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EXPERIMENTAL ARTICLES

The Effect of Mutations Affecting Synthesis of Lipopolysaccharides and Calcofluor-Binding Polysaccharides on Biofilm Formation by *Azospirillum brasilense*

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Abstract—The thickness and antigenic properties of biofilms produced by *Azospirillum brasilense* Sp245 and its mutants deficient in the synthesis of lipopolysaccharides (Lps) and calcofluor-binding polysaccharides (CBPS) at the interface between water and hydrophilic or hydrophobic solid surfaces were compared. The mutants deficient in acidic LpsI synthesis produce thicker biofilms on hydrophilic surfaces. Biofilms produced on hydrophobic surfaces by bacteria that are unable to synthesize CBPS are less pronounced. Defects in CBPS production in *Azospirillum* mutants with impaired flagellar motility can cause adverse effects on the cell ability to attach to hydrophobic and hydrophilic surfaces. The loss of the neutral LpsII antigen by the mutants capable of producing CBPS does not affect their behavior on hydrophobic surfaces, which is probably due to the compensatory increase in the total polysaccharide production. The fundamental change in the Lps structure correlates with the activation of biofilm formation by the relevant mutants on hydrophilic and hydrophobic surfaces.

Key words: Azospirillum brasilense, biofilms, lipopolysaccharides, mutations, antibodies, calcofluor.

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In natural habitats, biofilms, i.e., spatially and metabolically structured communities of microorganisms embedded in an extracellular polymeric matrix and located at the interface between two different phases, are the main form of existence of microorganisms [1]. For the gram-negative bacterium *Azospirillum brasilense*, an inhabitant of plant rhizosphere and many other ecological niches, both the specific characteristics of biofilm formation and the relative role of various surface polymers are poorly studied.

Lipopolysaccharides (Lps) are the dominant surface structures of the members of the genus *Azospirillum*. Strain *A. brasilense* Sp245 synthesizes LpsI containing an acidic O-specific polysaccharide (O-PS) and LpsII containing a neutral O-PS. Both O-PS are homopolymers consisting of D-rhamnose residues [2]. The use of polyclonal antibodies against the Lps of strain Sp245 revealed a common epitope shared by LpsI and LpsII, as well as an additional epitope of LpsII [3]. Members of the genus *Azospirillum* are also characterized by the production of surface polysaccharides (CBPS) that bind calcofluor, a vital fluorescent dye (Cal$^+$ phenotype) [2]. The absence of fluorescence in bacterial colonies grown on calcofluor-containing media (Cal$^-$ phenotype) is a convenient benchmark for selection of the mutants defective in their ability to synthesize relevant polysaccharides. The calcofluor-binding polysaccharides of *A. brasilense* are represented by exopolysaccharides (EPS) and capsular polysaccharides (CPS) [2]. The antigenic identity of Lps, EPS, and CPS of *A. brasilense* Sp245 was demonstrated [4].

The goal of the present work was to study the effect that several classes of *cal* and *lps* mutations exert on the properties of biofilms produced by *A. brasilense* at liquid phase–solid hydrophilic and liquid phase–solid hydrophobic surface interfaces.

MATERIALS AND METHODS

Bacterial strains. Biofilm formation was studied in *A. brasilense* Sp245 [5] and its mutants in Lps and CBPS synthesis [3, 6]. Strain Sp245.5, the spontaneous Cal$^-$ LpsI$^-$ LpsII$^-$ mutant, is a derivative of strain Sp245 (RP4) that lost RP4 and the 85- (p85) and 120-MDa (p120) resident plasmids [6]. The mutants KM252 (Cal$^-$ LpsI$^-$), KM127, KM134, KM348 (LpsI$^-$), KM139 (LpsII$^-$), and KM018 (Cal$^-$ LpsII$^-$ Mot$^-$ Swa$^-$, with the paralyzed polar and lateral flagella) contain a single omegon (artificial transposon) insertion in four different p120 loci [3]. To obtain antibodies against the

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polysaccharide and protein antigens of the cell surface (see below), strains *A. brasilense* Sp245 [5] and Cd [7] were used.

Assessment of the bacterial ability to form biofilms. The ability to form biofilms was studied according to the recommendations stated in [8]. During our preliminary experiments, we selected the optimal temperature, incubation time, and the composition of the nutrient medium (potato agar, malate- and glycerol-containing salt media [9], and LB [10] were tested) in order to obtain biofilms of wild type *A. brasilense* firmly attached at the interface between the liquid phase and hydrophilic or hydrophobic solid surfaces. Cultivation for 96 h in LB medium at 28°C resulted in Sp245 biofilms most suitable for analysis.

Overnight bacterial cultures grown in LB medium were diluted 100-fold with sterile medium. The aliquots (200 μl and 1 ml) of these suspensions were dispensed into polystyrene 96-well plates and into 5-ml glass test tubes, respectively, and incubated without agitation at 30°C for 96 h. Planktonic bacteria were then removed by aspiration. The plate wells and test tubes were carefully washed with water, supplemented with a 1% aqueous solution of crystal violet, and incubated at room temperature for 10 min. The solution was then removed, and the plates and test tubes were washed with water. In order to assess the biomass yield, the bound dye was dissolved in 200-μl (plate wells) or 2.5 ml (tubes) of an acetone : ethanol mixture (20 ml : 80 ml). Optical density of the solution was measured in plates as A_{570} using an AIF-Ts-01S plate reader (ILIP Corp., Russia) or in test tubes as A_{590} using a KFK-2 photocolorimeter (Russia).

Determination of the relative surface hydrophobicity of bacterial cells. The relative surface hydrophobicity of bacterial cells was determined by salting-out [11]. The wells of polystyrene plates were supplemented with 25-μl portions of phosphate buffer (PB, 2 mM, pH 6.8) with various $(NH_4)_2SO_4$ contents and with 25-μl aliquots of cell suspensions of overnight broth cultures grown in LB medium and washed with PB (A_{590} 1.2). The minimal concentration of ammonium sulfate at which bacterial cell aggregation occurred was determined [11]. The lower the salt concentration that promotes aggregation, the higher the cell hydrophobicity is. This phenomenon can be attributed to the lower density of negative charges (hydrophilic groups) or to the elevated density of hydrophobic sites on more hydrophobic surfaces [11].

Raising of antibodies. To perform immunization by injection of intact cells of strains *A. brasilense* Sp245 and Cd, cell suspensions with an optical density of A_{660} 0.5 (~10^9 cells/ml) were used. Rabbits were immunized every four weeks by multiple intracutaneous injections of 1-ml portions of the bacterial suspension mixed 1 : 1 with complete Freund's adjuvant. Antibodies (Ab 1) against the Lps of *A. brasilense* Sp245 were raised as described previously [12].

Solid-phase indirect enzyme immunoassay (EIA) [13] was performed on polystyrene 96-well plates. To detect the strain-specific polysaccharide antigens, antibodies against the Lps of Sp245 (Ab 1) were used. To detect the surface protein antigens, polyclonal antibodies raised against *A. brasilense* Cd intact cells (Ab 2) were used. The Ab2 antibodies that recognize the whole pool of the surface Cd antigens interact with the proteins of strain Sp245 rather than with its polysaccharide determinants [12]. The concentrations of both Ab 1 and Ab 2 (50 μg/ml) were the same in all experiments. Horseradish peroxidase-conjugated goat anti-rabbit antibodies (Sigma, United States) (1 μg/ml) served as secondary antibodies.

The enzyme immunoassay of biofilms formed on polystyrene plates was performed in plate wells after cultivation. After removal of bacterial suspensions, the wells were treated with 100-μl aliquots of a 0.05% polyethylene glycol 2000 (PEG-2000) solution in order to block the free sites on polystyrene plates. The blocking solution was removed after 40 min. Then, 50-μl portions of the relevant antigen-recognizing antibodies dissolved in the phosphate buffered saline (PBS; pH 6.8) with 0.02% Twin-20 and 0.005% PEG-2000 (PBS-TP), were added. After 40–60 min of incubation, the wells were washed three times for 15 min with PBS containing 0.02 % Twin-20, supplemented with 50-μl portions of peroxidase-labeled anti-rabbit antibodies in PBS-TP, and incubated for 40 min. The wells were washed twice for 10 min and supplemented with 50-ml portions of the substrate in order to assess the peroxidase activity. The substrate consisted of 0.03% *o*-phenylenediamine and 0.02% hydrogen peroxide dissolved in 0.1 M sodium citrate buffer (pH 4.5). The optical density (A_{490}) was measured using an AIF-Ts-01S plate reader.

Similarly, the enzyme immunoassay of bacterial cultures grown in the liquid LB medium was carried out. In the latter case, the plates supplemented with 50-μl portions of the tested bacterial suspensions were agitated on a vibration shaker at room temperature for 30 min and then incubated without agitation at 4°C overnight (18 h).

Statistical analysis of the obtained quantitative results was performed using Microsoft Excel 2003 (11.6355.6360) SP1 software package. The confidence intervals were calculated for a significance level of 95%.

RESULTS AND DISCUSSION

Relative hydrophobicity of the cell surface of *A. brasilense* Sp245 and its CBPS and Lps mutants. It is well known that a number of physicochemical factors affect the initial stages of bacterial attachment to solid surfaces [1]. For example, hydrogen bonds and electrostatic interactions are the most essential factors for bacterial adsorption to hydrophilic surfaces,

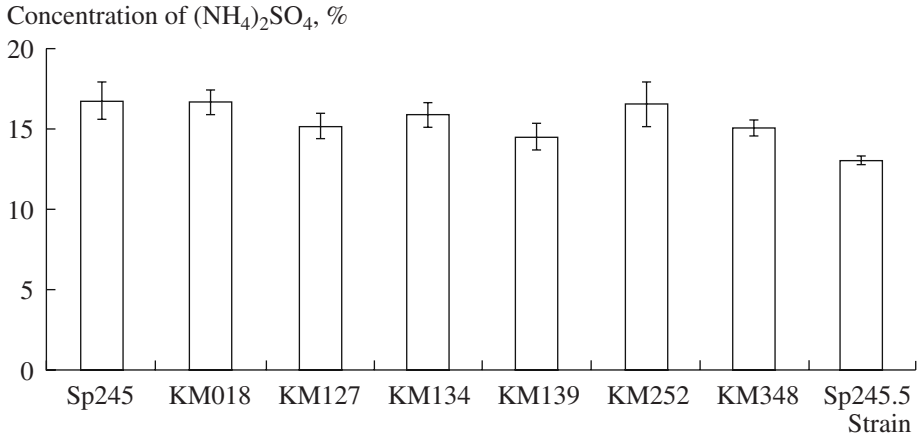

Fig. 1. Relative hydrophobicity of the surfaces of planktonic *A. brasilense* cultures grown in LB medium for approximately 20 h determined by the salt aggregation test method.

whereas hydrophobic interactions govern bacterial adsorption to hydrophobic surfaces.

In the present work, we assessed the total relative hydrophobicity of planktonic cells of *A. brasilense* Sp245 and its mutants incubated in liquid LB medium at 28°C for about 20 h. No differences in the hydrophobicity of the studied *Azospirillum* strains (with the exception of Sp245.5) grown under these conditions were revealed (Fig. 1). However, in the mutant *A. brasilense* Sp245.5, drastic changes in the Lps structure (as well as the loss of CBPS) [4] resulted in a small but statistically reliable increase in the cell surface hydrophobicity. This is demonstrated by a decrease in the minimal concentration of ammonium sulfate that causes Sp245.5 cell aggregation (Fig. 1).

It seems likely that the contribution of hydrophobic interactions to the attachment of the *A. brasilense* strains Sp245, KM018, KM127, KM134, KM139, KM252, and KM348 grown in the liquid LB medium to solid surfaces are almost identical. In the case of Sp245.5, initial interaction of cells with the hydrophobic surface possibly proceeds more rapidly as compared to other strains.

Evaluation of the thickness of bacterial biofilms formed on glass and plastic. After the first 24–48 h of incubation of bacterial cells in liquid LB medium, thin films formed in test tubes or plate wells. Microscopic observations revealed that these films consisted of individual cell aggregates (microcolonies) which could be easily removed by aspiration and washing. After 72–96 h of incubation, the microcolonies merged to form a solid, thick, and smooth biofilm.

The biomass contents in the biofilms produced by *A. brasilense* Sp245 and its mutants at the liquid phase (LB)–solid hydrophilic surface (glass) and liquid phase (LB)–solid hydrophobic surface (polystyrene) interfaces were determined by crystal violet staining of the studied cultures.

On the hydrophilic surface, *A. brasilense* strains KM127, KM134, KM348 (LpsI$^-$ mutants), and KM252 (Cal$^-$ LpsI$^-$ mutant) formed similar biofilms that were thicker than those produced by the wild type strain. This can be attributed to the change in the cell charge of the studied mutants due to the loss of acidic LpsI.

The strains Sp245, KM124, KM134, KM139, and KM348 did not differ with respect to the thickness of the films formed on hydrophobic solid surfaces, which corresponds to their similar hydrophobicity patterns.

The biofilms formed on polystyrene surfaces by strains KM018 and KM252 (Cal$^-$ LpsII$^-$ Mot$^-$ Swa$^-$ and Cal$^-$ LpsI$^-$ mutants, respectively) were thinner than those produced by other strains. Both these mutants are characterized by their inability to synthesize CBPS.

As compared to other strains, the ability of KM018 mutant with paralyzed flagella to form biofilms on glass surfaces was significantly lower (Fig. 2).

Biofilms formed by the Cal$^-$ LpsI$^-$ LpsII$^-$ mutant Sp245.5 with the drastically changed structure of the cell surface [6] were thicker than those formed by strain Sp245 and its omegon mutants (Fig. 2).

Comparative analysis of bacterial films by enzyme immunoassay. To analyze biofilms formed on polystyrene surfaces by *A. brasilense* Sp245 and its mutants, an indirect enzyme immunoassay using the antibodies against the polysaccharide (Ab 1; Fig. 3) and protein (Ab 2; Fig. 3) antigens of *Azospirillum* was performed.

The total levels of polysaccharide epitopes revealed in biofilms formed by strain Sp245, as well as by strains KM127, KM134, KM348 (LpsI$^-$ mutants), and KM252 (Cal$^-$ LpsI$^-$ mutant) on hydrophobic surfaces differed insignificantly (Fig. 3). At the same time, the interaction between planktonic cells of the LpsI$^-$ mutants and antibodies was less efficient, than in the case of the wild type strain (data not shown). Taking into consideration similar amounts of biomass in the films formed by Sp245, KM127, KM134, and KM348 (Fig. 2), one can

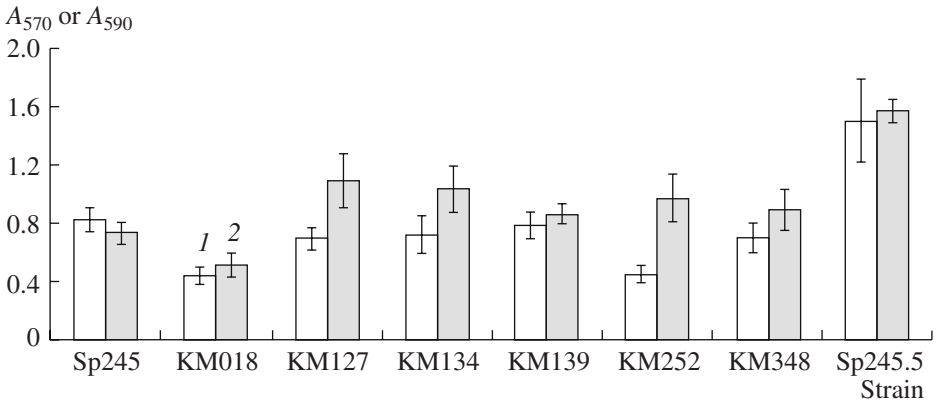

Fig. 2. Relative amount of biomass in the biofilms formed by *A. brasilense* strains at the (*1*) liquid LB–polystyrene surface (A_{570}) and (*2*) liquid LB–glass (A_{590}) interfaces, determined by crystal violet staining of the studied cultures.

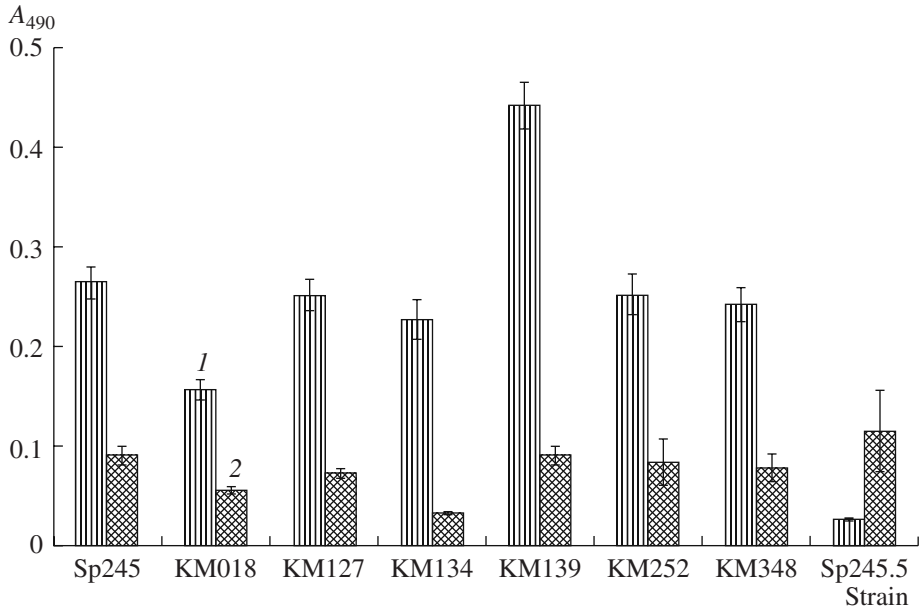

Fig. 3. Comparative analysis of polysaccharide and protein antigens in the biofilms formed by *A. brasilense* strains at the liquid LB–polystyrene surface interface carried out by indirect enzyme immunoassay using (*1*) antibodies against the Lps of strain Sp245 (Ab 1) or (*2*) antibodies against intact cells of strain Cd (Ab 2).

assume that the interaction of cells with a solid surface activates polysaccharide synthesis. In the case of KM252, it is obvious that polysaccharide production intensifies even more, since the biofilms produced by this mutant on polystyrene surfaces are thinner than those formed by other LpsI$^-$ mutants (Fig. 2).

The total amount of polysaccharide antigens produced by strain KM139 (LpsII$^-$ mutant) in broth culture (data not shown) and in biofilms is significantly higher than in other strains (Fig. 3).

Since the mutant Sp245.5 lipopolysaccharides hardly interact with Ab 1 [4], the indirect enzyme immunoassay was useless in this case. A weak positive reaction in Sp245.5 biofilms may be due to enhanced nonspecific sorption of antibodies in the folds of the matrix (a characteristic trait of this mutant).

The differences between strains Sp245, KM127, KM139, KM252, KM348, and Sp245.5 with respect to the production of protein antigens detected with Ab 2 in the biofilms produced by these strains are insignificant (Fig. 3). In the mutants KM018 and KM134, the amount of exposed protein antigens was lower than in the other strains (Fig. 3); however, in the case of KM018, this decrease correlated well with the smaller thickness of the biofilms formed by this strain (Fig. 2).

As a result of this work, it was established that the *A. brasilense* strains that lost the acidic polysaccharide antigen (LpsI) form thick biofilms on hydrophilic surfaces. Biofilms formed on solid hydrophobic surfaces by the mutants deficient in CBPS synthesis are thinner. Defects in CBPS production by *Azospirillum* strains with impaired flagellar motility can cause adverse effects on the cell ability to attach to hydrophobic and hydrophilic surfaces. The loss of the neutral LpsII antigen by the mutants capable of producing CBPS does not affect significantly their behavior on hydrophobic surfaces, which is probably due to the compensatory increase in the total polysaccharide production. A drastic change in the structures of polysaccharide antigens resulting in the production of Lps which are more heterogeneous in length correlates well with the activation of the biofilm formation on various solid surfaces.

Thus, lipopolysaccharides and calcofluor-binding polysaccharides contribute to the structural organization of biofilms formed by the *Azospirillum* strains at the liquid phase–solid hydrophilic and liquid phase–solid hydrophobic surface interfaces.

It has been shown that spontaneous (as in strain Sp245.5) [6] or induced (as in strains KM018, KM127, KM134, KM139, KM252, and KM348) [3] changes in the composition and/or structure of *A. brasilense* Sp245 plasmids profoundly affect the social activity of these bacteria and, consequently, the process of biofilm formation.

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