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SHORT COMMUNICATIONS

Plasmid Rearrangements and Alterations in *Azospirillum brasilense* Biofilm Formation

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The genome of *Azospirillum brasilense* a bacterium capable of mutually beneficial association with plants is represented by a chromosome, as well as by numerous plasmids [1]. Type strain *A. brasilense* Sp7 contains plasmids of 90 (pRhico), 115 (p115), and over 300 MDa. Multiple open reading frames apparently responsible for the synthesis of major cell surface components have been revealed in pRhico [2]. Almost nothing is known of the functions of other Sp7 plasmids. However, plasmid rearrangements have been shown to be accompanied by alterations in a number of traits of strain Sp7 [3–5]. Strain Sp7-S lacking the p115 plasmid has been obtained as a result of Sp7 cultivation at elevated temperature [3]. After several years of storage, Sp7-S spontaneous variants R, RS, and S, which contain pRhico derivatives with altered molecular mass and structure, were isolated [4]. A spontaneous S-variant Sp7.K2 isolated upon multiple freezing–thawing of Sp7 culture has also lost its p115 [4]. *Azospirillum brasilense* strain Cd lacking p115 was isolated from roots of bermuda grass inoculated with *A. brasilense* Sp7 culture [6]; analysis of polymorphism of the total DNA restriction fragments length evidences the strain similarity to Sp7 [5]. Spontaneous plasmid rearrangements in these Sp7 derivatives were accompanied by increased swarming rate and variations in colony morphology and resistance to ampicillin and surface-active agents [4, 5].

Since bacteria exist in the natural environment mainly as part of structured communities embedded into a polymeric extracellular matrix and located on a phase interface [7], the aim of the present work was to analyze the effect of spontaneous plasmid rearrangements on the efficiency of *A. brasilense* biofilm formation.

To evaluate azospirilla biofilm thickness, bacteria were cultured and stained with crystal violet as described previously [8]. Incubation for 96 h in LB medium at 28°C turned out to be optimal for Sp7 biofilm formation. At the interface "liquid LB medium–solid hydrophilic surface (glass)", strains Sp7, Sp7.2, Sp7.3, Sp7.4, and Sp7.6 formed biofilms of approximately equal thicknesses. The biofilms of strains Cd and Sp7.K2 were significantly thinner, and in variants Sp7.1 and Sp7.5 they were slightly less pronounced than in Sp7 (see table). The biomass amount in the films of strains Cd, Sp7.K2, and Sp7.1–Sp7.9 formed at the interface between liquid medium and hydrophobic polystyrene was considerably lower than in Sp7 films (see table).

The process of colonization of the roots of wheat seedlings, the associative partner of these bacteria, by strain Sp7 and its derivatives was also investigated. Sterilized wheat seeds were incubated for 3 days on a solid malate–salt medium (MSM) for azospirilla [8]. Inoculation was performed by incubating the seedlings for 3 h with gentle shaking (25 rpm) with suspensions of overnight *A. brasilense* cultures grown in MSM and washed with 50 mM phosphate buffered saline (PBS, pH 7.2) (A_{590} = 0.5). The seedlings were then placed into sterile PBS for several minutes of washing. One seedling was taken for microscopy analysis, and the rest were transferred to test tubes with nitrogen-free mineral medium for plants and grown for 1–7 days. Biofilm images were obtained with a phase contrast microscope equipped with a digital video camera and with a scanning electron microscope. Four to five hours after inoculation, strain Sp7 actively colonized root tips, hairs, and root fractures. After 1 day, Sp7 films became more compact and multilayered, with cell aggregates starting to form. The overall picture of wheat root colonization by Sp7 derivatives was similar to that of the wild type strain; however, at the initial stages of biofilm formation, bacterial films were thinner (especially in the cases of Sp7.K2 and Cd), single cells could be detected, and no colonization was observed in surface regions remote from the root tip. The R-variants under study, Sp7.1 and Sp7.4, were between Sp7 and Sp7.K2, Cd in terms of initial wheat root colonization intensity (figure). After 24 h, all the strains under study formed cellular aggregates on wheat roots; most of them were located on root hairs and tips and in places of root hair initiation, and visible interstrain differences disappeared.

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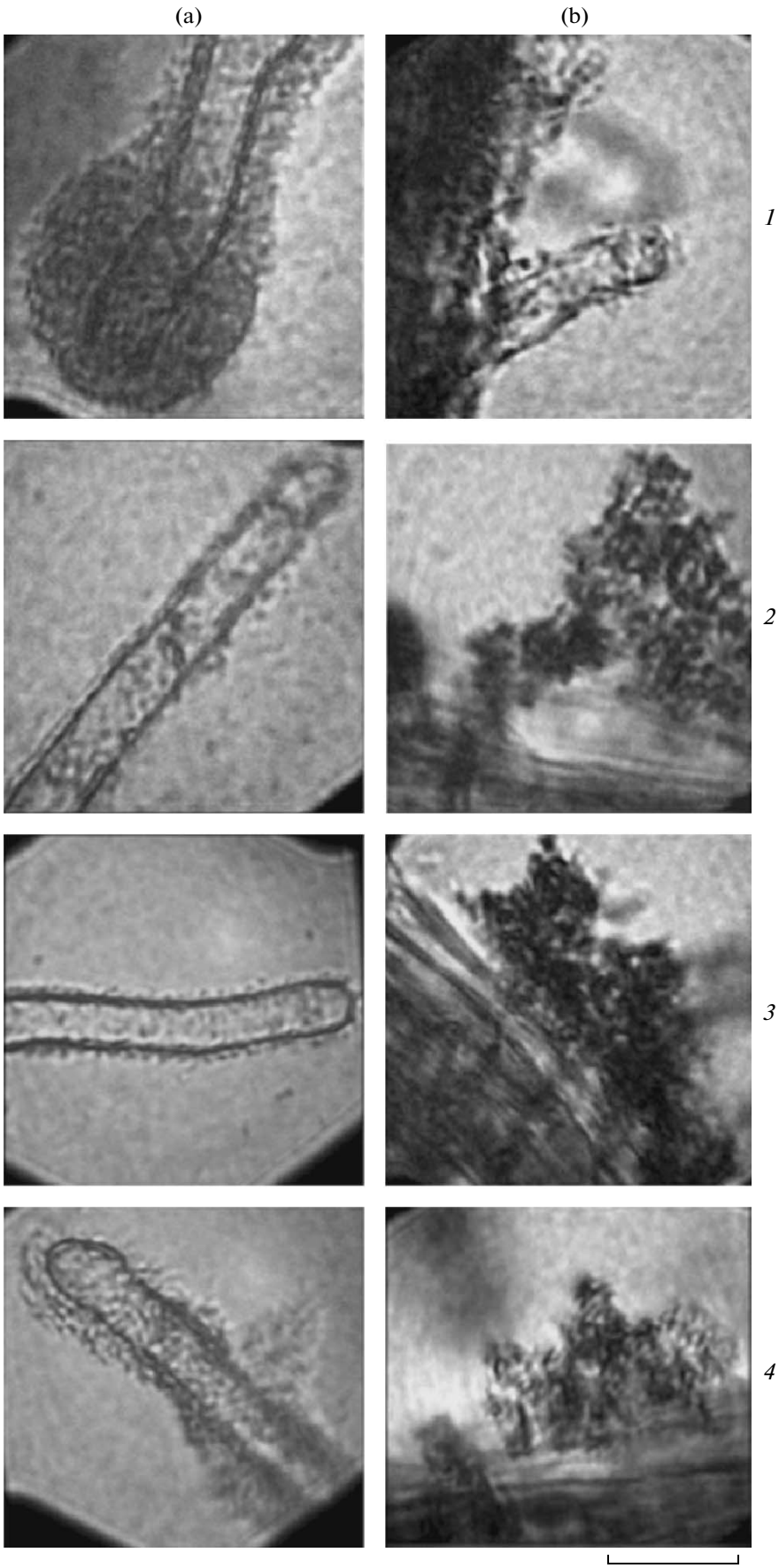
Plasmid composition, colony morphology, and relative biomass content in biofilms of related *A. brasilense* strains

A. brasilense strain	Plasmid molecular mass, MDa	Morphology of colonies on solid MSM	Expression of biofilms* formed during 96 h at 28°C on the surface of	
			glass (A_{590})	polystyrene (A_{570})
Sp7	90, 115, >300	R	0.36 ± 0.03	1.34 ± 0.08
Cd	90, >300	S	0.14 ± 0.01	0.24 ± 0.02
Sp7.K2	94, >300	S	0.19 ± 0.02	0.32 ± 0.02
Sp7.1	124, >300	R	0.24 ± 0.02	0.23 ± 0.03
Sp7.2	131, >300	R	0.40 ± 0.01	0.37 ± 0.04
Sp7.3	121, >300	R	0.30 ± 0.03	0.24 ± 0.02
Sp7.4	131, >300	R	0.33 ± 0.02	0.33 ± 0.02
Sp7.5	94, >300	RS	0.25 ± 0.02	0.30 ± 0.04
Sp7.6	90, >300	S	0.29 ± 0.04	0.23 ± 0.02
Sp7.7	107, >300	S	0.28 ± 0.02	0.36 ±0.03
Sp7.8	124, >300	S	0.25 ± 0.02	0.40 ± 0.05
Sp7.9	94, >300	S	0.24 ± 0.03	0.69 ± 0.09

* The biofilms were stained with crystal violet, bound dye was extracted with the acetone–ethanol mixture, and A_{590} (tubes; a KFK-3 photocolorimeter) or A_{570} (plates; an AIF-Ts-01S analyzer) of the solution were determined [8]. Confidence intervals are given for a 95% significance level.

Phase contrast microscopy of the roots of the seedlings sprouts of the soft spring wheat of Saratovskaya 29 variety after 4–5 (a) and 48 h (b) of inoculation with *A. brasilense* Sp7 (*1*), Cd (*2*), Sp7.K2 (*3*), and Sp7.1 (*4*). The scale bar corresponds to 10 μm.


(a)
(b)
1
2
3
4

Thus, spontaneous rearrangements in plasmid content have a negative effect on *A. brasilense* biofilm formation on hydrophobic and (less frequently) hydrophilic abiotic surfaces. Derivatives of Sp7 lacking p115 and containing the modified pRhico are less active in root colonization during the first hours of interaction. It appears that coordinated expression of the complete set of plasmid genes affecting a wide spectrum of cell structures and functions is important for more rapid adaptation of *A. brasilense* Sp7 to the new environment and for a more successful realization of the complex program for the development of a biofilm microbial community.

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