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EXPERIMENTAL ARTICLES

Resting Forms of *Sinorhizobium meliloti*

N. G. Loiko[a, 1], N. A. Kryazhevskikh[a], N. E. Suzina[b], E. V. Demkina[a], A. Yu. Muratova[c], O. V. Turkovskaya[c], A. N. Kozlova[a], V. F. Galchenko[a], and G. I. El'-Registan[a]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

[c] *Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410015 Russia*

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Abstract—The ability of the symbiotrophic rhizobium *Sinorhizobium meliloti* P221 to produce cells having all the properties of resting forms (RFs) during the development cycles of the culture or after addition of the threshold concentrations of anabiosis autoinducers was demonstrated. The numbers, properties, and ultrastructure of *S. meliloti* resting forms depended on the conditions of growth and poststationary-phase incubation. In the four-month poststationary-phase, cultures grown in media deficient in some nutrient elements and energy sources (nitrogen, phosphorus, or oxygen), numerous cells (24–76% of the number of CFUs in the stationary-phase cultures) exhibiting a high degree of heat resistance and reversibly inhibited metabolic activity (the absence of endogenous respiration) were detected. According to their ultrastructure, all the resting forms detected in starving cultures were divided into three groups: (1) cystlike resting cells (CRCs) with thick cell envelopes and compacted nucleoids, (2) CRCs containing numerous (up to three-quarters of their volumes) polyhydroxyalkanoate inclusions, and (3) RFs similar to *Azotobacter* cysts. The resting forms obtained in the culture grown at high concentrations (5×10^{-5} M) of C_{12}-AHB, a chemical analogue of microbial anabiosis autoregulators, were incapable of endogenous respiration and retained the colony-forming ability. The CFU number after plating of these resting forms was twice as high as in the control culture; the heat resistance of these cells (55°C, 10 min) was an order of magnitude higher. The bacterial cells obtained from the resting forms either had a mixed (Swa^+Gri^+) type of motility in semisolid agar, typical of the dominant phenotype of the parent cells, or switched to the Gri^+ type. Emergence of different motility phenotypes depended on the conditions of RF formation. More severe stress conditions of RF formation induced the emergence of the Gri^+ type of cell motility. The results obtained can be used for development of a new generation of bacterial preparations based on bacterial CRCs which are able to preserve their viability for a long time and are highly resistant to stress impacts.

Keywords: Sinorhizobium meliloti, cystlike resting cells, biodiversity of resting forms, types of cell motility, bacterial preparations.

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The relationship between higher plants and symbiotrophic soil microorganisms is a complex scientific problem of urgent interest, which is focused on the symbiotic mechanisms, as well as on the biochemistry and genetics of nitrogen fixation [1–4]. However, elucidation of the survival strategies of the symbiotrophs, including root nodule-forming rhizobia, at the stage of free-living heterotrophic cells constantly exposed to various stresses and unfavorable environmental conditions, is an equally important issue [5, 6]. In recent years, data on some organic and inorganic factors providing for survival of rhizobia in soils and for their competitiveness in plant colonization became available [7–9]. The results of these investigations play an important role in the development of high-efficiency bacterial preparations of nitrogen-fixing bacteria providing an inexpensive and environmentally friendly way of enhancing soil fertility, eliminating the need for nitrogen fertilizers, which improves the quality of agricultural produce [10, 11]. In addition, symbiosis between plants and symbiotrophic nitrogen-fixing bacteria (root nodule-forming *Azorhizobium, Rhizobium, Mesorhizobium, Sinorhizobium*, and *Bradyrhizobium* or associative *Azospirillum, Agrobacterium, Arthrobacter*, and *Klebsiella*) provides significant adaptive advantages during growth under unfavorable or changing ambient conditions, which determines their efficacy in soil bioremediation [12].

One of the main adaptive strategies of microorganisms in response to unfavorable conditions is the ability of bacterial cells to develop stress-resistant resting

[1] Corresponding author; e-mail: loikonat@mail.ru.

states [13]. The formation of resting forms (RFs) necessary for survival of a population under unfavorable growth conditions has been shown for a number of non-spore-forming gram-positive and -negative bacteria, as well as for spore-forming bacteria under conditions inhibiting spore formation [14–18]. The formation of RFs by the typical growth-inducing rhizobacteria of the genus *Azospirillum* was studied as well [19, 20]. It was found that two *A. brasilense* strains exhibiting different types of relationships with their plant partners (endophytic and nonendophytic) produced resting cells under different conditions; the morphotypes of their resting cells differed as well. Both strains produced simple RFs in the form of cyst-like resting cells (CRCs) or differentiated cells morphologically similar to *Azotobacter* cysts [20]. Data on the resting cells formed by root nodule-forming rhizobia, as well as on their properties and growth conditions, are scarce. To date, only a few works have been reported in which mutations in some genes of rhizobia affecting the adaptation and survival of bacteria under stress impacts were studied [6, 21, 22].

The goal of the present work was to obtain data confirming the formation of resting cells by the symbiotrophic bacterium *Sinorhizobium meliloti* P221 as an adaptive response to unfavorable conditions, as well as to study the morphological and physiological diversity of these resting cells.

MATERIALS AND METHODS

The bacterium *Sinorhizobium meliloti* P221 isolated from the preroot zone of *Phragmites australis* L. (common reed) [12] was the subject of this study. Bacteria were cultivated in a medium (pH 6.8) containing the following (g/l): yeast extract, 1.0; mannitol, 2.0; K_2HPO_4, 0.2; KH_2PO_4, 0.2; $MgSO_4$, 0.2; and $CaCl_2$, 0.02. Bacterial cultures were incubated at 28°C in 250-ml flasks with 50 ml of the medium on a shaker (140–160 rpm). The inoculum (a 24-h culture) was added to the initial optical density (OD) of cell suspensions 0.05 ($\lambda = 540$ nm; $l = 10$ mm; Specord, Germany). For long-term storage, columns of agarized LB medium were stab-inoculated.

Resting forms of *S. meliloti* were obtained in the bacterial cultures grown under different stress conditions, i.e., in media deficient in nutrient elements (nitrogen, phosphorus, or oxygen), or in the presence of exogenic anabiosis autoinducers initiating an increase in the RF concentrations under stress conditions or during soil drying.

In our work, we used five modifications of the base medium in order to induce starvation stress: (1) the concentration of yeast extract was decreased to 0.1 g/l (lim N), (2) the concentrations of K_2HPO_4 and KH_2PO_4 were decreased to 0.04 g/l (lim P), (3) the concentration of yeast extract was decreased to 0.1 g/l and the concentrations of K_2HPO_4 and KH_2PO_4 were decreased to 0.04 g/l (lim N, P), (4) the level of aeration was lowered (the flasks were supplemented with another 50 ml of the medium and the cultures were incubated without agitation) (lim Î), (5) a combination of variants (3) and (4) (lim N, P, O). The obtained 48-h cultures were stored at 4°C or at room temperature (20°C) for a long time (seven months or longer).

Another way of obtaining the resting forms included the use of autolysing suspensions with a high cell density (space exhaustion stress). The cells from the stationary growth phase (48 h) were precipitated by centrifugation (5000 *g*, 15 min); the pellet was resuspended in a supernatant volume 20 times less than the initial volume before centrifugation. The obtained cell suspensions were incubated under static conditions at room temperature for four months or longer.

The third way of obtaining the resting forms included supplementing the prestationary cultures with a chemical analogue of microbial anabiosis autoinducers, C_{12}-AHB, as ethanol solutions to the final concentrations from 5×10^{-5} to 10^{-3} M; the final ethanol concentration did not exceed 0.5% (vol/vol) and did not affect the experimental results. The cultures supplemented with C_{12}-AHB were incubated under static conditions at room temperature for one month or longer.

The viability of vegetative and resting cells was determined by the number of colony-forming units (CFUs) obtained by plating of the cell suspensions from respective dilutions on agar media.

The social motility of rhizobia was assessed by their ability to produce colonies within 2–13 days after stab inoculation of the semisolid agarized media (0.3–0.6% agar).

Microscopic examinations were carried out using a Reichert microscope (Austria) equipped with a phase contrast device. Electron microscopic observations were carried out using a JEM-100B electron microscope (JEOL, Japan) at an accelerating voltage of 60 kV, as described in [20].

Respiration activity was detected in an LP7E polarograph (Czech Republic) in a 10-ml oxygen cell using the Sholz–Ostrovskii method [23].

Heat resistance was determined by counting the cells (CFUs) that remained viable after heating of cell suspensions (0.5 ml) in an ultrathermostat at 55°C (10 min) and 60°C (5 min).

For activation of resting forms, 0.5 ml of the stored culture or cell suspension was added to a flask with 49.5 ml of the physiological saline (pH 7) or 10^{-4} M of an indole-3-acetic acid (IAA) solution in the saline and incubated at 20°C and agitation for 1–2 h. Tenfold dilutions of the culture were then plated onto solid agarized (1.5% agar) LB medium or the base medium for CFU enumeration.

Statistical analysis was performed by the standard mathematical methods using the Microsoft Excel XP software package. The presented results are averages of

Table 1. Population dynamics of viable *S. meliloti* resting cells obtained in different media after storage at 20 and 4°C

Time of storage	Number of viable cells, CFU × 10^9/ml (% of the CFU number at the stationary growth phase (48 h))									
	Standard medium		limN		limP		limN, P		limO	limN, P, O
	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	20°C
Control (stationary culture, 48 h)	12.5 ± 0.3 (100.0)	16.0 ± 0.1 (100)	3.5 ± 0.1 (100)	3.3 ± 0.2 (100)	7.7 ± 0.3 (100)	8.2 ± 0.5 (100)	3.1 ± 0.2 (100)	3.4 ± 0.4 (100)	0.59 ± 0.05 (100)	0.80 ± 0.02 (100)
2 months	2.4 ± 0.4 (19.2)	6.0 ± 0.2 (37.5)	2.4 ± 0.1 (68.6)	2.6 ± 0.2 (78.8)	2.4 ± 0.2 (31.2)	5.0 ± 0.3 (64.9)	2.2 ± 0.2 (71.0)	2.4 ± 0.1 (70.6)	0.25 ± 0.05 (42.4)	0.65 ± 0.1 (81.3)
4 months	0.3 ± 0.05 (2.4)	0.1 ± 0.01 (0.6)	2.1 ± 0.2 (60.0)	2.5 ± 0.3 (75.8)	2.2 ± 0.2 (28.6)	3.2 ± 0.3 (41.6)	1.2 ± 0.08 (38.7)	2.1 ± 0.1 (61.8)	0.14 ± 0.02 (23.7)	0.60 ± 0.05 (75.0)

three independent measurements. The standard deviation was calculated. The differences between the data sets were considered reliable at a probability criterion $P > 0.95$.

RESULTS

Analysis of the laboratory *S. meliloti* cultures grown in the standard medium and stored for 6–12 months at room temperature (20°C) revealed the presence of small amounts (0.01–0.20%) of intact refractory cells of a much smaller size in these cultures. The ability of these cells (CFUs) to remain viable for a long time, as well as their morphological properties, allowed us to consider them as resting forms. In order to increase their amount so as to study their properties and identify them as resting forms, we employed the previously developed methods of obtaining of resting forms described in [24, 25] and based on the imitation of natural stress conditions [14–18].

Formation of *S. meliloti* resting forms under starvation stress induced by modification of the cultivation conditions. Nutrient and energy limitations inducing the formation of resting surviving cells are the main stress factors for soil bacteria.

The first experimental variant inducing the massive production of resting forms by non-spore-forming bacteria was based on the depletion of nutrient elements (nitrogen or phosphorus) and on lowering of the level of aeration. Rhizobia were grown in media deficient in the following elements: (1) nitrogen (lim N), decreased tenfold; (2) phosphorus (lim Ð), decreased fivefold; (3) nitrogen and phosphorus (lim N, P), decreased ten- and fivefold, respectively; (4) aeration level (lim O); and (5) nitrogen and phosphorus (decreased ten- and fivefold, respectively), as well as aeration level (lim N, P, O). The poststationary cultures grown under these conditions were stored at 20 or 4°C for four months or more. Microscopic examinations revealed that the growth of bacterial cells in deficient media resulted in a considerable increase (up to 70–80%) in the amount of nondividing refractory cells that were able to remain viable for long periods of time (four months and more) (CFUs) (Table 1). It should be noted that, while the amount of cells (CFUs) in the stationary-phase cultures (48 h) grown in nutrient- and oxygen-deficient media was lower than that in the control culture, the number of resting forms was considerably higher. For instance, the number of CFU in the four-month cultures of the experimental variants decreased by 24–76% (of the maximum in the stationary-phase culture), whereas, in the control cultures grown under standard conditions, the number of CFUs decreased by two orders of magnitude (Table 1). The following variants showed the best RF production: limN (4 and 20°C); limN, P (4°C); and lim N, P, O (20°C). Another factor affecting the amount of cells surviving for long periods of time was the incubation temperature of poststationary cultures. The amount of viable cells (CFUs) in the cultures grown in the same medium and stored at a low temperature (4°C) was much higher. In absolute numbers (cells/ml) of resting cells with the colony-forming ability, the variants lim P (20°C), lim N (4°C), limP (4°C), and lim N, P (4°C) were the best.

In addition, a decrease in the number of viable cells revealed during storage of *S. meliloti* resting forms was due to their depth of dormancy. The reactivation procedures, including washing of resting cells in order to remove the amphiphilic anabiosis autoregulators, as well as incubation of the resting forms in a 10^{-4} M phytohormone (indole-3-acetic acid (IAA)) solution, resulted in a 1.5- to 2-fold increase in the number of CFUs (Table 2).

Electron microscopic observations revealed three types of *Rhizobium* resting forms with an ultrastructure differing significantly from that of the vegetative cells (Fig. 1). The resting forms of the first type detected in the lim N, P and lim Î media were similar to the cyst-like resting cells described for many non-spore-forming bacteria [14–18] (Figs. 1c and 1d). These forms were characterized by thick cell envelopes, the presence of a pronounced capsular layer and extracellular electron-dense granules attached to it, easily distinguishable periplasmic space with low electron density, fine-granular lumpy cytoplasmic structure, and condensed DNA in the nucleoid (Figs. 1c and 1d). The resting forms of the second type detected in the cul-

Table 2. Viability of the resting cells obtained in different deficient media after seven-month storage at 20°C and reactivation of RF, CFU/ml (% of the CFU number before reactivation)

Reactivation procedure	Medium variants			
	lim N	lim P	lim N, P	lim N, P, O
Control (before reactivation)	6.6×10^8 (100)	8.2×10^8 (100)	5.1×10^8 (100)	6.5×10^7 (100)
Washing in saline solution, pH 7.0, 1 h	7.0×10^8 (107)	8.5×10^8 (104)	5.7×10^8 (112)	7.3×10^7 (112)
Washing in saline solution, pH 7.0, 2 h	1.0×10^9 (152)	8.7×10^8 (106)	6.8×10^9 (133)	7.8×10^7 (120)
Washing in saline solution, pH 7.0 + IAA (10^{-4} M), 1 h	1.1×10^9 (167)	9.6×10^8 (117)	0.83×10^8 (163)	8.4×10^7 (129)
Washing in saline solution, pH 7.0 + IAA (10^{-4} M), 2 h	1.3×10^9 (197)	1.0×10^9 (122)	1.2×10^9 (235)	9.3×10^7 (143)

Table 3. Heat resistance of *S. meliloti* resting forms obtained in different deficient media after four-month storage

Conditions of RF obtaining		Number of viable RFs, CFU/ml (% of the CFU number before heat treatment)		
Medium	*T*, °C	Before heat treatment	After heat treatment	
			55°C, 10 min	60°C, 5 min
Control, exponential phase cells	20	1.2×10^9 (100)	6.1×10^3 (5×10^{-4})	5×10^1 (4×10^{-6})
lim N	20	1.0×10^9 (100)	1.9×10^6 (0.19)	9.0×10^2 (9.0×10^{-5})
	4	2.5×10^9 (100)	5.5×10^6 (0.22)	9.2×10^2 (3.7×10^{-5})
lim P	20	2.4×10^9 (100)	6.3×10^5 (0.023)	8.0×10^2 (3.3×10^{-5})
	4	7.5×10^9 (100)	2.2×10^5 (0.003)	8.0×10^2 (1.1×10^{-5})
lim N,P	20	2.2×10^9 (100)	5.1×10^5 (0.023)	9.8×10^2 (4.5×10^{-5})
	4	3.2×10^9 (100)	9.5×10^5 (0.029)	4.5×10^2 (1.4×10^{-5})
lim O	20	0.3×10^9 (100)	4.4×10^4 (0.018)	2.5×10^2 (0.8×10^{-4})
lim N, P, O	20	0.8×10^9 (100)	1.5×10^5 (0.020)	2.1×10^2 (2.6×10^{-5})

tures grown in the lim N; lim N, P; and lim O media and stored for a long time were characterized by thick electron-dense cell envelopes and by the presence of numerous (up to three-fourths of their volumes) polyhydroxyalkanoate inclusions (Figs. 11c–11e). Interestingly, the amount of inclusions in the resting forms obtained in the cultures grown in the nitrogen-deficient media was especially high; at the same time, these resting forms exhibited the highest colony-forming activity and heat resistance (Fig. 1e). The resting forms of the third type detected in the cultures grown in the lim N, P, O medium were morphologically similar to *Azotobacter* cysts (Fig. lf) [26]. The resting forms of this morphotype had well-pronounced intine- and exinelike layers outside the outer cell membrane. The cytoplasm was lumpy, and the nucleoid was poorly visible. No inclusions were detected. It should be noted that, in the *S. meliloti* cultures grown in different media and subjected to long-term storage, several RF morphotypes were detected, which indicates the heterogeneity of the resting cell population.

The surviving cells obtained in poststationary cultures stored for two to seven months did not exhibit any metabolic activity (anabiosis), since endogenous respiration was not detected (according to polarographic measurements).

Finally, the higher stress resistance was another important property of the surviving *S. meliloti* cells. The resting cells of *S. meliloti* stored for four months exhibited enhanced heat resistance, which was found to be two orders of magnitude higher than that of the vegetative cells (Table 3). The degree of heat resistance depended mainly on the composition of the growth medium. The resting forms obtained in the lim N medium exhibited the highest heat resistance.

Hence, the surviving *S. meliloti* cells of different morphotypes formed in the development cycles of their cultures and capable of retaining their colony-forming capacity, as well as characterized by specific ultrastructural organization, heat resistance, and metabolic activity that cannot be detected experimentally, may be considered as typical resting forms.

Formation of *S. meliloti* resting forms in concentrated cell suspensions. Space exhaustion stress occurring when a critical cell density is achieved is among the stresses "prearranged" in the cycles of culture development [15, 16]. This situation was simulated by obtaining 20-fold concentrated suspensions of *S. meliloti* cells. Autolysis of some part of the population was observed; other cells remained intact and acquired refractivity. The number of viable cells

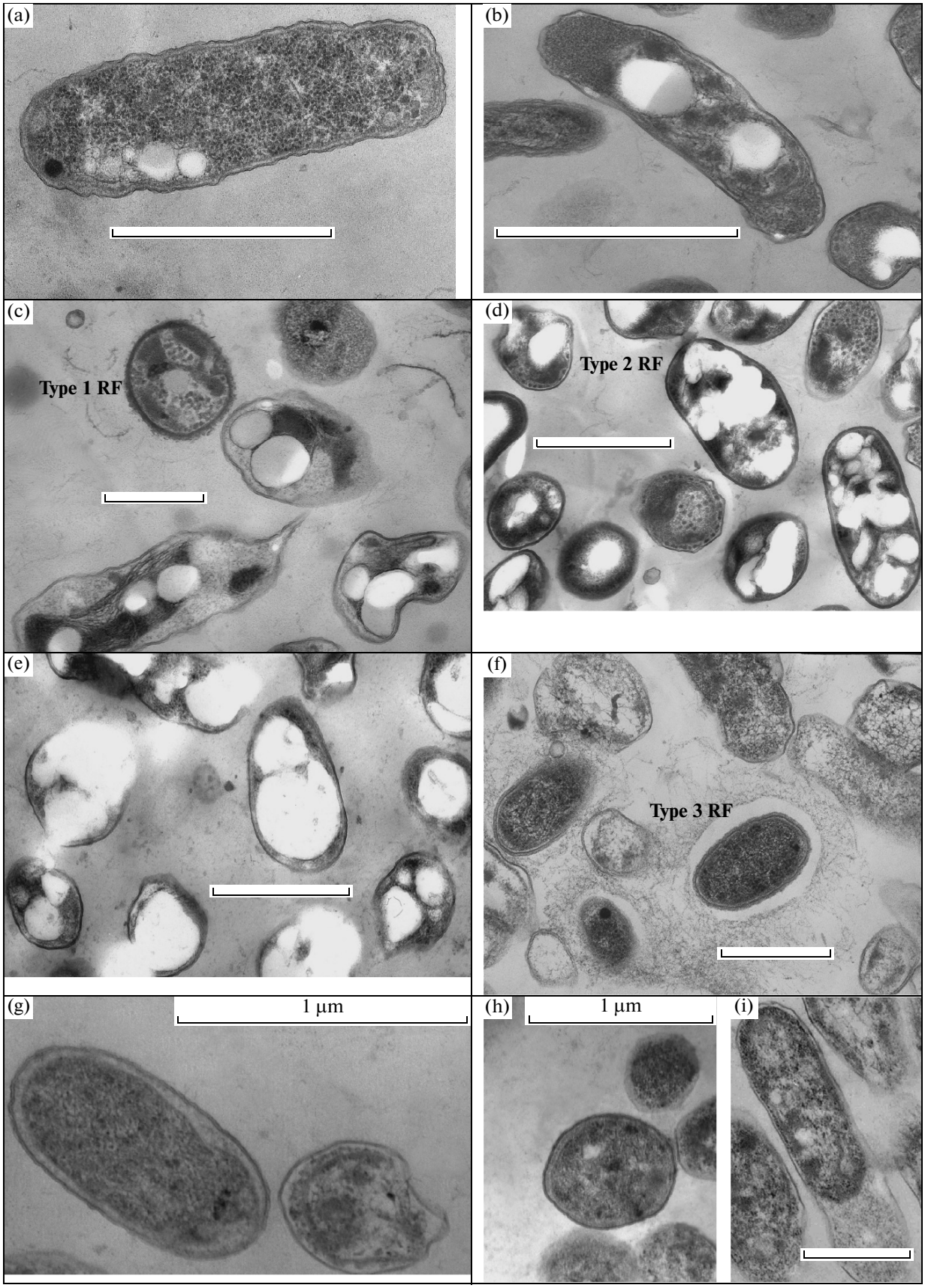

Fig. 1. Electron microphotographs of *S. meliloti* cells: vegetative cells, 15 h (a); stationary-phase cells, 48 h (b); RF, obtained after four-month storage in deficient media: lim N, P, 20°C (c); lim O, 20°C (d), lim N, 4°C (e); lim N, P, O, 20°C (f); and RF, obtained after three-month storage in 20-fold concentrated cell suspension (g, h, i). Scale bar, 1 μm.

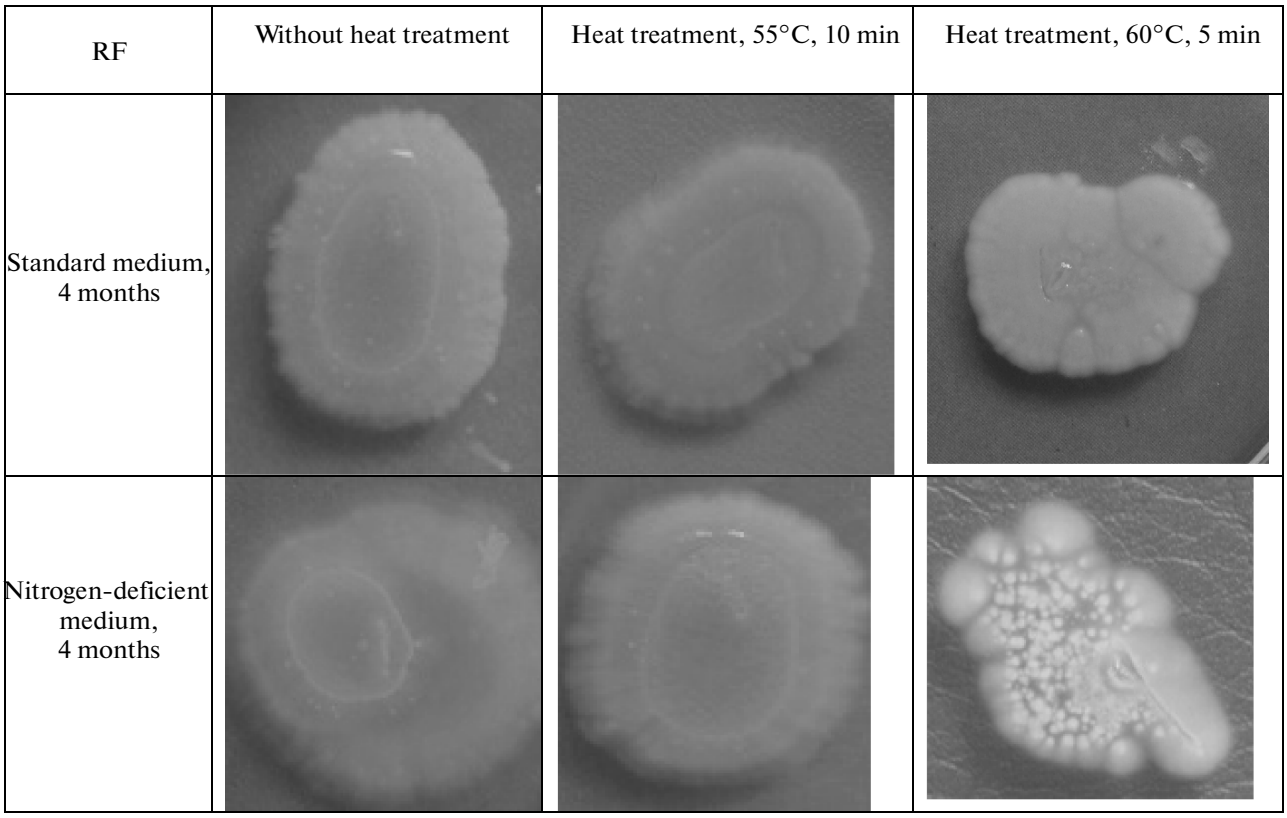

Fig. 2. Motility of *S. meliloti* RF obtained in the standard and nitrogen-deficient medium after four-month storage, before and after heat treatment.

(CFUs) in concentrated cell suspensions after two-month storage at 20°C was found to be one order of magnitude lower than the CFU number in the poststationary control culture (Table 4). Heat treatment of the cell suspensions resulted in a sharp decrease in the number of surviving cells (Table 4). After heating of concentrated cell suspensions at 55°C for 10 min, the number of viable cells was 5.2×10^3 CFU/ml, while, in the growth media that were not subjected to concentration, it was as high as 7.1×10^4 CFU/ml. Electron microscopic observations demonstrated that the cell suspension (three-month storage) consisted of intact cells with (1) an enlarged periplasmic space of their resting forms, which were found to be similar to the resting forms of the first type (Figs. 1g and 1h), or (2) the protoplast contracted at one pole (Fig. 1i).

Hence, although the formation of surviving cells in the concentrated suspensions of *S. meliloti* cells was detected, the number of these cells was extremely low, and they had no heat resistance.

Formation of *S. meliloti* resting forms in the presence of exogenic anabiosis autoinducers. The third way of obtaining *S. meliloti* resting forms included imitation of the ecological situation with soil drying, which results in increased concentrations of the autoregulators, including anabiosis autoinducers belonging

Table 4. Viability and heat resistance of *S. meliloti* cells obtained in concentrated suspensions, CFU/ml (% of the initial CFU number)

Observation time	Stationary-phase culture (without concentration)	20-fold concentrated cell suspension
1 h after concentration of a one-day culture	5.0×10^9 (100)	2.0×10^{10}(100)
2 weeks	3.1×10^9 (62.0)	8.6×10^8 (4.3)
1 month	1.8×10^9 (36.0)	3.8×10^8 (1.9)
2 months (without heat treatment)	1.0×10^9 (20.0)	2.7×10^8 (1.4)
2 months (after heat treatment at 55°C for 10 min)	7.1×10^4 (7.1×10^{-3}*)	5.2×10^3 (1.9×10^{-3}*)

Note: *, % of the CFU number before heat treatment.

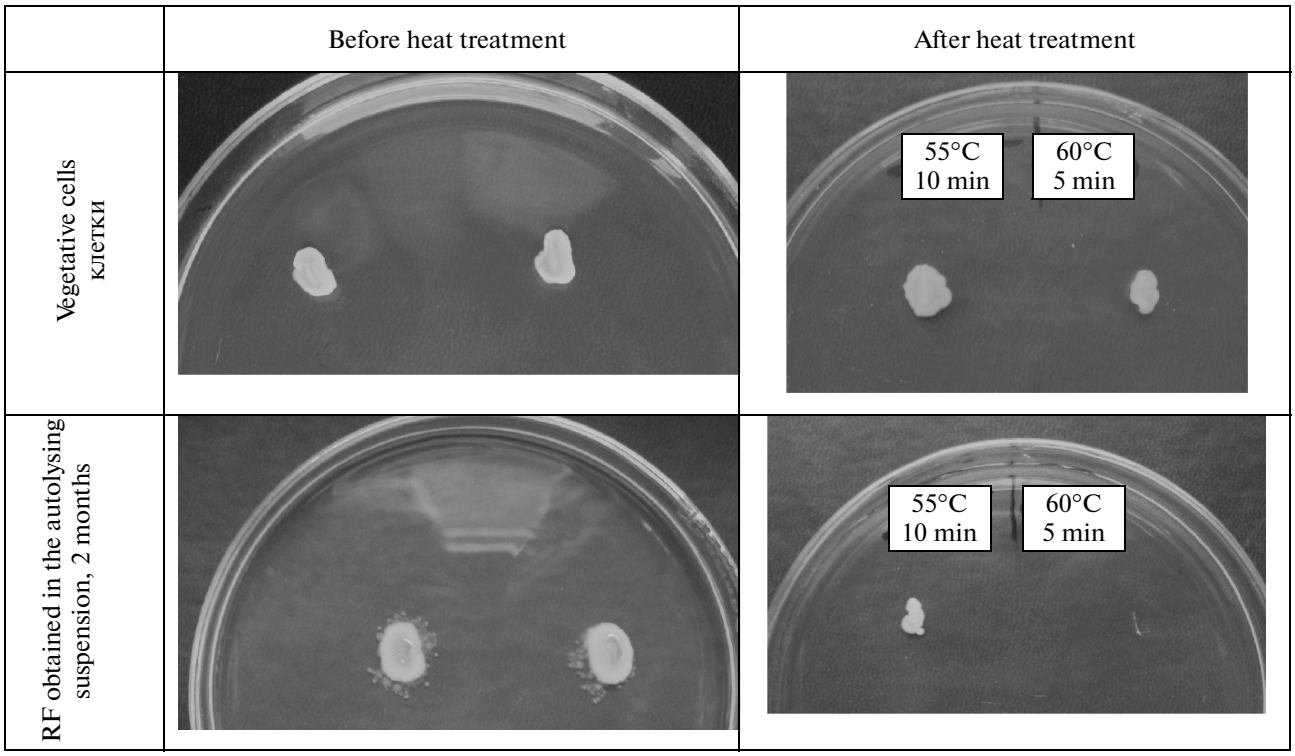

Fig. 3. Motility of stationary-phase *S. meliloti* cells (5 days) and RF obtained in the 20-fold concentrated autolysing suspension after two-month storage, before and after heat treatment.

(according to their chemical structures) to alkylhydroxybenzenes (AHB) [13]. The formation of resting forms was induced by the introduction of a chemical analogue of microbial autoregulators (C_{12}-AHB) into the prestationary culture to the final concentrations of 10^{-3}, 5×10^{-4}, 10^{-4}, and 5×10^{-5} M. The effects exerted by the autoregulator were found to be dose-dependent (Table 5). When the maximum concentration of C_{12}-AHB (10^{-3} M) was applied, the cells lost their ability to form colonies after a 30-min exposure, while remaining intact. These cells may be considered as mummified forms, described in [27]. At a C_{12}-AHB concentration of 5×10^{-4} M, the cells lost their ability to form colonies after one-month incubation, probably due to the transition to the temporary uncultivable state previously described for the *Micrococcus luteus* resting forms [28].

When the lowest concentration of C_{12}-AHB (5×10^{-5} M) was applied, the number of cells able to form colonies after one-month incubation was twice as high as that in the control culture (Table 5) and their heat resistance (55°C, 10 min) was five times higher (Table 6). Exposure to 60°C for 5 min was found to be fatal for the cells from both the control and the experimental cultures. However, this temperature induced reactivation of uncultivable resting forms in the variant with the C_{12}-AHB concentration of 5×10^{-4} M. It should be noted that these reactivation procedures, which include washing of uncultivable cells in physiological saline and incubation of resting forms in the IAA solution, were ineffective in this case, whereas, in

Table 5. Viability of *S. meliloti* cells after treatment with C_{12}-AHB, CFU/ml (% of the CFU number at 30-min exposure)

C_{12}-AHB concentration, M	Time of exposure		
	30 min	5 days	1 month
Control	6.9×10^9 (100)	2.3×10^9 (33.3)	4.2×10^8 (6.1)
10^{-3}	0	0	0
5×10^{-4}	1.0×10^6 (100)	6.0×10^5 (60.0)	0
10^{-4}	5.9×10^9 (100)	2.1×10^8 (3.6)	5.1×10^7 (0.9)
5×10^{-5}	6.7×10^9 (100)	3.2×10^9 (47.8)	7.7×10^8 (11.5)

Table 6. Heat resistance of *S. meliloti* RF obtained after treatment with C_{12}-AHB and stored for 1 month

AHB, M	Number of viable cells, CFU/ml (% of the CFU number before heat treatment)		
	Before heat treatment	55°C, 10 min	60°C, 5 min
Control.	4.2×10^8 (100)	4.5×10^4 (0.01)	0
10^{-3}	0	0	0
5×10^{-4}	0	0	6.7×10^3
10^{-4}	5.1×10^7 (100)	2.2×10^4 (0.04)	0
5×10^{-5}	7.7×10^8 (100)	4.0×10^5 (0.05)	0

the other variants (10^{-4} and 5×10^{-5} M of C_{12}-AHB), a 1.5- to 2-fold increase in the number of CFUs was detected. The intact cells obtained under the influence of C_{12}-AHB (5×10^{-4}–5×10^{-5} M) and stored for one month exhibited no endogenous respiration that could be revealed experimentally, which indicated that their metabolic activity was inhibited; the specific features of their ultrastructural organization were similar to those of CRCs.

Thus, in the presence of high concentrations of anabiosis autoinducers, *S. meliloti* produced CRCs with properties similar to those of bacterial resting forms.

Motility patterns of the cells obtained from the resting forms. Motility plays an important role in colonization of plant roots by rhizobia. Therefore, in the last series of experiments, the motility of proliferating cells and of the cells obtained from *S. meliloti* resting forms was studied. For this purpose, the cells were inoculated into semisolid agar media imitating the gel-like structures of the polysaccharides secreted by plant roots. In the preliminary experiments exploring the motility patterns of bacteria of the dominant *S. meliloti* phenotype in semisolid media with different agar concentrations (0.6, 0.5, 0.4, and 0.3%), we demonstrated that cell motility was virtually absent in the media with the agar concentration of 0.5% and higher, while, at agar concentrations below 0.4%, the rate of cell distribution was very high. The medium with 0.4% agar, in which vegetative *S. meliloti* cells exhibited swarming motility with formation of regular ring-shaped structures (the swarming Swa^+ phenotype) [29], was found to be optimal. On the periphery of the ring-shaped structures, fine-granular forms were observed, which can be considered a manifestation of the Gri^+ (granular inclusions) phenotype, i.e., bacteria distributed in the form of granular inclusions (microcolonies). Thus, the proliferating cells of the dominant *S. meliloti* phenotype exhibited the mixed (Swa^+Gri^+) motility pattern.

The study of the distribution in semisolid agarized media of the cells obtained from the resting forms from four-month cultures grown in the standard and nitrogen-deficient media revealed that their type of social motility remained the same; however, the number of microcolonies increased (Fig. 2). The microcolonies were clearly visualized not only at the periphery, but also inside the ring-shaped structures. The distribution rate of bacterial cells grown from the resting forms (determined by the diameter of the swarming rings after 2-day incubation), was lower by 10–30% than the distribution rate of the bacterial cells obtained by inoculation of proliferating cell cultures. Importantly, heat treatment of the resting forms at 55°C for 10 min before inoculation did not result in any significant changes in the type and rate of motility, whereas heating at 60°C for 5 min induced the process of cell transition to the Gri^+ phenotype. After heating of the vegetative cells, the type of motility remained unchanged; however, the rate of their spreading in the semisolid medium decreased significantly (Fig. 3).

In the case of the resting cells obtained in the concentrated autolysing suspensions after two-month storage, a pronounced manifestation of the Gri^+ phenotype was observed during growth in a semisolid nutrient medium (0.4% agar). Figure 3 shows that numerous microcolonies spread far beyond the swarming ring. The rate and dynamics (an increase in the diameters of the swarming rings with time) of the formation of the swarming rings were considerably lower than in the cells grown from the resting forms obtained in the standard medium and stored for the same time period (two months) (Table 7). The resting forms obtained in the concentrated suspensions were found to be most heat-sensitive and, after heating at 60°C for 5 min, completely lost their capacity for germination in semisolid agar (Fig. 3). These facts agree well with the previously demonstrated (Table 4) low level of heat resistance of these resting forms.

Table 7. Motility of rhizobia in the semisolid medium (0.4%) inoculated with the *S. meliloti* RF obtained in concentrated autolysing cell suspensions

Time of storage	Time of colony formation in semisolid agar, days	Diameter of zones of cell spreading, mm	
		Stationary-phase culture	20-fold concentrated cell suspension
2 weeks	3	11.0	7.5
	6	14.0	9.5
	13	20.0	12.0
1 month	3	11.0	6.5
	6	13.5	9.0
	13	17.5	11.5
2 months	3	4.5	4.0
	6	7.5	5.5
	13	9.0	6.0

Table 8. Motility of rhizobia in the semisolid agar medium (0.4%) inoculated with the *S. meliloti* RF obtained after treatment with C_{12}-AHB

RF age	Diameter of zones of cell spreading after 2 days, mm				
	Control	C_{12}-AHB concentration, M			
		10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}
30 min	16.5	0	15.0	25.0	15.0
5 days	15.0	0	15.0	19.5	14.0
1 month	10.0	0	0	15.0	10.0

During germination of the resting forms obtained in the cultures with C_{12}-AHB, the Gri^+ phenotype was rarely observed. The distribution rate of the swarming rings depended on the autoregulator concentrations and the time of storage of the RF cultures obtained under the influence of C_{12}-AHB (Table 8). At high C_{12}-AHB concentrations (10^{-3} M), the cells lost their colony-forming capacity both on solid (Table 6) and semisolid agar media. The resting cells obtained in the cultures with 5×10^{-4} M of C_{12}-AHB after one-month storage were not able to germinate in the semisolid medium. However, the rate of spreading of the bacterial cells grown from the resting forms obtained in the cultures with 10^{-4} M of C_{12}-AHB was 30–50% higher than that of the cells grown from the resting forms obtained in the cultures with 5×10^{-5} M of C_{12}-AHB and from the resting forms in the control cultures (Table 8).

Hence, the resting *S. meliloti* cells obtained under different conditions differed in their viability, heat resistance, and ultrastructure and were able to form colonies with different phenotypes (types of motility) during their germination in semisolid agar.

DISCUSSION

The present work demonstrated for the first time the ability of the symbiotrophic rhizobium *S. meliloti*, similarly to other non-spore-forming bacteria, to produce cells with all the properties of resting forms, including prolonged maintenance of viability under autolysis-inducing conditions (storage in growth media at room temperature), absence of metabolic activity (endogenous respiration), resistance to stress impacts (heat treatment), and changes in the ultrastructural organization indicating significant intracellular structural rearrangements. RF formation by rhizobia, as well as by other non-spore-forming bacteria [14–18], is an extreme stress response on their part to unfavorable conditions, such as depletion of nutrient sources and/or energy in a deliberately modified media, extremely high cell density due to the artificial concentration of cell suspensions, and high levels of long-chain AHBs (microbial anabiosis autoinducers). The amount, properties, and polymorphism of the obtained *S. meliloti* resting forms depended on the culture growth conditions (modifications of the media), the conditions of their poststationary incubation, and AHB concentrations. It should be noted that the RF properties depend on their ultrastructure. For instance, the resting forms containing numerous polyhydroxyalkanoate inclusions (Figs. 1d and 1e) exhibited higher heat resistance than the classical cystlike resting forms (Fig. 1f). These results correspond to the literature data on the low heat resistance of *Azotobacter* cysts [26]. Most resting forms with the protoplast contracted at one pole (Fig. 1i) obtained in concentrated long-stored suspensions showed low viability and heat resistance.

The following results of this study should be noted due to their importance for microbial ecology. First, it was demonstrated that *S. meliloti* is able to produce several types of resting forms depending on the conditions of culture development. These resting forms differ not only in their ultrastructure, but also in viability and heat resistance, as well as in the phenotypes developing during the germination of resting cells in semisolid agar. In total, this promotes realization of different ecological functions and ensures the species survival under unfavorable conditions. Two types of resting *S. meliloti* cells were found to be morphologically similar to the previously described resting forms of the associative exosymbiont *Azospirillum brasilense*: (1) RF similar to *Azotobacter* cysts and (2) simple cystlike resting cells (CRCs) [20]. It should be noted that the intraspecific polymorphism of resting cells is also typical of other symbiotrophs, for example, lactic acid bacteria: *Lactobacillus plantarum*, in addition to cystlike forms, produces surviving L-type cells [30]. It is possible that the enhanced polymorphism of resting cells of symbiotrophic bacteria is an additional advantage enabling these bacteria to use their adaptive mechanisms more extensively, i.e., to survive periodi-

cal changes of hosts and to retain their capacity for plant colonization after being released into soil.

Second, it should be noted that not all the methods of obtaining rhizobial RF described in this work were efficient. For example, although incubation of the concentrated *S. meliloti* cell suspensions resulted in the RF formation, the number and heat resistance of these resting forms were found to be extremely low. The pronounced change of the cell phenotypes (phase variation) during germination is the characteristic trait of these resting forms. Other microorganisms, e.g., *B. cereus, M. luteus, P. aurantiaca*, etc., have been shown to produce heat-resistant cells with a high CFU titer in concentrated suspensions [15, 18]. It may be suggested that nutrient-deficient media imitate the situation in which symbiotrophic bacteria have to adapt to unfavorable conditions after being released into soil during transfer to another plant host and therefore produce resistant and viable cell forms. During the concentration of cell cultures, the situation in which a critical population density is achieved may be a signal for the bacteria to switch to another phenotype (the proportion of Gri^+ cells increases) in order to establish symbiotic relationships with their plant host.

Third, during the germination of resting forms, the obtained *S. meliloti* cells exhibited motility in semisolid agar, which indicated that, after a prolonged period of dormancy, they retained their ability to colonize plants under favorable conditions. This is important due to the fact that rhizobia "recognize" their host by using the biochemical components of root excretions and use chemotaxis (movement of a bacterial cell toward the chemical stimulus [31]) in order to move towards root cells, inoculate these cells, and begin reproducing. Although cell motility and chemotaxis are not directly associated with root nodule formation, any abnormalities in these processes may impair the competitive ability of rhizobia [32]. For *Rhizobium leguminosarum* bv. *viciae*, it was shown that the growth conditions, including unfavorable ones, directly affect the ability of these microorganisms to colonize plant roots [33, 34]. The adaptation of rhizobia to various ambient conditions by using alternative mechanisms responsible for collective cell spreading in semisolid media (swarming or collective migration accompanied by the formation of microcolonies) may significantly enhance the adaptive capacity of these bacteria. In this connection, the ability of the studied bacteria to produce swarming rings or microcolonies in semisolid media in the course of collective migration is of particular interest. The regulation of the processes responsible for realization of a particular type of social motility in rhizobia, first of all in the preroot zone, is an important issue. The answer to this problem may promote the development of modern agrobacterial biotechnologies.

The results obtained can be used for development of a new generation of bacterial preparations based on bacterial CRCs with long-preserved viability and highly resistant to stress impacts. During germination in soil, the cells obtained in deficient media or in cultures with anabiosis autoinducers will switch to a phenotype that is better adapted to stress conditions. Moreover, these resting cells will retain their viability and high colonizing activity much longer.

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