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EXPERIMENTAL ARTICLES

Production of Resting Forms by the Gram-Negative Chemolithoautotrophic Bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum*

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Abstract—The haloalkaliphilic chemolithoautotrophic gram-negative bacteria *Thioalkalivibrio versutus*, strain AL2, and *Thioalkalimicrobium aerophilum*, strain AL3, were shown to possess the capacity to produce resting forms, namely cystlike refractile cells (CRC), whose production was controlled by the level of the d_1 extracellular factors exhibiting the function of anabiosis autoinducers. The conditions were elucidated that promote the formation of CRC in the developmental cycles of the cultures studied, in condensed cell suspensions undergoing autolysis, and under the action of exogenously introduced chemical analogues of anabiosis autoinducers (alkylhydroxybenzenes). The peculiarities of the fine structure of the resting cells obtained were studied. Distinctions were revealed (with respect to viability and thermotolerance) between the CRC formed under different conditions. The relationship between the growth strategy and survival strategy of extremophilic bacteria is discussed taking into account the effect of the d_1 autoregulatory factors. A new model of CRC formation is proposed: CRC production in the life cycle of bacteria developing under conditions of increased concentration of anabiosis autoinducers.

Key words: resting forms, non-spore-forming bacteria, *Thioalkalivibrio, Thioalkalimicrobium*, cyst-like cells, autoinducers of anabiosis.

Investigations that were carried out earlier showed that the life cycle of microorganisms incapable of forming specialized resting forms (such as endospores, cysts, or exospores), as well as of spore-forming bacteria grown under conditions repressing sporulation, culminates in the formation of cystlike refractile cells (CRC) [1–3]. The abundance of CRC formed depends on the cultivation conditions, particularly on nitrogen and/or phosphorus limitation, which enhances lipogenesis and the level of biosynthesis of alkylhydroxybenzenes, which play the role of anabiosis autoinducers in a number of microorganisms [1] and regulate CRC formation. It is possible that in natural ecotopes, locally increased concentrations of extracellular autoinducers of anabiosis in microzones also result in the production by non-spore-forming microorganisms of resting forms similar to CRC, which promotes their survival under natural conditions. One of the mechanisms increasing the concentration of extracellular inducers of anabiosis may be autolysis of a part of a microbial population, resulting in the release from lysed cells of autoinducers, which exert their effect on the cells remaining intact [4]. From this point of view, the stage of autolysis is prerequisite for the formation of resting forms and thus for the realization of the survival strategy of a microbial population. Our experiments showed that the formation of resting forms is influenced by the autolysis rate [4], which under natural conditions must depend both on environmental factors and on the character of microorganism growth. It may be suggested that *r* strategists, which survive due to a high growth rate under favorable conditions, also exhibit a high death rate under unfavorable conditions, which should lead to the rapid establishment of a high concentration of low-molecular factors and metabolites released from cells undergoing autolysis and involved in the regulation of the production of resting forms by the surviving part of the population. The slowly growing K strategists are more resistant to changes in the environment and for a longer time retain a constant, although low, population density, mainly represented by cells whose dormancy is not deep. An interesting experimental model which might be used to verify the above hypothesis is the extremely haloalkaliphilic bacteria *Thioalkalivibrio versutus*, strain AL2, and *Thioalkalimicrobium aerophilum*, strain AL3, which do not produce known specialized resting forms, such as spores or cysts, and differ in their growth strategy [5]. Earlier, we found that these bacte-

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ria have a system of autoregulation of culture development; this system includes the production of anabiosis autoinducers (d_1 factors), which provide for restriction of population density and formation of refractile anabiotic cells in short-term experiments with the introduction of a preparation of d_1 factor in cell suspensions [6].

The aim of this study is to elucidate the conditions of the production of resting forms in the developmental cycles of two sulfur-oxidizing haloalkaliphilic bacteria, *Tv. versutus* and *Tm. aerophilum*, and to reveal a possible relationship between the strategy of their survival and the strategy of their growth.

MATERIALS AND METHODS

The subjects of this study were the haloalkaliphilic chemolithoautotrophic sulfur-oxidizing bacteria *Thioalkalivibrio versutus*, strain $AL2^T$, and *Thioalkalimicrobium aerophilum*, strain $AL3^T$, recently isolated from soda lakes [7, 8]. The bacteria were grown on a medium with the following composition (g/l): Na_2CO_3, 24; NaCl, 10; K_2HPO_4, 1; KNO_3, 1; before sterilization, the pH of the medium was adjusted to 10.2 with $NaHCO_3$. After sterilization, the medium was supplemented with the following separately sterilized components: (1) trace element solution by Pfennig and Lippert [9] (1 ml/l for strain AL2 and 2 ml/l for strain AL3); (2) $MgCl_2 \cdot 6H_2O$ (0.5 mM for strain AL2 and 1 mM for strain AL3); and (3) $Na_2S_2O_3 \cdot 5H_2O$ (40 mM for strain AL2 and 80 mM for strain AL3). Cultivation was performed in 250-ml flasks containing 50 ml of medium or in 2000-ml flasks containing 450 ml of medium on a shaker (140 rpm) at 28°C. As the inoculum, stationary-phase cultures were used in an amount of 10–15 vol %. The cultures were maintained at 4°C in liquid media supplemented, after substrate exhaustion, with $MgCl_2$ (0.1 ml of 10% $MgCl_2$ per 10 ml of the culture); culture transfers were performed once every three months.

To prepare agarized media, equal volumes of salt base of the medium and 3.5–4% agar solution were mixed and supplemented with separately sterilized components: trace elements, $MgCl_2 \cdot 6H_2O$, and $Na_2S_2O_3 \cdot 5H_2O$ (at the same concentrations as for the liquid medium).

Viability of bacteria in various physiological states was determined by the end-point dilution method in liquid medium or by plating diluted suspensions onto solid medium and counting CFU numbers.

Microscopic examinations were carried out using an Amplival (Germany) microscope equipped with a phase-contrast device.

For electron-microscopic studies, sedimented cells were resuspended in 0.6 M NaCl, fixed with a 2.5% solution of glutaraldehyde in cacodylate buffer (pH 7.2), and then with a 0.5% solution of Ru tetroxide (Figs. 1c, 1d, 2a, 2b) or a 1% solution of OsO_4 (Figs. 1a, 1b, 2c, 2d) in the same buffer containing 2% NaCl for 1 h at room temperature. The specimens were embedded in Epon. Ultrathin sections were stained with lead citrate and examined under a JEM-100C electron microscope (Japan) at a magnification of 20000–30000×.

Endogenous respiration of cells was determined with a Clarke electrode connected with a 5-ml thermostatted cell and a recorder (Yellow Spring Instr., Ohio, United States).

To determine the protein content, cells were sedimented by centrifugation, washed twice with 0.6 M NaCl to remove thiosulfate, and resuspended in 1 ml of 1 N NaOH, after which protein was determined by the method of Lowry *et al.* [10].

To obtain resting forms in cell suspensions undergoing natural aging, the suspensions were incubated under conditions promoting autolysis (at 20°C without agitation) for a month or longer.

To obtain CRC in thickened suspensions undergoing autolysis, stationary-phase cells were concentrated 10-, 20-, and 30-fold by centrifugation at 2500 *g* for 30 min under sterile conditions and then resuspended in their own culture liquid. These suspensions were incubated for a month or longer with periodical determination of the number of viable cells.

To determine thermostability of vegetative cells and resting forms, aliquots of cell suspensions were heated in a U-10 ultrathermostat at 60 or 80°C for 15–30 min and plated onto solid media for subsequent CFU count. The resistance to the combined effect of osmotic shock and low pH values was determined from the number of cells retaining viability after cell incubation in distilled water (pH 5.5) or in 0.005 M HCl (pH 2.4) for 1.5 h.

In some experiments, resuscitation of resting cells was performed by transferring 0.5 ml of the cellular suspension to 100 ml of the salt base of the cultivation medium (pH 10.2) with further incubation under constant mixing for 2 h or longer.

The effect produced on the bacteria by the d_1 factor chemical analogues (alkylhydroxybenzenes differing in the length of the alkyl radical, C_{12} AHB and C_7 AHB) was studied by their introduction into suspensions of stationary-phase (48-h) cells in the form of ethanol solutions; the concentration of ethanol in the suspension was 5%.

The results of experiments were statistically processed by standard methods.

RESULTS

Production of Resting Forms in the Developmental Cycle of the Studied Bacterial Cultures

Bacteria of the genus *Thioalkalimicrobium* are typical *r* strategists, surviving in the community at the expense of a high growth rate and metabolic activity but rapidly dying off under starvation or other unfavorable conditions. On the contrary, the slowly growing representatives of the genus *Thioalkalivibrio*, exhibiting high efficiency of growth, lower maintenance expendi-

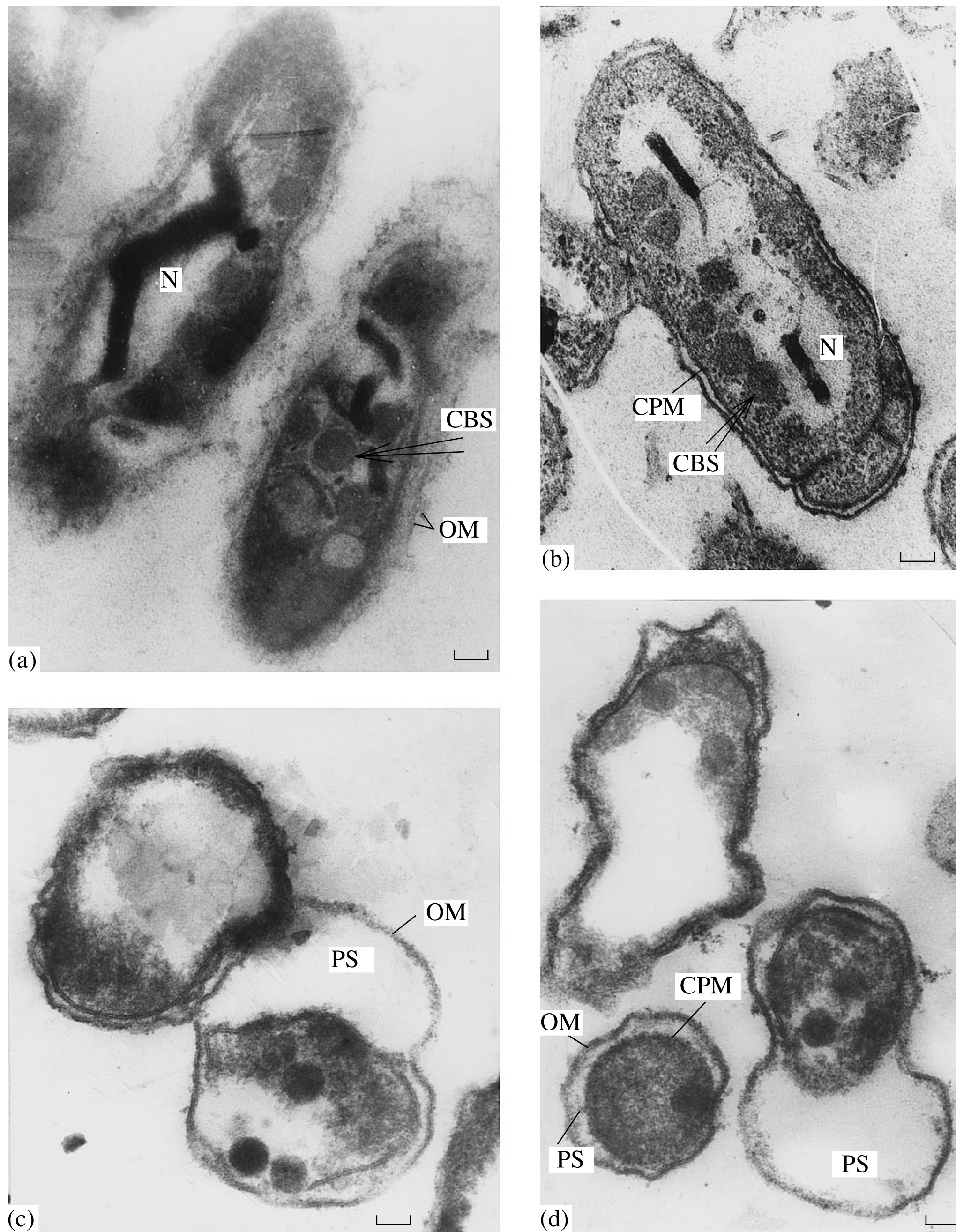

Fig. 1. (a) Cells from a stationary-phase 48-h culture of *Tv. versutus*, strain AL2. Here and in other figures, the bar represents 0.1 μm. (b) Cells from an exponential-phase 8-h culture of *Tm. aerophilum*, strain AL3. (c) Cells of a culture of *Tm. aerophilum*, strain AL3, stored in the growth medium of 1 month. (d) Cells of a culture of *Tm. aerophilum*, strain AL3, stored in a 20-fold concentrated suspension for 1 month. OM, outer membrane; PS, periplasmic space; CPM, cytoplasmic membrane; CBS, carboxysomes; N, nucleoid.

tures, and more diverse metabolism, are close to K strategists, adapted to long-term adverse impacts [5, 7, 8]. During the storage of a liquid culture of *Tv. versutus*, strain AL2, viable cells were found in it after a year and sometimes longer, whereas maintenance of *Tm. aerophilum*, strain AL3, required culture transfers every 3 to 6 months, depending on the initial cell titer. Lack of specialized resting forms in these microorganisms and the presence of an autoregulatory system, involving the production of anabiosis autoinducers (d_1 factors) [6], provided grounds to hypothesize the possibility of formation in their developmental cycle of CRC ensuring the survival of these bacteria. Examinations under a phase-contrast microscope showed that, during storage of both cultures, most cells in them lysed, and the remaining ones decreased in size and acquired a certain

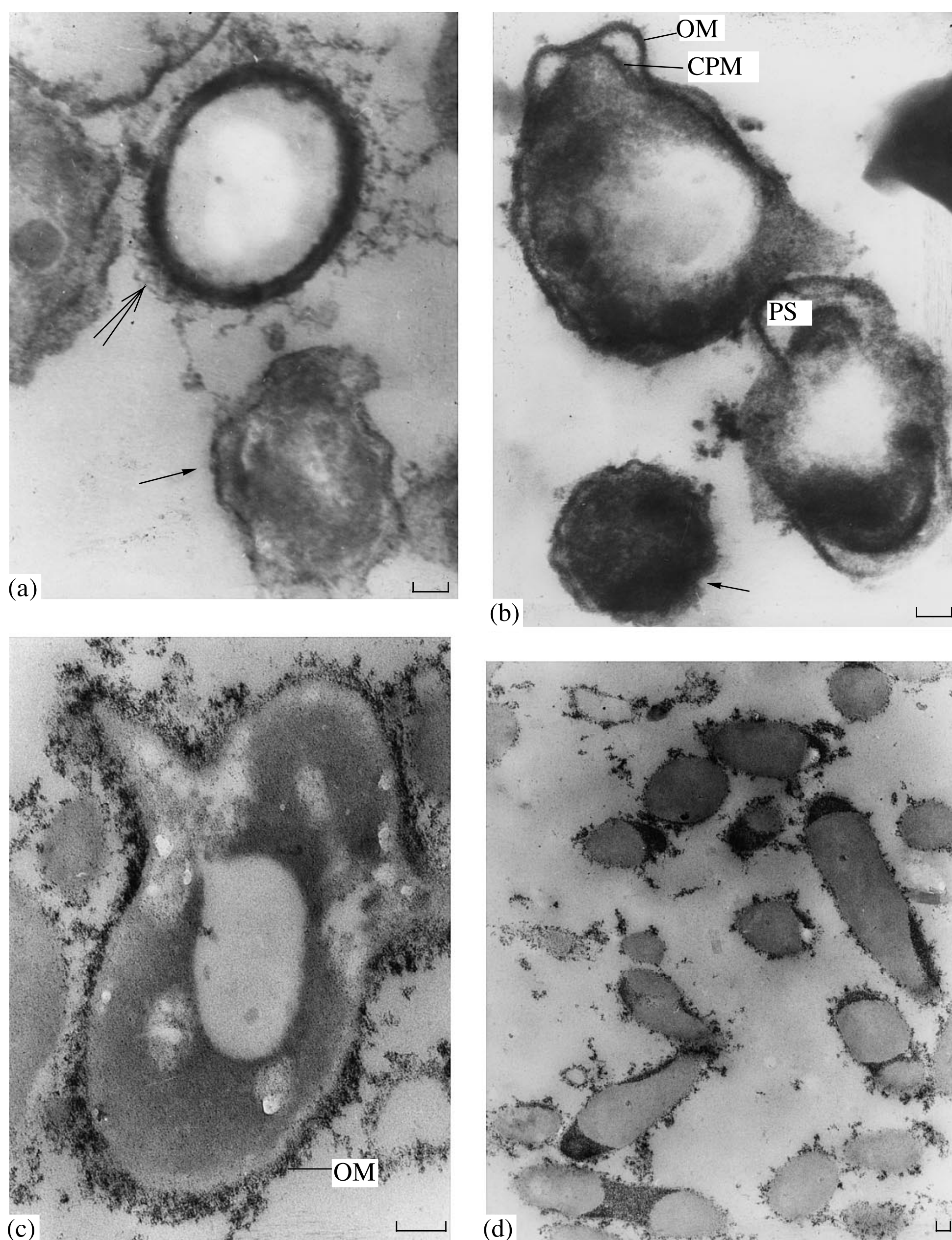

Fig. 2. (a) Cells from a culture of *Tm. aerophilum*, strain AL3, stored in the growth medium for 3 months (arrow shows cell of the 1st type; binary arrow shows cell of the 2nd type). (b) Resting forms of *Tv. versutus*, strain AL2, obtained upon the addition of 2×10^{-6} M water-soluble C_{12} AHB (chemical analogue of anabiosis autoinducer) to a suspension of stationary-phase cells. (c, d) Cells of *Tv. versutus,* strain AL2, unable to produce colonies; these cells were obtained upon the addition of 5×10^{-3} M ethanol-soluble C_{12} AHB (chemical analogue of anabiosis autoinducer) to a suspension of stationary-phase cells. OM, outer membrane; PS, periplasmic space; CPM, cytoplasmic membrane.

degree of refractility, which is a specific morphological feature of this type of resting form. Electron-microscopic examinations revealed ultrastructural changes in these cells as compared to vegetative cells. Two-day-old stationary-phase cells of strain AL2 (Fig. 1a) had a cell wall structure typical of gram-negative bacteria and cytoplasm of rather homogeneous density with inclusions typical of this organism, i.e., carboxydosomes (Fig. 1a, binary arrow). The nucleoid was located in a zone with a low electron density and a fine-granular structure; against the background of this zone, DNA fibrils formed a cord of a high electron density (Fig. 1a). Vegetative cells of an exponential-phase culture AL3 had a structure typical of gram-negative bacteria (Fig. 1b). Suspensions of old cultures of strains AL2 and AL3, stored for 1 month, contained, along with typical lysed cells, appearing as sheaths, intact cells with altered ultrastructural organization (Fig. 1c). The

nucleoid could not be revealed in these cells, and their cytoplasm lost its granular structure and became less electron dense, as compared to vegetative cells. In many of the intact cells, shrinkage of the protoplast and increased periplasmic space (especially at one of the cell poles) were observed.

Electron-microscopic examinations of cell suspensions of strain AL3, in which the number of viable cells remained relatively high even after three months of storage, revealed one more subpopulation of intact cells, distinct from the above-described subpopulations. Its cells, which were rounded, exhibited thick cell walls and unstainable cores of their protoplasts; they also possessed capsules (Fig. 2a) that resembled dehydrated protoplasts of mutant forms of *Bacillus subtilis* endospores with impaired synthesis of cortex [11].

Cell suspensions of strains AL2 and AL3, stored for a month or longer and containing a small fraction of intact cells, did not exhibit a polarographically detectable level of endogenous respiration or of respiration in the presence of exogenously added thiosulfate.

Great changes were observed during the storage of liquid cultures of strains AL2 and AL3 grown under standard conditions in the amount of viable cells, which was determined by the end-point dilution method and, in parallel, by CFU count after plating onto solid media (Table 1, the Control column). Note the significant differences between the changes in the numbers of viable cells observed for the two stains. After a month of storage of strain AL2, the decrease was not drastic (15% of the cells retained viability), suggesting a low intensity of autolytic processes; by contrast, for strain AL3 a decrease by several orders of magnitude was observed, with a parallel decrease in the dry cell mass (data not shown), indicating actively proceeding autolysis (Fig. 3). To increase the amount of intact resting cells possessing CRC features (retaining viability, changed ultrastructural organization, lack of polarographically detectable respiration), we set up model experiments on their formation in concentrated suspensions undergoing autolysis.

Formation of Resting Cells in Concentrated Suspensions Undergoing Autolysis

Upon concentration of cell suspensions of stationary-phase bacterial cultures, the rate and extent of autolysis, dependent on the level of extracellular d_2 factors (autolysis autoinducers), determine the increment in the concentration of extracellular d_1 factors (anabiosis autoinducers), which promote the formation of CRC by the other part of the population. In a previous publication [6], we reported on the occurrence of the d_1 and d_2 factors in strains AL2 and AL3, on the productivity of the cultures in this respect, and on the sensitivity of cells of the strains to these factors.

In the experiments described in this section, we examined the possibility of the formation of resting

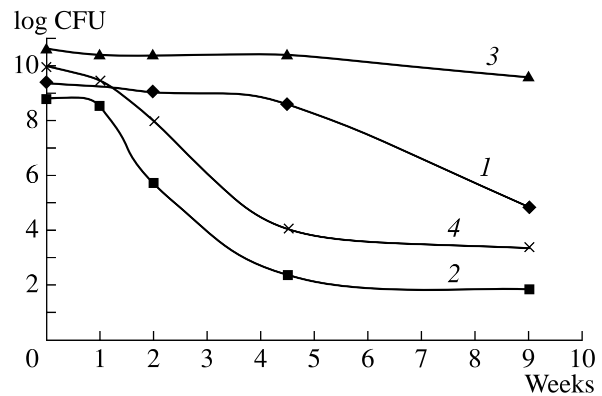

Fig. 3. Time course of the decline in the cell number in cell suspensions of haloalkaliphilic bacteria during a 9-week storage: (*1*) a culture of *Tv. versutus*, strain AL2, developmental cycle; (*2*) a culture of *Tm. aerophilum*, strain AL3, developmental cycle; (*3*) a culture of *Tv. versutus*, strain AL2, 20-fold concentration; (*4*) a culture of *Tm. aerophilum*, strain AL3, 20-fold concentration.

forms in suspensions undergoing autolysis that were concentrated 10-, 20-, and 30-fold in native growth medium; these conditions suggested development of spontaneous autolysis without exogenous inducers and simulated the natural situation of increased population density in near-bottom layers of water bodies. Determination of the number of viable cells in control variants (nonconcentrated suspensions) and in concentrated suspensions stored for two months showed that an essential difference between strains AL2 and AL3 with respect to the rates and intensities of autolysis was also observed in concentrated suspensions (Table 1, Fig. 3).

At the initial stage (first week of incubation), the main part of the population underwent autolysis, both in control and experimental variants. In concentrated suspensions of both strains, the absolute value of the number of autolysed cells was significantly greater than in the controls (by an order of magnitude in the 20× and 30× variants). As a result, the amount of regulators released from cells and promoting resting cell formation was greater in the experimental variants than in the controls. Due to this fact, immediately after two weeks of incubation, the CFU number in all concentrated suspensions was significantly higher than in the control variants. After two months of storage of strains AL2 and AL3, the amount of viable cells in experimental variants was, respectively, five and two orders of magnitude higher than in the controls. With respect to maintenance of viability, 20-fold concentration was optimal for both strains. Phase-contrast microscopy of concentrated cell suspensions revealed viable cells in amounts sufficient for analyses. These cells were characterized by a decrease in size and an increase in refractility. Suspensions of both strains did not exhibit a polarographically detectable level of endogenous respiration or of respiration in the presence of exogenously added thiosulfate.

Table 1. Viability of bacteria in liquid cultures (control) and concentrated suspensions stored under conditions favoring autolysis, CFU/ml (% of initial cell number)

Time	Control	10-fold concentration	20-fold concentration	30-fold concentration
		***Tv. versutus*, strain AL2**		
0*	$(2.5 \pm 0.2) \times 10^9$ (100.0)	$(2.0 \pm 0.3) \times 10^{10}$ (100.0)	$(4.4 \pm 0.4) \times 10^{10}$ (100.0)	$(4.5 \pm 0.6) \times 10^{10}$ (100.0)
1 week	$(1.9 \pm 0.2) \times 10^9$ (76.0)	$(1.3 \pm 0.2) \times 10^{10}$ (65.0)	$(2.6 \pm 0.4) \times 10^{10}$ (59.1)	$(2.6 \pm 0.4) \times 10^{10}$ (57.8)
2 weeks	$(1.1 \pm 0.1) \times 10^9$ (44.0)	$(1.1 \pm 0.2) \times 10^{10}$ (55.0)	$(2.5 \pm 0.5) \times 10^{10}$ (56.8)	$(2.5 \pm 0.3) \times 10^{10}$ (55.6)
1 month	$(3.8 \pm 0.4) \times 10^8$ (15.2)	$(0.9 \pm 0.2) \times 10^{10}$ (45.0)	$(2.4 \pm 0.3) \times 10^{10}$ (54.5)	$(2.2 \pm 0.3) \times 10^{10}$ (48.9)
2 months	$(6.7 \pm 0.9) \times 10^4$ (0.003)	$(4.8 \pm 0.7) \times 10^8$ (2.4)	$(3.9 \pm 0.47) \times 10^9$ (8.9)	$(7.7 \pm 0.9) \times 10^8$ (1.7)
		***Tm. aerophilum*, strain AL3**		
0*	$(6.2 \pm 0.8) \times 10^8$ (100.0)	$(3.6 \pm 0.5) \times 10^9$ (100.0)	$(9.8 \pm 0.8) \times 10^9$ (100.0)	$(1.7 \pm 0.2) \times 10^{10}$ (100.0)
1 week	$(3.5 \pm 0.5) \times 10^8$ (56.0)	$(5.7 \pm 0.7) \times 10^8$ (15.8)	$(3.0 \pm 0.4) \times 10^9$ (30.6)	$(5.9 \pm 0.8) \times 10^9$ (34.7)
2 weeks	$(5.3 \pm 0.4) \times 10^5$ (8.5×10^{-2})	–	$(9.3 \pm 0.8) \times 10^7$ (0.9)	–
1 month	$(2.1 \pm 0.3) \times 10^2$ (3.4×10^{-5})	$(3.1 \pm 0.3) \times 10^3$ (8.6×10^{-5})	$(1.1 \pm 0.1) \times 10^4$ (1.1×10^{-4})	$(2.3 \pm 0.4) \times 10^3$ (1.4×10^{-5})
2 months	$(6.5 \pm 1.2) \times 10^1$ (1.0×10^{-5})	$(5.2 \pm 0.4) \times 10^2$ (1.4×10^{-5})	$(2.3 \pm 0.4) \times 10^9$ (2.3×10^{-5})	$(1.2 \pm 0.2) \times 10^2$ (0.7×10^{-6})

* The number of viable cells immediately after concentration; for the control variant, stationary-phase 48-h culture.

Electron-microscopic examinations revealed a similarity between the ultrastructure of strain AL2 and AL3 cells obtained in 20-fold concentrated suspensions (Fig. 1d) and the ultrastructure of cells formed in the natural developmental cycles of these bacteria and stored for 1 month (Fig. 1c). A specific feature of such cells was cytoplasm shrinkage and formation of a site with extended periplasmic space at one of the cell poles; the nucleoid could not be revealed. The electron density of the cytoplasm varied. In concentrated suspensions of strain AL3, rounded cystlike cells occurred which were analogous to those shown in Fig. 2a.

An important property of resting forms, essential for the survival of the microbial population, is their increased resistance to conditions unfavorable for growth and to adverse impacts. It should be noted that the maintenance of viability and an intact state of cystlike cell in cell suspensions undergoing autolysis suggests their resistance to lytic enzymes, whereas vegetative cells are highly sensitive to extracellular autolytic enzymes and hydrolases released from the cells into the medium during autolysis.

A vivid indicator of the resistance of resting cells of both strains was their thermotolerance (Table 2), most dramatically demonstrated by heating at 80°C, which was fatal for the entire population of vegetative cells from the stationary phase (control). Prolongation of the temperature shock (60°C) from 15 to 30 min resulted in a decrease in the number of viable vegetative cells by an order of magnitude, but confidently increased the number of resting cells of strain AL2 that were capable of germination; this phenomenon may be viewed as activation of the resting part of a microbial population, a process analogous to activation of spore germination [12]. A fact deserving special attention is the different thermotolerance of resting forms of strains AL2 and AL3. After a 30-min exposure to a temperature of 60°C, the viability of cystlike cells of strain AL3 (of both those formed in the developmental cycle and those formed in suspensions undergoing autolysis) decreased insignificantly, whereas the viability of cystlike cells of strain AL2 decreased by four orders of magnitude.

Resting forms of the haloalkaliphilic bacteria under study also exhibited an increased tolerance to osmotic and acidic shocks. After 2 h of incubation in water at pH 5.5, viability was retained by 20.3% of the cells of 2-day-old stationary phase cultures of strain AL2 and by 80% of the cells in suspensions concentrated tenfold

Table 2. Thermostability of CRC obtained in the developmental cycle and in concentrated suspensions after a month of storage, CFU/ml (% of initial cell number)

Treatment	Control (2-day stationary-phase cultures)	CRC (after a month of storage) obtained			
		in developmental cycle	in suspensions concentrated		
			10-fold	20-fold	30-fold
***Tv. versutus*, strain AL2**					
No treatment	$(2.5 \pm 0.2) \times 10^9$ (100.0)	$(3.8 \pm 0.4) \times 10^8$ (100.0)	$(0.9 \pm 0.2) \times 10^{10}$ (100.0)	$(2.4 \pm 0.3) \times 10^{10}$ (100.0)	$(2.2 \pm 0.3) \times 10^9$ (100.0)
60°C, 15 min	$(1.4 \pm 0.2) \times 10^9$ (5.6×10^{-3})	$(3.0 \pm 0.5) \times 10^4$ (7.9×10^{-3})	$(4.8 \pm 0.6) \times 10^5$ (5.3×10^{-3})	$(1.2 \pm 0.2) \times 10^6$ (5.0×10^{-3})	$(1.1 \pm 0.1) \times 10^6$ (5.0×10^{-3})
60°C, 30 min	$(6.3 \pm 0.7) \times 10^4$ (2.5×10^{-3})	$(4.1 \pm 0.8) \times 10^{45}$ (1.1×10^{-2})	$(1.2 \pm 0.2) \times 10^6$ (1.3×10^{-2})	$(4.3 \pm 0.6) \times 10^6$ (1.8×10^{-2})	$(1.3 \pm 0.2) \times 10^6$ (6.0×10^{-3})
80°C, 15 min	0 (0)	$(5.0 \pm 0.7) \times 10^0$ (81.3×10^{-6})	$(2.0 \pm 0.4) \times 10^3$ (2.2×10^{-5})	$(7.9 \pm 0.7) \times 10^4$ (3.3×10^{-4})	$(4.4 \pm 0.4) \times 10^4$ (2.0×10^{-4})

***Tm. aerophilum*, strain AL3**

	Control (2-day stationary-phase cultures)	CRC (after 2 months of storage) obtained	
		in developmental cycle	in a 20-fold concentrated suspension
No treatment	$(6.2 \pm 0.8) \times 10^8$ (100.0)	65.0 ± 12.0 (100.0)	$(2.3 \pm 0.4) \times 10^3$ (100.0)
60°C, 30 min	$(2.0 \pm 0.5) \times 10^3$ (3.2×10^{-4})	37.5 ± 7.0 (57.7)	$(1.5 \pm 0.2) \times 10^2$ (6.5)

and stored for a month. Incubation in an HCl solution with pH 2.4 for 1.5 h revealed a similar tendency: viability was retained by 0.038% of the control cells of strain AL2 and 2% of the cells in suspensions concentrated 20-fold and stored for a month.

It should be recalled that populations of resting cells are heterogeneous with respect to the extent of dormancy; this general rule holds for the cystlike forms of the haloalkaliphilic bacteria studied. Special resuscitation procedures promote germination of cells occurring in the state of deep dormancy; this can be seen from an increase in the CFU number. In our experiments, we used a resuscitation procedure simulating a possible natural ecological situation: before plating, we resuspended resting cells in a large excess of mineral medium. During this procedure, part of the intracellular amphiphilic d_1 factors are released from the cells into the medium [2], and this disturbs the dormancy of the cells. Resuspension of cystlike cells of strain AL3 (obtained upon 20-fold concentration) in a large volume of fresh mineral medium (0.5 ml of concentrated suspension in 100 ml of mineral base) and subsequent 2-h incubation resulted in a 4.6-fold increase in the number of germinating cells.

Formation of Resting Cells under the Effect of Exogenously Introduced Chemical Analogues of d_1 Factor

In an earlier conducted work [6], we demonstrated the formation of refractile cells of the CRC type in stationary-phase (48-h) cultures of strains AL2 and AL3 under the effect of chemical analogues of the d_1 factors (anabiosis autoinducers). Further experiments showed that the viability of these refractile cells varied from analogue to analogue and depended on its amount and the pattern of its dispersion in the course of introduction into cell suspension. The forms obtained under the action of C_{12} AHB lacked an experimentally detectable level of endogenous respiration, and their viability and thermotolerance after a month of storage were at levels characteristic of CRC obtained in the developmental cycle (data not shown). Resuscitation procedures failed to increase the number of germinating cells. The ultrastructure of such cells (Fig. 2b) was similar to that of cells that form in the developmental cycle and retain their viability during prolonged storage in native medium (Fig. 1c) or are obtained after concentration of suspensions (Fig. 1d).

Of particular interest was the structure of cells that were obtained in the presence of high concentrations of the ethanol-soluble C_{12} AHB and did not show any evidence of viability under any conditions. Introduction of

Table 3. Effect of a water-soluble form of the chemical analogue of d_1 factor, C_7 AHB, introduced together with the inoculum, on the viability of strain AL3

d_1 analogue concentration, M	Viability, CFU/ml (% of the control), after	
	1-month storage	1.5-month storage
Control (without d_1 analogue)	$(6.9 \pm 0.9) \times 10^2$ (100.0)	$(3.0 \pm 0.5) \times 10^2$ (100.0)
5×10^{-5}	$(1.9 \pm 0.1) \times 10^3$ (275.4)	$(7.1 \pm 0.7) \times 10^2$ (236.7)
5×10^{-6}	$(3.5 \pm 0.4) \times 10^2$ (50.7)	$(0.5 \pm 0.08) \times 10^2$ (16.7)
10^{-6}	$(2.8 \pm 0.3) \times 10^3$ (405.8)	$(5.0 \pm 0.4) \times 10^2$ (166.7)

this analogue into cell suspensions in concentrations higher than 5×10^{-4} M (for strain AL2) and 2×10^{-4} M (for strain AL3) resulted, within 20 min, in the development of cell refractility, irreversible inhibition of metabolic activity, and changes in the ultrastructure of cells (Figs. 2c, 2d). The cytoplasm of such cells had a low electron density. The structure of the outer and cytoplasmic membranes was impaired, and this was the most probable cause for the irreversible loss of viability. Such cells, which completely lose their growth capacity but retain morphological integrity, were earlier described by us as *mummified* [13].

The models of CRC production considered up to this moment (in this and other works) simulated development of microbial cultures under natural conditions: (1) exogenous introduction of d_1 factors or their analogues simulates the increase of autoregulators occurring upon soil drying; (2) C/N imbalance or limitation by N, P, or O_2 enhances biosynthesis of d_1 factors [1]; (3) autolysis of a part of the microbial population results in an increase in the content of d_1 factors in the medium [4]. In the present work, we used one more model: development of bacterial cultures under conditions of an increased concentration of anabiosis autoinducers. The introduction of C_{12} AHB in concentrations of 10^{-7} to 10^{-5} M into fresh nutrient medium simultaneously with the inoculum resulted in the inhibition of growth and development of both cultures studied (data not shown). The introduction of another chemical analogue of the d_1 factor, C_7 AHB, in concentrations of 10^{-6} to 5×10^{-5} M did not inhibit the growth of the cultures but, on the contrary, stimulated it (especially in a concentration of 5×10^{-6} M): the development of the cultures culminated in the formation of resting cells in amounts that were larger than in the control cultures (Table 3); these cells possessed all of the aforementioned characteristics of resting forms. These results can be explained by metabolic changes occurring during the development of cultures in the presence of autoregulator concentrations that are higher than the natural level. Another plausible explanation is a changeover of the initial phenotype to another one, characterized by higher resistance and a more pronounced tendency toward the transition to the resting state. The latter supposition is supported by the data of our earlier investigations that showed that the phenotypic variability of the *Bacillus cereus* strain 504 is influenced by anabiosis autoregulators [14].

DISCUSSION

Comparison of the processes of resting cell formation by *Tv. versutus*, strain AL2, and *Tm. aerophilum*, strain AL3, which realize different growth strategies, showed that both the absolute and specific amount of resting cells formed by strain AL3 were several orders of magnitude lower. However, the percent of cells remaining viable after thermal treatment was considerably higher for strain AL3; i.e., the resting cells formed by this strain exhibited higher resistance. Thus, strains AL2 and AL3, which differ in their growth strategy, are also different with respect to their survival strategy. The bacterium *Tm. aerophilum* AL3 is an *r* strategist characterized by a high growth rate and rapid autolysis at the dying-off stage of culture development; as distinct from *Tv. versutus* AL2 (K strategist), it forms a low number of resting forms, but they are highly resistant. *Tv. versutus* AL2 form a large number of resting cells, but only a small fraction of these cells are resistant to adverse impacts. These data suggest that, in natural environments, the populations of the *r* strategist *Tm. aerophilum* AL3 survive due to the high resistance of CRC rather than to their number. Another possible hypothesis implying different productivity of *Tv. versutus* AL2 and *Tm. aerophilum* AL3 with respect to extracellular d_1 factors is disproved by the results of our earlier study [6], which showed that the amounts of extracellular d_1 factors in culture liquids of these two bacteria differ insignificantly, equalling 10.3 ± 0.5 U/g protein (9.3 ± 0.4 U/l) for AL2 and 7.2 ± 0.3 U/ g protein (8.7 ± 0.4 U/l) for AL3. The same work also showed that AL3 cells were more sensitive to d_1 factors and various AHB homologs, which are chemical analogues of anabiosis autoinducers. Our experiments on the formation of resting forms in concentrated suspensions showed that in 40% of AL3 suspensions whose initial titer was higher than 1.25×10^9/ml, intact refractile cells that did not exhibit viability even after resuscitation procedures could be detected after a month of storage. The ultrastructure of these cells, examined in thin sections, was very similar to the above-described ultrastructure of refractile cells retaining viability. Similar experiments with concentrated AL2 suspensions with a high initial cell titer revealed cystlike cells that invariably retained viability during the course of at least 3 months of storage (the observation period). It can be assumed that, in K strategists, not only the growth, but also the dying-off process proceeds more slowly; therefore, maturation of resting forms, coupled with dying off, requires more

time as compared to *r* strategists. This correlates with the more efficient metabolism of K strategists. It should be taken into account that massive autolysis results in the liberation not only of d_1 factors, but also of d_2 factors (free unsaturated fatty acids), which are autolysis autoinducers and, as shown previously [4, 15–17], regulate the rate of cell self-degradation and influence the rate of liberation of intracellular autoinducers of anabiosis and, hence, the amount of resting forms produced. That is why the amount of resting forms in concentrated suspensions of AL2 cells increased drastically (by five orders of magnitude) as compared to the amount formed in the developmental cycle.

It should be mentioned that autolysis results in the liberation not only of free unsaturated fatty acids but also of other low-molecular-weight intracellular autoregulators, including those increasing cell osmoresistance, as has been demonstrated for halophilic archaea [18]. It can be assumed that this mechanism is supplementary to the mechanism that involves anabiosis autoinducers. In our investigations, the cystlike cells formed in concentrated suspensions acquired combined resistance to both osmotic shock and changes in the pH of the medium; such a combined resistance is ecologically important for haloalkaliphilic bacteria.

Considering our data on the ultrastructure of the cystlike cells of *Tm. aerophilum* AL3 and *Tv. versutus* AL2, we would like to draw attention to the fact that the cells exhibiting characteristics of resting forms and retaining viability during prolonged storage decreased in size and had an irregular shape due to an uneven profile of the outer membrane and to irregular shrinkage of the protoplast, resulting in the formation of a voluminous periplasmic space. Cells of a similar structure, possessing a voluminous periplasmic space, were earlier reported to be produced by gram-negative bacteria and were assigned to spheroplasts, i.e., cell forms arising upon partial degradation of the cell wall [19]. In the opinion of Kats [19], spheroplasts of this kind are likely cell forms produced by gram-negative bacteria that can survive unfavorable conditions and rapidly resume vegetative growth.

Another type of resting cell that we revealed in the bacterium AL3 (cells with a thickened cell wall and electron-transparent cytoplasm) is most probably characterized by a deeper level of dormancy, which allows these cells to retain viability during prolonged storage. Earlier, we found analogous cystlike cells in cell suspensions of *Bacillus cereus* stored for 3 years after growth under conditions of repressed sporulation, as well as in cell suspensions of *Micrococcus luteus* (unpublished data).

Thus, our investigation showed that the bacteria *Tm. aerophilum* AL3 and *Tv. versutus* AL2 are capable of producing CRC-type resting forms during the developmental cycles of their cultures and in concentrated suspensions undergoing autolysis. These resting forms are characterized by morphological alterations (smaller cell size and increased refractility), a sharply decreased level of metabolic activity (lack of polarographically detectable endogenous or exogenous respiration), an increased resistance to extreme impacts, an ultrastructural organization specific to CRC, and retained viability over long periods of time.

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