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EXPERIMENTAL ARTICLES

Reproductive Resting Forms of *Arthrobacter globiformis*

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Abstract—Submerged cultures of *Arthrobacter globiformis* grown in media unbalanced with respect to carbon and nitrogen sources were found to contain cells exhibiting features typical of resting forms: long-term viability, specific ultrastructure, dormant metabolism, and thermoresistance. Such cells were produced not only in the collection strain VKM B-1112, but also in the *A. globiformis* strains isolated from 2- to 3-million-year-old permafrost sediments.

Key words: cystlike refractile dormant cells, *Arthrobacter globiformis*, dormancy autoinducers, submerged culture

It is known that representatives of the genus *Arthrobacter* usually prevail among bacteria isolated from various types of soil [1, 2]. These bacteria are characterized by low growth rates, the ability to accumulate reserve substances, the slowing down of indigenous metabolism in response to detrimental environmental conditions, as well as by enhanced tolerance to starvation, drying [1, 2], and long-term exposure to low temperatures in permafrost sediments [3].

The high survival of *Arthrobacter* species under unfavorable conditions is of particular interest, since so far, arthrobacters have not been known to form specialized dormant cells in their life cycle. However, some data available in the literature indicate the formation of large spherical or lemon-shaped arthrobacter cells (the so-called cystites), which are considered either as involutionary forms produced in response to unfavorable growth conditions [4] or as resting forms [5]. In this case, however, no convincing evidence has been presented that such cells are true resting forms. Unlike cystites, cells grown under conditions of severe carbon deficiency have small sizes and an ultrastructure somewhat different from that of cells grown in chemostat culture [6]. Soina *et al.* suggested that, under conditions of severe carbon and energy deficiency, arthrobacter cells transit into a starvation-resistant state resembling dormancy.

Methods developed to enhance the biosynthesis of dormancy autoinducers, or d_1 factors, allowed us to reveal the formation of cystlike refractile cells (CRCs) in cultures of non-spore-forming bacteria *Micrococcus luteus*, *Pseudomonas carboxydoflava*, *Escherichia coli*, and *Methylococcus capsulatus* [7–10]. CRCs, which exhibited all of the features of dormant cells, were referred to as a new type of resting microbial form. It can be suggested that not only the aforementioned bacterial species, but all (or nearly all) non-spore-forming bacteria, including arthrobacters, can form dormant cells of the CRC type, providing for bacterial survival under unfavorable environmental conditions.

The present work was aimed at studying the effect of cultivation conditions on the ability of arthrobacters to form resting cells in submerged cultures.

MATERIALS AND METHODS

Experiments were performed with the strain *Arthrobacter globiformis* B-1112 isolated from soil and strains 235-2 and 348-10 of the same species isolated from the late Pliocene permafrost, which is 2–3 million years old and which showed no evidence of thawing as a result of geological events or climatic fluctuations [11]. The first strain was obtained from the All-Russia Collection of Microorganisms (VKM), and two other strains were obtained from the Collection of Microorganisms of the Microbial Ecology Center, University of Michigan.

Strains were cultivated in liquid media differing in the content of C, N, and P sources.

Control medium 1 contained (g/l) glucose, 10; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.1; K_2HPO_4, 1; $CaCl_2$, 0.2; and $MgSO_4$, 0.1 (pH 7–8). A nitrogen-deficient variant 1 of this medium contained a lower amount of $(NH_4)_2SO_4$ (0.05 g/l). A nitrogen-deficient carbon-excessive variant 2 of medium 1 contained a lower amount of $(NH_4)_2SO_4$ (0.05 g/l) and a greater amount of glucose (20 g/l). A nitrogen- and carbon-deficient variant 3 of medium 1 contained lower amounts of glucose (2.0 g/l) and $(NH_4)_2SO_4$ (0.05 g/l).

Control medium 2 contained (g/l) glucose, 10; L-asparagine, 1.0; and K_2HPO_4, 0.5 (pH 7.5). A carbon-excessive nitrogen-deficient variant 1 of this medium contained a greater amount of glucose (100 g/l) and a

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Table 1. Number of viable *A. globiformis* cells (expressed in CFU/ml and % of the initial number) in suspensions stored in variants of medium 1 for four months at room temperature

Storage time, months	Medium 1 (control)	Variant 1 of medium 1	Variant 2 of medium 1	Variant 3 of medium 1
0	$(6.2 \pm 0.3) \times 10^8$ (100)	$(5.0 \pm 0.2) \times 10^8$ (100)	$(5.8 \pm 0.2) \times 10^8$ (100)	$(5.2 \pm 0.2) \times 10^8$ (100)
1	$(3.0 \pm 0.1) \times 10^8$ (48)	$(2.0 \pm 0.1) \times 10^8$ (40)	$(3.0 \pm 0.1) \times 10^8$ (52)	$(2.5 \pm 0.2) \times 10^8$ (48)
2	$(1.5 \pm 0.1) \times 10^8$ (24)	$(2.0 \pm 0.1) \times 10^8$ (40)	$(2.6 \pm 0.2) \times 10^8$ (45)	$(2.4 \pm 0.2) \times 10^8$ (46)
4	$(2.1 \pm 0.1) \times 10^6$ (0.3)	$(9.0 \pm 0.1) \times 10^6$ (0.2)	$(1.2 \pm 0.1) \times 10^6$ (0.2)	$(2.4 \pm 0.2) \times 10^8$ (46)

lower amount of L-asparagine (0.1 g/l). A phosphorus-excessive nitrogen-deficient variant 2 of medium 2 contained a greater amount of K_2HPO_4 (5.0 g/l) and a lower amount of L-asparagine (0.1 g/l).

Strains were cultivated in flasks containing 50 ml of medium at 28°C on a shaker (140 rpm). The growth media were inoculated with stationary-phase cultures to give an initial turbidity of 0.2 unit. Culture turbidity was measured at λ = 600 nm in 10-mm-pathlength cuvettes using a Specord spectrophotometer.

The formation of dormant bacterial forms was judged from the increased viability of the culture, decreased endogenous respiration, as well as from the increased thermoresistance of cells and specific changes in their morphology.

The number of viable cells was determined by plating cell suspensions onto nutrient agar and expressed via colony-forming units (CFU).

The endogenous respiration of cells was measured on an LP7 polarograph using a Clark-type oxygen electrode with a 1-ml measuring cell [12]. Aliquots of cell suspensions 0.1 ml in volume were mixed with 0.9 ml of 0.1 M phosphate buffer (pH 7.25), and the amount of oxygen consumed by cells was measured for 15 min.

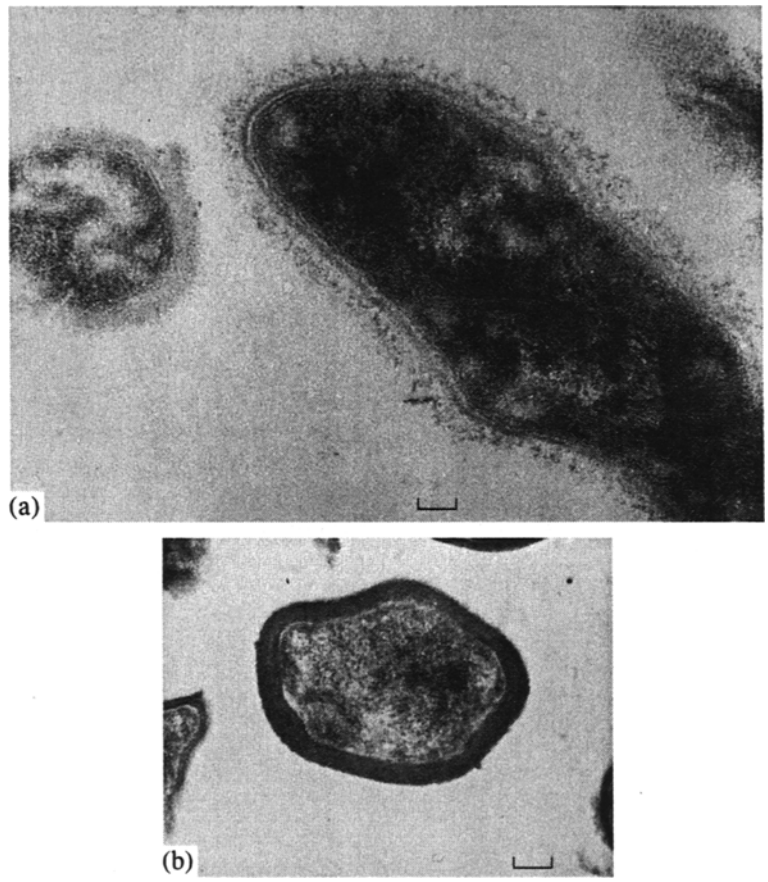

Fig. 1. Thin sections of *A. globiformis* B-1112 cells: (a) a vegetative stationary-phase cell grown in a mineral medium (control); (b) a resting cell from a suspension stored in the C- and N-deficient mineral medium at room temperature for four months. Bars represent 0.1 μm.

Table 2. Effect of heating at 80°C for 10 min on the number of viable cells in cell suspensions of different bacteria (data are expressed as CFU/ml and % of the initial number)

Culture	Number of viable cells	
	before treatment	after treatment
Vegetative *A. globiformis* cells (control)	$(5.2 \pm 0.2) \times 10^8$ (100)	$<10^3$
A. globiformis stored in variant 3 of medium 1 for 2 months	$(2.4 \pm 0.2) \times 10^8$ (100)	$(1.6 \pm 0.2) \times 10^8$ (67)
Resting *M. luteus* cells [9]	$(6.0 \pm 2.3) \times 10^6$ (100)	$(2.0 \pm 0.3) \times 10^6$ (33)
Resting *B. cereus* cells [9]	$(3.0 \pm 0.7) \times 10^8$ (100)	$(1.0 \pm 0.3) \times 10^8$ (33)

Table 3. Effect of incubation in the revival medium on the number of viable cells in *A. globiformis* suspensions stored in variants of medium 2 for two months at room temperature (data are expressed as CFU/ml and % of the initial number)

Storage medium	Number of viable cells		
	before incubation in revival medium		after incubation in revival medium
	before storage	after storage	
Medium 2 (control)	$(1.2 \pm 0.1) \times 10^8$ (100)	$(3.2 \pm 0.3) \times 10^6$ (3)	$(3.6 \pm 0.3) \times 10^6$ (3)
Variant 1 of medium 2 (P > N)	$(3.2 \pm 0.2) \times 10^8$ (100)	$(3.1 \pm 0.3) \times 10^6$ (1)	$(3.9 \pm 0.3) \times 10^7$ (12)
Variant 2 of medium 2 (C > N)	$(1.8 \pm 0.2) \times 10^8$ (100)	$(8.0 \pm 0.1) \times 10^5$ (0.4)	$(1.4 \pm 0.2) \times 10^7$ (8)

The sensitivity of determination of the respiration rate varied from 1 to 15 natoms O/min, depending on the culture.

To determine the thermoresistance of cells, cell suspensions were heated at 80°C for 10 min, and the number of cells remaining viable after such treatment was estimated as described above.

Microscopic observations were carried out under an Amplival phase-contrast microscope (Germany).

For electron microscopic studies, cells were fixed with a 2.5% solution of glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 3 h and refixed with a 0.5% solution of ruthenium tetroxide in the same buffer at room temperature for 1 h [13]. The material was then embedded in epon resins, cut into thin sections, contrasted with lead citrate, and examined in a JEM-100B electron microscope (Japan) at an instrument magnification of 30000×.

RESULTS AND DISCUSSION

The formation of dormant cells can be induced by increasing the concentration of factor d_1, a dormancy autoinducer, in bacterial cultures. Based on our knowledge of the nature of dormancy autoinducers, which are alkyl-substituted hydroxybenzenes, and the ways of their biosynthesis [14, 15], we assumed that an increased level of factor d_1 can be achieved by using media unbalanced with respect to the C, N, P, and O sources. Earlier, the effect of nutritionally unbalanced media on the formation of resting forms was studied in experiments with *Micrococcus luteus* and *Bacillus cereus* [8, 9].

In experiments with *Arthrobacter globiformis* strains, the modification of media with respect to C, N, and P contents was found to affect the number of viable cells in all the strains stored for 1–4 months at room temperature.

The cultivation and subsequent storage of *A. globiformis* B-1112 in nitrogen-deficient variant 1 and nitrogen-deficient carbon-excessive variant 2 of medium 1 led to a drastic decrease in the cell viability (down to 0.2%) by the fourth month of storage (Table 1). In the case of storage in control medium 1, cell viability was somewhat higher (0.3% of the maximum number of viable cells observed in the stationary growth phase). The phase-contrast microscopic examination of cells stored in these media showed that most of them had a ghostlike appearance.

The maximum viability was observed when arthrobacter cells were grown and then stored in the carbon- and nitrogen-deficient variant 3 of medium 1: 46% of the initial number of cells remained viable by the fourth month of storage at room temperature (Table 1). Similar results were obtained in experiments with the strains *A. globiformis* 235-2 and 348-10 isolated from permafrost: the number of viable cells remained at a level of 40–50% after 2–3 months of storage. Cells that were transferred onto nutrient agar readily restored their viability without any special efforts, so that normal colonies were observed after 3 days of cultivation on agar plates under standard conditions.

The high level of cell viability in the variant 3 of medium 1 implied that the majority of cells in this medium were resting forms (Table 1). Microscopic examination showed that more than 70% of the cells in this population were intact, without any evidence of autolysis. These cells were round and had a diameter of 0.3–0.4 μm. The refractility of cells could not easily be observed, probably, because of their small sizes.

Electron microscopic examination confirmed that most cells kept in the variant 3 of medium 1 were not autolysed. Unlike vegetative cells (Fig. 1a), intact dormant cells had a lower electron density of ribosomes in the cytoplasm, the indistinct nucleoid, and the thickened cell walls (Fig. 1b). The same features are typical of the dormant forms of other bacteria [8–10]. On the other hand, the dormant cells of *A. globiformis* described here differed from the *Arthrobacter* cystites (cells formed in media with a high C/N ratio), having small sizes and lacking reserve polymers of the glycogen type [16], which occupy a disproportionate share of the volume of cystites, imparting them with a lemonlike shape.

Thus, the nutritional imbalance C >> N does not always favor the formation of resting forms in arthrobacter cultures. At the same time, carbon deficiency evidently promotes the formation of such forms in *A. globiformis* cultures. These forms can be revived without using any special treatments.

It should be noted that colonies grown on nutrient agar after plating the *A. globiformis* cultures stored in variant 3 of medium 1 for four months at room temperature were heterogeneous in size but homogeneous in other features. The colonies that were visualized on the third day after plating had a diameter of up to 0.4 cm. At the same time, one week after plating, we observed the formation of new colonies whose diameter was less than 0.1 cm and did not increase throughout the incubation period. Examination under a phase-contrast microscope showed that these two types of colonies contained cells which were completely identical in morphology. These findings indicate that dormant cells in stored cultures are heterogeneous in the degree of dormancy and the ability to revert to vegetative forms; this is of much importance for elucidating the mechanisms of regulation of the microbial activity in soil.

Resting *A. globiformis* cells retained their viability for 4–6 months in the C- and N-deficient medium containing 0.2% glucose and 0.05 g/l $(NH_4)_2SO_4$ (such a medium is considered unsuitable for storage). These cells also exhibited two other features typical of resting microbial forms: increased thermoresistance and the absence of endogenous respiration. The respiration of dormant cells could be restored by 3-day incubation in fresh nutrient medium.

The thermoresistance of resting cells, whose viability was 46% after four months of storage, was estimated by heating them at 80°C for 10 min. After such treatment, cell suspensions retained their viability at a level of 67% (Table 2). The thermoresistance of resting *A. globiformis* cells turned out to be higher than that of not only vegetative arthrobacter cells (both rodlike and coccoid arthrobacter cells can withstand heating to only 55°C [2]) but also the resting forms of *M. luteus* and *B. cereus* [17], which retained their viability after the treatment at a level of 33% (Table 2).

In medium 2 variants deficient in nitrogen but sufficient in C (variant 1) or P (variant 2), the percentage of cells that remained viable after two months of storage at room temperature was 0.4 and 1%, respectively (Table 3).

Unlike the CRCs formed in the variants of medium 1, the CRCs formed in the L-asparagine-containing variants of medium 2 required a special medium for their revival: 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% yeast autolysate. After a 40-min incubation in this medium, the number of viable cells in variants 1 and 2 of medium 2 increased about 15-fold, whereas the number of viable cells in the nutritionally balanced control medium 2 did not rise (Table 3).

Thus, storage in nutritionally unbalanced media promotes the formation of *A. globiformis* cells with features typical of resting microbial forms—long-term viability during storage for up to four months, experimentally undetectable levels of metabolism and endogenous respiration, and increased thermoresistance. Unlike the resting forms of *M. luteus* and *B. cereus* [17], resting arthrobacter cells are formed after a more prolonged storage of cell suspensions (for up to 1 month). This may be explained by the special survival strategy of arthrobacters, which survive in soil for a long time under conditions of severe competition with other soil bacteria due to their low growth rates and energy requirements.

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