Curr Microbiol (2011) 62:1554–1559
DOI 10.1007/s00284-011-9896-2

Biofilm Formation by *Paenibacillus polymyxa* Strains Differing in the Production and Rheological Properties of Their Exopolysaccharides

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Received: 20 December 2010 / Accepted: 3 February 2011 / Published online: 18 February 2011

Abstract We evaluated the ability of several strains of the rhizobacterium *Paenibacillus polymyxa*, differing in the yield and rheological properties of their exopolysaccharides, to form biofilms on abiotic surfaces. Of these strains, *P. polymyxa* 1465, giving the highest yield of extracellular polysaccharides and the highest kinematic viscosity of the culture liquid and of aqueous polysaccharide solutions, proved to be the most active in forming biofilms on hydrophobic and hydrophilic surfaces. Enzyme-linked immunosorbent assay with rabbit polyclonal antibodies developed to isolated exopolysaccharides of *P. polymyxa* 1465 and 92 was used to detect *P. polymyxa*'s polysaccharidic determinants in the composition of the biofilm materials.

Introduction

In natural ecosystems and at industrial and healthcare facilities, microorganisms exist not as free-living cells suspended in their environment (plankton) but mainly as an organized community attached to various biotic and abiotic surfaces. Such communities are called biofilms [5]. The development of biofilm communities is a major strategy used by bacteria for survival in the external milieu. In biofilms, bacteria are stuck to each other by complex intercellular linkages and are functionally similar to multicellular organisms [12]. Usually, cells in biofilms are embedded in an extrapolymeric matrix that ensures biofilm stability and safety from external stresses [2, 18]. The matrix is formed from a mixture of components, including exopolysaccharides (EPSs), proteins, nucleic acids [25], glycosyl phosphate-containing biopolymers (e.g., teichoic acids), glycoproteins, and (in certain bacteria, e.g., bacilli) polyglutamic acid and other biopolymers [1, 15]. A key structural component of biofilms, which has received close attention in the past decade, is the extracellular polymeric substance called the exopolysaccharide matrix [18, 20, 24]. In different bacterial species, this matrix differs in physical properties and chemical composition; as a rule, however, it is an anionic polymer. The EPS of the matrix consists mostly of homo- and heteropolysaccharides. By now, the EPS composition of several bacteria has been identified [3, 4, 11, 13]. Possibly, EPSs play various roles (depending on the environmental conditions) in the structure and functions of biofilm communities [12].

The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* can penetrate root tissues [17] and can colonize plant roots with the formation of biofilms [22]. This ability is of decisive importance for improving plant resistance to biotic and abiotic stresses [10, 23]. *P. polymyxa* exoglycans, synthesized in large amounts, are thought to be important for colonization by the bacteria of the roots of their plant partner [9, 22].

Previously, we investigated the ability of *P. polymyxa* strain 1465, which produces high-molecular-weight exoglycans, to attach to the roots of wheat seedlings. We showed that the use of enzyme-linked immunosorbent assay (ELISA) with anti-EPS antibodies (Abs) offers promise for the evaluation of *P. polymyxa* colonization of wheat roots [27]. Here we examine the formation of *P. polymyxa* biofilms on hydrophobic and hydrophilic surfaces, and we establish the extent of involvement

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in this process of the exoglycans synthesized by these bacteria.

Materials and Methods

Strains and Growth Conditions

Paenibacillus polymyxa 1465 (ATCC 8523) and 1460 (ATCC 1041) were obtained from a culture collection held in Brno (Czech Republic). *P. polymyxa* 92 (VNIISHM 92) was isolated from wheat roots by Dr. Yu.M. Voznyakovskaya (All-Russia Research Institute for Agricultural Microbiology, Russian Academy of Agricultural Sciences, Pushkin-8, St. Petersburg, Russian Federation). Bacteria were grown with rotary shaking (220 rpm) at 30°C for 2 days in a liquid nutrient medium of the following composition (g l^{-1}): yeast extract, 4; Na_2HPO_4, 1.1; KH_2PO_4, 0.5; $MgSO_4 \times 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.1; $CaCO_3$, 0.2; glucose (or sucrose), 30; distilled water, up to 1 l (pH 7.2–7.5).

Azospirillum lipoferum type strain Sp59b [21] was grown with rotary shaking (220 rpm) at 30°C for 24 h in Day and Döbereiner's [6] liquid synthetic malate medium modified to the following (g l^{-1}): KH_2PO_4, 2.0; K_2HPO_4, 3.0; $CaCl_2$, 0.02; NaCl, 0.1; $MnSO_4 \times H_2O$, 0.01; $Na_2MoO_4 \times 2H_2O$, 0.002; $MgSO_4 \times 7H_2O$, 0.2; NH_4Cl, 2.5; malic acid, 5.0; distilled water, up to 1 l. The pH of the medium was brought to 6.8–7.0 with 20% NaOH. Before being sterilized, the medium was supplemented with 1 ml of a vitamin solution (thiamine, 0.4 g l^{-1}; biotin, 0.4 g l^{-1}; pyridoxal, 0.2 g l^{-1}) and 10 ml of an iron chelate solution ($FeSO_4 \times 7H_2O$, 2.0 g l^{-1}; nitrilotriacetic acid, 5.6 g l^{-1}).

EPS Isolation

The total EPSs of *P. polymyxa* were isolated as described by Yegorenkova et al. [27] and were lyophilized in a BENCHTOP 2 K lyophilizer (VirTis, NY, USA). For measurement of the intrinsic viscosity of aqueous polysaccharide solutions, the EPSs were suspended in distilled water and 0.1% solutions were made. The kinematic viscosity of the culture liquid and the EPS solutions was determined with an Ostwald capillary viscosimeter (Type VPZh-2, inside capillary diameter of 0.73 or 0.99 mm) thermostatted in a 20°C water bath (accuracy of ±1°C).

Ion-exchange chromatography was done on a DEAE-Toyopearl 650 M anion-exchange column (35 × 1.5 cm, $V_0 = 40$ ml). Neutral and weakly acidic components were eluted with Tris–HCl (0.01 M, pH 7.2), and acidic components were eluted with a solution of NaCl in the same buffer with a continuous concentration gradient of 0.01–1.0 M. The resultant fractions were concentrated by rotary vacuum evaporation (40°C) and were analyzed.

Evaluation of *P. polymyxa*'s Ability to Form Biofilm

The ability of the bacteria to form biofilm on abiotic surfaces (glass and polystyrene) was evaluated as recommended by Ferrieres and Clarke [8]. Cultures were grown for 24 h in the liquid nutrient medium containing 3% glucose (or sucrose), diluted 100-fold with sterile nutrient medium, and poured into small glass test tubes (1 ml per test tube) or into 96-well polystyrene plates (200 µl per well). The control test tubes and wells received only the nutrient medium. The cultures then were incubated unshaken at 30°C for 72–96 h. After incubation, the cultures were carefully withdrawn with a pipet and the test tubes (or plates) were washed twice with water. Each test tube received 2 ml, and each well received 200 µl, of 1% (w/v) crystal violet, and the test tubes and plates were allowed to stand for 15 min at room temperature. The solution then was withdrawn with a pipet, and the test tubes (or plates) were carefully washed with water. Dye that had bound to the cells adsorbed on the walls of the test tubes and the wells was dissolved into acetone–ethanol (20:80; 2.5 ml for the test tubes and 200 µl for the plates). Biofilm-forming ability was evaluated by the intensity of the solution color. For the plates, the absorbance (A_{570}) was read on a Multiskan Ascent analyzer (Thermo, Finland); for the test tubes, the absorbance (A_{590}) was read on a Specol 221 spectrophotometer (Carl Zeiss, Germany).

Production of Abs

Polyclonal Abs were raised against the isolated EPSs of glucose-grown *P. polymyxa* 1465 (EPS_{1465}) and 92 (EPS_{92}). Rabbits were immunized three times at 2 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 1:1 (v/v) with Freund's complete adjuvant, and for the subsequent injections, Freund's incomplete adjuvant was used. Blood was taken 1 week after the last immunization. The immunoglobulin G fractions were obtained from the antisera by ammonium sulfate precipitation followed by dialysis against phosphate-buffered saline.

Indirect ELISA

This was performed in 96-well enzyme immunoassay plates. ELISA of the formed biofilms was conducted in the plate wells after the bacteria had been cultivated in them. After removing bacterial suspensions, we placed 100 µl of 0.05% polyethylene glycol 20000 (PEG) in each well to block free binding sites on polystyrene. This solution was replaced by 50 µl of the appropriate primary Abs (EPS_{1465} or EPS_{92} Abs) diluted in PBS–0.02% Tween 20–0.005%

PEG (for prevention of nonspecific Ab sorption). After incubation for 40–60 min, the wells were washed three times with 100 µl of PBS–0.02% Tween 20, and 50 µl of peroxidase-labeled goat antirabbit Abs (Sigma, USA; 2 µg ml^{-1}) in PBS–0.02% Tween 20–0.005% PEG was placed in each well. After 40-min incubation, the wells were washed twice and peroxidase activity was estimated by adding to each well 50 µl of a substrate mixture of 0.03% *o*-phenylenediamine and 0.02% hydrogen peroxide in 0.1 M sodium citrate buffer (pH 4.5). The enzyme reaction was stopped with 100 µl of 1 N H_2SO_4. The absorbance (A_{490}) was read on the Multiskan Ascent analyzer. EPS_{1465} Ab ELISA of the EPSs of several glucose- or sucrose-grown *P. polymyxa* strains was run in the same way. Data were processed with Excel 2003 software (Microsoft Corp., USA); 95% confidence intervals are given.

Results and Discussion

Evaluation of *P. polymyxa*'s Ability to Form Biofilm

We first selected incubation conditions for *P. polymyxa* biofilm formation at the boundary between the liquid and the solid surface. When cultures were grown shaken in liquid medium under standard growth conditions, no biofilm was observed. Under unshaken conditions, strains 1465 and 92 developed complete biofilms on glass (test tubes and petri dishes; Fig. 1a, b) and plastic (polystyrene plates; Fig. 1c) surfaces at 72–96 h. A less distinct biofilm was produced by *P. polymyxa* 1460.

Figure 2 shows the results of evaluation of *P. polymyxa*'s biofilm-forming ability, which were obtained by staining the bacteria with crystal violet. The ability to attach to the hydrophobic and hydrophilic surface decreased in the order strain 1465 > strain 92 > strain 1460, regardless of whether the bacteria had been grown with glucose or with sucrose (Fig. 2). This difference was more noticeable on the hydrophilic surface. A large body of research shows that biofilm formation depends on the physical–chemical properties of the colonized material. Factors affecting biofilm development include the hydrophilic–hydrophobic, electrostatic, and bactericidal properties of the surface and the ratio between them [14, 19]. For example, from adsorption studies and image analysis, Deo et al. [7] found that the adhesion of *P. polymyxa* to hematite and corundum was greater than that to quartz. On the basis of Fourier-transform infrared spectroscopy studies conducted with *P. polymyxa* and the three mineral samples, those authors concluded that the electrostatic forces play a major role in the case of hematite and corundum, whereas in the case of quartz, chemical forces are possibly the

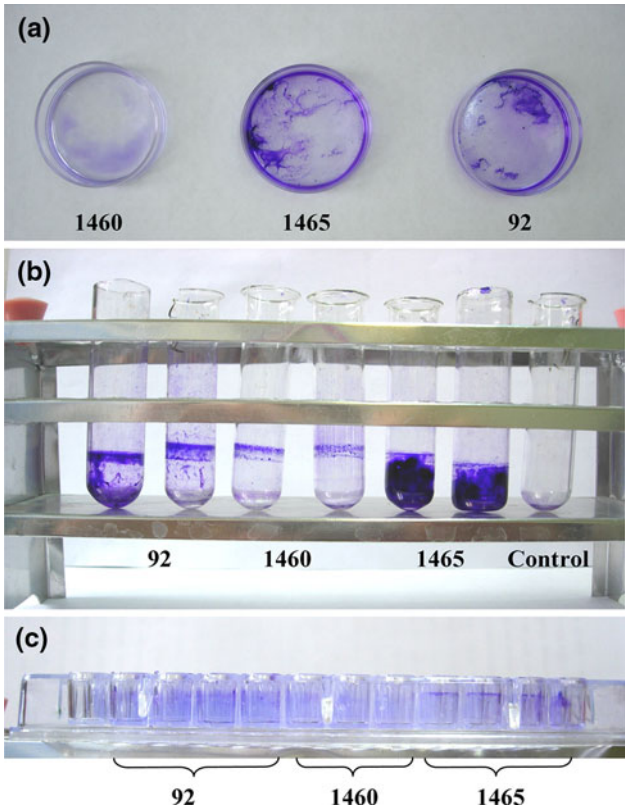

Fig. 1 Crystal violet staining of biofilms formed by *P. polymyxa* 92, 1460, and 1465 on hydrophilic (**a**, **b**) and hydrophobic (**c**) surfaces

governing factor. They also concluded that proteins are specifically adsorbed on quartz, whereas polysaccharides are adsorbed on hematite and corundum.

We found that strain 1460 differed essentially from the other biofilm-producing strains. It showed a lower EPS yield and lower kinematic viscosity, which may account for its decreased ability to form biofilms. For example, when

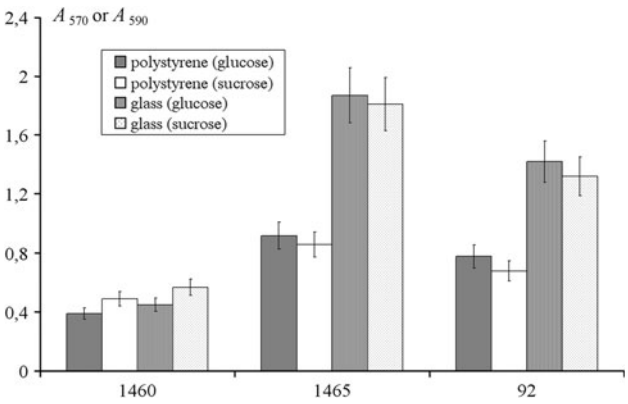

Fig. 2 Evaluation of the ability of *P. polymyxa* 92, 1460, and 1465 to form biofilms on hydrophilic and hydrophobic surfaces by using crystal violet staining. A_{570} is the absorbance of samples in polystyrene plates, and A_{590} is the absorbance of samples in glass test tubes

bacteria were grown with glucose, the viscosity of 0.1% EPS (EPS_{GL}) solutions averaged 4.5 $mm^2\ s^{-1}$ for strain 1465 and was not greater than 1.3 $mm^2\ s^{-1}$ for strain 1460. On the average, the production of *P. polymyxa* 1460 EPS (EPS_{1460}) was three times lower than that of EPS_{1465} for both glucose- and sucrose-grown bacteria. It should be borne in mind that for glucose-grown strains 1465 and 92, high-molecular-weight acidic fractions were essentially predominant (Fig. 3a), whereas for strain 1460, the predominance of a neutral fraction was observed in both sucrose- and glucose-supplemented media (Fig. 3b). The elution profiles for EPS_{1465} and EPS_{92} were similar; therefore, we present one chromatogram for both (Fig. 3a). When *P. polymyxa* was grown with glucose and sucrose, the culture liquid viscosity was approximately the same for both strain 1465 and strain 92. The reason is that despite the predominance of a neutral fraction in the sucrose-supplemented medium, the yield of the polysaccharide increases considerably, leading to high viscosity. Earlier [26], it was found that when the carbon source in the growth medium was changed from glucose to sucrose, biopolymer yield in *P. polymyxa* 1465 increased from 2.1 to 12.3 g l^{-1} of EPS in glucose and sucrose, respectively.

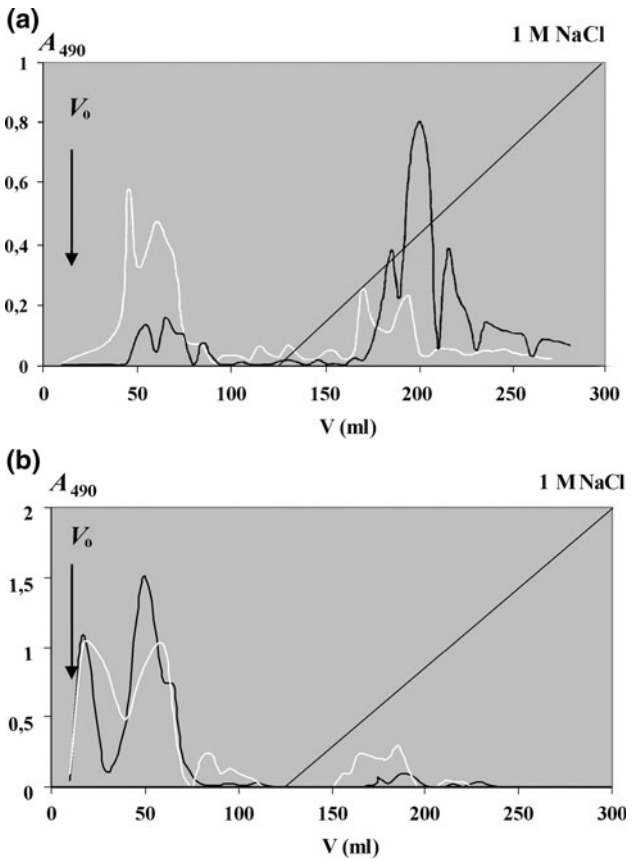

Fig. 3 Ion-exchange chromatography (DEAE-Toyopearl 650 M, column size of 35 by 1.5 cm) of the EPSs of *P. polymyxa* 92 (**a**) and 1460 (**b**) grown with sucrose (*light curve*) and glucose (*dark curve*)

An important addition to what has been said is that strain 92 was isolated from the roots of wheat and that strain 1465, on our evidence, was active in colonizing the roots of 3-day-old wheat seedlings in model experiments [27].

Use of ELISA to Study Biofilms

Several investigators consider the exopolysaccharide matrix to be one of the principal structural components of biofilms. More specifically, Smirnova et al. [19] found by cytochemical studies that the matrix of biofilms developed by *Salmonella typhimurium* includes acidic mucopolysaccharides, revealed by alcian blue staining, and cellulose, stained with Congo red. The involvement of lipopolysaccharides and calcofluor-binding polysaccharides in the structural organization of *Azospirillum* biofilms was established by Sheludko et al. [16]. According to the data of Timmusk et al. [22], the extracellular polysaccharides of *P. polymyxa* participate in biofilm formation on the roots of *Arabidopsis thaliana*.

In order to evaluate the contribution of *P. polymyxa* EPSs to biofilm development, we used ELISA with rabbit polyclonal Abs raised by us against isolated EPSs of *P. polymyxa* 1465 and 92 (Abs_{1465} and Abs_{92}). Prior to this, we had evaluated the antigenic properties of the EPSs from *P. polymyxa* 92, 1460, and 1465 grown in different media, by using ELISA with Abs_{1465}. It was found that of the preparations compared, EPS_{1465} and EPS_{92} were the most closely related and had almost the same number of common antigenic determinants in their acidic fractions (Fig. 4). Abs_{1465} interacted very weakly with EPS_{1460GL}, and the ELISA values for $EPS_{1460SUC}$ were close to the control ones, in good agreement with the earlier immunodiffusion results [26]. Therefore, we subsequently used only strains 1465 and 92 for ELISA.

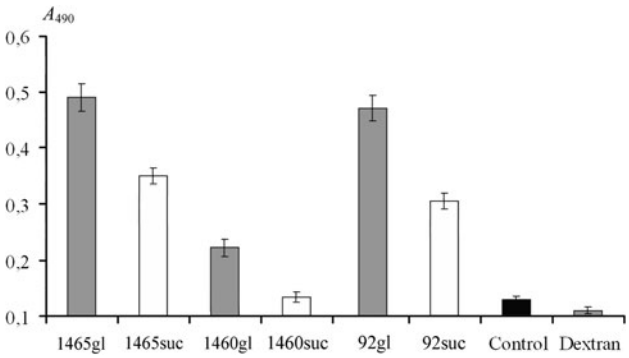

Fig. 4 ELISA of the EPSs of glucose- and sucrose-grown *P. polymyxa* 92, 1460, and 1465 by using Abs_{1465} (concentration of 100 μg ml^{-1})

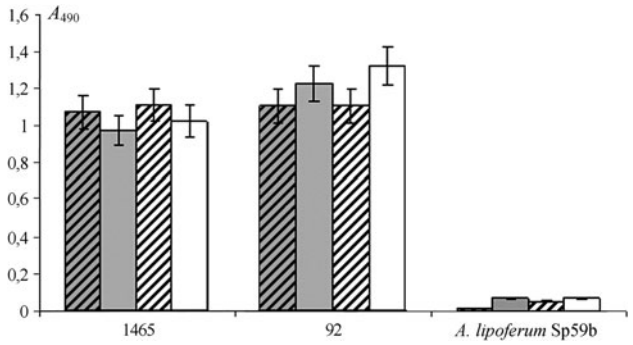

Fig. 5 Revelation of polysaccharidic antigens in *P. polymyxa* biofilms on hydrophobic surfaces by using ELISA with Abs_{1465} and Abs_{92}. *Gray bars*, growth with glucose; *white bars*, growth with sucrose; *hatched bars*, Abs_{1465}; *unhatched bars*, Abs_{92}

By ELISA, we revealed specific EPS determinants of *P. polymyxa* in the composition of the biofilm materials. The level of revealing such determinants in the biofilms produced by glucose- and sucrose-grown strains 1465 and 92 was quite high, and the differences between the strains were slight (Fig. 5). The level of Ab interaction with the biofilm of *A. lipoferum* Sp59b (control) was close to the background values.

Abs_{1465} and Abs_{92} interacted with the common antigens of the biofilms developed by strains 1465 and 92. This finding confirms the earlier conclusion that there is no pronounced strain specificity of the EPS antigenic determinants within the *P. polymyxa* species [26].

Analysis of the obtained results makes it possible to conclude that the exoglycans of *P. polymyxa* are involved in biofilm formation by these bacteria on solid hydrophobic and hydrophilic surfaces. Because *P. polymyxa* has been reported to develop biofilms in the process of root colonization [22], it is evident that the production of extracellular polysaccharides can also be conducive to successful bacterial colonization of plant roots.

Acknowledgments Thanks are due to Mr. Dmitry N. Tychinin (this institute) for translating the original manuscript into English.

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