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EXPERIMENTAL ARTICLES

Characterization of *Bacillus cereus* Dissociants

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Abstract—The autoregulation of the phenotypic (populational) variability of the *Bacillus cereus* strain 504 was studied. The isolated colonial morphotypes of this bacterium were found to differ in their growth characteristics and the synthesis of extracellular proteases. The phenotypic variabilities of vegetative proliferating cells and those germinated from endospores and cystlike refractory cells were different. Bacterial variants also differed in the production of the d_1 and d_2 factors (the autoinducers of anabiosis and autolysis, respectively) and sensitivity to them. The possible role of these factors in the dissociation of microorganisms is discussed.

Key words: populational variability, microorganisms, colonial morphotypes, *Bacillus cereus*, resting forms, autoinducers of dormancy.

The ability of microorganisms to form resting forms capable of surviving unfavorable environmental conditions and their populational variability, which serves to produce a bacterial variant (or clone, serotype, subpopulation, etc.) adapted to given environmental conditions as much as possible, are important evolutionary mechanisms providing for species conservation in nature. The relationship between these mechanisms is poorly understood.

It is known that microbial populations are heterogeneous and contain cells that differ in the colonial, morphological, physiological, and biochemical properties. This phenomenon is called phenotypic variability or phenotype metastability [1]. Populational variability is typical of enterobacteria [2], pseudomonads [3], actinomycetes [4, 5], bacilli [6, 7], and other bacteria. The dissociation frequency varies from 10^{-2} to -10^{-4} per one cell division [2]. The frequency of reversion to the original phenotype is also high.

Recently, a great deal of interest has been focused on the genetic mechanisms responsible for populational variability, which are related to the reversible modification and rearrangement of DNA. These are the switching of the expression of certain genes, the intrapopulation transfer of genetic information by phages and plasmids, frame-shift mutations, and other genetic rearrangements influencing DNA transcription [1, 2]. Taking into account that resting (anabiotic) forms may be considered to be dedifferentiated cells, we believe that their entering a new proliferative cycle should involve considerable modifications and rearrangements of the above-mentioned types.

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The adaptation of saprophytes to varying environmental conditions, the virulence and persistence of pathogenic microorganisms, symbiotic interactions, and other processes are related to the dissociation of bacterial populations. In light of this, the dissociation phenomenon is an interesting subject of investigation to researchers working in the fields of microbiology, medicine, biotechnology, ecology, and bacterial evolution.

Our earlier investigations into the mechanisms of the formation of resting cystlike refractory cells (CRCs) showed that the germination of the *Bacillus cereus* 504 CRCs, whose formation was induced by anabiosis autoinducers, gave rise to a great number of dissociants (variants) differing from the dominant variant in some colonial and morphological characteristics [8]. This finding prompted us to investigate the regulation of the populational variability of bacteria by two autoregulatory factors, the d_1 autoinducer of anabiosis, which represents alkyl-substituted hydroxybenzenes (AHBs), and the d_2 autoinducer of autolysis, which represents free unsaturated fatty acids (FUFAs).

The present work was undertaken to isolate and describe the colonial morphotypes of *B. cereus* 504, to compare their growth and biochemical characteristics, and to investigate the role of the resting state and of the d_1 autoregulatory factor controlling this state in the dissociation of the population of this bacterium.

MATERIALS AND METHODS

The spore-forming *Bacillus cereus* strain 504 was obtained from the All-Russia Collection of Microorganisms (VKM). The strain was maintained on potato agar slants. To obtain colonial variants of this strain and

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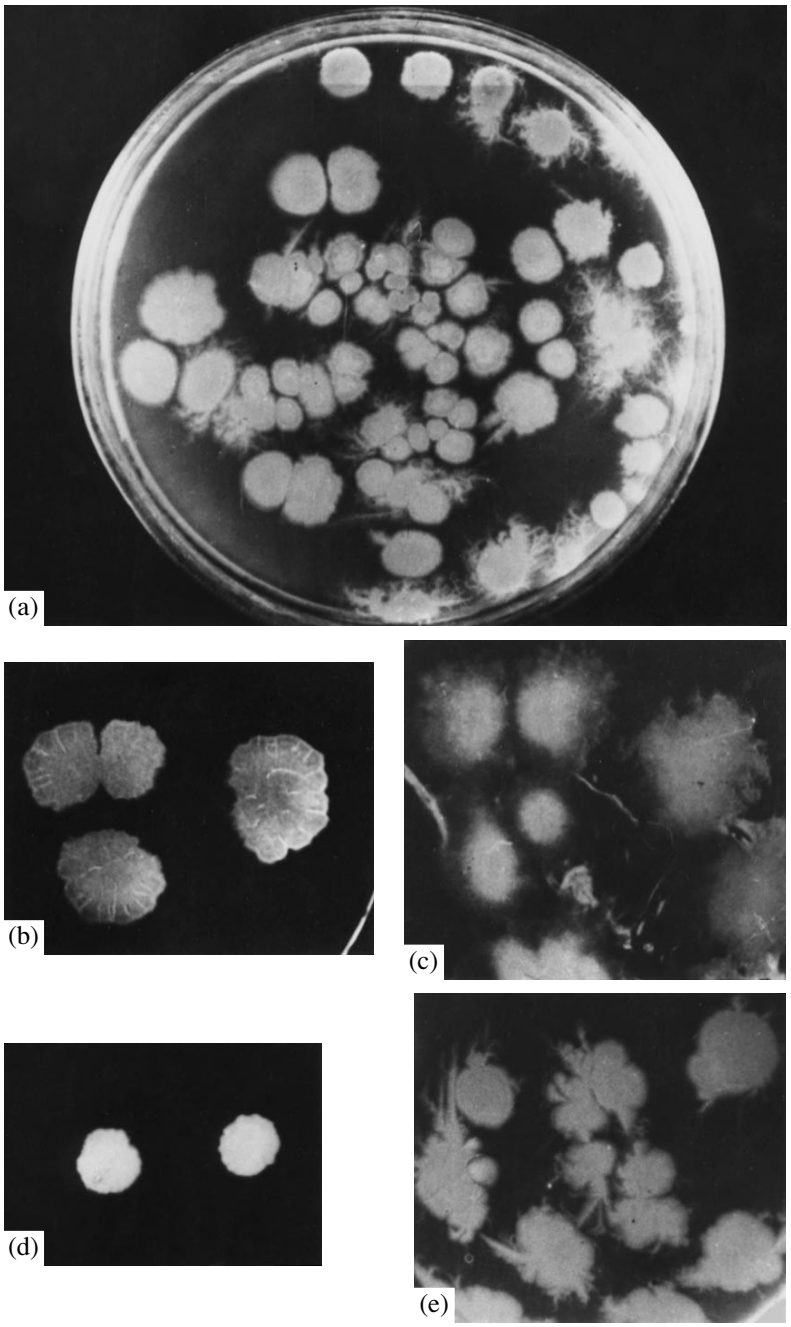

Fig. 1. Photographs of the *B. cereus* 504 variants observed after the plating of the CRCs induced by nitrogen deficiency: (a) entire plate; (b) dominant variant; (c) transparent variant; (d) white variant; and (e) mycoid variant.

to study their stability, it was subcultured on solid media nine or more times using 50 plates in each of the subcultures. Individual colonies were picked up by an inoculating loop and each of them was spread, using a spatula, over the surface of fresh agar plates to give from 10 to 80 colonies on one plate. Batch cultures were plated by the serial dilution method.

Resting forms were obtained using three different media. Endospores were obtained by cultivating *B. cereus* 504 in medium 1 containing (g/l) $(NH_4)_2HPO_4$, 1.0, KCl, 0.2, $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.0017; glucose, 2; nutrient broth, 20%. To obtain CRCs, strain 504 was cultivated in medium 2 of the same mineral composition as medium 1 but supplemented with 40–60 g/l glucose and 0.3 g/l bactopeptone. To obtain stationary-phase cells capable of long-term storage, strain 504 was cultivated in medium 3 of the same composition as medium 1 but containing 40–60 g/l glucose. The pH of the growth media after their sterilization was 7.0 ± 0.1. The media

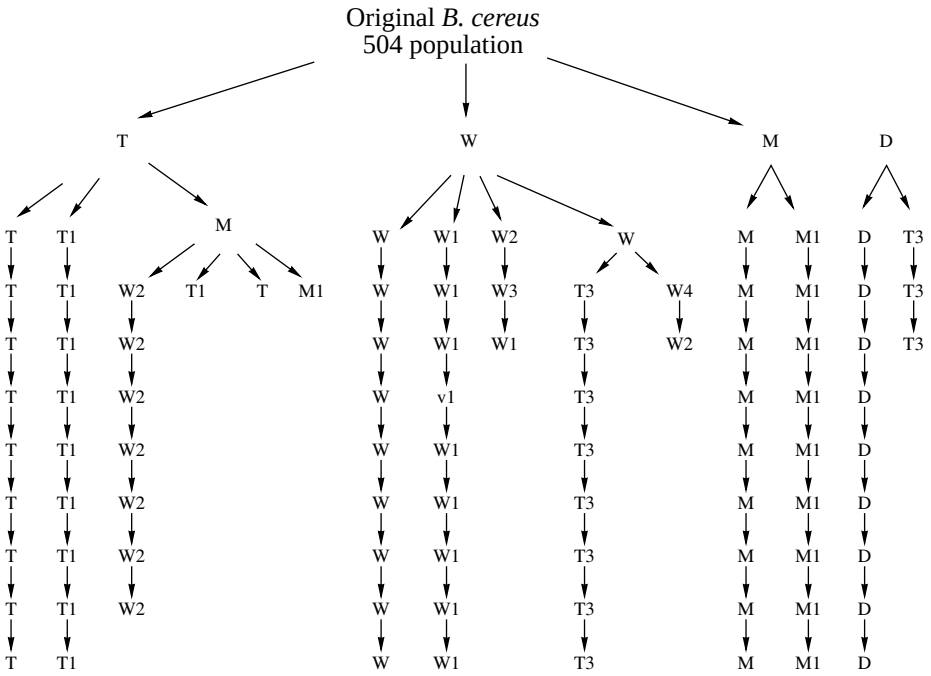

Fig. 2. Scheme illustrating the dissociation of *B. cereus* 504. The subtypes of the dissociants are designated by the respective letter (D, dominant; M, mycoid; T, transparent; and W, white) with serial numbers.

for obtaining endospores and long-storage stationary-phase cells were inoculated with 5- to 6-day-old suspensions of endospores produced in the culture incubated in medium 1. The medium for obtaining CRCs was inoculated with vegetative cells taken from the linear growth phase. The inocula were added in amounts to give an initial optical culture density of 0.2. The optical density of cultures was measured at λ = 600 nm using a Specord spectrophotometer and 10-mm-path-length cuvettes. The formation of CRCs was induced by adding the d_1 autoregulatory factor, which was preliminarily isolated from *B. cereus* 504, to the linear-phase culture of this bacterium grown in medium 1.

The d_1 autoinducer of anabiosis and the d_2 autoinducer of autolysis were isolated as described by Osipov *et al.* [9]. The content of the d_1 and d_2 factors was expressed in units of biological activity (U), which were defined as the amount of the respective factor inhibiting the endogenous respiration of linear-phase cells by 50%. The endogenous respiration was measured using an LP7E polarograph equipped with a Clark-type oxygen electrode with a 1-ml measuring cell [10]. As chemical analogues of the autoregulatory factors, we used 4-hexylresorcinol (M_r = 196; p*K*a = 9.0) and $C_{18:1}$ fatty acid, respectively. The analogues were added to the measuring cell of the oxygen electrode as alcohol solutions in amounts not allowing the alcohol concentration in the measuring cell to exceed 5 vol %.

To determine the proteolytic activity of cultures, they were grown for 7 days in tubes containing 30 ml of nutrient broth under stationary conditions or in 250-ml flasks which contained 100 ml of the broth and were shaken at 250 rpm. The cultivation temperature was 29°C. Proteolytic activity was measured as described in the laboratory manual of Gracheva *et al.* [11] and expressed as the amount (mg) of amine nitrogen formed during a 1-h hydrolysis of 5% gelatin solution by 1 ml of the culture liquid at 40°C at pH 7.3–7.5.

RESULTS AND DISCUSSION

The phenomenon of the increased morphological variability of *B. cereus* 504 was first observed in experiments on the germination of the CRCs of this bacterium, whose formation was induced by the d_1 autoinducer of anabiosis. Since our main task was to elucidate the relationship between the state of metabolic rest (anabiosis) of bacteria and their ability to realize their potential capacity for phenotypic variability, we investigated the variability of populations of various types of resting forms. The dissociation of *B. cereus* into colonial-morphological variants was observed in experiments with the lyophilized cultures of this bacterium obtained from the VKM and with the cultures maintained for a long time on potato agar slants. The variability of the stationary-phase *B. cereus* cells stored for a long time [12] or cells resuscitated from the CRCs produced spontaneously in medium 2 [8] or induced by the d_1 factor was greater than the variability of the vegetative cells of this bacterium grown in a submerged or surface culture (Fig. 1a).

Table 1. Characteristics of the *Bacillus cereus* 504 dissociants and their subtypes

Dissociant	Subtype	Characteristics of colonies
Dominant	D	Circular, slightly raised, dull, yellowish colonies with a pastelike consistency
Mycoid	M1	Gray–white, dull, branched colonies with pastelike consistency
	M2	Similar to M1 but less branched
Transparent	T1	Circular, colorless, uneven-edged colonies with pastelike consistency
	T2	Circular, colorless, shiny, even-edged, mucoid colonies
	T3	Colorless colonies with pastelike consistency and a structure typical of the mycoid variant
	T4	Yellowish flat colonies with pastelike consistency and a size larger than that of T1–T3
White	W1*	Circular, deep white, shiny, even-edged, mucoid colonies
	W2**	Circular, deep white, dull, even-edged, dense colonies
	W3	Circular, fairly raised, deep white, shiny, even-edged, mucoid colonies

* Variant W1 produced endospores under standard conditions.
** Variant W2 produced no endospores under standard conditions.

Table 2. The percentage of the *B. cereus* 504 variants produced under different cultivation conditions

Cultivation mode	Dissociant, %				
	dominant	mycoid	transparent	white	percentage of minor variants
Submerged (the linear growth phase)	99.6	0.02	0.01	0.01	0.04
Superficial	85.1	12.2	1.0	1.7	14.9

Table 3. The effect of the growth phase on the dissociation of the submerged *B. cereus* 504 culture

	Exponential phase	Linear growth phase	Stationary phase
Total bacterial count, CFU/ml	$(47.6 \pm 2.3) \times 10^6$ (100)	$(49.2 \pm 2.6) \times 10^7$ (100)	$(50.1 \pm 2.4) \times 10^7$ (100)
Minor variants, CFU/ml	$(18.0 \pm 0.9) \times 10^3$ (0.038)	$(21.0 \pm 1.2) \times 10^4$ (0.043)	$(35.0 \pm 1.8) \times 10^4$ (0.07)

It should be noted that not all colonies with an altered morphology are true colonial morphotypes. For instance, if bacterial cells are plated to give a high density of colonies (about 200 colonies on one petri dish), some colonies will have an altered morphology presumably due to a deficiency of nutrients [13] or the action of bacterial exometabolites. In our experiments, the transfer of such colonies onto fresh solid media caused their reversion to the original morphotype. At the same time, such transfer of true colonial morphotypes led to their dissociation with a certain frequency but with the retention of the dominant morphotype.

The stable *B. cereus* 504 dissociants obtained by routine methods [13, 14] according to the scheme presented in Fig. 2 had the following colonial characteristics: four dissociants were transparent, two dissociants were mycoid, three dissociants were white, and one dissociant was dominant (Fig. 1, Table 1). Some morphotypes had intermediate characteristics; for instance, morphotype M2 possessed the characteristics of dominant and mycoid variants, T3 possessed the characteristics of transparent and mycoid variants, and W2 possessed the characteristics of white and dominant variants. The formation of intermediate variants was presumably due to the simultaneous expression of genes responsible for the formation of different morphotypes or to the transfer of genetic material between them.

All of the isolated dissociants had a small amount (no more than 5%) of cells of the other variants, illustrating the continuous phenotypic dissociation of microbial cultures [6, 13, 14]. Some variants, namely, the transparent variant T4 and two white variants (the spore-forming variant W1 and the non-spore-forming variant W2), were unable to revert to the original form. The percentage of such variants was less than 0.1%. For this reason, they were not investigated further. The white variant W3 taken for analysis had the standard developmental cycle typical of all dissociants.

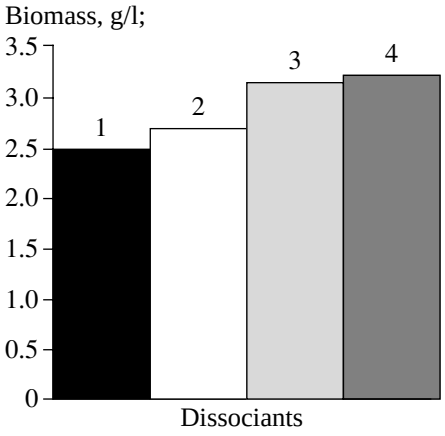

Fig 3. The biomass of the *B. cereus* 504 dissociants in the stationary growth phase: (1) dominant; (2) mycoid; (3) transparent; and (4) white.

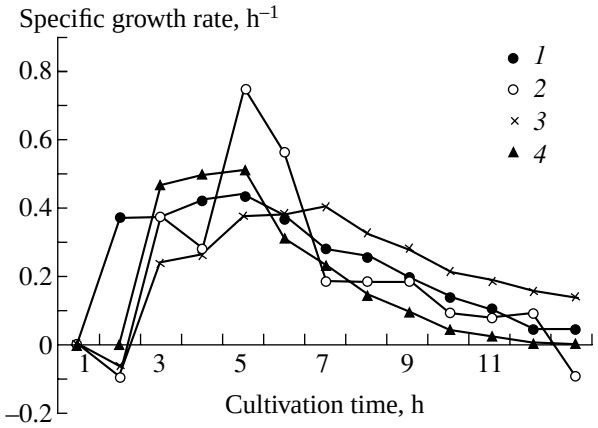

Fig. 4. The specific growth rate of the *B. cereus* 504 dissociants: (*1*) dominant; (*2*) mycoid; (*3*) transparent; and (*4*) white.

When plated on potato agar, *B. cereus* 504 usually gave rise to 85% dominant, 12% mycoid, 1% white, and less than 1% transparent variants. In a submerged batch culture, the fraction of the minor variants of *B. cereus* 504 was considerably lower than during bacterial growth on solid media (Table 2). Furthermore, the fraction of minor variants in the submerged culture was greater in the stationary growth phase than in the linear or exponential growth phases (Table 3). This fact may be due to the accumulation of the d_1 and d_2 autoregulatory factors in the stationary-phase culture [15–17] and changes in the ratio of these factors [16] (relevant data will be considered below). It should be noted that the phenomenon of the increased content of the minor variants of streptomycetes in senescent cultures was defined by Krasilnikov as the "age-related variability" [13].

The dissociants obtained differed in the specific growth rate and the biomass yield measured under standard cultivation conditions (Figs. 3 and 4). The maximum specific growth rates of all variants, except the white variant, were nearly the same, but the times in

Table 4. The activity of the extracellular proteases of the *Bacillus cereus* 504 dissociants

Cultivation	Parameter	Dissociant			
		dominant	mycoid	white	transparent
	Optical culture density	0.8	0.8	0.8	0.5
Without aeration	Protease, units/ml	7.7	3.6	4.7	14.8
	Protease, units/g dry cells	15.4	7.1	9.4	29.7
With aeration	Optical culture density	1.2	1.5	1.4	1.0
	Protease, units/ml	11.1	3.0	4.8	17.8
	Protease, units/g dry cells	22.3	5.9	9.5	35.6

Table 5. The production of the autoregulatory d_1 and d_2 factors by the *B. cereus* 504 dissociants

Dissociant	Concentration of autoregulatory factors, units/l		Ratio of factors	Specific concentration of factors, units/g dry cells		Ratio of factors
	d_1	d_2	d_1/d_2	d_1	d_2	d_1/d_2
Dominant	12.4	10.3	1.2	4.1	6.4	0.6
Mycoid	10.1	5.9	1.7	3.6	2.1	1.7
White	6.5	21.6	0.3	2.1	6.2	0.3
Transparent	19.4	8.1	2.4	6.3	3.6	1.75

Table 6. The sensitivity of the *B. cereus* 504 dissociants to the chemical analogues of the autoregulatory d_1 and d_2 factors

Dissociant	Concentration of factors corresponding to one unit of biological activity	
	d_1, $\times 10^{-5}$ M	d_2, $\times 10^{-9}$ M
Dominant	32.5	12.6
Mycoid	30.0	3.6
White	37.0	10.8
Transparent	95.0	11.1

which the specific growth rates of various variants attained their maxima were different. This time comprised 3–4 h for the dominant bacterial variant and 5–7 h for the transparent variant. For the white and mycoid variants, these times were 5–6 and 3–5 h, respectively. Thus, the transparent variant exhibited the slowest increase in the growth rate; however, the subsequent decrease in the growth rate was also slower in this variant than in other variants.

At was found that the *B. cereus* variants exhibited not only physiological and morphological, but also biochemical differences. The proteolytic activity of the transparent *B. cereus* variant grown microaerobically reached 35.6 U (1.5–2 times that of the dominant variant). The proteolytic activity of the white and mycoid variants was, respectively, 1.6–2.3 and 2.1–3.7 times lower than that of the dominant variant. Under better aeration, the proteolytic activity of the dominant and transparent variants increased, whereas that of the white variant did not change and that of the mycoid variant decreased. The proteolytic activity of the variants grown aerobically showed no correlation with their growth parameters (Table 4).

The characteristics of spore formation in the bacterial variants grown in submerged cultures in medium 1 were different. The 2-day-old culture of the mycoid variant contained 100% sporulating cells, and a complete release of mature spores was observed on the 3rd day. To compare, the 2-day-old transparent variant grown under the same conditions had 20% sporulating cells, and mature spores were released on the 6th day. The 2-day-old dominant and white variants had 50–60% sporulating cells, and mature spores were released on the 4th day.

Thus, the *B. cereus* variants considerably differ in their growth rates and their ability to transform to resting forms and to synthesize inducible extracellular proteases, which represent the adaptive responses of bacterial populations to the altered environment.

The accumulation dynamics of the d_1 and d_2 autoinducers of anabiosis and autolysis and the proportion between them are important factors responsible for the autoregulation of the growth of bacterial populations [16, 17]. The d_1 and d_2 autoinducers control the formation and germination of resting bacterial forms and the proliferation and autolysis of vegetative cells. In our experiments, the concentrations of the extracellular d_1 and d_2 factors were measured in the early stationary phase, where they were at maxima [16, 17]. The results summarized in Table 5 show that both the total (U/l) and specific production of the d_1 factor (the latter was expressed in U/g dry cells) were maximum in the transparent variant (19.4 U/l and 6.32 U/g dry cells, respectively) and minimum in the white variant (6.5 U/l and 2.12 U/g dry cells). At the same time, the production of the d_2 factor was maximum (21.6 U/l and 6.61 U/g dry cells) and minimum (5.9 U/l and 2.12 U/g dry cells) in the white and mycoid variants, respectively. The concentration of the d_1 and d_2 factors and the proportion between them correlated, to a certain degree, with the growth parameters of the dissociants (Table 5 and Fig. 4).

Of interest was the question of whether or not the intensity of the biosynthesis of the autoregulatory factors by various dissociants correlates with their sensitivity to these factors. This sensitivity was evaluated by measuring the degree of the inhibition of the endogenous respiration of exponential-phase cells to the chemical analogues of the d_1 and d_2 factors, AHB and FUFA, respectively. As can be seen from the data presented in Table 6, the mycoid variant, which synthesized the d_2 factor at the lowest rate, turned out to be the most sensitive to it. Conversely, the transparent variant, which synthesized the d_1 factor at the highest rate, was the least sensitive to its action. These data suggest that there is a correlation between the extracellular concen-

Table 7. Proportion of *B. cereus* 504 variants formed in the first passage of germinated resting forms

Resting form	Variant, % of the total number of colonies				
	dominant	mycoid	white	transparent	fraction of minor variants
Endospores stored for 14 days	89.4	10.0	0.6	0	10.6
Stationary-phase cells stored for 3 months	58.8	37.2	4.0	0	41.2
CRCs induced by nitrogen deficiency (1 month of storage)	51.0	46.0	3.0	0	49.0
CRCs induced by the d_1 factor (6.5 units/ml) after 1.5 months of storage	45.1	28.3	29.3	0.2	55.1

tration of the autoregulators and the preferential development of a particular variant. As was shown earlier, the proportion between the d_1 and d_2 factors controls changes in the physiological activity of bacterial cells. The function of this regulatory system depends on the concentration of the autoregulatory factors in the culture and on the physicochemical environmental conditions [16]. The different contributions of the dissociants to the extracellular pool of the d_1 and d_2 factors is essential to the proper functioning of the autoregulatory system under discussion, which controls the development of bacterial populations.

The investigation of the dissociation of physiologically different cells (vegetative cells and various resting forms) constituted the last part of this work. It showed that resting forms produced the same minor variants as vegetative cells, albeit in larger amounts. In the order of increasing percentage of minor variants produced, the resting forms ranked as endospores, stationary-phase cells residing in the state of proliferative rest, cystlike refractory cells formed under nitrogen deficiency, and CRCs formed in response to the action of the native autoregulatory d_1 factor isolated from *B. cereus* (Table 7). Thus, the proportion of minor variants was always higher after plating resting forms (as compared to prolifirating forms); among resting forms, metabolically resting (anabiotic) cells produced higher proportion of minor variants than stationary-phase cells residing in the state of proliferative rest. Noteworthy is a higher degree of instability of the dominant variant in the first subculture obtained by the germination of CRCs as compared with the subculture obtained by the germination of endospores. This may be due to the fact that, unlike the formation of CRCs, the formation of endospores is associated with considerable changes in the cell morphology and in the genetic material package. The dense package of genetic material in the endospores may decrease the recombinational variability of the genome, which, in the final analysis, determines the conversion of the dominant bacterial variant to minor variants.

The mechanisms underlying the regularities revealed should be investigated in terms of the mechanism of action of the d_1 autoregulatory factor. Investigations showed that AHBs (the chemical analogues of the d_1 factor in some bacteria and yeasts) are natural structural modifiers of membranes [17] and, therefore, may influence lipid–protein interactions in the membrane-associated replicative centers. On the other hand, AHBs possess the properties of chemical chaperones [17, 18] and, hence, can influence the conformation of protein molecules and their functional activity [18, 19]. Inasmuch as the σ-factors of RNA polymerases, which control the expression of some regulons, are proteins, there is a possibility that they can also be modified by AHBs. AHBs are also able to directly interact with DNA [20] and, therefore, the possibility of their involvement in the modification of genetic material during the formation of endospores and CRCs cannot be excluded. This consideration shows that there may exist several mechanisms responsible for the populational variability of microorganisms. In addition to the control exerted by autoregulatory factors, noteworthy is the fact that just as the cell differentiation in higher eukaryotes is implemented through the stage of proliferative rest [21], so the dissociation of bacterial populations is always implemented through the stage of rest, either proliferative or metabolic.

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