ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 5, pp. 559–568. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © A.Yu. Pogorelova, A.L. Mulyukin, L. P. Antonyuk, V.F. Galchenko, G.I. El'-Registan, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 5, pp. 618–628.

EXPERIMENTAL ARTICLES

Phenotypic Variability in *Azospirillum brasilense* Strains Sp7 and Sp245: Association with Dormancy and Characteristics of the Variants

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Received November 01, 2008

Abstract—The state of metabolic dormancy in diazotrophic bacteria *Azospirillum brasilense* Sp7 (non-endophytic strain) and Sp245 (endophytic strain) was found to be associated with phenotypic variability. The latter manifested itself in the extension of the spectrum of *A. brasilense* phenotypic variants resulting from plating of cyst-like resting cells (CRC) on solid media and was more pronounced in strain Sp7. The major colony's morphological variants of strain Sp7 were (1) the dominant S type; (2) the highly pigmented Pg type; (3) the R type; (4) the Sm type, forming small colonies; and (5) the Sg type, forming segmented colonies. In addition to their colony morphology, the variants differed in the phenotype stability during transfers on the standard solid medium and in their motility in semisolid agar. The occurrence frequency of the phenotypic variants depended on the conditions and duration of incubation (storage) of the CRC of strain Sp7, as well as on heat treatment (at 55 and 60°C for 10 min) of the cells prior to inoculation. The maximum frequency of S ⟶ Pg transitions (up to 74%) was observed during the germination of CRC stored in a spent culture medium at –20°C for 4 months; the maximum frequency (up to 100%) of S ⟶ Sm transitions was observed after inoculation of the CRC subjected to heat treatment . The Pg variants were the most stable, whereas other types reverted rapidly to the S or Pg variant. The S variant grown in semisolid agar exhibited the mixed type of motility (Swa^+Gri^+, swarming and migration in the form of microcolonies); the Pg and Sg variants showed the Swa^+Gri^- (swarming) phenotype and the Sm variant was nonmotile (Swa^-Gri^- phenotype). The spectrum of phenotypic variants of the endophytic strain Sp245 was narrower than that of strain Sp7 and was represented by S, Sm, and M (mucoid) variants that differed in the patterns of cell motility: the dominant S type displayed the swarming pattern (Swa^+Gri^-), the mucoid M type showed the mixed type (Swa^+Gri^+) of motility, and the Sm variant was nonmotile. The differences between the nonendophytic strain Sp7 and the endophytic strain Sp245 in their capacity for phenotypic dissociation and cell motility in semisolid media may reflect their ability to adapt to changing ambient conditions and specificity of plant–microbial interactions.

Key words: phenotypic variability, cyst-like resting cells, azospirilla, motility of the phenotypic variants.

DOI: 10.1134/S0026261709050051

Gram-negative plant growth-stimulating bacteria of the genus *Azospirillum* are of particular interest in research of symbiotic relationships between bacteria and high plants [1–4]. This interest is due to their ability to inhabit various ecological niches and survive under unfavorable conditions due to their flexible survival strategies [2]. One of them is the acquirement of a dormant stress-resistant state resulting in the formation of cyst-like resting cells (CRC) of various morphological types [5–7]. It was demonstrated that the endophytic (Sp245) and non-endophytic (Sp7) strains of *A. brasilense* specifically interact with their plant partners and differ in the types of dormant cyst-like cells formed under the same cultivation conditions [7].

Another survival strategy of these bacteria under changing ambient conditions is phenotypic (phase) variability of microbial populations, which can be easily diagnosed by the morphology of colonies developing on standard solid media, or by the predominance of certain variants on selective media. This strategy is widely used by bacteria and can be easily revealed during the germination of cyst-like resting cells [8], which was demonstrated by plating of the dormant forms of bacilli, staphylococci, pseudomonads, and other bacteria onto solid media [9–11]. The intrapopulation variability of *Azospirillum* species was studied in detail [12–17], although not in relation to dormancy. When studying the phenotypic dissociation of azospirilla, the main emphasis was placed on patterns of cell motility in semisolid media, as well as on the specific character-

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istics of the cell surface structures, which determine the ability of bacteria to colonize the plant rhizosphere. The differences in the morphological types of the CRC formed by the nonendophytic (Sp7) and endophytic (Sp245) strains of *A. brasilense* under the same growth conditions [7] suggest that there may be interstrain differences in the mechanisms of phenotypic variation.

The aim of the present work was to study the spectra of the phenotypic variants in populations of *A. brasilense* strains Sp7 and Sp245 grown from the dormant cells of different types, as well as to isolate and describe the *Azospirillum* phenotypic variants.

MATERIALS AND METHODS

The subjects of this study were gram-negative bacteria *Azospirillum brasilense*, strains Sp7 (ATCC 29145) and Sp245 obtained from the strain collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov. In both cases, the dominant S variants were used as starters in experiments to produce dormant cells.

Bacteria were cultivated on a standard synthetic medium (pH 7.0) containing the following (g/l): malate, 3.0; yeast extract (Difco), 0.1; $K_2HPO_4$4, 3.0; KH_2PO_4, 2.0; NH_4Cl , 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.1; $CaCl_2$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.02; and $Na_2MoO_4 \cdot 2H_2O$, 0.002. To obtain the stationary-phase cells, the bacteria were cultivated in 250-ml flasks containing 50 ml of the medium on a shaker (120 rpm) at 32°C for 72 h.

Microscopic observations were carried out under a Zetopan light microscope (Reichert, Austria) equipped with a phase-contrast device.

The methods for obtaining and storage of the CRC were previously described in detail in [7]. The viability (CFU) of the vegetative and dormant cells of azospirilla was determined by plating the cell suspensions in respective tenfold dilutions on agarized medium (1.5% of agar) of the above composition. The plates were incubated at 32°C for 14 days. Heat resistance of the cells was determined by retention of viability after heating of the cell suspensions (0.7 ml) in an ultrathermostat at 55 or 60°C for 10 min.

When counting the colonies obtained, the percentage (variation index) of colonies differing from the dominant type in shape, size, consistence, and pigmentation was determined; these colonies were then subcultured. The phenotypic stability of the isolated clones was judged by their ability to maintain the characteristics of the properties of colony morphology after three successive transfers on solid media, as well as after successive subculturing in a liquid medium (until the stationary phase, 72 h), followed by a transfer into solid medium. The motility patterns were determined after stab-inoculation on the semisolid medium (0.5% agar) as described in [15].

The measurements were repeated three times in three independent series of experiments. The data were statistically analyzed by the Student's test. The level of the confidence interval was assumed equal to 0.05% ($P < 0.05$).

RESULTS

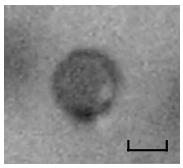
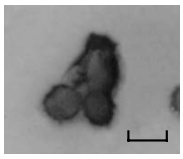
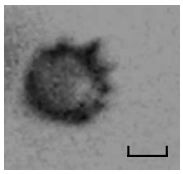
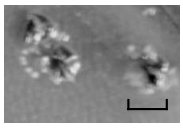
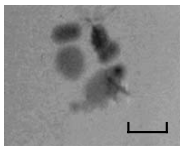
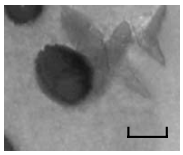
The capacity for phenotypic variation of both strains of *A. brasilense*, Sp7 and Sp245, was determined by the amount of colonies with distinct morphology, which appeared after plating of cyst-like resting cells (CRC) formed in the cultures grown in the standard medium with a fivefold decreased initial nitrogen content [7]. CRC were stored under different conditions: as suspensions of washed cells in saline solution (SS) incubated at 18–20°C (procedure 1); in a spent culture medium incubated at room temperature (procedure 2); in SS, stored at –20°C (procedure 3); and in a spent culture medium (storage at – 20°C) (procedure 4).

Phenotypic variation in *A. brasilense* Sp7. The phenotypic variability of the obtained populations depended on the duration and conditions of CRC storage. In the control, after plating of 3-day post-stationary cultures (the number of viable cells 2×10^8 CFU/ml) of strain Sp7 grown under nitrogen limitation, the S type variant predominated (96%); the highly pigmented Pg type and the R type were minor (3% and 1%, respectively) (Table 1).

Phenotypic variation in the populations obtained on solid medium from the 7-day CRC (at the beginning of CRC storage) was the same as in the control variants (stationary cultures) and did not depend on the conditions of CRC storage. For all the storage procedures, heat treatment of 7-day CRC at 55 and 60°C for 10 min resulted in a decrease in the CFU numbers by four orders of magnitude and altered the variation spectrum, as judged by the predominance of the Sm variant (Table 1) producing small colonies on the 3rd day of incubation and turning brown on the 10th day. The occurrence frequency of the Sm type ranged from 67 to 95%, depending on the procedure of CRC storage (for procedure 1, see Fig. 1; data for all the procedures are shown in Table 2). In the populations grown from the CRC subjected to heat treatment, apart from the Sm variant, Sg variants that formed segmented colonies (Table 1) and highly pigmented Pg variants were present (13 and 18%, respectively) (Fig. 1, Table 2).

An increase in the physiological age of the CRC up to 14 days resulted in further changing in the spectrum of phenotypic variation in the populations grown from these cells. In the procedures 1 and 4 of CRC storage, the proportion of Pg variants increased to 47 and 59%, respectively; in the case of procedure 1, R colonies were detected (11%). Heat treatment (at 55 and 60°C for 10 min) of these CRC also resulted in the predominance of Sm colonies. Analysis of the properties of long-stored (11 months under various conditions) CRC

Table 1. Description of the phenotypic variants of *A. brasilense* Sp7 (non-endophytic strain) and the optimal conditions for their formation

Type	Description	Variant appearance*	Optimal conditions for maximum appearance		Motility type
			CRC storage conditions	Emergence frequency	
S	Rounded pale-yellow colonies with smooth surfaces and edges		Stationary-phase cultures	96%	Swa^+Gri^+
Pg	Rounded highly pigmented dark-brown colonies with smooth surfaces and edges		Procedure 4 (storage for 4 months) without heat treatment	74%	Swa^+Gri^-
R	Rounded pale-yellow colonies with rough surfaces		Procedure 1 (incubation for 14 days)	11%	ND
Sm	Small (d 0.3–0.7 mm) cream-colored colonies with smooth surfaces and edges		Procedures 3 and 4 (storage for 4 months) after heat treatment at 60°C	100%	Swa^-Gri^-, 60% of the colonies of this variant merge on a solid medium
Sg	Oblong cream-colored segmented colonies		Procedure 1 (incubation for 4 months) without heat treatment	31%	Swa^+Gri^-
PgCr	Highly pigmented brown colonies producing extracellular crystals incorporated in the agar layer		Procedure 4 (storage for 4 month) without heat treatment	2%	Swa^+Gri^+

Note: * Scale bar, 5 mm.
ND, not determined.

demonstrated [7] that incubation of dormant cells in a spent culture medium at low temperatures promotes the preservation of their colony-forming ability, whereas the percentage of heat-resistant cells was higher in the populations stored in a spent culture medium at room temperature. The changes in the variation spectrum persisted.

In procedure 1, inoculation of 4-month CRC stored in saline solution at 18–20°C resulted in a decrease in the amount of viable cells by one order of magnitude, as well as in an increase in the proportion of Pg type cells (up to 18%) and the appearance of minor segmented colonies of the Sg type and the SmCr subtype forming small colonies and crystals incorporated in the agar layer, in addition to the dominant S variants. In this variant, the amount of CRC resistant to heating for 10 min at 55°C (but not at 60°C) was low; on solid medium, they all produced colonies of the Sm type (Fig. 1, Table 2).

In contrast to procedure 1, in 4-month suspensions of procedure 2 (spent culture medium, 18–20°C), a decrease in the CFU number (by two orders of magnitude) was observed simultaneously with a significant increase in the ratio of heat-resistant CRC (27% and 1.5% of the CFU titer in untreated suspensions after heating at 55 and 60°C, respectively). The variation

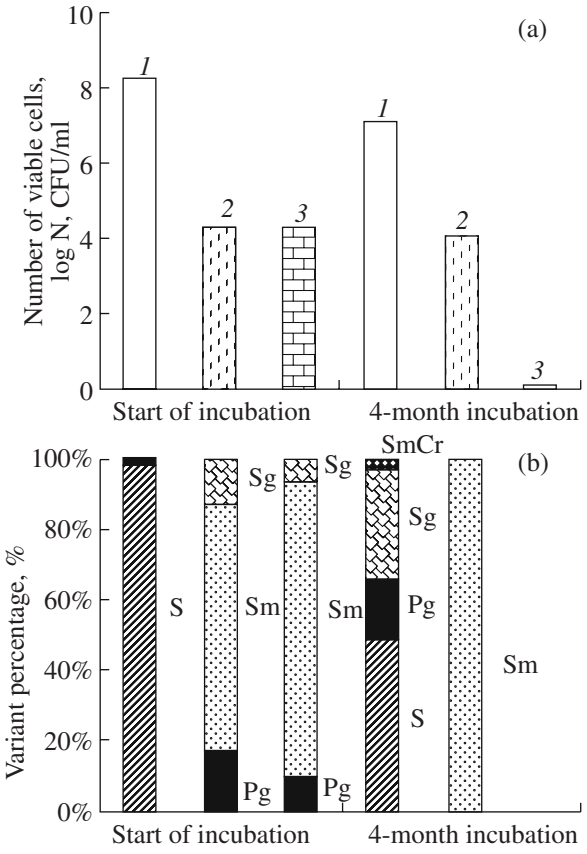


Fig. 1. Number of viable cells (log N, CFU/ml) in the control culture (*1*) and after 10-min heat treatment at 55 (*2*) and 60°C (*3*) and the occurrence frequency (%) of the variants under certain conditions of the germination of *A. brasilense* Sp7 CRC at the initial stage of growth and after 4 months of incubation in saline solution at 18–20°C (procedure 1).

spectrum of the stored CRC population was different: at the beginning of storage (7-day incubation) it was represented by the S, R, and Pg types (95, 3, and 2%, respectively); after 4 months, the proportion of Pg cells increased to 18% due to a decrease in the numbers of S cells and elimination of the R type. It is notable that, in populations grown from heated CRC, the Sm type was predominant, irrespective of the storage period (7 days or 4 months) of CRC (Table 2).

Similar changes in the variation spectrum of the populations grown from the CRC stored for 7 days and 4 months were observed in procedure 3 (SS, –20°C); the proportion of Pg cells in the population increased. After heating of the stored CRC, Sm cells were predominant in the cultures grown from them (Table 2).

The storage of CRC in the spent culture medium at –20°C (procedure 4) improved the preservation of CFU (57% of the CFU titer of a stationary culture; for comparison, the proportion of CFU for procedures 1, 2, and 3 was 5, 0.7, and 2%, respectively). The variation spectrum of the 4-month CRC population in variant 4 differed considerably from the variation spectra of the CRC populations stored under different conditions (procedures 1–3) and was represented, for the most part, by Pg colonies, while the amount of S cells was low; the minor variant PgCr, highly pigmented and producing extracellular crystals, was detected as well. After heating (at 55°C for 10 min) of the CRC (stored according to procedure 4), a new variant PgSm that produced small dark-brown colonies was detected in an equal proportion to the Sm variant (Table 2).

Hence, it was demonstrated that the germination of CRC of the nonendophytic strain *A. brasilense* Sp7 is associated with the enhanced phenotypic variability, whereas the percentage of the variants revealed by CRC plating under the same growth conditions depends on the conditions and length of storage of the dormant forms.

Phenotypic variation in *A. brasilense* Sp245. The relationship between changes in the variation spectrum of the populations of germinating CRCs and the conditions and duration of their storage was demonstrated for the endophytic *Azospirillum* strain Sp245. In the control cultures, when stationary-phase cells grown under nitrogen limitation were plated on solid medium (titer 1.1×10^8–1.6×10^8CFU/ml), similar to strain Sp7, the S variant was predominant (95%); slimy colonies of the minor mucoid M type were detected as well (5%) (Table 3). The numbers of viable CRC incubated in SS at room temperature (18–20°C) for 6 months decreased by two orders of magnitude (4.5×10^6 CFU/ml; 2.7–4% of the stationary cell number). After the germination of long-stored dormant cells on solid medium, Sm variants (35%), forming small colonies, were detected, in addition to the S and M variants (55 and 10%, respectively) (Table 3).

Heat treatment of the 6-month CRC suspensions of strain Sp245 (55°C, 10 min) resulted in a decrease in the number of viable cells to 1.1×10^4 CFU/ml (by two orders of magnitude as compared to untreated suspensions), as well as in an increased content of the Sm and M variants up to 70 and 20%, respectively, accompanied by a decrease in the proportion of the dominant S type to 10% (Table 3). Similar variant percentages were observed on the plates inoculated with the CRC populations heated at 60°C; however, the amount of viable cells was extremely low (100 CFU/ml). It should be noted that, unlike strain Sp7, the population spectrum of strain Sp245 was poor: other procedures for cultivation (on a nitrogen-limited synthetic medium) and CRC storage (in SS or a spent culture medium, at room temperature or in a frozen state), did not yield other variants than the S, M, and Sm types.

Thus, the nonendophytic and endophytic *A. brasilense* strains (Sp7 and Sp245, respectively) differ in the efficiencies of production of various dormant morphotypes under unfavorable conditions [7], as well as in the properties and diversity of the dormant forms. It was demonstrated by the analysis of the phenotypic variation in the populations grown from the dormant

Table 2. Amount of viable cells and the occurrence frequency of the variants during the germination of the cells of *A. brasilense* Sp7 incubated under different conditions

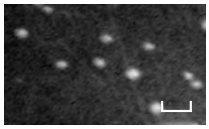
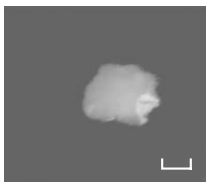
Incubation/storage procedure	Amount of viable cells, CFU/ml		Variant percentage, %	
Saline solution, 18–20°C	Start of incubation (7 days)			
	Without heat treatment	$(2.1 \pm 0.3) \times 10^8$	S type, Pg type	97 3
	Heat treatment at 55°C, 10 min	$(2.0 \pm 0.2) \times 10^4$	Sm type, Pg type, Sg type	67 17 13
	Heat treatment at 60°C, 10 min	$(2.0 \pm 0.1) \times 10^4$	Sm type, Pg type, Sg type	84 10 6
	4-month incubation			
	Without heat treatment	$(1.1 \pm 0.1) \times 10^7$	S type, Sg type, Pg type, SmCr type	48 31 18 4
	Heat treatment at 55°C, 10 min	$(1.4 \pm 0.1) \times 10^4$	Sm type	100
	Heat treatment at 60°C, 10 min	0	–	–
Spent culture medium, 18–20°C	Start of incubation (7 days)			
	Without heat treatment	$(2.1 \pm 0.3) \times 10^8$	S type, R type, Pg type	95 3 2
	Heat treatment at 55°C, 10 min	$(2.0 \pm 0.2) \times 10^4$	Sm type, Pg type, Sg type	72 18 10
	Heat treatment at 60°C, 10 min	$(2.0 \pm 0.1) \times 10^4$	Sm type, Pg type	90 10
	4-month incubation			
	Without heat treatment	$(1.6 \pm 0.3) \times 10^6$	S type, Pg type	75 25
	Heat treatment at 55°C, 10 min	$(4.3 \pm 0.4) \times 10^5$	Sm type, Pg type	88 12
	Heat treatment at 60°C, 10 min	$(1.9 \pm 0.2) \times 10^4$	Sm type, Pg type	83 17
Saline solution, –20°C	Start of incubation (7 days)			
	Without heat treatment	$(2.1 \pm 0.3) \times 10^8$	S type, Pg type	96 4
	Heat treatment at 55°C, 10 min	$(2.0 \pm 0.2) \times 10^4$	Sm type, Pg type	90 10
	Heat treatment at 60°C, 10 min	$(2.0 \pm 0.1) \times 10^4$	Sm type, Pg type	95 5
	4-month incubation			
	Without heat treatment	$(4.0 \pm 0.6) \times 10^6$	S type, Pg type	74 26
	Heat treatment at 55°C, 10 min	$(2.6 \pm 0.4) \times 10^3$	Sm type, S type	89 11
	Heat treatment at 60°C, 10 min	$(1.9 \pm 0.4) \times 10^3$	Sm type	100

cells of these bacteria, first of all qualitatively, by the diversity of colonial morphological variants revealed under standard conditions (Tables 1 and 3). It can be suggested that the wider variation spectrum of nonendophytic azospirilla (as compared to endophytic) increases their adaptive capacity during saprotrophic growth and in response to unfavorable conditions (simulated in this study by CRC heating). Changes in the variant percentage of *A. brasilense* observed as its dormant forms enter a new growth cycle may be regarded as one of the important mechanisms of bacterial adaptation to new growth conditions.

Table 2. (Contd.)

Incubation/storage procedure	Amount of viable cells, CFU/ml		Variant percentage, %	
Spent culture medium, –20°C	Start of incubation (7 days)			
	Without heat treatment	$(2.1 \pm 0.3) \times 10^8$	S type, Pg type	95 5
	Heat treatment at 55°C, 10 min	$(2.0 \pm 0.2) \times 10^4$	Sm type, Pg type	89 11
	Heat treatment at 60°C, 10 min	$(2.0 \pm 0.1) \times 10^4$	Sm type, Pg type	94 6
	4-month incubation			
	Without heat treatment	$(1.2 \pm 0.1) \times 10^8$	S type, Pg type, PgCr type	24 74 2
	Heat treatment at 55°C, 10 min	$(5.0 \pm 0.8) \times 10^3$	Sm type, PgSm type	52 48
	Heat treatment at 60°C, 10 min	$(4.1 \pm 0.3) \times 10^3$	Sm type	100

Table 3. Properties of the phenotypic variants of *A. brasilense* Sp245 (endophytic strain)

Type	Description	Variant appearance*	CRC storage conditions	Emergence frequency	Motility type
S	Rounded pale-yellow colonies with smooth surfaces and edges		Stationary-phase cultures on the standard medium and under a C : N unbalance	95%	Swa^+Gri^-
			CRC suspensions (growth at a C : N unbalance, transfer to saline solution, incubation for 6 months)	55%	
			The same variant after heat treatment at 55°C	10%	
Sm	Small semi-transparent colonies (d 0.3–0.7 mm) with smooth surfaces and edges		CRC suspensions (growth at a C : N unbalance, transfer to saline solution, incubation for 6 months).	35%	$Swa^-Gri^{+/-}$
			The same variant after heat treatment at 55°C	70%	
M	White colonies with slimy surfaces, raised center, and uneven edges		CRC suspensions (growth at a C : N unbalance, transfer to saline solution, incubation for 6 months).	10%	Swa^+Gri^+
			The same variant after heat treatment at 55°C	20%	

Note: * Scale bar, 3 mm.

Characterization of *A. brasilense* variants. The differences in the properties of the variants determine the tolerance range of bacterial populations with respect to many growth parameters. The following experiments were aimed to reveal the differences between *A. brasilense* Sp7 and Sp245 variants, especially with respect to their motility as an ecological trait determining their ability to interact with a plant partner. Importantly,

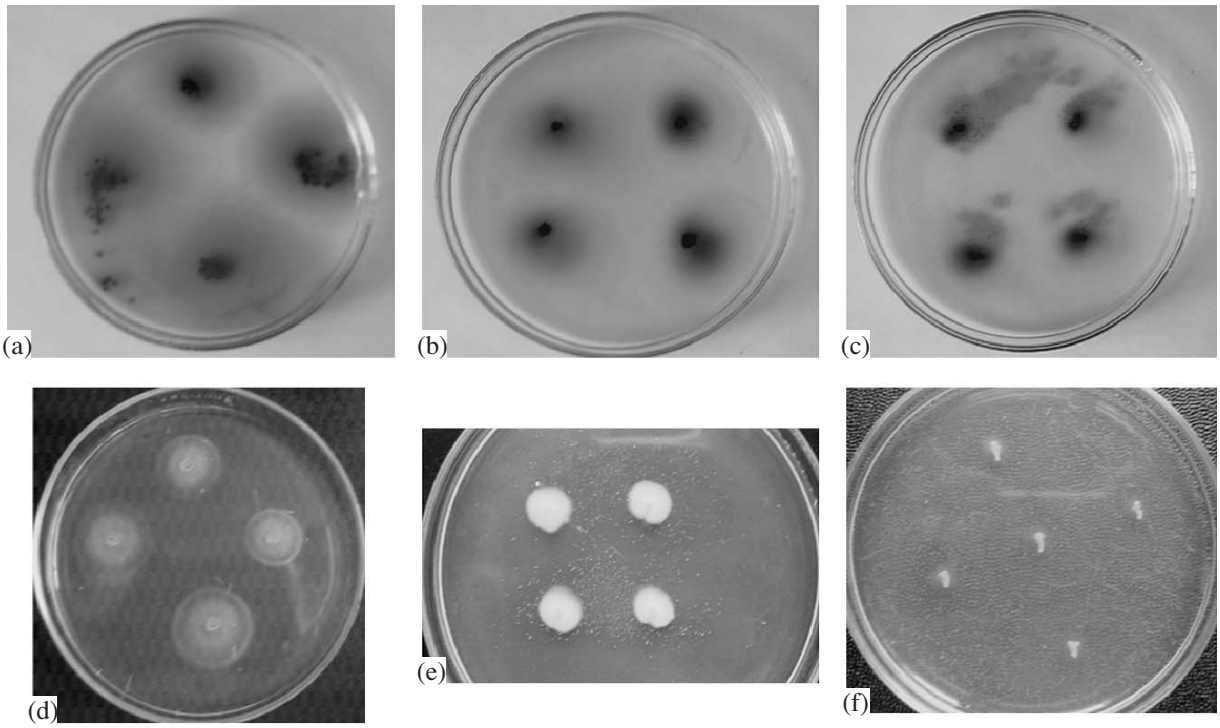


Fig. 2. Types of motility of *A. brasilense* variants in semisolid agar for strain Sp7: S type (a), Pg type (b), and PgCr type (c); strain Sp245: S type (d), M type (e), and Sm type (f). See Tables 1 and 2 for designations.

growth conditions have a selective effect on the development of certain variants, whereas the standard conditions reveal the stability of their properties.

In our experiments, we demonstrated that the strain Sp7 variants differed in the stability of their colonial morphology characteristics after successive culture transfers on solid media, as well as after successive subculturing in liquid medium (until the stationary phase, 3 days), followed by plating of culture aliquots on agar medium. For instance, after the first transfer on the solid medium, the Pg variant completely (100%) retained its main trait (the ability to produce a brown pigment); however, in the second and third transfers, 65- and 95-% reversions occurred, respectively, of the Pg variant to the dominant one. After cultivation in liquid medium with subsequent plating onto the solid medium, the frequency of Pg⟶S transitions was 67%. In the first transfer of the Sm variant on agar, only 5% of its ability to form small colonies was retained; reversion to the S and Pg types was 45 and 50%, respectively, and in subsequent transfers it was completely substituted by these types. A similar frequency of Sm⟶Pg and Sm⟶S transitions after a "liquid culture – solid medium" transfer was observed, which was assessed by colony counting after inoculation of 10^5-fold dilutions of the suspensions. However, after inoculation of less diluted (10^4-fold) suspensions, most colonies (91%) were of the Sm type, although the number of colonies per standard petri dish did not exceed 150. In both schemes of transfer, the Sg variant was instable and was substituted almost completely by the dominant S type or the Pg type after the second culture transfer.

Hence, during growth under the standard conditions, favorable for the development of the S type cells, the Pg variant survived more transfers than other variants; however, all of them finally reverted to the initial S type. The observed reversibility of transitions can be viewed as an argument eliminating the culture contamination with a foreign microflora and confirming that all these colonial morphologial variants belonged to *A. brasilense* Sp7.

The differences between *A. brasilense* Sp7 variants existed also in their motility in semisolid media (0.5% agar). The dominant S and PgCr variants that produced colonies of different morphologies were characterized by the mixed type of motility (Swa^+Gri^+, swarming and migration in the form of microcolonies) (Figs. 2a and 2c). The Gri type of motility of cell groups predominated in the PgCr variant (Fig. 2b); the distribution of cell clusters that stopped moving and formed brown colonies was irregular, suggesting the phenotypic heterogeneity of this variant. The Pg (Fig. 2b), Sg, and some minor variants (Table 1) were able only to swarm (Swa^+Gri^- type of motility). Although the Sm type did not exhibit stability of its colony morphology, it should be considered a variant, since it, unlike the others, was nonmotile (Swa^-Gri^-) in semisolid agar.

Similar differences in the motility types were found between the S, M, and Sm variants of the endophytic

strain Sp245 (Table 3). The dominant S variant was characterized by the Swa^+Gri^- type of swarming, while the mucoid (M) variant exhibited the Swa^+Gri^+ type of motility in which a swarming colony is surrounded by small colonies produced by migrant cell groups (Figs. 2d and 2e). Unlike these variants, the Sm cells were nonmotile in semisolid agar (Swa^-Gri^-); microcolonies formed in the agar layer along the stab line of inoculation (Fig. 2f). No super-swarming variants of the endophytic strain were detected.

Hence, the intrapopulation variants of the endophytic and nonendophytic strains of *A. brasilense*, appearing during the germination of cyst-like resting cells of these bacteria, differed both in their morphological properties and it the types of cell motility that determine their ability to colonize the plant rhizosphere.

DISCUSSION

The results of our investigation demonstrate that the symbiotrophic bacteria of the genus *Azospirillum*, as well as saprotrophic bacteria [8–11, 18, 19], exhibit dormancy coupled with an enhanced level of intrapopulation phenotypic variability. Both nonendophytic (Sp7) and endophytic (Sp245) strains of *A. brasilense* were able to produce cyst-like cells [5–7] that ensure species survival, and the germination of these cells and their entrance to a new growth cycle was accompanied by the intrapopulation variability. Both properties are constituents of one adaptation mechanism providing population (species) survival and development under new or unfavorable conditions.

The capacity for phenotypic variation depended on the conditions and duration of storage of the CRC of *A. brasilense* Sp7 and Sp245 (Fig. 1, Table 2). This conclusion consists of two statements. The first one is a confirmed fact that in symbiotrophs (introduced as new model objects), the CRC exhibit genotypic instability, which results in the phenotypic variability of the populations grown from these cells. This important property of cyst-like resting cells (as compared to other dormant forms, e.g., endospores), which promotes the species adaptation to ambient conditions, has been previously demonstrated when analyzing the spectra of colony morphology in the populations grown from the cyst-like resting cells of *Bacillus cereus* [9], *B. licheniformis* [19], *Pseudomonas aurantiaca, P. fluorescens* [11], etc. The second statement is based on the fact that, in this study, some variants of CRC storage conditions (spent culture medium, 18–20°C) allowed the maturation of dormant cells (including the structural reorganization of DNA), whereas other variants (SS, –20°C) impeded CRC maturation. While the changes in the DNA topology increase its stability in dormant cells, which was reported earlier [20–22], they also promote intragenomic alterations resulting in phenotypic variability. Thus, an enhanced phenotypic variability observed during the CRC germination can be considered a characteristic trait of dormant forms of this morphotype for both gram-negative and gram-positive bacteria [8–11, 18, 19]. Since cyst-like resting cells widely occur in natural populations [8, 23, 24] and are regarded as a form that ensures the survival of azospirilla in plant kernels between vegetation periods [25], enhanced phenotypic variability during the CRC germination is part of the total adaptive potential of populations.

This thesis was additionally confirmed by the results of our experiments indicating that unfavorable conditions (heating at 55 and 60°C for 10 min) inhibited almost completely the growth of the dominating phenotypes of strain Sp7, yielding S or Pg colonies, while the obtained population consisted of small Sm and PgSm colonies, which were not revealed in the populations grown from untreated suspensions (Table 2). Under similar conditions of heat treatment, elimination of the dominant S type of strain Sp245 and the predominance of the Sm variant were observed. Comparative analysis of changes in the number of CFU in the CRC suspensions subjected to heat treatment, as well as in the variation index determined by plating, did not allow us to conclude that heat treatment exerts a preferential selective or inducing effect on the development of the Sm variant. Noteworthy, the Sm variant that was nonmotile in semisolid agar (Tables 1 and 3, Fig. 2) was instable under standard growth conditions and reverted rapidly to the dominant S and Pg types. Hence, the capacity for motility in semisolid media, an important trait that determines the ability of bacterial cells to colonize the plant rhizosphere, was rapidly restored. We would like to emphasize that the application of heat treatment in our experiments resulted in the extension of the spectrum of phenotypic variation in bacterial populations grown from CRC due to the emergence of the Sm phenotype. Selection of favorable conditions for selective growth of a certain variant, which can remain latent under standard conditions, has a practical value for the search of the variants with desired properties.

The development of the phenotypic variants SmCr and PgCr from the cyst-like resting cells of strain Sp7 on the solid medium was accompanied by the formation of large extracellular crystals incorporated in the agar layer, which was previously observed in the case of *A. brasilense* Sp245 grown in a liquid malate-containing medium [26, 27]. These crystals were represented by struvite ($MgNH_4PO_4 \cdot 6H_2O$) kins [26], which contained Na^+, K^+, Ca^{2+}, Mn^{2+}, and $Fe^{2+/3+}$ as trace constituents and the anions Cl^-, HPO_4^{2-}, $H_2PO_4^-$, and SO_4^{2-} [27]. It seems likely that the struvite production by azospirilla is due to local alkalization of the medium and binding of ammonia nitrogen. It is quite possible that the ability to produce the extracellular crystals indicates the existence of a certain mechanism for ammonium ion detoxification in these *Azospirillum* variants. However, the question of the nature and role of crystal formation in the SmCr and PgCr variants of azospirilla requires additional study.

Reversible phenotypic transitions (phase variations) in bacteria are due to various genetic mechanisms [28]. The appearance of similar variants in different bacterial species and strains may be due to various intragenomic alterations. For instance, the phase transition accompanied by emergence of the nonmotile variant, as previously described for *A. lipoferum* [13] and other *Azospirillum* species, correlated with changes in the plasmid profile (disappearance of the 750- and 260-kbp plasmids in *A. lipoferum* 4B and *A. brasilense* WN1, respectively, and emergence of a changed (by the removal of a 160-kbp fragment) 1400-kbp plasmid in *A. irakense* KBC1 [17]). The S variant of *A. brasilense* Sp7 differed from the R variant in the lack of the 115-MDa plasmid [12]; the uncharacteristic R⟶S transition of this strain was associated with the redistribution of two O-specific polysaccharides on the cell surface in both variants depending on the culture age [16]. In addition, the differences were revealed between the parent strain *A. brasilense* Sp245 and its spontaneous mutants (deficient in the synthesis of lipopolysaccharides and calcofluor-binding polysaccharides and missing the RP4 plasmid and the resident plasmids p85 and p120) in their abilities to form biofilms [29].

Finally, another important result of this work is demonstration of the differences between the nonendophytic (Sp7) and endophytic (Sp245) strains of *A. brasilense* in the diversity of phenotypic variants, in addition to the previously described strain-specific peculiarities with regard to the CRC formation under similar cultivation conditions [7]. It seems likely that the enhanced phenotypic variability of the nonendophytic strain Sp7, resulting in the appearance of the wider diversity of variants during the CRC germination, provides the flexibility for the strain survival, taking into account the fact that, in the case of strain Sp7, the connection between its cells and the plant partner is less pronounced than in the case of the endophytic strain Sp245.

On the whole, the ability of the bacterium *A. brasilense* to produce various morphological types of cyst-like resting cells differing in the duration of viability and heat resistance [5–7], as well as in their capacity for phenotypic variation resulting in the appearance of colonial morphological variants with different physiological properties, broaden our understanding of the mechanisms that ensure the survival of these bacteria under natural conditions.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (projects nos. 07-04-01011 and 08-04-00164) and the grant of the President of the Russian Federation (project NSh-676177.2006.4).

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