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Research Paper

Lipopolysaccharides of *Pantoea agglomerans* 7604 and 8674 with structurally related *O*-polysaccharide chains: Chemical identification and biological properties

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ABSTRACT

Structurally related O-specific polysaccharide (O-antigen) and lipid A components were obtained by mild acid degradation of the lipopolysaccharides (LPSs) of two strains of bacteria *Pantoea agglomerans*, 7604 and 8674. Studies by sugar analysis along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy enabled elucidation of the following structures of the *O*-polysaccharides, which differ only in the linkage configuration of a side-chain glucose residue:

R = α-D-Glc*p* in strain 7604 or β-D-Glc*p* in strain 8674

Lipid A samples were studied by GC–MS and high-resolution ESI–MS and found to be represented by penta- and tetra-acyl species; lipid A of strain 8674 also included hexaacyl species. A peculiar feature of lipid A of both strains is the presence of the major *cis*-9-hexadecenoic (palmitoleic) acid, which has not been found in *P. agglomerans* strains studied earlier. The LPSs of both strains were pyrogenic, reduced the average adhesion and the index of adhesiveness and showed a relatively low level of lethal toxicity. O-antiserum against strain 7604 showed one-way cross-reactivity with the LPS of strain 8674, and O-antisera against both strains cross-reacted with LPSs of some other *P. agglomerans* strains but more strains were serologically unrelated. These structural and serological data indicate immunochemical heterogeneity of *P. agglomerans* strains and will find demand in classification of *P. agglomerans* by O-antigens.

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→6)-α-D-Glc*p*-(1→
R-(1→3)┘

1. Introduction

Pantoea agglomerans is probably one of the most widespread organisms in the world. Originally a plant bacterium, it is known both as an epiphytic microbe developing on the surface of plants and as an endophytic organism living inside the plants (Quecine et al., 2012; Walterson & Stavrinides, 2015). The bacterium also occurs abundantly in plant and animal products, in the body of arthropods and other animals, in water, soil, dust and air, and occasionally in humans (Loncaric et al., 2009; Völksch, Thon, Jacobsen, & Gube, 2009; Walterson & Stavrinides, 2015). It causes disorders in people exposed to inhalation of organic dusts (Rylander & Burrell, 1988) and diseases of crops (Medrano & Bell, 2007). On the other hand, *P. agglomerans* produces substances effective in the treatment of cancer and other diseases of humans and animals (Kohchi et al., 2006). It suppresses development of various plant pathogens by antibiotic production and/or competition (Pusey, Stockwell, Reardon, Smits, & Duffy, 2011; Walterson & Stavrinides, 2015), promotes plant growth by nitrogen fixation and other mechanisms (Jiang, Wu, Wang, & Feng, 2015; Quecine et al., 2012; Walterson & Stavrinides, 2015) and appears as a potentially efficient bio-fertilizer and bio-remediator (Walterson & Stavrinides, 2015; Xiong et al., 2014).

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The early taxonomy of *Pantoea* representatives was quite complex, with some of the first members of the group being classified as *Bacillus agglomerans* (Beijerinck, 1888) and *Enterobacter agglomerans* (Beijerinck, 1888; Tindall, 2014). Other names also associated with members of this group included *Bacterium herbicola* Löhnis 1911, *Pseudomonas herbicola* (Geilinger 1921), *Erwinia herbicola* (Düggeli) Dye 1964 and *Erwinia milletiae* (Kawakami and Yoshida 1920), which were later established as synonymous (Ewing & Fife, 1972; Tindall, 2014). Later, Beji et al. (1988) and Gavini et al. (1989) recognized *E. herbicola, E. milletiae,*\ and *E. agglomerans* as also being synonymous, leading to the transfer of these three groups to *Pantoea agglomerans* (Beijerinck, 1888; Gavini et al., 1989), which served as the nomenclatural type for the establishment of the genus *Pantoea* (Beijerinck, 1888; Ewing & Fife, 1972; Gavini et al., 1989; Tindall, 2014). Biochemical heterogeneity of *P. agglomerans* and related strains and species renders identification difficult, and the diversity of *Pantoea* spp. strains and their possible association with hosts and diseases remain poorly known.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, plays an essential role in taxonomy of pathogens and their interactions with animal and plant hosts (Dow, Newman, & von Roepenack, 2000; Newman, Dow, Molinaro, & Parrilli, 2007). The LPS consists of three structural parts: lipid A, core oligosaccharide and O-specific polysaccharide (OPS). Hydrophobic lipid A is responsible for a wide spectrum of non-specific pathophysiological reactions, such as fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death (Culbertson & Osburn, 1980). The OPS linked to lipid A via a core oligosaccharide is composed of oligosaccharide repeats (O-units), which usually include two to eight monosaccharide residues. The nature of OPS constituent monosaccharides, their ring form, sequence, type of linkages between them and substitution with non-sugar groups vary from strain to strain within most Gram-negative species, giving rise to enormous structural diversity. The fine structure of the OPS determines the serological specificity of bacteria, and therefore this surface polysaccharide is called O-antigen.

Until now, the LPSs of *P. agglomerans* have been only scarcely characterized both chemically and biologically. Different levels of acylation of lipid A have been shown (Tsukioka et al., 1997; Zdorovenko et al., 2017), and OPS structures of two *P. agglomerans* strains have been elucidated (Cimmino et al., 2008; Zdorovenko et al., 2017). In the present work, the LPSs of two new *P. agglomerans* strains, 7604 and 8674, were isolated and characterized chemically and biologically.

2. Materials and methods

2.1. Strains and growth of bacteria

P. agglomerans 7604 (isolated from rye seeds) and 8674 (type strain of the species, host plant is unknown) were obtained from the culture collection of the Department of Phytopathogenic Bacteria (D.K. Zabolotny Insitute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev). The cultures were grown at 28 °C for 24 h on a beef-extract agar medium. Cells were separated by centrifugation and dried with acetone and ether.

2.2. Isolation and characterization of lipopolysaccharides

LPS samples were isolated from bacterial cells in yields of 3.4 and 6.8%, respectively, by the phenol-water procedure (Westphal & Jann, 1965) followed by removal of proteins and nucleic acids by precipitation with aqueous 50% CCl_3CO_2H at 4 °C and ultracentrifugation at 105,000 × *g* (3 × 4 h).

Total carbohydrates, proteins, nucleic acids and 3-deoxyoct-2-ulosonic acid were quantified as described earlier (Zdorovenko et al., 2017). The LPS preparations from *P. agglomerans* 7604 and 8674 contained 31 and 42% carbohydrates, traces of proteins, 1.4 and 7.7% nucleic acids, 0.6 and 0.4 % 3-deoxyoct-2-ulosonic acid, respectively.

2.3. Degradation of lipopolysaccharides, isolation of lipid A and O-polysaccharides

A LPS sample (50 mg of each strain) was hydrolyzed with aqueous 2% AcOH at 100 °C for 1.5 h. A precipitate (lipid A) was separated by centrifugation (13,000 × *g*, 20 min), washed twice with distilled water and freeze-dried. A water-soluble carbohydrate portion was fractionated by gel-permeation chromatography on a column (56 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 0.05 M pyridinium acetate buffer (pH 4.5) monitored with a differential refractometer (Knauer, Germany) to give OPS samples of *P. agglomerans* 7604 and 8674 (11.5 and 14.5 mg, respectively).

2.4. Fatty acid composition

For fatty acids analysis, LPS samples were subjected to methanolysis with 1.2 M AcCl in methanol (80 °C, 45 min) followed by extraction with hexane and silylation with *N,O*-bis(trimethylsilyl)trifluoracetamide (80 °C, 5 min). The samples were analyzed by GC–MS using a Maestro system (Interlab, Russia) equipped with an Rxi®–5 ms column (25 m × 0.25 mm × 0.25 μm) (Restek, USA) using the temperature program of 135–320 °C at 7 °C min^{-1}. Ionization was performed by electron impact (energy −70 eV). Detection was carried out in full scan mode (SCAN). Fatty acid derivatives were identified using the available mass-spectra database NIST14.

2.5. Sugar composition

An OPS sample (0.5 mg from each strain) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h). Monosaccharides were identified as the alditol acetates (Sawardeker, Sloneker, & Jeanes, 1965) by GC on a HP-5 capillary column using a Maestro (Agilent 7820) chromatograph (Interlab, Russia) and a temperature gradient of 160 °C (1 min) to 290 °C at 7 °C min^{-1}. The absolute configuration of rhamnose was determined by GC of the acetylated (*S*)-2-octyl glycosides as described (Leontein & Lönngren, 1993).

2.6. Mass spectrometry

Lipid A samples were analyzed by negative-ion mode high-resolution electrospray ionization mass spectrometry (HR ESI MS) (Belyakov et al., 2010) using a Bruker micrOTOF II instrument. Interface capillary voltage was 3200 V, mass range from *m/z* 50 to 3000. A syringe injection was used for 1:1:0.1 acetonitrile/water/triethylamine solutions at a flow rate 3 μL min^{-1}. Nitrogen was applied as dry gas, and interface temperature was set at 180 °C. Internal calibration was done with Electrospray Calibrant Solution (Fluka).

2.7. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O and then examined as solutions in 99.95% D_2O. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer (Germany) at 30 °C using internal sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (δ_H 0.0, δ_C −1.6) as reference for calibration. 2D NMR experiments were performed using standard Bruker software, and Bruker TopSpin 2.1 program was used to acquire and process the NMR data. Spin-lock time of 60 ms and mixing time of 200 ms were used in TOCSY and ROESY experiments, respectively. A ^1H,^{13}C HMBC experiment was run with a 60-ms delay for evolution of long-range couplings.