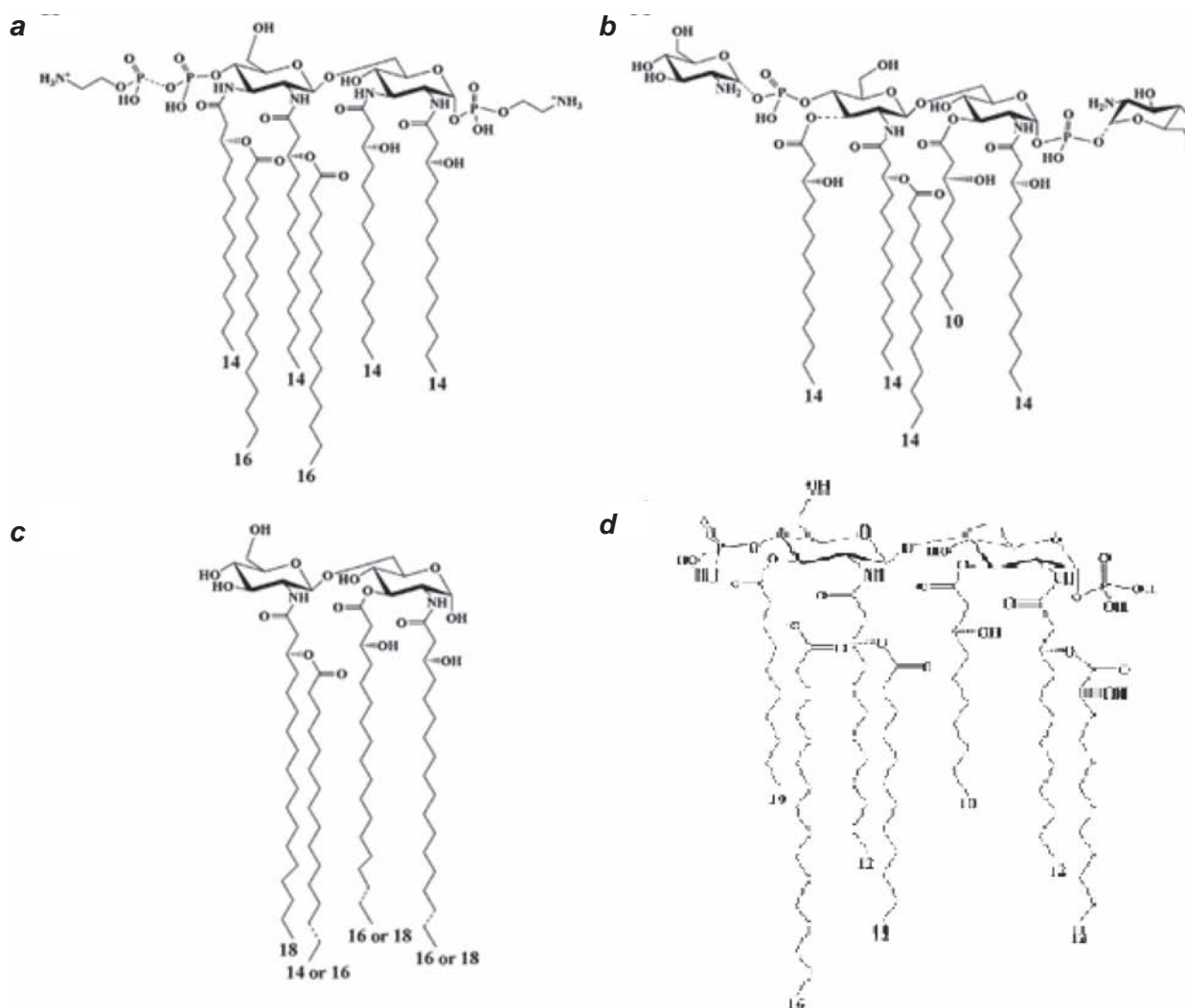

Fig. 3. Structures of lipid A variants: a – hexaacyl, b – pentaacyl, c – tetraacyl, d – heptaacyl [26]

[22]. The analysis of the published data indicates a variety of lipid A fatty acid compositions of LPS in different *P. agglomerans* strains.

The presence of only one 3-hydroxytetradecanoic acid, which acylates amino and hydroxy groups of glucosamine residues, is a distinctive feature of lipids A of the *Enterobacteriaceae* family members, whereas, for the majority of the studied gram-negative bacteria strains, the fatty acid composition is a species-specific characteristic [23].

Thus, despite a conservative structure, lipid A is characterized by considerable microheterogeneity, which depends on various factors, including bacterial adaptation to changing environmental conditions, incomplete biosynthesis, chemical modifications emerged in isolated lipids. Lipids A vary in the amount of fatty acids, their distribution along the disaccharide molecule of glucosamine, chain length and stereochemistry. The presence of charged groups in lipid A is essential for a variety of LPS biological activities, including the resistance of bacteria to antibiotics [24]. Thus, phosphate groups may be substituted with polar or other groups. Most common polar substituents of phosphate groups, which are usually present in non-stoichiometric amounts, are: secondary phosphate (lead to the formation of a diphosphate group), hydrogen, heptose, galacturonic acid, phosphoethanolamine, and 4-amino-4-deoxy-L-arabinose [12, 25]. These substituents affect the biological properties of not only LPS but also the entire bacterial cell. Thus, it was established [26, 27] that the substituents at 4′-phosphate of glucosamine II result in the bacterial resistance to some polycationic antibiotics, in particular, polymyxin B. In case, when the OH group at 4′-phosphate of glucosamine is not substituted, polymyxin B is attached to the OH group, and such bacteria are polymyxin sensitive. When there is substituent, such as 4-amino-4-deoxy-L-arabinose, at the OH group, polymyxin is not able to bind, and such bacteria become polymyxin-resistant. Since only one *P. agglomerans* strain among 14 studied showed resistance to polymyxin B [36], it can be assumed that lipid A of LPS isolated from this strain contains substituent 4-amino-4-deoxy-L-arabinose. LPS of the rest 13 strains presumably do not contain a substituent at C4′ of glucosamine II. This assumption proved to be correct when the lipid A structures of three *P. agglomerans* strains 8674[T,] 7604, and 7969 were analyzed by GC-MS and high-resolution ESI-MS [28, 29].

Usually, lipid A has a hexaacyl structure, in which 6 acyl chains of different lengths are esterified

with glucosamine disaccharide (Fig. 3). However, certain bacteria can synthesize under-acylated lipid A, for example, tetra- or pentaacyl forms [30, 31] or even over-acylated, such as the heptaacyl lipid A [31, 32].

Tsukioka et al. showed that lipopolysaccharide of *P. agglomerans* carries at least two types of lipids A with different levels of acylation [22]. One was the same as *Escherichia coli* 4′-monophosphorylhexaacyl-lipid A, and the other was similar to *Salmonella enterica* 4′-monophosphorylheptaacyl-lipid A. These LPS also differed in molecular weight: 5 kDa and 30-60 kDa, respectively.

Similar results were obtained by other researchers who detected two lipid A types in the LPS from *P. agglomerans* [33, 34]. They are characterized by low molecular weight and differ from the previously described structures only by the presence of an additional oxygen atom at the acyloxyacyl ester-linked side-chain of the distal portion of the molecule.

GC-MS and high-resolution ESI-MS analysis of lipids A of *P. agglomerans* 7604, 7969 and 8674[T] strains revealed their high heterogeneity [28, 29]. Lipid A from *P. agglomerans* 8674[T] was represented by hexa-, penta-and tetraacyl species [29]. The hexaacyl species included four 3-OH-C14:0 residues, one C14:0 residue and one C12:0 residue; the pentaacyl species was characterized by the absence of one C14:0 residue and the tetraacyl species did not contain two fatty acid residues, namely 3-OH -C14:0 and C14:0. In addition, peaks corresponding to hexa-, penta- and tetraacyl species, in which C14:0 or C12:0 residues were substituted with C16:1 or C18:1 residue, respectively, were observed. Moreover, in the hexaacyl species, additional peaks appeared, probably due to the substitution of C14:0 for C16:0 or 2-OH-C14:0, as well as corresponding monophosphorylated derivatives, were registered.

Analysis of the mass spectra of the lipid A of LPS from *P. agglomerans* 7969 showed two main peaks for diphosphorylated disaccharides, which correspond to hexa- and tetraacyl species, with the same residues as *P. agglomerans* 8674[T] strain. These data are consistent with previously published data on the structure of lipid A of *P. agglomerans* [22]. There are also peaks corresponding to two more hexaacylated species, in which C14:0 acids are likely to be replaced by C16:0 or 2-OH-C14:0; and peaks corresponding to monophosphorylated derivatives.

On the spectrum of *P. agglomerans* strain 7604, two species of lipid A were observed: pentaacyl with

four 3-OH-C14:0 residues and one C12:0 residue and tetraacyl - without one 3-OH-C14:0 residue [29]. Both derivatives demonstrated peaks appeared as a result of substitution C12:0 with C18:1, and peaks corresponding to monophosphorylated derivatives.

It can be concluded based on the obtained data on LPS lipid A structures of three *P. agglomerans* strains that they are heterogeneous, characterized by different degrees of acylation and do not contain any monosaccharides as substitutes at C4′ position. These findings were confirmed by the results of the studying the sensitivity of these strains to polymyxin B [35].

OPS structure. It is known that OPS structure is characterized by variability [26]. To determine OPS structure, researchers use the traditional methods, such as gas-liquid chromatography, periodate oxidation, methylation, determination of the absolute configuration of monosaccharides along with the most advanced ones, such as chromatography-mass spectrometry, NMR spectrometry, computer analysis [36]. The use of a combination of modern biochemical and biophysical methods enabled to characterize the unique structures of the OPS in many bacteria species [26, 37].

Typical components of OPS are monosaccharides, which occur in nature. Most of them exist in the form of pyranose, but some can be represented by furanoses (arabinose, ribose, xylose) or by both forms (galactose, fucose, paratose). In some OPS, ribose occurs in the form of pyranose, and N-acetylgalactosamine – in the form of furanose. An OPS usually contains N-acetyl and O-acetyl groups, less often – methyl group bound to hydroxyl or amino groups. In various OPS, the hexuronic acid may occur as an amide with an amino component, such as 2-amino-2-deoxyglycerol (GroN) or amino acid (L-lysine and its Nε-(1-carboxyethyl) derivative). Phosphate was found only in the form of a diester, including cyclic phosphate [26].

The O-antigen structures are mostly established for bacteria from the *Enterobacteriaceae* family. Thus, at present, the full O-antigen structures have been identified for more than 180 strains of *E. coli*, which is a type species of the *Enterobacteriaceae* family. They contain linear or branched tri-, tetra-, penta- and hexasaccharides; less commonly disaccharides or homopolysaccharides [26].

Data on the OPS structures of *P. agglomerans* representatives are limited. Thus, it was shown [34] that the OPS from *P. agglomerans* contain a branched heteropolymer with repeating pentasaccharide units, containing rhamnose, glucose, NAc-glucosamine and NAc-fucosamine residues (I).

A neutral OPS isolated from *P. agglomerans* strain FL1 was found to have a completely different structure. Thus, it is constituted by a homopolymer arranged as linear tetrasaccharide repeating units consisting of D-rhamnose residues [38]:

→2)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→
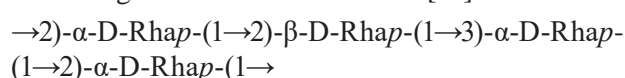
Hashimoto et al. showed that the structure of OPS of LPS from *P. agglomerans* IG1 (IP-PA1) is composed of linear tetrasaccharide repeating units consisting of glucose and rhamnose residues, in which 40% of one of the rhamnose residues is substituted with glucose [39]:

→2)-α-L-Rha*p*-(1→6)-α-D-Glc*p*-(1→2)-[β-D-Glc*p*-(1→3)]0.4-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→
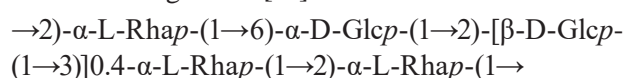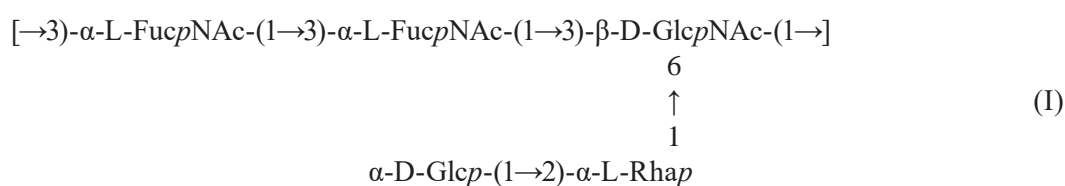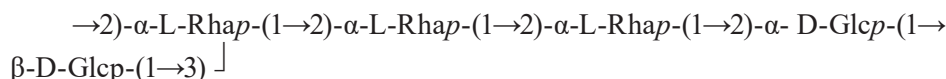
This means that if OPS consists, for example, of 10 repeating units, only in four of them rhamnose is replaced by glucose, and the rest are unsubstituted. This leads to one of the possible heterogeneities of the LPS molecule that plays a significant role in its biological activity.

A similar structure, where rhamnose residues were substituted with glucose, was identified in the OPS from *Shigella flexneri* serotype X [40]:
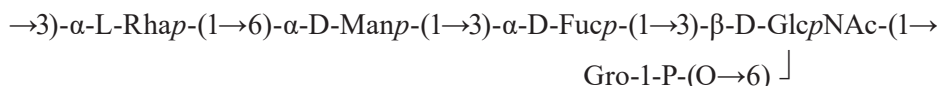
→3)-β-D-Glc*p*NAc-(1→2)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→
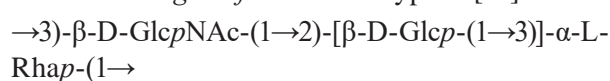
The structures of the OPS from *P. agglomerans* 8674[T] and *P. agglomerans* 7604 were shown to have similar features and constituted by branched pentasaccharides containing 3 rhamnose residues and 2 glucose residues, one of which was in the lateral position (II). The only difference was that in 8674[T]

[→3)-α-L-Fuc*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→]
                                                    6
                                                    ↑   (I)
                                                    1
                              α-D-Glc*p*-(1→2)-α-L-Rha*p*

P. agglomerans 8674ᵀ

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α- D-Glc*p*-(1→

β-D-Glcp-(1→3) ⌐

(II)

P. agglomerans 7604

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α- D-Glc*p*-(1→

α-D-Glcp-(1→3) ⌐

→3)-α-L-Rha*p*-(1→6)-α-D-Man*p*-(1→3)-α-D-Fuc*p*-(1→3)-β-D-Glc*p*NAc-(1→

Gro-1-P-(O→6) ⌐
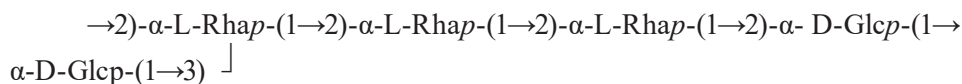
(III)

strain the glucose residues in the lateral position had β-configuration, and in 7604 strain - α-configuration [29].

It was reported that the main chain of neutral OPS isolated from *Serratia marcescens* 111 LPS (O29) has the same structural features; however, the lateral substituents were not identified in its structure [41].

The structure of OPS from *P. agglomerans* 7969 [28] differ greatly from the structures described above and is constituted by a linear tetrasaccharide consisting of rhamnose, mannose, fucose, and N-acetylglucosamine residues (III).

Its structure appeared to be heterogeneous due to non-stoichiometric (~25%) substitution at position 6 of N-acetylglucosamine residue with glycerol 1-phosphate. Earlier, this type of heterogeneity was identified in the OPS from *Proteus vulgaris* TG103 [42]. There has been no reported data on such structure so far. However, the rhamnose and glucosamine disaccharide fragments are quite common in gram-negative bacteria, for example, *Agrobacterium tumefaciens* F1, *Serratia marcescens* O17 and O19, *Klebsiella pneumonia* O12. In OPS from *Stenotrophomonas maltophilia* O6, this disaccharide is xylosylated at position 4 GlcNAc [43].

LPS functions. The structural components of the LPS molecule differ not only in structure but also in functions and biological activity. Thus, lipid A is responsible for the endotoxic activity of the molecule, while the O-specific polysaccharide is associated with the O-serological specificity of the bacterial cell. Therefore, subtle variations in LPS structures are the basis for the development of intraspecies serological classification schemes for gram-negative bacteria. The first serological classification scheme based on the structures of O-specific polysaccharides (10 structures) was proposed by Kauffmann and White for the *Salmonella* genus [44]. The authors showed that the serotypes of *Salmonella* genus members are determined by the presence of substituents at mannose or galactose. Thus, 3,6-dideoxy-hexoses, such as paratose (*S. paratyphi*), abequose (*S. abortus equi*, *S. typhimurium*), tyvelose (*S. typhi*, *S. trasbourg*) can be bound to the mannose as the side chain via the α-1,3 bond. Glucose attached to galactose by α-1,4 or α-1,6 bonds may be present as another side chain. In polysaccharides of group B *Salmonella*, abequose is often acetylated at C-2. Thus, in *S. typhimurium*, most abequose residues were found to be present in the form of O-acetyl derivatives.

Significant progress made in the last decades in studying OPS structures has allowed determining 46 serogroups for *Salmonella enterica*, 46 – for *Shigella representatives*, 76 – for *Proteus*, 61 – for *Providencia*, 39 – *Hafnia*. The most large-scale study has been carried out on *E. coli*, in which more than 180 serogroups have been identified [45].

A study of the serological activity of LPS from 14 strains of *P. agglomerans* by Ouchterlony double immunodiffusion between antisera to the studied strains and LPS isolated from the homologous strains demonstrated that LPS exhibit antigen activity [21]. However, it was found in serological cross-reaction tests between antisera and LPS from different strains that only some of them exert cross-reactivity. These findings enabled to divide, for the first time, the 14 studied *P. agglomerans* strains into 10 serogroups based on the LPS O-antigenic properties. This is the only study on the serological identification of *P. agglomerans* strains known currently [21].

LPS biological activity. The complex structure of the LPS molecule and its heterogeneity, as well as

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the presence of several LPS differing in molecular mass and structure within the same strain of bacteria, determine LPS polyfunctionality. On the one hand, LPS perform a biological protective function, are involved in cell adhesion, exhibit mitogenic activity and antitumor effect, and can serve as markers for bacterial strain identification. LPS can activate B- and T-lymphocytes, granulocytes and mononuclear cells and thus, are considered as potential immunomodulators. LPS, having antigenic activity, serve as biological recognition components, carriers of specific information determining the relationship between bacteria and macro-/microorganisms. On the other hand, as bacterial endotoxins, LPS in higher animal and human induce a wide range of pathophysiological reactions that can lead to septic shock, and be fatal in some cases. Owing to these properties, LPS contribute to the pathogenic potential of gram-negative bacteria, and thus, to the outbreak of severe infectious diseases. The pathogenesis of sepsis includes the following phases: neutrophil, monocyte and macrophage inflammatory reactions, intravascular coagulopathy, endothelial cell injury, and hypotension. Thus, LPS has a pleiotropic effect on tissues and organs that can lead to death [46]. Recently, researchers have found specific LPS receptors on the immune cell surface and blood proteins, which can recognize the minor differences in the LPS structure and make an organism to respond. Since the biological activities of LPS correlate with their structures, the latest findings on the LPS structures have enabled to investigate the mechanism of the LPS biological activity at the molecular and cellular level. Understanding these mechanisms is essential for solving some fundamental problems of immunogenesis and cell reception. The crucial point in the investigation was the discovery by Bruce Beutler in 1998 of the LPS receptor, Toll-like receptor, when he studied the effect of LPS on mice [47]. This receptor is similar to the protein coded by the Toll gene identified in Drosophila in 1995. LPS was shown to trigger an immune response via interaction with Toll-like receptor 4 (TLR4), which is a key receptor of innate immunity.

Identification of the LPS receptor was essential for understanding the mechanisms of innate immunity. In 2011, Bruce Beutler, Jules Hoffmann and Ralph Steinman received Nobel Prize in Physiology or Medicine for their discoveries of one of the molecular mechanisms for activation of innate immunity [48]. Toll-like receptor activation occurs when it binds to ligands, which can be certain moieties of bacteria, fungi or viruses. Fig. 4 presents the scheme, in which LPS of gram-negative bacteria is a ligand, and Toll-like receptor 4 (TLR4), which is a surface protein remotely related to the IL-1 receptor [48]. For the recognition of lipopolysaccharide, TLR4 require the presence of transport proteins such as lipopolysaccharide binding protein (LBP), soluble and membrane-bound CD14 proteins [49]. Genetic and biochemical analyses revealed that for the subsequent recognition of the LPS/CD14 complex by the TLR4 receptor, other proteins are also needed, in particular, TLR4-associated co-receptor MD-2 [50]. This protein belongs to the family of lipid-binding proteins [51, 52], which characterized by the β-sandwich structure. In the signaling pathway, TLR4 recognizes hexaacyl diphosphorylated domain of lipid A. The binding to LPS is achieved by intercalation of lipid A acyl chains into hydrophobic region of β-sandwich [53, 54]. This model was confirmed by structure analysis of MD-2 associated with lipid IVa, as well as a TLR4/MD-2 heterodimer in complex with the structurally related to lipid IVa synthetic compound E5564 [55, 56]. Activated by this complex macrophages or mononuclear phagocytes trigger the biosynthesis of various inflammatory mediators, such as TNF, IL-1, IL-6, IL-8, as well as adhesive molecules necessary for the adaptive immune response [57, 58].

A leading role in the LPS induction of most biological effects is performed by lipid A [58], because after removal of the core oligosaccharide and the O-specific polysaccharide from LPS, TLR4 is still able to recognize it. Tetraacyl and hexaacyl LPS from E. coli are the most studied. The cumulative effect of acyl groups in lipid A has been revealed: the more acyl residues and the longer the chains of acyl groups in lipid A structure, the more proinflammatory cytokines macrophages produce. Thus, a direct link between the biological activity of LPS and the chemical structure of lipid A was demonstrated.

The first data on the biological activity of LPS extracted from 10 strains of P. agglomerans isolated from dust, air, human and animal sources were presented by Dutkiewicz in 1976 [59]. Thomas in 1956 showed that P. agglomerans LPS mixed with adrenaline caused dermal hemorrhagic lesions in rabbits and death of the 10-day-old chicken embryos [60].

In further research, a significant increase in the number of polymorphonuclear leukocytes and alveolar macrophages was detected 24 h after guinea
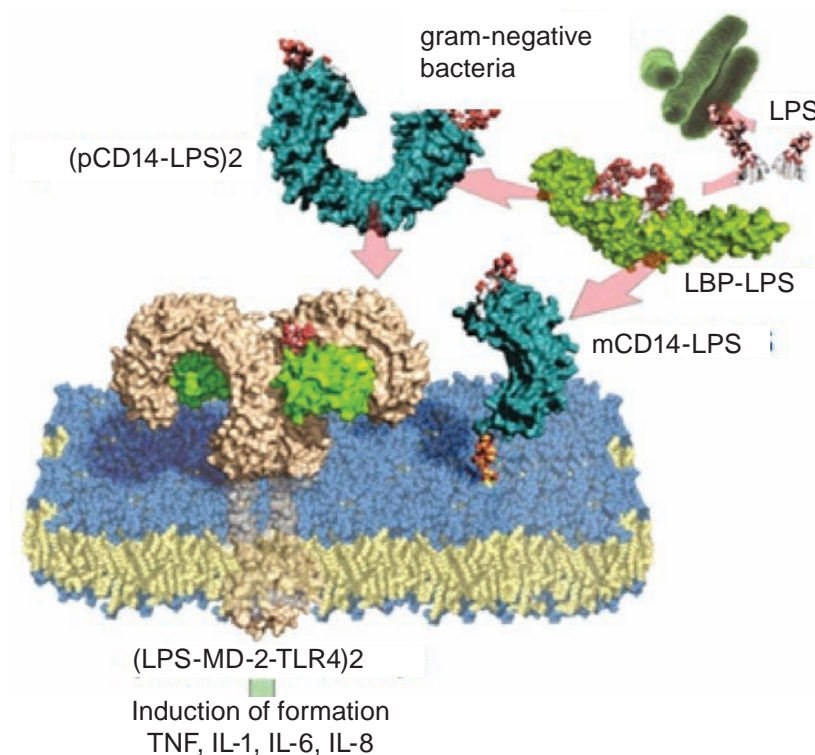
Fig. 4. TLR4 receptor recognition of lipid A [61]

pigs inhaled live cell suspensions or LPS from *P. agglomerans* isolated from cotton [61-63]. These LPS also caused the death of mice and were pyrogenic for rabbits [64].

In 1991, the compound, which activated macrophages after oral or subcutaneous administration, was discovered in an aqueous extract of wheat flour [56]. The active compound was identified as LPS isolated from *P. agglomerans* cells that were in symbiosis with wheat. The authors called this lipopolysaccharide as IP-PA1 (immunopotential from *P. agglomerans*, a former name: LPSp) and showed its effectiveness in various areas, such as healthy diet (to prevent or reduce metabolic syndrome), skin care products (to maintain healthy skin), as well as active ingredients in animal feed and aquaculture. Later, IP-PA1 administered orally or subcutaneously was shown to activate peritoneal macrophages and increase the activity of phagocytes through TLR4 signaling pathway [55, 56].

Iwamoto et al. demonstrated that intravenous co-administration of LPS from *P. agglomerans* with cyclophosphamide resulted in complete regression of mouse hepatoma MH134 [65]. In mice treated by this method, the researchers observed necrosis and inflammatory cell infiltrates (CD4+ and CD8+ T-lymphocytes, macrophages, neutrophils) in tumors and the related increase in serum cytokines (TNF-α, IL-1α, IL-6, G-CSF (granulocyte colonies stimulating factor)). It was concluded that treatment with LPS was effective mainly due to increased T-cell mediated antitumor immunity developed by the synthesis of TNF-α and other cytokines. These results were confirmed by Inagawa et al. [64, 66] who showed the complete regression of syndromal mouse tumors, Meth A fibrosarcoma, MH134 hepatoma, and Lewis carcinoma after combined treatment with intravenous administration of LPS with cyclophosphamide. The researchers indicated that the induction of TNF-α plays an important role in successful therapy and that subcutaneous drug administration was low toxic (by 230-380 times less) compared to intravenous administration.

Japanese researchers described this new medication, containing low molecular weight (5 kDa) LPS and with no side effect, as immunostimulator *P. agglomerans* 1 and suggested it for the treatment of a range of diseases including malignant tumors by oral or intravenous administration [47].

In addition, oral administration of IP-PA1 was shown to prevent diabetes, as well as infectious diseases caused by bacteria and viruses [56]. However, it is still not clear whether IP-PA1 administered orally, and absorbed in the intestine, enters into tis-

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sue macrophages. From drug delivery point of view, it is important to identify the IP-PA1-binding cells after IP-PA1 oral administration.

Dutkiewicz et al. reported that significant amounts of *P. agglomerans* were found in grain and flour dust in the form of globular nanoparticles measuring 10-50 nm, which can be described as "endotoxin super macromolecules" [67]. Their concentration in dust was found to range from 10^4 to 10^5 CFU/g and formed 73.2-96% of the total gram-negative bacteria in the air [67]. Therefore, researchers consider *P. agglomerans* as major causative agents of toxic pneumonia in agricultural workers. Indeed, *P. agglomerans* endotoxins were shown to cause inflammatory and fibrotic changes in lungs, to stimulate alveolar macrophages, to produce anion superoxide, interleukin-1 and chemotactic factors for other macrophages and neutrophils, and to increase the concentrations of pulmonary toll-like receptors and chemokines [67]. Researchers believe that LPS and proteins from *P. agglomerans* should be considered as major causative agents of occupational diseases, such as allergic dermatitis in farmers, and also allergic pulmonary disorders in cattle not only in the cotton industry but also for the grain industry and agriculture.

In 2016, researchers obtained impressive results demonstrating that the pretreatment with LPS from *P. agglomerans* (10 ng/ml) or with monophosphoryl lipid A for 18 h was effective in the prevention of Alzheimer's disease by enhancing phagocytosis of β amyloid by brain microglia [68]. Microglial cells were isolated from adult mouse brain.

Thus, the above results, indicating a high immunomodulatory activity of LPS, suggest their usage in the development of new drugs for various applications. However, therapeutic utilization of LPS is impeded by their high toxicity and pyrogenicity, as well as the insufficiency or inconstancy of their stimulating effect [69].

With regard to *P. agglomerans*, the LPS from the studied strains were characterized by a relatively low toxic effect (LD_{50} 420 and 147 µg/mouse), in contrast to LPS from other *Enterobacteriaceae* family members (LD_{50} range from 3.6 to 75 µg/mouse) [70]; however they induce pyrogenic reactions [70].

Since lipid A is responsible for endotoxic activity, the researchers studied the role of fatty acids, in particular, their length and distribution on glucosamine molecules. It was found that lipids A with short-chain fatty acids were less toxic, or completely non-toxic compared to lipids A with long-chain fatty acids [14, 71, 72]. Hexaacyl bisphosphorylated lipid A structure with an asymmetric (4+2) distribution of acyl chains attached to diglucosamine was shown to be the most active, while its various modifications significantly affect the biological activity of LPS. Such hexaacyl bisphosphorylated lipid A is characterized by a large tilt angle, a conical (hexagonal) molecular shape and high endotoxic activity. Hexaacyl monophosphorylated lipid A has a smaller tilt angle, and the conical shape is less expressed tending to be more cylindrical (cubic) shape. This correlates with less pronounced endotoxic activity. Penta- and tetraacyl lipid A or hexaacyl lipid A with symmetric acyl chain distribution (3/3) are characterized by a small tilt angle, a cylindrical (lamellar) shape and are endotoxically inactive, but can be antagonistic (Table) [73].

Thus, it was shown that lipid A conformation plays a significant role in the manifestation of the LPS biological activity. Thus, so-called endotoxically active conformation corresponds to the cubic and hexagonal supramolecular structures, while the lamellar structure is endotoxically inactive.

Although lipid A was found to play the main role in the manifestation of the LPS biological activity, the length of the OPS, as well as the molecular mass of LPS, is also important [22, 74-76]. Thus, the literature described the LPS structure from one of the *P. agglomerans* strains, which contained at least two types of lipids A with different level of acylation. One of them is similar to 4′-monophosphorylhexaacyl lipid A from *Escherichia coli*, and the other – to 4′-monophosphorylheptaacyl lipid A from *Salmonella enterica*. These LPS also differed in molecular weight: low 5 kDa and high 30-60 kDa, respectively. Hexaacyl lipid A was found to have high activity, including endogenous induction of TNF, while heptaacyl lipid A had lower activity. These LPS from *P. agglomerans* exhibited beneficial therapeutic effects, which were not observed for other LPS. It was suggested that these effects could not be attributed solely to the structure of lipid A. The length of the OPS chain and the LPS composition may also contribute greatly to the biological activity of lipid A.

Thus, Kohchi et al. [7] found that the OPS chain of IP-PA1 from *P. agglomerans* is shorter and characterized by a much lower molecular weight than the LPS of the type *E. coli* O:113 strain. Therefore, LPS IP-PA1 easily forms small-diameter mi-

Chemical composition, molecular conformation and biological activity of different lipids A

Conformation	Supramolecular structure	Fatty acid composition	Toxicity	Species of bacteria
	Lamellar	3-OH-C14:0 (2) 3-OH-C10:0 (2) C12:1 (1)	Not toxic	*Rhodobacter capsulatus*
		3-OH-C14:0 (2) 3-OH-C16:0 (2)		*Rhodospirillum fulvum*
	Cubic	3-OH-C14:0 (2) C12:1 (2)	Moderately toxic	*Escherichia coli*
	Hexagonal	3-OH-C10:0	Hight toxic	*Rhodocyclus gelatinosus*
		3-OH-C14:0 (2) C12:0 C16:0		*Salmonella minnesota*

celles, which can easily cross the mucous membrane and/or skin, enhancing positive effect of treatment after oral or subcutaneous administration.

Pupo et al. [77] demonstrated that LPS from *E. coli* containing short and long polysaccharide chains exhibited various biological activities on human macrophages. An essential role in the interaction of S-LPS with host cells is played by the LPS-binding protein. These results suggest that the polysaccharide part may affect the biological activity of LPS through serum factor.

Kadowaki et al. [78] divided the LPS IP-PA1 into two fractions: high-molecular (30-60 kDa) and low-molecular (5 kDa). The first fraction contains long-chain OPS and the last one contains short oligosaccharides. These fractions induced different NO production in RAW264.7 cells.

The described results support the involvement of the LPS polysaccharide region in its biological effects.

Thus, the reported results [59, 62, 63, 78] indicated that the potential of biological activity of the LPS preparations from *P. agglomerans* was very similar to that of the LPS from most *Enterobacteriaceae* species (for example, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Salmonella typhi*, *Salmonella typhimurium*) or *Pseudomonadaceae* (for example, *Pseudomonas putida*), and was higher compared to the LPS from other gram-negative bacteria, such as *Agrobacterium* sp. and *Xanthomonas sinensis* [62, 63] or *Acinetobacter calcoaceticus* and *Alcaligenes fecalis* [79].

The data found in literature and our own experimental results indicate the importance of the study of lipopolysaccharides of gram-negative bacteria, including *P. agglomerans,* a poorly-studied member of the *Enterobacteriaceae* family. Discovery of the heterogeneity of LPS, distinguished by unique structures of O-specific polysaccharides, lipids A, characterized by different degrees of acylation, as well as serological activity and endotoxic properties not only contribute to the biological characteristics of the species but also could stimulate the development on their base of new drugs of various applications.

Further research focused on the clarification of the following aspects could be promising:

- the role of LPS in the pathogenesis of infectious diseases - this will contribute to the development of a new effective approach to combating diseases caused by gram-negative bacteria;

- the ability of modified lipopolysaccharides to modulate both the innate and adaptive immune response;

- the possibility of using synthetic polysaccharides as components of vaccines, adjuvants, anticancer drugs, nonspecific stimulants.

ЛІПОПОЛІСАХАРИДИ *Pantoea agglomerans*: СТРУКТУРА, ФУНКЦІОНАЛЬНА ТА БІОЛОГІЧНА АКТИВНІСТЬ

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В огляді наведені дані літератури, а також результати власних експериментальних досліджень ліпополісахаридів (ЛПС) грамнегативних бактерій. Основну увагу автори приділяють *Pantoea agglomerans*, представнику родини *Enterobacteriaceae*. Вперше описано унікальні структури О-специфічних полісахаридних ланцюгів їхніх ліпополісахаридів, які можуть бути як розгалуженими, так і лінійними тетра- і пентасахаридними ланцюгами, що повторюються. Показана гетерогенність як самої молекули ЛПС, так і присутність в бактеріальній клітині декількох ЛПС, які відрізняються структурою ліпідів А, О-специфічних полісахаридних ланцюгів, серологічною активністю, а також ендотоксичними властивостями, зокрема токсичністю і пірогенністю. Така гетерогенність є одним із механізмів поліфункціональності ЛПС. На основі О-антигенності ЛПС вперше було проведено серотипування штамів *P. agglomerans* і віднесено їх до 10 серогруп. Висока імуномодулювальна активність ЛПС *P. agglomerans* дозволяє припустити використання їхніх олігосахаридних фрагментів для створення кон'югованих вакцин проти захворювань, спричинених грамнегативними бактеріями.

К л ю ч о в і с л о в а: *Pantoea agglomerans*, О-специфічний полісахарид, ліпід А, серологічна активність, TLR 4 рецептор, біологічна активність.

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