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EXPERIMENTAL ARTICLES

Changes in Biofilm Formation in the Nonflagellated *flhB1* Mutant of *Azospirillum brasilense* Sp245

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Abstract—Bacteria *Azospirillum brasilense* Sp245 with mixed flagellation are able to form biofilms on various surfaces. A nonflagellated mutant of this strain with inactivated chromosomal copy of the *flhB* gene (*flhB1*) was shown to exhibit specific traits at the later stages of biofilm formation on a hydrophilic (glass) surface. Mature biofilms of the *flhB1*::Omegon-Km mutant Sp245.1063 were considerably thinner than those of the parent strain Sp245. The biofilms of the mutant were more susceptible to the forces of hydrodynamic shear. *A. brasilense* Sp245 cells in biofilms were not found to possess lateral flagella. Cells with polar flagella were, however, revealed by atomic force microscopy of mature native biofilms of strain Sp245. Preservation of a polar flagellum (probably nonmotile) on the cells of *A. brasilense* Sp245 may enhance the biofilm stability.

Keywords: *Azospirillum brasilense*, biofilms, motility, flagella, insertional mutations, *flhB* gene

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Azospirilla are capable of stimulating the growth and development of a wide variety of plants with which they form associations [1]. The motility of *Azospirillum brasilense* depends on a single polar flagellum (Fla) that is formed on both liquid and solid media and on numerous lateral flagella (Laf); formation of the latter is only induced at an elevated medium density [2, 3]. Apart from motility, the polar flagellum is used by azospirilla for their adherence to the surface of plant roots [4].

Biofilm formation by azospirilla at the root surface appears to be an important prerequisite for the successful functioning of the plant–*Azospirillum* association. A bacterial biofilm is a spatially and metabolically structured community of microorganisms enclosed in the matrix and attached to one another and to the phase boundary [5–8]. It is known that if suspended ("planktonic") bacteria are incorporated into a biofilm, they lose flagella-dependent motility. At the same time, inactivation of the genes that are responsible for motility impedes biofilm formation by microorganisms [5–8].

Biofilm formation by bacteria with diverse physiological features and modes of interaction with the colonized object reveals a number of common stages, such as cell adherence to the surface with subsequent formation of microcolonies, a monolayer, and a multilayer biofilm. While aging and depleting carbon and energy sources, a biofilm undergoes disintegration (or dispersal). As a result, bacteria revert to a planktonic lifestyle, which implies seeking new habitats [5–9]. The diversity of regulatory mechanisms of biofilm formation, as well as of the structural elements of biofilms, proved commensurate with the diversity of biofilm-forming bacterial species and even strains. In other words, "there are as many different biofilms as there are different bacteria" [9].

Very scarce data are available concerning biofilm formation and the contributions of various cell surface components, including flagella, to this process in the genus *Azospirillum*. Spontaneous or inducible changes in the composition and/or function of the plasmids of the *A. brasilense* Sp245 strain that disrupt the synthesis of lipopolysaccharides (LPSs) and of Calcofluor-binding extracellular polysaccharides significantly affect the efficiency of formation of *Azospirillum* biofilms on abiotic surfaces [10, 11]. Inactivating the plasmid genes that code for the hypothetical TAD pili in *A. brasilense* Sp245 results in suppressing biofilm formation [12].

The goal of the present work was to conduct a comparative study of biofilm formation by the cultures of *A. brasilense* Sp245 and by its insertion mutant *flhB1* that has lost the capacity to synthesize the polar and the lateral flagella.

MATERIALS AND METHODS

Bacterial strains. In this work, we used the strain *A. brasilense* Sp245 isolated from wheat roots [13] and its *flhB1*::Omegon-Km insertion mutant Sp245.1063 with the *Fla*$^-$*Laf*$^-$ phenotype (Km^R) [14]. This mutant

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contained a single insertion of the artificial Omegon-Km transposon in the chromosome copy of the *flhB* gene (*flhB1*) that codes for a component of the flagellar protein export complex. Despite the presence of a second *flhB* gene copy in the plasmid gene cluster apparently responsible for Laf formation [12], the *flhB1*::Omegon-Km mutant Sp245.1063 has completely lost the capacity for synthesizing both Fla and Laf [14]. Plausibly, the induction of Laf synthesis involves a hypothetical multisensory hybrid histidine kinase/response regulator that is encoded by the AZOBR_150176 gene. This gene is transcribed in the same direction as *flhB1* and is adjacent to its 3' terminus. Omegon insertion into the *flhB1* gene could also impede the expression of the AZOBR_150176 gene in Sp245.1063 cells due to the polar effect [14].

Cultivation media. *A. brasilense* cultures were grown on the malate–salt medium (MSM) or the LB medium [16] at 30°C. If necessary, the media were supplemented with 50 μg/mL kanamycin (Km) and 1 mg/mL Calcofluor (Sigma, United States).

Determining the growth rate of bacteria under aeration. Overnight (18 h) bacterial cultures were diluted to A_{590} values of 0.05–0.10 (l = 0.5 cm) in 100 mL of sterile MSM or LB medium and transferred to 250-mL conical flasks. For intense aeration, the flasks were placed on the horizontal platform of an Excella E24 shaker–incubator (New Brunswick Scientific, United States) and incubated at 140 rpm at a temperature of 30°C. Optical density of the bacterial culture at 590 nm (l = 0.5 cm) was measured every two hours.

Determining the relative hydrophobicity of bacterial cells. Salting out was used for this purpose [17]. The wells of a polystyrene plate contained 25 μL of serial dilutions of ammonium sulfate solution in 2 mM phosphate buffer (PB; pH 6.8) and 25 μL of bacterial suspensions in PB (A_{590} = 1.2) that were prepared from 18-h submerged cultures in LB; the cells were prewashed with the buffer. The minimum sulfate ammonium concentration, at which bacterial aggregation occurred, was determined. The lower the aggregation-causing salt concentration, the more hydrophobic the cells were [17].

Assessment of the growth of planktonic cultures and biomass accumulation in biofilms. The optimum conditions for biofilm formation by *A. brasilense* Sp245 were selected by us earlier [10]. Overnight (18 h) bacterial cultures were diluted with sterile LB medium to A_{590} values of 0.05–0.10 (l = 0.5 cm), and aliquots of the suspensions were transferred to 5-mL glass test tubes (2 mL) or were placed in 30-mm petri dishes (4 mL). Sterile cover slips (24 × 24 mm) were located at their bottom. The system was statically incubated at 30°C. In a different series of experiments, test tubes with bacterial suspensions were incubated with shaking at a rate of 140 rpm.

Before staining the biofilms, planktonic cultures were sampled from the test tubes to determine the optical density (A_{590}; l =0.5 cm) of the liquid cultures that surrounded the biofilms. Aqueous solution of crystal violet (1%, 2 mL) was cautiously added to the test tubes. They were then incubated at room temperature for 10 min. The solution was removed, and the test tubes were gently washed with water. To assess bacterial biomass, the biofilm-bound dye was dissolved in 2.5 mL of ethanol. Optical density of the solution was measured at A_{590} (l = 0.5 cm).

After removing the planktonic culture, the cover slips with biofilms were used for microscopy.

Phase contrast microscopy of the biofilms. A Leica LMD 7000 device (Leica, Germany) was used for phase contrast light microscopy. Prior to microscopy, cover slips were placed on concave slides with the cover slip's biofilm side facing the slide. To measure the thickness (height) of the biofilms, the microscope was first focused on the "lower surface" of the biofilm (facing the glass). The focal distance (Z_1) in micrometers was recorded. Thereupon, the microscope was focused at the "upper surface" (Z_2). The biofilm thickness was determined using the formula $Z = (Z_2 - Z_1) \times (n/n_1)$, where n is the refraction index of glass (1.5) and n_1 the refraction index of air (1.0).

Atomic force microscopy of the biofilms. To prepare samples for atomic force microscopy (AFM), the bacterial cells that formed biofilms on the cover slip were washed with 50 mM phosphate buffer (PB, pH 7.0), dehydrated with 75% ethanol for 10 min, and air-dried. The topography of the biofilm surface was investigated with a SolverBio atomic force microscope (NT-MDT, Russia); the maximum size of the scanned area was 60 × 60 μm. The measurements were performed in the semi-contact mode of surface scanning, using NSG 10 silicon cantilevers (NT-MDT, Russia) with an elasticity modulus of 11.8 N/m and an average curvature radius of the pointed end equaling 10 nm. The size of the scanned areas (micrometers) is shown on Fig. 4, the scanning resolution was 1024 × 1024 points, and the scanning frequency was 1 Hz. Scanning was performed at room temperature in the air. To increase the contrast ratio and to rule out the influence of the sample surface tilt relative to the probe, the images obtained were processed using the NOVA software package (NT-MDT, Russia).

Transmission electron microscopy of biofilms and bacteria from liquid cultures. Prior to transmission electron microscopy of the biofilms formed on the surface of the medium in test tubes or on cover slips, the planktonic bacterial cultures were removed and the biofilms were gently washed two times with PB (pH 7.0). Thereupon, the biofilms were harvested by aspiration and suspended in PB (pH 7.0). The biofilm washings obtained and 18-h liquid cultures were used for preparing the samples for microscopy. The bacteria were applied to formvar supports. After 20 min, excess liquid was removed with filter paper and the preparations were air-dried. Electron microscopy of the bac-

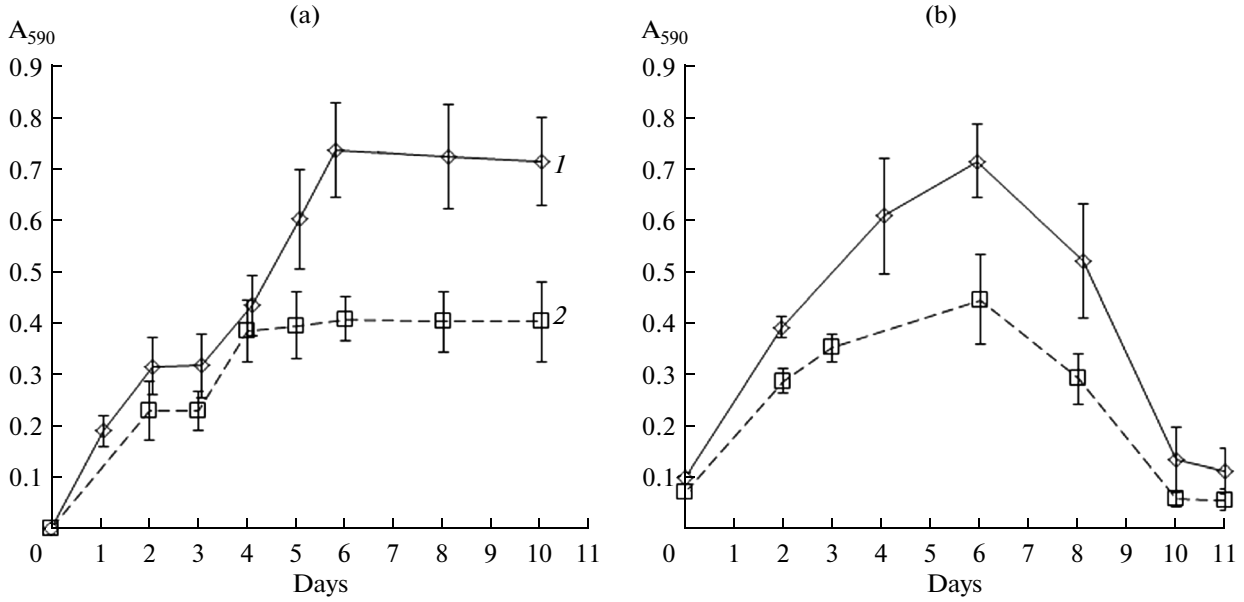

Fig. 1. Dynamics of biomass accumulation in the biofilms formed by *A. brasilense* Sp245 (*1*) and *A. brasilense* Sp245.1063 (*2*) on the glass surface (a) and changes in the density of planktonic cultures (b) of these strains during long-term incubation in LB under stationary conditions. A_{590} is the optical density of desorbed crystal violet after staining the biofilms (a) or the optical density of planktonic cultures (b).

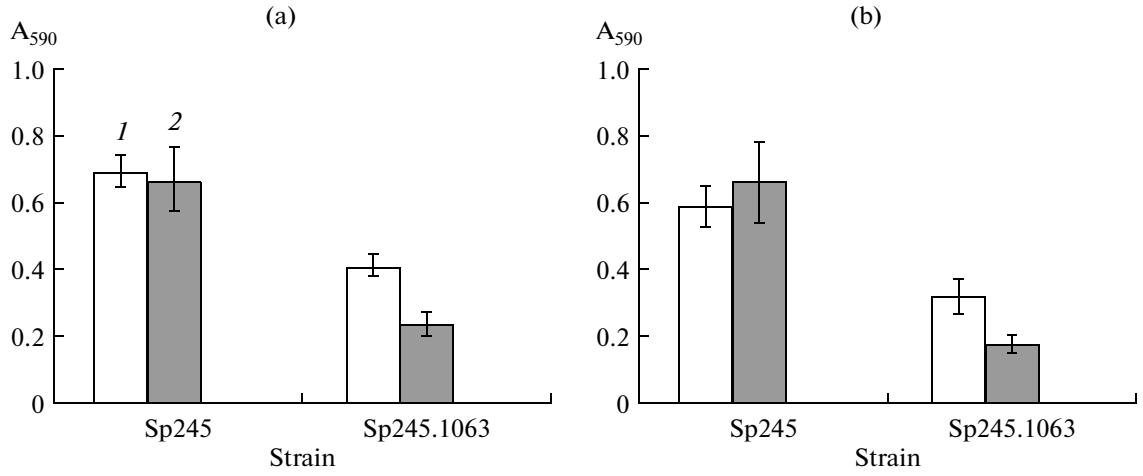

Fig. 2. Biomass accumulation in biofilms formed by *A. brasilense* Sp245 and *A. brasilense* Sp245.1063 on the glass surface during 6 days of incubation in LB (a) or MSM (b) under stationary conditions (*1*) or with stirring (140 rpm) (*2*). A_{590} is the optical density of desorbed crystal violet after staining the biofilms.

terial cells was carried out with a Libra 120 (Carl Zeiss, Germany) device at an accelerating voltage of 120 kV.

Statistical treatment of the results. Crystal violet staining of the biofilms to assess the bacterial biomass and the measurement of optical density of the solution of the desorbed dye were performed 30 to 50 times for each variant of the experiment. As for the other quantitative assays, at least five independent experiments were carried out, each of them in two repeats. The results were statistically processed using the Microsoft Office Excel 2007 software package. Confidence intervals were determined for the 95% level of significance.

RESULTS AND DISCUSSION

Dynamics of biofilm formation by *A. brasilense* Sp245 and Sp245.1063. Staining bacteria with crystal violet was used to compare the dynamics of biomass accumulation in the films formed by *A. brasilense*

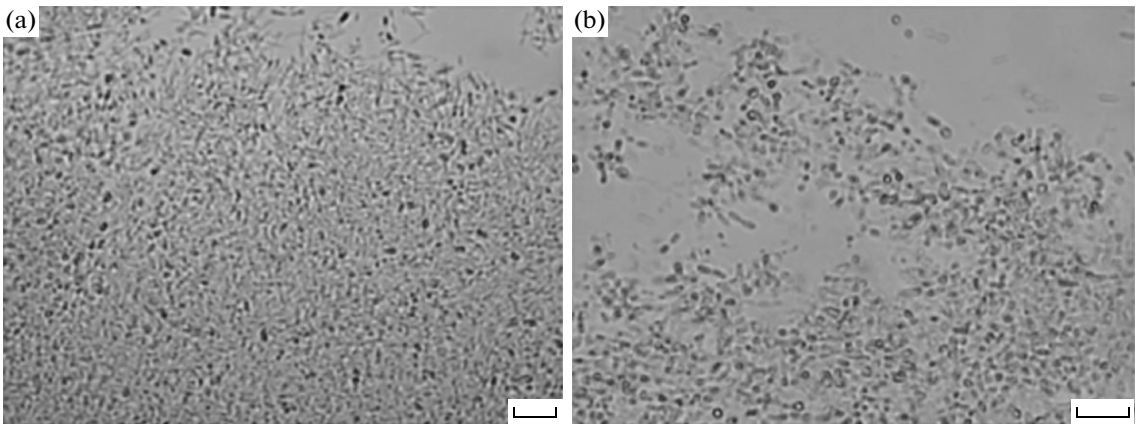

Fig. 3. Phase contrast microscopy of biofilms formed by *A. brasilense* Sp245 (a) and *A. brasilense* Sp245.1063 (b) on the glass surface during 6 days of incubation in liquid LB medium. Scale bar, 10 μm.

Sp245 and its mutant Sp245.1063 at the liquid–solid hydrophilic surface (glass) interface. Thin films appeared during the first day of incubating bacteria in liquid LB medium; microscopy revealed scattered cell aggregates (microcolonies) that were easily removed by aspirating the suspension and washing the test tubes with water. After 24 h, the number of glass surface-adhering bacteria increased, which manifested itself in an increase in biofilm biomass (Fig. 1a). For instance, the biofilm biomass of the strain Sp245 increased 1.5-fold after 2 days of incubation, compared to the data obtained after 1 day of incubation (Fig. 1a). On days 2–3 of incubation in liquid LB medium, the biomass level of the test tube surface-attached azospirilla reached a stable plateau, and no significant differences between the strains were detected (Fig. 1). The process of bacterial adherence to the glass surface was obviously completed during this period. By day 3 of incubation, bacterial microcolonies merged into a film with a flattened surface, and biomass accumulation commenced. With the Sp245.1063 mutant, the biomass quantity in the biofilm was stabilized after 4–5 days of incubation; thereafter, it remained unchanged during the whole cultivation period (Fig. 1). The thickness of the biofilms formed by the parent strain (Sp245) reached a plateau at a later point—after 6 days of incubation (Fig. 1).

The biofilm height for the *A. brasilense* Sp245.1063 mutant and the *A. brasilense* Sp245 parent strain cultivated in LB medium was 23.7 ± 2.5 and 33.7 ± 3.5 μm, respectively, on day 6 of incubation. Therefore, the biofilm thickness of the Sp245.1063 mutant was 70.3 ± 7.5% of that of the parent Sp245 strain. These data are consistent with the results of determining the relative biomass amount by straining biofilms with crystal violet (Fig. 1a). By day 6 of incubation, optical density of the dye desorbed from the biofilms of the Sp245.1063 mutant was 58.6 ± 4.4% of that of the parent strain (Fig. 1a).

Hence, the process of biofilm formation in the wild type and the mutant strains of azospirilla at the liquid–solid hydrophilic surface boundary includes several stages under stationary cultivation conditions, such as attachment, adherence, growth, and biomass stabilization.

The initial stages of biofilm formation, i.e., attachment and adherence of the bacteria to the glass surface, are likely to proceed irrespective of the ability of the cells to synthesize flagella. By day 3 of incubation, the biomass of flagella-free mutant cells adhering to the glass surface did not significantly differ from that of the parent strain (Fig. 1). Subsequently, however (after 5 days of incubation), the biofilm of the flagella-free mutant was considerably less thick than that of strain Sp245.

Under stationary conditions, the density of planktonic cultures of *A. brasilense* Sp245 and Sp245.1063 began to decrease after 8 days of cultivation, even though the biofilm thickness of these strains did not change (Fig. 1b). In terms of growth, the planktonic culture of Sp245.1063 tended to lag behind that of Sp245 as early as on days 2–3 of cultivation. The biofilms of this strain still retained their thickness, which was close to that of Sp245 until day 4 of incubation (Fig. 1a). It seems likely that the cells of the nonmotile Sp245.1063 mutant precipitate at the solid–liquid phase boundary. However, since they are loosely bound to biofilm-incorporated bacteria, they are readily washed away during the staining procedure. Starting from day 5 of incubation, the biofilm thickness of the flagella-free mutant was appreciably below that of strain Sp245 (Fig. 1).

Importantly, under intense aeration both *A. brasilense* strains exhibited an almost identical growth rate in liquid LB and MSM media. Only after 48 h of cultivation in the LB medium was the optical density of the culture of strain Sp245 somewhat below that of strain Sp245.1063, which was probably due to

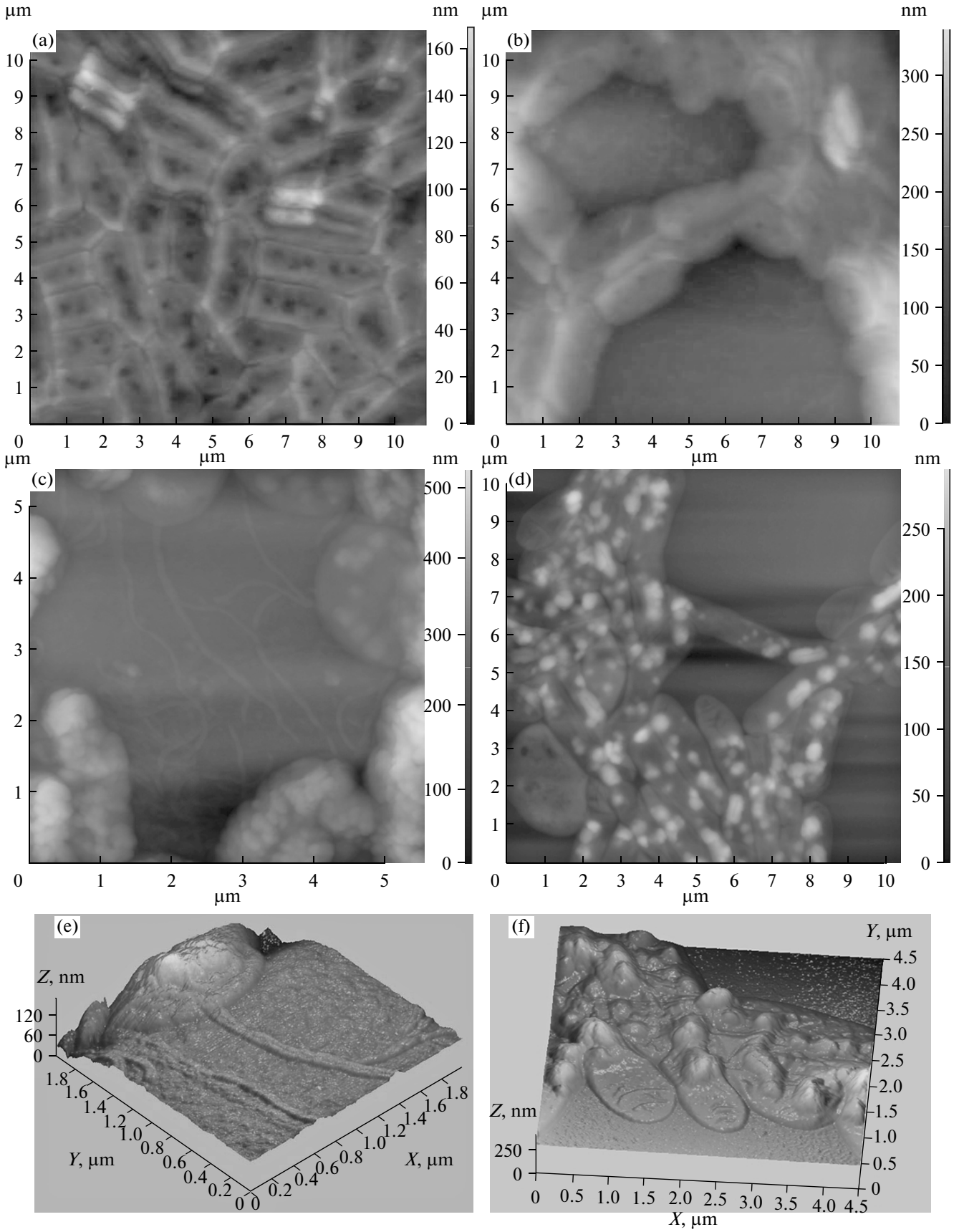

Fig. 4. AFM images of biofilms formed by *A. brasilense* Sp245 (a–c, e) and *A. brasilense* Sp245.1063 (d, f) on the glass surface during 6 days of incubation in liquid LB medium. The images in (e) and (f) were obtained via three-dimensional reconstruction.

cell aggregation being more manifest in the wild type strain than in the mutant.

Despite the difference between planktonic cultures of Sp245 and Sp245.1063 in terms of their growth, the onset of the increase in biofilm thickness followed the same pattern in both strains (Fig. 1). This process started on day 3 of incubation after stabilization of the biomass of the azospirilla adhered to the glass surface (Fig. 1a). Interestingly, there was no significant difference between the tested strains with respect to the thickness of their films (Fig. 1). Probably, the increase in the biomass of *Azospirillum* biofilms largely depends on the density of the bacterial population that adheres to the substratum, regardless of the growth rate of planktonic cells. To a large extent, it is the surface structures of bacterial cells that provide for the further development of biofilms. This conclusion is supported by comparative studies on the dynamics of biofilm formation in strain Sp245 and its flagella-free mutant.

The following series of experiments was aimed at comparing biofilm formation by azospirilla on the glass surface during their cultivation under stationary conditions and with intense stirring (140 rpm). With strain Sp245.1063, the thickness of the 6-day-old biofilm varied depending on cultivation conditions. If the bacteria were incubated in LB medium under stirring, the amount of the dye desorbed after staining the biofilms of Sp245.1063 was 40–50% below that found under stationary conditions (Fig. 2). However, with the parent strain (Sp245) stirring did not significantly influence biomass accumulation in the biofilms (Fig. 2).

Thus, in contrast to the wild-type strain, biofilm formation in the *A. brasilense* Sp245.1063 mutant was more sensitive to the hydrodynamic shear force resulting from stirring at the liquid phase–solid surface boundary. Presumably, the density of intercellular contacts, which influences the resistance of biofilms to this factor, decreased in the Sp245.1063 mutant strain, in contrast to the parent strain.

Importantly, the Sp245.1063 mutant retained the capacity for synthesizing wild-type lipopolysaccharides and extracellular polysaccharides that bind Calcofluor. The relative hydrophobicity of the cell surface of *A. brasilense* strains Sp245.1063 and Sp245 was approximately equal. The minimum ammonium sulfate concentration that caused bacterial aggregation was 17.7 ± 0.1 and $16.4 \pm 1.4\%$, respectively. However, this mutant lacks the capacity for synthesizing polar and lateral flagella.

Comparative analysis of *A. brasilense* strains Sp245 and Sp245.1063 by phase contrast microscopy. Phase contrast microscopy of 18-h liquid cultures of *A. brasilense* Sp245 and Sp245.1063 revealed interstrain differences in terms of bacterial behavior. In the liquid medium, motile cells of the strain Sp245 formed small aggregates. Within 10–15 min, the number of such aggregates in the area with the maximum concentration of motile Sp245 cells (due to aerotaxis) rapidly increased. The polar flagellum-devoid mutants are capable neither of aerotaxis (because of a lack of the motility organelles) nor of quick intercellular aggregation.

By day 4–6 of cultivation, the biofilms of *A. brasilense* Sp245 were composed of compactly adjoining cells. The biofilms of the Sp245.1063 consisted of loosely packed bacteria (Fig. 3). The polar flagellum of *A. brasilense* Sp245 is covered with a polysaccharide sheath with the antigen determinants identical to those of the LPSs [18], while the cell surface hemagglutinin is characterized by an affinity for the O polysaccharide of the LPSs of this strain [19]. Hence, bacterial aggregation might be promoted by the interaction between the polar flagellum and hemagglutinin. Presumably, the flagellum-mediated interaction between bacteria proceeds during the initial stages of biofilm formation. The question to be raised is whether biofilm-enclosed bacteria retain the flagellum during the growth of the biofilm and stabilization of its biomass.

Atomic force and electron microscopy of the biofilms formed on a glass surface by *A. brasilense* strains Sp245 and Sp245.1063. On days 4–6 of incubation, the outer layer of the biofilm of *A. brasilense* Sp245 was composed of densely adjoining bacterial clusters (Fig. 4a). On average, each cluster was made up of 5 ± 1 cells that formed longitudinal contacts. The biofilm of the *A. brasilense* Sp245.1063 mutant consisted of sparsely arranged bacterial cells.

AFM of the biofilms revealed Sp245 strain cells with a polar flagellum-like filament at their poles (Figs. 4c, 4e). Fig. 4 and Figs. 5a–5c represent flagellum images obtained using AFM of the native biofilms and by electron microscopy of the cells removed by washing from the biofilms growing on the glass surface. The pictures demonstrate that the morphology of the flagellum and its arrangement on the bacterial cell are similar in the biofilms and the 18-h liquid culture of the strain Sp245 (Fig. 5d). Importantly, cells with a polar flagellum were in close contact with biofilm-forming bacteria in fixed native biofilms of the strain Sp245 (Fig. 4c). Thus, these individuals were incorporated in the biofilm.

Samples for AFM or electron microscopy of native biofilms and of those removed from the glass surface by washing were taken both during the biomass accumulation period (after 4–5 days of incubation) and from mature biofilms (after at least 6 days, i.e., during the period that corresponds to the biofilm biomass stabilization phase; Figs. 1, 4, and 5). In the case of the strain Sp245, cells with the flagellar filament were detected in all microscopically investigated samples. This attests to the retention of its synthesis, not only during the biomass accumulation stage, but also in mature biofilms. As it was to be expected, the cells of the biofilms of the $Fla^{-}Laf^{-}$ mutant (Sp245.1063) lacked flagella (Fig. 4).

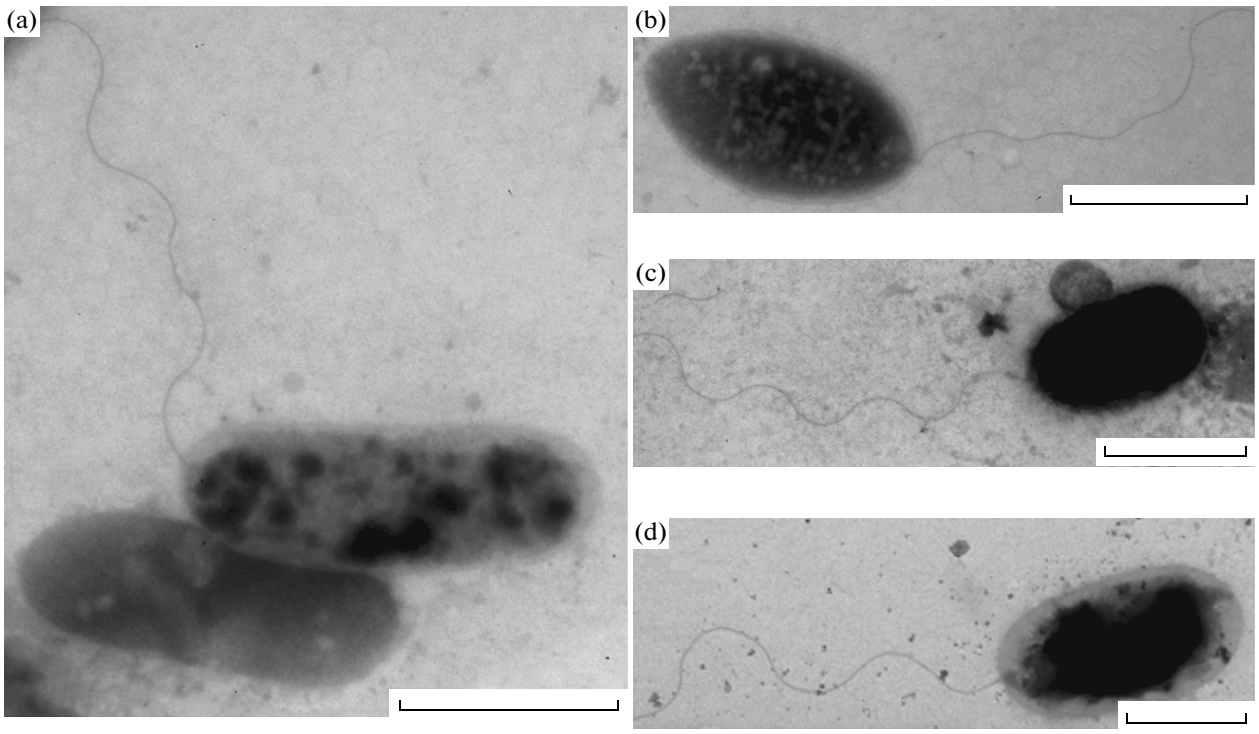

Fig. 5. Transmission electron microscopy of *A. brasilense* Sp245 from 6 (a) and 8 (b, c) day biofilms removed from the glass surface by washing and from 18-h liquid cultures (d). Cultivation medium: LB (a, c) or MSM (b, d). Scale bar, 1 μm.

Similar to the Fla$^-$Laf$^-$mutant, the cells in the biofilms of the *A. brasilense* strain Sp245 lacked lateral flagella. In all likelihood, the activity of the polar flagellum in the near-surface liquid layer enables wild-type cells to form a biofilm on the glass surface without spending additional energy on synthesizing lateral flagella. Moreover, the option cannot be ruled out that azospirilla use an alternative mechanism of collective motility, i.e., spreading associated with the formation of microcolonies [20] and other extracellular organelles such as hypothetical TAD pili [12].

Biofilm-forming microorganisms use flagella during the initiation of this process in order to attach the cells to the substratum. Bacterial motility is of relevance to the architecture of biofilms. It is mandatory for the bacteria that are liberated from mature biofilms [6–8]. Nonetheless, transition from the planktonic to the biofilm lifestyle involves suppressing the functioning and synthesis of flagella in a large number of bacteria [5–8]. It was revealed that the bacteria of the strain *A. brasilense* Sp245, which use the polar flagellum for active motility and adherence to colonized surfaces, continue the synthesis of this organelle when incorporated in a mature biofilm. It seems likely that this capacity enables azospirilla to retain the biofilm integrity under the influence of hydrodynamic shear force at the interface between the liquid and the solid medium. As was noted above, cultivation with stirring negatively influenced biomass accumulation in the biofilms of the flagellum-free strain *A. brasilense* Sp245.1063.

Thus, inactivation of the chromosome copy of the *flhB* gene (encoding a component of the flagellum-exporting complex of *A. brasilense* Sp245), which disrupts formation of the polar and lateral flagella, had a negative effect on the ability of the mutant Sp245.1063 cells to form biofilms on a hydrophilic surface. This results in decreasing the thickness of mature biofilms and lowering their resistance to hydrodynamic shear forces.

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