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EXPERIMENTAL ARTICLES

Formation of Polar Bundles of Pili and the Behavior of *Azospirillum brasilense* Cells in a Semiliquid Agar

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Abstract—This paper describes the formation of single polar bundles of pili on *Azospirillum brasilense* cells, the twitching motility of cell aggregates, and a new type of social behavior—the dispersal of bacterial cells in semiliquid agar associated with the formation of granular inclusions (the so-called Gri^+ phenotype)—which is an alternative to swarming (the Swa phenotype). The wild-type *A. brasilense* cells occurring in a semiliquid agar may show either the Swa^+Gri^-, or Swa^-Gri^-, or Swa^-Gri^+ phenotype. The formation of single polar flagella (Fla) or polar bundles of pili may reflect two alternative states of *A. brasilense* cells. The components of the Fla system may be involved in the regulation of the phenotypic variation of azospirilla.

Key words: polar bundle of pili, twitching motility, phenotypic variation, *Azospirillum brasilense*.

Azospirillum brasilense is a gram-negative plant-growth stimulating bacterium whose motility is provided by two types of flagella [1, 2]. In liquid media, individual *A. brasilense* cells move (the Mot^+ phenotype) due to rotation of single polar flagella (Fla) [1]. At concentrations of Bacto agar in the medium exceeding 0.4%, *A. brasilense* cells produce many lateral flagella (Laf) [1, 2]. When the wild-type *A. brasilense* cells are inoculated into media containing from 0.2 to 0.6% agar, they disperse using Fla and Laf, provided that the latter are produced. This process is accompanied by the formation of specific regular circular structures [2–4]. The flagella-aided dispersal of bacteria in the bulk and on the surface of semiliquid agar is known as swarming (the Swa^+ phenotype) [5]. It should be noted that *A. brasilense* cells cannot swarm on the surface of solid media containing more than 0.6% of agar.

Earlier, we reported on the Omegon Km^R mutants of *A. brasilense* Sp245 which were unable to swim and swarm [3]. The analysis of these mutants showed that Laf and Fla are produced independently and that the activity of both Fla and Laf are necessary for bacterial swarming in semiliquid media. One of these mutants with an Omegon-Km insertion in the 120-MDa plasmid, *A. brasilense* SK048 Fla^-, was investigated in more detail [4]. It was found that this mutant, when inoculated in a semiliquid medium, spreads with the formation of granular inclusions or microcolonies (the Gri^+ phenotype).

The aim of the present work was to investigate the behavior of nonswarming *A. brasilense* Sp245 mutants and some selected clones of *A. brasilense* Sp245 and SK048 in order to ascertain factors responsible for the Gri^+ phenotype.

MATERIALS AND METHODS

Bacterial strains. The wild-type strain *Azospirillum brasilense* Sp245 was a gift from J. Döbereiner, EMBRAPA-CNRS, Rio-de-Janeiro, Brazil. The Km^R mutants of this strain—Fla^- SK048, Mot^- SK248, Fla^- Laf^- SK051, BK570 (this mutant shows normal flagellation and, when placed in media containing 0.2–0.3% agar, forms swarming rings with diameters about 2.5 times larger than in the case of the wild type Sp245), the Fla^-leaky Laf^- mutants SK454 and SK586, the Laf^-leaky Fla^-/Mot^- mutants SK005 and SK531, and the Fla^-/Mot^-leaky mutant SK039—were described earlier [3]. The mutant SK048 carries a unique Omegon-Km insertion [6] in its 120-MDa plasmid [4]. The strains SK051, SK248, and BK570 carry different cointegrate molecules formed by fusions of an 85-MDa plasmid with the Omegon vector pJFF350 [7]. In the mutants SK039, SK454, SK531, and SK586, the Omegon-Km insertion is located in the chromosomal *Xho*I fragment about 23 kbp in size [3]. In the mutant SK237,

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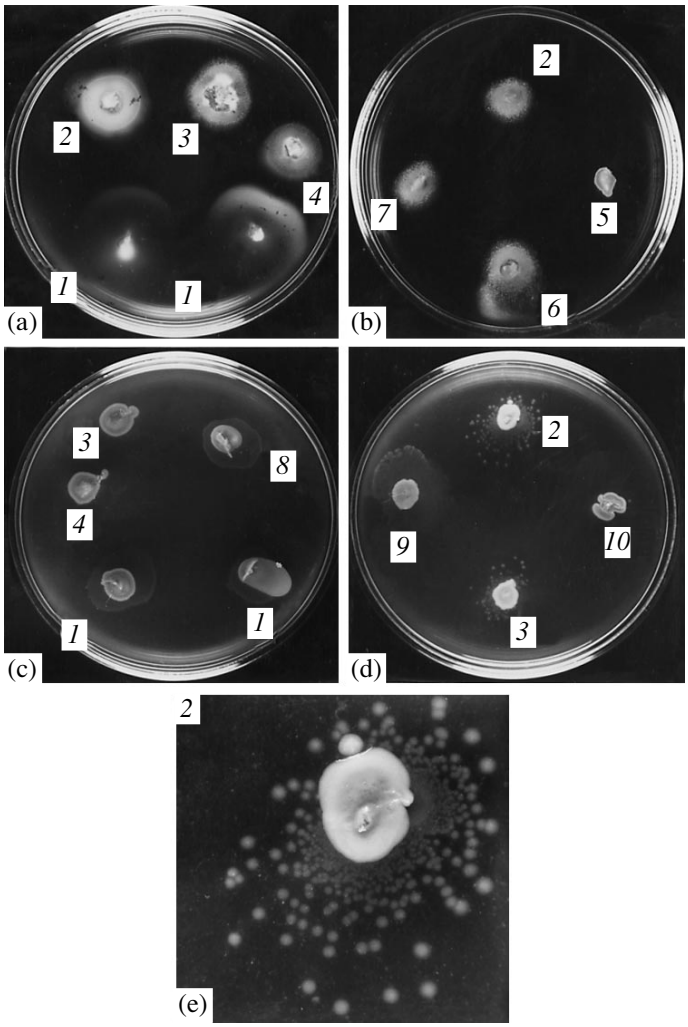

Fig. 1. Two-day-old *A. brasilense* colonies grown at 30°C on the semiliquid MSM medium containing either (a, b) 0.5 or (c, d, e) 0.9% agar: (*1*) Sp245, (*2*) SK048; (*3*) SK039; (*4*) SK005; (*5*) SK531; (*6*) SK454; (*7*) SK051; (*8*) BK570; (*9*) SK248; and (*10*) SK586. Photograph e shows a magnified view of strain SK048, which is capable of dispersing from the inoculation stab with the formation of microcolonies.

the Omegon-Km insertion is located in the chromosomal *Xho*I fragment about 29 kbp in size [3].

Nutrient media and cultivation conditions. The bacteria were grown in a malate-containing saline medium (MSM) of the following composition (g/l): malate, 5; K_2HPO_4, 0.5; KOH, 4; $FeSO_4 \cdot 7H_2O$, 0.2, NaCl, 0.1; NH_4Cl, 1; and Na_2MoO_4, 0.002 [8]. After autoclaving, the medium was supplemented with sterile solutions of $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ to final concentrations of these salts of 0.2 and 0.02 g/l, respectively. In experiments with sugars and amino acids, these compounds were added to semiliquid MSM medium instead of malate to final concentrations of 1 mM. The Omegon mutants were grown in the presence of 50 μg/ml kanamycin (Km). Experiments with calcofluor were carried out using TSA medium (Serva) containing (g/l) casein peptone, 15; soybean peptone, 5; NaCl, 5; and Bacto agar, 20. Calcofluor (Fluorescent brightener no. 28), purchased from Aldrich, was dissolved in distilled water and added to the TSA medium to a final concentration of 0.1%. The pH of all media was adjusted to 6.8–7.0.

The motility of bacteria and the morphology of their dispersal zones were examined by the phase–contrast microscopy of colonies grown at 30°C for 24–72 h on the MSM medium containing from 0.2 to 0.9% Bacto agar. Alternatively, azospirilla were streaked onto the surface of the MSM medium containing 1.8% agar and incubated for 42–72 h. The inoculum represented 42-h-old *Azospirillum* cells washed off from the agar-solidified MSM medium.

Transmission electron microscopy. 20-μl aliquots taken from an 18-h liquid or semiliquid bacterial culture or from a suspension of 48-h cells washed off from

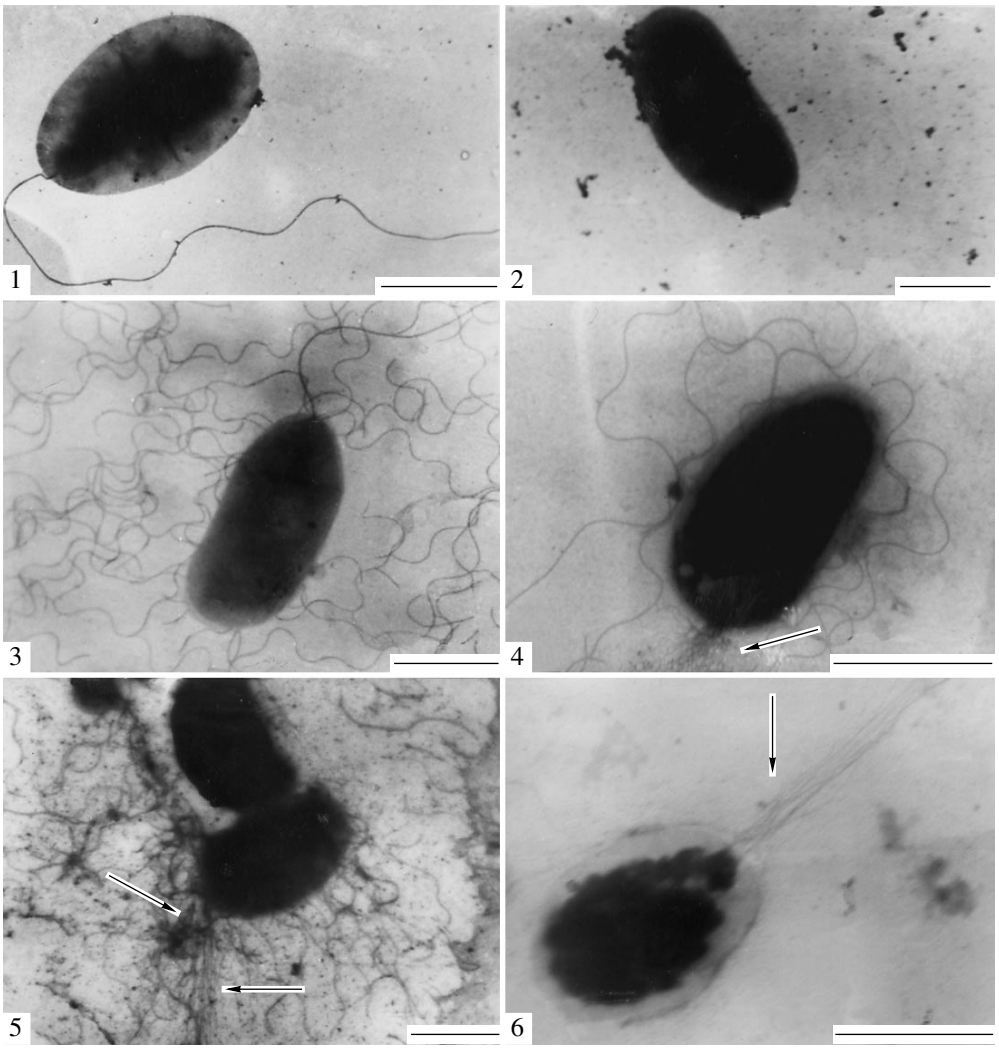

Fig. 2. The morphology of *A. brasilense* Sp245 cells and their Omegon mutants as observed under a transmission electron microscope: (1, 3, 5) Sp245; (2, 4) SK048; and (6) BK570. Bacteria were grown (1, 2, 6) in the liquid MSM medium for 18 h or (3, 4, 5) on the solid MSM medium for 42 h. Specimens for microscopy were prepared as described in the *Materials and Methods* section. The bars represent 1 μm. The arrows point at bundles of pili formed at one pole of cells.

the solid MSM medium were applied onto a Formvar-coated grid. The specimens thus prepared were kept for 20 min, dried on a sheet of filter paper (Whatman no. 1), washed with distilled water, and again dried. The specimens were then contrasted with a 2% solution of uranyl acetate for 1–5 min and examined under a Tesla BS-500 electron microscope (Czech Republic) at an accelerating voltage of 50 kV.

RESULTS AND DISCUSSION

The Gri$^+$ phenotype of Omegon *A. brasilense* mutants defective in the formation and function of lateral and/or polar flagella. The existence of Gri$^+$ phenotype in a population of the Fla$^-$Swa$^-$ mutant *A. brasilense* SK048 [4] suggested that it may result from a pleiotropic mutation induced by the Omegon-Km insertion into plasmid p120.

The present study showed that the Swa$^-$ mutants of strain Sp245 defective in flagellation or motility due to the Omegon-Km insertions into plasmid or chromosomal DNA may also have the Gri$^+$ phenotype. Indeed, unlike the swarming strains Sp245 (Figs. 1a, 1c, culture 1) and BK570 (Fig. 1c, culture 8), the Swa$^-$ mutants stab-inoculated into media containing 0.2–0.9% agar either produced colonies along the stab (Fig. 1b, culture 5; Fig. 1c, cultures 3, 4; Fig. 1d, culture 10) or formed granular dispersal zones (Fig. 1a, cultures 2–4; Fig. 1b, cultures 2, 6, 7; Fig. 1d, cultures 2, 3). Usually, the Mot$^-$ mutant SK248 remained in the stab, but might also disperse with the formation of granular or granular–branchy dispersal zones (Fig. 1d, culture 9). In the media containing more than 0.6% agar, azospirilla (including the wild type) either remained in the stab or dispersed over the petri dish bottom, provided that it was wet (Figs. 1c, 1d).

After 72 h of incubation, the diameter of the granular dispersal zones formed in the MSM medium containing 0.4% agar by the flagellation-defective Gri^+ *A. brasilense* mutants varied from 7 to 24 mm. In the medium containing 0.2% agar, the diameter of these zones was slightly larger.

In the media containing more than 0.4% agar, the cells of strains Sp245, SK039, SK048, and SK248 had many lateral flagella [3, 4, 9]. The number of such flagella on the cells of mutant SK454 was low. The diameter of the granular dispersal zones did not depend on the number of lateral flagella. For instance, the Fla^- leaky Laf^- mutant SK454 and the Fla^-/Mot^-leaky mutant SK039, as well as the Fla^- and Fla^-Laf^- mutants SK048 and SK051, formed granular dispersal zones of about the same diameter.

Strain Sp245 and its mutants grown on the solid MSM medium formed similar colonies and produced similar *O*-specific [10] and calcofluor-binding polysaccharides. The only exception was strain SK051, which, when grown on TSA medium containing calcofluor, did not fluoresce. These data suggest that the new type of mutant dispersal does not result from the impaired synthesis of cell-surface polysaccharides.

We failed to reveal any abiotic factors that would be able to influence the dispersal of *A. brasilense* cells in semiliquid media: irrespective of the agar concentration and the presence of malate, leucine, proline, tryptophan, arabinose, and rhamnose, the nonswarming Omegon mutants of azospirilla exhibited either the Swa^-Gri^- or the Swa^-Gri^+ phenotype.

Investigation of the Gri^+ phenotype of *A. brasilense* Sp245 and the phenotypic variation of strains Sp245 and SK048. The possibility could not be excluded that the new type of bacterial dispersal described above is an inherent property not only of the Omegon mutants of *A. brasilense* but also of some wild-type clones of this species. To verify this supposition, 2200 individual colonies of *A. brasilense* Sp245 grown on the solid MSM medium for 42 h were separately transferred onto the semiliquid MSM medium containing 0.4% agar and incubated at 32°C for 48 h. Among the 1814 grown colonies, six (i.e., 0.3%) had the Swa^-Gri^+ phenotype (it should be noted that the phenotype in these six clones was not so distinct as in SK048 and other mutant strains) and 81 (i.e., 4.5%) had the Swa^-Gri^- phenotype. The other clones dispersed with the formation of swarming rings from 4 to 20 mm in diameter.

To study the phenotypic stability of these clones, three Gri^+ and several Swa^- clones of Sp245 were transferred to the semiliquid MSM medium either directly or after intermediate subculturing on the solid MSM medium. In the case of the direct transfer, two of the three Gri^+ clones gave rise to Swa^+ offspring in the first subculture on the semiliquid medium and the third clone gave rise to Swa^+ offspring in the second subculture. When the tested clones were intermediately subcultured on the solid medium, all three Gri^+ and all spontaneous Swa^- clones gave rise to only Swa^+ offspring. Therefore, the phenotypes Swa^-Gri^+ and Swa^-Gri^- are unstable, at least under laboratory conditions. The dominant phenotype in *A. brasilense* Sp245 populations is Swa^+Gri^-.

However, the possibility cannot be excluded that the mode of *A. brasilense* dispersal in the plant root exudates in the form of microcolonies or cell aggregates may be dominant. It should be noted in this regard that, typically, azospirilla reside on the plant roots as cell aggregates [11], which are presumably formed with the involvement of capsular polysaccharides, exopolysaccharides, and outer-membrane proteins [12, 13].

The study of the behavior of *A. brasilense* SK048 in the semiliquid agar MSM medium showed that this mutant strain produced 10% of Swa^-Gri^- colonies (the offspring of these colonies had either the Swa^-Gri^+ or Swa^-Gri^- phenotype) and 90% of colonies with the Swa^-Gri^+ phenotype.

The motion of *A. brasilense* Sp245 cells was strongly suppressed, if they were grown in liquid nonaerated cultures. The effect of anoxic conditions on the occurrence rate of the Gri^+ and Swa^- phenotypes was studied in the following experiments: 1-ml aliquots of the serial dilutions of nonaerated Sp245 cultures were mixed with a molten MSM medium containing 0.4% agar and poured into petri dishes. The incubation of these petri dishes at 32°C gave rise to three colonial phenotypes: the sessile Swa^-Gri^- phenotype (259 clones), the dull Swa^-Gri^+ phenotype (34 clones), and the Swa^+Gri^- phenotype (2942 of the 3235 clones examined).

In other words, the cultivation under anoxic conditions slightly increased the occurrence rates of colonies with the Swa^-Gri^- phenotype (8%) and the Swa^-Gri^+ phenotype (1%). These data suggest that oxygen is necessary for the formation and/or functioning of flagella. Reportedly, the rotation of the flagella of *A. brasilense* cells is driven by the Na^+ pump [14]. The subculturing of several sessile Swa^-Gri^- and dull Swa^-Gri^+ colonies on solid and semiliquid agar media in the same way as described above showed that these two phenotypes are unstable.

Similar experiments with the mutant strain *A. brasilense* SK048 showed that 4 of the 680 clones (i.e., 0.6%) grown in the semiliquid agar MSM medium for 72 h exhibited a mixed phenotype: the granular dispersal zone of these four clones extended to form either small swarming rings (2 colonies) or swarming sectors (2 colonies). After the intermediate subculturing of these 4 clones on the solid MSM medium, they all gave rise to offspring with the Swa^-Gri^- phenotype after 48 h of incubation in the semiliquid MSM medium and with the Gri^+ phenotype after 72 h of such incubation. Among the remaining 676 colonies, 35 colonies had the Swa^-Gri^- phenotype and 641 colonies had the Swa^-Gri^+ phenotype.

The formation of single polar flagella or polar bundles of pili is indicative of two alternative states of *A. brasilense* cells. According to some data in the literature, the aggregation and dispersal of bacterial cells may be mediated by extracellular organelles such as pili [15, 16].

The transmission electron microscopy of *A. brasilense* SK048 cells grown on the solid MSM medium revealed polar bundles of filamentous structures which were thinner than flagella (the so-called bundle-forming pili, Bfp) (Fig. 2, specimen 4). It should be noted that about 10% of the SK048 cells grown in liquid aerobic cultures had more or less long single flagella, but none of the SK048 cells could produce such flagella during their growth on the solid MSM medium.

Cells of other Omegon mutants, as well as of the wild-type strain Sp245, also produced flagella (Fig. 2, specimens 5, 6) during growth in liquid or on solid media. The formation of either Fla or Bfp presumably reflects two alternative states of *A. brasilense* cells, since we could never observe Fla and Bfp on the same cell. This assumption is also confirmed by the fact that the cultivation of azospirilla under nonaerated conditions, which are unfavorable to Fla functioning, slightly augmented the occurrence rate of the Swa^-Gri^+ phenotype.

In all Omegon mutants of strain Sp245 with the phenotype close to Swa^-Gri^+, Fla either was not produced or was motionless, suggesting that Fla itself or some components of the Fla system may be involved in the regulation of the phenotypic variation of *A. brasilense* cells. Indeed, the occurrence rate of the Swa^-Gri^+ phenotype among Fla^- mutants was considerably higher than among the wild-type Sp245 cells.

As was shown earlier for some gram-negative bacteria, Bfp are required for the twitching motility and aggregation of bacterial cells [5, 15, 16]. Twitching motility depends on the presence of the so-called type IV pili (Bfp is a variant of such pili) [15–17], which are capable of cyclic contraction by a hitherto unknown mechanism [15].

Under laboratory conditions, the twitching motility of bacteria, e.g., pseudomonads, can be detected by stabbing them deeply (to the petri dish bottom) into a 1% agar medium and observing the formation of diffusive granular dispersal zones [17]. In the present work, the twitching motility of *A. brasilense* cells was observed not only at the petri dish bottom (Figs. 1d, 1e) but also on the surface of solid media. For instance, the twitching motility of aggregated *A. brasilense* SK048 cells was observed under a phase-contrast microscope in a drop of a liquid squeezed from the solid growth medium by pressing a cover glass.

To conclude, the present paper describes the formation of Bfp and the twitching motility of azospirilla. The two types of cell motility, one of which is dependent on flagella and the other is dependent on pili, obviously increases the adaptive capacity of this bacterium.

The derivatives of strain Sp245 described in this paper may be used for mapping the *bfp* loci in the *A. brasilense* genome and for studying the mechanisms of the phenotypic variation of this bacterium with respect to its locomotion.

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REFERENCES

1. Krieg, N.R. and Döbereiner, J., Genus *Azospirillum* (Tarrand, Krieg, and Döbereiner, 1979), *Bergey's Manual of Systematic Bacteriology*, Krieg, N.R. and Holt, J.G., Eds., London: Williams & Wilkins, 1984, pp. 94–104.
2. Moens, S., Michiels, K., Keijers, V., Van Leuven, F., and Vanderleyden, J., Cloning, Sequencing, and Phenotypic Analysis of *laf1*, Encoding Flagellin of the Lateral Flagella of *Azospirillum brasilense* Sp7, *J. Bacteriol.*, 1995, vol. 177, pp. 5419–5426.
3. Scheludko, A.V., Katsy, E.I., Ostudin, N.A., Gringaus, O.K., and Panasenko, V.I., Novel Classes of *Azospirillum brasilense* Mutants Defective in the Production and Functioning of Polar and Lateral Flagella, *Mol. Genet. Mikrobiol. Virusol.*, 1998, no. 4, pp. 33–37.
4. Katsy, E.I. and Shelud'ko, A.V., Mapping of the *fla* Locus in Plasmid with a Molecular Weight of 120 MDa in *Azospirillum brasilense* Sp245 Bacteria, *Genetika*, 1999, vol. 35, no. 10, pp. 1367–1372.
5. Henrichsen, J., Bacterial Surface Translocation: A Survey and a Classification, *Bacteriol. Rev.*, 1972, vol. 36, pp. 478–503.
6. Fellay, R., Krisch, H.M., Prentki, P., and Frey, J., Omegon-Km: A Transposable Element Designed for *In Vivo* Insertional Mutagenesis and Cloning of Genes in Gram-Negative Bacteria, *Gene*, 1989, vol. 76, pp. 215–226.
7. Katsy, E.I., Borisov, I.V., and Shelud'ko, A.V., Effect of the Integration of Vector pJFF350 into a 85-MDa Plasmid of *Azospirillum brasilense* Sp245 on Bacterial Flagellation and Motility, *Genetika*, 2001, vol. 37, no. 2, pp. 183–189.
8. Döbereiner, J. and Day, J.M., Associative Symbiosis in Tropical Grasses: Characterization of Microorganisms and Dinitrogen Fixing Sites, *Int. Symp. on Nitrogen Fixation: Interdisciplinary Discussion*, Newton, W.E. and Nijmans, C.J., Eds., Wash. State Univ. Press, 1976, vol. 2, pp. 518–538.
9. Shelud'ko, A.V., Derivation and Characterization of *Azospirillum brasilense* Sp245 Mutants Defective in the Formation and Functioning of Polar and Lateral Flagella, *Cand. Sci. (Biol.) Dissertation*, Saratov: Russian Res. Inst. "Mikrob," 2000.
10. Katzy, E.I., Matora, L.Yu., Serebrennikova, O.B., and Scheludko, A.V., Involvement of a 120-MDa Plasmid of *Azospirillum brasilense* Sp245 in the Production of Lipopolysaccharides, *Plasmid*, 1998, vol. 40, pp. 73–83.

11. Murthy, M.G. and Ladha, J.K., Differential Colonization of *Azospirillum lipoferum* of Roots of Two Varieties of Rice, *Biol. Fertil. Soils*, 1987, vol. 4, pp. 3–7.

12. Burdman, S., Yurkevitch, E., Schwartsburd, B., Hampel, M., and Okon, Y., Aggregation in *Azospirillum brasilense*: Effects of Chemical and Physical Factors and Involvement of Extracellular Components, *Microbiology*, 1998, vol. 144, pp. 1989–1999.

13. Burdman, S., Yurkevitch, E., Schwartsburd, B., and Okon, Y., Involvement of Outer-Membrane Proteins in the Aggregation of *Azospirillum brasilense*, *Microbiology*, 1999, vol. 145, pp. 1145–1152.

14. Wood, C.C., Ritchie, R.J., and Kennedy, I.R., Membrane Potential, Proton and Sodium Motive Forces in *Azospirillum brasilense* Sp7-S, *FEMS Microbiol. Lett.*, 1998, vol. 164, pp. 295–301.

15. Knutton, S., Shaw, R.K., Anantha, R.P., Donnenberg, M.S., and Zorgani, A.A., The Type IV Bundle-forming Pilus of Enteropathogenic *Escherichia coli* Undergoes Dramatic Alterations in Structure Associated with Bacterial Adherence, Aggregation, and Dispersal, *Mol. Microbiol.*, 1999, vol. 33, pp. 499–509.

16. Debabov, V.G., The Life of Bacteria beyond Laboratories, *Mol. Biol.* (Moscow), 1999, vol. 33, no. 6, pp. 1074–1084.

17. Glessner, A., Smith, R.S., Iglewski, B.H., and Robinson, J.B., Roles of *Pseudomonas aeruginosa las* and *rhl* Quorum-sensing Systems in Control of Twitching Motility, *J. Bacteriol.*, 1999, vol. 181, pp. 1623–1629.