ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 1, pp. 33–41. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © A.L. Mulyukin, N.E. Suzina, A.Yu. Pogorelova, L.P. Antonyuk, V.I. Duda, G.I. El-Registan, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 1, pp. 42–51.

EXPERIMENTAL ARTICLES

Diverse Morphological Types of Dormant Cells and Conditions for Their Formation in *Azospirillum brasilense*

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Received March 25, 2008

Abstract—Differences in generation of dormant forms (DF) were revealed between two strains of non-spore-forming gram-negative bacteria *Azospirillum brasilense*, Sp7 (non-endophytic) and Sp245 (endophytic strain). In post-stationary ageing bacterial cultures grown in a synthetic medium with a fivefold decreased initial nitrogen content, strain Sp7 formed two types of cyst-like resting cells (CRC). Strain Sp245 did not form such types of DF under the same conditions. CRC of the first type were formed in strain Sp245 only under phosphorus deficiency (C > P). The endophytic strain was also shown to form structurally differentiated cells under complete starvation, i.e. at a transfer of early stationary cultures, grown in the media with C > N unbalance, to saline solution (pH 7.2). These DF had a complex structure similar to that of azotobacter cysts. The CRC, which are generated by both azospirilla strains and belong to distinct morphological types, possessed the following major features: absence of division; specific ultrastructural organization; long-term maintenance of viability (for 4 months and more); higher heat resistance (50–60°C, 10 min) as compared with vegetative cells, i.e. the important criteria for dormant prokaryotic forms. However, CRC of non-endophytic strain Sp7 had higher heat resistance (50, 55, 60°C). The viability maintenance and the portion of heat-resistant cells depended on the conditions of maturation and storage of CRC populations. Long-term storage (for 4 months and more) of azospirilla DF populations at –20°C was optimal for maintenance of their colony-forming ability (57% of the CFU number in stationary cultures), whereas the largest percentage of heat-resistant cells was in CRC suspensions incubated in a spent culture medium (but not in saline solution) at room temperature. The data on the intraspecies diversity of azospirilla DF demonstrate the relation between certain type DF formation to the type of interaction (non-endophytic or endophytic) with the plant partner and provide more insight into the adaptation mechanisms that ensure the survival of gram-negative non-spore-forming bacteria in nature.

Key words: Azospirillum brasilense Sp7 and Sp 245, cysts, cyst-like cells, dormancy morphotypes, ultrastructure.

DOI: 10.1134/S0026261709010056

The genus *Azospirillum* of the class *Alphaproteobacteria* currently comprises of 11 species, with some of them described only recently [1]. Among azospirilla, the species *Azospirillum brasilense* attracts most attention as a model object in studies for associative and endophytic rhizosymbioses formed by bacteria and higher plants [2–5]. Azospirilla, representatives of growth-stimulating rhizobacteria, are characterized by the large size of their genome: from 4800 kbp in *A. irakense* to 9700 kbp in *A. lipoferum* [6]. High information capacity and plasticity of the genome enables them to occupy various ecological niches and to exist under unfavorable conditions due to flexible strategies of survival [3].

The overwhelming majority of *A. brasilense* isolates inhabit the roots of cultivated and wild plants from tropical, temperate and northern latitudes [1–3]. Under natural conditions, azospirilla have two lifecycle phases associated with a change in climatic conditions and vital functions of a host plant. Thus, the phase of pronounced activity of azospirilla concurs with the vegetation period of a colonized plant, while the phase of dormancy (in winter periods) concurs with the phase of dormancy of a host plant. The main array of experimental data on physiology, biochemistry, genetics and ecology of *A. brasilense* was obtained in experiments with vegetative actively growing cells [2–5]. As to the dor-

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mant forms (DF) of these bacteria, they have been described only in few publications. There are works [7, 8] on cyst formation in azospirilla and a publication [9] with experimental evidence of survival of *A. brasilense* and *A. lipoferum* in a dormant state within the seeds of forage herbs. It should be noted that encysted dormant forms of *A. brasilense* were obtained in the experiments [7, 8] only for strain Sp7 grown on solid, semisolid, and in liquid media with fructose and the minimal content of a nitrogen source, while the structural type especially resembling the cysts of *Azotobacter vinelandii* [10] was revealed in dried pigmented colonies [8].

It should be emphasized that azospirilla strains differ in their interactions with plant partners (associative and endophytic) and contribution to plant growth and development [2–5]. Bacterization of seeds with non-endophytes, e.g. *A. brasilense* Sp7, rarely results in an increase in crop yields. Among endophytes, *A. brasilense* Sp245, the strain providing significant stimulation for host plant growth and development, has been studied best [2–5]. Both strains colonize wheat plants during the vegetation period, but the population of strain Sp245 is localized mainly inside the root while the population of Sp7 is localized on its surface only [11, 12]. It is also known that azospirilla have different localization in ecotopes not only in the vegetative but also in the dormant phase of development: some strains survive the period of winter dormancy in seeds [9] while other strains survive it in soil [13].

In light of the demonstrated ability of *A. brasilense* Sp7 to produce cyst-like cells in colonies or flocks, it is worthwhile to pose a question on survival strategies of this non-endophytic bacterium under different growth conditions (as well as of the other, endophytic strain Sp245 with unknown ways of survival). At the same time, the survival strategies of these strains associated with entering dormancy, particularly in the natural habitats, may be different.

The goal of this work was the comparative study of the formation of dormant cells by two strains of *A. brasilense*, Sp7 and Sp245, having distinct types of interaction with plants, under different conditions.

MATERIALS AND METHODS

The objects of research were gram-negative bacteria *Azospirillum brasilense* Sp7 (ATCC 29145) and Sp245 from the collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov).

Bacteria were grown in a basal synthetic nutrient medium containing (g/l): malate, 3.0; yeast extract (Difco), 0.1; K_2HPO_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; $Na_2MoO_4 \cdot 2H_2O$, 0.002; pH 7.0. Bacteria were cultivated in 250-ml flasks (with 50 ml of the medium) at 32°C in a shaker (120 rpm) for 36–48 h to the stationary phase.

The nutrient medium was modified to obtain azospirilla DF by a fivefold decrease in nitrogen (NH_4Cl, 0.3 g/l) or phosphorus (K_2HPO_4, 0.6 g/l; KH_2PO_4, 0.4 g/l) content. Another approach to produce DF was based on complete starvation by shifting the stationary phase culture cells, washed three times from the growth medium, to saline (0.9% NaCl) solution (SS). Dormant azospirilla cells formed in post-stationary cultures were incubated (stored) for 4 months or more: in the spent (native) culture medium at room temperature (variant 2) or at –20°C (variant 4) and as a suspension in SS under the same temperature conditions (variants 1 and 3, respectively).

The viability (CFU) of azospirilla was determined by inoculation of 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 3–7 days. Another method involved treatment of 5-μl-aliquots of suspensions of dormant *A. brasilense* cells with a two-component dye, Live/Dead Baclight kit (Molecular Probes Inc.), according to the manufacturer's recommendations. Stained specimens were examined in an Axioplan fluorescent microscope (Carl Zeiss, Germany) with the 100 × 1.3 objective. At least 20 fields of vision were viewed for cell counting.

Heat resistance of the cells was determined as the number of viable cells (CFU) remaining viable after heating of the cell suspensions (0.7 ml) in an ultrathermostat at 50, 55, or 60°C for 10 min.

Microscopic observations were carried out in a Zetopan light microscope (Reichert, Austria) equipped with a phase-contrast device. For electron microscopic studies, precipitated cells were fixed in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, then washed three times in the same buffer and postfixed in 1% OsO_4 solution in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration, the material was embedded in Epon 812 resin. Ultrathin sections were contrasted for 30 min in 3% uranyl acetate solution in 70% alcohol and additionally stained with lead citrate according to Reynolds at 20°C for 4–5 min. The sections were examined in a JEM-100B electron microscope (Japan) at the accelerating voltage of 80 kV.

Measurements were repeated three times in three independent series of experiments. The results show the averaged values. The data were statistically analyzed by the Student's test ($P < 0.05$).

RESULTS

Since the efficiency of CRC formation is determined, among other factors, by cultivation conditions [14, 15], the composition of the nutrient media was modified in the first series of experiments with the strains *A. brasilense* Sp245 and Sp7 in order to create the unbalance of nitrogen or phosphorus sources: C > N or C > P. As has already been shown for other bacteria,

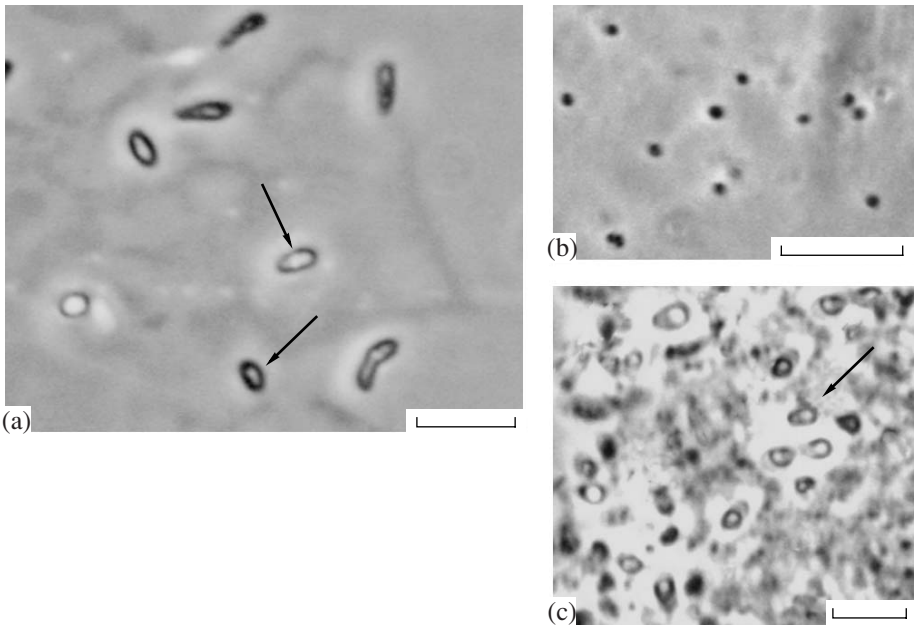

Fig. 1. CRC of *A. brasilense* under phase-contrast microscope: (a) Sp7 and (b) Sp245 in post-stationary (3-day) cultures and (c) suspensions of Sp245 cells starved in saline solution. Scale bar: 10 μm.

such an unbalance intensifies the biosynthesis of anabiosis autoinducers (alkylhydroxybenzenes), which facilitate the formation of cyst-like resting cells (CRC) [14, 15].

A. brasilense Sp245 and Sp7 were cultivated in the basal and modified media; the grown cultures were then incubated for 4 months or more at room temperature (18–20°C). Comparative analysis of these long-stored cultures revealed the differences between strains Sp245 and Sp7 in their ability to form DF under the same cultivation conditions. Microscopic observations showed that the highest yield of morphologically differentiated forms (40–50% of total cell number), resembling refractile CRC of some non-spore-forming bacteria [14, 15], was observed for strain Sp7 (non-endophyte) in post-stationary cultures grown in the medium with a fivefold decreased initial in nitrogen content (Fig. 1a). In the stored cultures of strain Sp7 grown in a complete medium or under a decreased phosphorus content, the portion of such CRC was much less and did not exceed 3–5%. No dividing and motile cells were revealed in any of the variants. In the other azospirilla strain, Sp245 (endophyte), analogous CRC were formed only in post-stationary cultures grown on the medium with a lower phosphorus content; they accounted for 35–45% of the total number of cells in the stationary phase culture (Fig. 1b). In the cultures of strain Sp245 grown on a complete medium or at a lower nitrogen content, the portion of CRC was much lower (1–3%) and most cells (85%) showed signs of autolysis.

Strains Sp7 and Sp245 exhibited the greatest differences in their adaptation responses to complete starvation. Under these conditions, strain Sp245 could form DF that morphologically resembled azotobacter cysts [10]. Luxurant formation of DF of this morphotype (Fig. 1c) was observed when *A. brasilense* Sp245 was cultivated on a medium with a five times decreased content of NH_4Cl till the onset of the stationary phase (28 h); then the cells were washed from the growth medium and transferred to a starvation medium (saline solution, pH 7.2) and resultant cell suspensions were incubated for 4 months or more at room temperature.

More noticeable differences between the DF of *A. brasilense* Sp7 and Sp245 were revealed in their ultrastructural organization. It should be noted that the ageing cultures of both azospirilla strains contained no dividing cells and cells with flagella. CRC of strain Sp7 (the medium with a low nitrogen content, 4 months of incubation, T = 18–20°C) had the following features differentiating them from vegetative cells (Fig. 2a): thickening of cell envelopes, presence of intracellular inclusions of two types: polyhydroxyalkanoates and polyphosphates, compaction of the nucleoid with electron-dense DNA strands, and inhomogeneous texture of the cytoplasm (Figs. 2, 3). In addition to the above general characteristics, CRC of strain Sp7 differed in the structure of cell envelopes, basing on which it was possible to distinguish two structural types of CRC. The first morphotype of CRC was characterized by (1) easily distinguishable periplasmic space with low electron density (Fig. 2b); (2) additional layers in electron-dense covers, well visible in some cells (Fig. 2c); (3) a pronounced capsular layer; and (4) extracellular electron-dense melanin-like granules attached to the capsular layer.

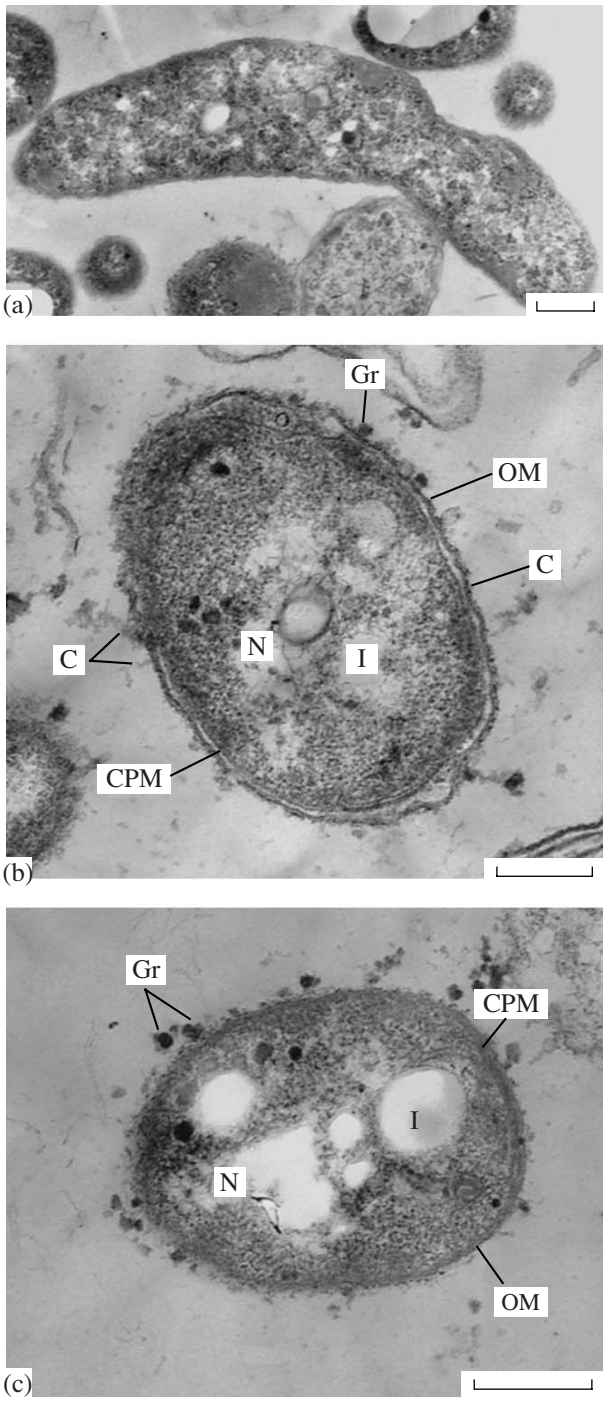

Fig. 2. Ultrathin sections of *A. brasilense* Sp7 vegetative cells (a) in 36-h cultures and CRC of the first type (b, c) in 4-month cultures grown on the medium with C > N unbalance. Designations: C, capsule; OM, outer membrane; CPM, cytoplasmic membrane; N, nucleoid; I, inclusions; Gr, granules on cell surface. Scale bar: 300 nm.

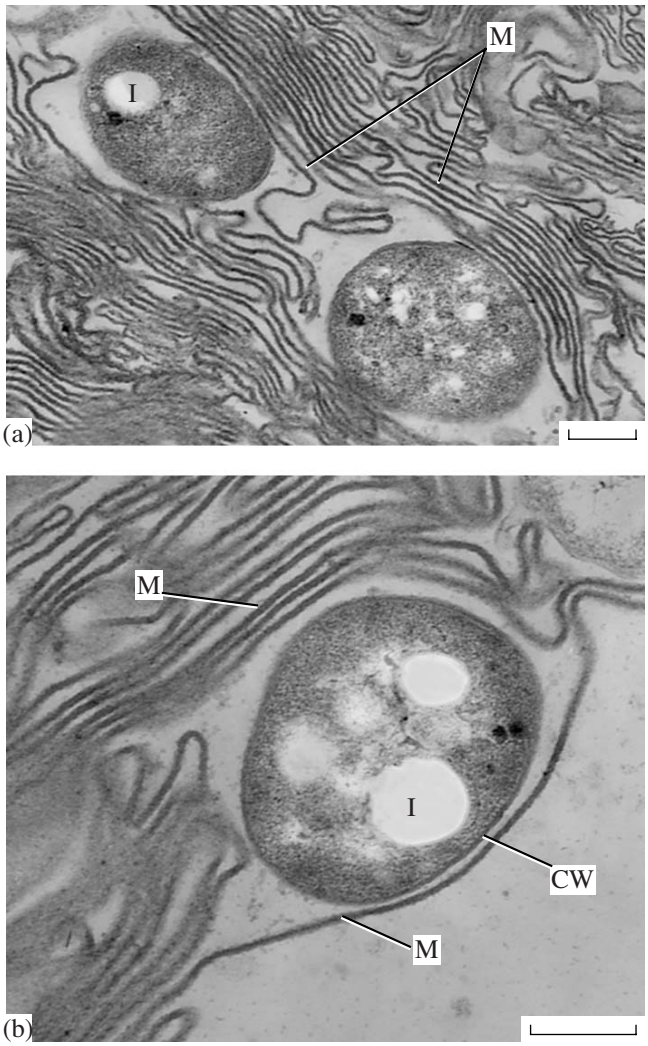

Fig. 3. Ultrathin sections of Sp7 CRC of the second type (a, b) in long-incubated cultures (C > N unbalance, 4 months). Designations: M, extracellular thread-like matrix; CW, cell wall; I, inclusions. Scale bar: 300 nm.

Of great interest is the structural organization of Sp7 CRC of the second type formed in cell clumps in the cultures grown under a decreased initial nitrogen content and stored under static conditions (without agitation, T = 18–20°C). Intact CRC of *A. brasilense* Sp7 of this type had no capsular layer but were enclosed in a vast matrix synthesized de novo and represented by the structures forming numerous folds or piles (Fig. 3a, b). Apparently, this matrix formed by the exometabolites of azospirilla provides additional protection for the dormant cells of non-endophytic bacteria from damage, which is in agreement with the higher heat resistance of the DF obtained under these cultivation conditions (see below, Table 1).

The additional test for heterogeneity of DF populations of *A. brasilense* Sp7 was based on fluorescence microscopic analysis using the Live/Dead stain. Azospirilla DF differed from each other in the pattern of fluorescence (intensity and color). For example, the population of DF formed under C > N unbalance contained, besides single CRC with green fluorescence, some cells with yellow fluorescence, including those within the cell aggregates (photographs not presented).

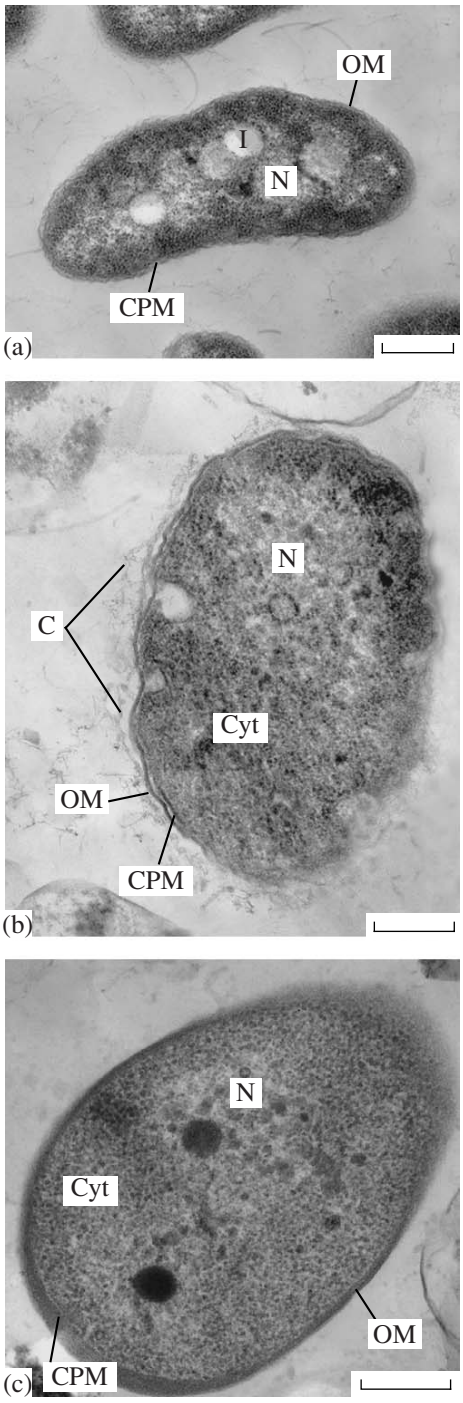

Fig. 4. Ultrathin sections of *A. brasilense* Sp245 vegetative cells (a) in 36-h cultures and CRC (b, c) in four-month cultures grown on the medium with phosphorus limitation. Designations: Cyt, cytoplasm; other designations see in Fig. 2. Scale bar: 300 nm.

Some DFs were impermeable to the components of the Live/Dead kit, i.e. emitted no fluorescence and were seen only under phase contrast. The fluorescence pattern in some CRC after Live/Dead staining was different from the pattern of "live" and "dead" cells; hence,

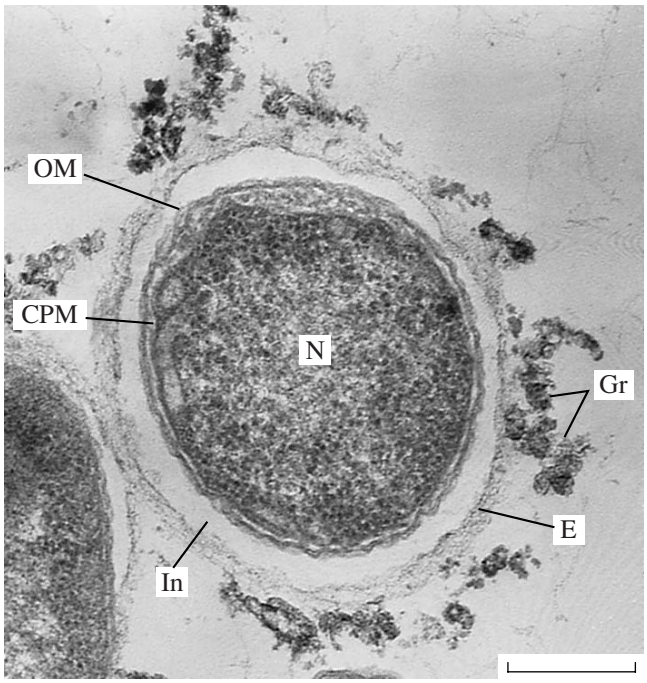

Fig. 5. Cysts of *A. brasilense* Sp245 in starved suspensions of cells, originally grown in C > N medium, washed, and transferred into saline solution. Designations: E, exine; In, intine; other designations see in Fig. 2. Scale bar: 300 nm.

their impermeability for the components of the dye could be due to the thickening of cell envelopes, emergence of the capsular layer, and/or presence of an external matrix enclosing the dormant cells. The differences between DF in the Live/Dead staining reflect the heterogeneity in the barrier functions of outer cell envelopes and membranes. Thus, the DF population of the nonendophytic strain Sp7 is characterized by diversity of CRC morphotypes differing in structural organization.

Electron microscopic studies of DF formed after prolonged (4 months) incubation of *A. brasilense* Sp245 cultures grown in the phosphorus-limited medium also showed ultrastructural heterogeneity of the dormant cells. Intact Sp245 cells of the first morphological type were characterized by the presence of (1) capsular layer; (2) thickened cell envelopes; (3) pronounced periplasmic space with appearance of sublayers; and (4) finely granular texture of the cytoplasm, where the nucleoid was poorly visible (Fig. 4b, 4c). This type of DF differed from vegetative cells (Fig. 4a) and resembled the first type of CRC of strain Sp7. However, the outer layers of DF of strain Sp245 were less differentiated, the cytoplasm contained fewer inclusions, and the capsular layer contained no granules. As a whole, Sp245 DFs of the first morphotype were similar in structure to CRC of pseudomonads [15].

Starved suspensions of *A. brasilense* Sp245 were shown to contain dormant forms analogous to azotobacter cysts [10] (Fig. 5). DF of this type had well distinguishable intine- and exine-like layers outside the

Table 1. Heat resistance of cyst-like cells of *A. brasilense* Sp7 and Sp245

Strain (type of dormant cells)	Number of viable cells (CFU/ml)			
	Before heat treatment (control)	After heat treatment (10 min)		
		50°C	55°C	60°C
A. brasilense Sp7 (CRC in the medium with C > N unbalance)	$(2.1 \pm 0.3) \times 10^8$	$(1.4 \pm 0.3) \times 10^6$	$(2.0 \pm 0.2) \times 10^4$	$(2.0 \pm 0.1) \times 10^4$
A. brasilense Sp245 (CRC in the medium with phosphorus limitation)	$(3.1 \pm 0.3) \times 10^7$	$(2.1 \pm 0.4) \times 10^5$	<10	<10

Table 2. Viability and heat resistance of *A. brasilense* Sp7 cysts at storage for 4 months

Variant no.	Culture storage (incubation) conditions	Number of viable cells (CFU/ml)		
		Before heat treatment	After heat treatment (10 min)	
			55°C	60°C
–	Control (before storage)	$(2.1 \pm 0.3) \times 10^8$	$(2.0 \pm 0.2) \times 10^4$ (0.01%)	$(2.0 \pm 0.1) \times 10^4$ (0.01%)
	Cultures after 4-month storage (incubation)			
1	Saline solution, 20°C	$(1.1 \pm 0.1) \times 10^7$	$(1.4 \pm 0.1) \times 10^4$ (0.13%)	0
2	Spent culture medium, 20°C	$(1.6 \pm 0.3) \times 10^6$	$(4.3 \pm 0.4) \times 10^5$ (27%)	$(1.9 \pm 0.2) \times 10^4$ (1.2%)
3	Saline solution, –20°C	$(4.0 \pm 0.6) \times 10^6$	$(2.6 \pm 0.4) \times 10^3$ (0.65%)	$(1.9 \pm 0.4) \times 10^3$ (0.5%)
4	Spent culture medium, –20°C	$(1.2 \pm 0.1) \times 10^8$	$(5.0 \pm 0.8) \times 10^3$ (0.004%)	$(4.1 \pm 0.3) \times 10^3$ (0.003%)

Note: 3-day culture of *A. brasilense* Sp7 was used as a control. The percentage of heat-resistant cells out of the number of viable cells in suspension before heat treatment is given in brackets in the last two columns.

outer cell membrane, beneath which there was an expanded periplasmic space filled with electron-dense granular material. The cytoplasm was lumpy and the nucleoid was poorly visible. DF of strain Sp245 differed from azotobacter cysts in the absence of polyhydroxyalkanoate inclusions. Thus, endophytic bacteria *A. brasilense* Sp245 were also able to form DF of different morphological types, albeit under conditions different from those required for strain Sp7.

Besides morphological and ultrastructural differences, dormant cells of *A. brasilense* Sp7 and Sp245 differed in heat resistance (Table 1). CRC of *A. brasilense* Sp7 (starting nitrogen deficiency) were more resistant to heating at 55 and 60°C (10 min) than DF of strain Sp245. Vegetative cells of azospirilla (control) did not survive such heat treatment.

Dormant cells of the non-endophytic strain Sp7, being more diverse morphologically and having a higher thermal resistance than DF of *A. brasilense* Sp245 (endophyte), were interesting for further experiments in order to determine the conditions important for long-term maintenance of their colony-forming ability. The cells of *A. brasilense* Sp7, grown on the medium with a C > N unbalance, were kept for 4 months under the following conditions: as suspensions of washed cells in saline solution (SS) incubated at room temperature of 18–20°C (variant 1); in a spent culture medium incubated at room temperature (variant 2); in SS, storage at –20°C (variant 3); and in a spent culture medium, storage at –20°C (variant 4).

Variant 4 (storage in a spent culture medium at –20°C) was optimal for long-term maintenance of colony-forming DF (1.2×10^8 CFU/ml, i.e., 57% of the CFU number in the stationary culture); however, the percentage of heat-resistant cells was low: 0.004% of CFU before the heat treatment (Table 2). Under incubation of *A. brasilense* Sp7 DF in the spent culture medium at room temperature (variant 2), the CFU number decreased (0.8% of the CFU titer of the stationary phase) with a simultaneous increase in the number of heat-resistant cells. In this variant, the portion of DF resistant to heating at 55 and 60°C was 27% and 1%, respectively, of the control (before heat treatment) (Table 2). During CRC incubation in SS at 20°C for 4 months, the number of viable DF was 1.1×10^7 CFU/ml (5% of the stationary culture), which was higher by an order of magnitude as compared with DF storage in the spent culture medium under the same temperature conditions (variant 2) or in SS but at –20°C. At the same time, the portion of heat-resistant cells was low in both variants (1 and 3) for the storage of dormant forms of azospirilla in SS suspensions (Table 3).

Thus, different storage conditions for DF populations ensured either the high titer of viable cells or their heat resistance "quality", despite the reduction in the

quantity of DF able to form colonies on the standard solid medium.

DISCUSSION

The results of our investigation demonstrate that both non-endophytic (Sp7) and endophytic (Sp245) strains of *A. brasilense* are able to produce dormant cells in their development cycles, with the features necessary and sufficient to qualify them as resting forms of prokaryotes [16]. They are characterized by the absence of division; long-term (4 months or more) preservation of viability; enhanced heat resistance as compared with vegetative cells; and the specificities of ultrastructural organization more significant than the structural differences between dividing and stationary cells, which points to a cytodifferentiation in these bacteria.

It should be noted that the few studies on the differentiated and dormant forms of *A. brasilense* offer no distinct terminology for the state of cell dormancy. Thus, the term C-forms, which is applied to define rounded immobile coccoid cells, is used by some researchers for the description of probably dormant forms of *A. brasilense* [17–20] and by other researchers for the description of physiologically specialized cells, which are also immobile and rounded [12, 21]. Thus, immobile, enlarged and rounded forms of *A. brasilense* with nitrogenase activity [21] should not be referred to as dormant forms, because the latter are characterized by either an absent or undetectable level of metabolism. Besides, the studies on localization of the endophytic strain *A. brasilense* Sp245 in the interior of wheat roothair cells by the FISH method showed that bacterial C-forms had the same fluorescence intensity as the vegetative cells [11]. This is their distinction in principle from DF which are not revealed by the FISH method [22] due to the limitations associated with the low content of rRNA in dormant forms and/or difficult penetration of oligonucleotide probe through cell envelopes [23]. Thus, terminologically, C-forms of azospirilla include the cells different in physiological state, stability and metabolic activity, and are intended either for survival [17–20] or for protection of nitrogenase from oxidation [21]. It should be noted that the formation of dormant cells, differing in structural organization and metabolic activity (cysts), and of C-forms depends on many factors: cultivation conditions [7, 8, 19, 21], interrelations with the plant partner [12, 21], and the properties of bacteria themselves [24, 25].

Substantial results of the present work are as follows: (1) evidence of the ability for both azospirilla strains to generate dormant forms destined for species survival, in accordance with all criteria accepted in sporology [16]; (2) the demonstrated diversity of DF morphotypes differing in ultrastructural organization and resistance to damaging factors (heat resistance); and (3) elucidation of the differences between azospirilla strains in their ability for generation of DF of various morphotypes under the same cultivation conditions. Both strains were able to form DF of the CRC type, although under different conditions: under C > N unbalance for non-endophytic Sp7 and under phosphorus deficiency for endophytic Sp245. Both strains proved capable of producing differentiated DF similar to azotobacter cysts, but in strain Sp245 they were formed under starvation at the transfer of the cells grown on the medium with C > N unbalance to saline solution, while in strain Sp7 they were formed under drying of the colonies or aggregates [7, 8], i.e. in conditions different from those used in the present work. Note that dormant forms have not been described previously for the endophytic strain Sp245.

Formation of several types of DF in both *A. brasilense* strains, those with simpler organization (CRC type) as well as differentiated ones, structurally close to cysts, which differ not only in the details of ultrastructural organization but also in thermal resistance, is evidence for intraspecies diversity of the dormant forms. It is inherent to many (if not all) microorganisms, as has been previously demonstrated for streptomycetes, myxobacteria, pseudomonades, and bacilli [14, 15, 26].

Thus, the same species of azospirilla is potentially able to form several DF types, although they are developed under different conditions. The conditions for obtaining the azospirilla DF used in this work (decreased concentrations of the phosphorus source or of the initial nitrogen level) were selected as close to natural situations as possible. Probably, different responses of strains Sp7 and Sp245 to a deficiency in these nutrient elements revealed in the present work provide the flexibility for species survival as cysts or as CRC. Taking into account the peculiarities of azospirilla localization in natural systems [11, 12], one may suppose that the induction of cytodifferentiation is not only determined by external conditions but is also coupled with the differences in the pattern of interaction with a plant.

The obtained CRC of the first type in both *A. brasilense* strains are analogous to the previously described dormant forms of gram-negative bacteria (*Pseudomonas*) [15]. The novelty of our results consists in detection of the heterogeneity of the CRC population of strain Sp7 in fine structures and in the characterization of the second CRC morphotype with less markedly thickened cell envelopes, enclosed in a vast structured extracellular matrix. The latter was revealed in Sp7 but not in Sp245 and, apparently, gives an advantage to the first (non-endophytic) azospirilla strain under stress resistance, which is in agreement with the data on DF viability maintenance after heat treatment (Table 1). The nature of the matrix in the CRC of *A. brasilense* Sp7 is still unknown and requires further investigation. As a working hypothesis, it may be suggested that it is formed of extracellular polymers: a lipopolysaccharide–protein complex (LPPC), a polysaccharide–lipid complex (PSLC), lectin (a surface glycoprotein) [4, 27,

28], and free polysaccharides. The behavior of polymers at isolation of LPPC preparations with the molecular mass of about 5 MDa [28] suggests a possibility of LPPC self-assemblage in aqueous solutions. The LPPC of *A. brasilense* Sp7 specifically interacts with lectin, and the lectin affinity to LPPC is higher than the affinity to the haptens of this lectin, L-fucose and D-galactose [4]. It is probable that such high-specific and numerous interactions between extracellular polymers allow *A. brasilense* Sp7 to form a well-structured and extensive matrix.

One more aspect of the problem of bacterial survival under natural conditions should be mentioned. The necessary properties for DF of microorganisms are preservation of the proliferative ability and gaining resistance to damaging factors. Our experiments showed that maintenance of CFU and the percentage of heat-resistant cells in ageing DF populations of azospirilla had an opposite dynamics dependent on the storage temperature and storage medium. In variant (2) incubation (4 months) of Sp7 CRC in the spent culture medium at 20°C, the number of cells forming colonies on the standard medium decreased with the simultaneous increase in the portion of heat-resistant forms (55°C, 10 min), probably due to maturation of dormant forms in the presence of substances with the functions of anabiosis autoinducers and stress protectors. This polymodal effect is typical of microbial autoregulators represented by alkylhydroxybenzenes (AHB) and occurs in a number of bacteria [29], including *A. brasilense* (unpublished data). In another variant (1) of Sp7 CRC incubation, also at room temperature but in saline solution, the portion of heat-resistant forms was less by two orders of magnitude, apparently because the extracellular autoregulators were removed in the course of DF washing from the native medium. The need for specific conditions and time for CRC maturation and development of their resistance was confirmed in experiments with the storage of DF suspensions in a frozen state, which excluded or greatly decreased the possibility of biochemical processes.

High preservation (57%) of the colony-forming ability for CRC stored at –20°C in the spent culture medium (variant 4), in contrast to variant (3) with suspensions frozen in SS, suggests the necessity for certain protectors from freezing–thawing stress. However, an increase in the amount of heat-resistant cells was not observed in the absence of maturation during storage in the growth medium at –20°C (Table 2). Reduction of the number of DF capable of quick reactivation in "maturing" populations is explained by the increase in the degree of dormancy in these DF. Reversion of such DF to growth needs additional procedures for reactivation, as have been previously demonstrated for long-incubated CRC of micrococci and pseudomonades [14, 15].

The demonstrated diversity of azospirilla DF and dependence of their proliferative potential and resistance to unfavorable factors on storage conditions, simulating natural conditions, demonstrate the flexibility of the survival strategies of these bacteria on plant roots (probably in caryopsis) and in soils, which is particularly important at alternating cold and warm seasons.

The other salient feature of *A. brasilense* Sp7 CRC as dormant bacterial forms ensuring survival and preservation of the species under non-growth conditions was their high phenotypic variability, which will be considered in the next paper.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project no. 07-04-01011) and Grant of the President of Russian Federation (project NSh-676177.2006.4).

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