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EXPERIMENTAL ARTICLES

Composition and Immunochemical Characteristics of Exopolysaccharides from the Rhizobacterium *Paenibacillus polymyxa* 1465

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Abstract—Exopolysaccharides (EPS) synthesized by *Paenibacillus polymyxa* 1465 in the course of batch cultivation were proven to contain neutral and acidic fractions. EPS are heterogeneous polysaccharides, represented by a complex of macromolecules with molecular mass of 7×10^4 to 2×10^6 Da. The acidic component was shown to be predominant in EPS preparations isolated from bacteria cultivated on glucose, which corresponds to a higher viscosity of EPS water solutions. The exoglycans were shown to contain glucose, mannose, galactose, and uronic acids. Polyclonal rabbit antibodies against the isolated *P. polymyxa* 1465 EPS preparations were used in a comparative immunodiffusion analysis of a number of *P. polymyxa* strains.

Key words: Paenibacillus polymyxa, exopolysaccharides, viscosity, monosaccharide composition, immunodiffusion analysis.

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The efficiency of *Paenibacillus polymyxa* in formation of associative relationships allows these soil nitrogen-fixing rhizobacteria to promote growth and development of a broad spectrum of plants. This is related to ability of these bacteria to fix nitrogen and mobilize phosphate, produce phytohormones, antibiotics, and a wide range of lytic enzymes, as well as to their high adaptive capacity [1, 2]. *P. polymyxa* are reported to invade root tissues [3] and colonize plant roots with biofilm formation [4]. In the process of ecological niche occupation, intense growth and spore forming ability, which allow for high competitive power and survival under stressful conditions, are of great importance [2, 5].

Paenibacillus polymyxa bacteria are capable of synthesizing a significant amount of neutral and acidic exopolysaccharides (EPS) [6–9]. Surface localization of extracellular polysaccharides provides for their mediation between *P. polymyxa* and other micro- and macroorganisms. In addition, a thick layer of EPS on the surface of bacteria may cover other cellular structures and thus be responsible for the immunochemical characteristics of the bacteria [10]. In a number of works *P. polymyxa* EPS were shown to be biologically active, displaying hemagglutinating and antiviral activity and enhancing nonspecific organism responsiveness [11]. Therefore, these biopolymers are of topical interest.

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Earlier, *P. polymyxa* was shown to be highly adhesive [12]. Rare literature data prove the important role of exoglycan synthesis in the processes of *P. polymyxa* interaction with plants [4, 9, 13] and cell adhesion to various substrates [14].

The aim of this work was to study physicochemical and immunochemical characteristics of isolated bacterial EPS of *P. polymyxa* strain 1465.

MATERIALS AND METHODS

In this study we used several strains of *Paenibacillus polymyxa* (previously *Bacillus polymyxa* [15]): 1465 (ATCC 8523), 1460 (ATCC 1041), and 1459 (ATCC 842) from the Czech collection of microorganisms (Brno); 88A (TsMPM B-4556) from the collection of microorganisms of Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences; and 92 (VNIISHM 92), isolated from wheat roots by Yu.M. Voznyakovskaya (All-Russia Research Institute for Agricultural Microbiology, St. Petersburg, Pushkin). For experiments on determination of growth dynamics, exoglycans yield, and kinematic viscosity of 0.1% EPS solutions, bacteria were cultured under strictly identical conditions for 7 days on a rotary shaker at 30°C in 500-ml flasks containing 150 ml of the medium. The medium contained the following (g/l): yeast extract, 4; Na_2HPO_4, 1.1; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.1; $CaCO_3$, 0.2; 30 g/l of glucose; distilled water to 1 l; pH 7.2–7.5.

Apart from glucose, sucrose was also used as a carbon source. Sugar solutions were sterilized separately and introduced into the media before inoculation. The samples were collected immediately after inoculation, after 6 h of incubation, and then every day over the course of a week. The number of viable cells was determined by plating serial tenfold dilutions on agarized cabbage medium, and was expressed as base ten logarithms of cell number per ml (log N) [16].

Isolation of the total EPS preparations was performed according to the technique described in [17]. After purification procedure, the samples were freeze-dried on a BENCHTOP 2K ES VirTis (United States) instrument.

To measure intrinsic viscosity of the polysaccharide water solutions, EPS preparations were suspended in distilled water to form 0.1% solutions. The kinematic viscosity of EPS solutions was determined using a type VPZh-2 Ostwald viscometer with the inner capillary diameter of 0.73 or 0.99 mm in a water bath at 20 ± 1°C.

EPS concentration in the culture liquid was measured by weight; the total amount of carbohydrates in the samples was determined by spectrophotometric analysis after treatment with phenol and sulfuric acid [18]; protein content was measured using the modified Bredford procedure [19] and expressed as a percent of supposedly dry matter weight.

Experimental data were subjected to statistical analysis [20].

Gel filtration of the EPS preparations was carried out on a Sepharose CL-4B (Pharmacia, Sweden) column (46×2.5 cm, $V_0 = 30$ ml) in 0.025 M NH_4HCO_3 solution (pH 8.3). Gel filtration was also used to evaluate EPS molecular mass. The columns were calibrated with dextrans of known molecular weights of 2×10^4, 7×10^4, 1×10^5, 2.29×10^5, 5×10^5, and 2×10^6. Detection was carried out with an LKB 2142 (LKB, Sweden) differential flow refractometer. Additionally, carbohydrate elution profiles were recorded from optical density of the fractions after treatment with phenol and sulfuric acid [18].

Ion exchange chromatography was performed on a DEAE-Toyopearl 650M anion exchanger (35×1.5 cm, $V_0 = 40$ ml). Neutral and weakly acidic components were eluted with Tris–HCl buffer (0.01 M, pH 7.2), and the acidic ones with NaCl solution in the same buffer; the salt concentration was continuously increased from 0.01 to 1.0 M. The fractions were concentrated using a rotary evaporator at 40°C and then analyzed.

Gel electrophoresis of EPS preparations was carried out in a 12.5% polyacrylamide gel with sodium dodecyl sulfate. The gels were stained with the silver nitrate reagent [21].

Hydrolysis of EPS was performed according to the following scheme: lyophilized preparations were dissolved in 4 N trifluoroacetic acid (TFA) and heated in sealed ampoles for 3 h at 100–105°C. To remove TFA remains, the hydrolysates were evaporated to dryness, water being added at times. Then hydrolysates were used for chromatography on paper or cellulose plates and to prepare alditol acetates for gas–liquid chromatography (GLC).

Neutral sugars, uronic acids, and amino sugars were identified by means of ascending paper chromatography or TLC on cellulose plates in pyridine–ethyl acetate–acetic acid–water, 5 : 5 : 1 : 3. Neutral sugars and uronic acids were detected with anisidine phthalate solution in butanol with subsequent heating at 115°C for 5 min. To detect amino sugars, chromatograms were developed with ninhydrin solution in acetone according to [22]. Colorimetric detection according to Dische [23] was also used to determine uronic acids content.

After complete EPS hydrolysis, reduction with $NaBH_4$, and acetylation, quantitative analysis of monosaccharide composition was carried out by GLC of corresponding alditol acetates according to [24]. The samples were analyzed on a Hewlett–Packard 5890 instrument equipped with an Ultra 2 stationary phase capillary column using a temperature gradient from 180°C (1 min) to 290°C at 10°C/min.

Polyclonal antibodies were obtained against total EPS preparation of glucose-grown *P. polymyxa* 1465. Rabbits were immunized 3 times with two-week intervals by successive injections of 0.5, 1.0, and 1.5 mg of EPS into popliteal lymph nodes. For the first injection, the antigen was mixed with Freund's complete adjuvant in the ratio of 1 : 1, while for the following injections Freund's incomplete adjuvant was used. Animals were exsanguinated one week after the last immunization. Immunoglobulins G were obtained from antisera by precipitation with ammonium sulfate [25]. Double immunodiffusion was carried out according to [26] in 1% agarose gel using Tris–glycine–barbiturate buffer (pH 8.8) on degreased glass plates.

RESULTS AND DISCUSSION

P. polymyxa 1465 grown in liquid medium with glucose accumulated 2.1 g/l of EPS (EPS_{Glu}) on average (see Fig. 1). When sucrose was used as a carbon source, the biopolymer yield increased considerably, the highest yield of EPS being 12.3 g/l (EPS_{Suc}). A study of EPS accumulation dynamics showed that exoglycan synthesis occurred in all the growth phases starting with the logarithmic one (Fig. 1). EPS enrichment was lagging behind the culture growth and reached its maximum during the late stationary growth phase. Moreover, EPS concentration didn't decrease by the end of fermentation, which suggests a lack of EPS reutilization by *P. polymyxa* under these conditions.

Lyophilized EPS preparations were white fibrous substances readily soluble in water. At a concentration of 0.1%, EPS formed homogeneous and clear solutions stable within several days. The kinematic coefficient of viscosity was found to depend on the culture growth phase and on the carbon source (see curves *3* and *4* in

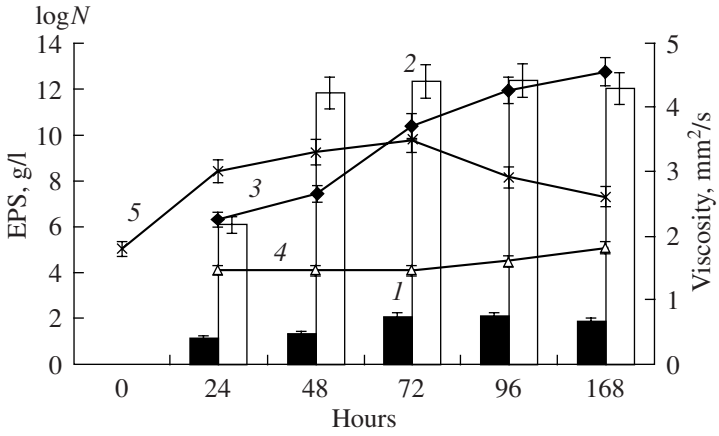

Fig. 1. EPS accumulation dynamics (*1* and *2*), viscosity of EPS 0.1% solutions (*3* and *4*), and growth dynamics (*5*) of *P. polymyxa* 1465 during seven-day incubation on glucose- and sucrose-containing media. *N* is the number of cells per ml. Cultivation in the media containing glucose (*1, 3*, and *5*) and sucrose (*2* and *4*).

Fig. 1). EPS solution viscosity increased in the course of cultivation. Glucose substitution with sucrose in the media led to a decrease of EPS preparation viscosity by a factor of 3 (1.4 instead of 4.5 mm^2/s).

Gel chromatography on a column calibrated with dextrans showed that the EPS_{Glu} preparation was heterogeneous and contained a mixture of polysaccharides of various molecular mass from 7×10^4 to 2×10^6 Da. The predominant fraction corresponded to the range from 5×10^5 to 2×10^6 Da (fraction *1* at Fig. 2). Two smaller peaks were eluted in the ranges of 100–200 kDa (peak *2*) and 20–70 kDa (peak *3*).

Ion exchange chromatography of total EPS_{1465} preparations on a DEAE-Toyopearl 650M column (35 × 1.5 cm) revealed their heterogeneity in terms of charge (Fig. 3a). Ion exchange chromatography resulted in separate fractions of polysaccharide-containing polymers, varying in negative charge density and designated as neutral and acidic ones. Neutral and weakly acidic components were eluted with Tris–HCl buffer (0.01 M, pH 7.2) and acidic ones with NaCl solution in the same buffer, with the salt concentration continuously increasing from 0.01 to 1.0 M. As follows from the data presented in Fig. 3a (curve *2*), total EPS_{Glu} preparations were composed of a minor (neutral) and a major (acidic) fractions. When the carbon source was replaced with sucrose, the EPS contained predominantly the neutral fraction (curve *1*), and 0.1% water solutions, as was mentioned above, were less viscous compared to those isolated from bacteria grown on glucose (Fig. 1, curves *3* and *4*). Thus, increase of the contribution of the neutral fraction, with lower molecular weight and viscosity, in the total EPS composition caused reduction of kinematic viscosity of the bacterial EPS solutions.

PAGE-SDS separation data confirmed the heterogeneity of the EPS preparations. As follows from Fig. 3b, preparations of EPS synthesized by bacteria on both sucrose (track *1*) and glucose (track *2*) are represented by a wide range of molecules of various size. EPS preparations obtained from *P. polymyxa* 1465 grown on different carbon sources were characterized by similar electrophoresis profiles, typical for polysaccharide-containing polymers. The observed differences apparently were due to the different ratio of molecules of various size and electrophoretic mobility in the EPS under study. For example, the presence of two distinct bands in the top part of the EPS_{Suc} electrophoresis picture indicates that the least mobile molecules were predominant in the preparation. Molecules with higher mobility were more abundant in the EPS_{Glu} preparations and formed an intense band in the middle part of the electrophoresis picture. Identification of the whole complex

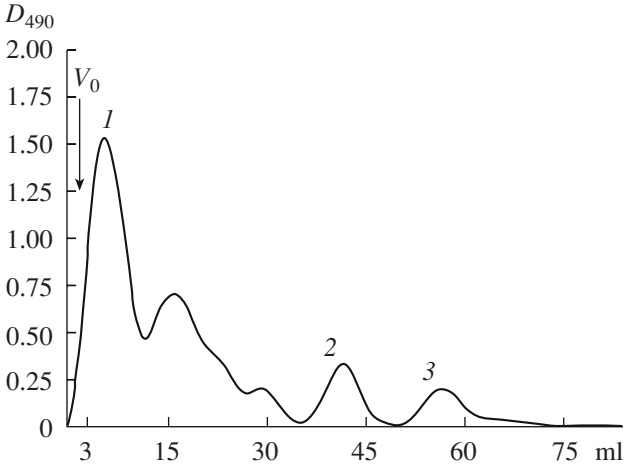

Fig. 2. Gel filtration elution profile of *P. polymyxa* 1465 EPS registered on a Sepharose CL-4B column (46 × 2.5 cm), detection of carbohydrates. Fraction *1*, molecular mass of 5×10^5 to 2×10^6 Da; fraction *2*, molecular mass of 100–200 kDa; fraction *3*, molecular mass of 20–70 kDa.

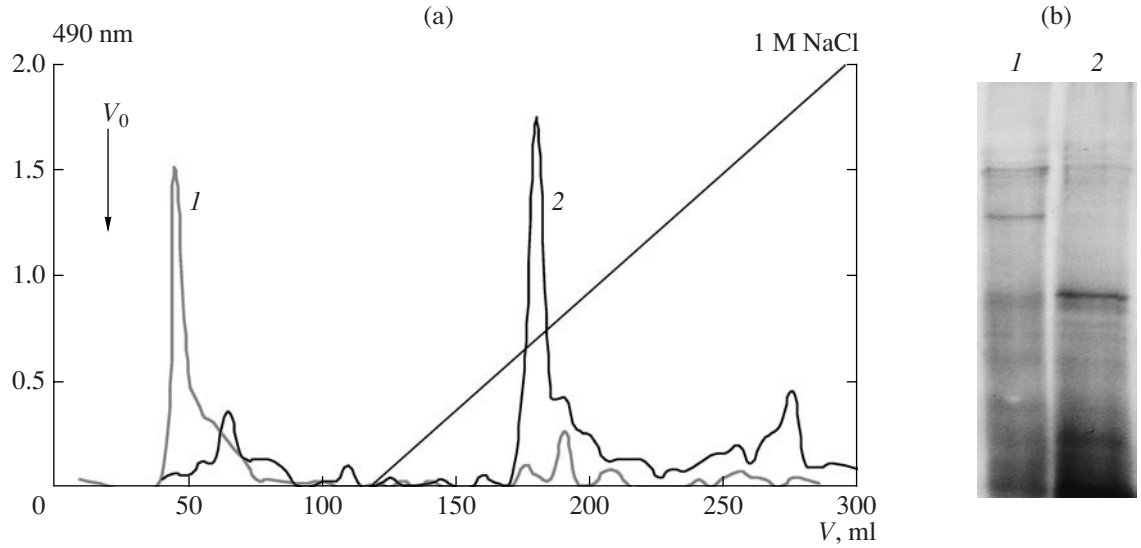

Fig. 3. Ion exchange chromatography on a DEAE-Toyopearl 650M column, 35 × 1.5 cm (a) and PAGE analysis (b) data obtained for EPS preparations of *P. polymyxa* 1465 grown in media containing glucose (*1*) and sucrose (*2*).

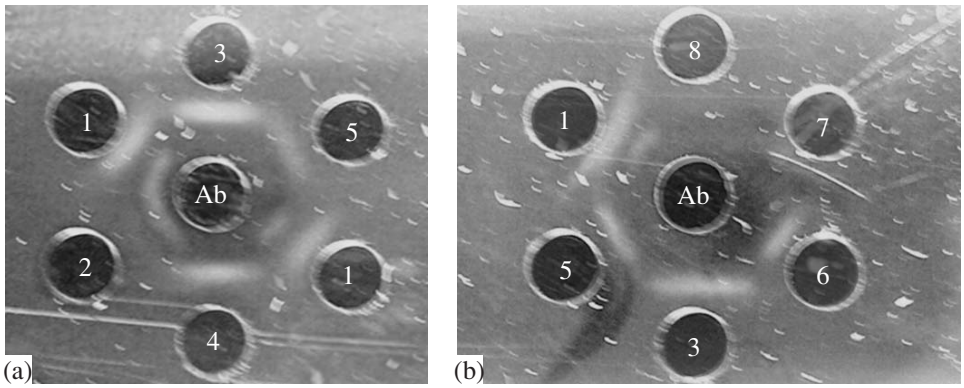

Fig. 4. Results of a comparative immunodiffusion analysis of EPS preparations isolated from various *P. polymyxa* strains, grown in media containing glucose (1–5) and sucrose (6–8): 1465 (1, 6), 1460 (2, 7), 92 (3, 8), 1459 (4), and 88A (5). Ab against EPS isolated from *P. polymyxa* grown on glucose-containing medium were used to detect antigens.

of polysaccharides in the EPS of strain 1465 is in accordance with literature data for a number of different *P. polymyxa* strains [7, 8, 27].

Analysis of the chemical composition of *P. polymyxa* 1465 EPS showed that the preparation contained 72% carbohydrates and 1.6% protein. Alditol acetates GLC analysis on complete acid hydrolysis of EPS_{Glu} polysaccharides revealed glucose, mannose, and galactose in the ratio 2 : 2 : 1, which changed depending on the carbon source in the medium. Uronic acids were revealed in the preparations by TLC and the Dische colorimetric method. Preliminary TLC and amino acid analysis data suggest the presence of galactosamine in the complexes under study.

To evaluate the relation of *P. polymyxa* 1465 EPS to the exopolysaccharides of other strains of this species, comparative immunochemical analysis was used. Rabbits were immunized with the total $EPS_{1465Glu}$ preparation to yield polyclonal antibodies (Ab). In spite of literature data, implying rather low immunogenicity of the compounds of this kind [27], we managed to obtain highly immunogenic EPS preparations; the Ab-specific antibodies titer in the antiserum against EPS preparation determined by the immunodiffusion reaction was 1 : 128. Two precipitation bands were formed by $EPS_{1465Glu}$ with Ab (Figs. 4a and 4b, well 1), which indicated the presence of two fractions with different antigen properties. The intensity of precipitation bands formed by the antigen varied and was higher for the band corresponding to the acidic fraction of the $EPS_{1465Glu}$.

We used Ab to study immunochemical properties of eight EPS preparations isolated from five *P. polymyxa* strains cultivated in various media (Fig. 4). An interaction was revealed between Ab and EPS of strains 1460, 92, 1459, and 88A when grown on glucose (Figs. 4a and 4b; wells 2, 3, 4, and 5). Cross reactivity was observed for one of the two precipitation bands characteristic of strain 1465, namely, the one corresponding to the acidic fraction. Arrangement of the bands formed by different EPS suggests partial identity of their antigen determinants (Fig. 4). It is important to say, that, particularly in the presence of glucose, strains 1465 (Fig. 3a, curve *2*), 92 (data not shown), 88A, and 1459 [8, 27] produce EPS with significantly dominating acidic fractions.

No interaction was detected between the antibodies and EPS produced in sucrose-containing media (Fig. 4b, wells 7 and 8). $EPS_{1465Glu}$, which formed a weak precipitation band with the antibodies (Fig. 4b, well 6), was an exception. The most pronounced precipitation band was formed by $EPS_{1465Glu}$ (Fig. 4a, well 2), in contrast to all other strains grown on glucose. The strain differed from the other tested ones in its low EPS yield, low (at background level) viscosity of EPS solutions, and significant predominance of neutral fractions when grown on both glucose and sucrose. Results of immunochemical analysis suggest the absence of expressed strain specificity of EPS antigen determinants within the species *P. polymyxa*. Besides, determinants characteristic of acidic fractions of the studied EPS are predominantly synthesized in glucose media, while neutral ones are produced mostly in sucrose media. The pattern of immunodiffusion precipitation bands corresponding to the acidic fraction of the EPS under study correlates with viscosity coefficients of the EPS water solutions.

To conclude, the batch culture of *P. polymyxa* 1465 produces EPS heterogeneous in molecular mass and charge; cultivation conditions influence the fraction composition and rheological and antigenic properties of the secreted EPS. The presence of glucose, mannose, galactose, and uronic acids in the EPS composition is typical of *P. polymyxa*. The results obtained in the study widen the picture of composition and property variations in *P. polymyxa* exoglycans. Antibodies against the EPS may be used in ecological studies for testing natural *P. polymyxa* isolates.

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