Microbiology, Vol. 73, No. 6, 2004, pp. 650–653. Translated from Mikrobiologiya, Vol. 73, No. 6, 2004, pp. 758–762.

EXPERIMENTAL ARTICLES

Influence of the Incubation Temperature on the Reaction of Oligotrophic Bacteria to Stress

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Received May 27, 2003

Abstract—Representatives of five genera of psychroactive oligotrophic bacteria, *Arcocella, Renobacter, Spirosoma, Caulobacter*, and *Methylobacterium*, were for the first time shown to be capable of growing at a negative temperature (–2°C). Long-term cultivation (for 116 days) at a low temperature under limitation by the carbon source is stressful for oligotrophic bacteria and leads to the death of a part of the cell population. The number of viable cells of *Caulobacter crescentus* decreased by two to three orders of magnitude. Over the studied period of time, *Renobacter vacuolatum* cells retained viability at a low temperature, whereas, at room temperature, the titer of colony-forming cells decreased by two orders of magnitude under starvation stress.

Key words: oligotrophic bacteria, temperature, starvation, stress, survival.

The slow-growing oligotrophic bacteria are widespread and even predominate in natural ecosystems; they show increased resistance to stressor factors [1]. They are capable of growing at low temperatures (6–8°C); some of them prefer a temperature of 8–10°C. Psychrotolerance was found to be characteristic of many soil forms of oligotrophs, although their growth is more active at a higher temperature (28°C), especially on methanol-containing media [2]. An increased level of adaptation to stressful conditions is characteristic of the oligotrophic bacteria with a high content of lecithin in their membranes. Rapid cell response to stressor factors, which is manifested in changes in the membrane lipid composition and element homeostasis, leads to protective cell adaptation [3]. The physiological and biochemical activity of psychrophiles was reported to correlate with the specific chemical composition of cell membranes, which determines the microorganism response to the growth temperature. With increasing growth temperature, the respiration activity of these microorganisms decreases; cell damage and death have been observed, as well as changes in membrane fluidity and permeability and in the content and ratio of phospholipids; the RNA synthesis increases, whereas the protein synthesis decreases [4]. Nevertheless, the survival of bacteria under conditions of negative temperatures and prolonged starvation is still a poorly studied problem [5, 6].

The subject of this study was the growth and survival of representatives of five genera of psychroactive oligotrophic bacteria (*Arcocella, Renobacter, Spirosoma, Caulobacter*, and *Methylobacterium*) at negative temperatures and during long-term exposure to low temperatures under conditions of nutrient limitation.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The bacterial cultures *Arcocella aquatica* NP-502, *Renobacter vacuolatum,* and *Spirosoma linguale*, all from the collection of the Institute of Microbiology, and *Caulobacter crescentus* ATCC 15252 were maintained on PYG medium (peptone, yeast extract, and glucose, 0.1% each), whereas the culture of *Methylobacterium organophilum* NP-220, from the collection of the Institute of Microbiology, was maintained on Hirsch mineral medium supplemented with 1% methanol [7].

At a negative temperature (–2°C), these microorganisms were grown in Petri dishes with 50 ml of liquid PYG or Hirsch media containing 1% glycerol without forced aeration under stationary conditions. At a higher glycerol concentration, the culture growth was inhibited. The same bacterial cultures grown for 26 days at room temperature served as controls.

Oligotroph survival at limited nutrient supply (starvation stress) was studied under the following conditions. Bacteria were grown in 100-ml glass flasks closed with sterile rubber stoppers on a shaker (250 rpm) at 7 or 28°C for 116 days on medium containing (per liter of distilled H_2O) Na_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $Mg_2SO_4 \cdot 7H_2O$, 0.05 g; microelements [7]; and Na glutamate, 15 or 150 mg. Samples (2.0 ml) were taken with a syringe through the rubber stopper on the 7th, 14th, 21st, 35th, and 116th days of incubation.

Measurement of the culture growth. Bacterial growth was judged from changes in optical density measured on a spectrophotometer at $\lambda = 600$ nm. The growth dynamics was assessed in terms of percent increase of the optical density. Cell morphology was

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Table 1. *R. vacuolatum* and *C. crescentus* growth in liquid Na glutamate–containing medium at different temperatures (OD units)

Culture age, days	*R. vacuolatum*				*C. crescentus*			
	Incubation temperature, °C							
	28		7		28		7	
	Na glutamate concentration, mg/l							
	15	150	15	150	15	150	15	150
0	0.08	0.09	0.08	0.09	0.08	0.10	0.08	0.10
7	0.15	0.28	0.12	0.21	0.25	0.28	0.18	0.20
21	0.20	0.35	0.18	0.27	0.28	0.35	0.30	0.37
116	0.21	0.37	0.24	0.40	0.30	0.38	0.28	0.35

Table 2. Changes in the cell titers of *R. vacuolatum* and *C. crescentus* (CFU/ml) after long-term starvation (116 days) at different temperatures

Experimental conditions	Na glutamate concentration, mg/l	*R. vacuolatum*		*C. crescentus*	
		0 days	116 days	0 days	116 days
28°C	15	5.8×10^7	6.0×10^5	3.5×10^7	4.5×10^5
	150	7.1×10^7	11.0×10^5	4.5×10^7	9.9×10^5
7°C	15	5.0×10^7	3.0×10^7	3.5×10^7	2.0×10^4
	150	7.1×10^7	2.5×10^7	4.5×10^7	12.0×10^4

examined under a light microscope equipped with a phase-contrast accessory.

Gaseous phase analysis. CO_2 was measured on an LKhM8MD chromatograph at room temperature (thermal conductivity detector; 1.5 m × 3 mm column; Porapak Q 80–100 mesh; hydrogen as carrier gas; flow rate, 40 ml/min). Oxygen was determined on the same chromatograph at room temperature (thermal conductivity detector; 2.5 m × 3 mm column; molecular sieve 13 × 0.25 mm; argon as carrier gas; flow rate, 40 ml/min).

Survival of microorganisms after exposure to stressful conditions was determined by the conventional method, i.e., by plating dilutions of cell suspensions onto solid nutrient media in Petri dishes.

Each experiment was run in triplicate and repeated twice sequentially; the results were averaged.

RESULTS AND DISCUSSION

All of the studied cultures proved to be capable of growing at a negative temperature (–2°C), which is in agreement with our previous data on the growth of oligotrophic bacteria at low temperatures (7–8°C) [3]. Figures 1 and 2 show the growth kinetics at different temperatures. The growth of *Renobacter vacuolatum* and *Methylobacterium organophilum* was significantly inhibited by the negative temperature (Figs. 1a, 1b). No cell lysis or spheroplast formation testifying to bacterial cell death were revealed by microscopic examination. After two weeks of growth at –2°C and until the end of the experiment, the optical density of the suspension of *Caulobacter crescentus* remained the same as at room temperature (Fig. 1c). The growth patterns of *Arcocella aquatica* and *Spirosoma linguale*, which are close in their compositions of cell phospholipids and membrane lipids, were similar (Figs. 1d, 1e). At the negative temperature, both *Arcocella aquatica* and *Spirosoma linguale* reached the stationary growth phase by the 15th to 20th day of cultivation; the optical density curves of *S. linguale* cultures were similar at both temperatures, although an insignificant growth delay was observed at the negative temperature. At room temperature, a decrease in the optical density due to cell lysis was observed under a phase-contrast microscope in the third week of culture growth. Thus, negative temperatures are presumably not stressful for the oligotrophic bacteria, which explains their wide distribution in natural ecosystems.

Tables 1 and 2 and Figs. 2a and 2b show the parameters of growth and survival of two oligotrophic cultures, *R. vacuolatum* and *C. crescentus*, that differ in their growth patterns at low temperatures. These results were obtained during long-term cultivation (116 days) under starvation stress on media containing 15 and 150 mg/l of Na glutamate as a single carbon source.

The highest growth rate and most intense release of carbon dioxide were observed from the 7th to 21st days of the growth of both bacteria under stressful conditions (carbon deficiency and low temperature). We have previously shown that oligotrophic bacteria are capable

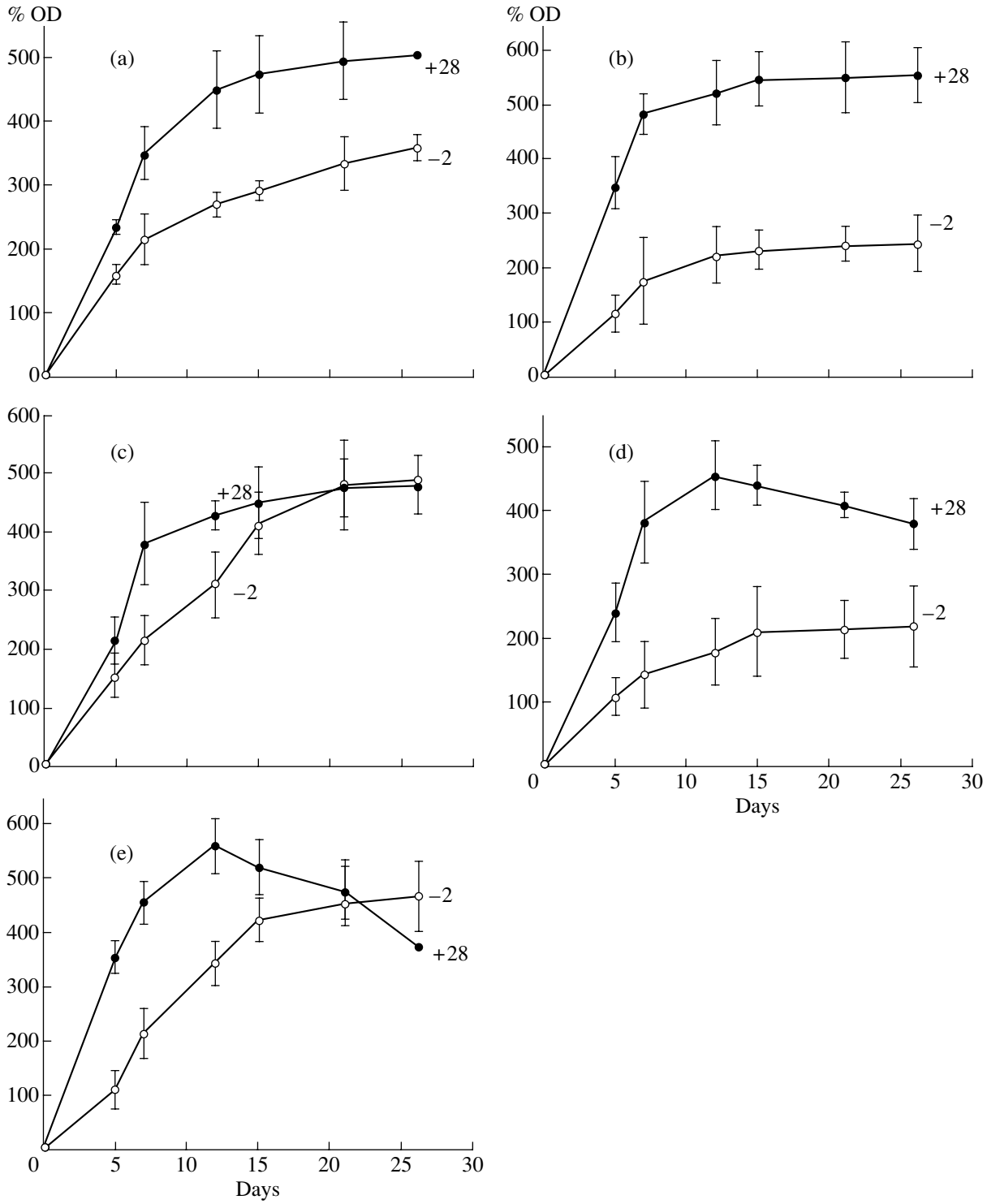

Fig. 1. Growth kinetics of oligotrophic bacteria at different temperatures (–2 and 28°C): (a) *R. vacuolatum*; (b) *M. organophilum* NP-220; (c) *C. crescentus*; (d) *A. aquatica*; (e) *S. linguale.*

of utilizing an intracellular reserve compound, poly-β-hydroxybutyrate [8]. This may explain the slow culture growth (Table 1) and CO_2 release after the exhaustion of a small amount of nutrients present in the medium (Figs. 2a, 2b). This assumption is in agreement with *R. vacuolatum* behavior after complete exhaustion of nutrient substances in the medium and reserve compounds in the cells [9]. High rates of growth and respiration were observed in both cultures grown on the medium with the higher content of the carbon source (150 mg/l).

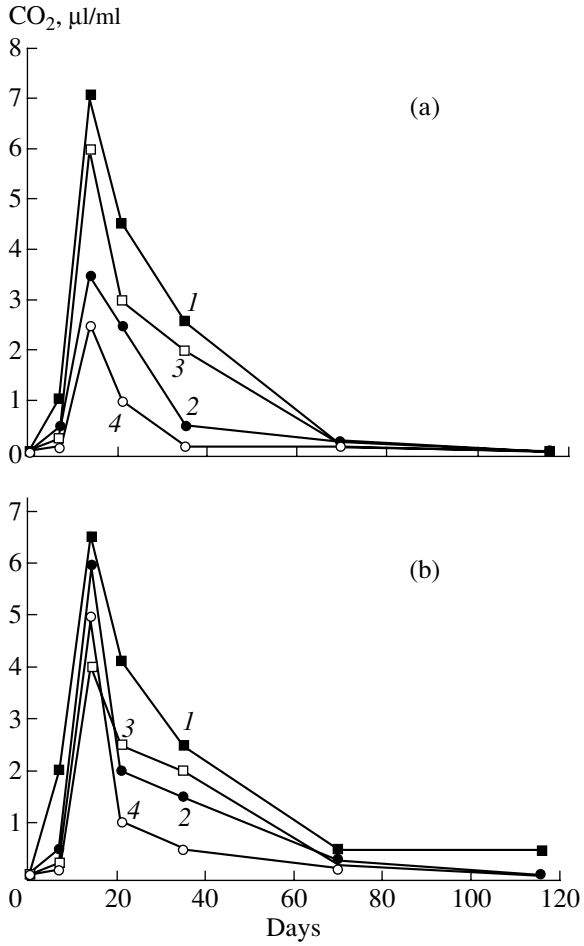

Fig. 2. Kinetics of carbon dioxide release by (a) *R. vacuolatum* and (b) *C. crescentus* cultures grown under carbon source deficiency at different temperatures: (*1*) 28°C, 150 mg/l; (*2*) 28°C, 15 mg/l; (*3*) 7°C, 150 mg/l; (*4*) 7°C, 15 mg/l.

R. vacuolatum and *C. crescentus* differed in the response to temperature: in the former organism, growth and respiration rates decreased at a low temperature, whereas, in the latter, these parameters were little dependent on the temperature.

After 116 days of cultivation, the number of cells was lower than that determined immediately after inoculation (Table 2). Although the optical density and cell morphology remained unchanged (no spheroplasts, cell debris, or other signs of cell death were observed), the number of colony-forming units decreased significantly. The number of viable cells of *C. crescentus* decreased by two and three orders of magnitude after cultivation at room temperature and at 7°C, respectively (Table 2). The *R. vacuolatum* cells were much more resistant to low temperature. Under conditions of decelerated metabolism, the number of viable cells of this organism decreased twofold (Table 2). Since at room temperature the rates of all processes are significantly higher, the bacteria utilized the energy sources much more rapidly under stressful conditions, which resulted in a noticeable decrease in the viable cell titer.

Our results confirmed psychrotolerance of the oligotrophic bacteria of the five taxonomic groups studied. They were for the first time shown to be capable of growing at a negative temperature (–2°C). The results obtained suggest that the growth of oligotrophs within a wide range of temperatures, including negative temperatures, promotes their adaptation to other stressor factors, particularly starvation (15 mg/l). The examined cultures differed in the response to long-term starvation, especially at low temperature, most probably because the degree of intracellular reserve reutilization is different in different microorganisms. The data obtained extend our knowledge on oligotroph resistance to limiting ambient factors and on the oligotroph role in ecosystems.

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