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EXPERIMENTAL ARTICLES

The Role of Alkylhydroxybenzenes in the Adaptation of *Micrococcus luteus* to Heat Shock

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Abstract—The response of the gram-positive bacterium *Micrococcus luteus* to heat shock (45°C, 15 min) and the adaptogenic activity of alkylhydroxybenzenes (AHBs), which are extracellular growth-regulating substances of these bacteria, were studied. The perception of stress and the postshock behavior of *M. luteus* cells proved to depend on the growth phase and medium. The magnitude of the stress response was more pronounced in cultures grown on synthetic medium than in cultures grown on rich medium (nutrient broth). During exponential or linear growth, the cells were more sensitive to the temperature effect than during decelerated growth. In linearly growing *M. luteus* cultures, the amount of total intra- and extracellular alkylhydroxybenzenes, the anabiosis inducers, increased in response to heat shock. AHB redistribution between cells and culture liquid occurred in the course of stress and after stress. In micrococci exposed to heat shock, an increase in the AHB concentration both in cells and in culture liquid is likely a defense reaction of stress resistance. This conclusion was confirmed in experiments with the addition 30 min before the heat shock of a chemical analogue of the anabiosis inducer, C_7-AHB (12 mM), which protected *M. luteus* cells so that their intense growth was observed after shock without any lag. The protective effect of AHBs is a result of their ability to form complexes with enzyme macromolecules and stabilize them. The data obtained extend the knowledge of the stress-protective functions of low-molecular-weight autoregulators and of the role of intercellular communications in the stress response of bacterial cultures.

Key words: heat shock, stress, alkylhydroxybenzenes, micrococcus, protection, chemical chaperons, thermostable enzymes.

Adaptation of microorganisms to environmental changes, both normal and abnormal for a certain species, has long been attracting researchers' attention in connection with different scientific and practical tasks. The principles of the cell stress response to sublethal and lethal actions have been studied [1–3]. Cell viability was found to depend on the type and intensity of the stressful action. At the first stage of the stress response, cell metabolic activity is inhibited and the regulons are switched on that control expression of stress proteins and proteins with chaperon functions. In recent years, the role of intercellular communication and extracellular autoregulators in the adaptive response of microorganisms has attracted considerable interest [4, 5].

We were interested in the autoregulatory metabolites of both affected and intact bacteria, which participate in the cell defense reaction and control cell structural reorganization and subcellular component stabilization. These properties are characteristic of the microbial autoinducers of anabiosis (d_1 factors), which were found in microorganisms of different taxa, pro- and eukaryotes. In some bacteria and yeasts, these are derivatives of alkylhydroxybenzenes (AHBs) [6–8].

The mechanism of the AHB effect on the cell is polymodal. These substances are membranotropic; they induce polycrystallization of the membrane lipid stroma, which leads to changes in membrane functional activity [9, 10]. On the other hand, AHBs act as chemical chaperons: they bind nonspecifically via noncovalent physicochemical bonds to protein macromolecules and thus change the conformation of the latter and stabilize them [11]. When accumulated to threshold concentrations in growing microbial cultures, AHBs arrest cell division and promote culture transition to the stationary phase. Further increase in the AHB concentration leads to the development of an anabiotic state of the cells and formation of resting forms [8, 12]. Thus, AHBs control the first stages of microbial cell response to physiological stress, which regularly occurs in the ontogenesis of microbial cultures (for example, starvation stress and stress caused by space limitation when the critical population density is reached). The involvement of AHBs in the adaptation of dividing cells (growing cultures) to abrupt changes in the ambience (heat shock, irradiation, etc.) still remains an open question. Under these conditions, AHBs are also expected to play a protective role given their protective functions in rest-

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ing microbial cells, where they were shown to modify the macromolecule and membrane structure [9–11] and act as antioxidants [10, 13].

In this study, we aimed to determine the role of AHBs in the adaptation of vegetative cells of growing bacterial cultures to sublethal effects of the ambience. In particular, the dynamics of extracellular and intracellular AHB accumulation was studied in a *Micrococcus luteus* culture exposed to heat shock. The protective role of exogenous AHBs in the cell heat response was also studied. AHBs were found to increase thermostability of enzyme proteins in vitro by forming complexes with them; this is evidently one of the mechanisms of the protective effect of AHBs.

MATERIALS AND METHODS

The subject of this study was the strain *Micrococcus luteus* NCIMB 13267. In *M. luteus*, the anabiosis inducers are represented by alkylhydroxybenzenes belonging to the class of alkylresorcinols [8], which can be quantitatively determined by a sensitive method [14]. The bacteria were grown on nutrient broth and on synthetic medium of the following composition (g/l): lithium lactate (Sigma), 5; NH_4Cl, 4; KH_2PO_4, 4. The medium also contained microelements and growth factors (mg/l): $MgSO_4$, 50; $FeSO_4$, 20; $MnCl_2$, 20; $ZnSO_4$, 0.4; $B(OH)_3$, 0.5; $CuSO_4$, 0.05; Na_2MoO_4, 0.2; thiamine, 40; methionine, 20; pH was adjusted with NaOH to 7.2–7.4. The cells were grown in 250-ml or 750-ml flasks containing 50 and 250 ml of medium, respectively, on shakers (200 rpm) at 28–30°C. A linear-growth-phase culture served as inoculate, added to obtain an initial optical density (OD) of the cell suspension of 0.2 units (Specord M-400, Jena, Germany; λ = 650 nm; l = 10 mm). The d_1 autoregulatory factor chemical analogue used in this study was alkylresorcinol C_7-AHB (Sigma).

A growing *M. luteus* culture was heated in an ultrathermostat at 45°C to expose the cells to heat shock. The state of the culture was evaluated from changes (as compared to the control culture) in the optical density and in the number of colony-forming units (CFU), which was estimated after plating tenfold serial dilutions of cell suspensions onto solid medium (nutrient agar).

Microbial AHBs were determined in the culture liquid (CL) and in cells. The biomass was separated from the CL by centrifugation at 6000 *g*. AHBs were three times extracted from the CL by *n*-butanol (1 : 1, vol/vol), which was followed by distillation on a rotary evaporator at 60°C. The residue was redissolved in 1 ml of a chloroform–methanol (1 : 1, vol/vol) mixture. AHB extraction from cells was performed by the Folch method, i.e., with a chloroform–methanol (1 : 1, vol/vol) mixture and then with a chloroform–methanol (2 : 1, vol/vol) mixture. The combined extracts were evaporated until dry and dissolved in 1 ml of a chloroform–methanol (1 : 1, vol/vol) mixture.

The amount of AHBs was determined in a colorimetric reaction with a diazotized derivative of 3,3'-dimethoxybenzidine (Fast Blue B Salt diazotized (FBB), Sigma) [14]. The working reagent was obtained by dissolution of 5 mg of FBB in 10 ml of 5% acetic acid, followed by the addition of five volumes of *n*-propanol. Aliquots of preparations of AHBs extracted from cells or culture liquid were evaporated until dry, and 2 ml of the working FBB reagent was added. The reaction mixture was incubated in the dark for 1 h. The extinction of the colored solution was measured on a Specord spectrophotometer (λ = 480 nm; l = 10 mm) in the automatic double-beam regime using the working reagent as the control and 2,5-dibutylresorcinol to obtain the calibration curve.

Thermostability of trypsin (EC 3.4.21.4; Sigma) in complexes with AHBs was determined using C_7-AHB at concentrations ranging from 1.6 to 12.8 mM in the reaction mixture. The enzyme–AHB complex (in the control, the enzyme alone) was preincubated at room temperature for 10 or 60 min, which was followed by heating at 60°C for 10 or 20 min. After rapid cooling of the mixture to 40°C, the residual trypsin activity was measured by a modified Anson method [15] from the accumulation of casein hydrolysis products that could not be precipitated with trichloroacetic acid. The activity of native unheated trypsin was taken as 100%. The substrate was hydrolyzed under conditions optimal for trypsin (pH 7.8, 40°C) for 20 min. The reaction mixture contained 2 ml of an 0.002% solution of the enzyme (native or in a complex with AHB) and 2 ml of a 2% casein solution in 0.12 M phosphate buffer, pH 7.8; the enzymatic reaction was terminated by the addition of 4 ml of 5% trichloroacetic acid. The remainder of the precipitated substrate was removed by filtration repeated three times. The value of enzymatic activity was determined from the difference between the concentrations of the low-molecular-weight products (expressed in micromoles of tyrosine content) after and before hydrolysis.

The experiments were run in triplicate in each of three independent series. Average values are presented. The results were processed statistically using Student's *t*-test at $P < 0.05$.

RESULTS

In preliminary experiments, the conditions of heat treatment were optimized so that the growth of a *M. luteus* culture would be inhibited without cell death. Efficiency of the stressful impact was assessed from changes in the culture optical density and in the number of viable cells (CFU). After heat treatment (45°C for 15 min) of an exponential-phase culture grown on synthetic medium, the proportion of viable cells was 30.8% (2.4×10^8 CFU/ml versus 7.8×10^8 CFU/ml in

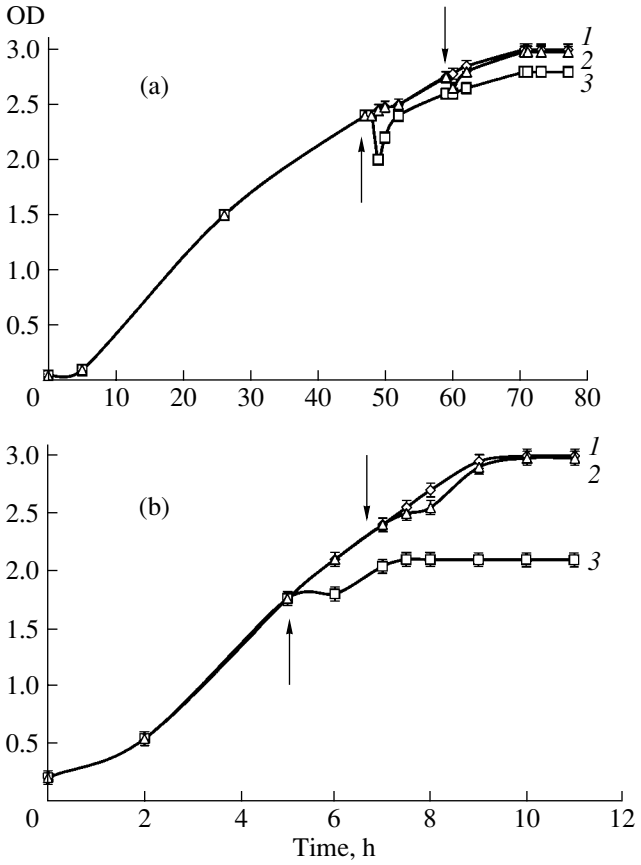

Fig. 1. Heat-shock (45°C, 15 min) sensitivity of *M. luteus* cultures grown on (a) synthetic medium and (b) nutrient broth, as dependent on the growth phase: (*1*) control (without heat treatment); (*2*) heating in growth retardation phase; (*3*) heating in linear growth phase. Arrows indicate the onset of heat treatment.

the control culture), whereas cell exposure to higher temperatures (60 or 70°C) for 10 min led to complete death of the culture. Therefore, in our further experiments, the cells were heat-treated at 45°C for 15 min. This treatment should be considered a shock since it was short-term (the time of heating was significantly lower than the cell cycle duration), which resulted in a partial decrease in CFU number in a culture growing on synthetic medium [1].

The culture response to the heat shock (45°C, 15 min) depended on the growth medium and the physiological age of the cells. After heat treatment of linear-phase cells growing on synthetic medium, OD decreased from 2.4 to 1.8 (by 25%), which was followed by growth resumption (Fig. 1a, curve *3*). The same treatment of a *M. luteus* culture growing on rich medium (nutrient broth) led to a stress response manifested in a temporary arrest of growth without any decrease in OD of the culture; however, this culture entered the stationary phase 2 h earlier than the control culture (Fig. 1b, curve *3*).

The *M. luteus* response to heat exposure also depended on the culture age. In a linear-phase culture grown on synthetic nutrient medium, the decrease in OD of the culture was more pronounced than in a decelerated-growth-phase culture (25 and 7%, respectively) (Fig. 1a). Similarly, after the same treatment (45°C, 15 min) of cells grown on rich medium, the micrococci of a linear-phase culture were more sensitive to the heat shock than cells of a decelerated-growth-phase culture: the growth delay was less pronounced in the latter culture (Fig. 1b).

Since the arrest of microorganism division is a genetically determined defense reaction in response to unfavorable environmental factors [1–3] and AHBs, whose concentration increases in growing microbial cultures, are likely involved in the stationary-phase regulation [8, 12], the AHB dynamics was expected to change in response to stress (high temperature). A linear-phase culture growing on nutrient broth was exposed to a temperature shock (45°C, 15 min) (Fig. 2a). As can be seen from Fig. 2b, the total amount of AHBs (per unit of culture volume) increased in the control both in cells and in culture liquid, which confirms previous data on these autoregulators [8, 12]. The dynamics of the total amount of AHBs changed after cell exposure to a heat shock (45°C, 15 min). After growth arrest, the increase in the amount of extracellular AHBs continued both in the control and in the experiment, whereas, in the cells subjected to shock, this parameter remained unchanged. In the postshock period, oppositely directed changes occurred in the levels of AHBs in the cells and culture liquid, which testifies to AHB redistribution between their extracellular pool and cells.

The amount of AHBs per unit of cell mass characterizes cell productivity under stressful conditions and AHB accumulation in cells (Fig. 2c), where they fulfill the adaptive function. In the control linear-phase culture, the specific content of extracellular AHBs remained unchanged, whereas the specific content of AHBs in cells increased. In the experiment, a delay in cell growth after the heat treatment of the culture was accompanied by a sharp increase in the specific content of extracellular AHBs, whereas within cells the AHB concentration remained at the level of the control. The postshock growth of the culture was accompanied by AHB redistribution: their amount decreased in culture liquid and increased symmetrically within cells to become significantly higher than in control cells. These data suggest that, under stressful conditions, the pool of extracellular AHBs serves for their redistribution between cells.

The dynamics of the overall AHB content (in CL and cells) in the control and heat-treated cultures of *M. luteus* can be seen from the table. After heat shock, the specific amount of AHBs increased by 35% as compared to the control, suggesting that the micrococcus cell productivity increased in response to stress.

The difference between the levels of overall AHBs and, importantly, between their concentrations in cells (Fig. 2c) of control and experimental cultures explains the fact that the experimental culture entered the stationary phase earlier than the control one (Fig. 2a).

Based on the above results, we suggested that an additional amount of exogenous AHBs, which enhances the overall pool of these substances (both extra- and intracellular) in a culture, may protect cells from further stressful actions. The AHB protective properties were demonstrated by preincubation of the linear-phase *M. luteus* culture with a chemical analogue of the autoregulatory d_1 factors, added before heat treatment (45°C, 15 min).

The biological effects of three chemical analogues of microbial autoregulators that differed in the degree of hydrophobicity and polarity of their molecules were compared. These were alkylresorcinols C_7-AHB and C_{12}-AHB with different alkyl radical lengths and *p*-hydroxyethylphenol (tyrosol), which differs in the number and position of hydroxyl-substituting groups [6–8]. The properties of the analogues that influence the efficiency of their protective action were compared, taking into account that all AHBs show antioxidant activity [10, 13] and chaperon properties [11].

The most advantageous was the use of C_7-AHB because, unlike tyrosol, this analogue functions as a scavenger of reactive oxygen species, including singlet oxygen, generated under oxidative stress [13]. On the other hand, unlike the long-chain AHBs, which inhibit the activity of the stabilized enzymes [10, 11], C_7-AHB complexes with enzyme proteins were stable, and their catalytic activity was increased [16].

The C_7-AHB concentrations were selected using a test for thermostability of a model enzyme, trypsin. The latter was incubated in the presence of C_7-AHB, and the trypsin–AHB complexes were heat-treated (60°C, 10 or 60 min). The residual enzymatic activity is represented in Fig. 3. Heating of unprotected trypsin for 10 or 60 min led to a decrease in enzymatic activity to 23 and 3%, respectively, as compared to that of the native enzyme (dotted line). In the complexes with C_7-AHB, trypsin thermostability was enhanced significantly and depended on ligand concentration and the time of complex preincubation. The enzyme–C_7-AHB complex activity ranged from 50 to 120% of the control within a range of AHB concentrations from 3.22 to 12.8 mM.

In the preliminary experiments, the C_7-AHB concentrations improving enzyme (trypsin) thermostability in vitro were shown to have no effect on the *M. luteus* culture growth. When added to a linear-phase culture (OD = 1.48) at a concentration of 4 mM, C_7-AHB caused no significant changes in the growth rate; 3 h after addition, the number of CFU in the experiment was on average 3.6% lower than in the control (without AHB addition). At a concentration of 12 mM, C_7-AHB exhibited a weak inhibiting effect; 3 h after its addition, the CFU number in the experiment was 7.3% lower than in the control.

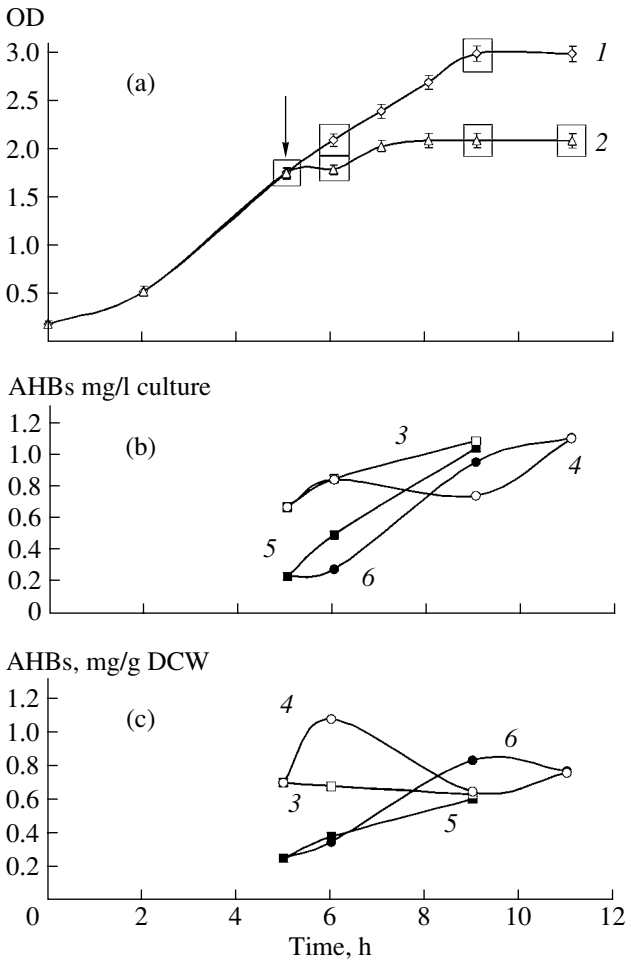

Fig. 2. Effect of heat shock (45°C, 15 min) on the dynamics (a) of *M. luteus* growth nutrient broth and of AHB accumulation in the culture as calculated (b) per unit of culture volume and (c) per unit of dry cell weight (DCW): (*1*) OD in control; (*2*) OD after heating; (*3*) content of extracellular AHBs in control; (*4*) content of extracellular AHBs after heat treatment; (*5*) content of intracellular AHBs in control; (*6*) content of intracellular AHBs after heat treatment. Arrow indicates the onset of heat treatment.

The C_7-AHB protective activity was studied when it was added at concentrations of 4–12 mM 30 min prior to the culture heat treatment (Fig. 4). In amounts of 4–8 mM, C_7-AHB showed no protective effect, whereas at a higher concentration, 12 mM, it exhibited a pronounced protective effect. After a temperature shock, no growth retardation characteristic of the control stress-exposed culture was observed in the experimental culture, though the growth rate was lower.

Thus, the AHB protective effect is observed when their concentration increases in a stress-exposed culture because their synthesis is stimulated in such cultures or because exogenous AHBs are added. The AHB protective effect is manifested in enhanced general resistance

Integral content of extra- and intracellular AHBs per unit of culture volume and of dry cell weight

Sampling points*	Integral content of AHBs			
	mg/ml culture		mg/g dry cells	
	control	experiment	control	experiment
Before heat treatment	0.91 ± 0.04	0.91 ± 0.04	0.95 ± 0.05	0.95 ± 0.05
After heat treatment	1.35 ± 0.06	1.14 ± 0.06	1.06 ± 0.05	1.43 ± 0.07
At the beginning of the stationary phase	2.13 ± 0.10	2.21 ± 0.11	1.24 ± 0.06	1.53 ± 0.07

* In Fig. 2a, the sampling points are indicated with squares.

of the cells and retention of their metabolic activity and proliferative ability.

DISCUSSION

The growth dynamics of *M. luteus* exposed to a heat shock (45°C, 15 min) was assessed from the cell suspension OD and the number of CFU. Our results showed that the microbial cell response to heat treatment depends on the medium composition and the physiological age of the culture. The response of the exponential-phase cells grown on the synthetic medium (Fig. 1a) was more pronounced (significantly reduced OD of the culture) than the response of heat-treated decelerated-growth-phase cultures grown on the complex organic medium. The sensitivity of the growing culture to a heat shock depends on the nutrient medium, probably for the following reasons. Nutrient broth contains amino acids and other metabolites that function as low-molecular-weight chemical chaperons [17] with heat-protective properties. In addition, reversible adhesion (RA) might occur, as in the case of bacillus cells [18]. The latter suggestion is supported by the extremely high rate of OD increase after removal of stress load, as well as by a decrease in cell RA on the rich medium as compared to the cells grown on minimal synthetic media [18]. Reversible cell adhesion on a solid surface or cell conglomerate formation is considered to be an adaptive cell response to stress: it is easier

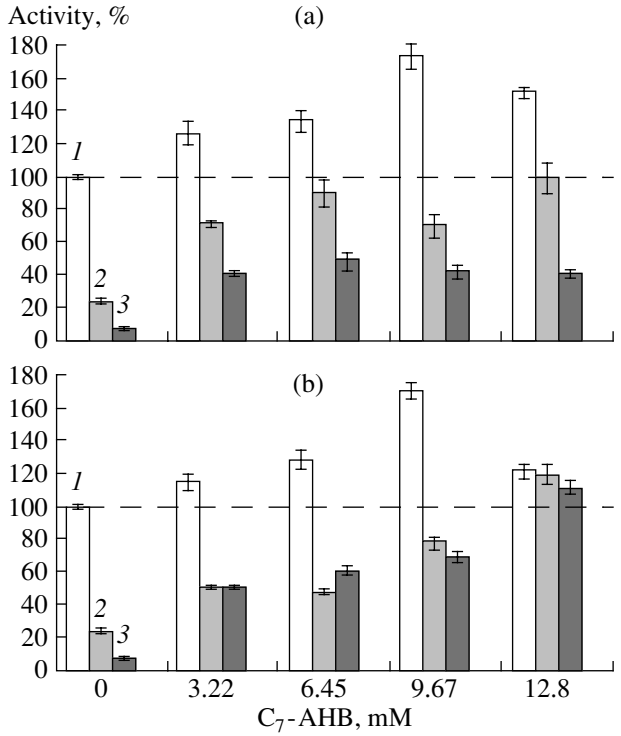

Fig. 3. Trypsin thermostability within a complex with C_7-AHB after preincubation for (a) 10 and (b) 60 min displayed as residual enzymatic activity: (*1*) without heating (control); (*2*) after heating at 60°C for 10 min; (*3*) after heating at 60°C for 20 min. The activity of native enzyme without preincubation with C_7-AHB and without heating was taken as 100% (dotted line).

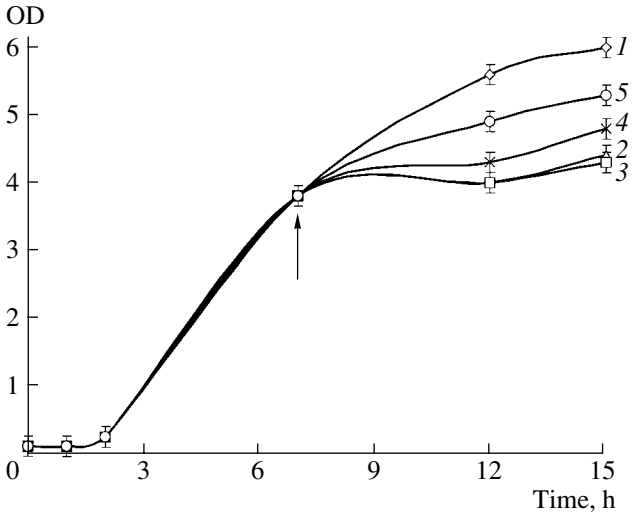

Fig. 4. Protective effect of C_7-AHB with respect to a heat-treated *M. luteus* culture: (*1*) control (growth without heat treatment); (*2*) heat treatment without AHB addition; and heat treatment after the addition of AHB to a concentration of (*3*) 4, (*4*) 8, and (*5*) 12 mM. Arrow indicates the onset of heat treatment.

to overcome the action of unfavorable factors in an immobilized state, which improves cell resistance [19].

The higher resistance to a heat shock characteristic of the older culture of *M. luteus* can be presumably explained by accumulation of both extra- and intracellular d_1 factors [8, 12], which are represented by alkylhydroxybenzenes in some bacteria, micrococci in particular [6, 8]. AHBs (d_1 factors) exhibit the properties of natural agents capable of modifying enzyme macromolecules [11, 16] and biomembranes [9, 10], and, therefore, their increasing concentrations enhance the stability of these cellular components and the cell in general. Note that the age-dependence patterns of the resistance of cultures grown on mineral and rich organic media were similar: under the same treatment, the exponential-phase micrococci were more sensitive to the heat shock than the decelerated-growth-phase cells (Fig. 1b). This phenomenon depends on AHB accumulation in cells with age, which confirms the adaptive properties of these autoregulators.

The adaptive response is considered to be the main mechanism underlying cell ability to overcome environmental changes. The principle of the phenomenon is that pretreatment with sublethal doses of some stressful agent increases the organism resistance against the same agent (and against many others) [1–3, 20]. In experiments with a recombinant strain *Aspergillus awamori* that produced the Ca^{2+}-dependent protein aequorin, it was found that an increase in exogenous AHB (hexylresorcinol) concentration in a growing microbial culture was recognized by cells as stress signal in response to which the content of Ca^{2+} increased in a cell (calcium response) to protect cells from the next stressful action [21]. On the bacterial model *M. luteus,* it has been established that, in response to a heat shock, (1) the productivity of a proliferating micrococcus culture with respect to the overall content of AHBs (in cells and CL) increased; (2) AHB redistribution occurred between the extracellular AHB pool and cells; (3) AHBs accumulated in cells, which resulted in an earlier (at lower cell density) onset of the stationary phase in the stress-exposed culture as compared to the control one. Our results suggest that stress stimulates synthesis of microbial AHBs, which participate in the response of a proliferating cell culture to unexpected stressful impacts. These results also confirmed the concept that the autoinducers of microbial anabiosis (d_1 factors) control a concentration-dependent transition of the culture to the stationary phase [8, 12]. The AHB involvement in the cell stress response was also confirmed in experiments where a chemical AHB analogue added to the culture before stress improved the *M. luteus* cell resistance.

The protective effect of AHBs is likely related to their polyfunctional activity. AHBs serve as (1) scavengers of reactive oxygen species (including singlet oxygen) generated under oxidative stress [13]; (2) agents stabilizing cell membrane structure [9, 10]; and (3) agents stabilizing enzyme proteins and increasing resistance of the latter to denaturing impacts within formed enzyme–AHB complexes [11, 16].

The latter function of AHBs as chemical chaperons was compared in different analogues and shown to depend on AHB structure and physicochemical properties [11, 16]. The results obtained in these works and our own studies showed that AHBs with different hydrophobicity show the same stabilizing effect but differ in the vector of changes in the model enzyme catalytic activity (stimulation or inhibition of the latter) [11]. The distinctions in the effects of the structurally different AHB ligands should be additionally studied.

Note that, apart from the above mechanisms of AHB protective effects (their role as antioxidants and natural agents that modify biopolymer and cell membrane structure), AHBs accelerate culture transition to the stationary phase. The latter is characterized by a higher general cell resistance because, at this growth phase, the *rpo*S regulon transcription is activated, and, therefore, a general cell response to stress is switched on [2, 3]. This AHB effect should also be additionally studied.

Thus, the adaptive role of microbial AHBs includes the following: (1) they function as chemical chaperons and stabilize the structure of subcellular components (biopolymers, membranes); (2) as shown in this study, AHBs regulate the population density and accelerate culture transition to the stationary phase; and (3) AHBs act as antioxidants scavenging reactive oxygen species [10, 13].

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