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EXPERIMENTAL ARTICLES

Formation of Resting Forms of *Arthrobacter globiformis* in Autolyzing Cell Suspensions

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Abstract—Under conditions of the spontaneous or induced autolysis of thick cell suspensions, *Arthrobacter globiformis* strains produced cells exhibiting features typical of resting microbial forms. The number of viable resting cells was greater under conditions of induced rather than spontaneous autolysis. The thermoresistance of the resting cells of *A. globiformis* strains isolated from 2- to 3 million-year-old permafrost was higher than that of the collection *A. globiformis* strain.

Key words: Arthrobacter globiformis, autolysis, resting forms

Under deleterious environmental conditions, soil microorganisms can transit to the state of dormancy, or hypometabolism [1], when the majority of cells in a population autolyse with the release of cell constituents into the environment. As was shown in model experiments with collection microbial cultures, the dormancy autoinducer (factor d_1) released from autolysed cells stimulates the transition of other cells of the population to resting forms, such as the cystlike refractile cells (CRCs) of the non-spore-forming bacteria *Micrococcus luteus*, *Escherichia coli*, and *Methylococcus capsulatus* [2–4].

A hypothesis has been proposed that the autolysis of microbial cultures is not merely the death of cells under unfavorable growth conditions but a prerequisite for the formation of dormant cells and, hence, species survival. Investigation of the mechanisms of transition of some microbial cells to the resting state under the action of factor d_1 released from autolysed cells may provide insight into the mechanisms of functioning of microbial complexes in nature. It seems expedient to perform such investigations using typical soil non-spore-forming bacteria of the genus *Arthrobacter*, since they are very resistant to deleterious environmental conditions and are known to form special forms, cystites or coccoid cells, which are considered to be resting forms [5–7]. It should be noted that no experimental evidence has been presented that such cells are true resting forms.

The aim of the present work was to study the formation of dormant forms in autolysing cell suspensions of the non-spore-forming bacterium *Arthrobacter globiformis*.

MATERIALS AND METHODS

Experiments were carried out using the strain *Arthrobacter globiformis* B-1112, which was isolated from the soil, and strains 235-2 and 348-10 of the same species, which were isolated from the late Pliocene permafrost, which is 2–3 million years old and has shown no evidence of thawing as a result of geological events or climatic fluctuations [7]. 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 of the University of Michigan.

Strains were cultivated at 28°C on a shaker (140 rpm) in flasks containing 50 ml of threefold diluted nutrient broth. This medium was 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 concentration of cells in suspensions subject to autolysis was 10- or 20-fold higher than in the early-stationary-phase culture. Such suspension were prepared as follows. Cells were aseptically precipitated by centrifugation at 4000 rpm for 20 min and suspended in one of the following media: (1) growth medium (threefold diluted nutrient broth); (2) phosphate buffers with different pH values containing 0.02% $CaCl_2$ to stabilize subcellular structures; (3) physiological saline solution with 0.02% $CaCl_2$; (4) tap water with 0.02% $CaCl_2$; and (5) growth medium with 1 mM oleic acid added in the form of an ethanol solution. Cell suspensions were kept at room temperature for 45 days.

The number of viable vegetative or dormant cells was determined by plating cell suspensions onto nutri-

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Table 1. Number of viable cells (expressed in CFU/ml and % of the initial number) in the spontaneously autolysing tenfold thickened suspensions of *A. globiformis* B-1112 cells stored at 28°C

Medium	Storage time, days			
	0	3	10	45
Control*	$(3.0 \pm 1.2) \times 10^8$ (100)	$(2.0 \pm 1.1) \times 10^8$ (65)	$(6.0 \pm 1.4) \times 10^7$ (20)	$(3.0 \pm 1.2) \times 10^6$ (1)
Nutrient broth with $CaCl_2$	$(3.1 \pm 1.8) \times 10^{10}$ (100)	$(1.6 \pm 0.5) \times 10^{10}$ (51)	$(5.6 \pm 1.2) \times 10^9$ (18)	$(3.1 \pm 0.7) \times 10^6$ (0.01)
Tap water with $CaCl_2$	$(1.7 \pm 0.6) \times 10^{10}$ (100)	$(1.3 \pm 0.3) \times 10^{10}$ (76)	$(1.1 \pm 0.2) \times 10^{10}$ (65)	$(4.0 \pm 1.3) \times 10^7$ (0.2)
Physiological saline with $CaCl_2$	$(6.2 \pm 1.1) \times 10^9$ (100)	$(3.5 \pm 0.8) \times 10^9$ (57)	$(1.1 \pm 0.2) \times 10^9$ (18)	$(1.9 \pm 0.4) \times 10^8$ (3)
Buffer with $CaCl_2$ (pH 6.5)	$(7.0 \pm 1.3) \times 10^9$ (100)	$(5.2 \pm 0.4) \times 10^9$ (74)	$(4.0 \pm 1.5) \times 10^9$ (57)	$(3.7 \pm 0.7) \times 10^8$ (5)
Buffer with $CaCl_2$ (pH 7.25)	$(4.9 \pm 1.2) \times 10^9$ (100)	$(3.2 \pm 0.6) \times 10^9$ (65)	$(2.8 \pm 0.2) \times 10^9$ (57)	$(5.8 \pm 1.0) \times 10^8$ (12)

* *A. globiformis* B-1112 cells grown in nutrient broth (without washing and thickening).

Table 2. Number of viable cells (expressed in CFU/ml and % of the initial number) in the spontaneously autolysing 20-fold thickened cell suspensions of *A. globiformis* strains stored at pH 6.5 at 28°C

Strain	Storage time, days			
	0	3	10	45
Arthrobacter globiformis B-1112	$(1.1 \pm 0.2) \times 10^{11}$ (100)	$(2.1 \pm 0.3) \times 10^{10}$ (20)	$(5.9 \pm 0.2) \times 10^9$ (6)	$(5.5 \pm 0.3) \times 10^9$ (5)
Arthrobacter globiformis 235-2	$(7.7 \pm 0.5) \times 10^{11}$ (100)	$(2.9 \pm 0.4) \times 10^{10}$ (38)	$(5.3 \pm 0.6) \times 10^8$ (1)	$(4.2 \pm 0.2) \times 10^8$ (1)
Arthrobacter globiformis 348-10	$(1.4 \pm 0.4) \times 10^{10}$ (100)	$(1.3 \pm 0.3) \times 10^{10}$ (10)	$(5.9 \pm 0.3) \times 10^8$ (0.4)	$(5.9 \pm 0.5) \times 10^8$ (0.4)

ent 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. The sensitivity of determination of the respiration rate varied from 1 to 15 natoms O/min [8].

The thermoresistance of cells was determined after storing them for 45 days. For this purpose, cell suspensions were heated at 80°C for 10 min, and viable cells were enumerated 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) and refixed with a 0.5% solution of ruthenium tetroxide in the same buffer at room temperature for 1 h [9]. 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). Instrument magnification ranged from 20000× to 30000×.

RESULTS AND DISCUSSION

Various detrimental factors, such as starvation, high cell concentrations, and freezing, can induce the autolysis of some microbial cells in a population and hence the release of the dormancy autoinducer (factor d_1) from autolysed cells. In our experiments, cell autolysis was either spontaneous or induced by the addition of a chemical analogue of an autolysis autoinducer (factor d_2) to cell suspensions.

Arthrobacter cells in autolysing suspensions were considered to be metabolically dormant if they (1) retained their viability during storage at room temperature for 45 days, (2) possessed an undetectable level of endogenous respiration, (3) were thermoresistant, and (4) had a specific ultrastructure.

Table 3. Changes in the number of viable cells and in the turbidity of spontaneously autolysing 20-fold thickened cell suspensions of *A. globiformis* strains stored at pH 6.5 at 28°C (data are expressed as the ratio CFU/OD_{540} in % of the initial values)

Strain	CFU/OD_{540}			
	Storage time, days			
	0	3	10	45
Arthrobacter globiformis B-1112	100/100	20/50	6/30	5/25
Arthrobacter globiformis 235-2	100/100	38/75	1/97	1/75
Arthrobacter globiformis 348-10	100/100	9/69	1/63	1/74

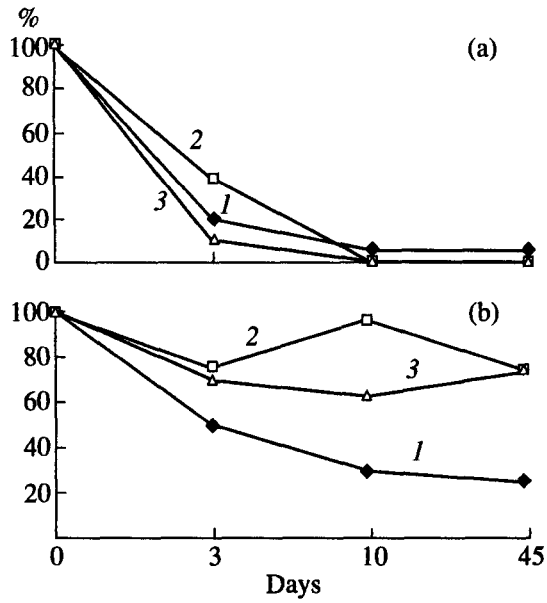

Fig. 1. Dynamics of (a) viable cell count and (b) turbidity of spontaneously autolysing 20-fold thickened cell suspensions of (*1*) *A. globiformis* B-1112, (*2*) *A. globiformis* 235-2, and (*3*) *A. globiformis* 348-10 stored at pH 6.5 at 28°C.

In spontaneously autolysing 10-fold and 20-fold thickened cell suspensions in phosphate buffer (pH 6.5 and 7.25) with 0.02% $CaCl_2$, the number of viable cells after 45 days of storage at room temperature comprised 5–12% of their initial number (Table 1). At the same time, in control (thin) autolysing suspensions, the number of cells that remained viable was less than 1% (Table 1). In the case of strains isolated from permafrost, the number of viable cell was still lower: 0.4% at pH 6.5 (Table 2).

It should be noted that the number of viable cells in autolysing suspensions is commonly estimated by measuring their turbidity. As can be seen from Fig. 1 and Table 3, data on the optical density of autolysing suspensions and their CFU values do not agree. Therefore, the number of viable cells in autolysing suspensions should not be estimated by measuring suspension turbidity.

Heating the suspensions of vegetative (control) cells at 80°C for 10 min caused their complete death (Table 4), indicating that such suspensions did not contain thermoresistant cells. At the same time, in the autolysing suspensions of *A. globiformis* B-1112 heated similarly, up to 40% of cells remained viable and, therefore, were thermoresistant (Table 4). In the case of strains 235-2 and 348-10 isolated from the permafrost, the percentage of thermoresistant cells was as high as 75%.

These data suggest that viable and thermoresistant *A. globiformis* cells in autolysing suspensions are resting forms, analogous to the CRCs of *A. globiformis* formed in response to altered cultivation conditions [10].

The thermoresistance of resting cells of *A. globiformis* strains isolated from permafrost was considerably higher than that of resting cells of the collection strain *A. globiformis* B-1112 (Table 4), although the latter strain produced 5–10 times more resting cells than the "permafrost" strains. The members of microbial communities exposed to a long-term influence of low temperatures in permafrost exhibit not only higher thermoresistance but also higher rates of viability, reproduction, and metabolic activity than species isolated from soil undergoing only repeated freezing–thawing cycles [11]. We may suggest that the long-term occurrence of bacteria in a supercooled state in permafrost gives rise to the selection of stress-resistant variants with an increased thermostability of some enzymes, as has been shown with respect to invertase [11, 12], and to some mechanisms preventing microbial death. It is these antistress mechanisms that may be responsible for the high thermoresistance of the resting forms of the arthrobacters isolated from permafrost.

In experiments on induced autolysis, the tenfold thickened suspension of *A. globiformis* B-1112 cells was supplemented with oleic acid (a chemical analogue of the autolysis autoinducer, or factor d_2) to stimulate autolysis and the release of the dormancy autoinducer (factor d_1) from cells. Oleic acid was added to the cell suspension at a concentration of 1 mM, which is close to the concentration of the natural d_2 factor in microbial cultures. These experiments showed that the number of viable cells in the case of induced autolysis is two orders of magnitude higher than in the case of spontaneous autolysis (Table 5), which implies the important role of the autolysis rate in the formation of resting forms in autolysing cell suspensions.

Cells that remained viable in autolysates stored at room temperature for a long time were metabolically

Table 4. Effect of thermal treatment on the number of viable cells in the autolysates stored for 45 days (data are expressed as CFU/ml and % of the initial number)

Strain	Before thermal treatment	After thermal treatment
Arthrobacter globiformis B-1112	$(5.5 \pm 0.3) \times 10^9$ (100)	$(2.2 \pm 0.1) \times 10^9$ (40)
Arthrobacter globiformis 235-2	$(4.2 \pm 0.2) \times 10^8$ (100)	$(3.2 \pm 0.2) \times 10^8$ (75)
Arthrobacter globiformis 348-10	$(5.9 \pm 0.2) \times 10^8$ (100)	$(4.1 \pm 0.2) \times 10^8$ (70)
Control*	$(3.1 \pm 0.2) \times 10^8$ (100)	$<10^3$

* Stationary-phase *A. globiformis* cells grown in nutrient broth.

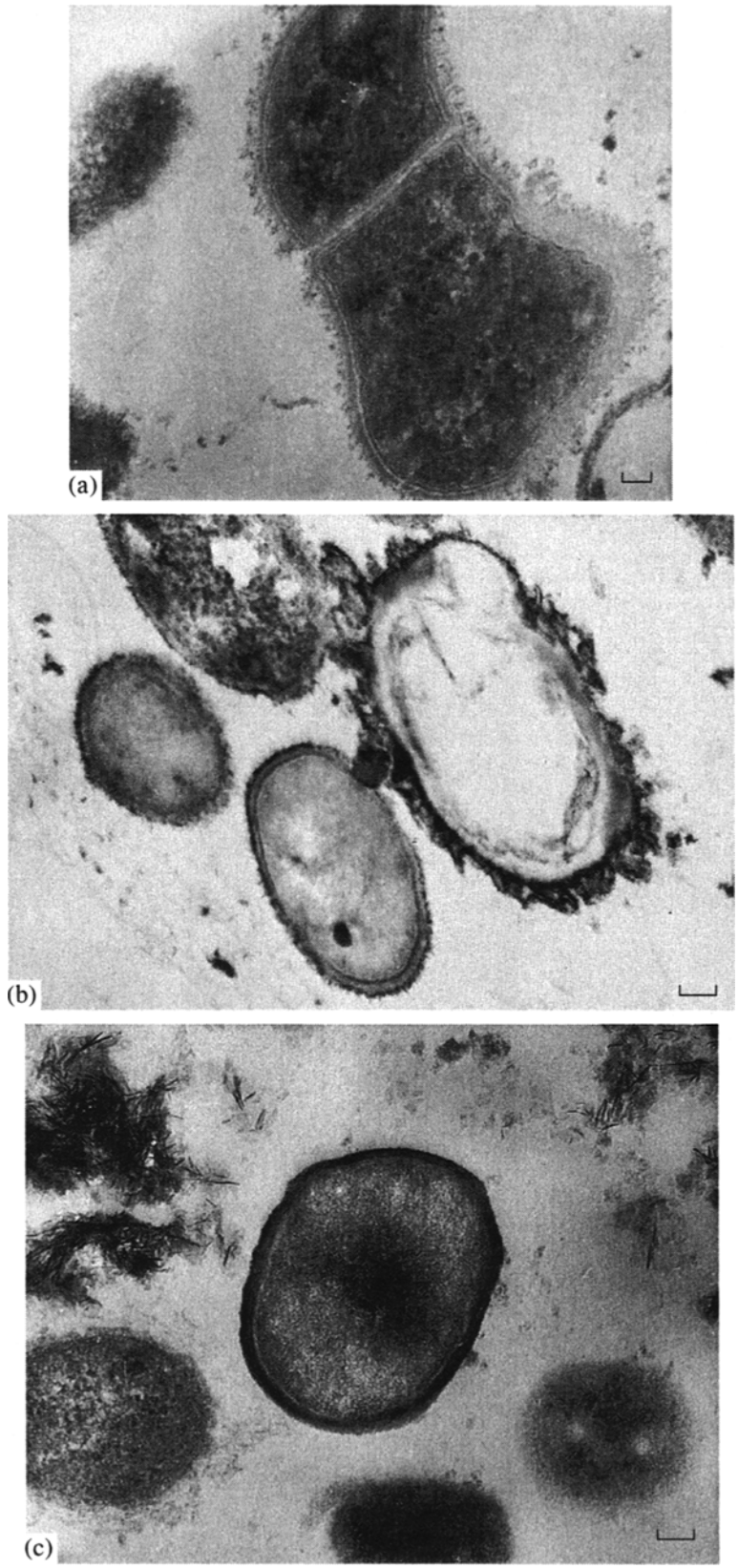

Fig. 2. Thin sections of *A. globiformis* B-1112 cells: (a) a vegetative linear-phase cell grown in nutrient broth (control); (b) an autolysed cell in the spontaneously autolysing 20-fold thickened suspension prepared using buffer with 0.02% $CaCl_2$ and pH 6.5; and (c) a resting cell formed in this suspension. Bars represent 0.1 μm.

Table 5. Effect of autolysis induced by oleic acid (an analogue of the d_2 factor) on the number of viable cells in tenfold thickened suspensions of *A. globiformis* B-1112 cells stored at pH 7.25 for 1 month (data are expressed as CFU/ml and % of the initial number)

Additive	Viable cell count
Oleic acid, 1 mM	$(5.8 \pm 0.4) \times 10^8$ (2.64)
Control*	$(2.2 \pm 0.2) \times 10^{10}$ (100.00)
Control**	$(4.0 \pm 0.2) \times 10^6$ (0.01)

* Cell suspension in nutrient broth without oleic acid before storage.

** The same suspension after one month of storage.

inactive, as was proven by the absence of endogenous respiration. Examination under a phase-contrast microscope showed that these cells were coccoid and had a small size (below 0.3 μm), so that their refractility was difficult to observe.

The electron microscopic studies of thin sections showed that about 85% of cells of both induced and spontaneous autolysates were typical autolysed cells, whereas the remaining 15% of cells looked like intact vegetative cells with a thick cell wall (Figs. 2a–2c). The cytoplasm of intact cells was homogeneous and had regions with a high electron density of ribosomes. Nucleoids were not observed. On the whole, the ultrastructure of these cells was similar to that of the resting forms of other non-spore-forming bacteria [3, 4].

All of these properties of cells, remaining viable in the autolysing suspensions of *A. globiformis* strains stored for a long time at room temperature, allow one to refer to them as resting microbial forms.

The data presented show that autolysis plays an important role in the formation of the resting forms of the typical soil bacterium *A. globiformis*. This substantially contributes to our knowledge of the mechanisms responsible for the formation of dormant forms of non-spore-forming bacteria and for the intrinsic regulation of the number of viable cells in soil microbial associations, which provide for their survival under deleterious environmental conditions.

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