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EXPERIMENTAL ARTICLES

On the Adaptive Nature of the Dissociation Process in *Bacillus thuringiensis*

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Abstract—The process of dissociation into variants that differ in colony morphology occurring in batch cloned cultures of two *Bacillus thuringiensis* strains belonging to different subspecies was studied at optimal and elevated temperatures. An increase in the cultivation temperature to 40°C resulted in an increase in the fraction of R variants to 100% after 72 h of cultivation of either of the strains. This increase was not due to the selection of forms with greater resistance to elevated temperature. The level of resistance to elevated temperature was determined by the strain genotype and did not correlate with morphological characteristics of the colonies.

Key words: Bacillus thuringiensis, dissociation, temperature resistance.

The variability type termed *dissociation* has for a long time been recognized by microbiologists, particularly those studying the species of enthomopathogenic bacilli *Bacillus thuringiensis*. Dissociation in this bacilli has been well studied at the phenotypic level; it has been described in various subspecies as the appearance among the forms producing smooth, glistening, opaque colonies (termed S variants) of forms producing colonies of a different morphology, namely, rough, transparent, gray colonies (R variants) [1]. It was shown that the changes in the colony morphology correlate with changes in a number of characteristics that are important from the point of view of industrial production of biopesticides, primarily, such characteristics as spore formation and toxin production, and hence, virulence [2, 3].

The role and mechanisms of dissociation in *Bacillus thuringiensis* have been insufficiently studied. It is believed that the primary role of this process is to increase the adaptation capacity of the population. Indeed, there are data showing that environmental conditions may affect the dissociation process in *Bacillus thuringiensis* [4]. Many researchers who studied analogous phenomena in other bacterial species consider them to be a result of the integration of various processes, primarily, DNA rearrangements (transpositions of mobile genetic elements, including temperate phages) and, more rarely, regulation at epigenetic level and selection of the arising variants under the action of environmental factors [5–7]. However, no special investigations of the adaptive significance of the dissociation process and of the role played in it by the genotype have been carried out.

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With the aim of verifying the supposition about the adaptive nature of the dissociation process in *Bacillus thuringiensis*, we investigated thermal resistance in R variants arising during cultivation at optimal and elevated temperatures. Temperature was chosen as the model factor because it can be stringently controlled in the experiment. In addition, it has long been known that the elevation of the temperature increases the dissociation rate [8]. The investigation of two groups of dissociants belonging to different subspecies gave us the opportunity to unravel the role of the genotype in the dissociation process.

MATERIALS AND METHODS

This work used *Bacillus thuringiensis* strain 49, which is the type strain of the subspecies *dendrolimus* and belongs to serotype 4a, 4b and strain *Bacillus thuringiensis* subsp. *thuringiensis* 2002 belonging to serotype I. Both strains were obtained from the collection of the Department of Microbiology, Irkutsk State University. The strains were grown on solid and liquid media of Luria and Bertain (LB). Cultivation was performed either at optimal (28°C) or elevated (40°C) temperature. On solid medium, both strains produced white, opaque, smooth colonies; the cells formed spores and crystals (S forms).

Cells or spores from individual colonies that exhibited pronounced morphological features typical of the variant under study were transferred to 100-ml flasks containing 15 ml of liquid LB medium and were cultivated for 12 h at the optimal temperature (28°C) without agitation. These 12-h cultures were diluted (1 : 750) with a fresh medium warmed to 28°C, and the number of viable cells was determined by plating aliquots onto solid an LB medium. The cultivation was continued at

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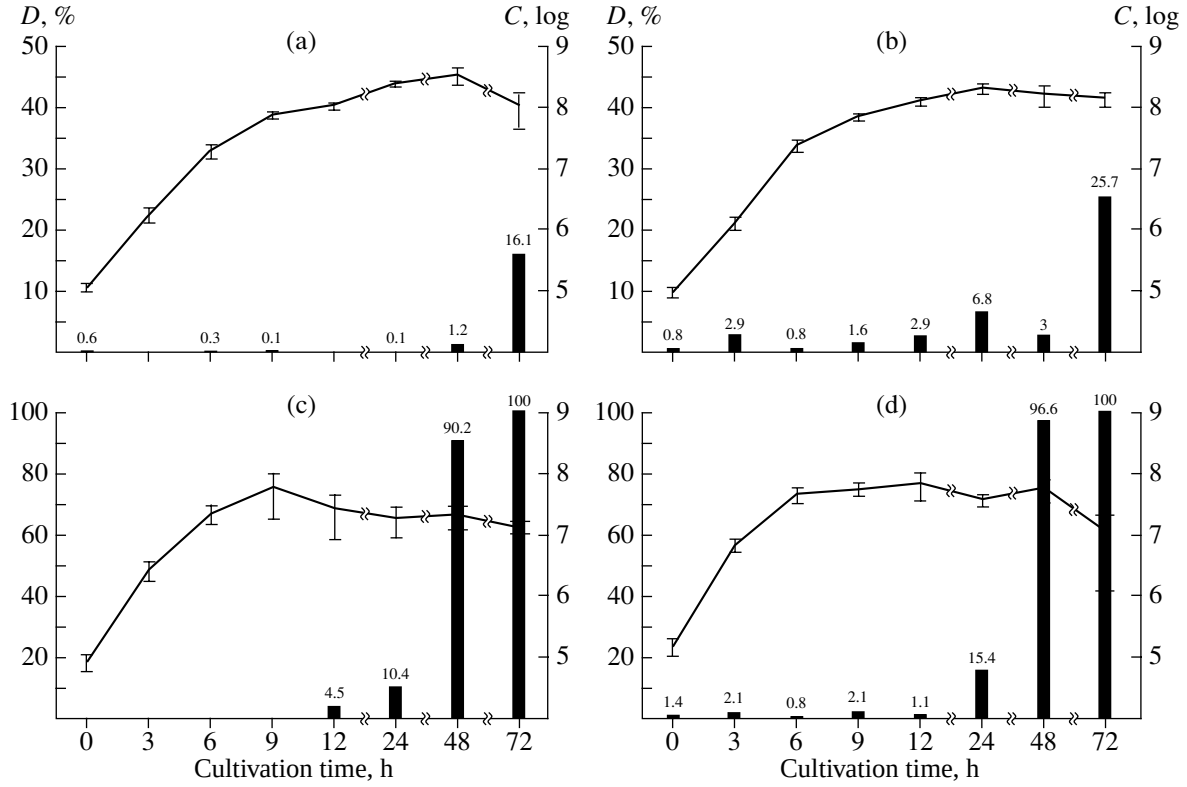

Fig. 1. Cell concentration (log *C*) and percent ratio of R variant (*D*, %) in the cultures of strain (a, c) 49 and (b, d) strain 2002 at (a, b) 28 and (c, d) 40°C.

28°C (control) and 40°C (experiment) under carefully standardized conditions. Periodic platings of culture aliquots were performed over 24–72 h; after 5 days of the growth of colonies, their morphology was examined, and their number was counted. Survival rate at 40°C was determined in short-term experiments as follows. Spores or cells from colonies of a pronounced morphological type were cultivated for 12 h onto a solid LB medium at 28°C; from these cultures, cell suspensions were prepared whose aliquots were plated on a solid LB medium to determine the number of viable cells. In parallel, platings were performed of aliquots of cell suspensions exposed to 40°C for 2 h. The growth parameters and survival rate were calculated according to commonly accepted formulas.

RESULTS AND DISCUSSION

The growth of batch cloned cultures of strains 49 and 2002 at 28 and 40°C and the dynamics of the ratios of dissociants are shown in Fig. 1.

During cultivation at the optimal temperature, the strains studied were very similar in their growth parameters, which were highly reproducible in six replicate variants. In the log phase, the generation time of strain 49 was 0.8 ± 0.11 h, and the generation time of strain 2002 was 0.8 ± 0.07 h. After 6–9 h of growth, transition to the stationary phase occurred. The process of cell death, as well as intraclonal selection, was noted only after 24–48 h of cultivation and was not very pronounced.

No pronounced strain differences were observed in the dynamics of the occurrence rates of R variants. In the original cultures of strain 49 (Fig. 1a) and strain 2002 (Fig. 1b), R variants were present in an amount of 0.6–0.8%. Since the inoculum was taken from individual colonies, these variants most probably arose in the course of the growth of colonies or overnight cultures. During further growth, the proportion of R variants fluctuated in both strains without visible regularity till the 48th hour of cultivation, averaging 0.3% for strain 49 and 2.7% for strain 2002. By the 72nd hour of cultivation, the fraction of R variants always increased in the cultures of both strains, averaging 25.7% in strain 2002 and 16.1% in strain 49. This increase in the occurrence rate of R variants by the end of cultivation can be explained by changes in the cultivation conditions caused by accumulation of metabolic products and a pH shift [4, 5]. In our experiments, the initial pH value was 6.8, whereas by the end of cultivation the pH value was 8.4 ± 0.04 for strain 2002 and 8.5 ± 0.40 for strain 49.

Thus, changes in the medium occurring in the course of cultivation, changes in the medium pH in particular, affect the dissociation process in *Bacillus thuringiensis*. However, this process is also controlled by the genotype. Thus, under the same cultivation condi-

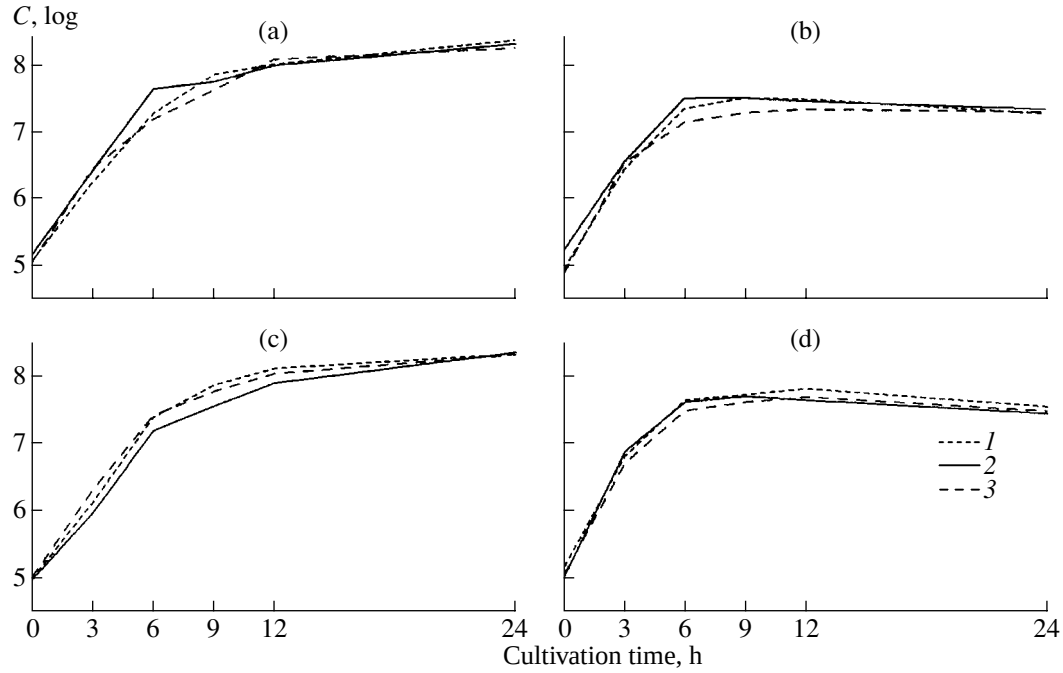

Fig. 2. Cell concentration (log C) in cultures of R variants of (a, b) strain 49 and (c, d) strain 2002 at (a, c) 28 and (a, d) 40°C. *1*, the wild type of strains 49 and 2002; *2*, R variant 49.0_1 or 2.12.4; *3*, R variant 49.0_2 or 2.24.12.

tions, the occurrence rate of R variants was significantly higher ($P < 0.05$) in strain 2002 (representing the subspecies *thuringiensis*) than in strain 49 (representing the subspecies *dendrolimus*).

Elevation of the cultivation temperature to 40°C (Figs. 1c, 1d) resulted in the same immediate reaction of both strains: an increase in the growth rate, which is characteristic of many microbial species. The logarithmic phase of growth lasted for 3 h in both strains. As compared to the optimal cultivation conditions, the generation time was lower and equaled 0.6 ± 0.05 and 0.6 ± 0.03 h for strains 2002 and 49, respectively. As distinct from the growth pattern observed under normal conditions, culture dying off and inrtraclonal selection began in the stationary phase of growth of both strains as soon as after 9–12 h. By the 72nd hour, the number of viable cells decreased by an order of magnitude in both cultures.

Only after 48 h of cultivation did the dynamics of occurrence of R variants observed at elevated temperature show a clear-cut difference from the control variant (Figs. 1c, 1d). In strain 2002, the fraction of R variants increased to 96.6% after 48 h and reached 100% after 72 h. A similar tendency was observed with strain 49 (Fig. 1c), in which the fraction of R variants increased to 10.4% after 24 h and also reached 100% after 72 h. Thus, elevated temperature induced the appearance of R forms both in strain 2002, belonging to the subspecies *thuringiensis*, and strain 49, belonging to the subspecies *dendrolimus*. The high frequency of R variants was apparently due to two factors: accumulation of metabolic products and elevated temperature. The fact that the increase in the proportion of R variants was observed only by the end of cultivation may mean that a certain period of culture development at elevated temperature is necessary in order to realize this the tendency.

Proceeding from the results obtained, two assumptions can be made: (1) the proportion of R variants in the culture increases as a result of selection processes, i.e., at an elevated temperature forms resistant to this factor are selected, and their concentration in the population increases at the expense of the dying off of the initially present S forms; (2) the proportion of R variants increases as a result of the switching off of the sporulation genes, which occurs due to the presence of

Parameters of growth of *Bacillus thuringiensis* strains 2002 and 49 and morphological variants derived from them in batch cultures at 28 and 40°C

Strain	Growth rate (v) and generation time (g) in batch culture			
	at 28°C		at 40°C	
	v, h^{-1}	g, h	v, h^{-1}	g, h
2002	1.32	0.75	1.8	0.56
2.24.12	1.21	0.83	1.97	0.5
02.12.4	1.32	0.75	1.84	0.54
49	1.22	0.81	1.64	0.61
49.0.1	1.37	0.72	1.8	0.55
49.0.2	1.24	0.8	1.45	0.68

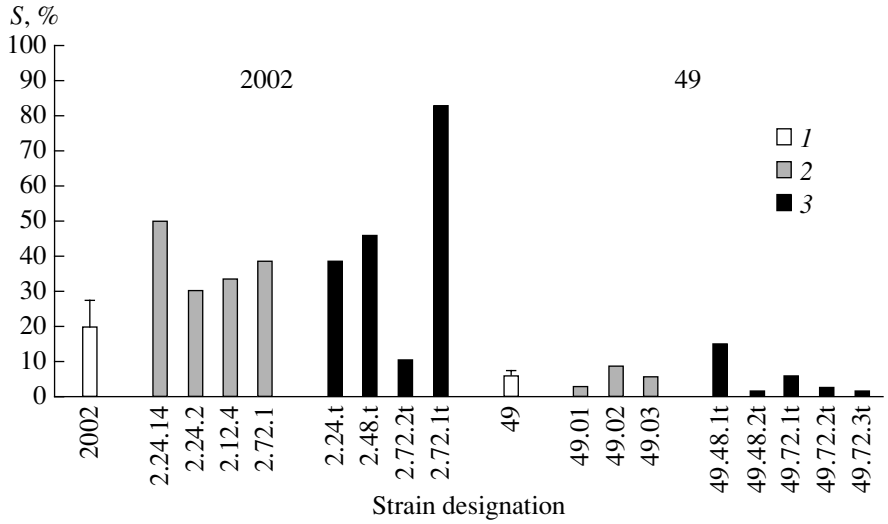

Fig. 3. Survival rate (*S*, %) after 2-h exposition at 40°C of cells of (*1*) the wild type and the derived R variants: (*2*) spontaneous and (*3*) thermally induced.

stress factors accumulating in the medium [9] or due to genome rearrangements. The acquired R state is stable and heritable and is preserved in many generations after the transfer of cells to optimal conditions.

Proceeding from the hypothesis about the adaptive nature of the dissociation process and the discovered increase in the fraction of R variants during cultivation at an elevated temperature, we expected that the forms arising at 40°C would exhibit increased resistance to elevated temperature as compared to the initial S forms and the R forms arising under normal cultivation conditions. To verify this suggestion, we investigated the growth patterns of randomly chosen R variants at various temperatures and also studied their resistance to temperature in short-term experiments. These studies used R variants obtained during cultivation at 28°C of strain 2002 (2.12.4, 2.24.2, 2.24.14, and 2.72.1) and strain 49 (49.0.1, 49.0.2, and 49.0.3) and R variants obtained during cultivation at an elevated temperature of strains 2002 (2.24.t, 2.48.t, 2.72.1t, and 2.72.2t) and strain 49 (49.48.1t, 49.48.2t, 49.72.1t, and 49.72.2t).The main criterion for the choice of variants was colony morphology, which always correlated with impairment of sporulation and crystal formation. Morphologically similar variants were obtained in different cultivation periods (the second figure in the variant designation reflects the time of cultivation at which the clone was obtained).

Investigations of the growth patterns in batch cultures at 28 and 40°C showed that R variants were not superior to the wild-type strains with respect to the growth rates observed at either normal or elevated cultivation temperature. The growth rates and generation times in the log phase (table) and the growth curves (Fig. 2) were very similar for R variants and the original S forms.

The growth parameter values observed in the log phase somewhat varied among different R variants and could be somewhat smaller or greater than the values characteristic of the original forms. No significant difference was observed between the ratios of S and R forms recorded at different temperatures, either after 24 h of cultivation (Fig. 2) or by the end of cultivation (48–72 h), i.e., in the presence of metabolic products in the medium. The growth rate observed in the cultivation period from 48 to 72 h was –0.78 h^{-1} for strain 2002; for variant 2.12.4, it was –0.76 h^{-1}.

Thus, the increase in the fraction of R variants observed in the cultures studied by the end of growth was not due to the greater growth rate of R variants. In these experiments, we did not observe revertant S variants in the cultures of R variants.

Short-term experiments set (in three replicates) to compare the resistance to elevated temperature of wild-type strains (S forms) and R variants derived from them under different cultivation temperatures also failed to reveal statistically significant differences (Fig. 3).

As can be seen from Fig. 3, R variants 2.24.14, 2.24.2, 2.12.4, and 2.72.1, derived from strain 2002 at optimal cultivation temperature, did not differ in their resistance to elevated temperature (40°C) from the wild-type strain or were slightly more resistant. A similar pattern of temperature resistance was observed for R variants that originated from the same strain at elevated temperature. One of the variants (2.72.2t) isolated from a 72-h culture grown at 40°C was more sensitive to elevated temperature than the original strain, and another one (2.72.1t) was more tolerant. An analogous pattern was observed for strain 49. The survival rate of the cells of this strain after 2-h exposition at 40°C was 6.0 ± 1.14%. The R variants derived from this strain could be either more or less resistant than the original

strain, without correlation with the conditions under which they were obtained. Thus, variant 49.0.1 exhibited higher sensitivity to temperature than the wild-type strain, and variant 49.0.2 was more resistant than the latter, although both variants were obtained under optimal cultivation temperature. Variant 49.72.2t exhibited a survival rate equal to that of the wild-type strain despite the fact that it was obtained at the end of cultivation of strain 49 at 40°C.

Based on the results obtained in this work, it cannot be unambiguously concluded that the increase in the occurrence rate of R variants observed during cultivation at elevated temperature results from the selection of forms that are more thermally resistant and that the R morphology correlates with resistance to elevated temperature. From the results presented in Fig. 3, it is evident that the resistance to temperature is determined by the genotype of a strain, regardless of the conditions under which it was obtained. Cells of strain 2002, belonging to the subspecies *thuringiensis*, are more resistant to elevated temperature than cells of strain 49, representing the subspecies *dendrolimus*. On the whole, the thermal resistance of R variants derived from strain 2002 was higher than that of R variants obtained from strain 49. Elevated temperature increases the frequency of appearance of variants that are diverse in their sensitivity to temperature; this diversity lies within the range of normal reactions of the genotype.

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