Microbiology, Vol. 71, No. 1, 2002, pp. 18–23. Translated from Mikrobiologiya, Vol. 71, No. 1, 2002, pp. 23–29.

EXPERIMENTAL ARTICLES

The Role of Bacterial Growth Autoregulators (Alkyl Hydroxybenzenes) in the Response of Staphylococci to Stresses

O. N. Il'inskaya*, A. I. Kolpakov*, M. A. Shmidt, E. V. Doroshenko***, A. L. Mulyukin***, and G. I. El'-Registan*****

**Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*

***Institute of Medical Microbiology, Giessen, Germany*

****Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

Received May 22, 2001

Abstract—The investigation of the response of a batch culture of *Staphylococcus aureus* to exogenous alkyl-substituted hydroxybenzenes (AHBs), chemical analogues of anabiosis autoinducers, showed that C_1-AHB at concentrations from 5 μM to 1.5 mM did not influence the culture growth, whereas the more hydrophobic C_6-AHB inhibited it at concentrations of 0.5 mM and higher. Either of the AHBs drastically enhanced the phenotypic dissociation of staphylococcal cultures, which manifested itself in an increase in the fraction of cells producing small nonhemolyzing colonies of G type when plated on solid media with erythrocytes. In a submerged staphylococcal culture, the relative number of cells producing G-type colonies varied from 10 to 90%, depending on the concentration of the AHB added. The growth of *S. aureus* in the presence of AHBs also enhanced cell tolerance to heat shock (heating at 45 or 60°C for 10 min). The role of AHBs, which are structural modifiers of membranes and possess chaperone activity, in the mechanisms responsible for cell tolerance and phenotypic dissociation of microbial populations is discussed.

Key words: alkyl hydroxybenzenes, anabiosis autoinducers, stress, stress tolerance, staphylococci, phenotypic dissociation, G-type colonies.

Alkyl-substituted hydroxybenzenes (AHBs), which are low-molecular-weight amphiphilic compounds synthesized by some microorganisms, are the principle natural growth autoregulators of microbial cultures. They are also called the d_1 factors of cell differentiation. Elevated concentrations of AHBs limit the growth of microbial cultures, and high AHB concentrations may cause a fraction of cells in a microbial population to transit to an anabiotic state [1, 2]. There is increasing evidence that AHBs are involved in the adaptive response of cells to stresses [3–5]. The development of hypometabolic conditions and ultimately anabiosis is provided by several mechanisms, one of which involves AHBs [6]. In this mechanism, AHBs act as membranotropic agents (which cause the polycrystallization of cellular membranes, affect their permeability to monovalent ions, and induce the dehydration of protoplasts [7]) and chemical chaperones (which stabilize the spatial structure of enzymes [3, 5]). In addition, AHBs may form complexes with biopolymers, including those of the cell wall, thus lowering the specificity of these polymers in enzymatic reactions and causing biological inactivity [6]. These effects enhance the tolerance and viability of cells in senescent and long-stored microbial cultures. The effect of d_1 factors, including AHBs, is not species-specific, due to which the action of intrinsic growth autoregulators on particular microorganisms can be simulated by the addition of exogenous AHBs. Of great practical interest is the study of the effect of exogenous AHBs, such as alkyl resorcinols, on pathogenic and opportunistic bacteria in relation to their antibiotic resistance.

The present study was performed with *Staphylococcus aureus*. Research interest to this species is stimulated by the fact that it is isolated, occasionally or permanently, from 15–30% of virtually healthy individuals, in which staphylococcal infections usually develop due to activation of autogenous microflora [8]. This fact calls for investigation of the mechanisms of the antibiotic resistance of staphylococci and their control.

We present here the results of the study of the effect of AHBs on the physiological characteristics of *Staphylococcus aureus*, the production of hemolysins by this bacterium, and the development of cellular response to heat shock.

MATERIALS AND METHODS

The strain of *Staphylococcus aureus* used in this study was obtained from the collection of microorganisms at the Institute of Medical Microbiology, Giessen,

0026-2617/02/7101-0018$27.00

Germany. The strain was grown in 250-ml flasks containing 50 ml of nutrient broth on a shaker (200 rpm) at 37°C. The inoculum (cells from the linear growth phase) was added to give an optical density (OD) of 0.2, which was measured in a Specord spectrophotometer using 10-mm-pathlength cuvettes.

Two chemical analogues of the d_1 autoregulatory factors of bacteria [1], hexylresorcinol or C_6-alkyl hydroxybenzene (C_6-AHB, M_r = 196) and methylresorcinol or C_1-alkyl hydroxybenzene (C_1-AHB, M_r = 124) with a purity of 99.9%, were used in the form of solutions in dimethylsulfoxide (DMSO). These substances were added in amounts such that the concentration of DMSO in the medium did not exceed 3%. The control reaction mixtures contained the equivalent amount of DMSO.

The growth-inhibiting activity of AHBs was evaluated by adding them to the growth media of staphylococci (nutrient broth or blood agar) together with the inoculum. Bacterial growth in nutrient broth was estimated by measuring culture turbidity at λ = 560 nm or by plating cell suspension dilutions on blood agar. In the latter case, the results were expressed as colony-forming units (CFU) per milliliter. The hemolytic activity of *S. aureus* was assayed on blood agar and expressed in the diameter of hemolysis zones around staphylococcal colonies.

Heat shock was induced by heating the aliquots of a 12-h linear-phase *S. aureus* culture at 45 or 60°C for 10 min. The protective activity of AHBs was evaluated from their effects on the colony-forming ability (estimated at 37°C) of *S. aureus* cells exposed to heat shock. Cells were grown in the presence of 50 μM C_6-AHB for 12 h (variant a), or C_6-AHB was added at the same concentration to 12-h-old cells (variant b). Variant c represented control cells grown in the absence of AHBs and not treated with them. Aliquots (0.1 ml) of these three variants of cell suspensions were added to a fresh nutrient broth and incubated for 8 h. The growth of these cultures was monitored as described above.

The results were statistically processed in terms of Student's *t*-test and standard deviation using the Microsoft Excel 2000 program. Data files were considered to be homogeneous if their standard deviations σ did not exceed 13%. The *t*-statistic was calculated for significance level $P < 0.05$.

RESULTS

Experiments showed that the effect of AHBs on the growth of staphylococci depended on the hydrophobic-

Table 1. The effect of AHBs on the number of *S. aureus* cells in a submerged culture capable of producing colonies on blood agar

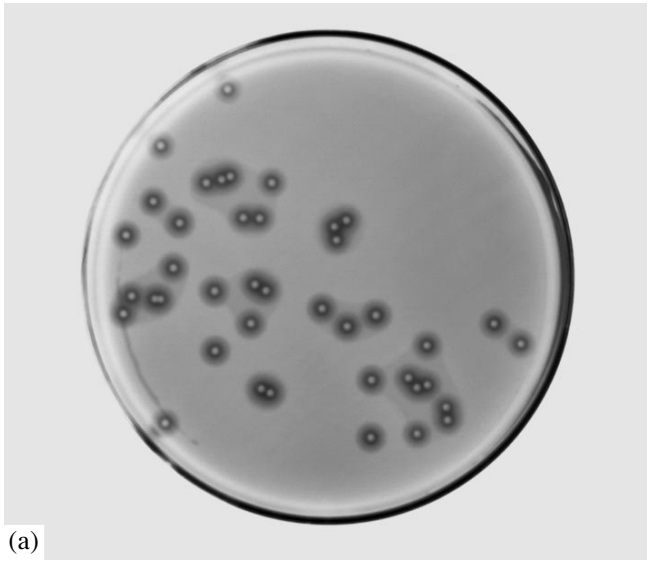
Days	Cell number, CFU $\times 10^7$/ml				
	Control	C_1-AHB, μM		C_6-AHB, μM	
	0	10	100	10	100
1	186 (0)	167 (0)	408 (0)	47 (0)	41 (0)
2	592 (35.7)	840 (20)	592 (13.5)	248 (9.4)	73 (9.5)
3	16 (22.5)	16.5 (20)	19.4 (16)	17.6 (29.5)	15.8 (25.3)
6	2.7 (7.4)	3.3 (40)	2.2 (15)	1.0 (81.8)	8.9 (46.1)
7	0.31 (12.9)	0.15 (53.3)	0.1 (69.8)	0.09 (33.3)	0.63 (90)

Note: Parenthesized is the percentage of cells producing G-type colonies.

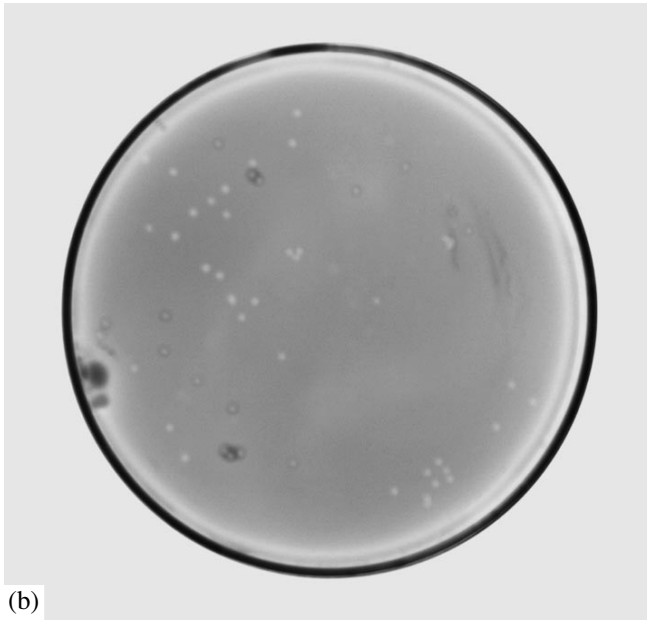
Table 2. The effect of C_6-AHB on the number of *S. aureus* cells that retained their colony-forming ability after exposure to 45 or 60°C for 10 min

Time, h	Variant c (control)		Variant a		Variant b	
	45°C	60°C	45°C	60°C	45°C	60°C
0	0.15	41	0.09	80	0.12	16.48
2	0.37	48	0.51	24.48	0.63	0
4	53	59.3	19.2	68	7.04	0
6	140.8	118.4	348.8	50.88	358.4	0
8	518.4	570	784	228.8	579.2	0

Note: Cell numbers for heat shocks at 45 and 60°C are given in CFU $\times 10^7$/ml and CFU $\times 10^2$/ml, respectively. Experimental variants are described in the Materials and Methods section.



(a)



(b)

Fig. 1. Colonies produced on blood agar by *S. aureus* cells grown in nutrient broth for 7 day in the (a) absence and (b) presence of 100 μM C_6-AHB.

ity of AHBs and their concentration. C_1-AHB stimulated the growth of staphylococci at low concentrations (Table 1) and inhibited it at high concentrations (data not shown). The inhibitory effect of C_6-AHB was more profound than that of C_1-AHB, which agrees with the data in the literature indicating that more hydrophobic AHBs exert more extensive effects on the functional (including the energy-yielding) activity of membranes [7, 9].

The inhibitory effect of AHBs is bacteriostatic. This follows from the observation that staphylococci whose division was arrested by AHBs did not autolyse for at least 12 h. The number of cells that retained their colony-forming ability decreased with increasing C_6-AHB concentration (Table 1). This can be explained by the facts that some AHB-treated cells transit to an anabiotic state [10] and that some morphologically intact cells irreversibly lose their growth ability [11].

On the other hand, the possibility cannot be excluded that some cells in an AHB-treated submerged *S. aureus* culture are resistant to AHB and continue growing. It should be noted in this regard that the long-term cultivation of microorganisms under unfavorable conditions, such as starvation, promotes the development of more adaptable cell variants, which, however, have lower growth rates [12]. In view of this, we analyzed the populational variability of staphylococci grown in the presence and absence of AHBs.

When plated on blood agar and incubated for 1 day, control (untreated with AHBs) cells primarily produced smooth, convex, dull, yellowish colonies surrounded by a zone of complete hemolysis (Fig. 1). But when staphylococci were cultivated in nutrient broth with AHB for 12 h, they predominantly produced small colonies without hemolysis zones (so-called G-type colonies). It should be noted that, in general, about 1% of fresh staphylococcal isolates, when grown on nutritionally deficient media lacking necessary growth factors (hemine, thiamine, pantothenic acid, and others), are able to produce such colonies [8]. In our experiments, the relative number of G-type colonies produced by the control cells reached a maximum (36%) on the second day of cultivation, i.e., when the bacterial population was also at a maximum (Table 1). When staphylococci were grown in nutrient broth in the presence of AHBs, the relative number of G-type colonies peaked on the 6th to 7th day of cultivation. The fraction of cells capable of producing such colonies under the action of 10 μM C_6-AHB and C_1-AHB was 82 and 40%, respectively. At a higher concentration of C_6-AHB and C_1-AHB (100 μM), the percentage of G-type colonies increased to 90 and 70%, respectively, and peaked on the 7th day of cultivation. It should be emphasized that AHBs caused no increase in the CFU number (except for the C_1-AHB at a concentration of 10 μM) but actively promoted the segregation of a subpopulation producing G-type colonies. At a high concentration (100 μM), both AHBs inhibited the growth of staphylococci and increased the number of cells capable of producing G-type colonies. Since these colonies are characterized by a slower growth rate and lower number of generations, as is evident from their small size, it can be suggested that they must have other distinguishing features.

Indeed, the hemolytic activity of cells capable of producing G-type colonies was obviously lower than that of normal cells. Figure 1 shows the colonies produced on blood agar by 7-day-old *S. aureus* cells grown in nutrient broth in the absence (Fig. 1a) and presence (Fig. 1b) of 100 μM C_6-AHB. It can be seen that the

clone segregated under the action of C_6-AHB completely lost its ability to synthesize hemolysins. This tendency was observed for aging cultures: thus, some senescent cells from the 7-day-old culture grown in the absence of C_6-AHB also had a diminished ability to synthesize hemolysins. The effect of AHBs on the phenotypic stability of staphylococci can be explained by taking into account two facts. First, as was shown for streptomycetes [13], bacilli [14], and some other microorganisms [12], any microbial population is heterogeneous and contains cells of several phenotypes differing in their growth and physiological characteristics [12–14]. Second, as was shown for *Bacillus cereus*, AHBs are able to induce the segregation of microbial populations into cell variants producing colonies of different morphotypes [14].

It can be suggested that the populational dissociation of staphylococci induced by AHBs is aimed at increasing their tolerance to stresses. The results of the following experiments confirm this suggestion. Three culture variants—a culture grown in the presence of 50 μM C_6-AHB for 12 h (variant a), a 12-h-old culture grown in the absence of AHB but supplemented with C_6-AHB immediately before heating (variant b), and a control culture grown in the absence of AHBs and not supplemented with them (variant c)—were exposed to heat shock at 45 and 60°C for 10 min. To avoid a possible protective effect of extracellular factors of shock tolerance (or adaptogens of direct and indirect action) [15], the heat shock tolerance of cells was evaluated from the degree of preservation of the proliferative potential of cells transferred to a fresh nutrient medium.

Severe heat shock at 60°C for 10 min completely arrested the growth of *S. aureus* cells, both control and AHB-treated, after the transfer to fresh nutrient medium (growth was estimated either turbidimetrically or from changes in the colony-forming ability of cultures). At the same time, heating at 45°C for 10 min did not kill staphylococci. The pretreatment of cells with C_6-AHB in both experimental variants (a and b) enhanced their heat shock tolerance (Fig. 2 and Table 2), presumably due to the AHB-dependent rapid repair of heat-induced damage or, more likely, to a smaller amount of the damage. The plated cells had normal morphology. All these results imply that C_6-AHB exerts a protective effect on staphylococci.

Some cells exposed to 60°C for 10 min retained their ability to produce G-type colonies, whose number was greater in variant a (growth in the presence of C_6-AHB for 12 h) than in the control variant c. After transferring these two culture variants into a fresh medium, the relative number of cells capable of producing G-type colonies gradually decreased (Fig. 3), indicating that they are less competitive than cells of the dominant phenotype growing under standard conditions.

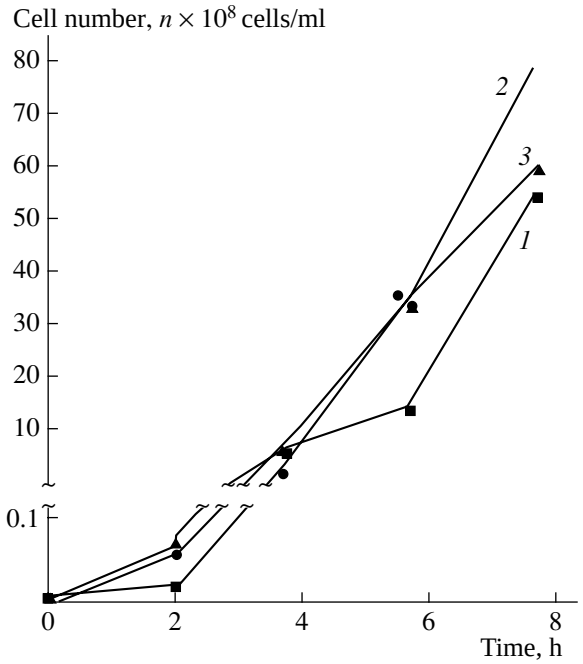


Fig. 2. Growth in fresh nutrient broth of *S. aureus* cells exposed to mild heat shock at 45°C for 10 min: (*1*) experimental variant c (control); (*2*) experimental variant a; and (*3*) experimental variant b. The experimental variants are described in the Materials and Methods section.

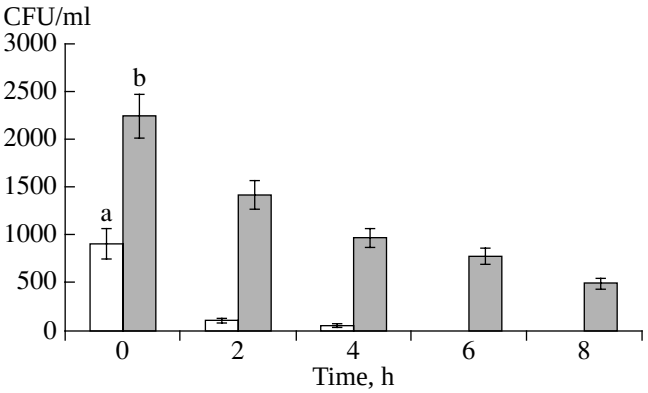


Fig. 3. The time dependence of the number of G-type colony-forming units after the transfer of heat-shocked (60°C; 10 min) *S. aureus* cells to fresh nutrient broth. Exposed to heat shock were (a) control *S. aureus* cells and (b) cells grown in the presence of 50 μM C_6-AHB for 12 h.

In general, C_6-AHB enhanced the tolerance of staphylococci to mild heat shock (exposure to 45°C for 10 min). Severe heat shock (exposure to 60°C for 10 min) drastically decreased the number of colony-producing cells, the decrease being more profound in the experimental variant b (the addition of C_6-AHB immediately before heating). These data indicate that a sharp increase in the C_6-AHB level acts as a stress factor.

DISCUSSION

It is known that cells universally produce heat shock proteins (HSPs) in response to various stresses, such as hyperthermia and exposure to reactive oxygen species, heavy metal ions, ethanol, and other toxic agents. In shocked cells, HSPs stabilize the altered tertiary structure of proteins, i.e., they act as molecular chaperones. In unshocked cells, HSPs play an important role in protein folding and the translocation of polypeptides through membranes [16]. It should be noted that HSPs are not exclusive stabilizers of biopolymers in cells. It was shown that the role of membrane lipids in the tolerance of yeasts to heat shock and exposure to ethanol is even more important than that of HSPs [17]. Hydrophobic toluene induces adaptive mutations in *Pseudomonas putida*, which are characterized by an altered phospholipid composition of the cytoplasmic and outer cell membranes of the mutants [18]. The tolerance of mutant *E. coli* cells to high pressures is considered to be due to specific changes in the stability of the Ca^{2+}-binding components of the cell surface [19], whereas the tolerance of *E. coli* cells to heat shock, antibiotics, and other factors is provided by extracellular dialyzable substances with adaptogenic functions [15]. These and some other observations indicate that small molecules can be very important for the stabilization of various cellular structures and for cell tolerance to extreme conditions. The study of alkyl hydroxybenzenes as low-molecular-weight chemical chaperones [1–6] may provide insight into the universal response of cells to stresses.

Elevated concentrations of AHBs in microbial cultures not only inhibit their growth [20] but also enhance the general tolerance of the stationary-phase cells, which is primarily due to the stabilizing effect of AHBs on cellular membranes and macromolecules [3, 5, 7] and the segregation (or the induction of growth) of minor cell subpopulations characterized by low growth rates, altered biosynthetic activity, and enhanced tolerance to unfavorable environmental conditions. The existence of a correlation between the effects of AHBs on the colony-forming ability and phenotypic dissociation of microbial populations suggests that AHBs not only stabilize various cellular structures and biopolymers but may also affect the cell genome. This problem is investigated in the accompanying paper.

ACKNOWLEDGMENTS

This work was supported by grant no. 01-04-48771 from the Russian Foundation for Basic Research, grant no. 015.11.01.13 within the scope of the Federal Program "Russian Universities–Fundamental Research," and by the DAAD (Deutscher Akademischer Austauschdienst, German Academic Exchange Service).

REFERENCES

1. Osipov, G.A., El'-Registan, G.I., Svetlichnyi, V.A., Kozlova, A.N., Duda, V.I., Kaprel'yants, A.S., and Pomazanov, V.V., On the Chemical Nature of an Autoregulatory d_1 Factor of *Pseudomonas carboxydoflava*, *Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 184–190.
2. Batrakov, S.G., El'-Registan, G.I., Pridachina, N.N., Nenasheva, V.A., Kozlova, A.N., Gryaznova, M.N., and Zolotareva, I.N., Tyrosol, the Autoregulatory d_1 Factor of *Saccharomyces cerevisiae*, *Mikrobiologiya*, 1993, vol. 62, no. 4, pp. 633–638.
3. Kolpakov, A.I., Il'inskaya, O.N., Bespalov, M.M., Kupriyanova-Ashina, F.G., Gal'chenko, V.F., Kurganov, V.I., and El'-Registan, G.I., Stabilization of Enzymes by Dormancy Autoinducers as a Possible Mechanism of Resistance of Resting Microbial Forms, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 180–185.
4. Il'inskaya, O.N., Kolpakov, A.I., Mulyukin, A.L., Dreier, F., and El'-Registan, G.I., The Effect of Membranotropic Microbial Autoregulators on the Growth of *ras*-transformed Fibroblasts, *Prikl. Biokhim. Mikrobiol.*, 2000, vol. 36, no. 5, pp. 549–554.
5. Bespalov, M.M., Kolpakov, A.I., Loiko, N.G., Doroshenko, E.V., Mulyukin, A.L., Kozlova, A.N., Varlamova, E.A., Kurganov, B.I., and El'-Registan, G.I., The Role of Microbial Dormancy Autoinducers in Metabolism Blockade, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 217–223.
6. El'-Registan, G.I., Gal'chenko, V.F., Il'inskaya, O.N., Kolpakov, A.I., Mulyukin, A.L., and Krylov, I.A., The Role of Chemical Chaperones in the Development of Anabiosis and Tolerance in Microorganisms, *XII yubileinaya konferentsiya "Fermenty mikroorganizmov"* (XII Jubilee Conf. "Microbial Enzymes"), Kazan, 2001, pp. 50–53.
7. Kaprel'yants, A.S., Suleimenov, M.K., Sorokina, A.D., Deborin, G.A., El'-Registan, G.I., Stoyanovich, F.M., Lille, Yu.E., and Ostrovskii, D.N., Structural and Functional Alterations in Bacterial and Model Membranes Induced by Phenolic Lipids, *Biol. Membr.*, 1987, vol. 4, no. 3, pp. 254–261.
8. *Meditsinskaya mikrobiologiya* (Medical Microbiology), Pokrovskii, V.I. and Pozdeev, O.K., Eds., Moscow: GEOTAR Meditsina, 1998, pp. 344–416.
9. Kozubek, A. and Tyman, J.H.P., Resorcinolic Lipids, the Natural Non-Isoprenoid Phenolic Amphiphiles and Their Biological Activity, *Chem. Rev.*, 1999, vol. 99, no. 1, pp. 1–31.
10. Mulyukin, A.L., Lusta, K.A., Gryaznova, M.N., Kozlova, A.N., Duzha, M.V., Duda, V.I., and El'-Registan, G.I., Production of Resting Forms of *Bacillus cereus* and *Micrococcus luteus*, *Mikrobiologiya*, 1996, vol. 65, no. 6, pp. 782–789.
11. Suzina, N.E., Mulyukin, A.L., Loiko, N.G., Kozlova, A.N., Dmitriev, V.V., Shorokhova, A.P., Gorlenko, V.M., Duda, V.I., and El'-Registan, G.I., Fine Structure of Mummified

Cells of Microorganisms Formed under the Influence of a Chemical Analogue of Anabiosis Autoinducer, *Mikrobiologiya*, 2001, vol. 70, no. 6, pp. 776–784.

12. Mil'ko, E.S. and Egorov, N.S., *Geterogennost' populyatsii bakterii i protsess dissotsiatsii* (Heterogeneity and Dissociation of Bacterial Populations), Moscow: Mosk. Gos. Univ., 1991.

13. Kuznetsov, V.D., Parallelism in Hereditary Variability and the Population Conception of Prokaryotic Species, *Zh. Obshch. Biol.*, 1987, vol. 48, no. 4, pp. 466–477.

14. Doroshenko, E.V., Loiko, N.G., Il'inskaya, O.N., Kolpakov, A.I., Gornova, I.B., Klimanova, E.V., and El'-Registan, G.I., Characterization of *Bacillus cereus* Dissociants, *Mikrobiologiya*, 2001, vol. 70, no. 6, pp. 811–819.

15. Nikolaev, Yu.A., The Involvement of Exometabolites in the Adaptation of *Escherichia coli* to Stresses, *Mikrobiologiya*, 1997, vol. 66, no. 1, pp. 38–41.

16. De Maio, A., Heat Shock Proteins: Facts, Thoughts, and Dreams, *Shock*, 1999, vol. 11, no. 1, pp. 1–12.

17. Swan, T.M. and Watson, K., Stress Tolerance in a Yeast Lipid Mutant: Membrane Lipids Influence Tolerance to Heat and Ethanol Independently of Heat Shock Proteins and Trehalose, *Can. J. Microbiol.*, 1999, vol. 45, no. 6, pp. 472–479.

18. Isken, S. and deBont, J.A.M., Bacteria Tolerant to Organic Solvents, *Extremophiles*, 1998, vol. 2, no. 3, pp. 229–238.

19. Hauben, K.J.A., Bernaerts, K., and Michiels, C.W., Protective Effect of Calcium on Inactivation of *Escherichia coli* by High Hydrostatic Pressure, *J. Appl. Microbiol.*, 1998, vol. 85, no. 4, pp. 678–684.

20. Svetlichnyi, V.A., Romanova, A.K., and El'-Registan, G.I., Investigation of the Content of Membranotropic Autoregulators in Lithoautotrophically Grown *Pseudomonas carboxydoflava*, *Mikrobiologiya*, 1986, vol. 55, no. 1, pp. 55–59.